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Efflux-mediated multidrug resistance in *Bacillus subtilis*: Similarities and dissimilarities with the mammalian system

(*bmr* gene/bacterial resistance/membrane transport/gene amplification)

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ABSTRACT **_Bacillus subtilis_ cells selected for their resistance to rhodamine 6G demonstrated a multidrug-resistance (MDR) phenotype resembling that of mammalian MDR cells. Like MDR in mammalian cells, MDR in bacteria was mediated by the efflux of the drugs from the cells. The bacterial multidrug efflux system transported similar drugs and was sensitive to similar inhibitors as the mammalian multidrug transporter, P-glycoprotein. The gene coding for the bacterial multidrug transporter, like the P-glycoprotein gene in mammalian MDR cells, was amplified in the resistant bacteria. On the other hand, the bacterial multidrug transporter showed no sequence similarity to P-glycoprotein but exhibited an obvious homology to tetracycline efflux pumps and carbohydrate–ion symporters. These results show that the transport of structurally unrelated molecules can be mediated by members of different families of membrane transporters.**

Mammalian cells selected in culture for resistance to various lipophilic cytotoxic drugs often demonstrate resistance not only to the selective agent but also to a large group of apparently unrelated toxic compounds. This phenomenon, called multidrug resistance (MDR), is based on active efflux of drugs from the cells, performed by a membrane ATPase pump, P-glycoprotein (reviews in refs. 1 and 2). In MDR cells, the *mdr1* gene coding for this protein is frequently amplified (1). Overexpression of the P-glycoprotein gene is believed to be responsible for clinical drug resistance in many tumors.

The most intriguing question in the area of MDR is the mechanism of extremely broad chemical specificity of P-glycoprotein. Its substrates have almost nothing in common, except that most of them bear positive electric charge and all of them are moderately hydrophobic. Some substances, such as reserpine and verapamil, are potent inhibitors of P-glycoprotein activity, apparently by a competition mechanism (3, 4).

P-glycoprotein belongs to a large family of membrane ATPase pumps both of eukaryotic and of prokaryotic origin (2). It is, however, the only member of this family with a proven ability to transport multiple drugs. Even close homologues of P-glycoprotein—the mammalian *mdr2* gene product (5) and the yeast STE6 protein (6)—do not share this property. Here we describe MDR in Gram-positive bacteria and characterize the gene of a bacterial multidrug transporter.¶

MATERIALS AND METHODS

Bacteria and Plasmids. *Bacillus subtilis* BD170 (*trpC2, thr-5*) and BD224 (*trpC2, thr-5, recE4*), *Escherichia coli* JM103, *B. subtilis* plasmids pCB20 (7) and pUB110, *E. coli* plasmids pUC19 and pBluescript KS(+) (Stratagene), and shuttle plasmid pMK3 (8) were used in this work.

Selection of Resistant Bacteria and Sensitivity Assay. BD170 bacteria were selected with rhodamine 6G in the liquid antibiotic medium 3 (Difco) supplemented with 0.4% glucose. Bacteria were grown at 37°C with the drug at 0.5, 1, 2, 3, and 4 μg/ml, consecutively. The cultures were diluted 1:100 with fresh drug-containing medium every 1–2 days. The drug concentration was increased every 1–2 weeks.

The sensitivity of bacteria to various drugs was assessed by inoculating overnight bacterial cultures (1:100) into 96-well plates containing serial 1:1.5 dilutions of the drugs in the liquid medium described above. The minimal concentration of drug completely inhibiting bacterial growth was evaluated by observing medium turbidity after 6 hr of incubation at 37°C. In some experiments the medium was supplemented with reserpine (5 μg/ml) or verapamil (25 μg/ml), which increased drug sensitivity (see *Results*). These compounds had no effect when the test was performed not in liquid medium but on agar plates, presumably due to their binding to the agar gel matrix. All the drugs were purchased from Sigma and used as 1 mg/ml stock solutions.

Accumulation and Efflux of Ethidium Bromide. Bacteria in logarithmic growth were collected by centrifugation, resuspended in Spizizen salt medium to an OD_{600} of 0.2, and placed in the fluorimeter cuvette. Ethidium bromide was added (2 μg/ml) and the change in fluorescence intensity (λ_{excite}, 530 nm; λ_{emit}, 600 nm) was recorded on a Hitachi MPF-4 spectrofluorimeter equipped with a plotter. Fluorescence intensity is proportional to the quantity of the intracellular dye, since ethidium fluorescence is dramatically enhanced by the binding to intracellular components, especially to nucleic acids. A similar assay was described previously (9). For measurement of ethidium efflux, bacteria were incubated with the dye (2 μg/ml) and reserpine (5 μg/ml) for 15 min, pelleted in a microcentrifuge, quickly resuspended in dye-free Spizizen medium, and placed in the fluorimeter cuvette. In some experiments protoplasts prepared from bacteria by lysozyme treatment (1 mg/ml, 1 hr, 37°C) were used. In these cases all measurements were performed in 0.5 M sucrose/20 mM $MgCl_2$/20 mM sodium maleate, pH 6.5. ATP was measured with an ATP monitoring kit (LKB).

DNA Manipulations and Cloning Experiments. Chromosomal DNA of *B. subtilis* was isolated as described (10). Plasmids were isolated from *E. coli* by the alkaline lysis procedure (11) and from *B. subtilis* by its slight modification. *E. coli* cells were transformed by the conventional $CaCl_2$ method. *B. subtilis* cells were transformed as described (12).

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Abbreviations: MDR, multidrug resistance; ORF, open reading frame; CCCP, carbonylcyanide *m*-chlorophenylhydrazone.

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¶The sequence presented in this paper has been deposited in the GenBank data base (accession no. M33768).

Conventional DNA manipulation procedures were performed (13). Transduction mapping of the *B. subtilis* genome was performed by using a collection of Tn*917*-transformed bacteria (14) and the phage AR9.

To clone the *bmr* gene from the genome of wild-type *B. subtilis*, 30 ng of DNA of BD224 bacteria was used as template in the polymerase chain reaction (PCR) (GeneAmp Kit; Perkin–Elmer/Cetus). The primers are shown in Fig. 4*b*. A single DNA product of the reaction was cloned into the *Sma* I cloning site of pBluescript KS(+). The insert was cut out by *Bam*HI and *Hin*dIII enzymes and recloned in pMK3.

Sequence Analysis. Chain-termination sequencing reactions on double-stranded templates were performed using a T7 sequencing kit (Pharmacia). Standard heptadecameric primers and synthetic heptadecamers complementary to already determined sequence were used as primers.

Sequences were compared with the Protein Identification Resource (PIR) data base (release 23) by the FASTA program. Alignments were generated by the DOOLITTLE program. Kyte–Doolittle hydropathy plots were constructed by the DNA STRIDER program.

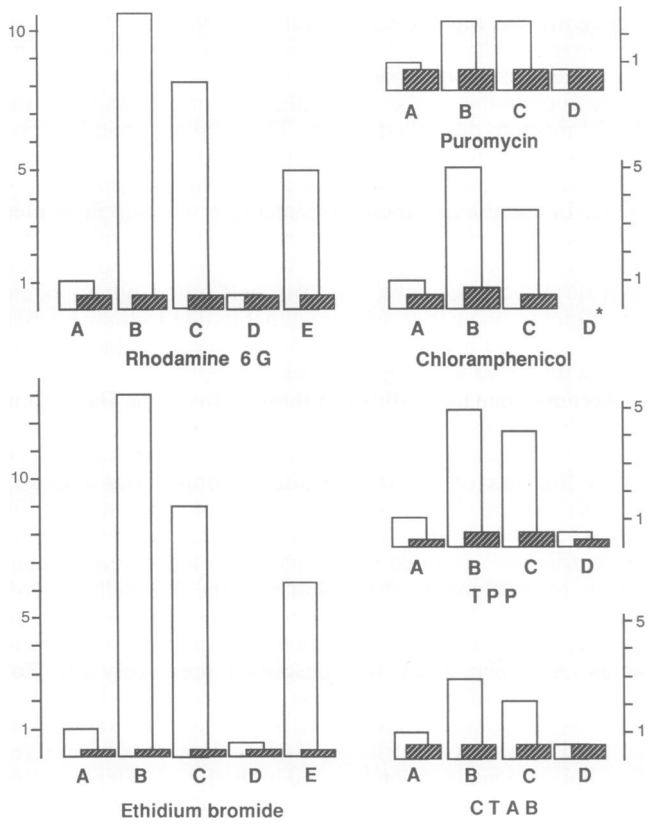


FIG. 1. Relative resistances to the drugs of the following *B. subtilis* strains: A, wild-type bacteria (BD170 or BD224); B, rhodamine-selected bacteria (1R4); C, bacteria bearing the resistance determinant in a plasmid (BD224/pEBR24); D, bacteria with the insertion-inactivated resistance determinant (BD170-1^{int}; star indicates that this strain is highly resistant to chloramphenicol due to integration of the Cm^r gene into the chromosome); E, bacteria bearing the isolated *bmr* gene in a plasmid (BD224/pMK3-bmr). Open bars, standard medium; hatched bars, medium containing reserpine (5 μg/ml). Relative resistances were determined by dividing the minimal inhibitory concentrations (MIC) by the MIC of the given drug for wild-type bacteria in standard medium. The latter had the following values (μg/ml) in this experiment: rhodamine 6G, 0.4; ethidium bromide, 2.0; cetyltrimethylammonium bromide (CTAB), 1.6; puromycin, 15; chloramphenicol, 0.6; tetraphenylphosphonium (TPP), 7.0 μg/ml. These values varied no more than 1.5-fold in different experiments. There were no detectable variations in the relative resistances of the bacterial strains.

RESULTS

We selected *B. subtilis* BD170 bacteria with stepwise increasing concentrations of one of the substrates of P-glycoprotein, rhodamine 6G. The selected cells able to grow in the presence of the drug at 4 μg/ml were subcloned and one of the clones, 1R4, was studied further.

The selected bacteria were resistant not only to rhodamine but also to some other known substrates of P-glycoprotein, such as ethidium bromide, chloramphenicol, and puromycin, as well as to tetraphenylphosphonium and cetyltrimethylammonium bromide, which to our knowledge, have not been shown to be transported by the mammalian MDR pump (Fig. 1). The sensitivity of the 1R4 bacteria to benzylpenicillin and tetracycline and their growth rate in drug-free medium did not change after the selection (data not shown).

Two inhibitors of mammalian P-glycoprotein, reserpine (Fig. 1) and verapamil (data not shown), significantly increased the sensitivity of wild-type bacteria to toxic compounds. Moreover, they completely reversed the MDR phenotype of 1R4 bacteria, just as they do with mammalian MDR cells.

The mechanism of resistance in the MDR bacteria also turned out to be the same as in MDR mammalian cells—namely, reduced accumulation of the drugs due to their accelerated efflux. Accumulation of the toxic dye ethidium bromide in 1R4 bacteria was significantly reduced in comparison with wild-type bacteria but could be restored by the addition of reserpine (Fig. 2*a*) or verapamil (data not shown). Both inhibitors also caused a slight increase in the accumulation of the dye in wild-type bacteria. The cells loaded with ethidium in the presence of reserpine gradually lost the dye when transferred to drug-free medium. The rate of dye efflux from 1R4 cells was 4–5 times higher than with wild-type cells (Fig. 2*b*). Reserpine dramatically decreased the efflux rate in both bacterial strains and abolished the difference between them.

Similar kinetics of ethidium accumulation and efflux were observed with protoplasts obtained by lysozyme treatment (data not shown), indicating that the reduced accumulation and increased efflux rate in the MDR bacteria were not caused by a modification of the bacterial cell wall. Efflux of ethidium bromide from the protoplasts of both 1R4 cells and wild-type cells completely stopped 10 sec after addition of the membrane protonophores carbonylcyanide *m*-chlorophenylhydrazone (CCCP, 2 μM) or pentachlorophenol (5 μM) (data not shown). The ATP pool in the protoplasts was not reduced significantly in such a short period after the addition of protonophores and it decreased only 50% after 3 min of incubation. These results are consistent with the hypothesis that the bacterial multidrug transporter, in contrast to P-gly-

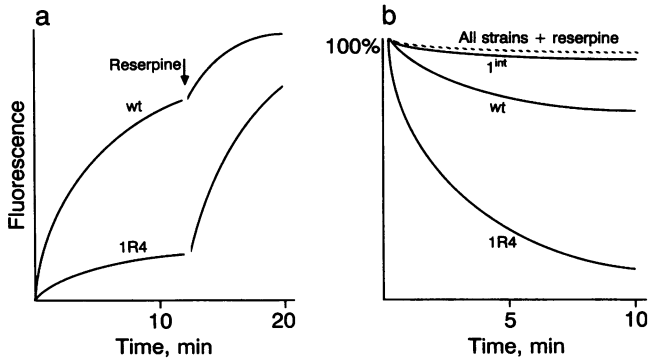


FIG. 2. Accumulation (*a*) and efflux (*b*) of ethidium bromide in *B. subtilis* strains BD170 (wild type, wt), 1R4, and BD170-1^{int} (1^{int}). Fluorescence is a measure of the quantity of cell-associated dye. Reserpine was added at 5 μg/ml.

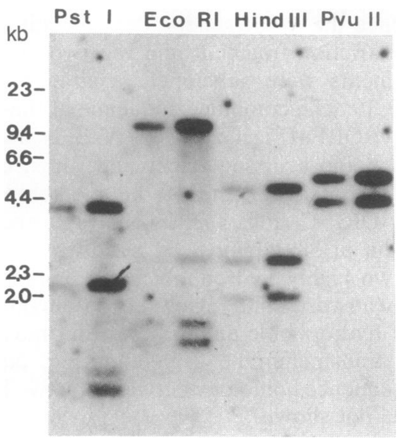


FIG. 3. DNA amplification in the rhodamine-selected cells as shown by Southern blotting of the chromosomal DNA of BD170 (left lane in each pair) and 1R4 (right lane in each pair) *B. subtilis* strains. DNA was digested with the indicated restriction enzymes. Plasmid pEBR1 was used as a hybridization probe. As judged by ethidium bromide staining, all the lanes contained equal amounts of DNA. Markers at left indicate positions and sizes of *Hin*dIII fragments of λ phage DNA.

coprotein, uses a transmembrane proton gradient, rather than ATP, as the energy source.

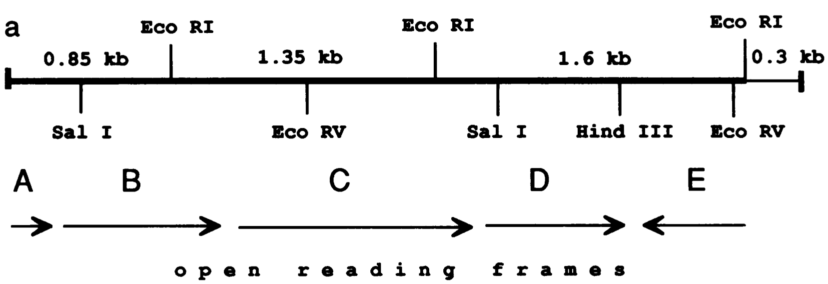
In all other respects the phenotype of the MDR bacteria described above is strikingly similar to the phenotype of mammalian MDR cells. By analogy with the mammalian system, we hypothesized that MDR bacteria arise as a result of amplification of the gene encoding the multidrug transporter. To clone this hypothetical gene, we constructed a DNA library of resistant bacteria in pCB20 plasmid (DNA was partially digested with *Sau*3A1 and the plasmid with *Bam*HI) and then selected the cells transformed with this library by plating them on agar containing ethidium bromide (15 μg/ml). Since the resistance gene presumably is able to confer the MDR phenotype when present in multiple copies, the plasmid bearing this gene should exert the same effect. *B. subtilis* strain BD224 was used as host for the library because it bears a *recE4* mutation preventing recombination of plasmid inserts with genomic DNA.

Four clones of bacteria resistant both to ethidium bromide and to erythromycin (pCB20 genetic marker) were selected. The plasmids isolated from these clones bore 10- to 12-kilobase (kb) DNA inserts yielding fragments of similar size when digested with restriction enzymes, thus presumably representing the same fragment of the *B. subtilis* genome. One of the plasmids, pEBR1, was analyzed further. Molecular hybridization experiments have shown that the fragment of DNA cloned in pEBR1 plasmid is indeed, as we assumed, amplified in 1R4 bacteria about 5–10 times, as estimated by the relative intensity of the hybridization signals (Fig. 3).

Only a part of the DNA fragment cloned in pEBR1 was essential for maintaining the resistance phenotype. The plasmid pEBR24, composed of the pUB110 *B. subtilis* plasmid and a 4.1-kb *Pvu* II restriction fragment of the pEBR1 insert, also conferred ethidium bromide resistance on wild-type bacterial cells. Cells transformed by pEBR24 were resistant to the same set of drugs as 1R4 cells, and their resistance could be completely reversed by reserpine (Fig. 1). The kinetics of ethidium bromide accumulation and efflux in BD224/pEBR24 bacteria were similar to those in 1R4 cells (data not shown).

Fig. 4*a* shows the restriction map of the pEBR24 insert. We have found that the substitution of the chloramphenicol-resistance (Cm^r) gene from *Staphylococcus* plasmid pC194 for the two central *Eco*RI fragments of the insert completely abolished the ability of pEBR24 to confer MDR. This modified plasmid was used to inactivate the genomic MDR determinant in wild-type bacteria (strain BD170) by homol-

a



b

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GGCGGATATGGATGGAGAAGTTTATACACATACACCCGCAGTCATTCATTCAGGTCCTGCCACAGCATATCGACATGCTTGTTCCCGCAAATGAATAAC
                        Primer 1
TGTCATAAGGCTTTAGAAAGATTTTGCAAATCCGTTGACTCTCCCCTAGGAGGAGGTCTTACAGTATAAGGGATACACACTTGAAAAAGGGGAAGTCAT
                                  "-35"                      "-10"                               R B S
   1 ATG GAG AAG AAA AAT ATT ACC TTA ACT ATA TTA TTA ACC AAT TTA TTT ATT GCT TTT TTG GGG ATC GGG CTT GTG
   1 Met glu lys lys asn ile thr leu thr ile leu leu thr asn leu phe ile ala phe leu gly ile gly leu val
     ATT CCA GTA ACG CCG ACC ATT ATG AAT GAA TTG CAT TTA TCG GGG ACC GCG GTC GGC TAT ATG GTT GCC TGC TTC
     ile pro val thr pro thr ile met asn glu leu his leu ser gly thr ala val gly tyr met val ala cys phe
 151 GCT ATT ACA CAG CTC ATT GTC TCA CCA ATA GCC GGA CGA TGG GTT GAT CGC TTC GGG CGC AAG ATC ATG ATC GTA
  51 ala ile thr gln leu ile val ser pro ile ala gly arg trp val asp arg phe gly arg lys ile met ile val
     ATC GGC CTG TTG TTC TTT AGT GTG TCG GAG TTT TTG TTC GGC ATT GGA AAA ACA GTT GAG ATC TTA TTT ATC ACC
     ile gly leu leu phe phe ser val ser glu phe leu phe gly ile gly lys thr val glu met leu phe ile thr
 301 CGT ATG CTG GGC GGT ATC AGC GCA CCG TTC ATT ATG CCC GGG GTC ACG GCT TTT ATT GCA GAT ATC ACG ACC ATT
 101 arg met leu gly gly ile ser ala pro phe ile met pro gly val thr ala phe ile ala asp ile thr thr ile
     AAA ACA CGG CCA AAA GCG CTC GGT TAT ATG TCA GCC GCT ATT TCA ACA GGA TTT ATT ATC GGC CCC GGC ATC GGG
     lys thr arg pro lys ala leu gly tyr met ser ala ala ile ser thr gly phe ile ile gly pro gly ile gly
 451 GGA TTT TTA GCA GAA GTC CAT TCC CGG CTG CCT TTT TTC TTT GCG GCA GCT TTT GCA CTG TTA GCA GCC ATT TTA
 151 gly phe leu ala glu val hys ser arg leu pro phe phe phe ala ala ala phe ala leu leu ala ala ile leu
     TCA ATC CTC ACG CTG CGC GAG CCG GAA CGA AAC CCT GAA AAT CAG GAA ATA AAA GGA CAG AAG ACA GGC TTT AAA
     ser ile leu thr leu arg glu pro glu arg asn pro glu asn gln glu ile lys gly gln lys thr gly phe lys
 601 CGA ATT TTT GCC CCC ATG TAT TTC ATA GCT TTT CTC ATT ATC TTA ATT TCG TCT TTT GGT TTA GCA TCA TTT GAA
 201 arg ile phe ala pro met tyr phe ile ala phe leu ile ile leu ile ser ser phe gly leu ala ser phe glu
     TCT TTA TTT GCA TTA TTC GTG GAT CAT AAA TTC GGA TTT ACG GCC AGC GAC ATT GCC ATT ATG ATT ACA GGA GGA
     ser leu phe ala leu phe val asp hys lys phe gly phe thr ala ser asp ile ala ile met ile thr gly gly
 751 GCG ATT GTT GGC GCC ATT ACG CAA GTC GTC TTA TTC GAC CGC TTC ACA AGA TGG TTT GGC GAA ATT CAT TTA ATT
 251 ala ile val gly ala ile thr gln val val leu phe asp arg phe thr arg trp phe gly glu ile hys leu ile
     CGG TAC AGC TTA ATT CTC TCG ACG AGT CTG GTA TTC TTG CTG ACA ACG GTA CAT TCA TAT GTT GCG ATT CTG CTG
     arg tyr ser leu ile leu ser thr ser leu val phe leu leu thr thr val hys ser tyr val ala ile leu leu
 901 GTG ACA GTC ACC GTA TTT GTC GGA TTT GAT CTC ATG CGG CCT GCG GTA ACG ACT TAC CTG TCA AAG ATT GCG GGA
 301 val thr val thr val phe val gly phe asp leu met arg pro ala val thr thr tyr leu ser lys ile ala gly
     AAT GAA CAG GGG TTT GCC GGC GGT ATG AAT TCA ATG TTT ACA AGT ATC GGC AAT GTA TTC GGG CCT ATT ATC GGC
     asn glu gln gly phe ala gly gly met asn ser met phe thr ser ile gly asn val phe gly pro ile ile gly
1051 GGA ATG CTG TTC GAT ATA GAT GTA AAC TAT CCT TTC TAC TTT GCA ACG GTC ACC TTA GCC ATA GGG ATT GCA CTG
 351 gly met leu phe asp ile asp val asn tyr pro phe tyr phe ala thr val thr leu ala ile gly ile ala leu
     ACC ATT GCT TGG AAA GCG CCT GCA CAT CTT AAA GCC AGC ACG TGA TAA GAAGCGCATTCTTTGTGTACTGCAAAGAATGCGCT
     thr ile ala trp lys ala pro ala his leu lys ala ser thr *** ***   Primer 2
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FIG. 4. (*a*) Restriction map and open reading frames (ORFs) of the pEBR24 insert. The DNA fragment shown by the thick line was sequenced. (*b*) Sequence of ORF C, the flanking DNA, and the ORF C-encoded protein. Putative ribosome-binding site (RBS) and putative elements of the promoter ("−10" and "−35") are indicated. Primers 1 and 2 were used for PCR amplification of the ORF C-containing DNA fragment from wild-type chromosomal DNA.

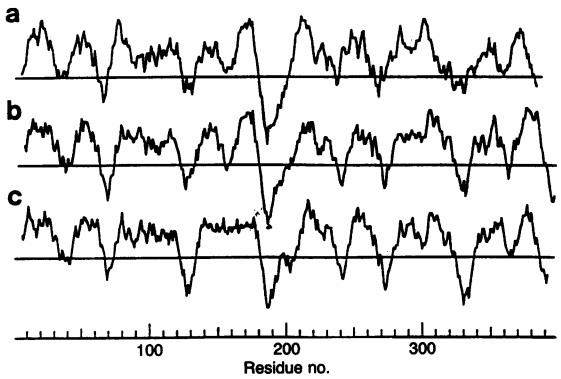

FIG. 5. Kyte–Doolittle hydropathy profiles of the ORF C-encoded protein (*a*) and the tetracycline efflux pumps encoded by transposon Tn*10* (*b*) and plasmid pBR322 (*c*).

ogous recombination. Cells were transformed with the insert cut from the modified plasmid by *Pvu* II and then selected for resistance to chloramphenicol (resistance to this drug provided by the Cm^r gene is much stronger than that provided by the MDR determinant). The cells selected by this procedure (BD170-1^{int}) were more sensitive to all the drugs of the MDR group (except chloramphenicol) than were the wild-type cells (Fig. 1). Efflux of ethidium bromide from these cells was at least 10 times slower than from wild-type cells (Fig. 2*b*). We conclude that the MDR determinant is active in wild-type cells and provides some resistance to the drugs even at a single copy.

A transduction analysis was used to map the MDR determinant on the *B. subtilis* chromosome. A collection of *B. subtilis* strains bearing the erythromycin-resistance (Em^r) gene in different positions of the chromosome (14) was transformed with the DNA of AR9 phage grown on BD170-1^{int} bacteria and then was selected with chloramphenicol. The only strain in which transduction of the Cm^r gene from BD170-1^{int} bacteria was frequently (in 85% of clones) associated with the loss of the Em^r phenotype was the strain bearing the Em^r gene at the 216-min position of the chromosome. Conversely, transduction of the Em^r gene from this strain to BD170-1^{int} cells led to the loss of the Cm^r phenotype in 90% of the clones. These results indicate that the MDR determinant in wild-type bacteria is located very close to 216 min of the *B. subtilis* chromosome.

To sequence the MDR genetic determinant, the left *Pvu* II–*Eco*RI restriction fragment and the two central *Eco*RI–*Eco*RI fragments were subcloned separately into *E. coli* plasmid pUC19. The combined sequence of these fragments contained five ORFs (Fig. 4*a*). ORFs A, B, D, and E encoded hydrophilic amino acid sequences and, thus, could hardly represent the potential gene of the membrane multidrug transporter. ORF C (Fig. 4*b*), in contrast, encodes a predicted protein of 389 amino acid residues that appears to consist of two highly hydrophobic domains of similar size separated by a hydrophilic region; each domain contains six stretches of hydrophobic amino acids that most likely correspond to transmembrane segments (Fig. 5*a*). There is significant sequence homology between the two halves of the protein (data not shown).

A comparison of the sequence of the ORF C protein with the sequence of P-glycoprotein revealed no significant similarity. On the other hand, this protein was closely similar to the tetracycline-efflux proteins encoded by *E. coli* transposons Tn*10* and Tn*1721* and plasmid pBR322 (Fig. 6). Their similarity is most evident in their hydropathy profiles, which are nearly identical (Fig. 5). Besides tetracycline-efflux proteins, the protein encoded by ORF C demonstrated highly significant similarity to some other *E. coli* membrane transporters: the arabinose and xylose transporters and enzyme II of the phosphotransferase system.

Using the PCR, we amplified the fragment of DNA shown in Fig. 4*b* from the genome of wild-type *B. subtilis* and cloned it into the *Sma* I site of *E. coli* plasmid pBluescript KS(+). Sequencing of the cloned PCR fragment showed that the wild-type ORF C and the upstream region of DNA did not differ from the respective sequences cloned previously from 1R4 cells. The cloned PCR fragment was recloned into shuttle vector pMK3 and then transfected into wild-type *B. subtilis* BD224. After transformation these cells exhibited resistance to ethidium bromide and rhodamine 6G that could be reversed by reserpine (Fig. 1). These cells were somewhat less resistant to the drugs than the rhodamine-selected cells. This may be either an indication of involvement of some other genes in the resistance of the rhodamine-selected cells, or, more likely, a result of lower expression of the *bmr* gene in the plasmid-transformed cells. In any case, this result indicates that the amplification of ORF C is responsible, at least partially, for the MDR phenotype of 1R4 bacteria. We

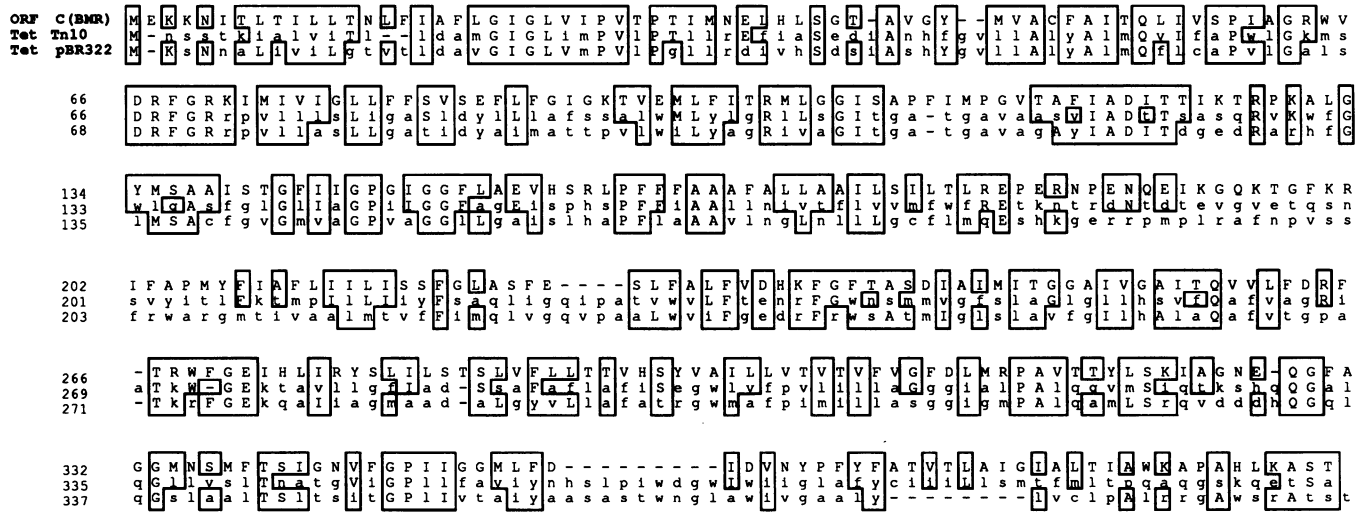

FIG. 6. Alignment of the sequences of the ORF C protein and the tetracycline efflux pumps (Tet) encoded by transposon Tn*10* and plasmid pBR322. The amino acid residues of the latter two proteins identical to the corresponding residues of the ORF C protein are shown by uppercase letters. Identical residues and conservative substitutions according to the groups (I, L, M, V), (A, S, T), (F, W, Y), (D, E), (N, Q), and (K, R) are boxed.

propose therefore to name this gene *bmr*, for *b*acterial *m*ultidrug *r*esistance.

DISCUSSION

Selection of *B. subtilis* cells with a cationic lipophilic drug, rhodamine 6G, led to establishment of a bacterial strain simultaneously resistant to at least six structurally unrelated toxic compounds. Our data suggest that the resistance is based on active efflux of the drugs, which was sensitive to the membrane protonophores CCCP and pentachlorophenol. An analogous phenotype has been described by Tennent *et al.* (15). Bacteria transformed with the *qacA* determinant derived from *Staphylococcus* plasmid were found to be resistant to a similar group of lipophilic toxic agents; the cells actively extruded the drugs in a CCCP-sensitive manner.

The genetic determinant that conferred MDR in the case of *B. subtilis* selected with rhodamine 6G was of chromosomal, rather than plasmid, origin. We found that these cells have acquired resistance as a result of amplification of the *bmr* gene. Examples of chromosomal DNA amplification in *B. subtilis* have been described (10, 16, 17).

The protein encoded by *bmr* displays a remarkable sequence similarity to tetracycline-efflux pumps (Tet proteins) and, to a lesser extent, to arabinose– and xylose–H^+ symporters. Outward transport of tetracycline by the Tet proteins is apparently coupled to inward transport of H^+ (18); the action of these proteins can be blocked by protonophores dissipating the transmembrane pH gradient (19). The high degree of sequence similarity of the predicted Bmr protein to membrane ion-dependent transport proteins, the ability of *bmr* to confer efflux-based MDR, the block of the drug efflux in the wild-type cells after the inactivation of this gene, and the strong sensitivity of the drug efflux to protonophores leave practically no doubt that *bmr* codes for a multidrug efflux transporter. It seems reasonable to suggest that the molecular mechanism of its action is similar to that of Tet proteins, namely H^+/drug antiport, though the chemical specificities are different. Tet proteins are specific to tetracycline, while *bmr* provides resistance not to this antibiotic but to a number of other drugs.

Most intriguing is the remarkable functional similarity between the bacterial and mammalian multidrug transporters. Many drugs can be transported by both Bmr protein and P-glycoprotein. Moreover, both membrane efflux pumps can be inhibited by the same substances, reserpine and verapamil. We failed, however, to detect even slight homology between the two transporters.

There are two possible explanations of the close functional analogy between the bacterial and mammalian multidrug transporters. According to the first hypothesis, the two proteins have similar "multidrug-binding" sites; these sites cannot be detected by the direct sequence comparison because they are formed by the spatial arrangement of the amino acid residues located discontinuously in the polypeptide chains.

An alternative hypothesis, having perhaps more interesting corollaries, assumes that multidrug transporters have no binding sites in the classic sense and that they recognize their substrates by physical rather than purely structural parameters. For example, they might transport to the external medium all moderately hydrophobic substances that occur in the highly hydrophobic membrane environment. The role of a "binding site" in such a transporter can be performed by a relatively hydrophilic part of the protein exposed into the lipid bilayer. In this case, the repertoire of substrates and competitive inhibitors should be determined mainly by the physical properties of the substances themselves and only slightly by the structure of transporter. If this hypothesis is correct, then the functional analogy between the structurally distinct bacterial and mammalian multidrug transporters is not surprising.

In conclusion, our results demonstrate that the transport of multiple drugs can be effected not only by P-glycoprotein or P-glycoprotein-related molecules but also by the bacterial Bmr protein belonging to another family of membrane transporters. Since the genetic and biochemical experiments are potentially easier to perform with bacteria than with mammalian cells, we anticipate that further study of the Bmr protein will contribute to the understanding of the mechanism of low-specificity membrane transport.

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