ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 1992, p. 484–485
0066-4804/92/020484-02$02.00/0

Vol. 36, No. 2

The Multidrug Efflux Transporter of *Bacillus subtilis* Is a Structural and Functional Homolog of the *Staphylococcus* NorA Protein

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Received 9 August 1991/Accepted 10 December 1991

The *Bacillus subtilis* multidrug efflux transporter Bmr demonstrates 44% amino acid sequence identity with a product of the *Staphylococcus aureus* gene *norA*, which is responsible for clinically relevant resistance to fluoroquinolones. We show here that overexpression of *bmr* in *B. subtilis* provides strong resistance to fluoroquinolones that can be reversed by reserpine, an inhibitor of Bmr.

Intrachromosomal amplification of the *Bacillus subtilis* gene *bmr* provides resistance to a number of structurally unrelated drugs (e.g., ethidium bromide, rhodamine, chloramphenicol, puromycin, and tetraphenylphosphonium) (4). The resistance is based on active efflux of drugs, which is sensitive to membrane protonophores. The Bmr protein is structurally similar to the tetracycline efflux transporters of the TetA, -B, and -C classes and demonstrates 24 to 25% sequence identity with these proteins.

Comparison of the Bmr sequence with the updated sequence data bases revealed its close similarity to that of the recently described NorA protein of *Staphylococcus aureus* (7). The *norA* gene is responsible for clinically relevant resistance of *S. aureus* to norfloxacin and some other fluoroquinolones, which also correlates with protonophore-sensitive efflux of drugs. Like Bmr, NorA is structurally similar to TetA, -B, and -C and demonstrates 24 to 25% sequence identity with these proteins.

The alignment of the Bmr and NorA sequences built by the GENEPRO program is shown in Fig. 1. The homology of these two proteins (44% identity) is much stronger than their homology with the Tet proteins. At the DNA level, identity of the *bmr* and *norA* coding sequences reaches 51%, while the flanking sequences of these genes are not homologous (data not shown).

We have found that the Bmr protein is similar not only structurally but also functionally to the NorA protein. In these experiments, we have compared the drug susceptibilities of *B. subtilis* BD224 (*trpC2*, *thr-5*, *recE4*) and bacteria of the same strain transformed with plasmid pBMR2. This *bmr*-expressing plasmid was constructed in two steps. First, the *bmr* gene was cut out by *Hin*cII enzyme from the pEBR24 plasmid (4), which contains a region of the *B. subtilis* chromosome. This *Hin*cII fragment was cloned into the *Bam*HI site of the plasmid pCB20 (6) to put *bmr* under control of the strong promoter *EU19035*. In the second step, to increase the copy number of the gene, the *Hin*dIII fragment of the resultant plasmid containing both the promoter and the *bmr* gene was cloned into the *Pvu*II site of the *B. subtilis* vector pUB110, resulting in plasmid pBMR2.

MICs of various drugs for *B. subtilis* BD224 and BD224/pBMR2 were determined by growing these bacteria in 96-well plates containing 1:2 serial dilutions of the drugs in 100 μl of LB medium (inoculum, 2×10^5 logarithmic-phase cells; incubation, 12 h at 37°C). The bacteria transformed with pBMR2, like the bacteria with an amplified *bmr* (4), demonstrated strong resistance to ethidium bromide, rhodamine, chloramphenicol, puromycin, and tetraphenylphosphonium (Table 1). These bacteria also exhibited resistance to acridine orange and netropsin. Most importantly here, the *bmr*-overexpressing bacteria were strongly resistant to several fluoroquinolones, including norfloxacin, ciprofloxacin, and temafloxacin. In full agreement with data on the *norA* gene (7), the *bmr* gene provided very weak if any resistance to the "old" quinolone antibiotics nalidixic and oxolinic acids (Table 1).

Previously we have shown (4) that the activity of the Bmr protein can be inhibited by nontoxic concentrations of reserpine, which is also an inhibitor of the adrenal catecholamine transporter (3), at least some tetracycline transporters (5), and the mammalian multidrug transporter P-glycoprotein (1). Table 1 demonstrates that the *bmr*-conferred resistance to fluoroquinolones, as well as that to the other drugs, can be reversed by reserpine.

Interestingly, neither *norA* (7) nor *bmr* (4) (Table 1) confers resistance to tetracycline, despite their homology to the genes of tetracycline transporters. The increased susceptibility

TABLE 1. Influence of reserpine (10 μg/ml) on resistance to various drugs of *bmr*-overexpressing *B. subtilis* BD224/pBMR2

Drug	Relative resistances[a] to:			
	BD224	BD224 + reserpine	BD224/pBMR2	BD224/pBMR2 + reserpine
Rhodamine 6G	1 (0.8)[b]	0.25	16	0.5
Ethidium bromide	1 (3.2)	0.125	32	1
Chloramphenicol	1 (1.6)	0.5	8	1
Tetraphenylphosphonium	1 (16)	0.25	32	1
Puromycin	1 (16)	0.5	8	2
Acridine orange	1 (6.4)	0.25	8	1
Netropsin	1 (6.4)	0.5	8	1
Tetracycline	1 (0.8)	0.25	1	0.25
Quinolones				
Norfloxacin	1 (0.1)	0.25	32	1
Ciprofloxacin	1 (0.02)	0.25	16	0.5
Temafloxacin	1 (0.02)	0.25	16	0.5
Nalidixic acid	1 (0.8)	1	2	1
Oxolinic acid	1 (0.04)	1	2	1

[a] Relative resistance is the ratio of the MIC of the drug to the MIC for the susceptible BD224 strain in the standard medium.

[b] The MIC of each drug for strain BD224 is given in parentheses (in micrograms per milliliter).

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Bmr     MEKKNITLTILLTNLFIAFLGIGLVIPVTPTIMNELHLSGTAVGYMVACFAITQLIVSPI   60
        : :    . .:  :.:. :::::::::: :    .: :.:. .:  :: ::..: :.::
Nor A   MNKQ---IFVLYFNIFLIFLGIGLVIPVLPVYLKDLGLTGSDLGLLVAAFALSQMIISPF   57

        AGRWVDRFGRKIMIVIGLLFFSVSEFLFGIGKTVEMLFISRMLGGISAPFIMPGVTAFIA   120
        .:   :. :.:. : :::. ::::::  :..:     : .:: .:: ::  .::::. ::
        GGTLADKLGKKLIICIGLILFSVSEFMFAVGHNFSVLMLSRVIGGMSAGMVMPGVTGLIA   117

        DITTIKTRPKALGYMSAAISTGFIIGPGIGGFLAEVHSRLPFFFAAAFALLAAILSILTL   180
        ::.    . :  ::::: : .:::.:::::::: :::  : ::.::.: ..:: : ::. .
        DISPSHQKAKNFGYMSAIINSGFILGPGIGGFMAEVSHRMPFYFAGALGILAFIMSIVLI   177

        REPERNPENQEIKGQKTGFKRIFAPMYFIAFLIILISSFGLASFESLFALFVDHKFGFTA   240
        .: .    :      .:     .   .. :. :::: ::.:. :.   :  ..
        HDPKKSTTSGFQKLEPQLLTKINWKVFITPVILTLVLSFGLSAFETLYSLYTADKVNYSP   237

        SDIAIMITGGAIVGAITQVVLFDRFTRWFGEIHLIRYSLILSTSLVFLLTTVHSYVAILL   300
         :: : ::::.: ::. :.  ::.: . : :.  :  ::. :  .. ::     :  : :
        KDISIAITGGGIFGALFQIYFFDKFMKYFSELTFIAWSLLYSVVVLILLVFANGYWSIML   297

        VTVTVFVGFDLMRPAVTTYLSKIAGNEQGFAGGMNSMFTSIGNVFGPIIGGMLFDIDVNY   360
        ..  ::.:::  :::.: : : :::  :::::: :: ::: ::  ::.:.: :::. .
        ISFVVFIGFDMIRPAITNYFSNIAGERQGFAGGLNSTFTSMGNFIGPLIAGALFDVHIEA   357

        PFYFA-TVTLAIGIALTIAWKAPAHLKAST                                 389
        : : :  :.::  . . :     : ::
        PIYMAIGVSLAGVVIVLIEKQHRAKLKEQNM                                 388
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FIG. 1. Alignment of sequences of Bmr (4) and NorA (7) proteins. Identical amino acids are indicated by colons, and homologous amino acids are indicated by dots. Residue 100 in the Bmr sequence (S) has been mistakenly published as T in the article cited in reference 4. The corresponding error in the nucleotide sequence (see "Nucleotide sequence accession number") has been corrected.

tibility of *B. subtilis* to tetracycline in the presence of reserpine (Table 1) most probably reflects inhibition of the reserpine-sensitive tetracycline transporter encoded by the cryptic chromosomal *tet* determinant (5).

In conclusion, these results indicate that the *bmr* gene is the *B. subtilis* structural and functional homolog of the *Staphylococcus norA* gene. Most probably, the homologs of *norA* and *bmr* are widespread among bacteria; the protonophore-sensitive norfloxacin-effluxing system has also been detected in *Escherichia coli* (2). It is conceivable that these homologs, like *bmr*, are sensitive to reserpine. If so, it would open the way to a search for clinically relevant reserpinelike agents capable of reversing the efflux-mediated fluoroquinolone resistance of staphylococci and other bacterial pathogens.

Nucleotide sequence accession number. The GenBank accession number for the Bmr sequence described in Fig. 1 is M33768.

This work was financed by grant MV-542 from the American Cancer Society.

I am grateful to O. Lomovskaya of the Massachussetts Institute of Technology for helpful discussions.

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