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Fluoroquinolone Resistance Protein NorA of *Staphylococcus aureus* Is a Multidrug Efflux Transporter

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The gene of the *Staphylococcus aureus* fluoroquinolone efflux transporter protein NorA confers resistance to a number of structurally dissimilar drugs, not just to fluoroquinolones, when it is expressed in *Bacillus subtilis*. NorA provides *B. subtilis* with resistance to the same drugs and to a similar extent as the *B. subtilis* multidrug transporter protein Bmr does. NorA and Bmr share 44% sequence similarity. Both the NorA- and Bmr-conferred resistances can be completely reversed by reserpine.

Fluoroquinolone resistance in several clinical isolates of *Staphylococcus aureus* is provided by the membrane protein NorA encoded in the bacterial chromosome (1, 2, 5, 7). This protein likely actively transports norfloxacin and several other fluoroquinolones out of bacterial cells, thus reducing the intracellular concentration of the drugs. NorA demonstrates a significant sequence similarity (24 to 25% identity) with tetracycline-efflux transporters (Tet proteins) of gram-negative bacteria (2, 7). Like the activity of Tet proteins, the drug-efflux activity of NorA depends on the transmembrane proton gradient and can be inhibited by membrane protonophores. NorA differs, however, from Tet proteins in substrate specificity and does not provide resistance to tetracycline (7).

Recently, we have shown (3) that NorA has another close bacterial homolog, the *Bacillus subtilis* protein Bmr, with which it shares 44% sequence identity. Bmr, like NorA, is a chromosome-encoded, highly hydrophobic protein with 12 putative transmembrane domains (4). Intrachromosomal amplification or overexpression of the *bmr* gene in a plasmid leads to multiple drug resistance in *B. subtilis* (3, 4). The *bmr*-overexpressing cells demonstrate resistance to a number of structurally unrelated compounds, including ethidium bromide, rhodamine 6G, tetraphenylphosphonium, puromycin, chloramphenicol, and acridine orange (3, 4) as well as norfloxacin and several other fluoroquinolones (3). The mechanism of Bmr activity is similar to that of NorA, i.e., it actively transports toxic compounds out of resistant cells and its function can be inhibited by protonophores (4).

The aim of the present study was to compare directly the substrate specificities of Bmr and NorA. In order to do that, the *bmr* and *norA* genes were each cloned into the same expression vector in *B. subtilis*. The construction of the *bmr*-expressing plasmid pBMR2 has been described previously (3). This plasmid is composed of the vector pUB110, a fragment of the plasmid pCB20 (6) containing a strong *B. subtilis* promoter, and the *bmr* gene cloned into the *Bam*HI site just downstream of the promoter. In the present study we destroyed the additional *Bam*HI site located in the vector part of pBMR2 by the cutting-filling-ligation reactions and then removed the *bmr* gene by *Bam*HI digestion and subsequent self-ligation of the plasmid. The resulting expression vector pBEV was used to clone the *norA* gene.

As a source of the *norA* gene, we used plasmid pK21 (1), which contains one of the variants of *norA*, *norA1199*. *norA1199* was cloned from a clinical isolate of *S. aureus*, SA-1199B (1). The plasmid was digested with the enzymes *Mnl*I and *Hin*PI. The resulting fragment of 1,575 bp contained, according to the sequencing data (2) (GenBank accession number M80252), the entire *norA1199*-coding region plus 18 bp upstream of it and 390 bp downstream of it. The fragment was purified from the agarose gel and was cloned via *Bam*HI linkers into the *Bam*HI site of the pBEV vector under control of the vector promoter. *B. subtilis* BD224 (*trpC2*, *thr-5*, *recE4*) was transformed with the resulting plasmid, pNOR1.

MICs of various drugs for the *B. subtilis* strains BD224/pBEV, BD224/pBMR2, and BD224/pNOR1 were determined by growing the bacteria in 96-well plates containing 1:2 or, in the experiments whose results are shown in Fig. 1, 1:1.5 serial dilutions of the drugs in 100 μl of LB medium (inoculum, 2×10^5 logarithmic-phase cells incubated at 37°C for 12 h).

For strain BD224/pBEV containing just the expression vector without any insert, MICs were the same as those of all tested drugs for strain BD224 (data not shown). Although strain BD224/pNOR1, which expresses *norA1199*, was not resistant to tetracycline or actinomycin D, it demonstrated significant resistance to a number of other drugs, including all the substrates of Bmr (Table 1). The spectra of substrate specificities of NorA and Bmr were found to be very similar, although not identical. We detected some quantitative differences in the resistances of BD224/pNOR1 and BD224/pBMR2 to norfloxacin, rhodamine 6G, and tetraphenylphosphonium (Table 1), but these differences were minor. These results indicate that in a *B. subtilis* background, NorA is not specific to fluoroquinolones and is able to protect cells from various compounds that have no apparent structural similarity. NorA1199 appears to have the same characteristics in *S. aureus*; norfloxacin-resistant isolate SA-1199B demonstrated eightfold resistance to ethidium bromide compared with that of the wild-type strain SA-1199 (data not shown).

The Bmr-conferred multidrug resistance can be reversed by the plant alkaloid reserpine, which inhibits the transporter function (3, 4). We found that the resistance conferred by *norA1199* appears to be less easily reversible by reserpine

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TABLE 1. Drug susceptibilities of the control *B. subtilis* (BD224), *bmr*-expressing (BD224/pBMR2), and *norA1199*-expressing (BD224/pNOR1) strains

Drug	MIC (μg/ml [relative resistance])		
	BD224	BD224/pBMR2	BD224/pNOR1
Norfloxacin	0.08 (1)	2.5 (32)	5 (64)
Rhodamine 6G	0.6 (1)	10 (16)	5 (8)
Ethidium bromide	3.1 (1)	100 (32)	100 (32)
Chloramphenicol	1.9 (1)	7.5 (4)	7.5 (4)
Tetraphenylphosphonium bromide	15.6 (1)	500 (32)	250 (16)
Puromycin	12.5 (1)	100 (8)	100 (8)
Acridine orange	3.1 (1)	25 (8)	25 (8)
Tetracycline	0.8 (1)	0.8 (1)	0.8 (1)
Actinomycin D	0.16 (1)	0.16 (1)	0.16 (1)

than is the resistance conferred by *bmr*. To achieve the same extent of inhibition of resistance, two to four times greater concentrations of reserpine were needed for BD224/pNOR1 than for BD224/pBMR2 (Fig. 1). Nevertheless, reserpine at a concentration of 20 μg/ml, which by itself does not affect bacterial growth, completely reversed the resistance of BD224/pNOR1 to ethidium bromide (Fig. 1) as well as to norfloxacin and tetraphenylphosphonium (data not shown).

The NorA1199 protein protected the bacteria from the multiple drugs by the same active efflux mechanism with which it reportedly protected *S. aureus* from norfloxacin. Figure 2 demonstrates the kinetics of accumulation and efflux of ethidium bromide in strains BD224/pBEV and BD224/pNOR1, which were determined as described previously (4). BD224/pNOR1 cells accumulated at least 10 times less of this dye than BD224/pBEV cells did. The uptake of rhodamine 6G by BD224/pNOR1 cells was also very low (data not shown).

The reduced accumulation of the drugs is apparently due to their active efflux from the NorA1199-expressing cells. BD224/pNOR1 bacteria effluxed ethidium bromide about eight times faster than BD224/pBEV cells did (Fig. 2). (The slow efflux of the dye from strain BD224/pBEV was apparently due to the activity of the Bmr protein expressed from

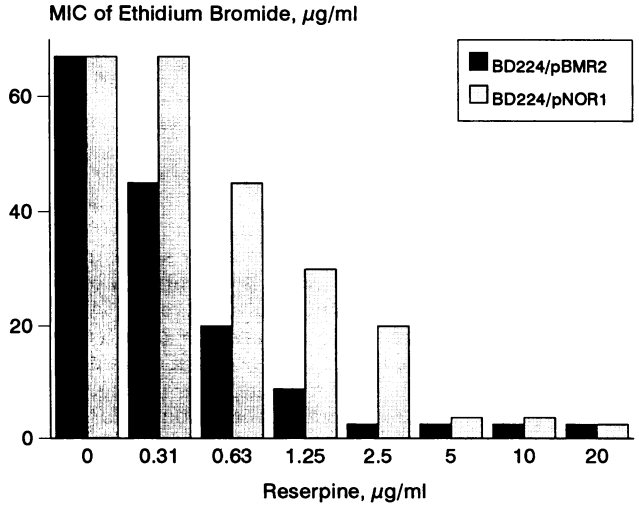

FIG. 1. Inhibition of the *bmr*- and *norA*-conferred resistances to ethidium bromide by reserpine.

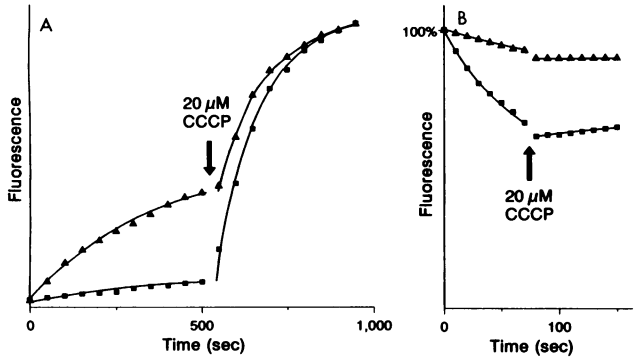

FIG. 2. Accumulation (A) and efflux (B) of ethidium bromide in strains BD224/pBEV (triangles) and BD224/pNOR1 (squares). Cell-associated dye was detected fluorimetrically as described previously (4). For the efflux measurements, bacteria were preloaded with the dye in the presence of reserpine and were then transferred to the dye-free medium.

the chromosomal copy of the *bmr* gene [4]). Addition of the membrane protonophore carbonylcyanide *m*-chlorophenylhydrazone (CCCP), which dissipates the membrane's electrochemical potential ($\Delta\mu H^+$), blocks the dye efflux and stimulates dye accumulation in both strains. In the presence of CCCP, bacteria of both types accumulate similar amounts of ethidium bromide (Fig. 2).

In conclusion, results of the present study demonstrate that the *Staphylococcus* protein NorA can provide resistance to various drugs, not just to fluoroquinolones. Like its homolog Bmr, NorA is a multidrug efflux transporter, apparently depending on $\Delta\mu H^+$ as a source of energy. NorA and Bmr are similar in their affinities to different drugs and to reserpine, which is surprising considering the 56% sequence divergence of these two proteins. Their physiological role and molecular mechanism of low substrate specificity remain to be understood.

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