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New Substrates on the Block: Clinically Relevant Resistances for EmrE and Homologues

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Transporters of the small multidrug resistance (SMR) family are small homo- or heterodimers that confer resistance to multiple toxic compounds by exchanging substrate with protons. Despite the wealth of biochemical information on EmrE, the most studied SMR member, a high-resolution three-dimensional structure is missing. To provide proteins that are more amenable to biophysical and structural studies, we identified and partially characterized SMR transporters from bacteria living under extreme conditions of temperature and radiation. Interestingly, these homologues as well as EmrE confer resistance to streptomycin and tobramycin, two aminoglycoside antibiotics widely used in clinics. These are hydrophilic and clinically important substrates of SMRs, and study of their mode of action should contribute to understanding the mechanism of transport and to combating the phenomenon of multidrug resistance. Furthermore, our study of one of the homologues, a putative heterodimer, supports the suggestion that in the SMR family, heterodimers can also function as homodimers.

The small multidrug resistance (SMR) family contains small hydrophobic proteins that reside mainly in the inner membrane of bacteria. Members of this family are practically absent from archaea, existing only in the membranes of halophilic and methanogenic archaea. These proteins extrude multiple cationic lipophilic substrates using proton gradients, thus causing resistance to a variety of toxic compounds. Since many of these compounds are routinely used as antibiotics and antiseptics, these transporters have been associated with the phenomenon of multiple drug resistance (MDR), which poses a serious problem in the treatment of infectious diseases and cancer (9, 19).

The SMR transporters function as homodimers or heterodimers. The homodimers confer multidrug resistance resulting from the expression of a single gene (meaning that the two protomers are identical). As was suggested by several studies, heterodimers confer multidrug resistance from the simultaneous expression of two genes that are located in adjacent pairs or at the most two genes apart on the chromosome (4, 11, 15, 22). The most characterized heterodimer is EbrAB from *Bacillus subtilis*. Biochemical studies show that the drug efflux pump is functional only when both components, EbrA and EbrB, are present. When each of the protomers is expressed alone, the resulting protein is inactive (15, 36). In spite of this observation, it was shown that under certain conditions EbrB is also functional when expressed alone, suggesting that its heterodimerization is not obligatory and that it can also function as a homodimer (12, 17).

The most studied member of the SMR family is EmrE, a homodimer from *Escherichia coli*, which provides a unique experimental paradigm for biochemical and biophysical studies of membrane-embedded ion-coupled transporters (1, 25–27, 33, 34). Nevertheless, there is no high-resolution three-dimensional structure of EmrE, and its low-resolution structural models are still controversial. Here, we expand the study of this family of proteins by identifying and characterizing new homologues of EmrE, one heterodimer and two homodimers. Since high stability is a prominent feature of integral membrane proteins of known atomic structure (24), the homologues were chosen from organisms living under extreme environmental conditions of heat and radiation. These proteins are expected to be more stable and therefore better suited for biophysical and structural characterization. We found that these homologues, as well as EmrE, confer resistance to two newly identified substrates, streptomycin and tobramycin, which are clinically relevant aminoglycoside antibiotics. We also found that, as for EbrAB, one of the protomers of the newly characterized heterodimeric homologue from *Deinococcus radiodurans* is not an obligatory heterodimer, since it is functional when it is individually expressed.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* strains BW25113 (5), HMS174 (DE3) (Novagen), BL21(DE3) (Novagen), and TA15 (8) were used throughout this work. BW25113 Δ*emrE* Δ*mdfA* cells were prepared as described by Datsenko and Wanner (5) and Tal and Schuldiner (30). For heat-induced overexpression and specific labeling with [^{35}S]methionine, TA15 and BW25113 Δ*emrE* Δ*mdfA* cells were transformed with the plasmid pGP1-2, which codes for the T7 polymerase under the inducible control of the λ p_L promoter (28). The plasmids used for gene expression were pT7-7 (16, 28) and pSN1 (32), a pACYC184 (3) derivative. pT7-7 confers resistance to ampicillin and carries a phage T7 RNA polymerase promoter. pSN1 confers resistance to chloramphenicol and contains a *trc* promoter. Tagged EmrE (16) (which, for the sake of simplicity, will be referred to as EmrE), MrSmr, TfSmr, and Rad1004/Rad1005 were fused at the C terminus to a Myc epitope followed by 6 His residues as described previously (16). Transfer of EmrE and cloned constructs from plasmid pT7-7 to pSN1 was performed using NdeI and ClaI restriction sites.

Construction of expression plasmids. The following genes were cloned using PCR. Mr*smr* (accession number ZP_04040476) was cloned using genomic DNA from *Meiothermus rubber* DSM 1279 as the template, and Tf*smr* (accession number YP_289048) was cloned using genomic DNA from *Thermobifida fusca* YX as the template; both strains were obtained from DSMZ GmbH (Deutsche Sammlung von Mikroorganismen

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und Zellkulturen [German Collection of Microorganisms and Cell Cultures]). *rad1004* (accession number AAF10579), *rad1005* (accession number AAF10580), and the gene pair *rad1004 rad1005* were cloned using genomic DNA from *Deinococcus radiodurans* R1 as the template (kindly provided by Shimshon Belkin, Department of Plant and Environmental Sciences, The Hebrew University of Jerusalem).

Primers corresponding to each of the sequences were designed to introduce restriction sites for the enzymes NdeI and EcoRI before and after the gene, respectively. The primers used were as follows: for Mr*smr*, 5′-GCACTACATATGAACGGATGG-3′ (sense) and 5′-GCAGTTAAGAATTCGAGGTGCAACCT-3′ (antisense); for Tf*smr*, 5′-GCACTACATATGCAGTGGCTG-3′ (sense) and 5′-GCAGTTAAGAATTCGCGGGCCGCGCC-3′ (antisense); for *rad1004*, 5′-GCTAAGCATATGAACGCATGGACAG-3′ (sense) and 5′-GCCGCTCGAGAAGCTTCAGAATTCCGCCGTCACCTTCAG-3′ (antisense); for *rad1005*, 5′-CGCGGTACCCGGGCATATGACGGCCCCGGC-3′ (sense) and 5′-GCAGTTGAATTCCCGATCCACAATC-3′ (antisense); and for the gene pair *rad1004 rad1005*, the *rad1004* sense and *rad1005* antisense primers. Each PCR product was purified, digested with the appropriate enzymes, and cloned first into the pT7-7 vector (containing or lacking the Myc His tag) predigested with NdeI and EcoRI and then into pSN1 vector predigested with NdeI and ClaI. The sequences obtained were the same as those in the GenBank database.

Resistance to toxic compounds on solid medium. *E. coli* BW25113 Δ*emrE* Δ*mdfA* cells were transformed with the indicated plasmids and were grown overnight at 37°C in LB medium with 34 μg/ml chloramphenicol (Cm). Five microliters of serial dilutions of the culture were spotted on LB-Cm plates containing 30 mM BisTris propane (pH 7.0) with 200 μM isopropyl-β-D-1-thiogalactopyranoside (IPTG) and with or without the addition of the indicated concentrations of the toxic compounds. Growth was analyzed after overnight incubation at 37°C. Each experiment was performed at least twice.

Resistance to toxic compounds in liquid medium. Overnight cultures of BW25113 Δ*emrE* Δ*mdfA* cells transformed with the indicated plasmids were diluted 100-fold into 3 ml LB-Cm medium and grown to early logarithmic phase in 1.5- by 15-cm glass tubes at 37°C. The cultures were then diluted to an A_{600} of 0.01 in LB-Cm medium containing 30 mM BisTris propane (pH 7.0), 200 μM IPTG, and the indicated concentrations of toxic compounds in a 96-well plate (Nunc) at 300 μl per well. The plate was placed in a Synergy 2 microplate reader controlled by Gen5 software (BioTek), and growth at 37°C was measured each hour for 6 h. Cell density was estimated from absorption at 600 nm. Data analysis for calculation of 50% inhibitory concentrations (IC_{50}s) was performed using Origin 8.0 software (OriginLab, Northampton, MA). The lowest r^2 value obtained in the fits was 0.99. Each experiment was performed at least twice.

Heat- or IPTG-induced overexpression and purification of His-tagged MrSmr and TfSmr. Heat-induced overexpression in *E. coli* TA15 cells was carried out essentially as described previously (7). IPTG-induced overexpression in *E. coli* BL21(DE3) or HMS174(DE3) cells was done as described previously (7), except that the growth was at 37°C, with no addition of kanamycin, and the carbon source was glucose. When the culture reached an A_{600} of 1.0, IPTG was added to 0.5 mM to induce T7 polymerase expression, and the cultures were further incubated at 37°C for 2 h.

His-tagged MrSmr and TfSmr were purified by solubilizing membranes in Na buffer (150 mM NaCl, 15 mM Tris-HCl, pH 7.5) containing 1% DDM (Glycon GmbH) for 20 min at 25°C (MrSmr membranes were also solubilized in SDS-urea buffer [2% SDS, 6 M urea, 15 mM Tris-HCl, pH 7.5]). After removal of unsolubilized material by centrifugation (4°C, 21,000 × *g*, 10 min), imidazole was added to 20 mM, and the solubilized membranes were incubated with Ni-nitrilotriacetic acid (Ni-NTA) beads for 1 h at 4°C. The unbound material was discarded, and the His-tagged protein bound to beads was washed three times with Na buffer containing 0.08% DDM and 30 mM imidazole (4°C, 1,700 × *g*, 2 min). The protein was eluted from the beads with 30 μl of sample buffer containing 450 mM imidazole. After incubation with shaking for 15 min at room temperature, the beads were sedimented by centrifugation (25°C, 3,800 × *g*, 1 min), and the eluted protein was loaded onto a 16% Tricine SDS-polyacrylamide gel and analyzed by Coomassie blue staining.

Specific labeling with [^{35}S]methionine. *E. coli* BW25113 Δ*emrE* Δ*mdfA* cells bearing the plasmids pGP1-2 and pT7-7 containing required constructs were used for protein labeling with [^{35}S]methionine essentially as previously described (29, 31).

Pulldown of Rad1004 by Rad1005. Untagged Rad1004 and Myc-His-tagged Rad1005 alone or together with untagged Rad1004 were specifically labeled with [^{35}S]methionine. Membranes were solubilized in Na buffer containing 1% DDM and were then incubated with Ni-NTA beads in the presence of 15 mM imidazole for 1 h at 4°C. Bound proteins were washed three times with Na buffer containing 0.08% DDM and 25 mM imidazole. The proteins were then eluted from the beads with sample buffer containing 450 mM imidazole, loaded onto a 16% Tricine SDS-polyacrylamide gel, and visualized with a FLA-3000 PhosphorImager (Fujifilm, Tokyo). The experiment was performed twice.

RESULTS

Identification and expression of EmrE homologues. We searched for homologues of EmrE in organisms living under extreme environmental conditions, as they are expected to be more stable and therefore better suited for biophysical and structural characterization. A BLAST search of the EmrE sequence against the whole genomes of thermophile bacteria yielded a list of 38 homologues. The closest to EmrE is MrSmr from *Meiotermus ruber*, with 42% identity and 68% similarity; another close homologue is TfSmr from *Thermobifida fusca*, which shows 38% identity and 58% similarity to EmrE (Fig. 1A). As for EmrE, a single gene encodes both, and the proteins are thus expected to function as homodimers. A screening of 174 fully sequenced bacterial genomes revealed many homologues of EmrE (23); one of them is Rad1004/Rad1005 from the radiation-resistant bacteria *Deinococcus radiodurans*. This protein pair is encoded by two consecutive genes in the bacterial genome; the first ATG codon of *rad1005* gene overlaps the termination codon of *rad1004*. Previous bioinformatics studies of the SMR family suggested that such contiguous genes encode heterodimers (23). The amino acid sequences of Rad1004 and Rad1005 are similar to each other and to that of EmrE (identities of 31% and 27% and similarities of 43% and 42%, respectively) (Fig. 1A). The positively charged residue distribution in each protomer is different, and according to the (K+R) bias analysis, the two protomers are expected to be antiparallel to each other (23) (Fig. 1B).

The genes were cloned from the bacterial genome into an expression vector, where the *rad1004* and *rad1005* genes were cloned either alone or together. The expression of MrSmr and TfSmr was tested by induction in several *E. coli* strains: HMS174(DE3) and BL21(DE3) (in both strains the induction was done using IPTG) and TA15 (induction was by heat as described previously 28). The proteins, which were fused in their C termini to a Myc epitope followed by 6 His residues, were purified on Ni-NTA beads and loaded on an SDS-polyacrylamide gel (Fig. 2A). TfSmr shows a sharp band, with a size similar to that of EmrE (~15 kDa), and its expression is similarly high in all the strains tested (about 1 mg/liter culture). MrSmr is also expressed in all strains but to a lesser extent; its band is smeared and displays an apparently higher molecular mass (Fig. 2A, shown only for strain TA15). The same profile was obtained when the MrSmr membranes were dissolved under other conditions (2% SDS–6 M urea–15 mM Tris-HCl [pH

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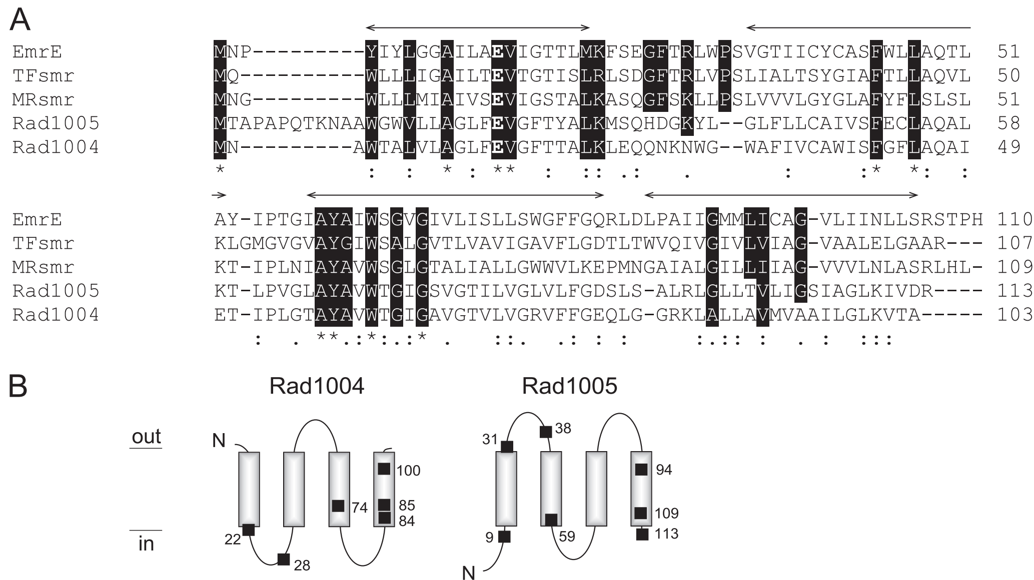

FIG 1 EmrE homologues. (A) Multiple-sequence alignment of amino acid sequences of EmrE homologues using ClustalX version 2.0 (http://www.clustal.org/clustal2/). Sequences were obtained from the National Center for Biotechnology Information protein database. Accession numbers: NP_415075, EmrE of *E. coli*; YP_289048, TfSmr of *T. fusca*; ZP_04040476, MrSmr of *M. rubber*; AAF10579, Rad1004 of *D. radiodurans*; AAF10580, Rad1005 of *D. radiodurans*. Strictly conserved residues are marked with an asterisk; conserved and semiconserved substitutions are marked by a colon and period, respectively. Signature conserved residues of the SMR family (6) are highlighted in black; the essential glutamate at position 14 (16, 35) is in bold. Arrows indicate the putative transmembrane domains of EmrE (TM1 to TM4). (B) Secondary structure model of Rad1004/Rad1005 based on the sequence alignment with EmrE and on the distribution of positively charged residues (squares).

7.5]) instead of 1% DDM Na buffer (not shown). The expression levels of the *Deinococcus radiodurans* genes were too low to be detected by Coomassie blue staining (not shown). Therefore, we specifically labeled the proteins with [^{35}S] methionine using the rifampin/T7 polymerase protocol (28). Each of the protomer lanes shows a band corresponding to the apparent mass of Rad1004 or Rad1005. The heterodimer lane shows two bands corresponding to the apparent mass of both Rad1004 and Rad1005-Myc-His (Rad1005MH) (Fig. 2B). To test whether Rad1004 and Rad1005 interact directly to form heterodimers, we solubilized membranes with DDM and isolated the heterodimers using Ni-NTA beads. Only one of the monomers (Rad1005) is tagged and therefore capable of binding to the beads. Indeed, when detergent-solubilized Rad1004 was incubated with the beads, it did not bind as shown by the fact that no radioactivity remained on the beads after washing (Fig. 2C, left lane), whereas Rad1005 bound quantitatively (Fig. 2C, right lane). Notably, the heterodimer bound to the beads (Fig. 2C, center lane) even though only Rad1005 was tagged and practically undetectable in the original membranes (Fig. 2B, lane 4, top arrow). In addition to each of the monomers a band corresponding to the undissociated dimer was detected (Fig. 2C, center lane, dashed arrow).

EmrE homologues confer resistance to EmrE substrates. The ability of the homologue proteins to confer resistance to the classical substrates of EmrE was tested by phenotype assay (Fig. 3A). MrSmr and TfSmr confer robust and similar resistance to acriflavine and methyl viologen that is similar to that conferred by EmrE. MrSmr shows high resistance to ethidium, to the same extent as EmrE, whereas TfSmr shows lower resistance, but still higher than the negative control (Fig. 3A). On the other hand, Rad1004/Rad1005 did not confer significant resistance, neither when the protomers were expressed alone nor when they were coexpressed (not shown).

EmrE homologues confer resistance to streptomycin and tobramycin. Since the Rad1004/Rad1005 heterodimer belongs to the multidrug transporter family, we decided to screen for other potential substrates. For that, we performed a disk assay, which allows fast screening of a number of compounds (see Table S1 in the supplemental material). The results suggested that streptomycin and tobramycin, two aminoglycoside antibiotics routinely used in the clinic, are good candidates for further exploration.

Following these results, we tested the resistance to these two antibiotics in a solid phenotype assay. Rad1005 confers high resistance to both of them, while the resistance conferred by Rad1004 and the heterodimer Rad1004/Rad1005 is very weak (Fig. 3B, bottom panel). MrSmr and TfSmr confer robust resistance, where TfSmr shows a slightly higher resistance (Fig. 3B, top panel). Robust resistance was also shown by another homologue of EmrE that was already characterized in our lab (21), BpSmr from *Bordetella pertussis* (see Fig. S1 in the supplemental material).

In the case of Rad1004/Rad1005, IC_{50}s were also determined after growing cells in liquid medium. The values for Rad1004 and the heterodimer Rad1004/Rad1005 were very similar to that for the negative control. However, the values for Rad1005 were ~1.5-fold (streptomycin) and ~1.7-fold (tobramycin) higher than that for the negative control (data not shown).

DISCUSSION

Microorganisms have developed various ways to resist the toxic effects of antibiotics and other drugs (10, 18), one of which is extrusion of drugs by multidrug transporters (13, 20). The SMR family contains small hydrophobic proteins that reside mainly in

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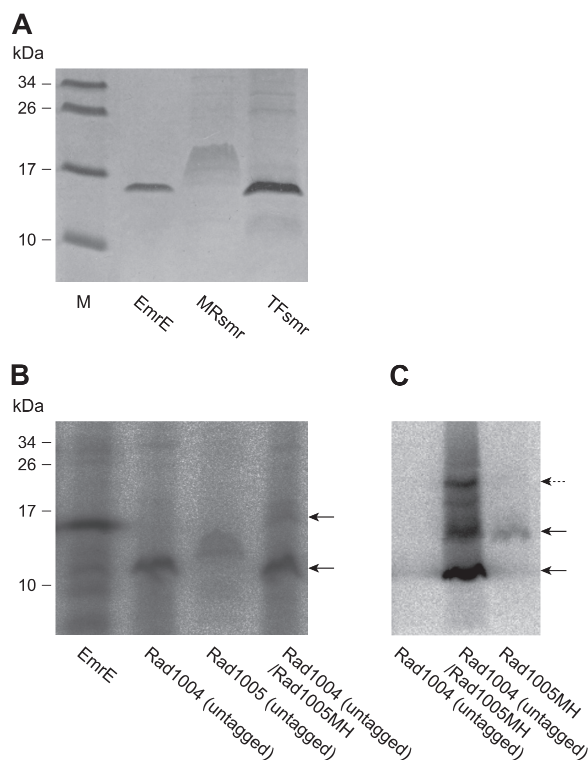

FIG 2 Expression profiles of homologue proteins. (A) Expression of MrSmr and TfSmr. Coomassie blue staining (with, for simplicity, only results for TA15) is shown. The same profile was obtained with the two other strains. M, marker. (B) Expression of Rad1004/Rad1005. Membranes bearing the indicated ^{35}S-radiolabeled proteins were prepared as described in Materials and Methods (the Rad1005 in the heterodimer [lane 4] was tagged for better resolution between the bands of Rad1004 [lane 2] and Rad1005 [lane 3], as they are of similar molecular mass). Both proteins are expressed in the heterodimer sample (arrows, lane 4). The tagged Rad1005 has a higher molecular mass than the untagged one, close to the expected molecular mass of EmrE (~15 kDa). (C) Pulldown of the untagged Rad1004 (lower arrow) by Myc-His-tagged Rad1005 (upper arrow). Detergent-solubilized ^{35}S-radiolabeled proteins were incubated with Ni-NTA beads. After the elution, untagged Rad1004 is detected only in the presence of Rad1005, demonstrating direct interaction. The amount of radioactivity loaded in the center lane is four times higher than that in the right one. The upper band (dashed arrow) in the center lane corresponds to the undissociated dimer.

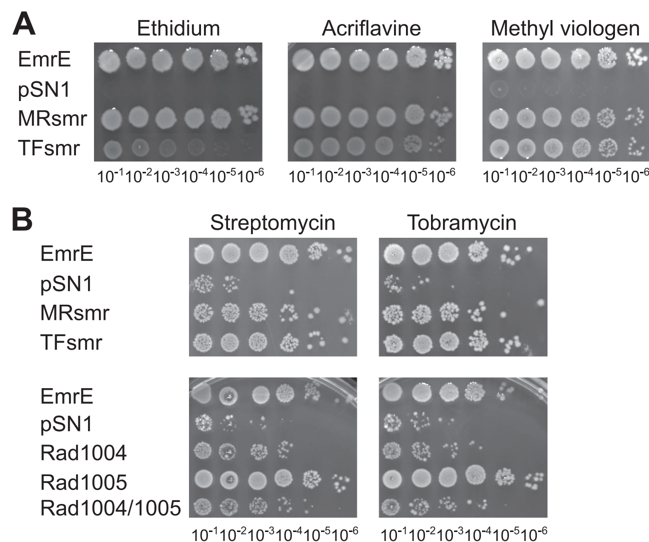

FIG 3 EmrE homologues confer resistance to drugs. (A) MrSmr and TfSmr confer resistance to substrates of EmrE. *E. coli* BW25113 Δ*emrE* Δ*mdfA* cells were transformed with the indicated constructs, and the experiment was conducted as described in Materials and Methods. Drug concentrations are 100 μg/ml ethidium bromide, 25 μg/ml acriflavine, or 0.3 mM methyl viologen. In control plates with no toxins, growth of all strains was similar (not shown). (B) EmrE homologues confer resistance to streptomycin and tobramycin. Details are as for panel A, except with the addition of 4.5 (top panels) or 2.5 (bottom panels) μg/ml streptomycin or 1.0 (top panels) or 0.55 (bottom panels) μg/ml tobramycin.

the inner membrane of bacteria and extrude multiple cationic lipophilic substrates using proton gradients. Some of these transporters function as homodimers, while others were suggested to function as heterodimers. The homodimers confer multidrug resistance from the expression of a single gene, whereas the heterodimers confer multidrug resistance from the simultaneous expression of two genes that are located in adjacent pairs or at most two genes apart on the chromosome. The most characterized protein of the SMR family is EmrE, a homodimer from *E. coli* that provides a unique experimental paradigm for biochemical and biophysical studies of membrane-embedded ion-coupled transporters (1, 25–27, 33, 34). Despite the wealth of biochemical studies, there is still very little structural information on EmrE. Proteins of organisms living under extreme environmental conditions are expected to be more stable and therefore better suited for biophysical and structural characterization. In this study, we identified and characterized three such homologues of EmrE: MrSmr and TfSmr, both putative homodimers from bacteria living under high-temperature conditions, and Rad1004/Rad1005, a putative heterodimer from bacteria living under conditions of high radiation. We found that the resistance profiles of MrSmr and TfSmr are similar to that of EmrE, since both proteins show some resistance to the classical substrates of EmrE, ethidium bromide, acriflavine, and methyl viologen. Furthermore, screening for resistance to other substrates revealed two additional drugs, streptomycin and tobramycin. MrSmr, TfSmr, BpSmr, and EmrE show high resistance to these antibiotics. These findings are new, since until now these two antibiotics were not known as substrates of EmrE. Rad1004/Rad1005 shows only feeble resistance to streptomycin and tobramycin. It is likely that this is because the heterodimer has an advantage in conferring resistance to other drugs that have not yet been identified. On the other hand, when Rad1005 is expressed alone, it confers high resistance to the above-mentioned antibiotics. This phenomenon of a heterodimer that can also function as a homodimer seems to be a general feature of the SMR family, as it was already demonstrated for EbrAB, a heterodimer from *B. subtilis* (12, 17). Previously, we showed that under certain conditions, EbrB is also functional when it is expressed alone, conferring resistance to ethidium bromide and acriflavine (17). The findings described here suggest that when Rad1004 and Rad1005 are coexpressed, they interact with high affinity. This heterodimerization is not obligatory, since in the absence of Rad1004, Rad1005 can also interact with itself (with a lower affinity), creating a functional homodimer.

A phylogenetic analysis exploring the evolutionary relationships of the SMR family members suggests that the heterodimers have emerged recently due to gene duplication of a homodimer homologue (2). This contention was already described in a model which suggests that the evolutionary challenge of recognition and

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transport of a wide spectrum of substrates might have selected for SMR heterodimers that originated from gene duplication of the more ancient homodimers. After gene duplication, a few mutations would enable the monomers within the heterodimer to assume either a parallel or an antiparallel orientation. In this manner, one protein with an only slightly modified sequence might extend the range of the substrate specificity (25). The findings that EbrB and Rad1005 are functional also as homodimers support the possibility that each of them is the ancient homodimer that underwent duplication and mutation and thereby gave rise to the second protomer in the pair, EbrA or Rad1004, respectively.

Tobramycin and streptomycin are both aminoglycoside antibiotics which are used in clinics. Until now, streptomycin was shown as a substrate only of the heterodimer YkkC/YkkD from *B. subtilis* (11), and some other aminoglycoside antibiotics were shown as substrates for another homologue of EmrE, from *Pseudomonas aeruginosa* (14). Our findings reveal a further substrate of this group, tobramycin, and show that aminoglycoside antibiotics are common substrates of the SMR family members. This is a highly significant addition for understanding of the mechanism of action of EmrE, since until now most (if not all) of its known substrates have been aromatic. Knowledge of the molecular mechanisms underlying microbial antibiotic resistance can help us fight the increasing numbers of drug-resistant bacteria more successfully.

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