Crystal Structures of QacR-Diamidine Complexes Reveal Additional Multidrug-binding Modes and a Novel Mechanism of Drug Charge Neutralization*

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The disturbing increase in multidrug-resistant bacteria has become an important and growing menace to human and animal health (1, 2). In bacteria, multidrug efflux transporters protect these organisms from myriad clinically relevant anti-

microbials by their ability to pump these compounds from the cell and thereby preventing their accumulation to toxic levels (1, 3–5). More specifically, antibiotic resistant strains of the Gram-positive bacterium Staphylococcus aureus have become a very serious health threat in the hospital environment (6). Some of these resistant strains harbor plasmid-encoded multidrug-resistant efflux pumps such as QacA, which confers resistance to quaternary ammonium compounds (QACs)³ (7–9). QACs comprise a broad spectrum of mono- and bivalent cationic lipophilic antimicrobials, antiseptics, and disinfectants.

The transcription of the qacA gene is controlled by the repressor QacR (10). QacR is a dimeric 188-residue (23-kDa) member of the TetR/CamR transcription regulator family (11). The signature elements of the TetR/CamR family are a homologous three helix N-terminal DNA-binding domain and diverse C-terminal domains that are utilized in binding protein-specific inducing compounds. Most TetR/CamR family members bind as dimers to ∼15-bp operator sites. However, QacR binds to an unusually long site in the qacA promoter (IR1, consisting of 28 bp) that overlaps the qacA transcription start site (10). QacR binds to IR1 as a dimer of dimers and likely functions as a repressor by preventing the RNA polymerase-promoter complex from entering a productive transcribing state rather than blocking RNA polymerase binding (10, 12, 13).

QacR is induced by multiple structurally dissimilar lipophilic monovalent and bivalent cationic compounds, which also serve as substrates of the QacA efflux pump. Upon binding one of these compounds in a one drug/QacR dimer stoichiometry, a coil-to-helix transition involving residues Thr-89 through Tyr-93 is triggered in the drug-bound subunit, resulting in the expulsion of residues Tyr-92 and Tyr-93 from the interior of the protein and the formation of the drug-binding pocket (Fig. 1) (14). This key conformational change brings about the elongation of helix α5 by one turn, which in turn causes the translational helix α6 toward the DNA-binding domain. Helix α6 engages in multiple interactions with helix α1 of the DNA-binding domain, and its movement results in the translation and rotation of the DNA-binding domain of the drug-bound subunit by 9.1 Å and 36.7°, respectively (14). The drug-free subunit undergoes a smaller translation (3.9 Å) and rotation (18.3°). The end result of these conformational changes is an increase in the center-to-center distance of the DNA recognition helices by over 9 Å, resulting in a structure that can no longer bind the IR1 site, thus allowing the qacA gene to be transcribed.

The drug-binding pocket of QacR is quite extensive and displays a volume of 1100 Å³ (14). This pocket has been de-

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The drug-binding pocket of QacR is quite extensive and displays a volume of 1100 Å³ (14). This pocket has been de-
scribed as having two independent but overlapping drug-binding sites that are designated the rhodamine 6G (R6G) and ethidium (Et) sites after their respectively bound drugs (14). A key feature of the binding pocket is the presence of several glutamates and a large number of aromatic residues. Combinations of these residues are used to define the specific drug-binding sites and provide maximal shape, chemical, and electrostatic complementarity (14). Hallmark residues of the R6G pocket include stacking residues Trp-61, Tyr-93, and Tyr-123. The carboxylate of residue Glu-90 appears to be crucial for charge complementation of the positively charged ethyl ammonium group of R6G, and residues Thr-89 and Gln-96 make other nondiscriminating contacts to polar groups of the drug. The key aromatic residues of the Et pocket are Tyr-103 and Tyr-123, and Phe-162 (from the other subunit). The positive charge of ethidium is complemented by the carboxylate of residue Glu-120. Residues Ile-199, Ile-100, and Gln-96 are also part of the Et-binding pocket. Thus, residues Tyr-123 and Gln-96 belong to both drug-binding pockets. The structures of QacR bound to malachite green and crystal violet revealed these monovalent cationic drugs bound between the RG6 and the Et pockets (contacting residues of each site), whereas berberine was bound in the R6G site but used the carboxylates of residues Glu-57 and Glu-58 to neutralize its positive charge (14). Dequalinium, which contains two positively charged aromatic quinolinium rings that are linked by a flexible 10-methylene carbon chain (Fig. 2A), was found to span both pockets. As observed for berberine, dequalinium does not use the Glu-90 residue of the R6G pocket to complement the quinolinium ring; rather residues Glu-57 and Glu-58 are involved. However, res-

**Fig. 1. Structure of a QacR-drug complex.** A ribbon diagram of QacR bound to pentamidine. The drug-bound subunit is colored aquamarine, and its secondary structural elements are labeled in black. The drug-free subunit is colored magenta. The pentamidine is displayed as a stick model and colored according to atom type with carbon (white), nitrogen (blue), and oxygen (red). The N and C termini are labeled.

**Fig. 2. Bivalent cationic ligands/drugs that bind QacR.** A, chemical structures of two classes of bivalent cationic drugs that are QacR inducers. Positively charged nitrogens and diamidine groups are marked appropriately with a plus (†). B, stereoview of the \( F_o - F_C \) difference electron density of hexamidine contoured at 3.0σ. The difference density is shown as a stick model and colored according to atom type with carbon (white), nitrogen (blue), and oxygen (red). The surrounding secondary structural elements are shown as light blue ribbons. C, stereoview of the \( F_o - F_C \) difference electron density of pentamidine contoured at 4.5σ. The difference density is shown as yellow mesh, and the structure of pentamidine is shown as a stick model and colored according to atom type with carbon (white), nitrogen (blue), and oxygen (red). The surrounding secondary structural elements are shown as light blue ribbons.
idues Trp-61 and Tyr-93 are still used in stacking interactions.

Whether other bivalent cationic compounds of similar size, shape, and flexibility utilize both, one, or neither subsite is unknown. Pentamidine and hexamidine, which feature these chemical characteristics, have been shown to be inducers of QacR in vivo as well as substrates of QacR in vivo and thus were selected for structural studies to address this issue (7).

The structures of these QacR-drug complexes revealed that pentamidine and hexamidine, which differ by only a single methylene carbon in their linkers, evoked the identical col-to-helix transition that is necessary for induction yet have very different binding modes. Although the hexamidine binding was different from the pentamidine binding, the former (longer) diamidine bound to QacR in a manner similar to dequalinium. Pentamidine, on the other hand, defines novel QacR drug-binding determinants, and the structure of the complex revealed that a formal negatively charged residue was not necessary to complement the positive charge of one of the benzamidine moieties. Thus, these structures reinforce and expand upon the idea of the plastic multidrug-binding pocket with a number of shared subsites that accommodate a large number of chemically and structurally dissimilar compounds.

**EXPERIMENTAL PROCEDURES**

**Preparation and Crystallization of the QacR-Drug Complexes—** A recombinant plasmid containing the qacR gene, in which both native cysteines are mutated (C72A, C141S) and a C-terminal hexahistidine tag is added, was used in this work (10, 12). The protein was overexpressed in the *E. coli* strain DH5α and purified from cell lysates by the use of nickel-nitrilotriacetic acid-agarose affinity resin – solution and reservoir buffer (2.95 M sodium acetate, pH 4.6). The protein-drug solution was subjected to reductive alkylation of the lysines, which allows the lysates by the use of nickel-nitrilotriacetic acid-agarose affinity resin – solution and reservoir buffer (2.95

**Data analysis**

| Parameter | QacR-Hexamidine | QacR-Pentamidine |
|-----------|-----------------|-----------------|
| Space group | P4$_2_2_2$ | P4$_2_2_2$ |
| Cell constants (Å) | $a = b = 171.8$ | $a = b = 172.1$ |
| Resolution (Å) | 48.98–2.90 | 86.03–2.62 |
| Completeness (%) | 98.3 | 96.0 |
| Overall R$_{work}$ (%) | 5.3 | 5.1 |
| Multiplicity | 3.7 | 3.7 |
| Overall I/σ(I) | 7.6 | 9.1 |
| Total no. of reflections | 127,316 | 190,973 |
| No. of unique reflections | 31,376 | 41,350 |
| High resolution shell (Å) | 3.98–2.90 | 2.78–2.62 |
| $R_a$ (%) | 40.5 | 30.9 |
| Electron density maps for guidance and the software package O (20). Water and sulfate molecules were added to each structure using the overlapping electron density criteria: $F_a - F_c > 3.0$ and $2F_c - F_a > 1.0$. The final model was verified by inspection of the simulated annealing composite omit maps and $F_a - F_c$ difference electron density (Fig. 2, B and C). The final R$_{work}$ and R$_{free}$ are listed in Table I for each QacR-drug complex. Ramachandran plot analysis was performed using the RAMPAGE server and revealed excellent stereochemistry (Table I) (21). The coordinates and structure factors have been deposited in the Protein Data Bank under accession codes 1RKW (QacR-pentamidine) and 1RPW (QacR-hexamidine).

**RESULTS**

**Structure of the QacR-Hexamidine Complex—** The structure of the QacR-hexamidine complex was determined by difference Fourier methods and refined to 2.90 Å resolution. The final R$_{work}$ was 22.9%, and R$_{free}$ was 28.0%. The structure revealed that hexamidine binds to QacR in a manner similar to dequalinium in that the drug was fully elongated, thus allowing the benzamidine groups to reach into the R6G and Et drug-binding pockets (Fig. 3). This may not appear too surprising given the similar end-to-end lengths of dequalinium (19.0 Å) and hexamidine (20.6 Å). However, the details of their binding modes are different.

As seen in the QacR-dequalinium structure, the positively charged ends of hexamidine were neutralized by the carboxylates of residues Glu-57 of the R6G pocket and Glu-120 of the Et pocket (Fig. 3). However, unlike dequalinium, the binding residue Glu-58 was not involved, as its carboxylate pointed away from the drug. Multiple QacR-hexamidine stacking interactions were found and involved R6G-binding pocket residues Trp-61 and Tyr-93 at one end of the symmetric hexamidine molecule and Et-binding pocket residues Tyr-123 and Phe-162 (from the other subunit) at the other end of the drug (Table II). The aliphatic side chain of Ile-124 was also within van der Waals distance of the aliphatic portion of hexamidine nearest

**Table I. Selected crystallographic data**

| Parameter | QacR-Hexamidine | QacR-Pentamidine |
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Pentamidine, on the other hand, defines novel QacR drug-binding determinants, and the structure of the complex revealed that a formal negatively charged residue was not necessary to complement the positive charge of one of the benzamidine moieties. Thus, these structures reinforce and expand upon the idea of the plastic multidrug-binding pocket with a number of shared subsites that accommodate a large number of chemically and structurally dissimilar compounds.
Novel Mechanism of QacR Drug Binding and Drug Neutralization

![Stereoview of the hexamidine-QacR-binding pocket](image)

**Fig. 3. Stereoview of the hexamidine-QacR-binding pocket.** For clarity, the side chains of only selected residues are shown (not shown are residues Asn-154 and Asn-157 from one subunit and Gln-165’ and Gln-166’ from the drug-free subunit) as sticks, whereby oxygen atoms are red, nitrogens are blue, carbons are gray, and the backbone of the drug-bound subunit is aquamarine with germane helices labeled in blue. Residue Phe-162’ from the drug-free subunit is labeled in green. Solvent molecules within the hydrogen-bonding distance of the protein or drug are shown as red spheres. W1 is 2.7 Å from the other oxygen of the hexamidine and 3.4 Å from the backbone amide of residue Asn-157; W2 is 3.3 Å from W3 and within 3.5 Å of the Asn-157 side chain; W4 is 2.6 Å from an amidinic nitrogen and 3.7 Å from W5; W5 is hydrogen-bonded to residue Glu-165’ (2.6 Å) and residue Asn-117 (2.7 Å). Also labeled are the R6G- and Et-binding pockets. This figure and figures 4–6 were created with Swiss-PdbViewer (33) and rendered with POVRAY (Persistence of Vision Raytracer, version 3.1, www.povray.org).

| QacR residue | Distance | QacR residue | Distance |
|-------------|----------|-------------|----------|
| Trp-61      | 3.9 Å    | Lys-60 (O)  | 4.8 Å    |
| Tyr-82      | 3.2 Å    | Glu-63      | 4.5 Å    |
| Ser-86      | 3.6 Å    | Gln-64      | 3.8 Å    |
| Gln-96      | 5.0 Å    | Tyr-93      | 3.8 Å    |
| Tyr-123     | 4.7 Å    | Water       | 3.9 Å    |
| Tyr-127     | 2.8 Å    | Tyr-162 (O) | 4.1 Å    |
| Ala-153 (O)| 3.0 Å    | Tyr-162     | 4.9 Å    |
| Asn-157     | 3.6 Å    |              |          |

*Electron density of the diamidine group at this site is weak, and the contact distances are best estimates.

* (O) designates contacts involving the polypeptide carbonyl oxygen atom.

"Residues from the drug-free subunit of the QacR dimer.

Table II

| QacR-drug contacts |
|--------------------|
| **Pentamidine Site 1** | **Pentamidine Site 2** | **Hexamidine Et site** | **Hexamidine R6G site** |
| QacR residue | Distance | QacR residue | Distance | QacR residue | Distance | QacR residue | Distance |
|-------------|----------|-------------|----------|-------------|----------|-------------|----------|
| Trp-61      | 3.9 Å    | Lys-60 (O)  | 4.8 Å    | Asn-117     | 4.5 Å    | Glu-57      | 4.2 Å    |
| Tyr-82      | 3.2 Å    | Glu-63      | 4.5 Å    | Glu-120     | 2.6 Å    | Trp-61      | 3.6 Å    |
| Ser-86      | 3.6 Å    | Gln-64      | 3.8 Å    | Tyr-123     | 4.4 Å    | Gln-64      | 3.2 Å    |
| Gln-96      | 5.0 Å    | Tyr-93      | 3.8 Å    | Ile-124     | 3.3 Å    | Tyr-93      | 3.8 Å    |
| Tyr-123     | 4.7 Å    | Water       | 3.9 Å    | Asn-154     | 3.1 Å    |             |          |
| Tyr-127     | 2.8 Å    | Tyr-162 (O) | 4.1 Å    | Asn-157     | 4.1 Å    |             |          |
| Ala-153 (O)| 3.0 Å    | Tyr-162     | 4.9 Å    |             |          |             |          |
| Asn-157     | 3.6 Å    |              |          |             |          |             |          |

The R6G pocket. Multiple polar contacts were observed between QacR and the drug. Within the R6G pocket, Gln-64 contacted one end of the drug while in the Et pocket Asn-117, Asn-154, Asn-157 and the backbone carbonyl oxygen of Phe-162’ contacted the other benzamidine moiety (Table II). In addition to these direct contacts, several water molecules were found in the drug-binding pocket that served in water-mediated protein-drug interactions. Water molecule W1 linked one of the hexamidine ether oxygens to the amide nitrogen of Asn-157. W4 contacted the nitrogen of the amidine group outside the Et pocket and connected it to Asn-154 and water molecule W5, which in turn formed hydrogen bonds to residues Asn-117, Glu-165’, and Gln-166’.

Structure of the QacR-Pentamidine Complex—The structure of the QacR-pentamidine complex, also determined by difference Fourier methods, was refined to 2.62 Å resolution resulting in an Rwork of 22.3% and an Rfree of 27.7%. The crystal structure of the QacR-pentamidine complex revealed that pentamidine, which differs from hexamidine by only a single methylene group, bound very differently to QacR when compared with hexamidine and dequalinium (Figs. 4–6). Unlike hexamidine and dequalinium, the pentamidine molecule was significantly twisted about its central linker, and as a result, the end-to-end length of the molecule was only 17.1 Å. In this conformation, pentamidine did not span the drug-binding pocket but rather bent into the core of the protein. In doing so, a novel drug-binding pocket was created (Table II). At one end of the pentamidine-binding site (designated as Site 2), the benzamidine moiety stacked with Tyr-93, the aromatic residue, which in part defined the R6G pocket (14). However, none of the other stacking residues of the canonical R6G pocket interacted with this end of the pentamidine molecule. Moreover, acidic residue Glu-63 (and not Glu57, Glu58, or Glu90) interacted with the positively charged benzamidine moiety. This was the first time that residue Glu-63 had been observed to play a primary role (i.e. that of charge neutralization) in drug binding. In addition, the side chain carbonyl oxygen of residue Gln-64 was also involved in binding.

Perhaps, the more striking aspect of the pentamidine binding was found at the other end of the molecule at binding Site 1 (Figs. 4 and 5). Although fully buried, there were no glutamates or aspartates within 6.0 Å of this positively charged benzamidine group. Rather, the positive charge of the drug was complemented by interactions with a number of nearby oxygen...
FIG. 4. **Stereoview of the pentamidine-QacR-binding pocket.** For clarity, the side chains of only selected residues are shown (not shown are residues Leu-54, Lys-60, and Gln-96) as sticks, whereby oxygen atoms are red, nitrogens are blue, carbons are gray, and the backbone of the drug-bound subunit is aquamarine with germane helices labeled in blue. Solvent molecules within the hydrogen-bonding distance of the protein or drug are shown as red spheres. The hydrogen-bonding network between W1, W2, and QacR is depicted with black dashed lines. W1 is 3.1 Å from the hydroxyl oxygen of residue Tyr-93, 3.4 Å from the hydroxyl of Tyr-123, and 2.8 Å from W2; W2 is hydrogen-bonded to the hydroxyl oxygen of residue Y123 (2.5 Å) and the backbone carbonyl oxygen of residue L54 (2.9 Å); W3 engages in a weak hydrogen bond with residue Asn-157 (3.6 Å), and van der Waals contacts with the pentamidine phenyl ring (closest approach 3.4 Å). The binding pocket subsites, Site 1 and Site 2, are labeled.

FIG. 5. **Close up stereoview of the Site 1 drug-binding site of pentamidine.** Interaction distances are given in angstrom. Hydrogen bonds are depicted by red dashed lines, van der Waals contacts are depicted by a black dashed line, a π-cation interaction is depicted by a green dashed line, and stacking interactions (closest contact) are depicted by purple dashed lines. Note that no negatively charged side chains are found within 6 Å of either of the amidinic nitrogens.

FIG. 6. **Stereoview of the superimposition of the three QacR-bivalent cationic drug complex structures.** Pentamidine is shown as yellow sticks, hexamidine is shown as green sticks, and dequalinium is shown as red sticks. The polypeptide backbone is shown as aquamarine ribbons with germane helices labeled in black. Subsites of the pentamidine-binding pocket, Site 1 and Site 2, and the R6G and Et subsites of the hexamidine- and dequalinium-binding pockets are labeled.
atoms. The closest drug-protein contacts were made with the backbone carboxyl oxygen of residue Ala-153 and the side chain hydroxyl oxygen atom of residue Tyr-127, both of which engaged in dipole-charge interactions with the amionic nitrogens. The side chain carboxyl oxygen of Asn-157 and the side chain hydroxyl oxygen of Ser-86 were also within proximity of the benzenoid moiety and contributed to the overall negative electrostatic character of Site 1 (Table II, Fig. 5). An energetically favorable cation stacking interaction was made between the aromatic side chain of residue Tyr-82 and one side of the benzamidine group (22, 23). Additional stacking interactions took place between the aromatic side chains of residues Trp-61 and Tyr-23 and one side of the benzamidine phenyl group. Interestingly, residue Trp-61 is one of the key residues that defines the R6G-binding pocket, whereas residue Tyr-123 contributes to the R6G and Et pockets (14).

As observed in all QacR-drug complexes, several solvent molecules were also found in the pentamidine-binding pocket but only near Site 1 (Fig. 4). Two of these water molecules contributed to a protein-water-drug interaction network whereby residue Tyr-93 of Site 2 is linked to Site 1 residue Tyr-123 by W1 (Fig. 4). Further, W1 hydrogen bound to W2, which also hydrogen bonded to the hydroxyl group of residue Tyr-123 and the backbone carboxyl oxygen atom of residue Leu-54. Thus, the solvent found near Site 1 serves to buttress the positions of key drug-binding side chains but clearly do not play a role in ligand specificity.

**DISCUSSION**

The multidrug-binding transcription repressor QacR has an exceptional ability to bind to a broad spectrum of mono- and bivalent cationic lipophilic compounds, which are also substrates of the QacA multidrug transporter (which QacR regulates) (7–9, 14). This ability is articulated through the multifaceted binding pocket of QacR, which is characterized by the presence of multiple glutamates, myriad aromatic residues, and several polar residues. Aromatic residues are expected to play a key role in drug binding in all multidrug-binding proteins, given their ability to stack with aromatic drugs and be involved in electrostatic interactions with charged drugs (22–25). Indeed, the crystal structure of the transcription activator, BmrR, with an aromatic monovalent cation (26, 27) and the recent moderate-resolution crystal structures of four drugs bound to the bacterial multidrug transporter AcrB, also showed the importance of such interactions (28, 29). The structures of the odorant-binding protein, a polyspecific multiligand-binding protein, in complex with multiple small nonpolar and aromatic compounds also demonstrate a central role for aromatic residues in ligand binding (30, 31).

The structures of the QacR-hexamidine and QacR-pentamidine complexes were only the second and third examples of bivalent cationic drug binding to this multidrug-binding protein. Hexamidine, similar to dequalinium, maintained an elongated conformation and thus readily spanned the R6G- and Et-binding pockets of QacR (Figs. 3 and 6). Similar to dequalinium, the two positive charges of the individual benzenoid groups were neutralized by the carboxylates of residues Glu-57 and Glu-120. However, there were differences in their binding mechanisms. Whereas the positions of the quinolinium ring of dequalinium and the benzamidine group of hexamidine in the R6G subsite were very similar, their locations in the Et-binding pocket were quite different, whereby the aromatic benzenoid group was found closer to helix α7 and about a turn of helix nearer the carboxyl terminus of that helix (Fig. 6). Despite their somewhat different locations, many of the protein-drug contacts were the same, such that the carboxylate of residue Glu-120 served to neutralize the positive charge of each drug, and the phenyl ring of Phe-162’ stacked with their aromatic rings. Regardless, the somewhat different binding modes of two compounds of similar end-to-end length and identical charge highlighted the plasticity of the multidrug-binding pocket of QacR.

The plasticity and promiscuity of the multidrug-binding pocket were further underscored by the structure of the QacR-pentamidine complex, in which an entirely novel manner of drug binding was observed (Figs. 4–6). Pentamidine was the first example of a drug that utilized neither the R6G nor Et subsites to bind QacR. Indeed the positive charge on the aromatic benzenoid group that was closest to the R6G site, which was designated Site 2 for pentamidine, was then neutralized by the carboxylate side chain of residue Glu-63. This marked the first time this acidic residue had carried out this function and added yet another QacR glutamate residue that was involved in drug binding, with the others being Glu-57, Glu-58, Glu-90, and Glu-120. More striking was the finding that the positive charge of the other aromatic benzenoid moiety, which was bound in Site 1, did not interact with the carboxylate of any acidic residue (Fig. 5). Rather, electrostatic neutralization was accomplished by drug interactions with the negative dipoles of several oxygen atoms from nearby side chains and the peptide backbone. A similar charge neutralization mechanism is readily envisioned for other multidrug-binding proteins including the transporters. For example, the structures of the AcrB-drug complexes, with the exception of AcrB-dequalinium, do not reveal any acidic residues within 6 Å of the bound positively charged cytotoxins, and hence their charge neutralization/complementarity is unclear (28). Perhaps several side chain or peptide backbone oxygens are proximal to the charge and thus carry out this function. That tyrosinyl hydroxyl oxygens can “neutralize” a buried charge is seen in the crystal structure of the multidrug-binding domain of BmrR (26). Moreover, peptide backbone carboxyl oxygens are particularly well suited to complement a formal positive charge as exemplified by the mechanism of potassium binding by the potassium channel (32).

In conclusion, the ability of QacR to bind two very similar aromatic diamidines in quite different ways underscored the plasticity of its multidrug-binding pocket and very likely those of other multidrug-binding proteins. Moreover, neutralization of the positive charge(s) of cationic drugs did not require the presence of proximal acidic residues but was affected by the electronegative dipoles of nearby oxygens. Thus, another layer of complexity is added to the binding pockets of multidrug-binding proteins, and any de novo structure-based efforts to design high affinity inhibitors of multidrug-binding proteins become yet more difficult.

**REFERENCES**

1. Levy, S. B. (2001) *Clin. Infect. Dis.* 33, Suppl. 3, S124–S129

2. Casewell, M., Friis, C., Marco, E., McMullin, P., and Phillips, I. (2003) *J. Antimicrob. Chemother.* 51, 159–161

3. Levy, S. B. (1992) *Antimicrob. Agents Chemother.* 36, 695–703

4. Saier, M. H., Jr., Paulsen, I. T., Slwinski, M. K., Pao, S. S., Skurray, R. A., and Nakaio, H. (1996) *FASEB J.* 12, 265–274

5. McKeegan, R. S., Borges-Walmsley, M. I., and Walmsley, A. R. (2003) *Trends Microbiol.* 11, 21–29

6. Hiratsuto, K., Okuma, K., Ma, X. X., Yamamoto, M., Hori, S., and Kapi, M. (2005) *Curr. Opin. Infect. Dis.* 15, 407–413

7. Mitchell, B. A., Brown, M. H., and Skurray, R. A. (1996) *Antimicrob. Agents Chemother.* 42, 475–477

8. Brown, M. H., and Skurray, R. A. (2001) *J. Mol. Microbiol. Biotechnol.* 3, 163–170

9. Littlejohn, T. G., Paulsen, I. T., Gillespie, M. T., Tennent, J. M., Midgley, M., Jones, I. G., Purewal, A. S., and Skurray, R. A. (1992) *FEMS Microbiol. Lett.* 74, 259–265

10. Gerkovics, S., Brown, M. H., Roberts, N. J., Paulsen, I. T., and Skurray, R. A. (1996) *J. Biol. Chem.* 271, 18665–18673

11. Aramaki, H., Yagi, N., and Suzuki, M. (1995) *Protein Eng.* 8, 1259–1266

12. Gerkovics, S., Brown, M. H., Schumacher, M. A., Brennan, R. G., and Skurray, R. A. (2001) *J. Bacteriol.* 183, 7102–7108
1. Schumacher, M. A., Miller, M. C., Grkovic, S., Brown, M. H., Skurray, R. A., and Brennan, R. G. (2002) *EMBO J.* 21, 1210–1218
2. Schumacher, M. A., Miller, M. C., Grkovic, S., Brown, M. H., Skurray, R. A., and Brennan, R. G. (2001) *Science* 294, 2158–2163
3. Raymont, I. (1997) *Methods Enzymol.* 276, 171–179
4. Leslie, A. G. W. (1992) *Joint CCP4 + ESF-EAMCB News. Protein Crystallogr.* 26
5. Collaborative Computational Project (1994) *Acta Crystallogr. Sect. D Biol. Crystallogr.* 50, 760–783
6. Kleywegt, G. J., and Jones, T. A. (1998) *Acta Crystallogr. Sect. D Biol. Crystallogr.* 54, 1119–1131
7. Brunger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grasse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) *Acta Crystallogr. Sect. D Biol. Crystallogr.* 54, 905–921
8. Jones, T. A., Zou, J. Y., Cowan, S. W., and Kjeldgaard, M. (1991) *Acta Crystallogr. Sect. A* 47, 110–119
9. Lovell, S. C., Davis, I. W., Arendall, W. B., III, de Bakker, P. I., Word, J. M., Prisant, M. G., Richardson, J. S., and Richardson, D. C. (2003) *Proteins* 50, 437–450
10. Dust, D. A. (1996) *Science* 271, 163–168
11. Tsuzuki, S., Honda, K., Uchimaru, T., Mikami, M., and Tanabe, K. (2000) *J. Am. Chem. Soc.* 122, 11450–11458
12. Neyfakh, A. A. (2002) *Mol. Microbiol.* 44, 1123–1130
13. Schumacher, M. A., and Brennan, R. G. (2002) *Mol. Microbiol.* 45, 885–893
14. Tsukazawa, I., Markham, P. N., Neyfakh, A. A., and Brennan, R. G. (1999) *Cell* 96, 353–362
15. Kleywegt, G. J., and Jones, T. A. (2000) *Acta Crystallogr. Sect. D Biol. Crystallogr.* 54, 905–921
16. Zheleznova, E. E., Markham, P. N., Neyfakh, A. A., and Brennan, R. G. (2001) *Acta Crystallogr. Sect. D Biol. Crystallogr.* 54, 905–921
17. Zhelaznova, E. E., and Brennan, R. G. (2001) *Acta Crystallogr. Sect. D Biol. Crystallogr.* 54, 905–921
18. Yu, E. W., Aires, J. R., and Nikaido, H. (2003) *J. Bacteriol.* 185, 5657–5664
19. Zheleznova-Heldwein, E. E., and Brennan, R. G. (2001) *Nature* 409, 378–382
20. Yu, E. W., McDermott, G., Zgruskaya, H. I., Nikaido, H., and Koshland, D. E., Jr. (2003) *Science* 300, 976–980
21. Yu, E. W., Mc Dermott, G., Zgruskaya, H. I., Nikaido, H., and Koshland, D. E., Jr. (2003) *Science* 300, 976–980
22. Yu, E. W., Aires, J. R., and Nikaido, H. (2003) *Acta Crystallogr. Sect. D Biol. Crystallogr.* 54, 905–921
