QacR Is a Repressor Protein That Regulates Expression of the Staphylococcus aureus Multidrug Efflux Pump QacA*

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Steve Grkovic‡, Melissa H. Brown, Natalie J. Roberts, Ian T. Paulsen§, and Ronald A. Skurray¶

From the School of Biological Sciences, University of Sydney, Sydney, New South Wales 2006, Australia

The Staphylococcus aureus QacA protein is a multidrug transporter that confers resistance to a broad range of antimicrobial agents via proton motive force-dependent efflux of the compounds. Primer extension analysis was performed to map the transcription start points of the qacA and divergently transcribed qacR mRNAs. Each gene utilized a single promoter element, the locations of which were confirmed by site-directed mutagenesis. Fusions of the qacA and qacR promoters to a chloramphenicol acetyl transferase reporter gene were used to demonstrate that QacR is a trans-acting repressor of qacA transcription that does not autoregulate its own expression. An inverted repeat overlapping the qacA transcription start site was shown to be the operator sequence for control of qacA gene expression. Removal of one half of the operator prevented QacR-mediated repression of the qacA promoter. Purified QacR protein bound specifically to this operator sequence in DNase I-footprinting experiments. Importantly, addition of diverse QacA substrates was shown to induce qacA expression in vivo, as well as inhibit binding of QacR to operator DNA in vitro, by using gel-mobility shift assays. QacR therefore appears to interact directly with structurally dissimilar inducing compounds that are substrates of the QacA multidrug efflux pump.

Closely following the discovery of mammalian P-glycoprotein (1, 2), the phenomenon of multidrug resistance was also described for a bacterial system, the Staphylococcus aureus QacA pump (3), and has since been found to be widespread among both Gram-negative and Gram-positive bacteria (4–6). Resistance involves the active transport of a structurally diverse range of toxic compounds, typically hydrophobic cations, from the cell by a single efflux system. In the case of P-glycoprotein, the ability to export many antiancer agents (7) has prompted investigations into its mode of action. Biochemical studies and the generation of mutants with altered drug binding capabilities have suggested P-glycoprotein interacts directly with various substrates (7, 8). Additionally, recent progress has been made toward determining the structure of P-glycoprotein (9). Despite these advances, the basis of multidrug recognition by P-glycoprotein is still not understood. The functional similarities of bacterial multidrug efflux systems with P-glycoprotein, together with their presence in significant human pathogens, such as S. aureus (3), Pseudomonas aeruginosa (10), Neisseria gonorrhoeae (11), and Mycobacterium tuberculosis (12), makes elucidation of their molecular mechanisms an important research goal. Some progress has been made toward delineating the significance that various motifs and individual amino acids hold for determining the specificity of transport and overall mechanism of action for the QacA (13) and Bacillus subtilis Bmr (14) multidrug transporter proteins (reviewed in Ref. 6), but the strong association of efflux pumps with the membrane makes isolation and in depth analysis of these proteins difficult.

Considerable effort has also been directed toward identifying the factors involved in regulation of multidrug transporter gene expression. For mammalian P-glycoprotein (15, 16), as well as the Bmr (17), and Escherichia coli EmrB (18) bacterial multidrug efflux systems, increased gene expression followed the addition of some of the structurally diverse compounds exported by these pumps. Indeed, a certain degree of regulatory control over the genes for membrane transport proteins is to be expected, given their toxic nature toward the cell if overexpressed, as has been observed for the Gram-negative bacterial tetracycline resistance gene, tetA (19). Control of tetA expression by the specific repressor protein, TetR, is the best understood example of the regulation of a gene encoding a drug transporter (19). Induction of tetA expression occurs when TetR binds a tetracycline/divalent metal cation complex, inducing a conformational change in the protein such that TetR no longer binds the tetA operator, thereby liberating the promoter site (20). A similar style of regulation may also be responsible for the induction of expression observed for some bacterial multidrug efflux genes. However, for this to be effective, both the transporter and the regulatory protein would need to recognize the structurally diverse compounds that these systems efflux from the cell. Despite this, regulation of some bacterial multidrug efflux genes involves specific trans-acting regulatory proteins, such as the B. subtilis BmrR (17), E. coli EmrR (18), and N. gonorrhoeae MrR (21) proteins. Importantly, the B. subtilis BmrR transcriptional activator protein binds directly to at least some of the compounds that both induce bmr expression and are also substrates for the Bmr transporter (17, 22, 23).

Analysis of the region immediately upstream from the S. aureus qacA gene revealed a putative regulatory element, qacR, previously called orf188 (24). Based on homology comparisons, QacR, together with TetR, belong to a family of regulatory proteins which all share common features associated with a multi-helical DNA-binding domain at their N-terminal ends and have highly divergent C termini postulated to be involved in the binding of inducing compounds (24, 25). In this paper, we make the first steps toward understanding how QacR regulates the expression of qacA, by demonstrating it is...
Regulation of qacA, a S. aureus Multidrug Efflux Gene

Table I

| Strain/plasmid/primer | Genotype/Description/Sequence | Ref. |
|-----------------------|------------------------------|------|
| **Strains, plasmids, and oligonucleotide primers** | | |
| S. aureus, rifampicin- and novobiocin-resistant laboratory strain | USK982 | (36) |
| E. coli, supE44 ΔlacZ169(δ80 lacZAM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 | DH5α | (30) |
| pSK1 | S. aureus multiresistance plasmid conferring trimethoprim, gentamicin and multidrug resistance | (24) |
| pUC18 | E. coli cloning vector | (50) |
| pK184 | E. coli cloning vector with p15A replicon, compatible with ColE1 origin containing plasmids | (27) |
| pTTQ18 | E. coli expression vector with IPTG-inducible tac promoter | (28) |
| pKK232–8 | E. coli promoter cloning vector containing a promoterless cat gene | (26) |
| pMal-p2 | E. coli expression vector with M13 origin for rescue of ssDNA | New England Biolabs |
| pSK201 | pKK232–8 with M13 origin from pMal-p2 for rescue of ssDNA | This study |
| pSK449 | 3.4-kb EcoRI-HindIII qacR-qacA fragment from pSK1 cloned into pBR322 | (3) |
| pSK616 | 3.4-kb EcoRI-HindIII qacR-qacA fragment from pSK449 cloned into pUC18 | This study |
| pSK4150 | P_mcat fusion, qacR in cis, PCR clone in pRK229–8 with the primers 899EcoRI and 409HindIII | This study |
| pSK4238 | qacR in pK184, PCR clone with the primers 678BamHI and 409HindIII | This study |
| pSK5202 | P_mcat fusion in pSK5201, PCR clone with the primers 671HindIII and 865BamHI | This study |
| pSK5203 | P_mcat fusion in pSK5201, PCR clone with the primers 672BamHI and 867HindIII | This study |
| pSK5206 | Site directed mutation in the pSK5203 P_m–10 region; from TATAAT to TGTTAC | This study |
| pSK5210 | PCR clone with the primers 678BamHI and 108HindIIIH6 of qacR-His tag in pTTQ18 for overexpression | This study |
| pSK5211 | Site directed mutation in the pSK5202 P_m–10 region; from TATCAT to AGATCT | This study |
| pSK5212 | P_mcat fusion, qacR in cis, PCR clone in pSK5201 with the primers 867HindIII and 108HindIIIH6 | This study |
| pSK5215 | P_mcat fusion, qacR in cis with one half of IR1 (Fig. 1) deleted, derived from pSK5212 | This study |
| Primer† | 409HindIII | 5’-GGGAACCTTGGTTCTCATAAGCGAG-3’ |
| 108HindIIIH6 | 5’-CCGAGCTTACTATGGTTGTTGGTTGGTTTTACTAAGTCATTTTAAAAATTTGG-3’ | |
| 133 | 5’TGGAGAATCCTGCTAAAC-3’ | |
| 262 | 5’-ATGGCGTGTTGTATAATGATG-3’ | |
| 557 | 5’-GATTCCTTATCTAATCGT-3’ | |
| 608 | 5’-GGCGCTATCTGAGG-3’ | |
| 671HindIII | 5’-CCGAGCTTCACCGTCCTTTAAAGATGTA-3’ | |
| 672BamHI | 5’-CCGGGATCCAGCGCTTTAATTAAATGATG-3’ | |
| 678BamHI | 5’-CCGGGATCTCTCTAGAGGGCTGTACGAGCTGTAATG-3’ | |
| 738 | 5’-CAACATACGCTGATTTTTCTTGC-3’ | |
| 865BamHI | 5’-CCGGGATCCTCTTTAATGAATCACTG-3’ | |
| 867HindIII | 5’-CCGGAGCTTATTTAAAAATGAAATCAG-3’ | |
| 899EcoRI | 5’-CCGGATCCTCGTTGACCTTACATC-3’ | |

† The number of each primer refers to the 5’ most base in each instance that corresponds to a nucleotide in the previously published qacR–qacA sequence (24), which is the same numbering as used in Fig. 1. Some primers contain additional nucleotides (underlined) at their 5’ ends for the incorporation of restriction endonuclease recognition sites, an improved ribosome binding site, or a hexahistidine encoding tag.

a negative regulator that both binds to an operator site adjacent to the qacA promoter and also appears to interact directly with a diverse range of inducing compounds.

**Experimental Procedures**

**Bacterial Strains and Plasmids**—The bacterial strains, plasmids, and primers used in this study are described in Table I. For all experiments performed in S. aureus, strain SK982 was the host; E. coli strain DH5α was used for CAT assays, QacR overexpression, and in all cloning manipulations. All strains were cultured at 37 °C in LB media containing, where appropriate, 100 μg of ampicillin, 50 μg of kanamycin, 30 μg of chloramphenicol, or 20 μg of gentamicin per ml. The plasmid pSK616, containing qacA–qacR DNA originally derived from pSK1 (24), was the starting material for all manipulations, unless otherwise specified. The vector pSK5201, used to generate promoter fusions to the chloramphenicol acetyltransferase cat reporter gene, was constructed by replacing the 1.4-kilobase pair Pm-HindI fragment in pKK232–8 (26) with the 1.9-kilobase pair Pm-HindI M13 origin-containing fragment from pMAL-p2 (New England Biolabs), to allow rescue of single-stranded DNA from subsequent constructs.

PCR utilizing the primers 671HindIII and 865BamHI (Table I), which contained additional sequences at their 5’ ends encoding HindIII or BamHI sites, respectively, were performed to generate a fragment encoding the qacR–qacA intergenic region (see Fig. 1). This was then cloned into pSK5201, producing pSK5202, with the predicted promoter for qacR, P_mcat. Placed upstream of the cat gene ribosome binding site. pSK5203 was constructed with cat gene expression under the control of the predicted promoter for qacA, P_mcat. Mutations were created in the putative −10 regions of the qacR (pSK5202) and qacA (pSK5203) promoters by site-directed mutagenesis producing pSK5211 and pSK5206, respectively. The primer used to mutate P_m–10 was 5’-GAG-TATTAAATGATCATCAGT3’ (corresponding to qacA sense nucleotides, mutations underlined), and that for P_m–10 was 5’-GGCATCTCTCTAGAGGGCTGTACGAGCTGTAATG-3’ (corresponding to qacA antisense nucleotides, mutations underlined). An additional plasmid, pSK5212, with qacR in cis to P_mcat controlling cat, was constructed by cloning the product of a PCR reaction with the primers 108HindIIIH6 and 867HindIII into pSK5201. To delete one half of the putative qacR operator sequence (nucleotides 818–835; Fig. 1), a 750-bp fragment from pSK5212 was obtained using the Sau3AI site separating the two halves of IR1 and another Sau3AI site in the polylinker adjacent to the 3’ end of qacR in pSK5212. This fragment was ligated into the BamHI site of pSK5201 and a resultant clone, with cat transcription under the control of P_mcat, designated pSK5215.

The plasmid pSK4238, which was constructed to enable the expression of qacR in trans, contained a PCR-generated qacR fragment preceded by a strong ribosome binding site inserted behind the lacZ promoter of pKL184 (27), a vector compatible with the pSK5201-based constructs. To facilitate the overexpression and purification of QacR, the primers 678BamHI and 108HindIIIH6 were used to obtain a PCR product that consisted of the qacR gene preceded by a strong ribosome binding site and also containing a hexahistidine-encoding sequence (His tag) at the 3’ end. This fragment was ligated into the E. coli...
Regulation of qacA, a S. aureus Multidrug Efflux Gene

Expression vector pT7Q18 (28) to produce pSK5210, with qacR expression placed under the control of the strong IPTG-inducible tac promoter. All clones produced from the products of PCR reactions were sequenced to confirm that no errors had been incorporated during amplification.

RNA and DNA Isolation and Manipulations—Total cellular RNA was extracted from 15 ml of Tris-HCl cell cultures of S. aureus GS252 (Fig. 1) that partially overlapped the most likely sequence for the qacA promoter (PqacA; Fig. 1) (24). Additionally, a potential promoter for qacA (bp 794–827 in Fig. 1) was identified, which would overlap IR1 and possibly subject qacR expression to auto-regulation (24). However, on further analysis of the qacR-qacA intergenic sequence, we identified another potential promoter-like element for qacR (PqacR; bp 702–729 in Fig. 1) that lies closer to the start of the gene and has its own distinct region of dyad symmetry (IR2; Fig. 1), suggesting that expression of qacR could be initiated from multiple promoters.

To ascertain the actual promoter(s) used by each gene, the tag for both qacA and qacR were determined in the S. aureus strain SK982 (36) harboring the qacA−, qacR− multiresistance plasmid pSK1 (24) using primer extension analysis. For each reaction, only one extension product was observed. The tag for qacA in S. aureus corresponded to a G at position 810 and that for qacR to be a C at position 693 (Fig. 2). Therefore, for each gene, transcription is initiated from a single promoter element,
each partially overlapped by potential operator sites for DNA-binding proteins (IR1 and IR2; Fig. 1). To confirm the same tsp was used when the cloned qac region was present in E. coli, primer extension analyses, using the same primers as described in Fig. 2, were performed on RNA isolated from E. coli strain DH5α harboring pSK616. The results obtained indicated that the same promoters were utilized in both species, although for both genes the tsp in E. coli was found to be an A, one nucleotide upstream from the tsp determined for S. aureus (data not shown). This finding may reflect a real difference, since A is the preferred start nucleotide for the initiation of transcription by E. coli α70 RNA polymerase (37). Alternatively, the observed change may merely be an artifact of preparing RNA from quite distinct species.

The expression of some regulatory genes, like tetR (19), from multiple, autoregulated promoters, suggested the originally proposed qacR promoter region (24) might also be functional as an inducible promoter. To test this, primer extension analysis using primer 557 (Fig. 1) was also performed on RNA isolated from cultures of S. aureus containing pSK1 that had been treated with Eb, a compound that acts as an inducer of QacR-mediated qacA gene expression (see below). A further tsp was not observed for qacR, supporting the finding that the qacR gene is transcribed from a single promoter. This was confirmed by failure to detect any primer extension product (data not shown) for another primer, 672BamIII (Fig. 1), which was at a closer, more ideal distance to the area at which transcription would be initiated for the originally proposed qacR promoter.

Various constructs in the promoter cloning and single-stranded DNA rescue vector pSK5201 were utilized to substan- tiate the location of the promoter elements. The plasmids pSK5202 and pSK5203 were constructed such that PqacR and PqacA, respectively, would be expected to control transcription of the cat reporter gene. Site-directed mutagenesis employing the primers described under “Experimental Procedures” was used to change 4 and 5 nucleotides of the putative PqacR and PqacA “−10” regions, from TATAAT and TATCAT to TGGTAC (pSK5206) and AGATCT (pSK5211), respectively, producing mutant sequences that were predicted to have severely reduced abilities to function as efficient promoters. Analysis of the above constructs by CAT assays demonstrated that the qacR promoter element was greater than 4-fold stronger than PqacA (Table II). The mutations generated in the nominated −10 regions reduced expression of the cat reporter gene 15- and 50-fold for the qacR and qacA promoter mutants, respectively, when compared with the wild-type promoter clones (Table II), thereby confirming the location of the promoters.

The qacR Gene Encodes a Repressor of qacA Expression That Acts via IR1—To analyze the potential regulation of qacA, a S. aureus Multidrug Efflux Gene

![Fig. 1. Regulatory region of the qacA and qacR genes.](Image 128x552 to 475x729)

**Fig. 1. Regulatory region of the qacA and qacR genes.** The respective promoters, PqacR and PqacA, their putative “−10” and “−35” regions, and the tsp for each gene are shown. Bold type delineates the most likely ribosome binding sites. Inverted repeats, IR1 and IR2, that partially overlap each promoter are indicated by bold arrows. Thin arrowed lines denote selected oligonucleotide primers (Table I) used in this work. The positions of only relevant restriction sites are shown. The deduced amino acid sequences for the amino termini of the respective gene products, QacR and QacA, are given adjacent to their coding strands. Boxed amino acids indicate the location of the putative helix-turn-helix motif in QacR. The numbering is according to the previously published sequence from the S. aureus multiresistance plasmid pSK1 (24).

![Fig. 2. Primer extension analysis of the qacA and qacR transcription start points in S. aureus.](Image 316x306 to 546x479)

**Fig. 2. Primer extension analysis of the qacA and qacR transcription start points in S. aureus.** Primer extension and sequencing reactions utilizing primer 899EcoRI for qacA and primer 557 for qacR were performed as detailed under “Experimental Procedures.” The corresponding DNA sequences, with the −10 region boxed, were shown for each gene. Nucleotides are numbered as described in Fig. 1. Primer extension analysis of qacA. S. aureus strain SK982 without (lane 1) and with (lane 2) the qacA+, qacR+ plasmid pSK1. Primer extension analysis of qacR; SK982 pSK1 uninduced (lane 3), Eb (50 μg/ml for 15 min) induced (lane 4), and SK982 alone (lane 5).
vector, pK184, lacking qacR, had no effect on transcription from P_{qacA} (data not shown). Taken together, these data indicated that the observed high level of repression afforded by pSK4238 was a result of qacR being placed under the control of the strong lac promoter, leading to abnormally high levels of QacR. The presence of pSK4238 was found to have no significant effect on the level of transcription from the qacR promoter in pSK5202 (Table II), indicating that the expression of qacR is not autoregulatory.

To investigate the potential of the region of dyad symmetry (IR1) overlapping the qacA promoter to act as an operator site, CAT assays were performed with cells carrying pSK5215, which possessed only one half of the dyad symmetry (bp 800–817, Fig. 1), the other being deleted as described under “Experimental Procedures.” Expression from the qacR promoter in pSK5215 was, relative to pSK5212 (which possessed the full IR1), restored to the level observed for pSK5203, which lacked qacR (Table II). Further experiments defining the role of IR1 as a potential operator to which QacR binds are detailed below.

Purification of QacR—To obtain large quantities of QacR for in vitro studies, the qacR gene was first cloned downstream from a strong ribosome binding site and the IPTG-inducible tac promoter in the E. coli vector pTTQ18, resulting in inducible overexpression of QacR (Fig. 3A, lane 2). Utilization of a His tag incorporated at the C terminus of QacR allowed purification close to homogeneity by Ni^{2+}-NTA chromatography (Fig. 3A, lanes 5 and 6). The purified QacR protein migrated in reducing SDS-PAGE gels with an observed molecular mass of approximately 23 kDa, the size predicted for a QacR monomer (24) with a hexahistidine tag. N-terminal amino acid sequencing of the first 12 residues of the purified protein confirmed its identity. The molecular mass of freshly purified QacR was determined by gel-filtration chromatography to be 22.5 kDa, in close agreement with the predicted molecular mass for a QacR-His tag monomer.

Long-term storage of the purified QacR-containing fractions at either 4 °C or room temperature resulted in the formation of higher molecular weight aggregates, the predominant band corresponding to the expected size for a QacR dimer (Fig. 3A, lane 7; QacRII). QacR monomer could be partially regenerated from these higher molecular weight forms by treatment with 7 mM guanidine-HCl followed by dialysis, but only with the addition of a reducing agent, 20 mM DTT (data not shown). This suggested that formation of disulfide bonds between the two cysteine residues in QacR (Cys^{72} and Cys^{141}) (24) was responsible for the oligomerization process. A recognized problem during the purification and storage of proteins is the formation of disulfide bonds resulting from the oxidation of free thiol groups catalyzed by divalent metal cations and dissolved oxygen (38). To minimize this problem, the major QacR-containing fractions were exchanged into GMS buffer containing 10 mM 2-mercaptoethanol immediately after their elution from the Ni^{2+}-NTA column, followed by freezing in single-use aliquots at ~70 °C.

When subjected to electrophoresis under native conditions, freshly purified QacR migrated as a monomer following incubation at 37 °C for 30 min in 20 mM 2-mercaptoethanol. However, incubation at room temperature in the absence of 2-mercaptoethanol resulted in the appearance of trace amounts of oligomeric forms. Thus the native PAGE and gel filtration data, taken in conjunction with the SDS-PAGE results presented in Fig. 3, indicate that QacR is purified from the cell as a monomer and the only oligomeric forms observed are the disulfide-bonded multimers, which appear following prolonged storage. Western blots using a 6×His monoclonal antibody to detect hexahistidine-tagged proteins were performed on crude cell lysates separated by electrophoresis through SDS-PAGE gels. Only monomeric QacR was detected (data not shown), confirming that the disulfide-bonded oligomers which form during storage of the purified protein (Fig. 3A) are unlikely to be physiologically relevant.

Involvement of Metal Cations in the Formation of Disulfide-Bonded Oligomers—Addition of EDTA to a final concentration of 5–10 mM immediately after elution of QacR from the Ni^{2+}-NTA column was able to partially suppress the formation of oligomers during storage (data not shown), supporting the involvement of divalent metal cations in catalyzing disulfide-bond formation between purified QacR molecules. To test the role of divalent metal cations, QacR monomer was incubated with various concentrations of NiCl_{2} and CuCl_{2}, which resulted in an increased rate of oligomer formation, reaching a maximum at 10 mM for CuCl_{2} and at 1 mM for NiCl_{2} (Fig. 3B). In particular, treatment with low concentrations of Ni^{2+} resulted in the appearance of a significant dimer band (QacRII; Fig. 3B), corresponding to the predominant oligomeric species observed after long term storage, which suggested that leaching of Ni^{2+} ions from the affinity column during the purification process could be contributing to the oligomer formation. To reverse the formation of the oligomers, heating in the presence of 100 mM DTT was required, confirming their disulfide-bonded nature. A faster migrating species, presumably representing QacR containing an intramolecular disulfide bond, was also reduced to the non disulfide-bonded monomer (Fig. 3B). Interestingly, addition of CuCl_{2} to a concentration of 1 mM or more largely abolished the intramolecular disulfide-bonded species, in favor of oligomeric forms (Fig. 3B). Two further metals tested, ZnSO_{4} and MgSO_{4}, had no effect on the formation of disulfide bonds (data not shown).

QacR Binds to a DNA Fragment Containing IR1 and the qacA Promoter—Gel-mobility shift assays were used to show QacR bound specifically to a DNA fragment corresponding to the qacA-qacR intergenic region (bp 672–867; Fig. 1), and not to a control fragment from the 3′ end of the qacA gene (Fig. 4). Only excess, unlabeled, intergenic competitor DNA, and not excess control DNA, was able to partially titrate out QacR from gel-mobility shift binding reactions (Fig. 4), confirming the specificity of QacR for the intergenic DNA fragment. Additionally, cleavage of the intergenic fragment at the DraI site (bp 751; Fig. 1), to yield two singly end-labeled fragments, resulted in only the qacA promoter-containing fragment being retarded (Fig. 4). This demonstrated that QacR is specific for the qacA
promoter and did not bind to a DNA fragment containing the qacR promoter and IR2, lending further support to the postulate that expression of qacR is not autoregulated. In addition, only the crude protein extract from IPTG-induced E. coli DH5α cells harboring the QacR overexpressing plasmid pSK5210, but not the control plasmid pTTQ18, was able to shift labeled intergenic DNA (data not shown), confirming that QacR alone is responsible for the observed retardation of intergenic DNA. The addition of CuCl_2 or NiCl_2 had no observable effect on gel-mobility shift binding reactions (data not shown), indicating that at concentrations which increased the rate of oligomer formation (Fig. 3B) these divalent metal cations do not appear to have any direct effect on the binding of QacR to IR1.

DNase I-footprinting experiments confirmed that purified QacR bound specifically to nucleotides in the inverted repeat (IR1) overlapping the qacA tsp (Fig. 5). As shown in Fig. 5B, QacR protected from DNase I digestion all the nucleotides in the IR1 region, with the exception of a few bases at each end, on both the qacA sense and antisense strands.

**Substrates of QacA Act as Inducers of qacA Expression**—The QacA protein confers resistance to a wide range of structurally dissimilar compounds from four distinct classes of chemicals; the dyes, biguanidines, diamidines, and QACs. To investigate if these substrates potentiate the expression of qacA, CAT assays after overnight incubation in sub-MICs of potential inducing compounds were carried out with E. coli DH5α cells harboring pSK4150, which contained the qacR gene in cis to the qacA promoter. A significant increase in CAT activity was observed for many of the QacA substrates (Table III). Conversely, except for Eb, which showed a small degree of induction independent of QacR, no increase in CAT activity was observed when the qacA promoter alone was tested against a range of inducing compounds (data not shown). Also tested for their ability to increase qacA gene expression were another diamidine, pro-madidine isethionate, and another QAC, cetyltrimethylammonium bromide, both of which showed no induction (data not shown). None of the compounds tested were able to overcome the strong repression of the qacA promoter in pSK5203 afforded by qacR being overexpressed in trans from the plasmid pSK4238 (data not shown).

**Structurally Diverse Substrates of QacA Inhibit Binding of QacR to a qacA Operator-containing DNA Fragment**—As for the in vivo induction of expression, many QacA substrates were also able to show an in vitro effect on binding of QacR to the qacA operator site. Fig. 6 demonstrates a strong dissociation of QacR from the operator-containing DNA fragment when any of the dyes Eb, rhodamine 6G, proflavin, or crystal violet were included in gel-mobility shift experiments. To dissociate QacR from the operator containing DNA, a concentration 50 times...
the MIC for S. aureus of the biguanidine compound chlorhexidine digluconate was required, whereas none of the diamidines, pentamidine isethionate (Fig. 6), propamidine isethionate, or diaminodiphenylamine-hydrochloric acid, as well as a QAC, cetyltrimethylammonium bromide (data not shown) had any effect. The QACs dequalinium chloride and cetylpyridinium chloride both strongly inhibited binding of QacR to the operator, at a sub-MIC, and 10-fold greater than the MIC for S. aureus, respectively (Fig. 6). Benzalkonium chloride was shown to partially inhibit binding of QacR to operator DNA at a concentration that was 4 times the MIC of this compound for S. aureus (Fig. 6). The addition of QacA substrates at the concentrations used in the above experiments to DNA alone did not affect its mobility.

**DISCUSSION**

This paper provides the first experimental evidence to demonstrate that the expression of the S. aureus multidrug efflux gene qacA is regulated by a repressor protein, QacR, the product of a divergently transcribed open reading frame. By both *in vitro* gel-mobility shift (Fig. 4) and DNase I protection experiments (Fig. 5), and *in vivo* analysis by deletion of one half of the dyad symmetry which resulted in constitutive expression of *qacA-cat* fusions (Table II), the operator site for QacR binding was shown to be correctly identified; located immediately downstream from the qacA promoter (IR1; Fig. 1). By analogy with studies on promoter/operator systems such as that of the *E. coli lac* operon (39, 40), the binding of QacR to its operator may not inhibit the binding of RNA polymerase, but would prevent the transition of the RNA polymerase-promoter complex into a productively transcribing state, insuring adequate repression of qacA transcription.

The inability of QacR to autoregulate expression of its own gene was demonstrated by qacR overexpressed in trans being able to completely abolish transcription from the qacA promoter, yet having no effect on its own promoter fused to a reporter gene (Table II). This finding was confirmed by showing that QacR did not bind to a DNA fragment containing the qacR promoter (Fig. 4). Most other members of the family of regulatory proteins sharing homology with QacR that are divergently transcribed appear to regulate the expression of their own genes, such as acrII-ORF1 from a *Streptomyces* antibiotic exporting complex (41), CamR, a repressor of n- camphor degra-
The ability of QacR to interact with multiple and chemically diverse compounds makes it an attractive candidate for future studies. Because of their location in the soluble cytoplasmic fraction of the cell, multidrug efflux regulatory proteins are much easier targets than the corresponding membrane bound transporter for initial studies directed at understanding how structurally diverse compounds are recognized by a single protein. Further work involving the demonstration of a direct interaction between QacR and inducing compounds, mutational analysis of individual residues, and refinement of the purification process toward x-ray crystallography studies on the structure of QacR bound to its operator DNA or inducing compounds is in progress.

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Regulation of qacA, a S. aureus Multidrug Efflux Gene

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