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Regulation of Bacterial Drug Export Systems

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INTRODUCTION

Drug-resistant microorganisms are a major worldwide health issue, as a number of important human pathogens have now acquired mechanisms that make them largely resistant to all currently available treatment regimens. The action of antimicrobial compounds can be negated at a number of points, including enzymatic inactivation, the employment of alternative metabolic pathways to bypass their activity, sequestration, reduced uptake, and alteration of the target site to render it not susceptible to the effect of otherwise toxic substances (28). A further resistance mechanism that has become increasingly important involves membrane-bound efflux pumps that transport toxic antimicrobial compounds from the cell. ATP-binding cassette (ABC) transporters power this process via the hydrolysis of ATP, whereas secondary transporters utilize the transmembrane electrochemical gradient, typically the proton motive force, to drive drug efflux (161, 173).

The first drug transport proteins to be identified were a family of tetracycline efflux pumps, which provide widespread resistance to tetracycline antibiotics in both gram-negative and gram-positive bacteria (25). More recently, a large number of multidrug resistance (MDR) transport proteins have been found to be involved in the export of a wide range of antimicrobial compounds (161, 173, 185). In contrast to the narrow substrate range of most transporters, including the tetracycline efflux determinants, individual MDR pumps are capable of exporting compounds that have few structural similarities. MDR determinants appear to contribute to the emergence of drug-resistant microorganisms via two mechanisms. First, they can confer low-level protection that facilitates the initial survival of the organism and thus provides it with the opportunity to subsequently acquire one of the high-level specific resistance mechanisms listed above. Alternatively, the MDR transporters themselves can furnish protection against clinically relevant concentrations of many antimicrobial compounds.

In addition to providing many pathogenic bacteria with protection against antibiotics, antiseptics, and disinfectants (161), drug transporters pose a number of additional medical challenges. Host-encoded antimicrobial compounds that are produced to combat infections caused by organisms such as *Esch-*

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erichia coli (143) and *Neisseria gonorrhoeae* (203) are also exported by some MDR pumps; in *Staphylococcus aureus*, the presence of an MDR transporter has been correlated with resistance to a cationic antimicrobial peptide (17, 87). Treatment of immunocompromised patients undergoing long-term antifungal therapy is often complicated by development of resistance to antifungal agents by the pathogenic yeast *Candida albicans* as a result of MDR transporter overexpression (84). Additionally, in human cancer cells, overexpression of an MDR transporter, P-glycoprotein, can provide high-level resistance to antitumor drugs, a finding which has been linked to the failure of chemotherapy (45).

Although these problems have generated a substantial degree of interest in drug transporters, the close association of efflux pumps with cellular membranes has severely hampered efforts to define the exact molecular mechanisms by which these proteins function. Only recently have high-resolution structures for any membrane transport proteins been elucidated, the *E. coli* proteins MsbA (23) and BtuCD (103), although more limited structural information has also been gained by employing electron microscopy of two-dimensional crystals, e.g., the single-substrate TetA tetracycline exporter has been shown to function as a trimer (240), the MDR transporters EmrE (216) and YvcC (22) form dimers, and P-glycoprotein functions as a monomer (180, 181).

In comparison to the limited achievements in understanding the structure-function relationships of the drug transporters themselves, research into the regulatory pathways that govern the expression of drug transporters has progressed relatively rapidly, in particular for a number of bacterial regulatory proteins. The antimicrobial pumps which are known to be subject to regulatory controls typically belong to either the major facilitator superfamily (MFS) or resistance, nodulation and cell division (RND) superfamily (Table 1), both of which primarily employ the proton motive force to energize drug efflux (161, 173). The requirement for regulatory controls to prevent excessive production of an integral membrane protein that utilizes the proton motive force is demonstrated by the deleterious effect of constitutive expression of the gram-negative tetracycline/H^+ antiporters TetA(B) and TetA(C), which, in the absence of tetracycline, place cells at a severe disadvantage when competing with nonconstitutively expressing strains (92, 142). Overproduction of TetA(B) can also be lethal to the cell if the gene is expressed from a strong promoter, resulting in nonspecific cation transport, loss of the membrane H^+ potential, and cell death (31, 57).

However, despite these observations, all members of a further family that utilize the proton motive force, the small multidrug resistance family, a subgroup of the drug/metabolite transport superfamily (67), do not appear to be subject to any regulatory controls that can alter the level at which these proteins are synthesized. It is therefore an intriguing question why some proteins that utilize the proton motive force to energize drug efflux are synthesized under strict regulatory controls, yet others appear to be expressed constitutively. This could well reflect currently unknown physiological roles for the unregulated pumps in normal cellular metabolism that require the constant low-level presence of such transporters. The supply of a small but continuous amount of a protein can be easily governed by mechanisms such as low-level production of the relevant mRNA and/or high turnover rates of the mRNA and/or the transport protein, without any need for additional, more complex regulatory controls.

For the bacterial drug efflux genes that are inducible, there are only a few documented cases for which translational controls have been shown to be the primary level at which expression is controlled. For example, translational attenuation has been proposed to modulate the synthesis of the gram-positive tetracycline resistance determinant TetA(K) (206), whereas a second gram-positive tetracycline resistance protein, TetA(L), is regulated by translational reinitiation (209). Experimental evidence suggests that the latter example involves tetracycline-induced stalling of ribosomes during the translation of a short leader peptide. This is likely to facilitate the transfer of ribosomes to the *tetA*(L) ribosome-binding site, an event which requires the presence of a stem-loop structure that is proposed to correctly orient the *tetA*(L) ribosome-binding site for ribosome transfer to occur (209).

In contrast to these determinants, expression of the majority of the bacterial drug transporter genes which are known to be subject to regulation is controlled by transcriptional regulatory proteins. Well-characterized proteins with a demonstrated role in controlling the expression of drug efflux genes encompass examples of both repressors and activators of target gene transcription, a process that can occur at either the local or global level. Local regulators of drug transporter genes include the *Escherichia coli* TetR repressor of tetracycline efflux genes (59) and three regulators of MDR transporter genes, the *Bacillus subtilis* BmrR activator (2), the *Staphylococcus aureus* QacR repressor (47), and the *E. coli* EmrR repressor (105). In some instances, local regulators appear to play only a modulating role, the principal factor controlling transcription instead being global regulatory proteins; e.g., increases in the expression of the *E. coli acrAB* MDR locus are mediated by the MarA, Rob, and SoxS global activators (7). Two-component regulatory systems are also increasingly being found to be associated with drug efflux genes (Table 1). Sequencing of entire bacterial genomes has identified a large number of additional MDR transporter homologs and their associated regulatory elements, although the functions of the majority of these systems remain to be experimentally investigated (162).

In general, the confirmed regulators of bacterial drug transporter genes belong to one of four regulatory protein families, the AraC, MarR, MerR, and TetR families, despite being from distantly related species, a classification which also shows little correlation with the family of the drug pump whose expression they control (Table 1). However, the assignment of these regulatory proteins to their respective families is based solely on similarities detected within their DNA-binding domains, which typically constitute only one third of each polypeptide. Like the majority of bacterial activators and repressors, the drug transport regulators identified to date all possess α-helix-turn-α-helix (HTH) DNA-binding motifs, which are embedded in larger DNA-binding domains that form a number of different structural environments, such as three-helix bundles and winged helix motifs (155). These serve to create a stable three-dimensional structure that buries the hydrophobic side chains of amino acids in the interior of the DNA-reading head but generally orients the second "recognition" helix of the HTH so that it fits into the major groove of B-DNA. Further detailed

TABLE 1. Transcriptional regulators that control expression of genes encoding bacterial drug efflux components

Organism	Regulatory protein(s)	Regulator family[a]	Function of regulator	Ligand(s) of regulatory protein[b]	Drug efflux gene(s) regulated[c]	Reference(s)
RND pump regulators						
Acinetobacter baumannii	Orf2-Orf3	Two-component system?		?	*adeABC**	111
Burkholderia pseudomallei	AmrR	TetR	Repressor?	?	*amrAB-oprA*	133
Escherichia coli	AcrR	TetR	Repressor	?	*acrAB**	107
	AcrS	TetR	Repressor?	?	*acrEF**	156
	BaeR-BaeS	Two-component system		?	*mdtABC*	12, 137
	EvgA-EvgS	Two-component system		?	*yhiUV*	144
	MarA/SoxS/Rob	AraC	Global activators	Rob? (MarA/SoxS^{NA})	*acrAB** and *tolC*	69, 70, 118, 177
	MarR	MarR	Repressor of *marA*	DNP, Pg, Sa, Md	*acrAB** and *tolC*, via MarA	6, 8, 120
Neisseria gonorrhoeae	MtrA	AraC	Global activator	HAs?	*mtrCDE**	184
	MtrR	TetR	Repressor	?	*mtrCDE** and *farAB*	106
Pseudomonas aeruginosa	MexR	MarR	Repressor	?	*mexAB-oprM**	32, 170
	MexT	LysR	Activator	?	*mexEF-oprN**	81
	MexZ	TetR	Repressor?	?	*mexXY*	4
	NfxB	LacI/GalR	Repressor	?	*mexCD-oprJ**	169
Pseudomonas putida	ArpR	TetR	Repressor?	?	*arpABC*	78
Stenotrophomonas maltophilia	SmeR-SmeS	Two-component system?		?	*smeABC*	100
MFS pump regulators						
Bacillus subtilis	BltR	MerR	Activator	?	*blt*	3
	BmrR	MerR	Activator	R6G, TPP, Ao, DEC, ABM, ADCP, DDPB	*bmr*	2, 227, 244, 245
	Mta	MerR	Global activator	?	*bmr* and *blt*	13, 43
Escherichia coli	EmrR	MarR	Repressor	CCCP, DNP, Eb, FCCP, Na, Sa, TCS	*emrAB** and *mcbABCDEFG*	20, 104, 105, 238
	EvgA-EvgS	Two-component system		?	*emrKY*	145
	TetR	TetR	Repressor	Tc	*tetA*	59, 60, 153
Staphylococcus aureus	ArlR-A1rS	Two-component system		?	*norA**, via 18-kDa protein	35
	QacR	TetR	Repressor	Bc, Be, Ch, Cv, Dc, Eb, Mg, Pf, R6G	*qacA*/*qacB*	47, 48, 197, 198

[a] Although two-component systems belong to a number of different families, they all consist of a transmembrane sensor of an external signal and a cytoplasmic response protein whose regulatory activities are modulated by reversible phosphorylation. Note that EvgA has been demonstrated to modulate the expression of both an RND type pump and an MFS member, *yhiUV* and *emrKY*, respectively.

[b] Ao, astrazon orange; ABM, 5-(1-adamanthylcarboxyethyl)-3-benzyl-4-methylthiazolium; ADCP, 4-amino-3,6-dimethylbenzo[*b*]cycloheptano[*e*]pyridinium; Bc, benzalkonium; Be, berberine; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Ch, chlorhexidine; Cv, crystal violet, Dc, dequalinium; DDPB, 5,6-dichloro-1,3-diethyl-2-(phenylaminovinyl)benzoimidazolium; DEC, diethyl-2,4′-cyanine; DNP, 2,4-dinitrophenol; Eb, ethidium bromide; FCCP, carbonyl cyanide *p*-(trifluoro-methoxy) phenylhydrazone; HAs, hydrophobic agents; Md, menadione; Mg, malachite green; Na, nalidixic acid; Pf, proflavine; Pg, plumbagin; R6G, rhodamine 6G; Sa, salicylate; Tc, tetracycline; TCS, tetrachlorosalicylanilide; TPP, tetraphenylphosphonium; ?, many of these regulatory proteins and two-component transmembrane sensors possess hypothetical ligand-binding domains for which ligands have yet to be identified. NA, not applicable, as the MarA and SoxS proteins do not possess ligand-binding domains.

[c] Drug efflux genes or operons marked with an asterisk (*) have been observed to confer elevated antimicrobial resistance in some clinical isolates due to regulatory mutations that result in overexpression of these determinants.

information on the structure and function of HTH motifs can be found in a number of excellent reviews (39, 54, 64, 155, 213).

Importantly, for four local regulators of drug efflux determinants, the portions of these proteins not involved in forming the DNA-binding domains have been demonstrated to be capable of directly binding substrates of their cognate pumps, which act as a signal to increase the synthesis of the relevant transport protein(s) in response to the presence of these toxic compounds. This finding has had important ramifications for the field of protein-drug interactions, since, in contrast to the membrane-bound transport proteins, which are notoriously difficult to purify and study in vitro, the soluble cytosolic regulators of drug resistance have provided much more amenable systems for the study of drug recognition and binding. For the TetR (60), QacR (198), and BmrR (245) regulatory proteins in particular, detailed X-ray crystallographic and biochemical data, combined with mutational studies, have been highly successful in providing a wealth of information on the molecular aspects involved in drug binding and the subsequent steps that result in the induction of target gene expression.

By concentrating principally on the better-characterized regulatory pathways, at both the local and global levels, this review is intended to illustrate the extremely varied nature of the transcriptional regulatory controls that act upon the genes encoding bacterial drug efflux systems. Particular emphasis is placed on the contributions that analysis of these regulatory pathways and their attendant proteins have made to the field of drug resistance as a whole. We also discuss the

insights that have been gained from evolutionary and medical perspectives.

TRANSPORTER OVEREXPRESSION AND ANTIMICROBIAL RESISTANCE

Almost all of the antimicrobial compounds, both synthetic and natural, which have been employed by humans to combat infectious bacteria are substrates of one or more drug efflux pumps, e.g., the fluoroquinolones; the antibiotics chloramphenicol, tetracycline, β-lactams, and aminoglycosides; and the antiseptics benzalkonium, cetrimide, and chlorhexidine. Tetracycline-specific pumps such as TetA(B) possess regulatory controls that are sensitively attuned for adjusting expression levels in response to the presence of tetracycline. In contrast, the vast majority of bacterial MDR genes need to be expressed at a level substantially greater than that observed in the wild-type organism before significant efflux of clinically relevant antimicrobial compounds occurs. This finding has lent considerable support to the proposal that MDR pumps in general have preexisting physiological roles, such as protection against low levels of toxic hydrophobic molecules encountered in the natural environment of many organisms or the transport of specific metabolites. However, for a limited number of the drug transporters identified to date, such as the tetracycline pumps and perhaps also the plasmid-encoded *S. aureus* MDR determinant QacA, which exports an impressive array of antiseptics, disinfectants, and related compounds (21), analysis of the pumps and their regulatory controls indicates that the efflux of medically relevant antimicrobial compounds is now the primary function of these systems.

For clinical isolates of the important human pathogens *E. coli*, *Neisseria gonorrhoeae, Pseudomonas aeruginosa*, and *S. aureus*, a large number of regulatory pathway mutations that have resulted in the overexpression of various MDR determinants and a concomitant elevated resistance to antimicrobial compounds have been characterized. For example, it has been well established that low-level multiantibiotic resistance in *E. coli* can arise from overexpression of the AcrAB-TolC MDR efflux complex as a result of increased production of the MarA global transcriptional activator protein (112, 149, 232). MarA and a number of other *E. coli* MDR regulators are discussed in greater depth later in this review. Overexpression of MDR transporters has now been identified as a major source of antimicrobial resistance in an alarmingly large number of pathogenic species. The most striking example of this phenomenon is the serious opportunistic pathogen *P. aeruginosa*, for which the hyperexpression of MDR pumps has been particularly important in the emergence of multiantibiotic-resistant strains.

P. aeruginosa MDR Pumps and Multiantibiotic Resistance

Mutations leading to the overexpression of pump complexes such as MexAB-OprM have now been identified as the predominant cause for acquisition of elevated resistance to structurally unrelated antibiotics by strains of *P. aeruginosa* (167). The *mexAB-oprM* operon encodes a tripartite pump complex that comprises the MexB cytoplasmic pump component, the MexA membrane fusion protein, and the OprM outer membrane channel (Fig. 1). Thus, the MexAB-OprM complex can simultaneously transport antibiotics across both the cytoplasmic and outer membranes, which provides a highly effective resistance mechanism when combined with the notoriously low permeability of the *P. aeruginosa* outer membrane (167). MexAB-OprM together with MexCD-OprJ, MexEF-OprN, and MexXY (which also utilizes the OprM outer membrane channel) form a family of related *P. aeruginosa* multidrug efflux pump complexes that have an extremely broad substrate range (134, 168). Each of the operons for these four pump complexes has a local transcriptional regulatory protein that is encoded in the immediately adjacent upstream region (Fig. 1). In stark contrast to the high degree of relatedness between the individual components of the pump complexes, the proteins encoded by each of these upstream genes exhibit no homology to each other; instead, they belong to four distinct regulatory protein families (Table 1).

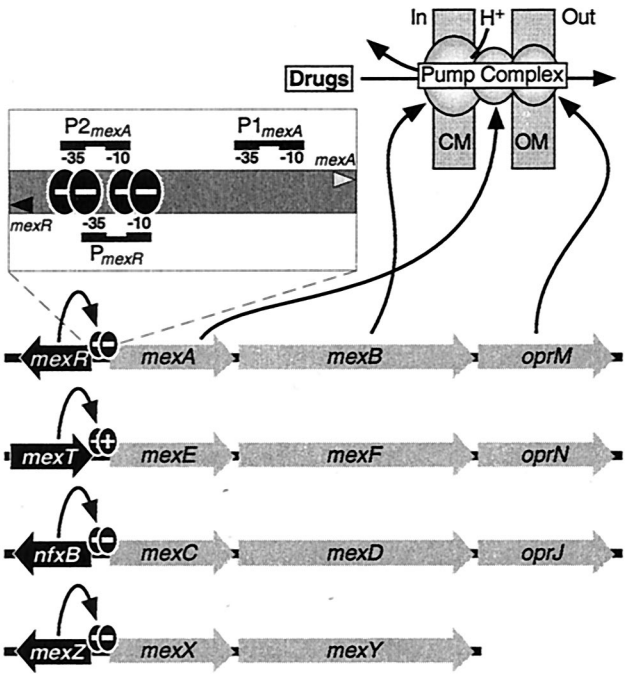

FIG. 1. Genetic organization of the *mexR-mexAB-oprM*, *mexT-mexEF-oprN*, *nfxB-mexCD-oprJ*, and *mexZ-mexXY* MDR loci from *P. aeruginosa*. Each operon contains genes (grey arrows) that encode a drug efflux complex and is regulated by the product of an upstream gene (black arrow) which either represses (−) or activates (+) operon expression, although this is yet to be confirmed for MexZ. The intergenic region separating the *mexR* and *mexA* genes is depicted in greater detail, with the positions of the binding sites for the two MexR dimers (black ovals) indicated relative to the −10 and −35 hexamers of the *mexR* promoter (P_{mexR}), the *mexA* promoter ($P2_{mexA}$), and a second potential *mexA* promoter ($P1_{mexA}$). A schematic representation of the MexAB-OprM tripartite complex, which can efflux drugs simultaneously across both the cytoplasmic (CM) and outer (OM) membranes, is also shown. MexB, the RND component of the pump complex, transports drugs across the cytoplasmic membrane in exchange for protons (H^+).

The regulation of the *mexAB-oprM* operon is the best-characterized example; the divergently encoded MexR, a member of the MarR family of proteins (Table 1), acts as a repressor of transcription of *mexAB-oprM* (170). MexR autoregulates ex-

pression of its own gene by repressing the *mexR* promoter (Fig. 1, P_{mexR}) in addition to controlling transcription from the *mexA* promoter (Fig. 1, $P2_{mexA}$) (32). A second promoter (Fig. 1, $P1_{mexA}$) has been proposed to be responsible for the relatively high constitutive level of *mexAB-oprM* expression, which contributes substantially to the high intrinsic resistance to antibiotics that *P. aeruginosa* exhibits. However, recent experiments with S1 mapping and reporter gene fusions have suggested that $P1_{mexA}$ is not a functional promoter (189). A large number of mutant strains that contain alterations to the *mexR* coding region and produce an inactive MexR repressor have been described; all of these mutations result in overproduction of the MexAB-OprM pump complex and enhanced efflux of a broad range of antibiotics, such as β-lactams, fluoroquinolones, tetracycline, and chloramphenicol (98, 186, 208, 246).

It has been proposed that the increased expression from the MexR-controlled promoter ($P2_{mexA}$) occurring in these strains is responsible for the observed MexAB-OprM hyperexpression (32). In some instances, the inability of the MexR derivatives to repress $P2_{mexA}$ has been confirmed to be due to the production of MexR proteins which are either unstable or compromised in their ability to dimerize or bind DNA (1). However, none of the antibiotics to which these mutants exhibit increased resistance induce expression, suggesting that the MexAB-OprM pump complex has evolved for other physiological roles, which may include the export of secondary metabolites (166) as well as a demonstrated role in the active efflux of an *N*-(3-oxododecanoyl)-homoserine lactone autoinducer signal associated with quorum sensing (163).

A recent crystal structure for apo-MexR has suggested that the MexR ligand may be an acidic peptide signaling molecule or the C terminus of a protein ligand, either of which could insert between the two winged-helix DNA-binding domains of a MexR dimer and induce a significant reduction in their spacing, thereby rendering the protein incapable of binding DNA (101). Further mutations that alter *mexAB-oprM* transcription but are located outside of *mexR* or the intergenic region (171, 208, 246) support the existence of unidentified regulatory proteins that influence the expression of this MDR pump complex, as does the linking of *mexAB-oprM* expression to the growth phase of cells (33). Interestingly, the MDR systems of *P. aeruginosa* have also been found to be subject to coordinate regulation, the expression of some systems decreased in response to increased levels of another (99).

Additional multiantibiotic-resistant pseudomonal mutants with an NfxC phenotype display resistance to chloramphenicol, fluoroquinolones, and trimethoprim due to overexpression of the *mexEF-oprN* MDR efflux operon (81). The locations of the mutations which produce this phenotype are unknown, but the overexpression is dependent on the presence of a functional MexT protein, which is a LysR-type transcriptional activator (Table 1) encoded by an upstream gene transcribed in the same direction as the *mexEF-oprN* operon (Fig. 1) (82, 123). As for MexR, none of the known substrates of MexEF-OprJ have any effect on the activity of MexT or expression of *mexEF-oprJ*, although simple overexpression of the MexT activator is sufficient to instigate transcription of this operon (81, 148). However, because most LysR-type regulators become active upon binding a cognate effector molecule, it has been suggested that the NfxC phenotype may result from the overproduction of a MexT effector molecule that normally induces production of the MexEF-OprN pump complex in response to the presence of its physiological substrate(s) (81). An alternative explanation involving mutations in an unidentified suppressor of *mexT* expression has also been put forward (123).

MexT may possess both repressor and activator functions, as it has been implicated in the downregulation of *oprD*, a gene which encodes a porin involved in the uptake of the β-lactam imipenem (148). Thus, the resistance to imipenem that *nfxC*-type mutants exhibit is due to reduced uptake of this antibiotic rather than its efflux by the overproduced MexEF-OprN pump complex. MexEF-OprN has also recently been proposed to influence the intracellular levels of *Pseudomonas* quinolone signal, a molecule involved in cell-to-cell signaling (83). This has been linked to the surprising finding that *nfxC*-type strains that overexpress MexEF-OprM, and hence have elevated antibiotic resistance, are actually less virulent due to a decrease in the production of extracellular virulence factors (83).

Overexpression of the *mexCD-oprJ* operon due to mutations in its divergently transcribed repressor of synthesis, NfxB (Fig. 1), can also confer multiantibiotic resistance on *P. aeruginosa* (169). NfxB autoregulates its own expression (204) and normally completely represses the *mexCD-oprJ* operon. Construction of *P. aeruginosa* strains in which three of the four *mex* operons are disrupted recently permitted the demonstration of *mexCD-oprJ* induction by acriflavine, tetraphenylphosphonium (TPP), ethidium bromide, or rhodamine 6G, all of which are substrates of this MDR pump complex (134). Although this result suggests a possible physiological role for MexCD-OprJ in the extrusion of toxic compounds, the mechanism by which the observed induction occurs needs to be identified before more concrete conclusions can be drawn.

Overexpression of the fourth *P. aeruginosa* multidrug resistance locus, MexXY, has been associated with aminoglycoside resistance (130, 233). In wild-type cells, MexXY also contributes to intrinsic antibiotic resistance by being inducible by the pump substrates tetracycline, erythromycin, and gentamicin, although again the mechanism by which this occurs is unknown (124). *mexXY* has been proposed to be regulated by MexZ, the product of a divergently transcribed gene (Fig. 1) that encodes a TetR family repressor (4). In addition to the four MDR transporter complexes discussed above, the extremely large *P. aeruginosa* genome appears to encode a number of additional potential efflux systems which show significant homology to known drug exporters (210).

Despite the recent demonstration that both MexCD-OprJ and MexXY are inducible by some antimicrobials, the majority of the currently available information indicates that the regulatory networks controlling the expression of MDR systems in *P. aeruginosa* are primarily intended to respond to physiological signals that are not related to the efflux of drugs. In support of this proposal is the identification of a number of *Pseudomonas putida* pump complexes that exhibit strong homology to MexAB-OprM. Although some of these systems are capable of exporting several structurally dissimilar antibiotics, the predominant physiological role of these efflux complexes appears to be the energy-dependent export of toxic organic solvents, which permits *P. putida* cells to grow in media containing relatively high concentrations of aromatic hydrocarbons (79, 175, 179). The identification of local and global regulatory

proteins, both of which are likely to be involved in controlling the expression of *P. putida* pumps in response to the presence of toxic organic solvents, provides strong evidence that aromatic hydrocarbon efflux is the authentic function of these systems (30).

In contrast to the *P. putida* organic solvent pumps, the four *P. aeruginosa* pump complexes discussed above export an extensive and overlapping array of structurally dissimilar drugs, although none of their local regulatory proteins appear to be involved in responding to the presence of these pump substrates and no alternative induction mechanisms have yet been elucidated. Similarly, ArpABC, another *P. putida* RND pump which can export structurally unrelated antibiotics, such as carbenicillin, chloramphenicol, erythromycin, and tetracycline, but does not contribute to organic solvent tolerance is also not induced by the addition of antibiotics or solvents (78). However, pseudomonal MDR pump complexes continue to have a substantial impact on antimicrobial resistance due to the many clinical strains which overexpress MDR transporters as a result of regulatory mutations, a trend which is becoming increasingly frequent in other established and emerging human pathogens.

Drug Transporter Expression in Other Pathogens and Antibiotic Producers

Increased synthesis of a chromosomal *S. aureus* MDR gene which encodes the MFS transporter NorA is particularly important in the emergence of fluoroquinolone-resistant clinical isolates of this species (76, 239, 241). The *flqB* mutation, a single T-to-G substitution in the 5′ untranslated region upstream of *norA*, has been shown to increase the half-life of *norA* mRNA 4.8-fold, resulting in NorA overproduction (37). It has been proposed that the increased mRNA stability is a consequence of changes to its secondary structure, which may affect an RNase III cleavage site involved in the degradation of this transcript (37). A single T-to-A change in the *norA* promoter region also appears to result in overexpression of NorA and subsequent fluoroquinolone resistance (75). Other mutations that produce increased resistance but lie outside the *norA* coding and promoter regions are currently uncharacterized (74, 136).

Although the regulation of *norA* expression is poorly understood, an unidentified 18-kDa protein has been demonstrated to bind to multiple sites in the DNA sequences upstream of the −35 region of the *norA* promoter (35). The binding of this 18-kDa protein is modified by ArlS, which appears to function as the transmembrane sensor of a two-component regulatory system, ArlR-ArlS. The first component of these systems is typically a transmembrane sensor protein that undergoes autophosphorylation upon binding a signal molecule present in the external environment (158). The phosphate is then transferred to the second component, a cytoplasmic response regulator which can be reversibly phosphorylated at a conserved aspartate residue (158). The activity of the response regulator is thereby altered so that it can then act to either stimulate or repress target gene transcription.

Although disruption of the gene for the ArlS transmembrane sensor resulted in upregulation of NorA expression and also altered the growth phase regulation of this transporter, it is not known if ArlS phosphorylates the 18-kDa protein directly or, alternatively, acts indirectly, e.g., via ArlR altering the transcription of a gene involved in the production of a ligand for the 18-kDa protein (35). The latter scenario could be related to the observation that *S. aureus* secretes a compound into the external medium that has the effect of lowering *norA* expression from the late logarithmic growth phase onwards (35). Although ArlR-ArlS also has a role in the regulation of virulence determinants in *S. aureus* (36), the expression of *norA* does not appear to be linked to that of known virulence determinants, suggesting that ArlR-ArlS modulates NorA expression in response to other physiological functions (35).

MDR pump overexpression has also made a substantial contribution to the emergence of many other bacterial species as serious human health threats. The intrinsic antibiotic resistance of the opportunistic pathogen *Stenotrophomonas maltophilia* has been attributed in part to MDR pumps (243), whereas elevated expression of the SmeDEF MDR pump in clinical isolates of this species has been correlated with increased levels of resistance to tetracycline, chloramphenicol, erythromycin, and quinolones (9). Although a second MDR pump homolog identified in this species, SmeABC, is not involved in drug efflux, the SmeC outer membrane component appears to be employed by another, as yet uncharacterized MDR pump which does transport antimicrobial compounds (100). The resistance of a clinical isolate of *Acinetobacter baumannii* to aminoglycosides and an extensive range of other antimicrobials has been confirmed to be due to an RND-type pump complex encoded by the *adeABC* gene cluster (111). While the basis for the increased contribution of the *adeABC* genes to drug efflux is unknown, three divergently transcribed genes which encode a putative two-component regulatory system (Table 1) and a transcriptional terminator-antiterminator have been proposed to be involved in the regulation of this MDR locus (111).

The inherent high-level resistance of the disease-causing bacterium *Burkholderia pseudomallei* to antimicrobial agents has also been partially attributed to an RND-type pump, AmrAB-OprA (133). Divergently transcribed from *amrAB-oprA* is the gene for AmrR, a TetR family protein proposed to be a transcriptional repressor of this operon. In the anaerobic pathogen *Clostridium perfringens*, the most widespread tetracycline resistance determinant is the plasmid-encoded *tet*(P) operon, comprised of two overlapping genes, which encode the TetA(P) tetracycline efflux pump and TetB(P), a protein likely to be involved in ribosome protection (72). Induction of this operon requires an unidentified, chromosomally encoded regulatory protein (73), whereas a transcriptional attenuation mechanism appears to prevent excessive production of TetA(P) in both the absence and presence of tetracycline (72). Although the mechanisms that lead to MDR overexpression and concomitant elevated antimicrobial resistance have yet to be identified in many of these species, it is immediately obvious that such processes represent one of the primary means by which bacteria subjected to drug exposure can acquire elevated resistance to these compounds.

For several antibiotic-producing bacteria, regulatory proteins have also been identified that control the synthesis of efflux pumps which are employed to provide protection against their own antibiotics. *Streptomyces* species, in particular, have been the object of considerable attention because these gram-

positive bacteria are responsible for the production of the majority of commercially important antibiotics. Uncharacterized regulators designated Pip proteins have been proposed to be involved in the regulation of drug transport genes in a range of *Streptomyces* species (187, 188). Isolation of the Pip protein from the genetically well-defined *Streptomyces coelicolor* indicated that it was a TetR family repressor that regulates the expression of the MFS antiporter Ptr, which confers resistance to the antibiotic pristinamycin I (34).

The self-resistance of *Streptomyces virginiae* to the antibiotic virginiamycin S is provided by the efflux pump VarS, production of which is governed by complex regulatory controls that include the transcriptional repressor BarA, which responds to a γ-butyrolactone autoregulator signal (138). Additionally, VarR, the product of a gene cotranscribed with *varS*, is a TetR family repressor that regulates *varS* transcription in a virginiamycin S-dependent manner (138). Another interesting example is the bacitracin peptide antibiotic-producing bacterium *Bacillus licheniformis*, which utilizes an ABC transporter, BcrABC, rather than a proton motive force-dependent exporter to provide self-resistance (164). An upstream two-component regulatory locus, *bacRS*, appears to control the expression of the *bcrABC* genes (139). The doxorubicin- and daunorubicin-producing organism *Streptomyces peucetius* also uses an ABC transporter, DrrAB, to confer self-resistance to these antibiotics (77). The role of activating expression of the synthesis and resistance operons for doxorubicin and daunorubicin in *S. peucetius* has been tentatively assigned to DnrI, which itself may be under the control of another positive transcriptional activator from the same gene cluster, DnrN (110).

Both the antibiotic biosynthesis genes and the self-resistance genes in all of these antibiotic-producing organisms appear to be under additional complex regulatory controls that respond to a number of environmental factors, in addition to linking their expression to differentiation events such as sporulation. In stark contrast to the majority of the drug pumps that confer antibiotic resistance to pathogenic bacteria, the expression of the transporters employed by antibiotic-producing organisms to provide self-resistance is intimately linked to the production, and hence the presence, of the relevant pump substrate(s). Understanding the function and regulation of these self-resistance mechanisms has considerable medical relevance, because in addition to aiding the exploitation of these species, their self-resistance mechanisms represent a pool of highly evolved determinants that can potentially be acquired by pathogenic bacteria.

Regulation of *N. gonorrhoeae* MDR Transporters

The *mtrCDE* operon of *Neisseria gonorrhoeae* encodes a multidrug efflux complex that is capable of exporting a range of MDR substrates that includes acriflavine, crystal violet, macrolides, and penicillin. However, the physiological role of this MDR pump is more likely to be reflected by its ability to extrude a range of structurally diverse hydrophobic agents, typically host-derived antimicrobial compounds such as fatty acids, bile salts, gonadol steroids, and antibacterial peptides, many of which coat mucosal sites colonized by *N. gonorrhoeae* (51). Transcription of *mtrCDE* can be increased by the addition of Triton X-100, an MtrCDE substrate which has a membrane-acting antimicrobial activity similar to that of the host-derived hydrophobic agents (184). Thus, in addition to being substrates of the efflux pump, hydrophobic agents are likely to act as natural inducers.

Analysis of the completed *N. gonorrhoeae* genome sequence identified the AraC family activator MtrA (Table 1), which was shown to be required for Triton X-100 enhancement of transcription (184). MtrA, like most AraC proteins (119), has an additional N-terminal domain which is likely to be involved in binding ligands, although it is currently unknown if this putative ligand-binding site plays a role in sensing the presence of toxic hydrophobic agents.

Divergently transcribed from *mtrCDE* is MtrR (156), a TetR family repressor (Table 1) that binds to a region which encompasses the *mtrCDE* promoter (106). Although MtrR appears to function solely as a modulator that is not involved in induction of *mtrCDE* expression (52, 106), mutations in either *mtrR*, the promoter for this gene, or the MtrR binding site can result in elevated *mtrCDE* expression and increased levels of resistance to hydrophobic agents (52, 106, 201, 202). Similar mutations have also been shown to produce increased resistance to the antibiotics azithromycin and erythromycin (242). However, disruption of MtrR binding alone does not fully explain the increases in *mtrCDE* transcription that result from some mutations that lie within the MtrR binding region and/or the *mtrR* promoter, suggesting the existence of other regulatory mechanisms and demonstrating the impact that *cis*-acting factors can have on efflux pump regulation (52).

A second *N. gonorrhoeae* efflux pump, FarAB, which utilizes the same outer membrane protein as the MtrCDE complex, MtrE (203), confers resistance to long-chain fatty acids (91). Surprisingly, although MtrR represses *mtrCDE*, it appears to enhance *farAB* expression (91), in addition to being either directly or indirectly responsible for controlling the expression of 14 other genes which may be linked to the establishment and/or maintenance of infections by *N. gonorrhoeae* (203). Overall, both the substrate range of the pumps and the available information on their regulation suggest that the function and expression of FarAB and MtrCDE are intimately linked to the virulence of *N. gonorrhoeae*.

The examples discussed to this stage have shown that the MDR systems of pathogenic bacteria are in general ill-adapted for the export of medically relevant drugs because of the requirement for mutations in their regulatory circuits before clinically significant resistance is observed. However, the following closer analysis of MDR regulatory controls from the model gram-negative and gram-positive organisms *E. coli* and *B. subtilis*, respectively, will demonstrate that this is not entirely the case. Additionally, the transcriptional repressors of tetracycline-specific pumps and the *S. aureus* plasmid-encoded QacA MDR determinant respond specifically to substrates of these pumps, and thus these regulatory proteins do not need to be bypassed before significant antimicrobial efflux can occur. These systems serve to illustrate in detail the better-characterized examples of proteins involved in the regulation of drug efflux genes, including examples of global activators and local activators or repressors.

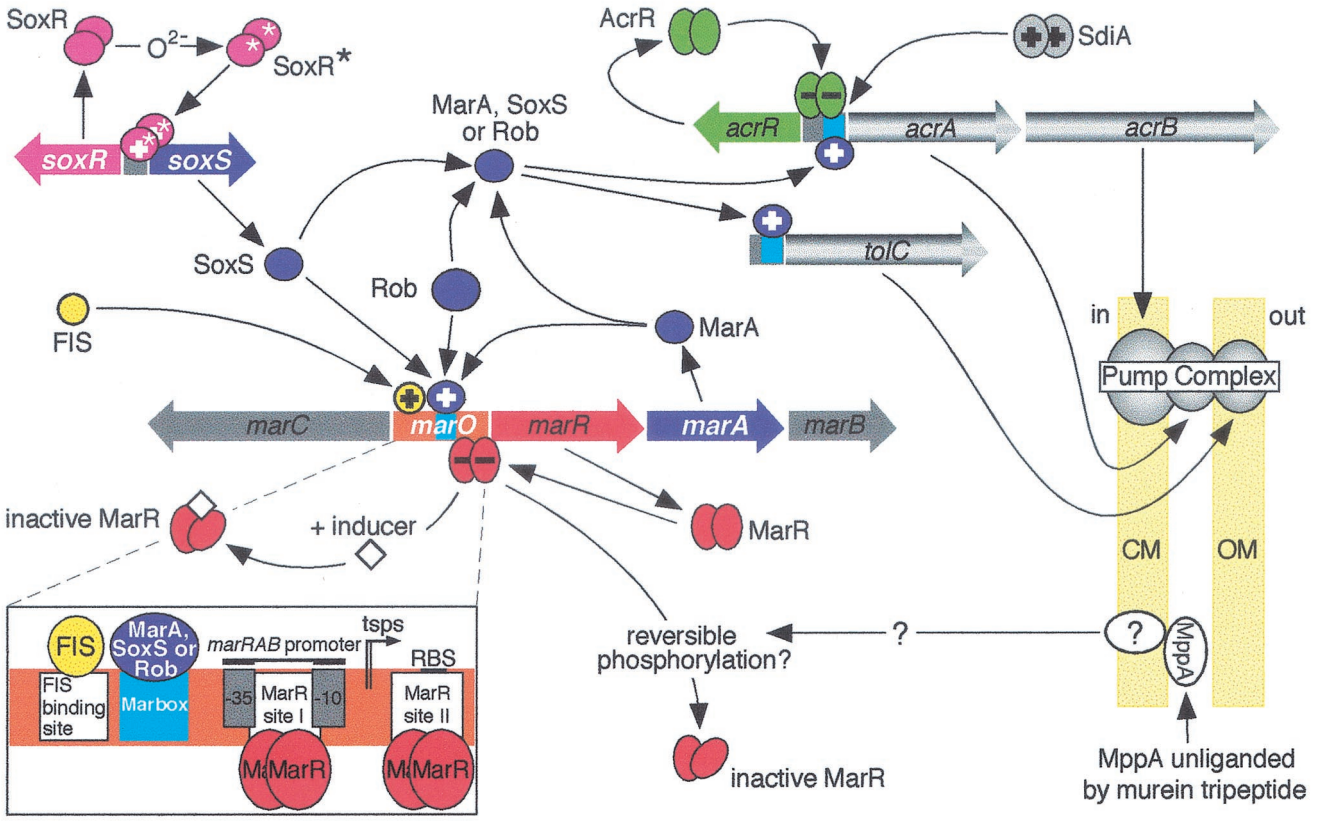

FIG. 2. Schematic representation of the known regulatory controls governing the expression of the *E. coli acrAB* and *tolC* genes. The AcrAB-TolC transport complex extrudes drugs across both the cytoplasmic (CM) and outer (OM) membranes (pale yellow shaded boxes). Excessive production of AcrA and AcrB is prevented (−) by the local dimeric repressor protein AcrR (green), whereas a regulatory protein involved in cell division, SdiA (grey), can increase (+) *acrAB* expression. However, activation of *acrAB* and *tolC* transcription occurs primarily because of the global regulatory proteins MarA, SoxS, and Rob (purple), any one of which can bind to a marbox (cyan) upstream of these genes. The intracellular level of MarA is controlled by MarR (red), a dimeric protein which binds to *marO* (orange) and represses (−) the expression of its own gene and the two others that constitute the *marRAB* operon. Binding of inducing compounds (diamonds) such as salicylate by MarR, in addition to the possible phosphorylation of MarR via a putative signal transduction pathway involving the periplasmic binding protein MppA, is proposed to transform MarR into a non-DNA-binding conformation, thereby permitting *marRAB* transcription to proceed. Hence, MarA protein is produced which can then bind as a monomer to the *marO* marbox upstream of the *marRAB* promoter, where it activates (+) transcription of *marRAB* and enhances the production of MarA. The ensuing highly elevated intracellular levels of MarA can then bind to marboxes adjacent to the promoters of *mar* regulon genes, such as *acrAB* and *tolC*, and activate their transcription. The MarA homologs SoxS and Rob can also bind to the *marO* marbox and activate *marRAB* transcription. The positive regulation by all three proteins on *marRAB* is enhanced by FIS (yellow), an accessory activating protein. SoxS is only produced upon conversion of the SoxR effector protein (magenta) into its active form (SoxR*) by superoxide-generating agents (O^{2-}). Rob, like SoxS, in addition to mediating increases in MarA synthesis, can also directly activate the expression of some genes that belong to the *mar* regulon, such as *acrAB*. The regulatory-protein-binding sites within *marO* are shown in finer detail in the bottom left corner. The positions of the −35 and −10 hexamers of the *marRAB* promoter, the ribosome-binding site (RBS) and transcription start points (tsps) for the *marRAB* operon are indicated. See text for other details. (Modified with permission from reference 49.)

CONTROL OF *E. COLI acrAB* MDR LOCUS BY GLOBAL ACTIVATORS

The *E. coli* AcrB RND pump functions as part of a tripartite complex which also consists of the membrane fusion protein AcrA (108) and the outer membrane channel protein TolC (11, 38), which is encoded in a remote part of the chromosome (Fig. 2). This MDR efflux system confers resistance to a diverse range of antimicrobials, such as dyes, detergents, fluoroquinolones, and many other lipophilic antibiotics, e.g., β-lactams, chloramphenicol, erythromycin, and tetracycline (108, 143, 150, 234). Immunoblotting has demonstrated that the AcrA protein was overexpressed in 9 of 10 *E. coli* clinical isolates which expressed high-level fluoroquinolone resistance (125). This has been attributed primarily to mutations that increase the production of the MarA global regulator, which modulates the expression of many genes, including *acrAB* and *tolC* (7, 149, 232). However, a number of insertions and single-amino-acid substitutions, duplications, and deletions that inactivate AcrR, a divergently transcribed local repressor of the *acrAB* operon (Fig. 2), have also been shown to result in enhanced expression of *acrAB* and increased fluoroquinolone resistance in clinical *E. coli* strains (71, 146, 230).

AcrR possesses a HTH DNA-binding domain that places it in the TetR family of repressors, together with the multidrug regulators MtrR from *N. gonorrhoeae*, QacR from *S. aureus*, and AcrS, a protein which is likely to control the expression of

AcrEF (109, 156), an additional MDR transporter from *E. coli* that is homologous to AcrAB (Table 1). Although AcrR represses both its own and *acrAB* transcription, it is not involved in the induction of *acrAB* and *acrR* expression in response to general stress conditions, such as 4% ethanol, 0.5 M NaCl, and entry into stationary growth phase; instead, these increases in transcription were attributed to an unidentified regulatory protein (107). Thus, it appears that the primary function of AcrR is to modulate *acrAB* expression, thereby preventing excessive production of the AcrAB pump, whereas MarA and related global regulators are primarily responsible for the actual induction of *acrAB* and *tolC* (Fig. 2; described in detail below).

A recent study has demonstrated that AcrAB is also positively regulated by SdiA, a protein which regulates cell division genes in a manner dependent upon quorum sensing (174). The link between AcrAB and quorum sensing, combined with the observation that some signal molecules utilized by bacteria for quorum sensing are similar to known AcrAB substrates, viz., the fluoroquinolones, led to the suggestion that AcrAB may have a physiological role in the export of non-freely diffusible quorum-sensing signals (174). Therefore, increases in AcrAB expression as growth rates slow (107, 176) may be related to increased levels of the quorum-sensing signals produced by *E. coli*.

MarA Global Activator

Although not involved in the response to the general stress conditions described above, transcriptional activation of *acrAB* expression is the predominant cause of multidrug resistance in strains that overexpress MarA or the closely related global regulators SoxS and Rob (7). The *mar* (multiple antibiotic resistance) regulatory locus (Fig. 2) consists of the *marRAB* operon and the divergently transcribed *marC*, which encodes a protein of unknown function, as does *marB* (7). The intracellular levels of the MarA global activator are controlled by the product of the first gene of the *marRAB* operon, MarR. Both of these proteins bind to *marO* (7), a region of DNA that separates the two transcriptional units and contains a large number of regulatory protein binding sites, within and around the *marRAB* promoter (Fig. 2).

MarA, a member of the AraC family of transcriptional activators (Table 1), activates its own transcription and that of a large number of *mar* regulon genes by binding to 20-bp DNA sequences known as marboxes that are located in the vicinity of the promoters for the target genes. For example, the MarA-binding site within the *marO* regulatory region is 16 bp upstream of the −35 region of the *marRAB* promoter (Fig. 2) (118, 177). Importantly, the *acrAB* promoter is also adjacent to a marbox at which MarA has been demonstrated to bind and activate transcription (7). In addition, overexpression of MarA or its homologs SoxS and Rob has also been demonstrated to result in increased synthesis of the TolC component of the AcrAB-TolC pump complex, which, in combination with the identification of a putative *mar*/*rob*/*sox*-box upstream of the *tolC* gene, strongly suggests that *tolC* also belongs to the *mar* regulon (11).

The transcriptional activation functions of MarA have also been proven to be global in nature by the demonstration that MarA can promote the transcription of genes encoding proteins of diverse functions, both in vivo and in vitro (7, 68). Gene array analysis of a strain constitutively expressing MarA has indicated that more than 60 *E. coli* genes are differentially regulated by this protein (15), whereas a second study employing an inducible MarA expression system identified an additional 67 MarA-regulated genes (165). However, although the total number of promoters directly activated by MarA has recently been estimated to be less than 40 (122), an even later report has shown that MarA is capable of activating a gene that possess a marbox which diverges substantially from the consensus sequence (14), suggesting that 40 may be an underestimate of the number of *mar* regulon promoters. One well-documented example of MarA activity is activation of transcription of *micF*, which produces an antisense RNA that downregulates the expression of *ompF*, a gene encoding an outer membrane protein that is a site for drug entry (29). Thus, the reduction in the rate of drug influx via OmpF, combined with the increased production of the AcrAB and TolC drug efflux proteins, represents a highly effective mechanism by which MarA can act to coordinate a response to the presence of toxic antimicrobials.

MarA transcriptional activation has several unusual features: it binds DNA as a monomer, and its degenerate 20-bp marbox binding sites are asymmetric, lacking any of the inverted or direct repeats characteristic of bacterial regulatory sequences. Additionally, MarA and the closely related SoxS protein are significantly smaller than other AraC family activators, such as *N. gonorrhoeae* MtrA, because of the complete lack of a MarA or SoxS ligand-binding domain (119). The crystal structure of MarA in complex with the marbox from the *marRAB* promoter has been solved (Fig. 3), revealing a protein possessing two separate HTH DNA-binding domains linked by a long α-helix, another highly unusual arrangement for a prokaryotic regulator (177). Since a typical HTH motif is only capable of recognizing 6 bp, in contrast to the requirement of an operator sequence of at least 11 to 12 bp for a DNA-binding protein to recognize sites that do not occur by chance in a bacterial genome (207), most bacterial regulatory proteins acquire two HTH motifs by forming oligomers. However, for MarA, the presence of two HTH motifs in a single polypeptide chain explains how this protein can function as a monomer. In order to facilitate MarA simultaneously contacting two successive major grooves with the recognition helices from its two HTH motifs, the DNA in the MarA-marbox complex is bent by approximately 35° (Fig. 3) (177). Alanine-scanning mutagenesis has confirmed that the N-terminal HTH of MarA, which contacts the more highly conserved portion of the marbox consensus sequence, contributes the majority of the important MarA-DNA interactions (42).

The orientations of marboxes and their distances from the −35 hexamers of *mar* promoters vary, but both are critical, presumably permitting the efficient formation of MarA-RNA polymerase-DNA ternary complexes (117). Mutagenesis has indicated that distinct solvent-exposed MarA residues are involved in the activation of transcription from each class of promoter, suggesting that MarA employs different mechanisms to promote RNA polymerase activity, depending on the orientation and location of each marbox (42). Utilization of nuclear magnetic resonance techniques to investigate the interaction of MarA with degenerate marboxes in solution revealed that por-

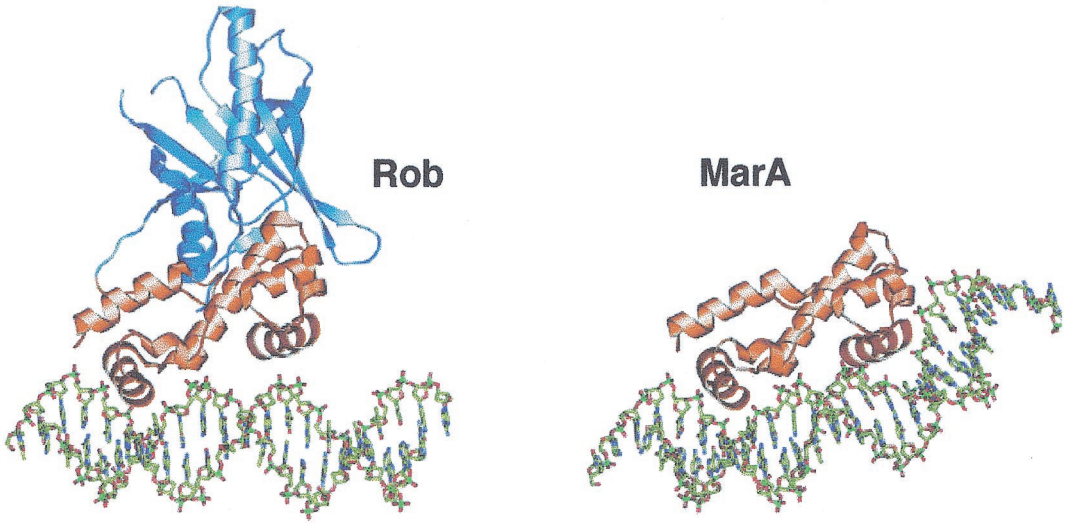

FIG. 3. Structures of Rob and MarA proteins bound to *micF* and *marRAB* marboxes, respectively. The highly homologous DNA-binding domains of Rob and MarA are colored orange, with their respective HTH recognition helices in brown and the additional C-terminal putative ligand-binding domain of Rob in blue. The N-terminal Rob HTH is inserted into the major groove, whereas the C-terminal HTH makes contacts only to the DNA backbone. In contrast, MarA induces a significant bend in the *marRAB* promoter to facilitate the placement of both HTH motifs in successive major grooves of *marRAB* marbox DNA. (Reprinted with permission from reference 89; kindly provided by Tom Ellenberger.)

tions of the DNA-bound form of MarA exist in a highly dynamic state (27). This led to the proposal that MarA possesses an inherent flexibility that grants it the ability to undergo significant rearrangements in order to accommodate variations in the DNA sequences of marboxes (27).

More recent evidence suggests that the primary mode of transcriptional activation by MarA first involves the formation of a complex between the activator and RNA polymerase, which can then scan the chromosome for *mar* regulon promoters more efficiently than RNA polymerase or MarA alone (116). The ability of a MarA-RNA polymerase complex to selectively identify marboxes that are adjacent to promoter sequences was suggested to be the mechanism by which MarA can distinguish real marboxes from the many marbox-like sequences present in the *E. coli* chromosome (116). A similar mechanism has also been proposed for the activity of the MarA homolog SoxS (see below) (46).

MarR, Antimicrobial-Sensing Repressor of *marRAB*

The MarR repressor, which is the product of the first gene in the *marRAB* operon, controls the intracellular levels of MarA and hence plays a crucial role in the MarA-mediated activation of *mar* regulon promoters (Fig. 2). MarR, like MarA, also binds within *marO* but at sequences distinct from the marbox, although a degree of competitive binding at *marO* between the two proteins does exist (118). MarR represses *marRAB* transcription by binding as a dimer to two distinct regions in *marO*, site I and site II, which are located downstream from the MarA binding site (Fig. 2) (120). MarR site I is positioned between the −35 and −10 regions of the *marRAB* promoter, an ideal binding site to prevent access by RNA polymerase, whereas site II does not appear to be required for repression or binding by MarR at site I (Fig. 2) (120).

A crystal structure obtained for MarR in the presence of the compound salicylate indicated that MarR contains a DNA-binding domain belonging to the winged-helix family (8), as was found for another MarR family member, the *P. aeruginosa* MexR protein (101). The MarR α3 and α4 helices constitute the HTH motif, whereas β-sheets contribute to the formation of the "wings." The location of the predicted α4 recognition helix, which is likely to make contacts to the DNA major groove, is in good agreement with mutagenesis studies that had previously assigned this region a role in DNA binding (5). In contrast to MexR, for which the proposed induction mechanism was suggested to be facilitated by the inherently high degree of flexibility observed for this protein (101), MarR appears to be a much more rigid protein whose structure is stabilized by a number of salt bridges (8), indicating that these two family members are likely to function by distinct methods.

Overall, MarA activates expression of the *mar* regulon, including *acrAB*, *tolC*, and *marRAB*, whereas MarR acts to downregulate this response by repressing the synthesis of MarA. Although overexpression of MarA from a plasmid is sufficient to activate the *mar* regulon genes (40), the addition of the antibiotics tetracycline and chloramphenicol (50), weak aromatic acids, such as salicylate, and a structurally diverse range of other compounds, such as the uncoupling agent carbonyl cyanide *m*-chlorophenylhydrazone and the redox-cycling compounds menadione and plumbagin (6, 200), have all been shown to cause induction of *mar* regulon expression. Two independent mechanisms for *mar* regulon induction have been identified, each of which has been proposed to act by suspending the repressor abilities of MarR. First, salicylate, plumbagin, 2,4-dinitrophenol, and menadione have been demonstrated both to induce the *marRAB* operon in vivo and also to interfere with the binding of MarR to *marO* DNA in vitro (6). This suggests that MarR can directly bind a broad range of ligands, the outcome of which is its dissociation from *marO*, MarA synthesis, and, hence, *mar* regulon activation (Fig. 2).

Interestingly, in order to produce the aforementioned crystal structure of MarR, high concentrations of the inducer salicylate were found to be necessary. Salicylate was observed to be bound at two sites on the surface of each MarR subunit, in the vicinity of the proposed α4 recognition helix (8). Although these observations were highly suggestive of a potential influence on MarR-DNA interactions, it remains to be seen if binding of salicylate at these sites has any effect on protein conformation or is even physiologically relevant (8).

A second mechanism by which the repressor activities of MarR appear to be interrupted involves the binding of the cell wall component murein tripeptide and its transfer into the cytoplasm by MppA, a periplasmic binding protein (97). An *mppA* null mutant exhibits phenotypes consistent with derepression of the *mar* regulon, a process that requires a functional MarA but also alters the expression levels of other genes which are not part of the *mar* regulon (97). It has been suggested that a low level of murein tripeptide in the periplasm is an indicator of stress, sensed by MppA, which activates a signal transduction pathway that ultimately produces phosphorylated, presumably inactive, MarR (97). However, the suggestion that MarR contains a functional aspartyl phosphorylation-dephosphorylation sequence (97) has been cast in some doubt by the MarR crystal structure (196). Hence, MarR is capable of sensing the presence of deleterious substances and/or environmental conditions by at least one and possibly two independent mechanisms, both of which would cause *marRAB* to be derepressed and the ensuing activation of the *mar* regulon, including *acrAB*.

MarA Homologs SoxS and Rob

SoxS, the effector of the *soxRS* global superoxide response (*sox*) regulon, and Rob, which binds the *E. coli* chromosomal origin of replication, are MarA homologs which, in addition to activating *marRAB* transcription by binding to *marO*, have also been shown to directly activate expression from promoters of genes belonging to the *mar* and *sox* regulons in vitro and in vivo (Fig. 2) (69, 70, 129, 215). Thus, it is not surprising that the majority of the residues identified in the MarA DNA-bound crystal structure as being important for forming DNA contacts represent amino acids that are conserved in the SoxS and Rob proteins (177). Elevated levels of SoxS and Rob have also been shown to specifically increase the transcription of *acrAB* (107, 234), which is the most important feature of the *mar* multidrug resistance phenotype, since even for strains constitutively expressing *marA*, *soxS*, or *rob*, deletion of *acrAB* results in hypersensitivity to many antimicrobial agents (129, 150, 215).

For SoxS to have a significant effect on *acrAB-tolC* transcription in wild-type cells, the expression of the *soxS* gene must first be activated by superoxide-generating agents via the conversion of SoxR, a divergently transcribed local transcriptional activator, into an active form (Fig. 2) (121, 147). Rob, on the other hand, has only a modest effect on the basal level of expression from the *marRAB* promoter, despite being naturally expressed at a relatively high level (121) and its ability to activate *acrAB* expression in a *mar* deletion strain (215). However, unlike SoxS and MarA, Rob contains an additional C-terminal domain, which the crystal structure of Rob complexed with DNA (Fig. 3) has revealed may be involved in the binding of an uncharacterized effector molecule with the potential to alter Rob activity (89). The Rob C-terminal domain has recently been demonstrated to be required for the apparent binding of the compound dipyridyl in vitro and also for the activation of Rob transcriptional stimulation by dipyridyl in vivo, although it is currently uncertain if this occurs via a direct interaction with Rob (182).

An unexpected finding from the Rob-DNA crystal structure (in this case with *micF* marbox DNA) was that, whereas the N-terminal HTH of Rob was inserted into the DNA major groove, the C-terminal HTH made contacts only to the DNA backbone (Fig. 3) (89). As a result, the Rob-bound *micF* marbox DNA was not bent, as had been observed for the MarA-bound *mar* marbox DNA (Fig. 3), although Rob does appear to bend the promoter DNA of some *mar* regulon genes in solution (70, 119). Because the *mar* and *micF* promoters used in the MarA and Rob crystallization studies represent different marbox promoter classes, it is possible that both of these proteins possess the ability to bind DNA with or without their C-terminal HTH inserted into the major groove. Although the role of the Rob C-terminal HTH is still in doubt (119), the use of alternative DNA-binding modes may provide a means by which these proteins can vary the activation mechanisms they employ to suit the different classes of promoters that they bind.

Yet another protein involved in the regulation of *acrAB-tolC* is FIS, a nucleoid-associated global regulatory protein that modifies transcriptional activity in response to various growth conditions and can also bind to a site within *marO* just upstream of the marbox (Fig. 2). FIS is proposed to limit the overall level of negative superhelicity and also stabilize the local DNA architecture of certain promoters (225), which, for the *marRAB* promoter, provides an additional twofold stimulation to MarA-, SoxS-, and Rob-mediated activation of transcription (Fig. 2) (121).

Regulatory systems akin to *mar* appear likely to be widespread, with evidence suggesting that many bacterial species contain closely related genes (26, 66, 90, 126). For example, in a clinical isolate of *Salmonella enterica*, increased quinolone resistance has been attributed to a point mutation that produced a constitutively active SoxR protein, presumably leading to elevated expression of *sox*/*mar* regulon genes, such as *acrAB* and *tolC* (86). *Enterobacter aerogenes*, an increasingly important cause of respiratory tract infections, also contains a *marRAB* operon analogous to that of *E. coli* which has been implicated in antibiotic resistance (24). Altogether, the activation of *acrAB* and *tolC* expression by several global regulators in response to a broad range of stress conditions suggests that the primary physiological function of the AcrAB-TolC complex in *E. coli* is the export of a wide variety of stress-related toxic compounds encountered by this organism in its normal environment, such as a demonstrated role in the efflux of fatty acids and bile salts (108, 143, 221).

Other Regulators of *E. coli* MDR Pumps

Neither MarR nor MarA has any effect on the expression of another chromosomally encoded *E. coli* multidrug pump, EmrAB, which, despite being an MFS transporter, also appears to form a tripartite complex with the TolC outer membrane porin in a manner analogous to the function of RND-

type pumps (96). Induction of *emrAB* is controlled by the local repressor EmrR, the product of the first gene of the *emrRAB* operon. Disruption of the *emrR* gene via a frameshift mutation has been demonstrated to be responsible for increased expression of EmrAB and elevated resistance to the antibiotic thiolactomycin (105). EmrR is a member of the MarR family of repressors (Table 1) and has been shown to be capable of repressing the *marRAB* promoter when overexpressed (211). Carbonyl cyanide *m*-chlorophenylhydrazone, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone, and 2,4-dinitrophenol, structurally unrelated substrates of the EmrAB pump which induce *emrRAB* expression (105), have been confirmed to be directly bound by EmrR, one ligand per repressor dimer (20).

EmrR acts by binding to an imperfect inverted repeat centered around the −10 region of the *emrRAB* promoter, an interaction that can be disrupted in vitro by addition of the ligands carbonyl cyanide *m*-chlorophenylhydrazone, 2,4-dinitrophenol, tetrachlorosalicylanilide, and nalidixic acid (238). Thus, like MarR, EmrR functions to sense the presence of toxic compounds, but differs in that EmrR exerts direct control over the expression of the EmrAB pump rather than acting indirectly through a global regulator. Interestingly, EmrR was originally described as MprA, the regulator of the plasmid-borne *mcb* operon, which encodes microcin B17 (104). The addition of compounds that inactivate EmrR repression of *emrRAB* transcription conversely resulted in EmrR-mediated repression of the main *mcb* operon promoter. Additionally, EmrR was required for positive regulation of a second *mcb* promoter that controls expression of microcin export and immunity genes, the products of which have also been implicated in the export of some fluoroquinolone antibiotics (104).

Two-component signal transduction systems involved in modulating the expression of several *E. coli* drug transporters have also been identified. Overexpression of the EvgA response regulator from the *evgSA* two-component system produces an elevated level of resistance to MDR substrates, including benzalkonium, crystal violet, deoxycholate, doxorubicin, erythromycin, rhodamine 6G, and sodium dodecyl sulfate (145). These increases have been attributed primarily to increased expression of the *yhiUV* MDR transporter (144), although the observed increase in resistance to deoxycholate is due in part to enhancement of the expression of another drug transport operon, *emrKY*, by EvgA (145). It is intriguing that *yhiUV* and *emrKY* encode an RND-type and an MFS transporter, respectively, both of which have been shown to require the TolC outer membrane channel (144).

An additional two-component regulatory system, BaeSR, has recently been demonstrated to activate the transcription of yet another *E. coli* MDR transporter, an RND-type pump complex encoded by the *mdtABC* operon, which confers resistance to bile salts, novobiocin, and deoxycholate (12, 137). Both *mdtB* and *mdtC* appear to encode RND pumps that interact, perhaps as heteromultimers, with the MdtA membrane fusion protein, whereas a fourth gene in the same operon, *mdtD*, encodes an MFS-type pump that is not required for drug resistance (12, 137). In addition to these *E. coli* examples, it is interesting that in several other species, such as *Acinetobacter baumannii*, *Stenotrophomonas maltophilia*, and *S. aureus* (Table 1), the expression of a number of MDR transporters also appears to be under the control of two-component regulatory systems which are designed to respond to specific external environmental stimuli (158). Thus, the two-component-regulated MDR transporters, by extension, are likely to perform specific export functions in response to these unknown signals.

A comparison of the nucleotide sequences upstream of known and hypothetical MDR transporters has suggested that the regulation of *E. coli* MDR efflux genes is likely to be considerably more complicated than the available experimental evidence indicates. Possible regulatory sequences that have been identified include a marbox and an AcrR-binding site upstream of *acrEF*, which encodes an AcrAB homolog, and an EmrR-binding site upstream of *acrAB* (178). Sequences upstream of the *marRAB* and *emrRAB* operons were also proposed to be bound by a hypothetical regulator (178). In combination with the available information on the local, global, and two-component regulatory systems known to control *E. coli* drug transport pumps, it would appear that MDR regulatory proteins in this organism are likely to be involved in a complex web of interactions governing the expression of their target genes. Although the individual pumps may have specific physiological roles, *E. coli* cells may be capable of coordinating the expression of at least some of these MDR transporters in response to multiple threats, making efficient use of their ability to extrude overlapping ranges of substrates.

RELATED LOCAL AND GLOBAL ACTIVATORS CONTROL MDR GENE EXPRESSION IN *B. SUBTILIS*

The chromosome of the gram-positive bacterium *Bacillus subtilis* encodes two highly similar MFS MDR transporters, Bmr and Blt. These proteins show 51% amino acid identity and confer very similar levels of resistance to an identical range of toxic substances when overexpressed (3, 141). They are each regulated by the product of an adjacent gene encoding a transcriptional activator belonging to the MerR family, BmrR (2) and BltR (3), respectively (Table 1). Although these activators have similar N-terminal DNA-binding motifs, their C-terminal domains, which are known to be responsible for inducer binding in this family of proteins (113, 212), show little homology to each other or to any other protein sequences currently in the DNA sequence databases, suggesting that distinct ligands are bound by each protein. However, the ligands for BltR have yet to be identified, which, in combination with the inability of known Blt substrates to induce expression of the *blt* gene, suggests that the multidrug-transporting abilities of Blt are likely to be entirely fortuitous (3).

Interestingly, the second gene in the *blt* operon, *SpAT* (Fig. 4A; formerly known as *bltD*), encodes an enzyme that acetylates polyamines, such as the natural cellular constituent spermidine. Despite this finding, alterations of polyamine levels had no influence on expression of the *blt* operon (115). Thus, although spermidine is a known substrate of Blt (237), the suggestion that the efflux of polyamines may be the primary role of Blt remains uncertain.

BmrR, Local Transcriptional Activator of *bmr* Expression

In contrast to Blt, Bmr is expressed under normal growth conditions, and disruption of its gene therefore produces cells that are hypersensitive to MDR substrates. In addition to its

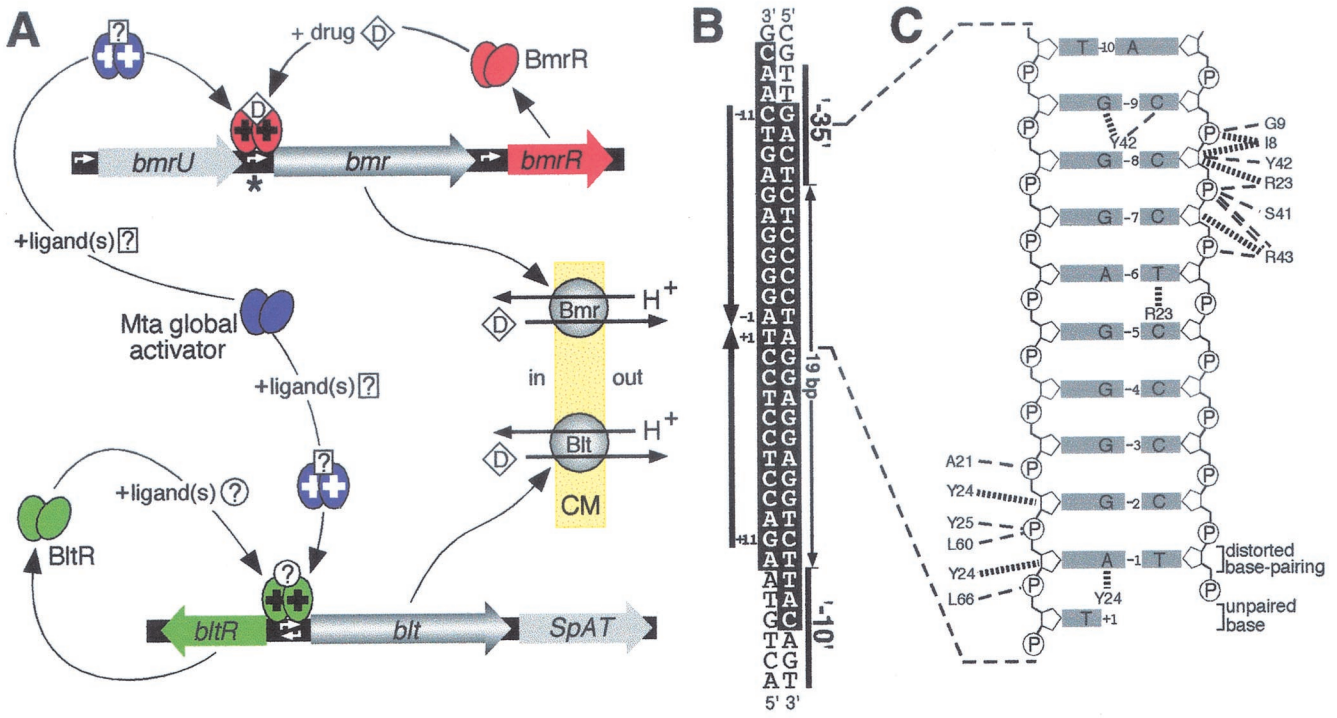

FIG. 4. (A) Regulation of expression of *B. subtilis* MDR genes *bmr* and *blt*. A BmrR dimer concurrently bound to both *bmr* promoter DNA and drugs (D) can correctly orient the −10 and −35 hexamers of this promoter to facilitate the binding of RNA polymerase. White arrows indicate the locations of promoters and also the direction in which transcription occurs from these sequences. Because many of the substrates of the Bmr MDR transporter are also ligands of BmrR (red ovals), activation (+) of *bmr* expression can occur in response to the presence of these deleterious compounds, permitting drug efflux across the cytoplasmic membrane (pale yellow; CM) in exchange for protons (H^+) to occur. The global regulatory protein Mta (purple ovals) and the local activator of the *blt* operon, BltR (green ovals), are likely to act in the same fashion as BmrR, although inducing ligands for these proteins have yet to be identified (?). Mta activates both the *bmr* and *blt* genes by binding to the same DNA sequence as the local regulators. The *blt* operon also encodes SpAT, a polyamine acetyltransferase, whereas Bmr expression can result from transcription initiated at either its own promoter or the promoter of *bmrU*, an upstream gene of unknown function. (B) The DNA sequence from the region indicated by an asterisk in A, which contains the *bmr* promoter (P_{bmr}). The −10 and −35 hexamers of P_{bmr} and the unusually large 19-bp spacing between these hexanucleotides are indicated, while the large arrows denote the imperfect inverted repeat (labeled −11 to +11) within P_{bmr} that constitutes the BmrR binding site (2). Bases protected from DNase I digestion by BmrR bound to P_{bmr} are highlighted in white. (C) DNA contacts made by BmrR to the half-site that encompasses positions −1 to −10 in B. Also shown is the thymine from position +1, which in the BmrR-DNA complex was observed to be no longer base-paired to its partner, whereas the adenine and thymine bases at position −1, although significantly displaced, still formed a distorted base pair. Thin dashed lines indicate hydrogen bonds, and thick broken lines indicate van der Waals interactions between BmrR amino acids and bases (grey boxes) or the phosphates (P) and deoxyribose rings (pentagons) that form the DNA backbone. Note that the sequence depicted in C differs at position −8 from that shown in B because the experimentally determined data best fit a GC base pair at this location rather than the AT that was actually present in the BmrR-DNA complex. (Panel A modified with permission from reference 49; panel C reprinted with permission from reference 245.)

own promoter, *bmr* can also be cotranscribed with *bmrU*, an upstream gene of unknown function (Fig. 4A), which suggests that Bmr and BmrU may have related but currently unknown physiological roles (115). However, more importantly from a drug resistance perspective, the local transcriptional activator, BmrR, can mediate increases in expression from the promoter immediately upstream of *bmr* after binding some of the synthetic substrates of the Bmr pump (Fig. 4A), such as astrazon orange, diethyl-2,4′-cyanine, rhodamine 6G, and TPP (2, 113, 114, 227). The drug-bound form of BmrR, in addition to possessing the ability to activate expression from the *bmr* promoter, also exhibits an improved affinity, in comparison to apo-BmrR, for *bmr* promoter DNA (2, 196, 227). Thus, it appears likely that the Bmr/BmrR system exists at least partially to provide protection against toxic, lipophilic, cationic substances that *B. subtilis* may encounter in its natural environment (2).

Like other MerR family members, BmrR binds as a dimer to an imperfect inverted repeat located between the −10 and −35 hexamers of a target promoter that exhibits an unusually large spacing of 19 bp (Fig. 4B) (2). This spatial arrangement places the −10 and −35 regions of the *bmr* promoter on opposite sides of the DNA helix, a conformation that is incompatible with RNA polymerase binding. In the case of *blt*, a 1-bp deletion that altered its promoter spacing to 18 bp was sufficient to cause a marked increase in expression (3). For promoters regulated by MerR and another family member, SoxR, 2-bp deletions in their spacer regions in each case produced a promoter that exhibited highly elevated transcription levels independently of their respective activator proteins (58, 157). Binding of the MerR activating ligand is known to have an effect similar to that of the 2-bp deletion, as it allows the protein to initiate the partial untwisting of promoter DNA, which suggested that activation by MerR involves the

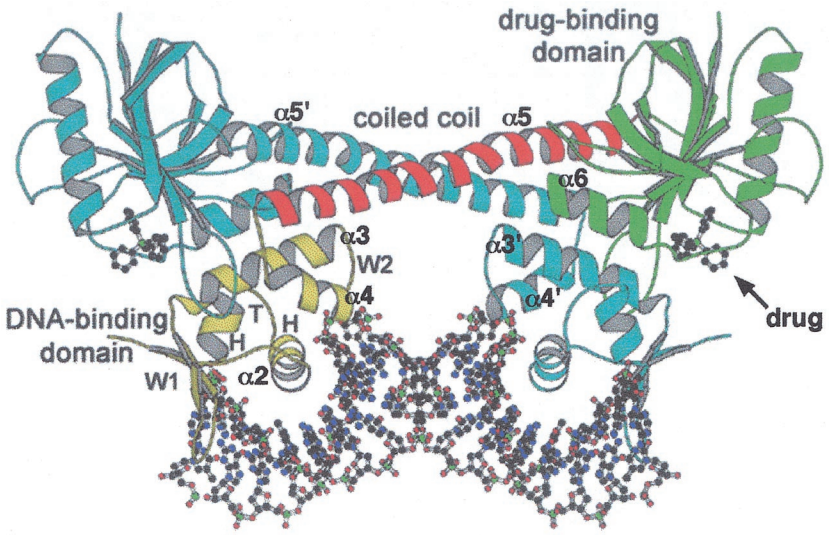

FIG. 5. Structure of a BmrR dimer in the drug- and DNA-bound tripartite complex. One polypeptide chain is colored yellow for the N-terminal winged-helix DNA-binding domain, red for the α5 linker helix, and green for the C-terminal drug-binding domain. The locations of selected helices are indicated for this polypeptide, as are the positions of the α3′, α4′, and α5′ helices in the second (cyan) polypeptide. Also labeled for the first monomer is the HTH (H, α1; T, turn; H, α2 recognition helix) and the two wings, W1 (sheets β2 and β3) and W2 (helices α3 and α4). The DNA and drug (tetraphenylantimonium) molecules are represented as a ball and sticks (carbon, black; nitrogen, blue; oxygen, red; and phosphorus/antimony, green). (Reprinted with permission from reference 245; kindly provided by Richard Brennan.)

production of a promoter with a more favorable spacing (212).

Structure of Tripartite BmrR–Drug-Activated DNA Complex

The finer details of the mechanism by which MerR family proteins activate expression from promoters under their control have recently been elucidated by determination of the crystal structure for the tripartite complex of BmrR bound concurrently to DNA and a drug (245). BmrR was found to consist of a DNA-binding domain which is linked to the C-terminal two-thirds of the protein responsible for ligand binding by a long helix, α5 (Fig. 5) (245). The tight packing of the drug-binding domain from each polypeptide against the DNA-binding domain of the second polypeptide in a BmrR dimer, in combination with an antiparallel coiled coil that α5 forms with α5′ from the other subunit, constitutes an extensive dimerization interface (Fig. 5). The BmrR DNA-binding domain belongs to the winged-helix superfamily and consists of an HTH (α1, the α2 recognition helix, and their connecting turn), and two additional wings, W1 and W2 (Fig. 5) (245). The combination of these DNA-binding elements ensures that BmrR makes a large number of contacts with *bmr* promoter DNA (Fig. 4C), however, at the same time, the promoter remains accessible for RNA polymerase binding, an essential feature for a transcriptional activator protein (245).

A startling revelation from the BmrR-drug-DNA structure was that the *bmr* promoter had been distorted to reposition the −35 and −10 sequences onto the same face of the DNA, so that both hexamers were now simultaneously available for RNA polymerase binding and subsequent initiation of transcription (245). The distortion of *bmr* promoter DNA was found to be facilitated by a novel mechanism that disrupted the base-pairing interactions of the 2 bp (AT) at the center of the pseudodyad within the *bmr* promoter (+1 and −1 bp in Fig. 4B and C). The AT base pair at position +1 was observed to become completely unpaired (Fig. 4C), with the adenine and thymine sliding away from each other, while the AT base pair at position −1, although significantly displaced, was still capable of forming a distorted base-pairing interaction (245). The combined effect produced an operator sequence that was "bunched up" in the middle, shortening the spacing between the −10 and −35 hexamers of the *bmr* promoter DNA by a distance equivalent to 2 bp (245). Interactions of BmrR residues Tyr24, Tyr25, Lys60, and Lys66 with the DNA backbone serve to stabilize the distorted base pair (Fig. 4C), maintaining *bmr* promoter DNA in this transcriptionally active configuration. Thus, drug-bound BmrR acts to facilitate the synthesis of Bmr, which can then extrude the deleterious compounds to ensure the continued survival of the cell.

Binding of Antimicrobial Ligands by BmrR

It has been demonstrated that the first 119 residues of BmrR, which comprise the N-terminal DNA-binding region of this protein, can be completely removed and the C-terminal portion expressed as a separate domain, BRC, which retains full dimerization and ligand-binding abilities (113). BRC has been shown to bind one ligand molecule for each BRC and two per BRC dimer (113), consistent with the two drug molecules, one per binding pocket, present in the full-length BmrR-drug-DNA complex (Fig. 5) (245). Prior to the recent work on the full-length protein, earlier X-ray crystallization studies on apo-BRC and BRC bound to the ligand TPP revealed a ligand-binding pocket that was lined with hydrophobic and aromatic amino acids, in addition to the unusual feature of a charged residue deeply buried in the core of the protein, specifically, Glu134 positioned at the base of the binding site (Fig. 6) (244).

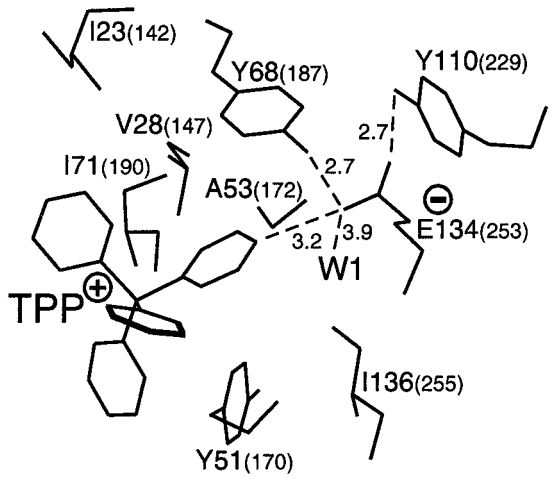

FIG. 6. Structure of a TPP molecule depicted relative to the location of BRC binding-pocket residues that interact with this ligand. The negatively charged Glu134 BRC residue (the equivalent BmrR amino acid number is shown in parentheses) provides the crucial electrostatic interaction with the delocalized positive charge carried by the TPP phenyl rings. Ile23, Val28, Ala53, Ile71, and Ile136 all form van der Waals contacts with TPP, whereas the aromatic side chains of Tyr51 and Tyr68 stack against TPP rings. Tyr68 also hydrogen bonds to Glu134 to stabilize its negative charge, as does Tyr110 and a water molecule (W1), which replaces the hydrogen bond of Tyr33 (BRC α2) that was broken because of the displacement of BRC α2 by the entry of TPP into the binding site. The stabilizing hydrogen bonds are indicated by broken lines, as is the crucial electrostatic contact, with distances given in angstroms. (Reprinted with permission from reference 244.)

Normally considered an energetically unfavorable position, the burial of the negatively charged Glu134 residue in the core of unliganded BRC is neutralized by hydrogen bonds to the hydroxyl groups of three BRC tyrosine residues, Tyr33, Tyr68, and Tyr110. Tyr33 is located in the short, flexible α2 helix of BRC (BmrR α6), which is positioned across the entrance to the binding pocket in the absence of a coactivator.

A negative residue (BRC Glu21), located on the surface of the protein adjacent to BRC α2, is thought to initially attract positively charged drugs to the entrance of the binding pocket (244). The BRC α2 appears likely to be highly flexible and fluctuate between a folded and partially unfolded conformation, facilitating entrance of the ligand into the binding site proper. However, the contribution of α2 (BmrR α6) to the specificity of ligand binding appears to be minimal, based on the mutagenesis of several BRC α2 residues, which was observed to have little effect (227). Upon ligand binding, displacement of BRC α2 removes the side chain of Tyr33 from the protein core, thus breaking one of the H bonds stabilizing BRC Glu134. However, a water molecule replaces Tyr33 and forms an H bond with Glu134 (Fig. 6), maintaining the stabilization of the internal BRC negative charge (244). Once the ligand has entered, binding occurs via an electrostatic contact between the negative charge of the carboxylate group of the BRC Glu134 residue at the base of the binding site and the partial positive charge carried by the phenyl rings of TPP (Fig. 6) (244). Van der Waals contacts between TPP and the surrounding hydrophobic amino acids that line the binding pocket strengthen the interaction, in addition to the stacking of TPP rings against aromatic side chains (Fig. 6) (244).

Model building with a second BmrR ligand, rhodamine 6G, demonstrated that the BRC binding pocket is capable of accommodating this compound as well, predicting a much closer electrostatic contact between the carboxylate group of the buried glutamate residue and the positive charge of the amino ethyl group in rhodamine 6G, explaining the 100-fold-greater affinity of BRC for rhodamine 6G than for TPP (244). Site-directed mutagenesis to replace the buried BRC Glu134 with a neutral alanine residue almost completely abolished the binding of five of the six BmrR ligands that were tested, confirming the importance of this electrostatic interaction (227). The binding of the sixth ligand was reduced by less than twofold, suggesting that although Glu134 is important, it is not essential for all BRC ligands (227). Changes to an other six BRC residues that the BRC-TPP structure had indicated were involved in ligand binding had lesser effects, consistent with the weaker nature of their interactions with TPP.

Interestingly, in a number of cases, although an alanine substitution decreased the binding affinities for some compounds, the same change failed to alter the binding of other drugs, whereas still other ligands exhibited an up to threefold increase in their binding affinities for that mutant (227). The inconsistent binding abilities that these mutants demonstrated towards different ligands are thought to reflect the intrinsic nature of the BRC binding site, by which differential interactions of binding pocket residues with various ligands permit the accommodation of structurally diverse drugs (227). The ability of individual compounds to form various interactions with different subsets of the residues lining the binding pocket lends support to the proposal that the ligand-binding sites of MDR proteins are likely to represent a compromise between maximizing their range of ligands and retaining high affinities for the individual compounds.

In addition to Glu253 (BRC Glu134), the structure of the BmrR-drug-DNA tripartite complex revealed that electrostatic interactions also occurred between the positively charged tetraphenylantimonium (a TPP analog) and two other negatively charged BmrR residues, Asp47′ (where the prime indicates the second subunit in a BmrR dimer) and Glu266 (245). This finding helps to explain the unexpected weak effect that the mutation of BRC Glu134 had on the binding of a single compound (227). Asp47′, located in the DNA-binding domain of the second monomer in a BmrR dimer, is of particular interest, because although this residue is absent in BRC, both BmrR and BRC have equal affinities for ligands (114), whereas the residue equivalent to BmrR Glu266 was disordered in the BRC-TPP complex (244). Because of the lack of a structure for the BmrR-DNA complex, it is also uncertain how drug binding is coupled to the transition of BmrR into a state in which it distorts *bmr* promoter DNA and activates *bmr* transcription. However, the unwinding of α6 in BmrR (BRC α2) upon drug binding has been proposed to act as the signal for conversion of BmrR into a transcriptional activator (244). The repositioning of α6 could influence DNA binding directly because of alterations in its interactions with α3′ and α4′ from the DNA-binding domain in the second polypeptide of a BmrR dimer (Fig. 5). Alternatively, unwinding of α6 is likely to affect the α5 linker helix (Fig. 5), which could therefore directly transmit a

signal from the drug-binding domain to the DNA-binding domain within each polypeptide (245).

Global *B. subtilis* MDR Gene Regulator Mta

In addition to their local activators, both *blt* and *bmr* are known to be subject to global regulation by Mta (Fig. 4), another MerR-like regulator (13). Although full-length Mta did not activate transcription of the *blt* or *bmr* gene, expression of MtaN, a truncated version containing only the N-terminal DNA-binding domain, resulted in increased *blt* and *bmr* expression. MtaN was found to bind directly to the *blt* and *bmr* promoter elements at the same position as the specific BltR and BmrR regulators (Fig. 4) (13). Presumably, deletion of the C-terminal ligand-binding domain of Mta mimics the in vivo effect of a bound inducer molecule. A crystal structure for apo-MtaN revealed a dimeric protein containing a winged HTH DNA-binding domain that is largely structurally comparable to that of BmrR (43). However, differences in the orientation of the α5 dimerization helix and the first wing of the DNA-binding domain suggest that MtaN interacts with DNA in a manner distinct from the transcriptional activation mechanism of BmrR (43).

The ligand-binding domain of Mta, unlike those of BltR and BmrR, shows significant homology to other proteins, most notably to thiostrepton-induced TipA, a global regulator that has been implicated in the control of multidrug transport in *Streptomyces lividans* (13, 61), although there are currently no clues as to the nature of the compound that binds to Mta and activates its global regulatory functions. Comparable to what has been demonstrated for *tipA*, *mta*, in addition to encoding a full-length Mta protein, is also likely to encode an individually expressed C-terminal domain that would sequester the putative Mta inducer in order to limit the positive-feedback loop controlling Mta expression (13).

In summary, despite the assignment of a putative physiological role for Blt, the different expression patterns of *blt* and *bmr* during normal growth, and the lack of any common BmrR and BltR ligands, the control of *bmr* and *blt* expression by Mta indicates that *B. subtilis* likely utilizes the very broad substrate specificities of these two MDR pumps as part of a global response to some stress, such as the presence of hydrophobic toxins. In addition, BmrR can independently activate *bmr* transcription in response to a wide range of toxic compounds. However, the genetic organization of these pumps indicates that they are also both likely to perform unidentified specific physiological roles. From an efficiency viewpoint, it certainly makes sense for cells to employ a single transport protein for multiple functions, provided that this can be achieved without causing any disruption to cellular metabolism.

TetR, EXQUISITELY SENSITIVE REGULATOR OF TETRACYCLINE EFFLUX GENES

The most common form of resistance to tetracycline in gram-negative bacteria is because of a group of related MFS TetA efflux pumps which export tetracycline complexed with a divalent metal cation, normally Mg^{2+} (25). Unlike the majority of genes for gram-positive tetracycline transporters, the expression of *tetA* genes in gram-negative organisms is controlled in each case by the product of a divergently transcribed gene, the TetR repressor (59). As for the TetA determinants, the TetR proteins whose genes have been sequenced were classified into eight classes on the basis of amino acid sequence similarity, TetR A to E, G, H, and J, all of which show greater than 40% homology (190). Interestingly, the *tetA*(Z) and *tetR*(Z) genes from the gram-positive bacterium *Corynebacterium glutamicum* also show strong similarity to their gram-negative counterparts (217).

By far the best-characterized TetR protein is the 207-amino-acid Tn*10*-encoded TetR(B), although a number of detailed structural analyses have also been carried out on the 218-amino-acid TetR(D) determinant encoded on the plasmid RA1, which has 63% amino acid identity with TetR(B) (59). In the absence of tetracycline, TetR binds to the *tet* operator sequences overlapping the promoter of the *tetA* gene and represses its transcription (Fig. 7A) (59). Induction occurs on the binding of a tetracycline-Mg^{2+} complex by TetR, which causes a conformational change in the protein so that it can no longer bind to the *tet* operator, freeing up the *tetA* promoter for transcription (60). TetR has a far greater affinity for tetracycline-Mg^{2+}, the intracellular form of the antibiotic, than the drug does for ribosomes, ensuring that expression of *tetA* occurs well before protein synthesis is inhibited, requiring only nanomolar concentrations of tetracycline-Mg^{2+} (59).

Contribution of *tet* Promoters and Operators

The oppositely orientated *tet* genes encoded by Tn*10*, *tetR*(B), and *tetA*(B) represent the system for which regulation has been best characterized. The locations of the *tetA*(B) promoter, P_{tetA}, and the two divergent *tetR*(B) promoters, P_{tetR1} and P_{tetR2}, which partially overlap both the *tetA*(B) promoter and each other, are illustrated in Fig. 7A. Although the fully induced transcriptional activity of P_{tetA} is 10-fold stronger than that of the combined *tetR* promoters, it is counterbalanced by a *cis*-acting sequence in the *tetA*(B) mRNA that reduces the efficiency of its translation by approximately 10-fold, and therefore, even under induced conditions, excessive synthesis of TetA(B) is prevented (59).

The TetR(B) protein is purified as homodimers that bind noncooperatively to two adjacent inverted repeats known as the *tet* operators O_1 and O_2, which overlap the *tetA*(B) and *tetR*(B) promoters (Fig. 7A) (128). Thus, TetR(B) binding to DNA can repress *tetA*(B) transcription and also autoregulate expression of its own gene, an essential aspect of the fine tuning of *tetA*(B) transcription by this system. Also of importance are the differences between the sequences of O_1 and O_2 (Fig. 7A), which explains the approximately fourfold-greater affinity that TetR(B) has for O_2 (80). Binding of TetR(B) at O_1 represses both genes, whereas if only O_2 is occupied, *tetA*(B) continues to be downregulated, but *tetR*(B) transcription can occur almost completely unhindered from P_{tetR2} (Fig. 7A) (128). Thus, small decreases in the amount of operator-bound TetR(B) will serve to increase the synthesis of the repressor but not TetA(B), preventing accidental synthesis of the transporter.

Induction of *tetA*(B) expression can therefore occur only in the presence of tetracycline, as the conversion of operator-bound TetR(B) into the non-DNA-binding, induced TetR-

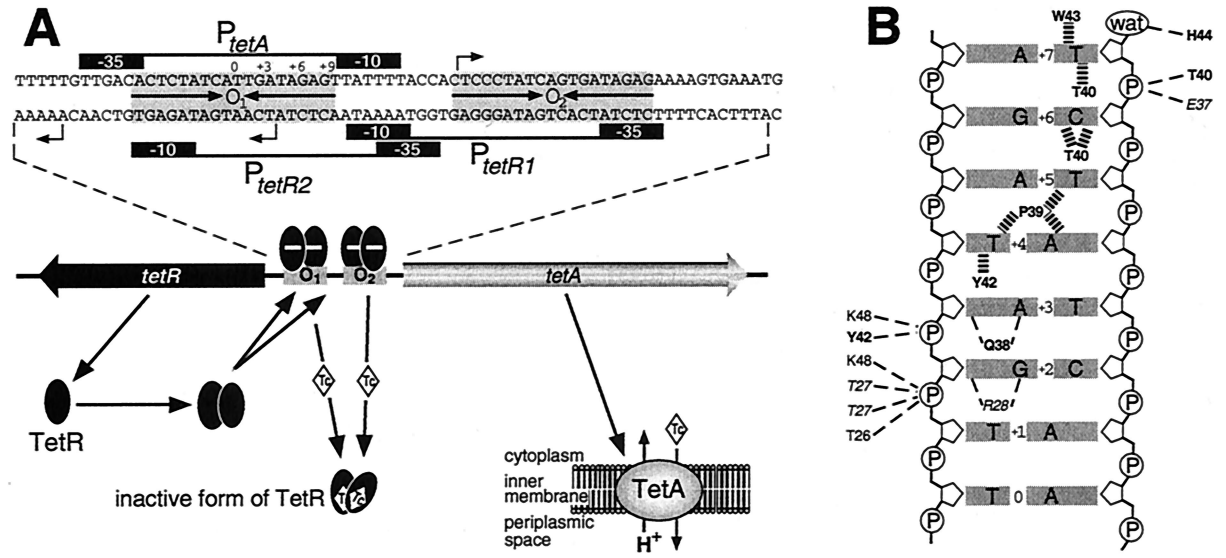

FIG. 7. Control of *tetA* transcription by TetR. (A) The DNA sequence of the Tn*10 tet* intergenic region containing the *tet* operators and promoters is shown, with the base pairs that form the O_1 and O_2 inverted repeats shaded light grey. The *tetA* promoter P_{tetA}, the two *tetR* promoters P_{tetR1} and P_{tetR2}, and their associated transcription start points (right angle arrows) are also indicated. The product of the *tetR* gene (black ovals) forms dimers and binds to O_1 and O_2, preventing the expression (−) of both genes. Binding by TetR of the intracellular form of tetracycline, a tetracycline-Mg^{2+} complex, results in a conformational change so that TetR can no longer bind the *tet* operators. The ensuing initiation of *tetA* transcription protects the cell from tetracycline because of subsequent production of the membrane-bound TetA protein, which exports tetracycline-Mg^{2+} complexes in exchange for protons (H^+). See text for other details. (B) DNA contacts formed by operator-bound TetR. The nucleotides from a *tet* operator half-site, representing the 0 to +7 positions of O_1 in A, are depicted as grey boxes attached to the phosphate-ribose backbone. The contacts made by the DNA-reading head of one monomer in a TetR dimer to the bases in that operator half-site are shown as thin dashed lines for hydrogen bonds and thick broken lines for van der Waals interactions. TetR amino acids from the HTH recognition helix (α3) are in boldface type, those from elsewhere in the HTH motif are in italics, and Thr26 and Lys48 are additional residues from outside the HTH which make *tet* operator DNA contacts. The proline (P39) in the TetR recognition helix transfers binding of the TetR reading head from nucleotides on one strand of the operator half-site to nucleotides on the other strand. A hydrogen bond formed between a water molecule (wat) and His44 in the crystal structure mimics an interaction that would otherwise take place between His44 and the DNA backbone phosphate at position +8 of O_1. (Panel B reprinted with permission from reference 153.)

tetracycline-Mg^{2+} complex is required for both O_1 and O_2 to be unoccupied by the repressor. The autoregulation of the *tetR*(B) gene ensures that only the minimum level of the repressor required to fully occupy O_1 and O_2 will be present in the cell, a situation that, combined with the extreme sensitivity of TetR(B) to the presence of tetracycline, provides for very fast and efficient induction of TetA(B) synthesis in response to the presence of the drug. The concomitant increase in TetR(B) expression ensures that first *tetA*(B) transcription and then that of *tetR*(B) can be rapidly brought to a halt once sufficient amounts of the TetA(B) protein have been produced to clear the antibiotic from the cell (59). Thus, by having synthesis of the repressor always occur both prior and subsequent to induction of TetA(B) expression, this regulatory system can maintain strict control of *tetA*(B) transcription at all times.

Structure of a TetR Dimer

Solving the crystal structure for the inducer-bound form of TetR(D) revealed a protein consisting of 10 α-helices, α1 to α10 (Fig. 8) (60). TetR homodimers contain two polypeptide chains, α1 to α10 from the first monomer and α1′ to α10′ from the second (60). The dimerization of TetR is principally achieved by the antiparallel helices α8 and α10, which interact with the symmetry-related α8′ and α10′ to form a four-helix bundle (Fig. 8) (60). The function of this four-helix bundle has been investigated by site-directed mutagenesis to alter residues in the hydrophobic core of the protein, which produced a TetR(B) derivative with altered dimerization specificity and confirmed the contribution of α8 and α10 to oligomerization (192). In addition to the hydrophobic core of the dimer, solvent-exposed residues on the periphery of the dimerization surface have also been demonstrated to be involved in protein-protein recognition by the four-helix bundle (191, 195).

TetR Binding to *tet* Operator

In each TetR polypeptide chain, the helices α1 to α3 form the DNA-reading head, which is connected to the core of the protein, α5 to α10, through α4 (Fig. 8) (60). Although the HTH motif common to many regulatory proteins comprises α2 and α3, α1 is important for the DNA-binding process because of its stabilizing effect on the HTH structure (153). This confirmed previous studies in which TetR variants that had been constructed with N-terminal deletions of various lengths were found to be incapable of binding DNA (18). Also contributing to DNA binding are the N-terminal residues of α4, which participate in the formation of the hydrophobic center of the DNA-binding domain, further stabilizing it in the operator-bound complex (153).

In the induced TetR-tetracycline-Mg^{2+} complex, the two

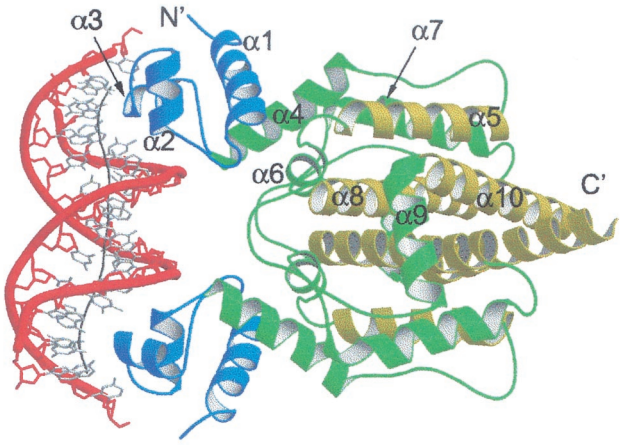

FIG. 8. Structure of the TetR dimer-*tet* operator DNA complex. The α-helices in each polypeptide are colored blue for the DNA-binding domain (α1 to α3), yellow for the rigid α-helices (α5, α8, and α10), and green for those that undergo conformational changes upon induction (α4, α6, α7, and α9). The α3 and α3′ recognition helices fit into successive major grooves of *tet* operator DNA, which is represented as red for the phosphate-ribose backbone and grey for the bases. A grey line also delineates the curvature of operator DNA induced by TetR binding. (Reprinted with permission from reference 153; kindly provided by Winfried Hinrichs.)

HTH DNA recognition helices in the TetR dimer, α3 and α3′, were found to be separated by 39.6 Å, preventing the repressor from binding to successive major grooves in B-form DNA, which are about 34 Å apart (60). This explained the observed inability of TetR to bind the *tet* operator and the subsequent induction of *tetA* expression that occurs in response to tetracycline. The recent publication of the crystal structure for a TetR(D)-DNA complex confirmed this, revealing that α3 and α3′ are separated by 36.6 Å when bound to a 15-bp *tet* operator fragment (−7 to +7 positions of O_1; Fig. 7A), compared to the TetR-tetracycline-Mg^{2+} complex, in which the gap between α3 and α3′ had increased by 3 Å (153).

Each of the HTH motifs in the TetR(D)-DNA complex were found to be bound to the corresponding major groove of operator DNA, whereas no contacts were made with the minor groove (Fig. 8) (153). Except for the central 3 bp, TetR contacted all 15 bp of the operator fragment, either via a series of hydrogen bonds to backbone phosphate groups and the bases themselves or by a lesser number of van der Waals interactions with the bases (Fig. 7B) (153). This proved to be in excellent agreement with data provided by the saturation mutagenesis of one *tet* operator half-site, which had previously indicated the importance of positions +2 to +7, with bp +1, +8, and +9 making lesser contributions to TetR binding and the central (position 0) base pair being required only for correct spacing of the two half-sites (Fig. 7B) (236).

Although the recognition helix of the TetR HTH is unusually short, the TetR(D)-DNA structure showed that TetR compensates by making a large number of contacts with the DNA (Fig. 7B), involving all recognition helix residues except Leu41, which forms part of the hydrophobic core stabilizing the three-helix bundle (153). Other TetR residues that form DNA contacts are located in the first helix of the HTH, the turn of the HTH, and both of the interhelical loops connecting the HTH to the adjacent helices (Fig. 7B) (153). Many of the TetR residues shown to contact DNA in this structure had been identified previously through mutagenesis (10, 53, 235).

Unlike many protein-DNA interactions, the TetR-DNA interface contains no water molecules, reflecting a high degree of structural complementarity between the DNA-binding domains and the *tet* operator (153). Contributing to this close fit is Pro39 in the recognition helix of the TetR HTH, a somewhat unusual feature, as proline residues occur infrequently in the α-helices of globular proteins because of their propensity to be helix breakers (229). Pro39 also plays an important role in TetR DNA recognition, as it contributes to the ability of TetR to make contacts to both sides of the *tet* operator strand by forming van der Waals interactions with each of the nucleotides at the +4 position and a single nucleotide at +5 bp (Fig. 7B) (153). This enables TetR to contact backbone phosphates and nucleotides on one side of the major groove (+2 and +4) before transferring via Pro39 to contact points on the other side of the groove (+4 to +7) (Fig. 7B), an arrangement previously predicted on the basis of methylation protection experiments that utilized mutations in both the *tet* operator and TetR (56).

TetR-Ligand Interactions

The results of a large number of biochemical, structural, and mutagenesis studies on TetR(B) and TetR(D) are in good agreement, which provides an exceptionally detailed picture of the induction process (55, 60, 135, 193, 194, 205). In addition to identifying amino acids involved in the induction process, random mutagenesis has even produced a TetR mutant that exhibits increased, not decreased, affinity for the *tet* operator upon inducer binding, which, in combination with the relative nontoxicity of tetracycline to eukaryotic cells, has facilitated its use to tightly regulate gene expression in eukaryotes (16, 44, 62).

The two tetracycline-Mg^{2+} complexes bound by a TetR homodimer were found to be located in binding tunnels buried in the core of the protein (60). Each of the two binding tunnels accommodates a single tetracycline-Mg^{2+} complex and is formed by helices contributed by both polypeptide chains, the first by α helices 4 to 8 and 8′ and 9′ and the second by 4′ to 8′ and 8 and 9 (Fig. 8). Although the structure of inducer-bound TetR revealed the specific interactions involved in tetracycline-Mg^{2+} binding, it failed to identify which of the two similar-sized openings in each tunnel had been used as the entry point to the complex by tetracycline-Mg^{2+} (60). Solving the structure for tetracycline-free TetR brought to light that the opening adjacent to the C terminus of α9′ (and α9 for the second tunnel) was twice as large as the opening C-terminal to α4 in the inducer-free TetR dimer (151).

Molecular modeling of the protein surface confirmed the opening next to the C terminus of α9 as the site of tetracycline-Mg^{2+} entry, indicating that the inducer would have a strong preference for attraction to the area surrounding the α9′ and α9 entrances (151). Because of the constricted nature of the binding tunnel, a model for induction could then be proposed in which the tetracycline A ring of the tetracycline-Mg^{2+} complex (Fig. 9A) is forced to enter through the α9′ opening end-on (151, 152). The subsequent events triggered by the

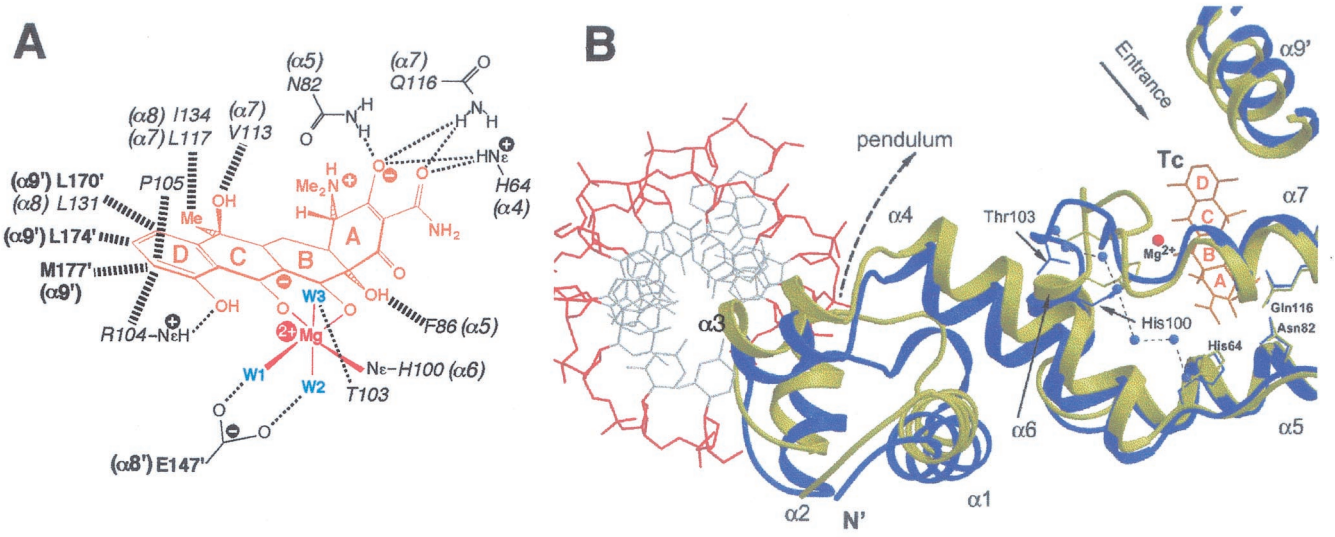

FIG. 9. (A) Diagrammatic illustration of specific interactions between TetR(D) binding-tunnel residues and a tetracycline-Mg^{2+} complex. Tetracycline is colored orange, with its four rings labeled A to D and the methyl groups shown as Me. The three water molecules coordinated by the Mg^{2+} atom (red) are represented by W1 to W3 (blue). Residues from the TetR dimer that contribute to the binding of this tetracycline-Mg^{2+} complex are shown in italics for those from the first polypeptide and in bold type for those from the second polypeptide. The α-helices in which these amino acids are located are also indicated except for the Thr103, Arg104, and Pro105 residues, which form part of the interhelical loop connecting α6 to α7. Thin dotted lines indicate hydrogen bonds, and thick broken lines show hydrophobic interactions. Equivalent residues from the other binding tunnel in a TetR dimer contact a second tetracycline-Mg^{2+} complex; see text for other details. (B) TetR conformational changes that occur upon binding a tetracycline-Mg^{2+} complex. The α1 to α8 helices of one monomer and the α9′ helix from the second polypeptide in a dimer are represented in blue for DNA-bound TetR and yellow for the induced tetracycline-Mg^{2+}-bound form. The DNA phosphate-ribose backbone is shown in red, bases in grey, tetracycline (Tc) in orange, the Mg^{2+} atom in red, and the chain of water molecules that constitute the water zipper in the induced conformation as blue spheres. The pendulum-like motion of α4 upon tetracycline-Mg^{2+} binding leads to significant displacement of the attached α1-α3 DNA-reading head, so that the α3 recognition helix can no longer contact the major groove of *tet* operator DNA at the same time as the α3′ recognition helix from the second polypeptide in a TetR dimer. The location of the entrance to the TetR binding tunnel is also indicated, whereas the sliding door motion of α9′ that closes this entrance in the induced form is also apparent. See text for other details. (Panel A reprinted with permission from reference 60; ©1994, American Association for the Advancement of Science. Panel B reprinted with permission from reference 153; kindly provided by Winfried Hinrichs.)

entry of tetracycline-Mg^{2+} into the binding site are listed below.

(i) Residues located at the base of the binding site, His64 (α4), Asn82 (α5), and Gln116 (α7), form tight hydrogen bonds to the side chains of the A ring of tetracycline in addition to a hydrophobic contact made by Phe86 (α5) (Fig. 9A). Water molecules displaced by the entry of the inducer are proposed to be released through the tunnel opening C-terminal to α4. (ii) The imidazole group of His100 (α6) binds to Mg^{2+}, which triggers the formation of a hydrogen bond between Thr103 and W3, one of the three Mg^{2+}-coordinated water molecules (Fig. 9A). The resulting 2.5-Å induced movement of Thr103 produces a partial unwinding of α6, which loses its last two residues, Leu101 and Gly102, because of their inclusion in the formation of a type II β-turn with residues His100 and Thr103 (Fig. 9B). Both Gly102 and Thr103 have now undergone a significant displacement from their original positions, an event that triggers the conformational changes associated with induction (Fig. 9B). (iii) Formation of the new β-turn displaces all residues of the adjacent interhelical loop, resulting in reorientation of Arg104 and Pro105 so that they can form part of the hydrophobic pocket surrounding tetracycline ring D (Fig. 9A). Val113, Leu131, Iso134, Leu170′, Leu174′, and Met177′ complete the region of nonpolar van der Waals contacts in this area which serve to guide the tetracycline-Mg^{2+} complex into its final position (Fig. 9A). Movement of these residues to accommodate tetracycline induces motions in α9′ and the loop connecting α6 and α7, which partially closes the binding tunnel entrance. (iv) By rotating 90°, the carboxylate group of Glu147′ (α8′) can hydrogen bond to Gly102 of the new β-turn and also to the remaining two Mg^{2+}-coordinated water molecules, W1 and W2 (Fig. 9A). (v) Formation of a salt bridge between Asp178′ (α9′) and Arg104 draws α9′ closer and completes the "sliding door" movement of α9′ residues, closing the entrance to the binding pocket. Determination of the interspin distances between nitroxide groups attached to residues near the tetracycline-binding tunnel confirmed the relatively large movement of α9′ that occurs during induction (224).

Conformational Changes in TetR That Transmit the Induction Signal

In the uninduced forms of TetR, the C-terminal residues of α6 form a hydrophobic contact region with the central part of α4, the helix connecting the core of the protein to the DNA-reading head (151, 153). Upon tetracycline-Mg^{2+} binding, the formation of the new β-turn at the C terminus of α6 creates space at the contact surface with α4, which moves to fill it (Fig. 9B). Because the C terminus of α4 is anchored by the hydrogen bonding of His64 to tetracycline ring A (Fig. 9A and B), the movement of α4 is similar to that of a hinge, serving to reposition the attached α1 to α3 helices in a pendulum-like motion

so that they adopt the non-DNA-binding configuration (Fig. 9B). The reorientation of the interhelical turn connecting α6 to α7 permits the formation of a chain of water-mediated hydrogen-bonds (water zipper) that forms along the length of α4 (Fig. 9B), which assists in locking the protein into a non-DNA-binding conformation by linking the DNA-binding domain to the tetracycline-Mg^{2+} binding site (151).

Of critical importance to the induction process are the hydrophilic interactions which coordinate the Mg^{2+} atom. Their importance has been validated by solving the structures of TetR-tetracycline-Mg^{2+} complexes in which Mg^{2+} has been partially or completely removed by the addition of a divalent metal cation-chelating agent (152). When only tetracycline occupied the binding tunnel, the structure of TetR was almost unchanged from that of inducer-free TetR, confirming the essential role of Mg^{2+} in initiation of the conformational reorganization associated with induction (152).

The reduced freedom imposed by the interaction of residues from both polypeptides with each tetracycline-Mg^{2+} complex combined with the hydrogen bond interactions formed by the water zipper serve to freeze TetR into its inducer-bound conformation. It has been suggested that the transition from a free to frozen state has a high entropic potential, which may be partially compensated for by increased motion in some parts of the protein (228). For example, the flexibility of the loop connecting α8′ to α9′ has been shown to increase upon ligand binding, contributing to an overall smaller change in entropy (228). Although this loop is highly variable between the different TetR classes both in length and in composition, deletion of this region produced mutants deficient in tetracycline binding and induction, consistent with a proposed role in the closure of the α9′ sliding door (19). Replacement of the loop with various numbers of alanine residues indicated that the loop has to be a minimum length to permit efficient inducer binding and the associated conformational changes (193). Engineered TetR derivatives that contain cysteine residues in mobile regions of the protein have also been used to confirm, by the formation of ligand-dependent disulfide bonds, that conformational changes in TetR upon tetracycline-Mg^{2+} binding occur both in vitro (222) and in vivo (223).

A new group of tetracyclines, the glycylcyclines, also demonstrate the highly specific nature of the TetR-ligand interaction. In addition to not being recognized by the TetA transporter, these tetracycline derivatives also cause reduced induction of its synthesis. The glycylcyclines contain a glycylamido substituent that causes steric hindrance with the TetR sliding door helix, α9′, thereby preventing the ligand from reaching the binding position within TetR that is necessary to trigger the conformational changes that are required for induction of *tetA* transcription (154).

QacR, REGULATOR OF *S. AUREUS qacA/B* MDR GENES

The nearly identical *S. aureus qacA* and *qacB* MDR genes encode the first bacterial MDR transporters to be described (218), their discovery closely following that of mammalian P-glycoprotein. Investigation of the *S. aureus* QacA and QacB MFS pumps has been stimulated by the prevalence of *qacA* and *qacB* genes on multiresistance plasmids, such as pSK1, which are commonly isolated from clinical strains of this important human pathogen (88, 160, 219, 220). QacA utilizes the proton motive force to drive the efflux of more than 30 different toxic monovalent or bivalent, cationic, lipophilic compounds which belong to 12 distinct chemical classes (102, 131, 132, 159). Like the majority of compounds exported by MDR pumps, the QacA substrates make ideal antimicrobials because their positive charge attracts them towards the interior of bacterial cells, which are typically negatively charged compared to the exterior environment, while the lipophilic nature of these molecules aids their passage through cellular membranes. In fact, many of the QacA substrates have either a current clinical application, such as the quaternary ammonium antiseptics benzalkonium and cetrimide (199), the antibacterial diamidine compound pentamidine (127), and the biguanidine antiseptic chlorhexidine (41, 65), or they have been used historically as antiseptics, e.g., the dye proflavine.

The *qacR* gene, which is divergently transcribed from all known *qacA/B* determinants, encodes a *trans*-acting transcriptional repressor of the *qacA/B* genes (47) (Fig. 10A). QacR contains a poorly defined N-terminal HTH DNA-binding motif that places it in the TetR family of regulatory proteins (Table 1) (183). The QacR DNA-binding site, IR1, is a relatively large operator sequence immediately upstream from the *qacA* promoter (P_{qacA}), consisting of 15 bp in each half-site, separated by a 6-bp spacer sequence. In the absence of QacA substrates, QacR binds IR1 and downregulates *qacA* transcription. However, the transcription of *qacA* has been demonstrated to increase in a *qacR*-dependent manner in response to a structurally diverse range of monovalent and bivalent compounds that represent almost all of the substrate classes exported by the QacA pump (Fig. 10A and B) (47; S. Grkovic, M. H. Brown and R. A. Skurray, unpublished data). A direct interaction of QacR with these inducing compounds was subsequently confirmed by the binding of QacR to IR1 operator DNA that had been disrupted in vitro by the separate addition of eight structurally diverse QacA substrates, including benzalkonium, dequalinium, ethidium bromide, chlorhexidine, and rhodamine 6G (47) Thus, like TetR, QacR acts as a sensor molecule that can facilitate increases in transcription in response to the presence of toxic compounds because the ligand-bound form of this protein is incapable of binding operator DNA (Fig. 10A).

Interestingly, analysis of P_{qacA}-reporter gene fusions (47) indicated that QacR does not repress transcription from P_{qacA} to anywhere near the same extent that the related regulator TetR represses transcription from the *tetA* promoter (59). Thus, besides inducing expression in response to substrates of the QacA pump, the basal level of *qacA* expression that is afforded by QacR can mediate protection against compounds which are substrates of the MDR pump but are not strong ligands of the regulatory protein. The demonstration that a simple 2-bp change in the central 6-bp spacer region of IR1 could produce an operator exhibiting greater affinity for QacR (48) supports the proposal that the QacR/IR1 system evolved to provide a substantial basal level of *qacA* transcription. In comparison, the highly specific nature of the TetA-TetR system does not require any significant level of *tetA* expression in the absence of tetracycline.

Gene fusions have also permitted demonstration that expression of *qacA* is induced in response to the plant alkaloid

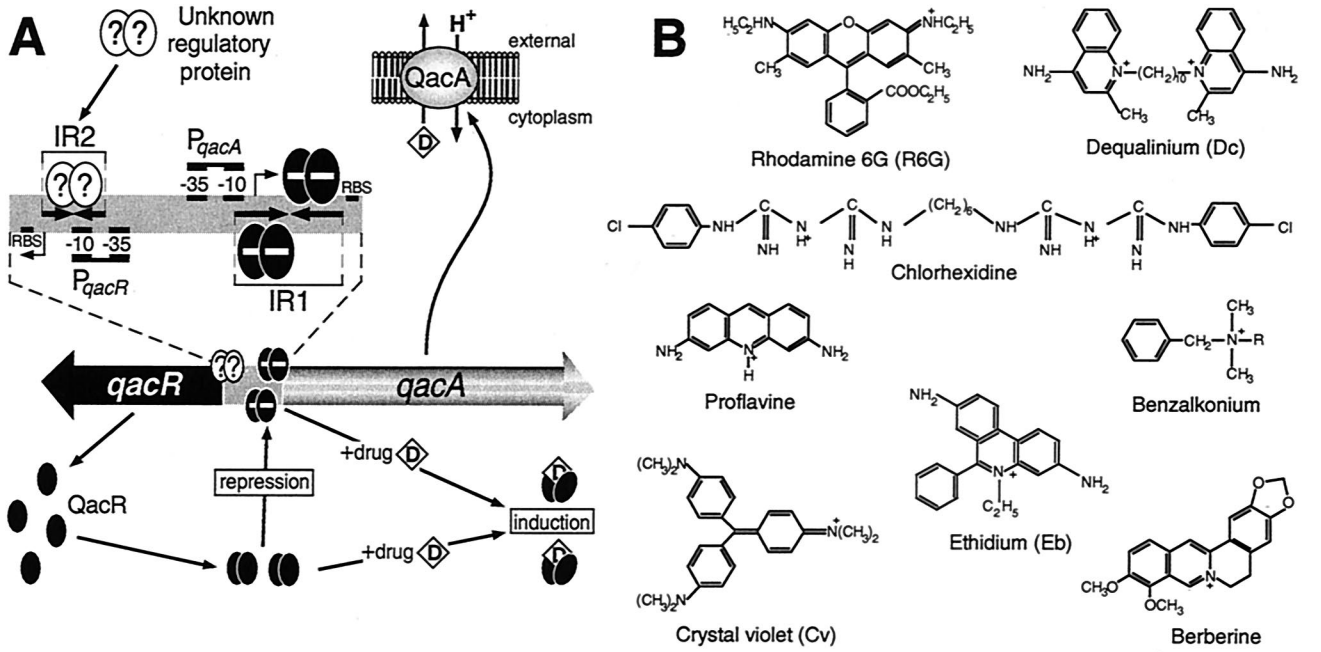

FIG. 10. (A) Model for regulation of expression of *S. aureus qacA* MDR gene. QacR (black ovals) represses (−) transcription from the *qacA* promoter, P_{qacA}, by binding as one dimer per IR1 half-site. Many of the lipophilic cationic drugs (D) exported from the cell by QacA in exchange for protons (H^+) are also ligands of QacR. Conformational changes that occur in a QacR dimer upon drug binding result in the ligand-bound form of this protein being incapable of binding IR1, thereby mediating increases in *qacA* transcription in response to the presence of transporter substrates. The locations of the ribosome-binding site (RBS) and transcription start point (right-angle arrows) are indicated for both the *qacA* and *qacR* genes. Preliminary results indicate that an unknown regulatory protein (?) indirectly influences *qacA* expression by binding IR2, which overlaps the *qacR* promoter, P_{qacR}. (B) Structurally diverse compounds that are both substrates of the QacA MDR pump and ligands of the QacR regulator. The chemical structures of representative compounds from four distinct chemical families are depicted. Dequalinium and benzalkonium (where R represents a mixture of alkyls, either $C_{12}H_{25}$, $C_{14}H_{29}$, or $C_{16}H_{33}$) are bivalent and monovalent quaternary ammonium compounds, respectively; rhodamine 6G, ethidium bromide, proflavine, and crystal violet are monovalent dyes; chlorhexidine is a bivalent guanidine; and berberine is a monovalent plant alkaloid.

berberine (S. Grkovic, M. H. Brown, and R. A. Skurray, unpublished data), which is an amphipathic, positively charged compound that shows some structural similarities with other QacR ligands (Fig. 10B). In contrast, all previously identified ligands of QacR had been synthetic substances that have been developed only recently. Additional evidence that berberine represents a natural MDR substrate is provided by the demonstration that this plant alkaloid is a substrate of both the plasmid-encoded QacA and the chromosomally encoded NorA *S. aureus* MDR pumps (63, 94, 95).

Taken together, these results are highly suggestive of a preexisting role for the QacA-QacR system in providing resistance to plant- and other naturally derived, cationic and hydrophobic antimicrobial compounds. Therefore, in order to combat antiseptics and disinfectants prevalent in the hospital environment, *S. aureus* appears to have recruited a system that was already well adapted for the export of such compounds. The continuing presence of the *qacA-qacR* locus on multiresistance plasmids associated with clinical *S. aureus* isolates (88; L. Worton, R. A. Skurray, and N. Firth, unpublished data) provides strong circumstantial evidence that the current function of this system in these strains is now predominantly the export of synthetic antimicrobial compounds. Also supporting this hypothesis is the increased substrate range of QacA compared to QacB (132, 159), which, in combination with the finding that QacB is encoded by the earliest known *qacA*- or *qacB*-carrying plasmid (160), suggests that *qacA* has more recently evolved from *qacB* in response to antimicrobial agents encountered in the hospital environment.

Although IR2, an inverted repeat that partially overlaps the *qacR* promoter (Fig. 10A, P_{qacR}), is very similar in sequence to the *tet* operators (Fig. 7A), overexpression of QacR or TetR in *trans* had no effect on transcription from P_{qacR}/IR2 in vivo (S. Grkovic, M. H. Brown, and R. A. Skurray, unpublished data), and purified QacR also failed to bind IR2-containing DNA in vitro (47). The failure to define a role for IR2 in the autoregulation of *qacR* expression is at odds with the results with other divergently transcribed members of the TetR family, which in general autoregulate the expression of their own genes (47). However, mutagenesis of IR2 indicated that this regulatory sequence has an alternative role that involves an as yet undefined chromosomally encoded protein (Fig. 10A) (S. Grkovic, M. H. Brown, and R. A. Skurray, unpublished data).

QacR DNA Binding

QacR, like other TetR family members, self-assembles into dimers. However, QacR-IR1 complexes were found to be equivalent in size to four QacR molecules bound to each IR1-DNA sequence even though IR1 contains only a single palindromic sequence (48). Furthermore, upon addition of QacR ligands, the four DNA-bound protein molecules were observed

to dissociate from IR1 DNA as dimers (48). This suggested that QacR does not self-assemble into tetramers, in addition to a pair of QacR dimers appearing to bind IR1 operator DNA without the requirement for any direct contacts between the two DNA-bound dimers (48).

The recent determination of the crystal structure for a QacR-IR1 complex confirmed and extended these findings, permitting the details of an unexpected and highly unusual mode of DNA binding to be elucidated (197). A pair of QacR dimers were found to be bound to opposite sides of a 28-bp DNA fragment so that successive major grooves could be contacted by two HTH motifs, one from each dimer (Fig. 11) (197). A four-helix-bundle dimerization domain was created by the two C-terminal QacR α-helices from each of the subunits in a dimer (Fig. 11; α8 and α9 from the first subunit, and α8′ and α9′ from the second subunit). Other than the three-helix bundle that forms the DNA-binding domain of each monomer (Fig. 11, α1 to α3), the four-helix bundle represented the only region of QacR that exhibited significant structural homology to TetR (197).

The combined contacts made by a pair of QacR dimers bound to IR1 DNA were in good agreement with the extended DNase I footprint observed for QacR-IR1 complexes (Fig. 12A) (47). One monomer of a QacR dimer (Fig. 11, green polypeptide of dimer 1) was found to make a large number of base and phosphate contacts to seven out of the eight positions immediately adjacent to the IR1 operator axis of symmetry (Fig. 12A, −1 to −8 bp) (197). This monomer has been designated the proximal (to the center of IR1) subunit to distinguish it from the second, distal dimer 1 subunit (Fig. 11, magenta polypeptide), which binds at a position more remote from the axis of symmetry. The contacts made by the proximal subunit of dimer 1 to 3 bp from the 6-bp IR1 spacer region (Fig. 12B, −1 to −3 bp) predominantly involved the phosphate backbone, which is consistent with the ability of QacR to bind to IR1 sequences in which all 6 of the central 6 bp have been altered (48). Although the base-specific contact made by the proximal subunit to position −3 involved a thymine, the proximal subunit of the second dimer would instead contact an adenine in the corresponding position of the wild-type IR1 sequence (+3 bp; Fig. 12A).

The contacts made by the distal subunit from dimer 1 (Fig. 11, magenta polypeptide) involved bases and the phosphate backbone in seven of the positions from +4 to +12 of IR1, i.e., in the half-site opposite that to which the proximal subunit of this dimer binds (Fig. 12) (197). Since the DNA-reading heads from the second QacR dimer have essentially the same interactions with IR1 DNA as the first dimer (197), the contacts illustrated in Fig. 12B for an IR1 half-site depict those formed by the proximal (green) subunit from dimer 1 and the distal (purple) subunit from QacR dimer 2 (Fig. 11). Of note is that the two HTH motifs within each QacR dimer make differential contacts to DNA sequences that lack any obvious twofold symmetry (Fig. 12), a highly unusual interaction for the combination of an inverted repeat sequence and a bacterial regulatory protein that binds to DNA in a dimeric form. However, closer inspection of the distal and proximal subunits illustrated in Fig. 12B, which are from different dimers, reveals that the four base-specific contacts made by each of their DNA-reading heads are very similar and hence constitute a pseudo-direct repeat (Fig. 12B). Thus, the DNA sequence that is bound by the two QacR polypeptides within an individual dimer contains a pseudo-inverted repeat (197).

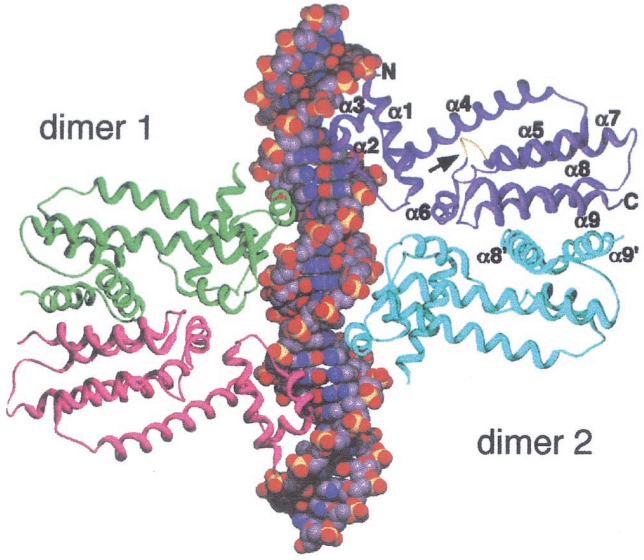

FIG. 11. Structure of the complex formed by a pair of operator-bound QacR dimers. The two QacR dimers bound to a symmetrical version of IR1 operator DNA are depicted as ribbons, whereas the DNA is shown with the phosphate, oxygen, carbon, and nitrogen atoms colored yellow, red, grey, and blue, respectively. The DNA-reading heads from the distal subunit (purple) of dimer 2 and the proximal subunit (green) of dimer 1 contact the first major groove, whereas the second major groove of IR1 DNA is contacted by the DNA-binding domains from the other two subunits, one from each dimer. The individual α-helices (α1 to α9) of the distal subunit from dimer 2 are labeled, as are the N and C termini of that polypeptide. Additionally, the α8′ and α9′ helices from the proximal subunit of dimer 2, which form the four-helix-bundle dimerization domain with α8 and α9, are also indicated. For the distal (purple) polypeptide of dimer 2, an arrow points to the yellow region at the N terminus of α5 that undergoes a coil-to-helix transition upon ligand binding. (Reprinted with permission from reference 197; kindly provided by Maria Schumacher; ©2002, Oxford University Press.)

The QacR-DNA structure indicated that the observed cooperative effect in the binding of a pair of dimers to IR1, which occurs in the absence of any direct dimer-dimer contacts (48), was the results of the successive major grooves of IR1 DNA being contacted by one HTH from each of the two DNA-bound QacR dimers (Fig. 11) (197). It was proposed that the binding of the first dimer requires an induced fit between the protein and IR1 DNA, which produces a significant increase in the width of the major groove and a corresponding undertwisting in QacR-bound DNA, thereby creating the correct conformation for binding of the second dimer (197). The flexibility of the α4 helix, which links the DNA-binding domain to the inducer-binding domain (Fig. 11), has also been suggested to play a role in DNA-binding cooperativity (197). Mutagenesis of the 6-bp IR1 spacer sequence (Fig. 12A) indicated that the proximal subunits of QacR dimers, which make contacts to this region, appear to be highly flexible in accommodating changes to the base composition of these central 6 bp (48). In contrast, the QacR-DNA structure clearly demonstrated that the failure of QacR to bind altered IR1 sequences in which the size of the

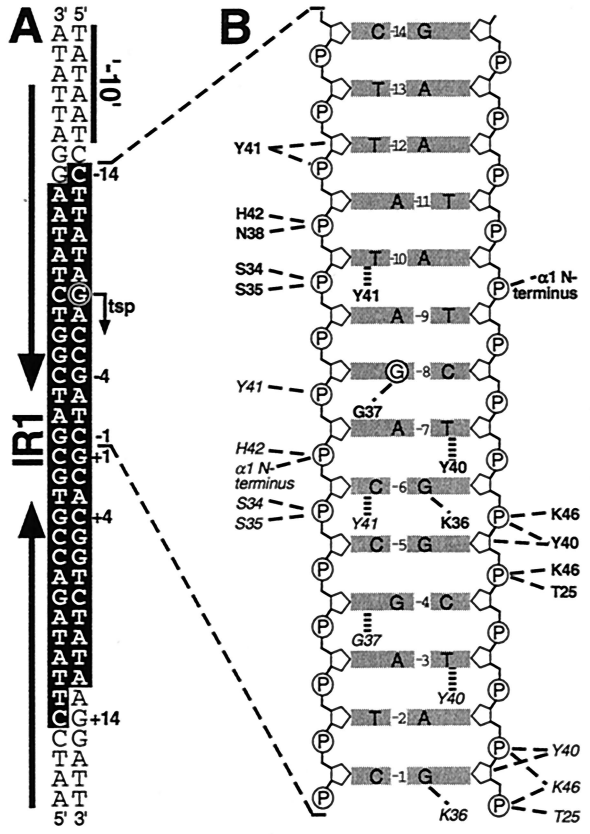

FIG. 12. DNA contacts formed by QacR to an IR1 half-site. (A) Sequence of the *qacA* −10 promoter region and the downstream IR1 operator, with the bases that QacR protects from DNase I digestion highlighted (white) against a black background, the *qacA* transcription start point (tsp) circled, and the location of IR1 indicated by bold arrows that flank the central 6-bp IR1 spacer region (47). (B) The DNA contacts made by a pair of operator-bound QacR dimers to a single IR1 half-site. The bp −1 to −14, which constitute the depicted operator half-site, are shown as a mirror image of A. The QacR residues in bold that contact the DNA in this half-site are from the distal subunit of dimer 2, whereas those in italics are from the proximal subunit of dimer 1 (Fig. 11, purple and green polypeptides, respectively). The contacts made to the bases and DNA backbone in the operator half-site by these amino acids are shown as thin dashed lines for hydrogen bonds and thick broken lines for van der Waals interactions. Also indicated is a DNA contact made by the N terminus of α1. (Panel B reprinted with permission from reference 197.)

6-bp spacer region had been increased or decreased by 2 bp (48) would be predominantly because the change in the spacing of the two IR1 half-sites significantly affected the ability of the four QacR DNA-reading heads to contact successive major groves.

Similar to TetR (59), the abnormally short recognition helix of QacR (α3; residues 36 to 42) is partially compensated for by the large number of residues from this helix that make contacts with operator DNA (Fig. 12B). In fact, the only QacR α3 residue that does not interact with DNA is Leu39, similar to Leu41 of TetR α3. However, whereas TetR employs residues from outside its recognition helix to make contacts to both bases and phosphates (Fig. 7B), the residues that QacR employs from outside α3, which includes the N terminus of α1, contact only phosphates (Fig. 12B). Therefore, in order to ensure that adequate base-specific contacts are made, QacR appears to additionally compensate for its short recognition helix by binding IR1 operator DNA cooperatively as a pair of dimers.

A critical aspect of the interactions between QacR and its operator appears to be the very small side chain of Gly37, which was proposed to facilitate the tight docking of the recognition helix to the DNA, provide room for the second QacR monomer to bind, and also permit the interactions of the Gly37 residues from the various subunits with the conserved guanine at positions −4, −8, +4, and +8 of IR1 (Fig. 12). The contact made to the −8 guanine of IR1 may have extra significance for the repression mechanism, as this base represents the transcription start point of the *qacA* gene (Fig. 12) (47). The importance of Gly37 and a number of other QacR HTH residues to DNA binding has been recently confirmed by mutagenesis (S. Grkovic, M. H. Brown, and R. A. Skurray, unpublished data).

Versatile QacR Multidrug-Binding Pocket

Structural determinations of QacR-ligand complexes, combined with the data available on ligand binding by BmrR, have produced major advances in our understanding of the molecular basis of multidrug recognition. In the case of QacR, each dimer was found to possess two drug-binding pockets, the first being formed by α4 to α8 from one subunit and α8′ from the other subunit, with the second ligand-binding site involving the reciprocal helices from each of these polypeptides. Although the two drug-binding tunnels possessed by a TetR dimer are also largely formed by the equivalent α-helices, the binding sites of these two related proteins show no significant similarities (198).

QacR-ligand structures have been obtained for a number of different ligands, including rhodamine 6G, ethidium bromide, dequalinium, crystal violet, and berberine. Whereas the monovalent compounds rhodamine 6G (Fig. 13A) and ethidium bromide (Fig. 13B) were found to bind to distinct but overlapping portions of an extended QacR ligand-binding pocket, the flexible linker carbon atoms of the bivalent compound dequalinium enabled the ring systems of this compound to be bound in both of these regions (Fig. 13C) (198). Four negatively charged glutamate residues lining the QacR ligand-binding pocket were observed to be available for forming electrostatic interactions with drugs, viz., Glu90 for neutralization of the positive charge of rhodamine 6G and Glu120 likewise for ethidium bromide, whereas one of the positive charges of dequalinium interacted with Glu57 and Glu58, while the second was neutralized by Glu120 (Fig. 13A to C). The single delocalized positive charge of crystal violet was also found to interact with two glutamates, Glu90 and Glu120 (Fig. 13D) (198). Additionally, the crystal violet structure demonstrated the versatility of the extended QacR ligand-binding pocket, as this less planer compound was bound at an intermediate position between the rhodamine 6G and ethidium bromide binding sites (Fig. 13D). The plant alkaloid berberine was essentially bound in the same manner and to the same portion of the extended QacR binding-pocket as rhodamine 6G (198).

Also lining the QacR drug-binding pocket are a large number of aromatic and hydrophobic residues which, similar to the

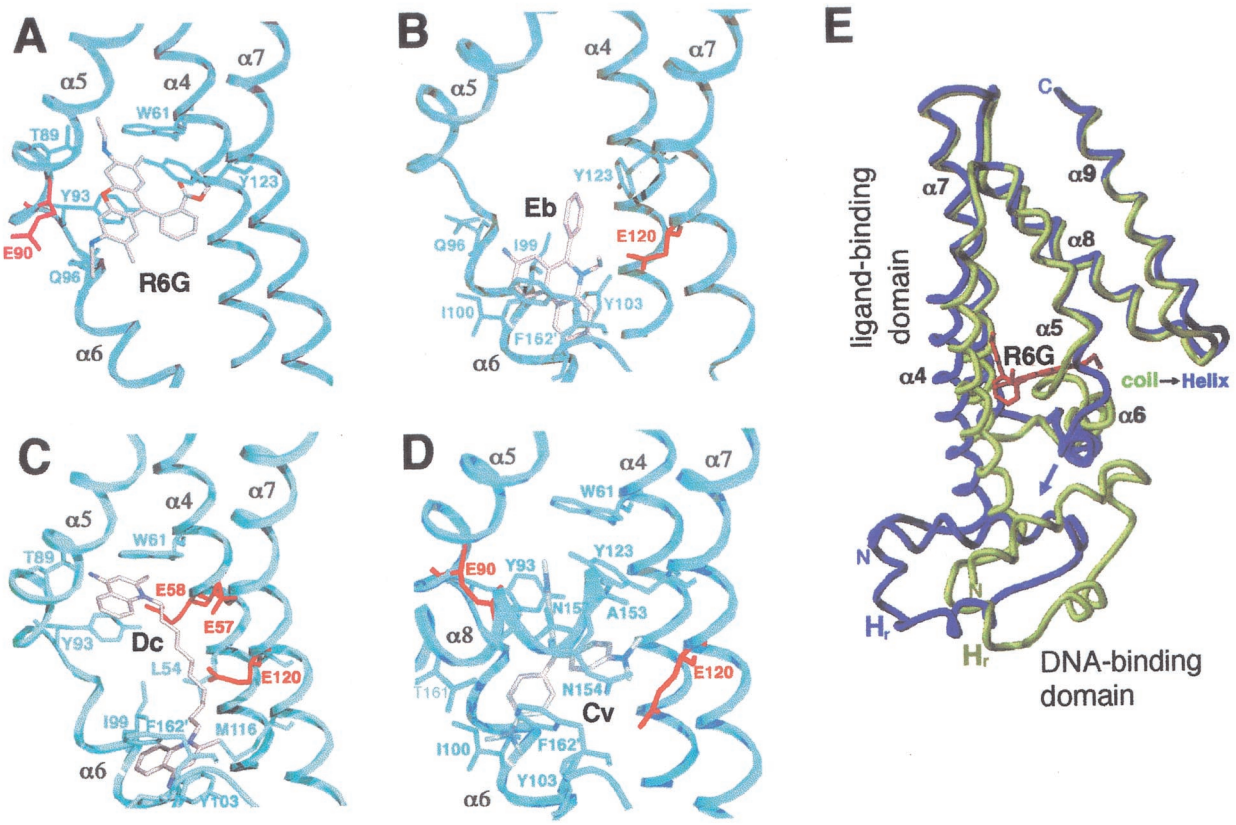

FIG. 13. (A to D) Binding of structurally diverse compounds in the extended QacR ligand-binding pocket. The key QacR residues and relevant α-helices in the ligand-binding site that interact with the compounds rhodamine 6G (R6G, A), ethidium bromide (Eb, B), dequalinium (Dc, C), and crystal violet (Cv, D) are shown in cyan, whereas the QacR glutamate residue(s) involved in electrostatic interactions with the positive charge(s) of each bound drug is colored red. The carbon, nitrogen, and oxygen atoms of each ligand are colored grey, blue, and red, respectively. (E) The QacR DNA-bound conformation (yellow) has been superimposed on the conformation of the QacR subunit to which a ligand is bound (blue). This illustrates the coil-to-helix transition that extends the N terminus of α5 by a turn and the concomitant shoving effect (blue arrow) of α6 against the DNA-binding domain, which produces a dramatic alteration in the position of this three-helix bundle. The location of rhodamine 6G (red) in the drug-bound structure is also indicated, as are α4 to α9, the HTH recognition helix (H_r), and the N and C termini of the protein. (Reprinted with permission from reference 198; kindly provided by Maria Schumacher.)

glutamates, present incoming drugs with a broad range of possibilities for the formation of hydrophobic interactions or the stacking of phenyl rings (Fig. 13A to D) (198). In addition, a number of polar residues, such as asparagine, glutamine, serine, and threonine, were observed to be available for interacting with various ligands as hydrogen bond donors or acceptors (Fig. 13A to D) (198). Therefore, the binding of structurally diverse ligands by QacR is facilitated by an incoming drug molecule being presented with a diverse array of potential electrostatic and hydrophobic interactions as well as many possibilities for the stacking of phenyl rings and the formation of hydrogen bonds, so that each ligand can form its own unique subset of interactions to maximize its binding.

A comparison of the ligand-binding domains of the QacR repressor and BmrR activator proteins reveals that although that of QacR is completely helical in nature (198), whereas BmrR is predominantly β-sheet (245), the two proteins have notable similarities, including the aromatic, hydrophobic, and negatively charged residues that line their binding pockets as well as the crucial role played by tyrosines (see below). One important difference between the two proteins is the observation that BmrR does not form any hydrogen bonds to the ligand TPP (Fig. 6). Although it is not known whether BmrR forms hydrogen bonds to ligands other than TPP, it has been suggested that by not employing distance- and orientation-dependent hydrogen bonds to contact ligands, multidrug-binding pockets can enhance their substrate range (115, 140). However, the available QacR-ligand structures clearly demonstrate that an array of residues which are capable of being hydrogen bond donors or acceptors can contribute substantially to multidrug-binding capabilities (198). These variations in the ligand-binding mechanisms used by individual multidrug-binding proteins may reflect the ancestry of each protein, e.g., QacR has a common ancestry with TetR, a protein that employs an extensive network of hydrogen bonds to form highly substrate-specific interactions with tetracycline-Mg^{2+} complexes (Fig. 9).

The proposal that multidrug transporters and their regulators originally evolved to be a general removal system for hydrophobic compounds may explain the broad ligand range of QacR (140). It has been suggested that because there are only a very few essential intercellular hydrophobic molecules, mul-

tidrug-binding proteins do not need to be very specific, as they only need to distinguish between hydrophilic compounds and the vast majority of hydrophobic ones (140). The structural data on the nature of the extended QacR binding pocket, which indicate that this protein is extremely well adapted for binding to a broad range of hydrophobic compounds, is in good agreement with this hypothesis. In contrast, transport machinery involved in the export of hydrophilic molecules requires highly specific interactions to prevent the undesirable efflux of structurally related compounds that are important cellular metabolites.

QacR Induction Mechanism

An intriguing feature of the ligand-binding process observed for QacR was a coil-to-helix transition, which, upon drug binding, resulted in extension of the C terminus of α5 by 4 residues (Fig. 11 and 13E) (198). This coil-to-helix transition was found to eject Tyr92 and Tyr93 from the hydrophobic core of QacR, thereby increasing the volume of the binding pocket available for drug binding; Glu90, previously positioned external to the ligand-binding pocket, was also now repositioned so that it could assist with drug binding (Fig. 13A to D). In the drug-free conformation, Tyr92 and Tyr93 were observed to act as drug "surrogates," ensuring that the hydrophobic core of QacR was stabilized in the absence of ligands. This situation has many similarities to that of BmrR, in which a flexible α-helix was found to permit the displacement of a stabilizing tyrosine, Tyr152, from the core of the protein upon drug binding.

In the case of QacR, the coil-to-helix transformation also served to switch the dimers into a non-DNA-binding conformation, thereby ensuring induction of *qacA* transcription. The mechanism by which this occurs involves the lengthening of the α5 C terminus, forcing the attached α6 helix downward (Fig. 13E). Since α6 is anchored to residues 12 to 23 of the DNA-binding domain, the translocation of this helix produces some dramatic changes in the orientation of the DNA-binding domains of both monomers (198). The result is an increase in the HTH center-to-center distance from 37 Å in the DNA-bound form of a QacR dimer to 48 Å in the ligand-bound form (Fig. 13E). Such a drastic change clearly prevents the ligand-bound form of QacR from binding to the successive major grooves of either B-form DNA, which are 34 Å apart, or QacR-bound DNA, which are 37 Å apart (197). This direct "shoving" mechanism observed for QacR is in marked contrast to the induction process of TetR, in which a small increase in the distance between the DNA-reading heads produced by changes transmitted through several helices is locked in place by the formation of a chain of water-mediated hydrogen bonds (Fig. 9B). The QacR induction mechanism appears to be a much more robust process, which may have granted the remainder of the QacR ligand-binding pocket the freedom to maximize the potential for interactions with structurally diverse ligands without compromising the mechanism or the efficiency of the induction switch.

In contrast to TetR, in which a tetracycline-Mg^{2+} complex is bound in each of the two drug-binding tunnels formed by a TetR dimer (60), the observed QacR-ligand binding stoichiometry was 2:1 despite the presence of two binding pockets in a QacR dimer (198). Closer inspection of QacR-ligand complexes revealed that the coil-to-helix transition that occurs upon ligand binding shifted the position of the adjacent C terminus of that subunit, such that it now blocked the entrance to the second ligand-binding pocket within the same dimer (198). This may give QacR an advantage over BmrR, which binds two drug molecules per dimer (Fig. 5) (245), as the ability of a single ligand bound to a QacR dimer to initiate the induction process may enhance the sensitivity of QacR to drugs, perhaps aiding the induction of expression in response to suboptimally bound ligands. The requirement for two unliganded QacR dimers to bind operator DNA before repression can be established could also have a role in enhancing the sensitivity of this regulator to the presence of inducing compounds.

OVERVIEW

A common theme in the regulation of membrane-bound drug transporter proteins is the need to prevent excessive expression, which is reflected by the fact that these proteins are notoriously difficult to overproduce for purification purposes (231). While it appears that some of the systems that are capable of exporting antimicrobial compounds do not need to be regulated because of a naturally low level of expression, the production of most is subject to some form of transcriptional and/or translational regulation, presumably as a safeguard against the deleterious effects of transporter overproduction. This situation is best exemplified by a number of transporter genes, which, although they are under the control of one or more inducible regulatory proteins, possess additional translational or transcriptional controls to provide added insurance that the fully induced level of expression cannot exceed a certain threshold (59, 72, 107). However, it is equally important to the cell that these transport proteins be available when required to perform their physiological roles. Thus, the expression of many drug transport proteins appears to be inducible, so that, in some instances, upregulation in response to the presence of natural or synthetic antimicrobial molecules has been demonstrated, although the expression of many other proteins is likely to be linked to presently unidentified physiological roles; for example, it has been proposed that the MDR pumps from *E. coli* and *P. aeruginosa* are involved in the export of quorum-sensing signals (83, 174).

Perhaps the most striking feature of bacterial drug pump regulation is the incredible diversity in the mechanisms that are employed to control their synthesis: each pump appears to be regulated in its own unique way. Additionally, for the MDR exporters, the majority of these determinants appear to be subject to multiple levels of control, possibly reflecting employment of their diverse substrate capabilities for multiple cellular functions. This is in marked contrast to the transcriptional regulation of the dedicated tetracycline transporters, which need to respond to only one substrate. As it was for TetR, when induction in response to antimicrobials has been clearly demonstrated for an MDR system, the common theme of requiring a protein that is capable of directly or indirectly sensing the presence of pump substrates has also been demonstrated. In comparison, the interaction of tetracycline with the ribosomal machinery involved in translation has provided an alternative method by which translational controls mediate

the induction of some tetracycline-specific pumps, particularly in gram-positive organisms.

An important aspect of research into the regulation of bacterial antimicrobial agent export systems has been elucidation of the ligand-binding mechanisms that are employed by some regulatory proteins to sense the presence of pump substrates. Structural analyses of the tetracycline-binding TetR repressor and the QacR and BmrR multidrug-binding regulators have been very successful in providing answers to questions about the mechanisms of protein-drug interactions that have not been forthcoming from the investigation of transport proteins. Of particular interest has been the demonstration of the principles of multidrug binding for the versatile QacR ligand-binding pocket; an extended ligand-binding site that possesses multiple but linked drug-binding pockets (198). These principles are also likely to apply to the MDR transporters themselves, as results from the mutagenesis of specific transporter residues, in addition to biochemical data, suggest that the MDR transporters human P-glycoprotein (214), *Saccharomyces cerevisiae* Pdr5p (85), *Lactococcus lactis* LmrP (172, 226), *E. coli* MdfA (93), and *S. aureus* QacA (132) are all likely to possess extended substrate-binding sites that are similar in nature to that of QacR.

The detailed pictures of protein-drug interactions for the multidrug-binding regulators as well as the tetracycline-specific system will have important applications in aiding the rational design of new antimicrobial compounds and the development of transporter inhibitors. The latter approach has received considerable attention, as the successful use of compounds that inhibit the function of drug efflux pumps may renew the usefulness of currently ineffective antibiotics (94).

Regardless of whether the primary function of a drug transport system is antimicrobial efflux or the export of such compounds merely occurs fortuitously, these proteins have been recruited by microbial pathogens in a highly successful effort to circumvent the relatively recent widespread use of antimicrobial compounds as therapeutic, prophylactic, and veterinary agents. In particular, the extensive use of antimicrobial compounds in modern hospitals has placed infectious bacteria under immense selective pressures. Investigations into the regulatory pathways controlling the expression of transport proteins capable of drug efflux have provided a fascinating picture of one way in which bacteria have responded to these selective pressures.

The various bacterial drug transport systems can be viewed as being at different stages along the path towards becoming dedicated systems for the provision of resistance to clinically relevant antimicrobial compounds. At one end of the spectrum, there are the acutely sensitive gram-negative TetA/TetR resistance determinants which have evolved specifically to provide the efficient removal of tetracycline. In contrast, at the other end of the scale are many MDR transporters whose broad substrate ranges are being increasingly exploited by microorganisms to cope with the elevated use of antimicrobial compounds. Although some of these MDR transporters are proposed to have had preexisting roles in providing protection against low levels of toxic compounds, many of their regulatory networks instead appear to be generally geared towards other physiological functions, such as the export of specific metabolites. Thus, mutations that disrupt the normal function of local and global regulatory networks, leading to the overexpression of chromosomally encoded MDR transporters in *P. aeruginosa*, *E. coli,* and *S. aureus*, have been required before significant levels of drug efflux occur. This is likely to represent the first step in a process that may culminate in these MDR loci evolving into systems that are specialized for drug efflux.

An interesting example of a drug efflux determinant that would seem to be further along this path, although less well adapted than the tetracycline-specific transport systems, is the plasmid-encoded *S. aureus qacA-qacR* locus. Evidence supporting this proposal is the apparent recent increase in the substrate range of QacA, the presence of the *qacA-qacR* locus on multicopy, transmissible, multiresistance-determining plasmids, and the ability of the QacR regulator to respond directly to the presence of antimicrobials, leading to an increase in the transcription of *qacA* without the need for the regulatory mutations that are characteristic of most other MDR loci involved in drug efflux. Further analysis of drug transporter regulatory networks will reveal whether these systems continue to evolve in order to match the new roles of their target genes, in addition to shedding light on preexisting physiological functions.

The increasing frequency with which bacteria are recruiting transport systems for the purpose of providing antimicrobial resistance makes it essential to gain a fuller understanding of the regulatory controls acting on their expression. This information should prove valuable in developing improved strategies to slow or circumvent the emergence of drug-resistant microorganisms.

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