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Structures of AcrR and CmeR: Insight into the mechanisms of transcriptional repression and multi-drug recognition in the TetR family of regulators

Mathew D. Routh^a, Chih-Chia Su^b, Qijing Zhang^c, and Edward W. Yu^{a,b,d,*}

^aMolecular, Cellular and Developmental Biology Interdepartmental Graduate Program, Iowa State University, IA 50011, USA

^bDepartment of Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames, IA 50011, USA

^cDepartment of Veterinary Microbiology and Preventive Medicine, College of Veterinary Medicine, Iowa State University, Ames, IA 50011, USA

^dDepartment of Physics and Astronomy, Iowa State University, Ames, IA 50011, USA

Abstract

The transcriptional regulators of the TetR family act as chemical sensors to monitor the cellular environment in many bacterial species. To perform this function, members of the TetR family harbor a diverse ligandbinding domain capable of recognizing the same series of compounds as the transporters they regulate. Many of the regulators can be induced by a wide array of structurally unrelated compounds. Binding of these structurally unrelated ligands to the regulator results in a conformational change that is transmitted to the DNA-binding region, causing the repressor to lose its DNA-binding capacity and allowing for the initiation of transcription. The multi-drug binding proteins AcrR of *Escherichia coli* and CmeR from *Campylobacter jejuni* are members of the TetR family of transcriptional repressors that regulate the expression of the multidrug resistant efflux pumps AcrAB and CmeABC, respectively. To gain insights into the mechanisms of transcriptional regulation and how multiple ligands induce the same physiological response, we determined the crystal structures of the AcrR and CmeR regulatory proteins. In this review, we will summarize the new findings with AcrR and CmeR, and discuss the novel features of these two proteins in comparison with other regulators in the TetR family.

Keywords

TetR family; *Escherichia coli* AcrR; *Campylobacter jejuni* CmeR; Transcriptional regulator; Multidrug resistance

1. Introduction

Bacterial infections are commonly treated with various classes of antibiotics. The clinical treatment is necessary for curing infectious diseases, but an unintended consequence of the treatment is the selection of bacterial pathogens with elevated levels of resistance to antibiotics.

Constant emergence and spread of antibiotic resistance has become a major threat to the health of humans and animals [1]. Bacterial organisms utilize multiple mechanisms to combat antibiotics and antimicrobial agents. One important mechanism that gives rise to multidrug resistance (MDR) is the expression of multidrug efflux transporters that are capable of reducing the intracellular concentration of toxic compounds [2–6]. The expression of these transporters is tightly controlled at the transcriptional level by regulators [2]. Many of these transcriptional factors are multidrug binding proteins, which recognize and respond to the same set of toxic chemicals that are exported by the transporters they regulate [7]. These transcriptional factors act as cytosolic chemical sensors and respond to threatening levels of toxic compounds [8,9].

In bacteria, transcriptional regulation involves either one-component or two-component regulatory systems. Two-component regulatory systems control protein expression through the function of a membrane-bound sensor kinase and a cytoplasmic response regulator, which is a DNA-binding protein [9–11]. The membrane-bound kinase is responsible for receiving external signals and transmitting the information into the cell by phosphorylating the DNA-binding protein. The phosphorylated DNA-binding protein then modulates gene transcription by interacting with its cognate DNA. A key feature of two-component regulatory systems is the phosphorylation between sensor kinase and response regulator. One-component bacterial transcriptional regulators modulate gene expression levels using a single two-domain protein where one domain receives signals and the other domain binds specific DNA sequences to regulate transcription [9]. Information flow between the two domains is through conformational changes, contrasting the phosphorylation events required in two-component systems. Structural analyses revealed that almost 95% of all known prokaryotic transcriptional factors employ the helix–turn–helix (HTH) motif to bind their target DNAs [9]. Prokaryotic transcriptional regulators are classified in families based on their functional and sequence similarities. One such family is the TetR family of transcriptional regulators [9]. Members of the TetR family are two-domain proteins which possess an N-terminal HTH DNA-binding motif and a C-terminal ligand regulatory domain. Many of these regulators control the expression of MDR efflux transporters that are required for bacteria to adapt to environmental stresses. These transporters protect bacterial cells from deleterious compounds by actively extruding these compounds as they enter the cells.

Understanding the molecular mechanisms of transcriptional regulation is vital due to the potential that these regulatory proteins can offer for new drug targets. Recently, the crystal structures of AcrR [12,13], a transcriptional regulator of the AcrAB efflux pump in *Escherichia coli*, and CmeR [14], a regulator that represses the expression of CmeABC in *Campylobacter jejuni*, have been determined. Induction of AcrR is initiated through an interaction of cationic and neutral ligands. In contrast, CmeR more favorably recognizes anionic and uncharged compounds. In this review we will describe the structural features of these two regulatory proteins and discuss the valuable insights that they provide for delineating the mechanisms of gene regulation and multidrug recognition by these TetR family regulators. An extensive and detailed review on the TetR family of regulators has been published elsewhere [9] and is not the focus of this paper.

2. The AcrR regulator

E. coli AcrB is a prototypical multidrug transporter that belongs to the resistance-nodulation-division (RND) superfamily of MDR pumps [15,16]. Of all currently characterized multidrug transporters, AcrB possesses the widest range of ligand recognition. It is capable of recognizing many structurally dissimilar compounds, including most of the currently administered antibiotics, chemotherapeutic agents, bile salts, dyes, and detergents [17,18]. This inner membrane efflux pump functions in conjunction with the periplasmic membrane fusion

protein, AcrA [19], and the outer membrane channel protein, TolC [20], to export a diverse range of compounds completely out of the bacterial cell.

The expression of AcrAB is modulated by the transcriptional regulator AcrR, whose open reading frame is located 141 bp upstream of the *acrAB* operon and is transcribed divergently [21]. Transcription of the *acrR* gene gives rise to a 215 amino acid protein, which shares N-terminal sequence and structural similarities to members of the TetR family [9]. The signatures of the TetR family of regulators include a homologous N-terminal three-helix DNA-binding domain and a diverse C-terminal ligand-binding domain [9]. Experimental evidence suggests that the 24 base pair palindromic inverted repeat (IR) sequence (5'

TACATACATTGTGAATGTATGTA3'), located between the *acrR* and *acrAB* genes and overlapping with the *acrAB* promoter, is the target DNA for AcrR [21,22]. It has been demonstrated through fluorescence polarization and gel filtration that AcrR binds to this IR as a dimer of dimers, with a dissociation constant (K_D) of 20.2 nM [12,22]. This suggests that the binding of AcrR to its IR resembles that of QacR, which binds a 28 bp IR1 sequence as a dimer of dimers, but is distinct from many other members of the TetR family where the interaction consists of a dimer bound to an ~15 bp IR [9,23–25]. The diverse C-terminal region of the TetR family of regulators possesses unique sequences, which allows different regulators in the family to accommodate specific sets of inducing ligands. Upon ligand binding, the AcrR regulator is presumed to dissociate from its target DNA to allow the expression of the AcrAB efflux complex which, in turn, protects the bacterial cell from toxic substances.

Recent studies indicate that AcrR interacts with the same set of antimicrobial agents as AcrB with strikingly similar affinities [22,26]. Su et al. [22] demonstrated that AcrR binds ethidium bromide (Et), proflavin (Pf), and rhodamine 6G (R6G) with dissociation constants of 4.2, 10.1, and 10.7 μ M [22]; while the K_D values for AcrB with these ligands are 8.7, 14.5, and 5.5 μ M [26], respectively. These affinities also coincide with those observed for QacR [27], BmrR [28], and TtgV [29]. The K_D s in this range may be optimal to initiate the expression of MDR pumps while the antibiotic concentration is at the sub-inhibitory level. Each AcrR monomer binds an inducing ligand, and thus a dimer can accommodate two identical molecules. Fluorescence polarization assays also suggest that AcrR binds many structurally unrelated ligands in distinct but possibly overlapping binding sites [22]. For instance, Et-saturated AcrR can accommodate Pf with equal affinity as apo-AcrR while Et and R6G seem to be competing for the same binding site [22]. The 1:1 ligand-to-monomeric AcrR stoichiometry is similar to that of TetR [23], but distinct from the 1:2 ligand-to-monomer ratio of QacR [24]. AcrR is unique in that its ligand binding mode is similar to TetR while its mode of DNA-binding is related to that of QacR. Exploration of the AcrR induction may provide us with new insight into the mechanisms that the TetR family utilizes to regulate genes.

2.1. Crystal structure of AcrR in space group of P222₁

The crystal structure of AcrR in space group P222₁ [12] is illustrated in Fig. 1a. It reveals a dimeric protein composed almost entirely of α -helices with an overall architecture similar to members of the TetR family, including TetR [24,30], QacR [23,31], EthR [32,33], CprB [34], CmeR [14], ActR [35], HapR [36], and IcaR [37]. Each subunit in the dimer comprises nine helices (α 1 to α 9 and α 1' to α 9'). Of this two-domain protein, helices α 1 to α 3 make up the N-terminal DNA binding domain while the larger C-terminal ligand-binding domain consists of helices α 4 through α 9. A high degree of conservation exists in the DNA-binding region among members of the TetR family of transcriptional regulators [12]. This can be attributed to the critical role of specific amino acids in contacting the phosphate backbone of the DNA strand and to the overall function of the TetR family proteins in transcriptional repression. When the N-terminal domain of AcrR (amino acids 12–62) is aligned against QacR and CmeR, 43% amino acid identity is observed in both cases [12]. Superimposition of this

domain to QacR reveals a very similar overall structure, which is reflected by an overall rmsd of 1.2 Å calculated over the C α atoms.

As the DNA-binding mode of AcrR is expected to be similar to that of QacR, a speculative model of DNA-bound AcrR (Fig. 1b) was generated by aligning its domains to those of DNA-bound QacR [12]. This model suggests that R45, G46, Y49, W50, H51 and K55 are important for IR binding. Among these residues, R45 interacts directly with four different bases of the DNA, confirming its critical role for IR recognition. Therefore, it is not surprising that a recent sampling indicated six of 36 isolated fluoroquinolone-resistant *E. coli* strains had a mutation at codon 45 (Arg→Cys), and all six R45C mutants showed evaluated resistance to multiple antibiotics [38].

The C-terminal regulatory domain of AcrR comprises six helices, including helices α 4 through α 9. Helices α 8 and α 9 form the majority of the dimerization surface with small contributions from helices α 6 and α 7, creating a 2002 Å²/monomer buried, mostly hydrophobic contact region [12]. Owing to differences in inducing ligands and varying specificity, the C-terminal domain of AcrR possesses little sequence homology to other members of the TetR family of transcription regulators. It is intriguing that superposition of AcrR [12], QacR [31], and CmeR [14] reveals significant topological similarities in the C-terminal domains. This, in part, may be attributed to a similar functional role that the C-terminal domains play in recognizing inducing ligands and transmitting the signal to the N-terminal DNA-binding regions. Like other members of the TetR family, a large internal cavity, with a total volume of 350 Å³ is formed in the C-terminal region of AcrR (Fig. 1c). This cavity, surrounded by helices α 4 through α 8 of each monomer, is predicted to form the multidrug-binding pocket in AcrR [12]. It should be noted that the C-terminal domain of apo-QacR does not have a ligand-binding cavity. A unique characteristic of AcrR is the presence of three openings to the drug-binding pocket. Two of the openings are located at the front and side surfaces of each monomer and appear to be orthogonal to each other. The loop between helices α 4a and α 4b contributes to form part of the openings. The other opening is located at the dimer interface and is partially blocked by the loop between helices α 8' and α 9' from the second subunit. It is likely that drug molecules may enter AcrR through the loop region between helices α 4a and α 4b.

Docking of ligands into the AcrR structure suggested that the large cavity created by helices α 4 to α 8 could accommodate different drugs, including Et, Pf and ciprofloxacin (Cip) [12]. In each case, the bound drug was completely buried in the AcrR molecule, and strong interaction was observed between bound drug and the regulator. Predictions also indicated that the binding sites for Et, Pf and Cip are distinct, but partially overlapped in each monomer of AcrR. Fig. 1d depicts the multi-drug binding site formed by the C-terminal domain of the regulator. The extensive binding pocket, which is created by helices α 4 through α 8, is mostly hydrophobic in nature, with W63, I70 and F114 predicted to make important hydrophobic contacts with the inducing ligands. In addition to these hydrophobic interactions, residues E67 and Q130 are predicted to make electrostatic interactions with bound drug to secure the binding. Among these amino acids, E67 seems to be of particular importance for drug recognition. It was found that a mutation of this residue by an alanine, E67A, abolished the binding of Pf, Et and R6G to the regulator [12].

2.2. Crystal structure of AcrR in space group of P3₁

Recently, a new crystal structure of AcrR with space group P3₁ [13], which is distinct from the P222₁ space group structure, was determined. A comparison of these two structures reveals considerable conformational changes at both the N-terminal and C-terminal regions, suggesting that these two structures represent different conformational states of AcrR. These crystal structures have provided valuable insight into the mechanisms of ligand binding and AcrR regulation.

The overall structure of AcrR with the space group $P3_1$ is quite similar to the $P222_1$ space group structure. A detailed comparison, however, reveals a significant change in conformation at the N-terminal DNA-binding domain. This change results in an overall rmsd of 2.8 Å calculated over the C α atoms at the N-terminal domains (residues 7–65), in contrast to the <0.7 Å rmsd of the C-terminal domains (residues 73–210) [13]. Fig. 2 illustrates a superposition of these two AcrR structures using the program ESCET [39].

Conformational changes between the $P3_1$ and $P222_1$ structures seem to be predominantly rigid-body translation and rotation at the N-terminal domain. These movements lead to a downward shift of the entire N-terminal DNA-binding domain of the $P3_1$ structure (with respect to the orientation shown in Fig. 1a) by 2.6 Å, and a rotation of 10° towards the subunit interface of the dimer when compared with that of the structure of $P222_1$ (Fig. 2). As a consequence of these movements, the two N-terminal domains of the AcrR dimer, in the $P3_1$ structure, move closer to each other by approximately 2 Å. The center-to-center distance between recognition helices $\alpha 3$ and $\alpha 3'$ (as measured by the distance between C α atoms of Y49 and Y49') decreases from 42 Å in the $P222_1$ structure to 39 Å in the $P3_1$ structure [13]. To bind two consecutive major grooves of B-DNA, the center-to-center distance has to be ~34 Å. This distance is thought to increase upon drug binding, which in turn inhibits the binding of the regulator to its operator DNA. With the observed center-to-center distances in the crystal structures, it is likely that the conformation of the DNA-bound form of AcrR is more similar to the $P3_1$ structure, while its drug-bound form is more closely related to the $P222_1$ structure. In addition to these differences, R45, an N-terminal amino acid previously identified to be critical for DNA binding and AcrR regulation [38], undergoes a significant conformational change. The C α –C α distance between R45 and R45' decreases from 40 Å in the $P222_1$ structure to 35 Å in the $P3_1$ structure [13].

When examining the C-terminal domain, the most striking conformational change involves the amino acid E67. This residue may act as a molecular switch that drives the change of conformations throughout the AcrR molecule. Superimposition of the two AcrR structures reveals that the C α atom of E67 shifts by 4.2 Å. This shift initiates considerable changes in the C-terminal domain of AcrR, including helix $\alpha 4a$ shifting towards the N-terminal domain by 2.3 Å and a local unwinding of the N-terminal end of helix $\alpha 6$ which shortens the helix by one turn. The local unwinding and overall change result in the disruption of the hydrogen bonded network connecting the N- and C-terminal domains. In the $P222_1$ structure, it was found that R106 is H-bonded with E67 in the drug binding site, and the C-terminal domain residue R105 is H-bonded with the N-terminal domain residues Q14 and D18, respectively (Fig. 1a) [12, 13]. These H-bonds are missing in the $P3_1$ structure.

Based on the $P3_1$ and $P222_1$ structures of AcrR, we suspect that the changes in conformation of the N-terminal DNA-binding and C-terminal drug-binding domains of AcrR are cooperative, due to the formation of H-bonds at the interface between these two domains (Fig. 1a). In the DNA-bound form of AcrR, the structure of the regulator may be closer to the $P3_1$ structure. Thus, the side chain of E67 may point outside the drug-binding pocket, exposed to the solvent. Drug-binding within the C-terminal domain may induce structural changes resulting in a conformation more closely related to the $P222_1$ structure, in which the side chain of E67 flips into the interior of the hydrophobic core. This change may also be accompanied by the formation of new H-bonds between E67 and R106, R105 and Q14, and R105 and D18. The crystal structures of both DNA-bound and drug-bound AcrR would be necessary to confirm the change in conformation upon DNA and drug binding.

3. The CmeR regulator

Campylobacter jejuni is the leading cause of food-borne enteritis to humans in the USA as well as other developed countries [40]. *C. jejuni* is able to infect animal hosts, and colonize the intestinal tracts of these animals. To withstand the various deleterious conditions both *in vitro* and *in vivo*, this Gram-negative microorganism harbors 13 putative MDR transporter genes (according to the genomic sequence of NCTC 11168) that may be used to extrude antimicrobial compounds that *Campylobacter* may encounter in the intestinal tract [41,42]. Some of these multidrug transporters have been linked to the intrinsic and acquired resistance of *Campylobacter* to various antibiotics [43]. Currently, CmeABC and CmeDEF (belonging to the RND-family of MDR proteins) are the only two, of the 13 predicted, antibiotic resistance transporters that have been functionally characterized in this Gram-negative microorganism [44–47].

A primary contributor to antibiotic resistance in *C. jejuni* is the CmeABC efflux system. CmeABC is a tripartate RND efflux transporter [45]. It consists of an inner membrane efflux pump, CmeB, a periplasmic membrane fusion protein, CmeA, and an outer membrane channel, CmeC. Together, these three proteins effectively mediate the extrusion of commonly used antibiotics, metal ions, and lipophilic compounds out of the bacterial cell [45–49]. Importantly, CmeABC is a key player in *C. jejuni* for resistance to bile salts, which are ubiquitously present in the intestinal tract. CmeABC deletion mutants are unable to colonize in the intestinal tract of chickens [50], indicating the essential role of CmeABC in adapting to the *in vivo* environment. Expression of the CmeABC efflux complex is inducible by bile salts [49] and potentially by other unidentified ligands. Understanding the regulation of *cmeABC* is an important step in the elucidation of the mechanism of multi-drug extrusion in *Campylobacter*.

Transcription of *cmeABC* is controlled by the transcriptional regulator CmeR [51]. The gene for *cmeR* is located immediately upstream of *cmeABC* and encodes a 210 amino acid protein that shares N-terminal sequence and structural similarities to members of the TetR family of transcriptional repressors [9,52]. CmeR is a two-domain protein with an N-terminal DNA-binding region and a predicted C-terminal ligand-binding domain. The 16 bp IR sequence, 5' **TGTAA-TAAATATTACA**3', located between *cmeR* and *cmeABC* is shown to be the operator site of CmeR binding and transcriptional repression [51]. Transcriptional repressors of the TetR family bind their IR operator sites which generally overlap the promoter sequences of the regulated genes. This interaction inhibits RNA-polymerase from binding or blocks the transcriptional initiation event to repress gene expression. Alterations that affect CmeR–operator binding, including deletions of CmeR and single nucleotide mutations in the operator site, releases the repression of CmeR and results in overexpression of CmeABC [51,53]. In addition, CmeR–DNA interactions are inhibited when inducing ligands, such as bile salts, interact with CmeR and cause a conformational change in the protein that renders it unable to bind to its operator DNA. Guo et al. [54] used DNA microarrays and real-time quantitative reverse transcription-PCR to analyze the regulatory network of *cmeR*. The results showed that in addition to repressing the transcription of *cmeABC*, CmeR functions as a pleiotropic regulator and modulates the expression of at least 28 other genes in *C. jejuni* [54]. The array of genes regulated by CmeR outlines the important role this regulator plays in the adaptive response to the intestinal environment.

3.1. Crystal structure of CmeR

Recently, the crystal structure of CmeR has been determined (Fig. 3a) [14]. This work revealed novel structural features of a TetR family regulator, and has brought new insights into the mechanisms of transcriptional regulation and ligand recognition. Like AcrR, CmeR is a dimeric two-domain molecule with an entirely helical architecture with similar topology to other

members of the TetR family. Distinct from the other members of the TetR family, CmeR exhibits a unique crystal structure that lacks the $\alpha 3$ helix (replaced by a random coil) which is involved in DNA recognition. Along with this unique characteristic, a large center-to-center distance (54 Å as measured by the separation between C α atoms of Y51 and Y51' from the other subunit) was observed between the two N-termini of the dimer. In addition, a large flexible ligand-binding pocket is found to form in the C-terminal domain of CmeR. Each monomer forms a 20 Å long tunnel-like cavity in the ligand-binding domain of CmeR and occupies a volume of about 1000 Å³ (Fig. 3b), which is approximately three times of that of AcrR. As CmeR recognizes anionic and neutral ligands, the structure offers the first glance on how anionic and uncharged ligands are bound by a regulator from the TetR family.

The crystal structure of the dimeric CmeR regulator is shown in Fig. 3a. This structure revealed that each subunit of CmeR is composed of nine α helices, in which the short recognition $\alpha 3$ helix, presumably formed by residues 47–53, is missing [14]. To facilitate comparisons with other TetR members, $\alpha 3$ was excluded from the numbering of the α -helical segments. Thus, helices $\alpha 1$, $\alpha 2$, and the random loop (residues 47–53) form the N-terminal DNA-binding domain, and helices $\alpha 4$ through $\alpha 10$ form the C-terminal ligand-binding domain.

The N-terminal DNA-binding domain of CmeR exhibits several distinct features compared with the rest of the TetR family members. First, helix $\alpha 1$, consisting of 23 residues, is relatively long among all structurally known TetR regulators. For example, the corresponding helices $\alpha 1$ in QacR [31], TetR [30], and EthR [32] are composed of only 16, 13, and 17 residues, respectively. As mentioned above, the structure of CmeR does not consist of the third N-terminal helix. This is, perhaps, the most striking feature that makes CmeR distinct from the other TetR family members. To date, the CmeR regulator is the only observed case of a random coil replacing helix $\alpha 3$ in a TetR family member. Presumably, the TetR regulators possess a HTH DNA-binding motif formed by helices $\alpha 2$ and $\alpha 3$. Owing to its important role in recognizing target DNA, helix $\alpha 3$ is named the “recognition helix” [9]. Thus, we reasoned that the flexible coil might need to transform into a helix when the regulator binds target DNA (Fig. 3c). Since CmeR is a pleiotropic regulator of a large set of genes and is predicted to bind multiple operator sites, with many of those not being of the consensus IR sequence located in the promoter region of *cmeABC* [54], it could be postulated that the flexibility of the DNA-binding domain permits CmeR to recognize multiple cognate DNA sites.

One other unique feature of the CmeR structure is its large center-to-center distance between the two N-termini of the dimer. This center-to-center distance (according to the separation between C α atoms of Y51 and Y51') was measured to be 54 Å [14]. The corresponding distances are 39 Å and 35 Å in the apo forms of QacR [31] and TetR [55]. These center-to-center distances increase upon ligand binding. For the ligand-bound dimers of QacR [31], TetR [30], EthR [33], and YfiR [56] these distances become 41 Å, 38 Å, 52 Å and 54 Å, respectively. Thus, the relatively large center-to-center distance observed for CmeR reflected the fact that CmeR was liganded [14]. Indeed, the crystal structure indicated that a glycerol molecule was bound in each subunit of the CmeR dimer (Fig. 3a) [14].

The C-terminal domain of CmeR consists of helices $\alpha 4$ through $\alpha 10$, with helices $\alpha 4$, $\alpha 5$, $\alpha 7$, $\alpha 8$ and $\alpha 10$ forming an anti-parallel five-helix bundle. In view of the crystal structure, helices $\alpha 6$, $\alpha 8$, $\alpha 9$ and $\alpha 10$ are involved in the formation of the dimer. Dimerization occurs mainly by couplings between pairs of helices ($\alpha 6$ and $\alpha 9'$, $\alpha 8$ and $\alpha 10'$, and their identical counter pairs). A surface area of 1950 Å² per monomer is buried in the contact region of the dimer [14]. The interaction surface is mostly hydrophobic in character. The C-terminal domain of CmeR is distinct in that helix $\alpha 9$, which is between the two anti-parallel helices $\alpha 8$ and $\alpha 10$, deviates from the direction of $\alpha 8$ by 40°. Thus, helix $\alpha 9$ bends toward the next subunit of the dimer, interacting with $\alpha 6'$ and $\alpha 7'a$ to secure interaction between the dimer.

The C-terminal domain forms a large tunnel-like cavity in each subunit of CmeR. This tunnel, surrounded by mostly hydrophobic residues of helices $\alpha 4$ – $\alpha 9$, opens horizontally from the front to the back of each protomer. The length of this tunnel is approximately 20 Å. Helices $\alpha 7$ and $\alpha 8$ from one subunit, and $\alpha 9'$ from the other subunit of the regulator make the entrance of the tunnel. Helices $\alpha 4$ – $\alpha 6$, however, contribute to form the end of this hydrophobic tunnel. Each hydrophobic tunnel, occupying a volume of about 1000 Å³, spans horizontally across the C-terminal domain and can be seen through from the front to the back of the dimer without obstruction. This unique feature, not found in other structures of the TetR family of regulators, highlights the flexibility of the CmeR regulator [14]. As indicated above, the crystal structure of CmeR revealed the presence of a glycerol molecule inside this large ligand-binding tunnel. Glycerol binds identically in each subunit, as indicated by the crystallographic two-fold symmetry of the CmeR dimer [14]. This ligand-binding mode is different from that of QacR in which one dimer of QacR binds one drug [31], but similar to that of TetR, which interacts with tetracycline in a manner of 1:1 monomer-to-drug molar ratio [30]. The volume of the ligand-binding tunnel of CmeR is large enough to accommodate a few of the ligand molecules. Additional water molecules fill the portion of the large tunnel that is unoccupied by ligand. The structure suggests that CmeR might be able to bind more than one drug molecule at a time, or possibly accommodate a significantly larger ligand that spans across the entire binding tunnel. Indeed, a docking study showed that the hydrophobic tunnel of CmeR should be able to accommodate large, negatively charged bile acid molecules, such as taurocholate and cholate [14]. Fig. 3d demonstrates the extensive predicted ligand-binding site, and important residues that are critical for ligand recognition in the tunnel. The bound bile acids are predicted to anchor to several hydrophobic, polar and positively charged residues, including H72, F99, F103, F137, S138, Y139, V163, C166, T167, K170 and H174. These anionic ligands were predicted to span almost the entire length of the ligand-binding tunnel of the regulator, respectively. The flexibility of this large ligand-binding tunnel suggests that CmeR is a multiple ligand binding protein [14].

4. Conclusions and perspectives

In the past decade, several crystal structures of the TetR family of regulators have been determined. These structures have allowed us to glance through the phenomenon of multidrug recognition. In particular, the structures of six QacR–drug complexes [30] and QacR simultaneously bound by two drugs [57] revealed the presence of multiple binding sites within an extensive, sizeable drug-binding pocket of the regulator. It is quite conclusive that QacR recognizes a combination of drugs, both positively charged and neutral, by using multiple, proximal and distinct drug binding sites. Based on the structures of AcrR, it is very likely this regulator employs a similar mechanism that QacR uses for drug binding. It is expected that AcrR binds cationic and neutral charged drugs by utilizing aromatic and acidic residues. The predicted drug-binding pocket at the C-terminal domain of AcrR indeed consists of multiple hydrophobic and aromatic residues with a buried glutamate critical for drug binding. A similar drug-binding pocket has been found in BmrR in which multiple aromatic side chains are stacked against the positively charged tetraphenylphosphonium ligand [58]. The charge of the bound ligand was further neutralized by negatively charged acidic residue(s) in the ligand-binding site [31,57–59].

In the case of CmeR, CmeR tends to bind anionic and uncharged ligands, including the large bile salts such as cholate and deoxycholate. Based on the crystal structure, the mechanisms by which CmeR employs to recognize anionic compounds seem to be an “analog” to those of QacR and AcrR. The C-terminal domain of CmeR forms a large, sizable drug-binding tunnel that occupies a volume of 1000 Å³ [14]. This tunnel, possibly consisting of multiple mini-pockets for different ligands, is rich in aromatic residues and contains three positively charged

amino acids (two histidines and one lysine). It is very likely that CmeR uses these positively charged residues, in an analogous manner, to recognize negatively charged ligands.

The structure of CmeR indicated that anionic bile acids, such as cholate and deoxycholate, are probably bound in the large hydrophobic tunnel via hydrophobic and aromatic stacking interactions. These bound ligands presumably are further neutralized by one or more of the positively charged residues, including lysine and histidine, in the binding tunnel to secure the binding. This binding mode is quite different from that shown in the MarR-salicylate structure in which the anionic salicylates are not bound in hydrophobic pockets, but in openings that are exposed to solvent [60]. Although the binding sites for salicylates possess positively charged arginines to neutralize the formal negative charge of salicylates, they do not contain any aromatic residue. Thus, the crystal structures of the CmeR–ligand complex and other regulatory proteins bound by anionic ligands are needed to provide a clearer understanding on the mechanisms of anionic ligand recognition utilized by these regulators.

CmeR possesses a very unique structural feature at the N-terminal domain, in which it does not have the recognition $\alpha 3$ helix. It is not yet known if this unique feature is related to its function. CmeR acts as a pleiotropic regulator and modulates the expression of many other genes in *C. jejuni*. As helix $\alpha 3$ is critical for contacting with target DNA, the lack of helix $\alpha 3$ in the structure of CmeR could explain the fact that CmeR bind its IR in a much weaker fashion when compared with the binding of TetR, QacR and AcrR to their target DNAs [51]. Detailed interaction between CmeR and target DNA awaits the atomic resolution structure of the CmeR–IR complex.

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Fig. 1.

Structure of AcrR. (a) Crystal structure of AcrR in space group of $P222_1$. Ribbon diagram of the AcrR homodimer generated by crystallographic symmetry. The hydrogen bonds at the interface between the C-terminal and N-terminal domains of each subunit AcrR, shown as dotted lines in the left subunit, are between Glu67 and Arg106; between Gln14 and Arg105; and between Asp18 and Arg105. These H-bonds are absent in the $P3_1$ structure. (b) Speculative model of AcrR in its DNA-bound form. The N- and C-terminal domains of the ligand-bound dimeric AcrR were individually aligned with those of the DNA-bound QacR (1JT0) to generate the DNA-bound form of AcrR. The model of the 24 bp IR model is shown as a space-filling model. It is expected that two dimers of AcrR (in orange and deep olive ribbons) bind one

double-stranded IR. (c) Electrostatic surface potential of one subunit of AcrR. This view shows the large cavity spanning from the side surface (front) to the subunit interface (back) of the C-terminal domain of one subunit of AcrR. Blue (+15 $k_B T$) and red ($-15 k_B T$) indicate the positively and negatively charged areas, respectively, of the protein. (d) Binding site prediction for AcrR. Residues, W63, E67, I70, F114 and Q130, which are predicted to be important for drug binding are shown as yellow sticks. The figure was prepared using PyMOL [<http://www.pymol.sourceforge.net>].

Fig. 2. Structural comparison of the $P3_1$ and $P222_1$ structures of AcrR. Superimposition of the dimeric AcrR structures was performed using the program ESCET (green, $P3_1$ structure; orange, $P222_1$ structure). Residue E67 in each subunit is shown as a stick model.

Fig. 3.

Structure of CmeR. (a) Crystal structure of CmeR. Ribbon diagram of the ligand bound CmeR homodimer generated by crystallographic symmetry. The bound glycerols were shown as space-filling models. (b) Electrostatic surface potential of one subunit of CmeR. This view shows the long tunnel spanning through the C-terminal domain of CmeR. Blue (+15 k_BT) and red (-15 k_BT) indicate the positively and negatively charged areas, respectively, of the protein. (c) Speculative model of CmeR in its DNA-bound form. The N- and C-terminal domains of the ligand-bound dimeric CmeR were individually aligned with those of the DNA-bound QacR (1JT0) to generate the DNA-bound form of CmeR. The two DNA recognition $\alpha 3$ helices (red) in the dimer of CmeR are included in the model. Each helix of the bound DNA is shown in orange thread. (d) Binding site prediction for CmeR. Residues, H72, F99, F103, F137, S138, Y139, L163, C166, T167, K170 and H174, which are predicted to be important for drug binding are shown in stick models.