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Characterization of the multidrug efflux regulator AcrR from *Escherichia coli*

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Abstract

The *Escherichia coli* AcrR represses transcription of the *acrB* gene, which encodes the multidrug efflux pump AcrB that extrudes a wide variety of toxic compounds, by binding its target operator DNA. Fluorescence polarization was performed using purified, recombinant AcrR that contains a 6xHis tag at the C-terminus and a fluorescein-labeled 28-base pair oligonucleotide bearing a predicted palindrome (IR) operator sequence. Binding of AcrR to the predicted IR sequence occurred with a dissociation constant (K_D) in the nanomolar range. Fluorescence polarization assays were also applied to characterize the affinity and specificity of AcrR interaction with three different fluorescent ligands, rhodamine 6G, ethidium, and proflavin. The K_D values for these ligands range from 4.2 to 10.1 μM, suggesting that AcrR is capable of recognizing a wide range of structurally dissimilar toxic compounds as it is in the case of the AcrB multidrug efflux pump. We found that the binding of rhodamine 6G to AcrR is inhibited by the presence of ethidium. In contrast, the dissociation constant of proflavin binding to AcrR was not affected by ethidium, a result suggesting that ethidium and proflavin are bound to distinct binding sites.

Keywords: TetR family; Transcriptional regulator; Multidrug resistance; AcrR; Fluorescence polarization; Simultaneous binding

The increase in bacterial resistance to multiple drugs has emerged as a major clinical problem. One important mechanism that gives rise to multidrug resistance (MDR) in bacteria is the expression of multidrug transporters that are often regulated at the transcriptional level by transcriptional regulators [1]. Many of these transcriptional regulators are multi-drug binding proteins, which recognize the same array of toxic chemicals extruded by the transporters that they regulate [2]. These regulators often act as cytosolic chemical sensors and respond to threatening levels of toxic chemicals. The results are the over-expression of MDR transporters, which, in turn, promote efflux from cells, thus protecting them from toxic substances.

Bacterial efflux transporters capable of transporting multiple toxic compounds fall into five classes: (i) the ATP-binding cassette (ABC) family [3]; (ii) the major facilitator superfamily (MFS) [4,5]; (iii) the resistance-nodulation-division (RND) family [6,7]; (iv) the small multidrug resistance (SMR) family [8]; and (v) the multidrug and toxic compound extrusion (MATE) family [9]. The ABC family transporters take the free energy generated from ATP hydrolysis to expel toxic substances out of cells [10]. Efflux transporters in the other families, however, utilize the transmembrane electrochemical gradient of protons or sodium ions to extrude these harmful substrates from cells [10].

Of all known MDR transporters, the *Escherichia coli* AcrB multidug efflux pump, which belongs to the RND family, shows the widest substrate specificity ranging from most of the current use antibiotics, disinfectants, dyes, bile salts, fatty acids, and detergents, to simple solvents [11]. AcrB is regulated by a global transcriptional activator MarA and a local transcriptional repressor AcrR [12]. The *acrR* gene is located 141 bp upstream of the *acrAB*

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operon and transcribed divergently [12]. It encodes a 215-amino-acid protein, which shares N-terminal sequence and structural similarities to members of the TetR family of transcriptional repressors [13]. Like other members in the TetR family, the N-terminal domain of AcrR contains a predicted DNA-binding helix-turn-helix (HTH) motif, while its C-terminal domain has unique sequences and is expected to form a multi-drug binding site for its inducing drugs.

As a member of the TetR family of transcriptional regulators, it is expected that binding of drug to the C-terminal ligand-binding domain of AcrR triggers conformational change in its N-terminal DNA-binding region. This change in conformation results in the release of AcrR from its operator DNA, and thus allows transcription from its cognate promoter.

At present, the AcrR palindrome (IR) operator site has not been identified. However, there is strong evidence that this IR is composed of a 24 bp sequence, 5′**TACATAC ATT**TGTG**AATGTATGTA**3′. Ma et al. [12] demonstrated that AcrR interacts with the *acr* promoter located in the section between *acrR* and *acrAB*. A comparison of nucleotide sequences upstream of multidrug resistance transport genes also predicted that the upstream region of the *acrAB* operon contains a candidate IR operator. This operator consists of an inverting 24 bp sequence, which is well conserved and overlaps with the *acrAB* promoter [14].

In this study, we used fluorescence polarization assay [15–17] to examine interaction between AcrR and its predicted target DNA. We also used this method to determine the affinities of three different AcrR drugs, ethidium (Et), proflavin (Pf), and rhodamine 6G (R6G). So far, all known members of the TetR family have been reported to bind target DNAs in the form of homodimers as basic units [13]. Thus, the DNA-binding data were fitted using an AcrR dimer as a fundamental unit. For drug binding, we fitted these data using a monomeric AcrR as a single structural unit since many of the TetR family members bind ligands in both subunits of the homodimers, although QacR only binds one drug molecule in the dimer [18]. The possibility of simultaneous binding of two different drugs by AcrR was also examined using this fluorescence polarization technique.

Results

DNA binding

Presumably, AcrR suppresses the expression of the AcrB multidrug efflux pump by binding its target DNA. As mentioned above, the predicted IR operator of AcrR is composed of an inverting 24 bp sequence. Fluorescence polarization-based assay was carried out to study the interaction between AcrR and a 28 bp DNA containing the IR sequence. Fig. 1A illustrates the binding isotherm of AcrR in the presence of 1 nM fluoresceinated DNA. The titration experiment indicated that AcrR binds the 28 bp operator, with a dissociation constant, K_D, of 20.2 ± 1.4 nM. This value is substantially lower than those of the CmeR ($K_D = 88$ nM) [19] and EthR ($K_D = 146$ nM) [20] repressors, but albeit higher than the values of TetR ($K_D = 0.2$ nM) [21], MtrR ($K_D = 0.9$ nM) [17], and QacR ($K_D = 5.7$ nM) [17] in the TetR family. It should be noted that this K_D is sensitive to the concentration of salts in titration buffers, and in some cases also the length of oligodeoxynucleotides. Doubling the salt concentration may increase the K_D by two orders of magnitude as shown in MtrR [17].

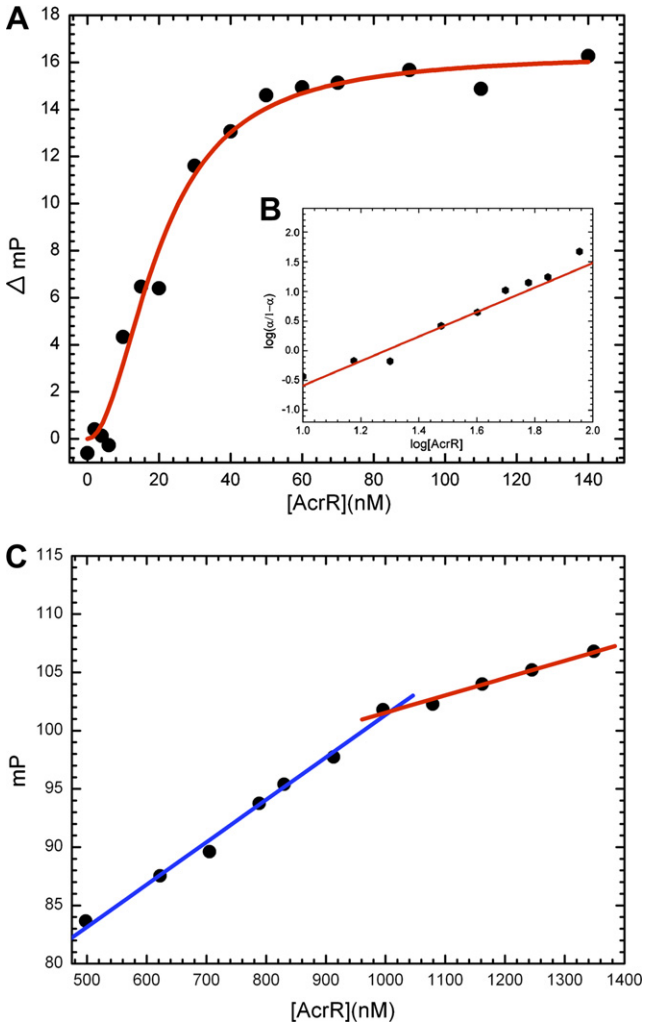

Fig. 1. AcrR binding to the 28-mer cognate oligodeoxynucleotide. (A) Binding isotherm of AcrR in the presence of 10 mM Na-phosphate (pH 7.2), 100 mM NaCl, 1 nM fluoresceinated DNA, and 1 μg of poly(dI–dC) as non-specific DNA. The sequence of one of the IR strands is 5′-TTTACATACATTTGTGAATGTATGTACC-3′. The binding curve suggests a K_D of 20.2 ± 1.4 nM. (B) Hill plot of the data obtained for IR binding to AcrR. α corresponds to the fraction of bound IR. The plot gives a slope of 2.06 ± 0.12, indicating a cooperative binding process with a stoichiometry of two AcrR dimers per one IR. The interception of the plot provides a K_D of 19.2 ± 1.0 nM for the IR binding. (C) Determination of the stoichiometry of AcrR–DNA binding. The inflection point at an AcrR dimer concentration of 1000 nM in the presence of 500 nM DNA, indicating the stoichiometry of 2:1 protein-to-DNA ratio.

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A Hill plot of the DNA-binding data yielded a Hill coefficient of 2 (Fig. 1B), suggesting a stoichiometry of two AcrR dimers per cognate DNA. The corresponding Scatchard plot showed a typical convex curve (see Suppl. Fig. S1), a result suggesting that more than one dimer of AcrR bind cooperatively to the DNA. To confirm the stoichiometry of AcrR and DNA binding, the protein was titrated into the binding buffer (containing 500 nM fluoresceinated DNA) until the concentration of AcrR (as dimer) reached 1500 nM (Fig. 1C). The result indicated that the inflection point occurs at a dimeric AcrR concentration of 1000 nM, suggesting a stoichiometry of two dimers of AcrR per cognate DNA.

Binding of AcrR ligands

The goal of this experiment was to determine the binding affinities of a variety of AcrR ligands. We initially measured the binding affinity of Et by AcrR. Fluorescence polarization measurements showed that the K_D of the AcrR–Et complex is 4.2 ± 0.3 μM (Fig. 2A). The Hill and Scatchard plots of the data (Fig. 2B and C) suggested that the protein employs a simple binding stoichiometry of 1:1 monomeric AcrR to Et molar ratio.

Fluorescence polarization was also used to observe the Pf and R6G binding (Fig. 3). The values of K_D were found to be 10.1 ± 0.8 μM and 10.7 ± 1.4 μM, respectively. QacR binds Et, Pf, and R6G with K_D values of 186, 42, and 0.8 μM, respectively [22]. Thus, AcrR appears to have stronger interaction with Et and Pf, but weaker with R6G.

Evidence of simultaneous binding of two drugs by AcrR

It has been shown that two different drugs bind simultaneously to two different sites in a large binding pocket of the QacR regulatory protein [22]. This raises the question whether AcrR can accommodate two different drugs at a time. We carried out a series of titration experiments that involved the initial saturation of AcrR with 100 μM of Et. To ensure that AcrR and Et formed a complex, the mixture of AcrR and Et was incubated for at least two hours before titration. The pre-formed AcrR–Et complex was then titrated with 1 μM of Pf. Fluorescence polarization assay was used to monitor the change in polarization of Pf. By using this method, we were able to provide evidence for the simultaneous binding of two drugs by AcrR. The polarization experiments indicated that AcrR binds Pf, with a K_D of 14.9 ± 0.9 μM, in the presence of 100 μM Et. Fig. 4A depicts the binding isotherm of the AcrR–Et complex in titrating with Pf. This value is very close to the K_D of Pf (10.1 ± 0.8 μM) in the absence of Et, indicating that the pre-bound Et does not affect the binding of Pf to the regulator.

We also studied the binding of R6G in the presence of 100 μM Et using the same method. Fluorescence polarization experiments indicated that AcrR–Et binds R6G with a K_D of 132.0 ± 9.1 μM (Fig. 4B). This K_D is about

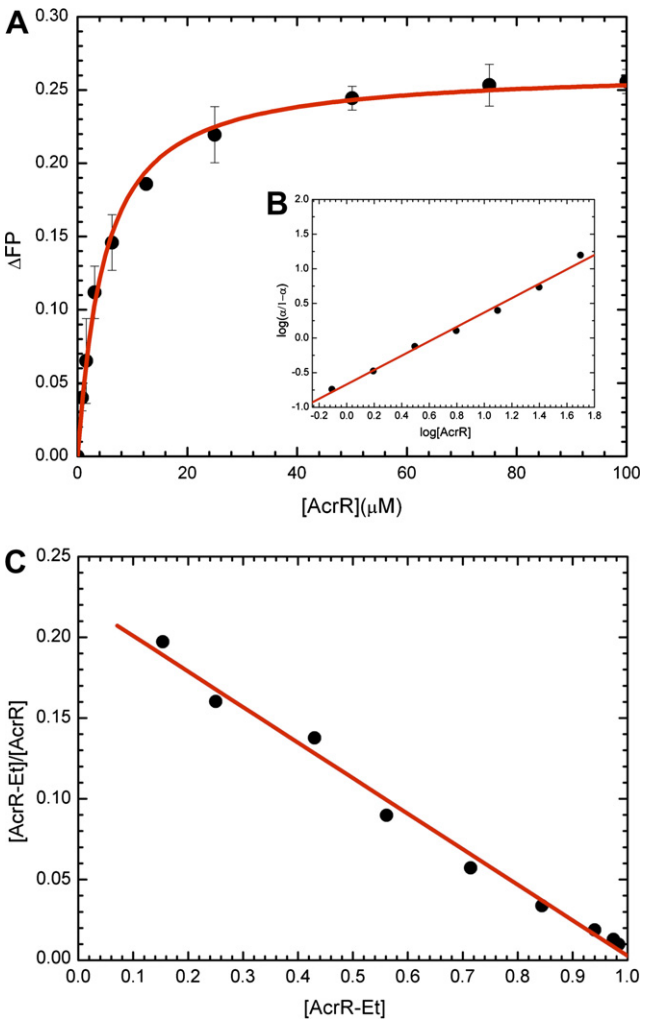

Fig. 2. Representative fluorescence polarization of AcrR with Et. (A) Binding isotherm of AcrR with Et, showing a K_D of 4.2 ± 0.3 μM. (B) Hill plot of the data obtained for Et binding to AcrR. α corresponds to the fraction of bound Et. The plot gives a slope of 1.02 ± 0.02, indicating a simple binding process with a stoichoimetry of one AcrR protomer per one drug molecule. The interception of the plot provides a K_D of 4.4 ± 1.0 μM. (C) Linearization of the data obtained for Et binding to AcrR in a Scatchard plot, indicating a K_D of 4.6 ± 0.3 μM.

13 times of that of R6G in the absence of Et, suggesting the presence of Et severely interferes with the binding of R6G in AcrR.

Discussion

Using recombinant AcrR protein that contains a 6xHis tag at the C-terminus, we confirmed that AcrR interacts with the 28 bp DNA, 5′TT**TACATACATT**TGTG**AATG TATGTA**CC3′. This DNA contains the inverting 24 bp IR. IR is a typical DNA sequence that forms binding sites for regulatory proteins in the promoter regions. A gel mobility shift assay, using a DNA fragment bearing the sequence of the promoter region between *acrR* and *acrAB*, has demonstrated that AcrR binds to the promoter sequence. Thus, our experimental data strongly suggest

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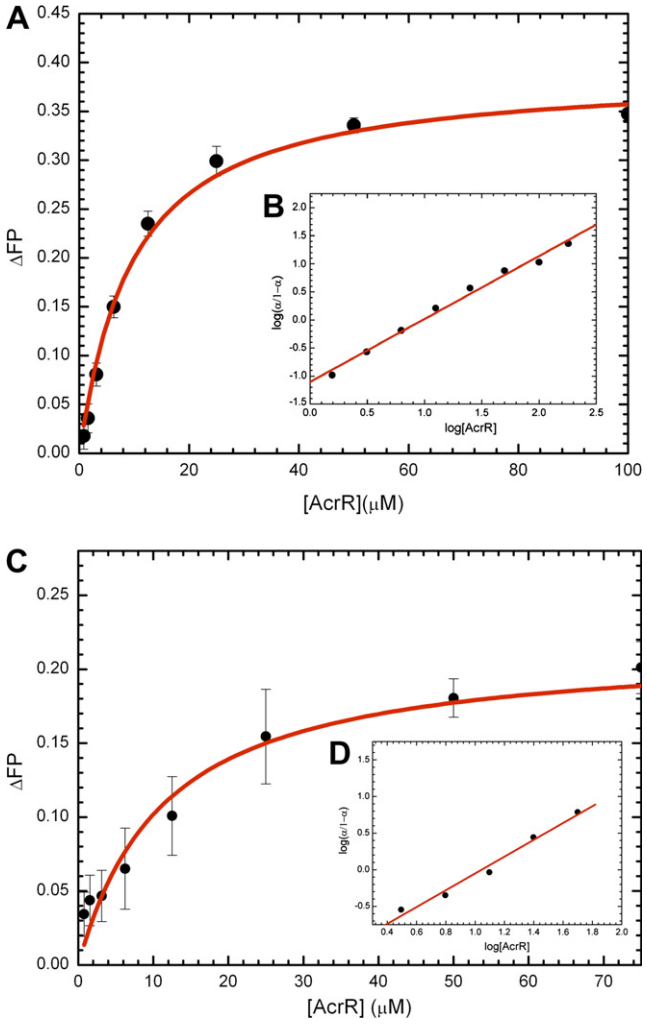

Fig. 3. Fluorescence polarization of AcrR with Pf and R6G. (A) Binding isotherm of AcrR with Pf, showing a K_D of 10.1 ± 0.8 μM. (B) Hill plot of the data obtained for Pf binding to AcrR. α corresponds to the fraction of bound Pf. The plot gives a slope of 1.12 ± 0.05, and the interception provides a K_D of 9.6 ± 1.1 μM. (C) Binding isotherm of AcrR with R6G, showing a K_D of 10.7 ± 1.4 μM. (D) Hill plot of the data obtained for R6G binding to AcrR. α corresponds to the fraction of bound R6G. The plot gives a slope of 1.14 ± 0.09, and the interception provides a K_D of 11.1 ± 1.2 μM.

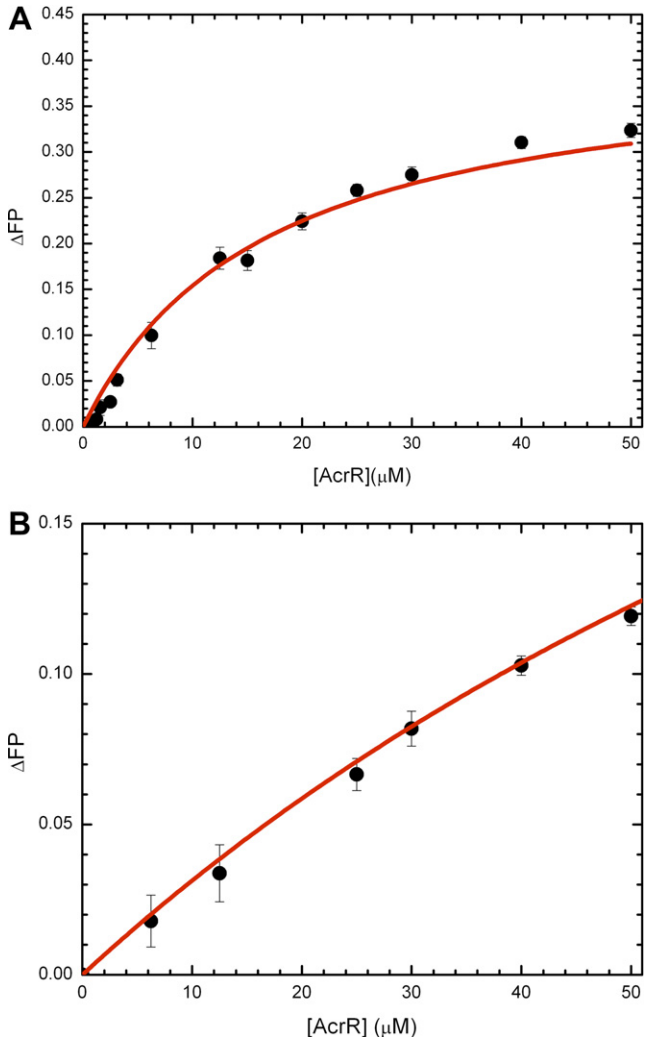

Fig. 4. Binding of ligands by AcrR in the presence of 100 μM of Et as determined by fluorescence polarization assay. (A) The change in fluorescence polarization signals (ΔFP) of Pf measured at an emission wavelength of 508 nm. The binding curve suggests a K_D of 14.9 ± 0.9 μM for Pf. (B) The change in fluorescence polarization signals (ΔFP) of R6G measured at an emission wavelength of 550 nm. The binding curve suggests a K_D of 132.7 ± 14.6 μM for R6G. The maximum concentration of the AcrR monomer was 50 μM.

that this predicted 24 bp IR, located at the promoter region of *acrR–acrAB*, is very likely to serve as the *acr* operator.

The affinity of AcrR for IR is in the nanomolar range (20.2 nM), and this dissociation constant is closer to that of QacR among members of the TetR family. The IR sequence of AcrR is similar in length to that of the long 28 bp IR1 recognized by QacR, but is different from the 15 bp *tetO* bound by TetR. TetR binds as a single dimer to the *tetO* operator [23], however, two dimers of QacR interact with one IR1 [24]. Based on the length of the IR sequence, we reasoned that AcrR might bind its operator as two homodimers in a way that is similar to the QacR–DNA binding. Our fluorescence polarization measurements suggested that two AcrR dimers interact with one 28 bp IR. Thus, it is very likely that two homodimers of AcrR bind the 24 bp target DNA cooperatively as shown in the structure of the QacR–DNA complex [24].

The affinities of AcrR to its drugs are in the micromolar region, and the dissociation constants are quite similar to those of QacR [25], BmrR [26], and TtgV [27]. Based on the fluorescence polarization assays, titrations of AcrR to the ligand-binding solutions give a stoichiometry of 1:1 monomeric AcrR-to-drug molar ratio. As the recent preliminary X-ray diffraction data suggest a dimeric assembly of the AcrR regulator [28], thus we believe that each C-terminal ligand-binding site of AcrR (each subunit of which contains one site) in the homodimer binds a drug molecule. This drug-binding mode is the same as that of TetR [29], but different from the ligand-binding mode of QacR which binds one drug molecule per homodimer [22]. In comparing the DNA and drug-binding modes of AcrR with those of

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TetR and QacR, AcrR is distinct in that its DNA-binding mode is similar to that of QacR, however, it employs the mode of drug-binding similar to that of TetR. Thus, the induction mechanism of AcrR may have unique features that have not been revealed by previous studies concerning other regulators in the TetR family.

To address the question whether AcrR allows simultaneous binding of two different drugs, we used a ligand-binding assay that utilizes the polarization of fluorescence. Our experimental results suggest that AcrR is most likely to bind Et and R6G competitively as the binding affinity of R6G is greatly decreased in the presence of Et. In the case of Et and Pf, Pf is bound by both apo-AcrR and AcrR–Et in equal affinity, indicating that Et and Pf may exhibit noncompetitive binding. If this is the case, it is anticipated that Et and Pf are bound to distinct and unoverlapped binding sites in the regulator. Indeed, a docking study using the most recent crystal structure of apo-AcrR determined in our laboratory suggests that Et and Pf bind independently at different places in the large multi-drug binding pocket of AcrR (see Suppl. Fig. S4). This simultaneous drug-binding phenomenon has been found in the MdfA transporter [30] and the recent study QacR repressor [22]. Thus, it is not surprising that AcrR is capable of accommodating two different drugs at a time.

Materials and methods

Expression and purification of recombinant AcrR. The cloning, expression, and purification of recombinant AcrR that contains a 6xHis tag at the C-terminus were done as described before [28]. The purified 6xHis AcrR protein was extensively dialyzed against buffer containing 10 mM Na-phosphate (pH 7.2) and 100 mM NaCl, and concentrated to 10 mg/ml.

Fluorescence polarization assay for the DNA-binding affinity. Fluorescence polarization assays were used to determine the DNA-binding affinities of the AcrR regulator. Both the 28 bp oligodeoxynucleotide and fluorescein-labeled oligodeoxynucleotide were purchased from IDT, Inc. (Coralville, IA). These oligodeoxynucleotides contain the predicted 24 bp IR site for AcrR binding. Their sequences were 5′-TT**TACATACATTT** GTG**AATGTATGTA**CC-3′ and 5′-F-GG**TACATACATT** CACA**AATG TATGTA**A-3′, where F denotes the fluorescein which was covalently attached to the 5′ end of the oligodeoxynucleotide by a hexamethylene linker. The 28 bp fluoresceinated ds-DNA was prepared by annealing these two oligodeoxynucleotides together. Fluorescence polarization experiment was done using a DNA-binding solution containing 10 mM Na-phosphate (pH 7.2), 100 mM NaCl, 1 nM fluoresceinated DNA, and 1 μg of poly(dI–dC) as non-specific DNA. The protein solution containing 500 nM dimeric AcrR and 1 nM fluoresceinated DNA was titrated into the DNA-binding solution until the millipolarization (mP) become unchanged. All measurements were performed at 25 °C using a Perkin-Elmer LS55 spectrofluorometer equipped with a Hamamatsu R928 photomultiplier. The excitation wavelength was 490 nm, and the fluorescence polarization signal (in ΔP) was measured at 520 nm. Each titration point recorded was an average of 15 measurements. Data were analyzed using the equation, $P = \{(P_{\text{bound}} - P_{\text{free}})[\text{protein}]^2/(K_D{}^2 + [\text{protein}]^2)\} + P_{\text{free}}$, where P is the polarization measured at a given total protein concentration, P_{free} is the initial polarization of free fluorescein-labeled DNA, P_{bound} is the maximum polarization of specifically bound DNA, and [protein] is the protein concentration. The titration experiments were repeated for three times to obtained the average K_D value. Curve fitting was accomplished using the program ORIGIN [31].

Fluorescence polarization assay for AcrR ligand-binding affinities. The experimental procedures for determining ligand-binding affinities of AcrR using the technique of fluorescence polarization were similar to that of the DNA-binding assay. The experiments were done using a ligand-binding solution containing 10 mM Na-phosphate (pH 7.2), 100 mM NaCl, and 1 μM ligand (R6G, Et, or Pf). The AcrR protein solution in 10 mM Na-phosphate (pH 7.2), 100 mM NaCl, and 1 μM ligand was titrated into the ligand-binding solution until the polarization (P) become unchanged. The excitation wavelengths were 527, 483, and 447 nm, respectively, for R6G, Et, and Pf. Fluorescence polarization signals (in ΔP) were measured at emission wavelengths of 550, 620, and 508 nm, respectively, for these ligands. Each titration point recorded was an average of 15 measurements. The titration experiments were repeated for three times to obtain the average K_D values. Curve fitting was accomplished using the program ORIGIN [31].

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.06.175.

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