**In vivo analysis of DNA binding and ligand interaction of BlcR, an IclR-type repressor from Agrobacterium tumefaciens**

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**Agrobacterium tumefaciens** BlcR represses transcription of the blcABC operon, which is involved in metabolism of γ-butyrolactone, and this repression is alleviated by succinate semialdehyde (SSA). BlcR exists as a homodimer, and the blcABC promoter DNA contains two BlcR-binding sites (IR1 and IR2) that correspond to two BlcR dimers. In this study, we established an *in vivo* system to examine the SSA-responsive control of BlcR transcriptional regulation. The endogenous blcR, encoded in the pAtC58 plasmid of *A. tumefaciens* C58, was not optimal for investigating the effect of SSA on BlcR repression, probably due to the SSA degradation mediated by the pAt-encoded blcABC. We therefore introduced blcR (and the blcABC promoter DNA, separately) exogenously into a strain of C58 cured of pAtC58 (and pTiC58). We applied this system to interrogate BlcR–DNA interactions and to test predictions from our prior structural and biochemical studies. This *in vivo* analysis confirmed the previously mapped SSA-binding site and supported a model by which DNA coordinates formation of a BlcR tetramer. In addition, we identified a specific lysine residue (K59) as an important determinant for DNA binding. Moreover, based on isothermal titration calorimetry analysis, we found IR1 to play the dominant role in binding to BlcR, relative to IR2. Together, these *in vivo* results expand the biochemical findings and provide new mechanistic insights into BlcR–DNA interactions.

**INTRODUCTION**

Many bacteria adapt to environmental changes via transcriptional regulation of specific genes. The IclR-type family of proteins, found in a broad range of bacteria, is a recently defined class of transcriptional regulators (Krell et al., 2006; Molina-Henares et al., 2006). IclR-type regulators are responsive to low-molecular-mass ligands, which usually serve as cues reflecting environmental or physiological changes, and subsequently alter their DNA-binding activities. Proteins of the IclR family are involved in regulation of diverse cellular processes, including metabolism (Sunnarborg et al., 1990; Brune et al., 2007), multidrug resistance (Guazzaroni et al., 2007b), aromatic compound degradation (Romero-Steiner et al., 1994; Tsoi et al., 1999), pathogenicity (Reverchon et al., 1991) and sporulation (Jiang & Kendrick, 2000; Traag et al., 2004; Yamazaki et al., 2003). Despite their important regulatory roles and the increasing evidence of their profound influence on a variety of cellular and physiological processes, there is a limited understanding of the molecular mechanism(s) by which IclR proteins function.

The BlcR (formerly AttJ) protein of *Agrobacterium tumefaciens* has served as an experimentally tractable model for molecular analysis of the IclR protein family. BlcR is a negative regulator of the blcABC operon, and its DNA binding ability is affected by succinate semialdehyde (SSA), its cognate effector (Carlier et al., 2004; Wang et al., 2006; Chai et al., 2007). The blcABC operon is responsible for the catabolism of γ-butyrolactone (GBL) (Carlier et al., 2004; Wang et al., 2006; Chai et al., 2007), and enables *A. tumefaciens* to utilize GBL, commonly found in plant extracts, as a carbon and energy source. Intriguingly, BlcC, a lactonase, also efficiently degrades the *A. tumefaciens* acylhomoserine lactone (AHL) quorum-sensing signal, and exerts a profound influence on this intercellular signalling process (Carlier et al., 2004; Zhang et al., 2002). In our recent structural and biochemical studies (Pan et al., 2011), we solved the crystal structure of the dimeric BlcR and identified residues important for SSA binding via mutagenesis. Two BlcR dimers bind to a DNA sequence, proximal to the blcABC promoter that contains two pairs.
of inverted repeats (IRs) with a short inter-IR gap. We also showed that BlcR tetramerizes on binding to DNA, and that DNA plays a scaffold role for BlcR tetramerization and thereby promotes BlcR–DNA interaction. Studies using a BlcR mutant (BlcRF147A), which is locked in a tetrameric form, support the allosteric mechanism of SSA action on BlcR via interfering with BlcR tetramerization, a process required for effective BlcR–DNA interaction. In this study, we have performed in vivo analyses to examine this molecular model of SSA-regulated BlcR transcriptional control.

**METHODS**

**Strains, plasmids and growth conditions.** The genome of *A. tumefaciens* C58 includes two large plasmids, pTiC58 and pAtC58. C58 At+/Ti− refers to a derivative in which pTiC58 has been cured and C58 p− refers to the strain with both pTiC58 and pAtC58 cured. All the bacterial strains and plasmids used in this study are listed in Table 1. Fragments containing *A. tumefaciens* C58 wild-type blcR or mutated versions of the blcR gene were amplified from plasmids pQE–BlcR or pTB146–BlcR (Pan et al., 2011) using Phusion DNA polymerase. PCR fragments were purified using a PCR purification kit (Qiagen), excised with Ndel and BamHI restriction enzymes and ligated into the compatibly cleaved vector pSRKGm so that the inserted gene is under the control of the lac promoter (Plac+, blcR, Gm±), termed pCL6, or pCL6–blcRmut. A DNA fragment of 800 bp containing the BlcABC promoter (PblcA) region was amplified from *A. tumefaciens* A6 genomic DNA and fused, with BamHI and PstI sites, to lacZ (P(blcA–lacZ, Sp)−) on the vector pRA301, to create pC8. The Pbla fragment was designed to insert the promoter region and ribosome-binding site with the start codon of the blcA gene in-frame with the lacZ coding sequence. Plasmids pCL8–49 bp, pCL8–50 bp, pCL8–52 bp and pCL8–53 bp were generated by incorporating site-specific point mutations in the inter-IR gap region of Pbla in accordance with our previous study (Pan et al., 2011) using the QuickChange kit (Stratagene). All plasmid constructs and mutations were confirmed by DNA sequencing. Plasmids were electroporated into *A. tumefaciens* using a standard method (Mersereau et al., 1990).

*A. tumefaciens* derivatives were grown in ATGN medium [10.7 g KH2PO4 l−1, 160 mg MgSO4 l−1, 10 mg CaCl2 l−1, 5 mg FeSO4 l−1, 2 mg MnSO4 l−1, 2 g (NH4)2SO4 l−1 and 0.5% (w/v) glucose] (Tempe et al., 1977) at 28 °C. Escherichia coli strains were grown in LB medium at 37 °C. Transformants with appropriate antibiotic resistance were selected [pCL6 or its derivatives with gentamicin (Gm) selection and pCL8 or its derivatives with spectinomycin (Sp) selection]. The final antibiotic concentrations were: for *A. tumefaciens*, 300 μg Gm ml−1, 300 μg Sp ml−1; and for *E. coli*, 100 μg ampicillin (Ap) ml−1, 30 μg Gm ml−1, 100 μg Sp ml−1.

**Assay of β-galactosidase activity (Miller, 1972).** Cells for β-galactosidase assay were cultured from a fresh single colony in 2 ml ATGN medium in the presence of desired amounts of SSA (pH 7.0). Sigma–Aldrich) and appropriate concentrations of IPTG at 28 °C to an OD600 of approximately 1.0 (the actual OD600 was recorded). Then, 0.1 ml cell culture was mixed with 0.9 ml Z-buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4, 50 mM β-mercaptoethanol, pH 7.0), 35 μl 0.05% SDS and 30 μl chloroform. The sample was manually shaken vigorously for 30 s to facilitate cell lysis and 100 μl of a 4 mg ml−1 solution of the colorimetric reagent ONPG (Sigma) was added to the lysed culture. The reactions were incubated at room temperature and timed from the point of ONPG addition until the solution was medium yellow or if no yellow colour appeared within several hours, and the reaction was terminated by adding 600 μl 1 M Na2CO3 and the reaction time recorded. After centrifuging the sample at 13 000 r.p.m. (18 000 g) for 5 min, 350 μl of the supernatant was transferred to a 96-well microplate. Sample A420 was recorded using Synergy HT Microplate Reader (BioTek). The β-galactosidase activity in Miller units (MU) is calculated as follows:

$$MU = 1000 \times \frac{A420}{OD_{600} \times \text{time (min)} \times f}$$

where $f = \frac{V_{\text{cell}}}{V_{\text{cell}} + V_{\text{z-buffer}}}$.

**Isothermal titration calorimetry (ITC).** To generate the DNA duplex, the single-stranded oligonucleotide and its complementary oligonucleotide (Integrated DNA Technologies) were incubated at 98 °C, and the temperature was slowly decreased to allow optimal annealing. ITC experiments were carried out at 25 °C in a VP-ITC titration calorimeter system (MicroCal). A total of 30 aliquots of 10 μl samples of ~200 μM BlcRF147A (in monomeric form) were injected into the DNA solution (~5 μM) at 420 s intervals. Proteins and DNA were analysed in 50 mM Tris/HCl, pH 7.5, 300 mM NaCl and 0.5 mM EDTA. Data were processed with Origin (OriginLab), and the baseline-corrected binding isotherm was used to derive thermodynamic parameters of the binding process.

**RESULTS**

**Induction of the blcABC promoter using *A. tumefaciens* C58 At+/Ti−**

To develop an appropriate *A. tumefaciens* derivative for in vivo analyses of the SSA response of BlcR, we evaluated the activity of the native blcR gene encoded on the resident pAtC58 plasmid using the PblcA–lacZ-containing reporter pC8. As shown in Fig. 1(a) (inset), when pC8 was introduced into *A. tumefaciens* C58 At+/Ti−, a minimal β-galactosidase activity (approximately 5 MU) was observed suggesting the possibility that the endogenous BlcR was repressing the Pbla–lacZ fusion on pC8. When using the isogenic *A. tumefaciens* C58 p− (cured of pAtC58) without resident blcR, a drastic increase in β-galactosidase activity was observed for this same PblcA–lacZ fusion plasmid [−95 MU, Fig. 1(a) (inset)], supporting the model that the pAtC58-encoded blcR repressed Pbla–lacZ activity. Next, we examined how SSA affected the repression of the pAtC58-encoded blcR on Pbla–lacZ. A range of SSA concentrations were added exogenously to C58 At+/Ti− at the time of inoculation, and β-galactosidase activity was found to increase with SSA concentration, to as high as sixfold at 2 mM SSA. This amount of expression is 30% that of the fully derepressed Pbla–lacZ activity in the C58 p− derivative, in which blcR is not present (~100 MU, with or without SSA) [Fig. 1(a) (inset)]. Above this SSA concentration there was substantial growth inhibition, suggesting a detrimental effect of excess SSA on cellular viability. To assess the SSA effect on blcR activity at high concentrations, we allowed *A. tumefaciens* cells to grow to a cell density of OD600 = 1.0, added a range of SSA concentrations to cell cultures and continued cell growth for three more hours before assaying for β-galactosidase activity. Although β-galactosidase activity appeared to
increase with SSA concentration, its maximal value of approximately 45 MU at 5 mM SSA was much less than that expected for full derepression. The inability of SSA to fully derepress the pAtC58-encoded blcR that expected for full derepression. The inability of SSA to approximately 45 MU at 5 mM SSA was much less than

### Table 1. Bacterial strains and plasmids used in this study

Ap', Gm' and Sp', resistance to ampicillin, gentamicin and spectinomycin, respectively.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)</th>
<th>Reference or source</th>
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<tr>
<td><strong>A. tumefaciens strains</strong></td>
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<tr>
<td>C58</td>
<td>Nopaline-type strain, pTiC58, pAtC58</td>
<td>Scialy <em>et al.</em> (1978)</td>
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<td>C58 At^+/Ti^-</td>
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<td>Our collection</td>
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<td>C58 At^+/Ti^- (pCL8)</td>
<td>Derivative of C58 At^+/Ti^- harbouring pCL8</td>
<td>This study</td>
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<td>C58 p^- (pCL8)</td>
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<td>C58 p^- (pCL8–blcRmut)</td>
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<td>C58 p^- (pCL8(n))</td>
<td>Derivative of C58 p^- harbouring pCL8 variants with altered inter-IR distance, n=+2, +1, −1, −2 bp</td>
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<td>pCL8(−2)</td>
<td>Derivative of pCL6 with an A base deletion in the IR gap region of PblcA</td>
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### Ectopic plasmid-borne expression of blcR provides SSA-modulated repression of the blcABC promoter

In order to circumvent any potential problems of blcABC-mediated SSA degradation or endogenous blcR expression control, we constructed a plasmid to express the blcR regulator from the lac promoter (P_{lac}−blcR, pCL6), which also carries the lac repressor (lacI) and therefore provides IPTG control of expression. To study BlcR function, we introduced this blcR expression plasmid into *A. tumefaciens* C58 p^- harbouring pCL8 (P_{blcA}−lacZ) and tested β-galactosidase activity as a function of IPTG concentration. Induction of blcR expression with 100 μM IPTG repressed the P_{blcA}−lacZ activity to a level (approximately 5 MU, Fig. 1b) similar to that observed in C58 At^+/Ti^- (pCL8) approximately 5 MU, Fig. 1(a) in which blcR is supplied from its native location on pAtC58, suggesting that expression of the pCL6-borne blcR was induced in a range at or above that of the wild-type copy. Note that this amount of the P_{lac} inducer had no effect on the strain harbouring the vector control instead of blcR (grey bars in Fig. 1b). Importantly, in direct contrast to the pAt-encoded blcR, activity of the pCL6-borne blcR induced with 100 μM IPTG was completely inhibited by 1 mM SSA (Fig. 2), a concentration below the threshold of 2 mM at which SSA is inhibitory to cell growth (Fig. 1a). Thus, unless stated otherwise in subsequent experiments, *A. tumefaciens* C58 p^- (pCL8 pCL6) was used to study the effect of SSA on BlcR activity, and 100 μM IPTG was used to induce expression of pCL6-borne wild-type or mutant blcR.
In vivo analysis of BlcR mutants compromised for SSA interactions

BlcR residues Y133, F147, T158, D210 and C220 are important for SSA binding, based on structural and biochemical studies (Pan et al., 2011). Individual mutation of these residues to Ala resulted in proteins that maintained wild-type repressive activity on \( P_{blcA} \) (Fig. 2). Consistent with our prior biochemical analyses, this suggests that these mutations did not weaken DNA binding of BlcR (Pan et al., 2011). Mutations of D210A or C220A abolish SSA binding to BlcR and the Y133A mutation decreases SSA binding affinity by approximately 25-fold (Pan et al., 2011). The three mutants maintained repression of \( P_{blcA} \) even in the presence of 2 mM SSA (Fig. 2). BlcRT158A appeared to partially respond to SSA, with 58 % (51 MU) and 68 % (75.2 MU) of the level of derepression of wild-type in the presence of 1 and 2 mM SSA, respectively. These findings suggest a weakened affinity of BlcRT158A for SSA. Interestingly, the approximately 50 % derepression displayed by BlcRT158A in the presence of 1 mM SSA correlates well with ITC analysis showing that this protein binds SSA with 50 % the affinity of wild-type BlcR [dissociation constant (\( K_d \) of 1.6 and 0.7 \( \mu \)M, respectively)] (Pan et al., 2011). Lastly, we have isolated a BlcR mutant that is apparently locked in a tetrameric form (BlcRF147A) which mimics the DNA-bound BlcR (Pan et al., 2011). Also in that study, we have shown that SSA-bound BlcRF147A retains a tight interaction with DNA based on ITC. Consistently, despite the presence of inducing levels of SSA (1–2 mM SSA), the \( blcR^{F147A} \) mutant continued to repress the \( P_{blcA} \) promoter, as indicated by low \( \beta \)-galactosidase activity (8.8 ± 0.3 MU). Taken together, these in vivo analyses are consistent with our structural and biochemical observations, providing additional support for the role of key SSA binding site residues for interactions between BlcR and SSA and in the tetramerization of BlcR in binding to its DNA target site.

Lysine 59 as the residue important for DNA binding

K59, a DNA-binding-promoting residue, is located on the potential DNA binding helix, and its side chain is solvent-accessible (Pan et al., 2011). To test whether K59 has a role
in BlcR–DNA interaction, this position was mutated to Ala (K59A) and the regulatory activity of BlcRK59A on PblcA was analysed using the PblcA–lacZ fusion. Induction of blcRK59A did not repress PblcA, as its associated β-galactosidase activity was the same as that of the control without blcR (94.8 ± 5.7 MU; Fig. 2). The observation that BlcRK59A did not repress the PblcA promoter suggests that K59 is an important residue for DNA-binding activity of BlcR.

The role of the BlcR target site associated with the blcABC promoter

The DNA sequence proximal to the PblcA promoter (Chai et al., 2007) plays an important role in binding BlcR by mediating formation of the DNA-binding, active, tetrameric form of BlcR (Pan et al., 2011). The 51 bp DNA sequence we have identified in the BlcR–DNA binding study consists of two IR pairs (Fig. 3a), with each IR functioning as a binding element for one BlcR dimer, and with the two IRs separated by three base pairs. Our prior ITC measurements have shown that when the inter-IR distance is increased or decreased by 2 or 1 bp (+2, +1, −1 and −2 bp), DNA fragments with such altered inter-IR distance do not bind the dimeric BlcR\textsuperscript{wt}. However, we have found that the modified binding site sequences (+2, +1 and −1 bp) retain high affinity to the preassembled, tetrameric BlcR\textsuperscript{F147A}.

To confirm these in vitro findings, we modified the promoter P\textsubscript{blcA} in pCL8 accordingly and examined the interaction of both BlcR\textsuperscript{wt} and BlcR\textsuperscript{F147A} with the modified P\textsubscript{blcA} promoters via β-galactosidase activity. When two base pairs (GG) were inserted into the space between the two IRs (Fig. 3a), repression by BlcR\textsuperscript{wt} on the modified P\textsubscript{blcA}+2 promoter was relieved by approximately 15-fold as compared with the wild-type P\textsubscript{blcA} (Fig. 3b). In contrast, repression by BlcR\textsuperscript{F147A} on the modified P\textsubscript{blcA}+2 promoter was relieved only modestly by around twofold, compared with the wild-type promoter. When the inter-IR distance was either increased or decreased by 1 bp, repression by BlcR\textsuperscript{wt} on both P\textsubscript{blcA}+1 and P\textsubscript{blcA}−1 was approximately 10-fold weaker than on P\textsubscript{blcA}. In comparison, repression activity by BlcR\textsuperscript{F147A} on both modified promoters remained relatively strong (Fig. 3b). Thus, repression activity of dimeric BlcR\textsuperscript{wt} was more significantly affected by the inter-IR spacing in P\textsubscript{blcA} than was tetrameric BlcR\textsuperscript{F147A}. These in vivo observations, along with the biochemical analyses, argue for the important structural role of DNA in orchestrating formation of the DNA-binding, active BlcR tetramer.
(a) DNA sequences of the BlcR binding site variants with altered inter-IR distance or switched IR1 and IR2. Dark- and light-grey boxes denote sequences that are symmetrically related in IR1 and IR2, respectively. Nucleotides that are symmetrically related are underlined in each variant. (b) Effects of altering the inter-IR distance on \( P_{\text{blcA}} \) on BlcR transcriptional activity. IPTG (100 \( \mu \)M) was included at the beginning of culture growth, strains were grown to OD600 ~1.0 and \( \beta \)-galactosidase activity was measured. No SSA was included in the experiments. As in Fig. 2, strain C58 p' (pCL8 pSRKGm), which does not contain the \( \text{blcR} \) gene, was included as the negative control, and its \( \beta \)-galactosidase activity was set to 100 %. At least three biological replicates were performed for each experiment, and SDs are shown as error bars. (c, d) ITC studies on binding of BlcRF147A with the 49 bp DNA (c) and 53 bp DNA (d). Binding of BlcRF147A to the 53 bp DNA (\( K_d = 5.64 \) nM and \( N = 1.1 \)) is similar to that of BlcRF147A protein with the cognate 51 bp DNA (\( K_d = 54 \) nM and \( N = 1.0 \)) (Pan et al., 2011).
When two base pairs (CA) were omitted from the original 3 bp inter-IR gap, wild-type BlcR did not repress expression from this promoter at all (Fig. 3b), suggesting that interaction between BlcR and the modified \( P_{\text{blcA} - 2} \) was completely abolished. Strikingly, BlcRF\(^{147A} \) also failed to inhibit \( P_{\text{blcA} - 2} \), in contrast to its inhibition of the other blcA promoters, wild-type and modified \( (P_{\text{blcA} + 2}, P_{\text{blcA} + 1} \) and \( P_{\text{blcA} - 1}) \). To extend our \textit{in vivo} observations, we deleted the two base pairs in the inter-IR region in the 51 bp DNA sequence used in our earlier biochemical studies and measured binding of this 49 bp DNA to BlcRF\(^{147A} \) by ITC. No interaction of the 49 bp DNA and BlcRF\(^{147A} \) was detected (Fig. 3c), in direct contrast to strong binding of other 51 bp derivatives (53, 52 and 50 bp; Fig. 3d for an example).

The above findings with BlcRF\(^{147A} \) provide information on BlcR recognition pattern in target DNA. To test the hypothesis that the sequence in IR1 has greater effect on BlcR–DNA interaction than that in IR2, we examined binding of BlcR to a 39 bp DNA fragment that included an intact IR1 but a 3’-truncated IR2 (see Fig. 3a). BlcRF\(^{147A} \) bound this 39 bp DNA with a similar affinity as the cognate 51 bp fragment \( (K_d 86 \pm 14 \text{ nM, Fig. 4(a)}; \) and \( 54 \pm 6 \text{ nM (Pan \textit{et al.}, 2011), respectively}). In a different experiment, when the positions of IR1 and IR2 were switched (Fig. 3a), the 51 bp variant did not bind to BlcR\(^{wt} \) nor to tetrameric BlcRF\(^{147A} \) based on ITC measurements (see Fig. 4b for an example). These findings further support the primary role of IR1 in the BlcR–DNA interaction.

**DISCUSSION**

BlcR belongs to the IclR-type transcriptional family, whose members are involved in a diverse range of important cellular processes. Mechanistic information on how IclR proteins function is scarce. BlcR of \( A. \text{tumefaciens} \) has served as a tractable system for biochemical and mechanistic investigations, with a well-identified target promoter and the SSA inducing ligand. Here, we have established an \textit{in vivo} system that allows us to examine the interaction of BlcR with the \( P_{\text{blcA}} \) promoter. We showed that the pAt-encoded resident \( \text{blcR} \) was not optimal for examination of the effect of SSA on BlcR function, because only partial derepression of the \( P_{\text{blcA}} \) promoter could be achieved by addition of exogenous SSA at concentrations not detrimental to cell growth. We therefore used the \( A. \text{tumefaciens} \) C58 p\^-\text{cured of pAtC58 (and pTiC58) expressing blcR from P_{lac} (pCL6) and the P_{blaA-lacZ} fusion plasmid (pCL8) to evaluate the influence of SSA on BlcR-mediated repression at non-inhibitory SSA concentrations. Induction of \( P_{\text{lac}}-\text{blcR} \) at 100 \( \mu \text{M IPTG, with 1 mM SSA to relieve this repression, was found to be optimal. We subsequently applied this \textit{in vivo} system to expand upon results from our earlier biochemical and structural studies and to reveal new mechanistic insights. In particular, K59 appeared to be important for BlcR to bind to the target promoter sequence, as its mutation abolished repression by BlcR. In investigating the role of the inter-IR gap in \( P_{\text{blcA}} \) on transcriptional activity of BlcR, we showed that the +2, +1 and -1 bp inter-IR modifications drastically reduced repression of BlcR\(^{wt} \) as expected, but only modestly affected the repressive activity of tetrameric BlcRF\(^{147A} \).
supporting our model of DNA in orchestrating formation of the DNA-binding-active, repressive tetrameric BlcR. Intriguingly, a 2 bp deletion in the inter-IR gap, however, abolished both BlcR<sup>F147A</sup> repression and the BlcR<sup>F147A</sup>-DNA interaction. We analysed the orientations of the BlcR-binding site between IR1 and IR2 in the spacing variants, but could not account for the differential effects of the inter-IR spacing on the DNA binding of BlcR<sup>F147A</sup>. For example, although the 2 bp shortening of the inter-IR spacing alters relative orientation between IR1 and IR2, a simple misalignment of the BlcR-binding site in IR1 and IR2 along the surface of the DNA structure cannot explain the resulting complete loss in DNA binding of BlcR<sup>F147A</sup>.

Palindromic sequences frequently function as binding sites for the transcription factors. For BlcR, there are two pairs of IRs with divergent sequences, with each IR functioning as a single BlcR-binding element for one BlcR dimer (Chai et al., 2007; Pan et al., 2011). Our findings using DNA fragments with the IR2 truncation or with the switched IR1/IR2 sequence suggested that IR1 has a greater effect on BlcR–DNA interaction than IR2 and support a model in which binding of one BlcR dimer to IR1 recruits a second BlcR dimer to interact with IR2 to form a functional DNA–protein complex. The primary role of IR1 in binding BlcR may be attributed to the additional pair of symmetry-related nucleotides compared with IR2 (Fig. 3a). The secondary role of IR2 relative to IR1 in the overall BlcR–DNA interaction is also consistent with studies of both transcriptional activity and ITC using the BlcR binding sequences with variable inter-IR distance. All modifications to the spacing between IR1 and IR2 decreased the binding and in vivo repression of wild-type BlcR. However, the tetrameric BlcR<sup>F147A</sup> mutant was able to bind to most of these spacing variants, suggesting that the failure of wild-type BlcR to bind was due to the inability of the altered sites to foster tetramerization. However, the –2 construct in which IR2 is moved two bases closer to IR1 could not be bound by BlcR<sup>F147A</sup> and wild-type BlcR. Each base pair of spacing alters the position of the BlcR-binding site on the face of the DNA double helix by 36 degrees. Apparently, the BlcR<sup>F147A</sup> tetramer can better tolerate shifting of the binding site in one direction over the other, probably because the narrower intervening sequence may be sterically blocked in the BlcR<sup>F147A</sup> fixed tetramer, whereas the outward flexing of the tetramer is better tolerated.

BlcR of <i>A. tumefaciens</i> and TtgV of <i>Pseudomonas putida</i>, the two IclR proteins whose biochemical mechanisms have been characterized at the molecular level, share similar characteristics but also differ in several aspects of their transcriptional regulation. Both target promoters (P<sub>B6A</sub> and P<sub>TtgC</sub>) contain two pairs of IRs arranged consecutively, suggesting a tetrameric binding of the regulator. We have shown in this study and prior work that BlcR exists as dimer in its unbound form yet binds to DNA as a tetramer and that tetramerization of BlcR is structurally orchestrated on the DNA via the specific arrangement of the two BlcR-binding sites (Pan et al., 2011). In contrast, TtgV is a tetramer but with two conformations, a less stable asymmetrical (T) state when in isolation and a more stable symmetrical (R) state when in complex with DNA (Guazzaroni et al., 2007b; Lu et al., 2010). Like BlcR, the TtgV monomer folds into two functional domains, the DNA-binding N-terminal domain (NTD) and the ligand-binding C-terminal domain (CTD), which are connected via a linker helix. The helix adopts a straight helical conformation in the symmetrical T state, while it becomes bent and distorted in the R state, resulting in an asymmetrical arrangement of the NTDs relative to their CTDs. The ability of the NTDs to assume different orientations allows TtgV to adopt a configuration that interacts effectively with the two consecutive IR pairs on DNA. Unlike in BlcR–DNA binding, the DNA-binding-induced conformational change to align TtgV NTDs with IR pairs does not affect the oligomeric state of TtgV, despite small structural adjustments in the tetrameric interface. Differences in the influence of the DNA target site on protein multimerization during BlcR–DNA and TtgV–DNA interactions may account for the divergent mechanisms by which the inducing ligand regulates the DNA-binding activity of BlcR and TtgV. Binding of SSA is proposed to interfere with the DNA-mediated tetramerization of BlcR and, as a result, the DNA-binding, active BlcR tetramer dissociates into the stable, inactive SSA-bound BlcR dimer. The proposed role of SSA in dissociating tetrameric BlcR may be explained by partial overlap of the SSA-binding site and the BlcR tetramerization interface. This model is supported by structural and mutational analyses of the BlcR<sup>F147A</sup> mutant protein, which is locked in a tetrameric state. The BlcR<sup>F147A</sup> tetramer binds DNA with 10-fold stronger affinity when compared with the wild-type. Intriguingly, F147 is situated within the SSA-binding site on BlcR: thus the parallel involvement of F147 in two important, yet distinct, functions provides a mechanistic link to relay SSA occupancy into changes in BlcR oligomerization. In contrast, the DNA-binding activity of TtgV is regulated via different mechanisms from that of BlcR and does not involve changes in oligomeric state, as TtgV is a stable tetramer. TtgV appears to recognize a wide range of chemical ligands (Guazzaroni et al., 2005) and may utilize different sets of amino acid residues to interact with different ligands (Guazzaroni et al., 2007a). One ligand-binding site is proposed to be near the TtgV tetramerization interface, and presumably the bound ligand prevents TtgV CTDs from undergoing the aforementioned conformational adjustments in the tetrameric interface observed for the TtgV–DNA complex. A second ligand-binding site is close to the linker helix, and binding of ligand to this site may prevent the linker helix from adopting the bent conformations, thereby dissociating TtgV from DNA. Thus, although transcriptional regulators of the IclR family may have structural and functional resemblances, they can also utilize different modes of action. These two regulators share the common feature of a tetrameric form which binds to a pair of palindromic DNA target sequences, but alteration of DNA binding activity by
inducing ligands can clearly occur via disparate mechanisms.

REFERENCES


