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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

Abbreviations: IR, inverted repeat; ITC, isothermal calorimetry; MU, Miller units; SSA, succinate semialdehyde.

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 ($BlcR^{F147A}$), 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 *Nde*I and *Bam*HI restriction enzymes and ligated into the compatibly cleaved vector pSRKGm so that the inserted gene is under the control of the *lac* promoter (P_{lac}–*blcR*, Gm^r), termed pCL6, or pCL6–$blcR^{mut}$. A DNA fragment of 800 bp containing the *blcABC* promoter (P_{blcA}) region was amplified from *A. tumefaciens* A6 genomic DNA and fused, with *Bam*HI and *Pst*I sites, to *lacZ* (P_{blcA}–*lacZ*, Sp^r) on the vector pRA301, to create pCL8. The P_{blcA} 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 P_{blcA} in accordance with our previous study (Pan *et al.*, 2011) using the QuikChange 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 KH_2PO_4 l^{-1}, 160 mg $MgSO_4$ l^{-1}, 10 mg $CaCl_2$ l^{-1}, 5 mg $FeSO_4$ l^{-1}, 2 mg $MnSO_4$ l^{-1}, 2 g $(NH_4)_2SO_4$ l^{-1} and 0.5 % (w/v) glucose] (Tempé *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 OD_{600} of approximately 1.0 (the actual OD_{600} was recorded). Then, 0.1 ml cell culture was mixed with 0.9 ml Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 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 Na_2CO_3 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 A_{420} was recorded using Synergy HT Microplate Reader (BioTek). The β-galactosidase activity in Miller units (MU) is calculated as follows:

$$\mathrm{MU} = 1000 \times \frac{A_{420}}{\mathrm{OD}_{600} \times \mathrm{time\ (min)} \times \mathrm{f}}$$

$$\text{where } \mathrm{f} = \frac{Vol_{\mathrm{cells}}}{Vol_{\mathrm{cells}} + Vol_{Z-\mathrm{buffer}}}.$$

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 $BlcR^{F147A}$ (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 P_{blcA}–*lacZ*-containing reporter pCL8. As shown in Fig. 1(a) (inset), when pCL8 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 P_{blcA}–*lacZ* fusion on pCL8. 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 P_{blcA}–*lacZ* fusion plasmid [~95 MU, Fig. 1(a) (inset)], supporting the model that the pAtC58-encoded *blcR* repressed P_{blcA}–*lacZ* activity. Next, we examined how SSA affected the repression of the pAtC58-encoded *blcR* on P_{blcA}–*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 P_{blcA}–*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 OD_{600}=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

Table 1. Bacterial strains and plasmids used in this study

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

Strain or plasmid	Relevant characteristic(s)	Reference or source
***A. tumefaciens* strains**		
C58	Nopaline-type strain, pTiC58, pAtC58	Sciaky *et al.* (1978)
C58 At^+/Ti^-	$pAtC58^+$, $pTiC58^-$ derivative of the nopaline-type strain C58	Our collection
C58 At^+/Ti^- (pCL8)	Derivative of C58 At^+/Ti^- harbouring pCL8	This study
C58 p^-	$pTiC58^-$, $pAtC58^-$ derivative of the nopaline-type strain C58	Our collection
C58 p^- (pCL8)	Derivative of C58 p^- harbouring pCL8	This study
C58 p^- (pCL8 pSRKGm)	Derivative of C58 p^- harbouring pCL8 and pSRKGm	This study
C58 p^- (pCL8 pCL6)	Derivative of C58 p^- harbouring pCL8 and pCL6-borne $blcR^{wt}$	This study
C58 p^- (pCL8 pCL6–$blcR^{mut}$)	Derivative of C58 p^- harbouring pCL8 and pCL6-borne *blcR* mutants $blcR^{F147A}$, $blcR^{T158A}$, $blcR^{D210A}$ and $blcR^{C220A}$	This study
C58 p^- (pCL8(n))	Derivative of C58 p^- harbouring pCL8 variants with altered inter-IR distance, $n=+2, +1, -1, -2$ bp	This study
C58 p^- (pCL8(n) pSRKGm)	Derivative of C58 p^- harbouring pSRKGm and pCL8 variants	This study
C58 p^- (pCL8(n) pCL6)	Derivative of C58 p^- harbouring pCL8 variants and pCL6-borne $blcR^{wt}$	This study
C58 p^- (pCL8(n) pCL6–$blcR^{F147A}$)	Derivative of C58 p^- harbouring pCL8 variants and pCL6-borne $blcR^{F147A}$	This study
***E. coli* strains**		
DH5α	Standard cloning host	Hanahan (1983)
Plasmids		
pSRKGm	pBBR1MCS-5-derived broad-host-range expression vector containing lac promoter and $lacI^q$, $lacZ\alpha^+$, Gm^r	Our collection
pCL6	pSRKGm carrying A6 *blcR* gene for expression, Gm^r	This study
pCL6–$blcR^{mut}$	Derivative of pCL6 with mutant *blcR* (K59A, Y133A, F147A, T158A, D210A and C220A)	This study
pRA301	Broad-host-range *Plac* expression vector, Sp^r	Our collection
pCL8	pRA301 carrying A6 *PblcA* region, Sp^r	This study
pCL8(+2)	Derivative of pCL6 with GC base insertion in the IR gap region of *PblcA*	This study
pCL8(+1)	Derivative of pCL6 with a G base insertion in the IR gap region of *PblcA*	This study
pCL8(−1)	Derivative of pCL6 with an A base deletion in the IR gap region of *PblcA*	This study
pCL8(−2)	Derivative of pCL6 with CA bases deletion in the IR gap region of *PblcA*	This study

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* on P_{blcA}–*lacZ* might reflect the contribution of SSA degradation by the pAtC58-encoded BlcABC degradation system.

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*.

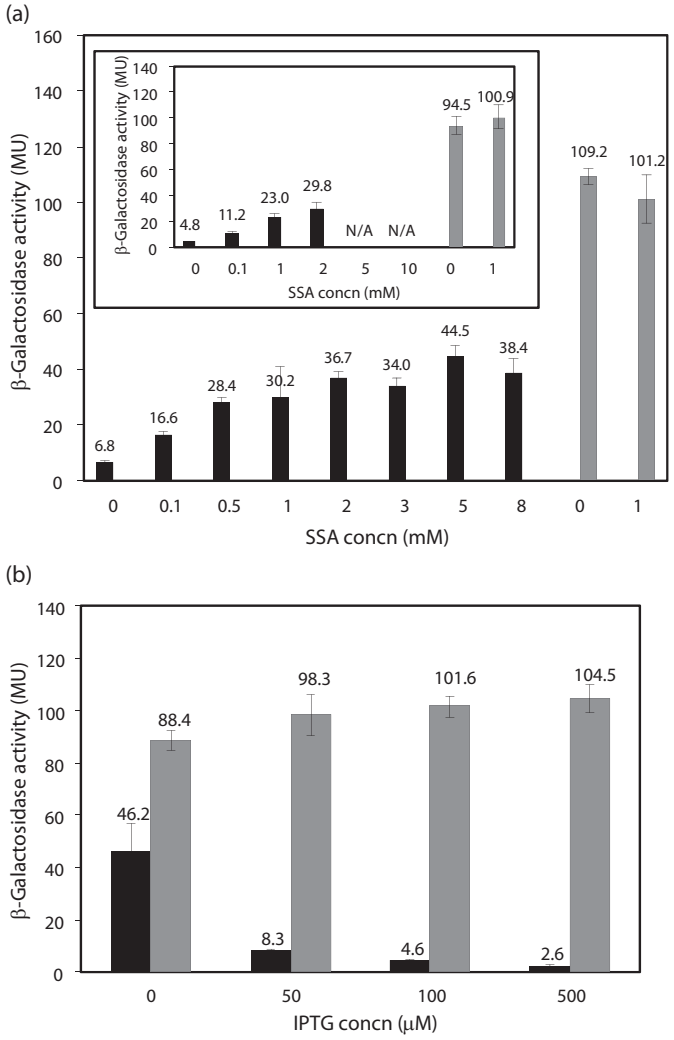

Fig. 1. (a) Effect of SSA on repression activity of resident *blcR* using C58 At^+/Ti^- (pCL8, P_{blcA}–*lacZ*) (black columns). *A. tumefaciens* was grown to OD_{600} ~1.0 and appropriate amounts of SSA were added. Cells continued to grow for three more hours before harvesting for the *β*-galactosidase assay. Inset graph: a range of SSA concentrations were included at the beginning of cell growth. Cells collected from cultures were used for *β*-galactosidase assays when their OD_{600} reached 1.0. N/A, cells did not grow. (b) IPTG-dose-dependent induction of pCL6-borne *blcR* (P_{lac}–*blcR*). Cultures of strain C58 p− (pCL8 pCL6) (black columns) for which different IPTG concentrations were added at the beginning of growth were assayed for *β*-galactosidase activity. At least three biological replicates were performed for each experiment in (a) and (b), and error bars indicate SD. Strain C58 p− (pCL8) (grey columns) was utilized as the negative control.

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). $BlcR^{T158A}$ 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 $BlcR^{T158A}$ for SSA. Interestingly, the approximately 50 % derepression displayed by $BlcR^{T158A}$ 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 μM, respectively)] (Pan *et al.*, 2011). Lastly, we have isolated a BlcR mutant that is apparently locked in a tetrameric form ($BlcR^{F147A}$) which mimics the DNA-bound BlcR (Pan *et al.*, 2011). Also in that study, we have shown that SSA-bound $BlcR^{F147A}$ 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 *β*-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

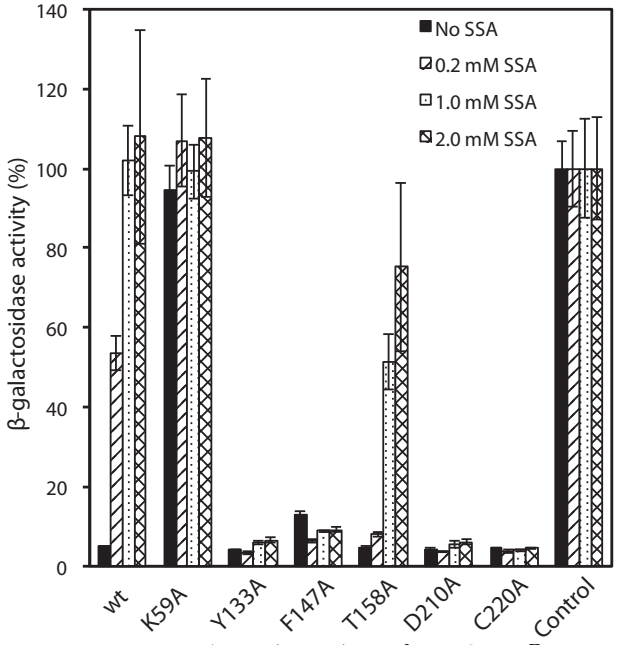

Fig. 2. *In vivo* mutational analyses on the SSA- and DNA-binding sites. IPTG (100 μM) and the indicated concentration of SSA were included at the beginning of culture growth, C58 p$^-$ (pCL8 pCL6) or C58 p$^-$ (pCL8 pCL6–*blcR*^{mut}) strains (black columns), were grown until OD_{600} ~1.0 and the *β*-galactosidase activity was measured. Strain C58 p$^-$ (pCL8 pSRKGm), which does not contain the *blcR* gene, was utilized as the negative control, and since its *β*-galactosidase activities under various concentrations of SSA were approximately 100 MU (± 10 MU), their activities were set to 100 % to facilitate data comparison and interpretation. At least three biological replicates were performed for each experiment, and error bars indicate SDs.

in BlcR–DNA interaction, this position was mutated to Ala (K59A) and the regulatory activity of BlcR^{K59A} on P_{blcA} was analysed using the P_{blcA}–*lacZ* fusion. Induction of *blcR*^{K59A} did not repress P_{blcA}, 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 BlcR^{K59A} did not repress the P_{blcA} 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 P_{blcA} 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^{wt}. However, we have found that the modified binding site sequences (+2, +1 and −1 bp) retain high affinity to the preassembled, tetrameric BlcR^{F147A}.

To confirm these *in vitro* findings, we modified the promoter P_{blcA} in pCL8 accordingly and examined the interaction of both BlcR^{wt} and BlcR^{F147A} with the modified P_{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^{wt} on the modified P_{blcA+2} promoter was relieved by approximately 15-fold as compared with the wild-type P_{blcA} (Fig. 3b). In contrast, repression by BlcR^{F147A} on P_{blcA+2} 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^{wt} on both P_{blcA+1} and P_{blcA-1} was approximately 10-fold weaker than on P_{blcA}. In comparison, repression activity by BlcR^{F147A} on both modified promoters remained relatively strong (Fig. 3b). Thus, repression activity of dimeric BlcR^{wt} was more significantly affected by the inter-IR spacing in P_{blcA} than was tetrameric BlcR^{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.

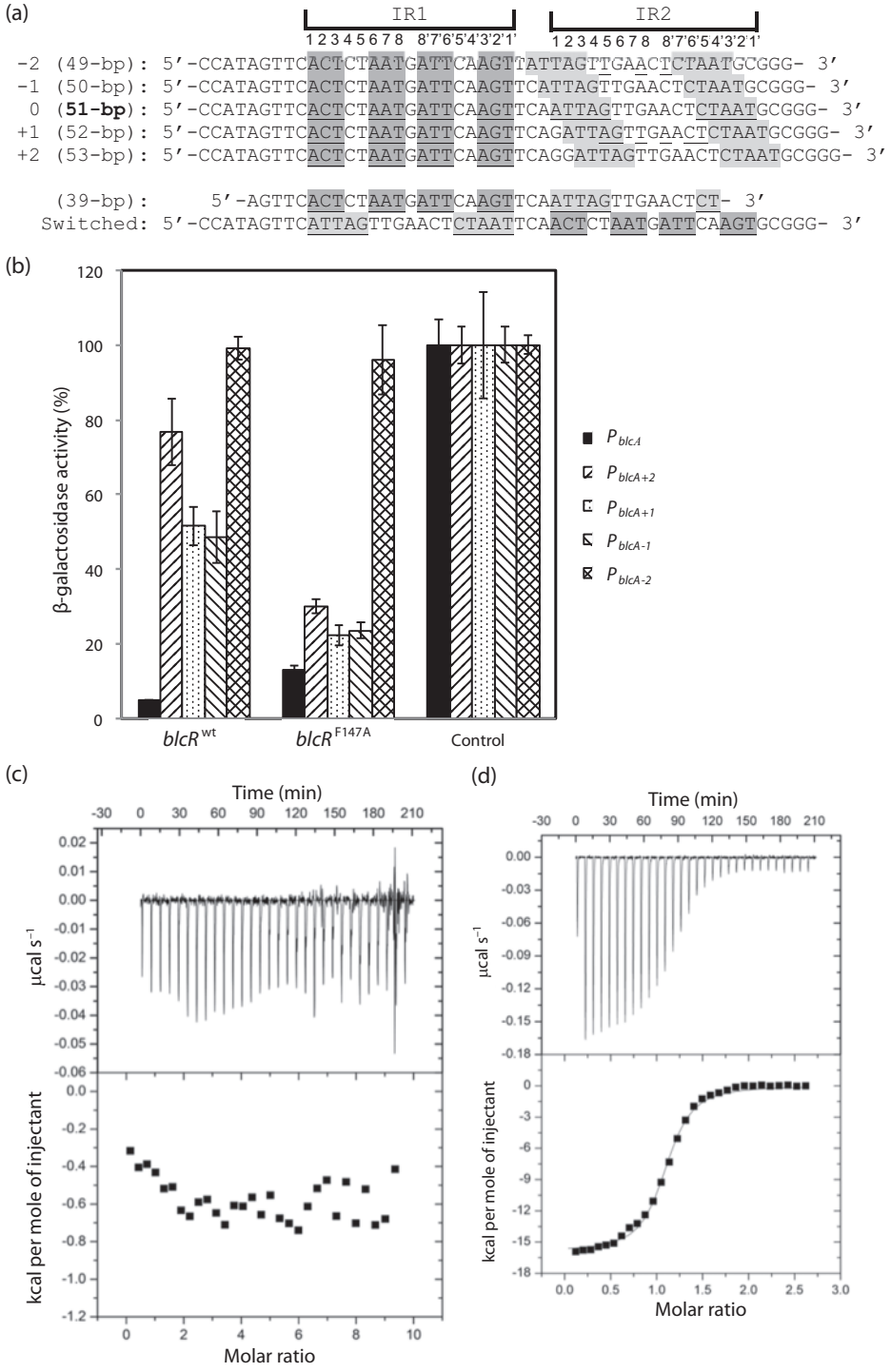

Fig. 3. (a) DNA sequences of the BlcR binding site variants with altered inter-IR distance or switched IR1 and IR2. Dark- and light-grey boxes denote sequences that are symmetrically related in IR1 and IR2, respectively. Nucleotides that are symmetrically related are underlined in each variant. (b) Effects of altering the inter-IR distance on P_{blcA} on BlcR transcriptional activity. IPTG (100 µM) was included at the beginning of culture growth, strains were grown to OD_{600} ~1.0 and β-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 *blcR* gene, was included as the negative control, and its β-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 $BlcR^{F147A}$ with the 49 bp DNA (c) and 53 bp DNA (d). Binding of $BlcR^{F147A}$ to the 53 bp DNA (K_d=64 nM and N=1.1) is similar to that of $BlcR^{F147A}$ 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_{blcA-2} was completely abolished. Strikingly, BlcR^{F147A} also failed to inhibit $P_{blcA-2,}$ in contrast to its inhibition of the other *blcA* promoters, wild-type and modified (P_{blcA+2}, P_{blcA+1} and P_{blcA-1}). To extend our *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 BlcR^{F147A} by ITC. No interaction of the 49 bp DNA and BlcR^{F147A} 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 BlcR^{F147A} 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). BlcR^{F147A} bound this 39 bp DNA with a similar affinity as the cognate 51 bp fragment (K_d 86±14 nM, Fig. 4(a); and 54±6 nM (Pan *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 BlcR^{F147A} 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. 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 *in vivo* system that allows us to examine the interaction of BlcR with the P_{blcA} promoter. We showed that the pAt-encoded resident *blcR* was not optimal for examination of the effect of SSA on BlcR function, because only partial derepression of the P_{blcA} promoter could be achieved by addition of exogenous SSA at concentrations not detrimental to cell growth. We therefore used the *A. tumefaciens* C58 p$^-$ cured of pAtC58 (and pTiC58) expressing *blcR* from P_{lac} (pCL6) and the P_{blcA}–*lacZ* fusion plasmid (pCL8) to evaluate the influence of SSA on BlcR-mediated repression at non-inhibitory SSA concentrations. Induction of P_{lac}–*blcR* at 100 μM IPTG, with 1 mM SSA to relieve this repression, was found to be optimal. We subsequently applied this *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

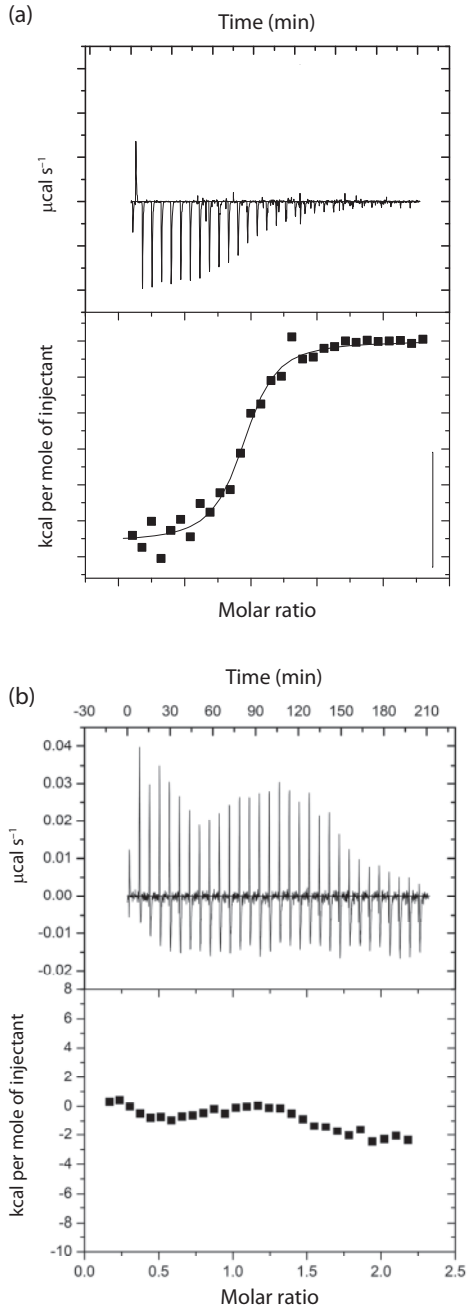

Fig. 4. IR1 has a dominant role over IR2 in the overall DNA binding of BlcR^{F147A}. (a) ITC studies using a 39 bp DNA fragment with truncated IR2, the sequence of which is shown in Fig. 3(a), titrated with BlcR^{F147A}. (b) ITC studies using a 51 bp DNA fragment with IR2 placed ahead of IR1, the sequence of which is shown in Fig. 3(a), titrated with BlcR^{F147A}.

BlcR. In investigating the role of the inter-IR gap in P_{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 BlcR^{F147A},

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 $BlcR^{F147A}$ repression and the $BlcR^{F147A}$–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 $BlcR^{F147A}$. 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 $BlcR^{F147A}$.

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 $BlcR^{F147A}$ 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 $BlcR^{F147A}$ 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 $BlcR^{F147A}$ 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 $BlcR^{F147A}$ fixed tetramer, whereas the outward flexing of the tetramer is better tolerated.

BlcR of *A. tumefaciens* and TtgV of *Pseudomonas putida*, 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_{blcA} and P_{ttgG}) 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 (R) state when in isolation and a more stable symmetrical (T) 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 $BlcR^{F147A}$ mutant protein, which is locked in a tetrameric state. The $BlcR^{F147A}$ 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.

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