Crystal structure of BrlR reveals a potential pyocyanin binding site

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Abstract

The transcriptional regulator BrlR from *Pseudomonas aeruginosa* is a member of the MerR family. Studies have shown BrlR can be activated by the secondary messenger 3′,5′-cyclic diguanylic acid (c-di-GMP) and contributes to P. aeruginosa biofilm tolerance. Previous structural analysis of BrlR-c-di-GMP complex has identified one hydrophobic pocket locating in its C-terminal GyrI-like domain. However, what kind of small molecule bound in this pocket remains unknown. Here, we report the apo structure of BrlR and identified the direct binding between GyrI-like domain of BrlR and *Pseudomonas aeruginosa* toxin pyocyanin. Furthermore, pyocyanin can enhance the binding between BrlR and DNA in vitro. These novel findings suggest BrlR functions as one shared receptor for c-di-GMP and pyocyanin. The structural analysis also provide an important basis for rational drug design combating the infection of P. aeruginosa.

Key words: BrlR, c-di-GMP, pyocyanin, *Pseudomonas aeruginosa*

Introduction

P. aeruginosa infections have complex pathophysiology and are difficult to eliminate [1]. Its intrinsic ability to develop resistance to antibiotics, formation of impenetrable biofilms and release of a large quantity of virulence factors all contribute to *P. aeruginosa* infections. In addition to secreted attacking proteins enzymes, including elastase, alkaline protease and LasA protease, many secondary metabolites also are released, which can cause serious damage to the host.

Pyocyanin is a blue redox-active secondary metabolite, which can easily penetrate biological membranes and cause damages [2, 3]. It is synthesized from chorismate through a series of complex steps mediated by gene products encoded by two phzABCDEFG operons and its precursors will be modified into the tricyclic compound by the phzH, phzM and phzS genes [4, 5]. The synthesis of pyocyanin is regulated by quorum sensing (QS) systems, but the details are not clear.

Pyocyanin was reported to kill fungal and *Caenorhabditis elegans* [6, 7]. Recent studies demonstrated that pyocyanin is required for lung infection in mice [8, 9]. And large quantities of pyocyanin can be readily recovered from the sputum of patients with CF infected by *P. aeruginosa* [10]. It has been reported that pyocyanin interferes with multiple cellular functions that it can cause oxidative stress, inactivate of V-ATPase, decrease mitochondrial and cytoplasmic aconitase activity and ATP levels [2, 11].

Many researches about pyocyanin focus on its role in pathogenesis, yet the regulation and signal transduction pathway it participates have long been ignored. Until now, only few pyocyanin receptors are reported. The arylhydrocarbonreceptor (AhR) from human

is a highly conserved ligand-dependent transcription factor, which can sense pyocyanin, induce detoxifying enzymes and modulate immune cell differentiation and responses [12-14]. RmcA from *P. aeruginosa* is a multiple domain protein with both phosphodiesterase (PDE) and diguanylate cyclase (DGC) domain, whose activity is modulated by pyocyanin through its PAS domain [15].

BrlR protein is a transcription regulator in *P. aeruginosa*. It can only be detected in biofilm cells and activated by bacterial secondary messenger c-di-GMP [16]. The activated BrlR can upregulate the expression of multidrug efflux pumps, such as MexAB-OprM and MexEF-OprN efflux pumps. The knock-out of BrlR leads to the susceptibility of the biofilm when the bacteria were treated with five different classes of antibiotics [17]. BrlR belongs to the MerR protein family and consists of three domains with the middle coiled-coil region flanked by N-terminal HTH_MerR domain and GyrI-like domain at the C-terminus. MerR protein family have been demonstrated as multidrug binding (MDR) proteins, and some of them can bind potential multidrug molecules directly. In our previous study, we determined the complex structure of BrlR-c-di-GMP and identified one conserved drug-binding pocket. However, so far, there is no report regarding which small molecule can bind into this pocket and what is the common link between biofilm and multidrug resistance in *P. aeruginosa*. In this study, we demonstrate that BrlR from *P. aeruginosa* is a pyocyanin receptor and identify the GyrI-like domain as potential binding site for pyocyanin.

Materials and Methods

Protein expression and purification

The expression and purification of full length *brlR* gene and 119-end fragment (GyrI-like domain) were the same as previous protocol [18]. Briefly, the genes encoding the corresponding sequences were amplified by polymerase chain reaction (PCR) and inserted into the pET-28a vector (Novagen). The *E. coli* BL21 (DE3) cells were used as host for expression. The proteins were obtained by two-step purification procedure (Ni-affinity and gel filtration purification). The N-terminal His-tag of BrlR removed by thrombin protease before gel filtration was used for crystallization and The N-terminal His-tag of BrlR left intact through the purification was used for western blot (WB). The protein was then concentrated in gel filtration buffer (20 mM Tris pH 7.5, 150 mM NaCl, 5% Glycerol). Protein purity was checked by Coomassie Brilliant Blue-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The mutagenesis of BrlR (Y40N, R86A, and W150A) was performed by overlap-extension PCR and the expression and purification of these mutant proteins were the same as the wild type.

Crystallization, data collection, structure determination and refinement

Purified BrlR proteins were concentrated to approximately 10 mg/mL in the gel filtration buffer and screened for crystallization using commercial available kits (Molecular Dimension). Sitting-drop vapor diffusion method was used in 24-well Itelli-plate to optimize the crystals at 20 °C under condition (10% v/v 1,4-Dioxane, 0.1 M MES pH 6.5, 1.6 M Ammonium sulfate). The grown crystals were dehydrated by 1.8 M Lithium sulfate and macromolecular crystal annealing (MCA) was performed to improve the resolution.

The diffraction data were collected at the beamline BM14 at the European Synchrotron Radiation Facility (ESRF). The data were indexed, integrated and scaled with HKL2000 [19]. Phaser (PHENIX) was used to do the molecular replacement [20]. The coordinate of BrlR from c-di-GMP complex structure (PDB: 5XQL) was used as the search model. Coot was used to modify the result model and phenix.refine was used for refinement [20, 21]. The structure of BrlR was deposited in RCSB with a PDB code 5YC9. Crystallographic statistics are summarized in Table 1. All the structure figures were prepared with PyMOL (http://www.pymol.org).

Electrophoretic mobility shift assay (EMSA)

According to previous papers, three DNA probes are designed and labeled with Cyanine 5 (Cy5) fluorescent dye (Thermofisher Scientific) (Supplementary table1) [17, 22]. The DNA probes (0.5 pmol), BrlR proteins (25 pmol) and different concentration of pyocyanin (Everon Life Sciences) or c-di-GMP (Labex Corporation) were incubated for 30 min at 25°C in binding buffer (10 mM Tris-HCl pH 7.5, 50 mM KCl, 1 mM EDTA, 1 mM DTT and 5% glycerol) with a total volume of 20 µl. Then, the samples were subjected to electrophoresis with a 6% polyacrylamide glycine gel using 0.5×TBE running buffer on ice for 80 min. Imaging and data analyses were performed on a LI-COR imaging system (LI-COR Biosciences).

Ligand trapping assay

In order to check the binding between BrlR and its ligands (c-di-GMP and pyocyanin), the ligand trapping assay were performed with a 5 KD cutoff centrifugal filter. BrlR proteins were incubated with its ligand in a 1:2 ratio for 20 min at RT. Then, BrlR

proteins with various ligands were concentrated by centrifugal filter at 4 °C for 30 min. The flow-through was collected to measure the concentration of un-trapped ligand and the buffer with ligand was used as control experiment. Every binding assay was repeated at least three times.

Binding affinity measurement

The T_m value of BrlR was determined by thermo-denaturation assay with 2 °C increment from 50 °C to 68 °C. Briefly, 20 μg protein was incubated at denaturing temperature for 3 min followed by centrifugation for 5 min. Then, the supernatant was collected for concentration measurement. The T_m (57.5 °C) was obtained by fitting the denature curve. The BrlR-pyocyanin binding affinity was measured by ligand concentration-dependent thermo-denaturation assay. The 0.05 μM BrlR protein was incubated with various concentrations of pyocyanin (0.5 μM, 1 μM, 5 μM, 10 μM, 20 μM) and denatured at 57.5 °C for 3 min. The supernatants were collected for western blot (WB). The protein was detected by using primary mouse anti-His antibody (1:5000, Clontech) and secondary HRP conjugated Rabbit anti-mouse antibody (1:2000, Cell signaling). The image was analyzed using image J and Kd was calculated with fitted curve.

Result

Tetrameric apo BrlR structure

The crystal structure of apo BrlR was determined at 3.3 Å, with four BrlR molecules in the asymmetry unit. There are two disordered regions (residues 27-40 and 137-144) in each protomer due to the poor density around these areas (Fig. 1A). According to our

previous paper, residues (27-40) take part in c-di-GMP binding and residues (137-144) are located near the drug-binding pocket [18]. Therefore, both areas show the binding potential for small molecules duo to their flexibility.

The overall structure of apo BrlR is much similar to the c-di-GMP bound form. No dramatic conformation difference is observed after c-di-GMP binding and some minor differences may be due to the crystal packing artifact (Fig. 1B, 1C, 1D). Like the c-di-GMP bound form, apo BrlR structure can be clearly divided into three parts: N-terminal HTH_ MerR domain, middle coiled-coil region and C-terminal GyrI-like domain (Fig. 1C). The apo BrlR exists as tetramer in solution which have been revealed by our previous gel filtration experiment [18].

The c-di-GMP binding did not change the overall conformation and oligomerization state of BrlR (Fig. 1E and F). Therefore, how the c-di-GMP stimulates the BrlR remains elusive. There are two c-di-GMP binding sites identified in our previous paper [23]. In order to check which c-di-GMP binding site is real, residues involved in c-di-GMP binding were mutated. Their c-di-GMP and DNA binding abilities were tested using ligand trapping assay and EMSA assay respectively. The residue Tyr40 in first binding site was mutated to Asn and this Y40N protein lost its c-di-GMP binding ability shown by ligand trapping assay (Fig. 2A). At the same time, its DNA binding potential was also decreased in the presence of c-di-GMP (Fig. 2B). In contrary, R86A mutation in the second site has no effect on c-di-GMP binding and DNA binding (Fig. 2). Therefore, there is only one c-di-GMP binding site in BrlR.

Pyocyanin binds BrlR in the GyrI-like domain

The similarity and conservation of GyrI-like domain between BrlR and other MDR proteins discussed in our previous paper indicates BrlR can bind flat shape molecules (Fig. S1). For detailed analysis, we compared the GyrI-like domain of BrlR with SAV2435 (PDB code: 5KAU) carefully. The GyrI-like domain of BrlR and SAV2435 align well, with a root-mean-square deviation (RMSD) value of 2.3 Å over 165 residues (Fig. 3A). Like SAV2435, the drug-binding pocket of BrlR GyrI-like domain is deep and hydrophobic, which indicates that this site may have the same preference for small molecules with aromatic rings. Unlike BmrR and SAV2435, which are reported to bind many molecules, no small molecules are reported to bind the GyrI-like domain of BrlR so far. We noticed that the central xanthene ring of rhodamine 6G (RH6G) in SAV2435 complex structure is very similar to pyocyanin, which exists in large quantities in *P. aeruginosa*.

It is reported that exogenous added pyocyanin can affect the expression of dozens of genes: 22 genes are upregulated; 29 genes are downregulated [24]. It may be due to the chemical property of pyocyanin served as a redox-active molecule or its directly binding to some transcription regulators that cause this regulation. However, no transcription regulators are identified as pyocyanin receptors so far. We noticed that among the upregulated genes, eight of which encode putative transporters (i.e. the RND transporters mexGHI-opmD, PA3923-3922-opmE and the putative Major Facilitator Superfamily transporter PA3718). The BrlR gene expression level is also upregulated with 2.3 fold. We found this regulation pattern is somewhat similar to the BrlR gene does. In addition to itself, BrlR will activate genes encoding the MexAB-OprM and

MexEF-OprN multidrug efflux pumps after stimulation by c-di-GMP [17]. In such condition, we hypothesize that BrlR can bind pyocyanin and trigger its gene regulation function.

To better characterize the binding property of BrlR towards pyocyanin, we modelled the pyocyanin into the surface cavity of GyrI-like domain in silicon according to the binding mode of RH6G in SAV2435 (Fig. 3B and C). The result shows both RH6G and pyocyanin are buried in the cavity of the drug-binding pocket, with several aromatic residues around them. Like Trp34 in SAV2435, Trp150 form stack interaction with pyocyanin in this model. At the same time, Tyr249 form hydrogen bond with pyocyanin, which will play a role in its binding specificity (Fig. 3C). In order to confirm this hypothesis, we tested the binding between BrlR protein and pyocyanin using ligand trapping assay. As is shown in Fig. 3D, it clearly show that the BrlR can bind the pyocyanin and reduce its chance to flow through the filter membrane. The binding also change solution color from blue to green especially in the more concentrated condition (Fig. S2). To test whether pyocyanin binding pocket is located in our predicted site in the C-terminal Gyr-I like domain, we used the C-terminal part of BrlR for this binding assay and it shows it binds pyocyanin much better than full length protein, which indicates the full length protein may be in a more regulated conformation (Fig. 3D). The binding affinity between BrlR and pyocyanin is about 0.75 μM (Fig. 3F), which is comparable to the c-di-GMP binding affinity (2.2 μM) shown by previous study [16]. However, the binding between W150A mutation protein and pyocyanin is decreased as is shown by ligand trapping assay (Fig. 3D), which suggests the predicted binding site

is correct.

Pyocyanin can enhance the DNA binding of BrlR

It was reported that BrlR can bind to its own promoter (P*brlR*) and the promoter of *mexA* (P*mexA*) and *mexE* (P*mexE*) [17]. While c-di-GMP can enhance this binding, we speculate that pyocyanin could function in the same way. To characterize the effect of pyocyanin binding, we performed the EMSA assays of BrlR with Cy5 labeled P*brlR*, P*mexA* and P*mexE* under different concentration of pyocyanin (Fig. 4). While the DNA binding of apo BrlR is weak, it is clearly shown that increased pyocyanin concentration can enhance the binding of BrlR towards P*brlR*, P*mexA* and P*mexE*. Because BrlR can non-specifically bind many dyes (data not shown), we use unlabeled P*brlR* to show its specificity (Fig. 4B). In our assays, high concentration of unlabeled P*brlR* can successfully compete the BrlR protein, which indicates the labelling dye does not disturb our EMSA assays. The failure of obtaining the complex structure of BrlR and pyocyanin makes the activation mechanism still unknown. In our size-exclusion chromatography (SEC) experiments, the apo and pyocyanin bound form BrlR share the same elution time, which suggests that pyocyanin does not change the oligomeric sate of BrlR, just like the c-di-GMP (Fig. S3). The W150A mutation which lost the pyocyanin binding shows a compromised binding ability to P*brlr* DNA in the presence of pyocyanin (Fig. 4C). These data shown above indicate the pyocyanin can bind and regulate the transcription factor BrlR.

Discussion

The secondary messenger c-di-GMP can promote the biofilm formation in *P.*

aeruginosa and the BrlR protein can only be detected after the biofilm formation. The binding of pyocyanin or c-di-GMP by BrlR leads to the expression of *BrlR* gene and BrlR regulated multidrug efflux pump genes [17]. It seems that both these two small molecules contribute to the multidrug resistance of *P. aeruginosa*. Actually, the low concentration of c-di-GMP and pyocyanin can act cooperatively to enhance the BrlR binding to its promoter though the enhancement is not dramatic in our experiment condition (Fig. 4F). However, this synergetic enhancement can be significant in vivo duo to the optimal condition. Duo to the lacking of BrlR-ligand-DNA ternary complex structure, the molecular mechanism of BrlR stimulation by c-di-GMP or pyocyanin remains elusive.

In summary, our data suggest that BrlR is a novel receptor for both c-di-GMP and pyocyanin. As an unusual transcription regulator, BrlR has involved two separate binding site towards c-di-GMP and pyocyanin. The next step study will focus on the physiological significance of this binding between BrlR and pyocyanin. It also shows the co-receptor BrlR will be a promising drug target for the cure of intractable infection of *P. aeruginosa*.

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Figures

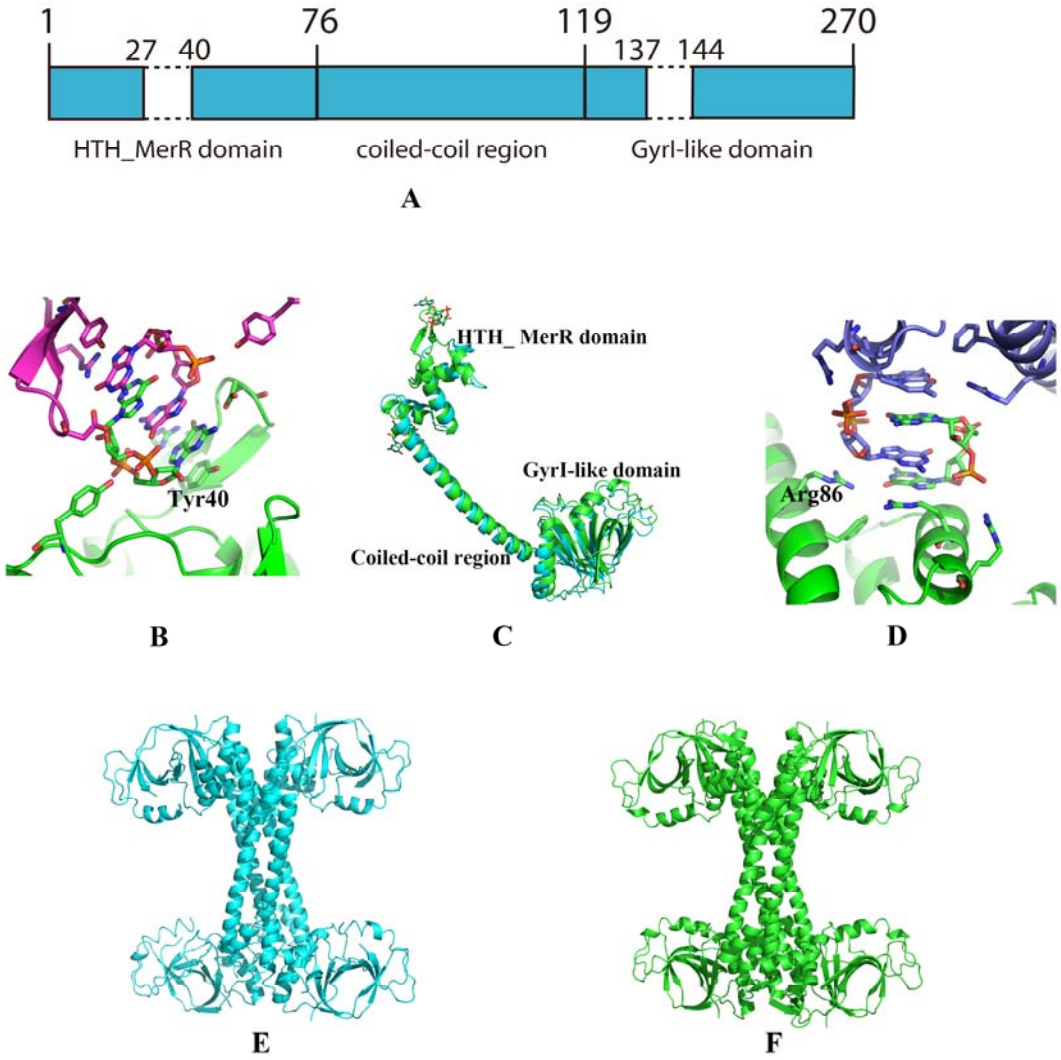

Figure 1. Overall structure of apo BrlR structure. (A) Domain structure of BrlR. Dash line indicates the disordered regions in our apo BrlR structure. (B, D) c-di-GMP binding sites of BrlR. Residues and c-di-GMP from another tetramer are colored in magenta and slate. The annotated residues are for the mutation analysis. (C) Superposition of cartoon presentation of apo BrlR monomer with BrlR monomer from c-di-GMP complex (PDB: 5XQL). No obvious conformation change is observed between the two forms. Apo BrlR monomer can be clearly divided into three parts: N-terminal HTH_ MerR domain (1-76 aa), middle coiled-coil region (77-119 aa) and C-terminal GyrI-like domain (120-270 aa). (E, F) Cartoon presentation of tetramer of apo BrlR (in

cyan) and BrlR tetramer from c-di-GMP bound form (in green). Just like the c-di-GMP bound form, the HTH_ MerR domain, middle coiled-coil region and GyrI-like domain all take part in the massive interaction among the four BrlR protomers in the apo form, while the relative position of these four chains has only a minor difference.

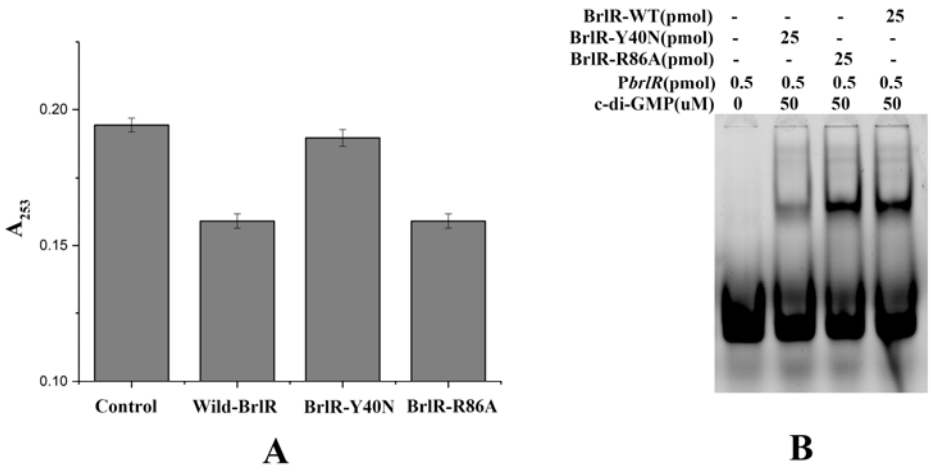

Figure 2. Analysis of c-di-GMP binding site. (A) Ligand trapping assay for c-di-GMP and BrlR. The ligand in flow-through was measured. While the wild BrlR and R86A mutation could bind c-di-GMP, Y40N almost lost its c-di-GMP binding ability. (B) EMSA assay for c-di-GMP and BrlR. While the DNA binding ability of wild BrlR and R86A mutation are enhanced by c-di-GMP, DNA binding ability of the Y40N mutation is still weak.

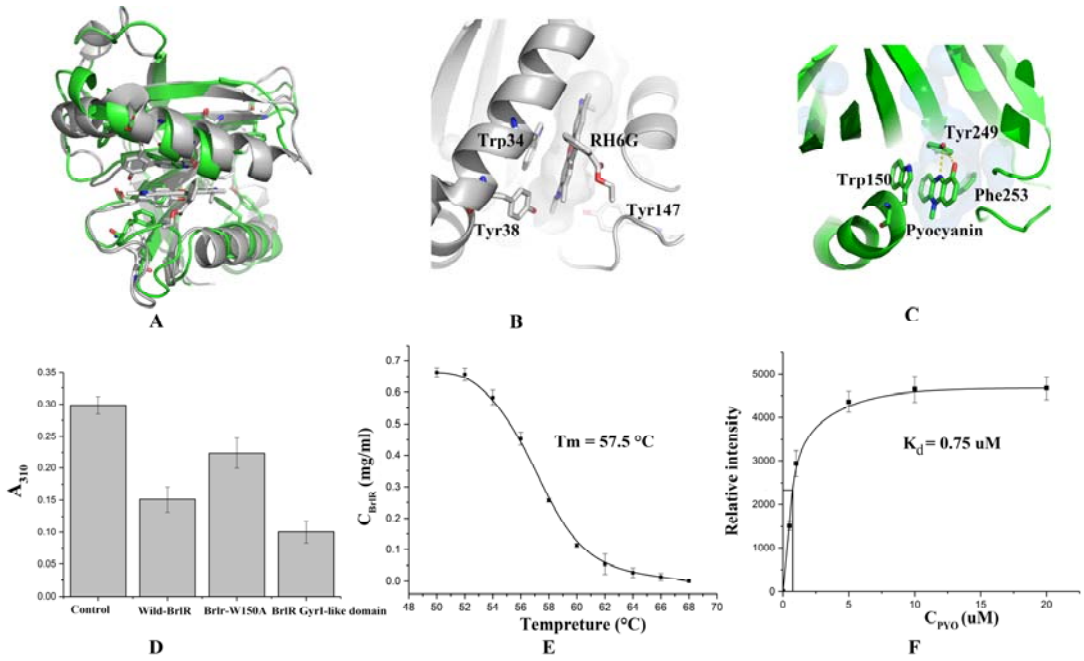

Figure 3. Pyocyanin binds BrlR in the GyrI-like domain. (A) Superposition of cartoon presentation of GyrI-like domain of BrlR with RH6G bound SAV2435 (PDB: 5KAU). BrlR is in cyan and SAV2435 is in grey. Aromatic residues around the drug-binding pocket and RH6G molecule are rendered as stick. The overall fold of the GyrI-like domain of BrlR is quite similar to SAV2435 and the shape of the pocket is almost the same. (B) RH6G binding site in SAV2435. The cavity is shown in surface; key residues for RH6G binding and RH6G are rendered as stick. (C) Predicted pyocyanin binding site in BrlR GyrI-like domain. The cavity is shown in surface; predicted key residues for pyocyanin binding and pyocyanin are rendered as stick. The yellow dash lines indicate the possible interaction between pyocyanin Tyr249, which may play a role in binding specificity. (D) Ligand trapping assay for BrlR and pyocyanin. While the GyrI-like domain of BrlR can even bind pyocyanin tighter, the full length W150A mutation binds less pyocyanin. (E) Denature curve of BrlR. (F) Ligand concentration-dependent thermo-denaturation assay for BrlR and pyocyanin. Data are calculated on relative intensity of bands detected by WB and analysis using ImageJ. K_d stands for the dissociate constant.

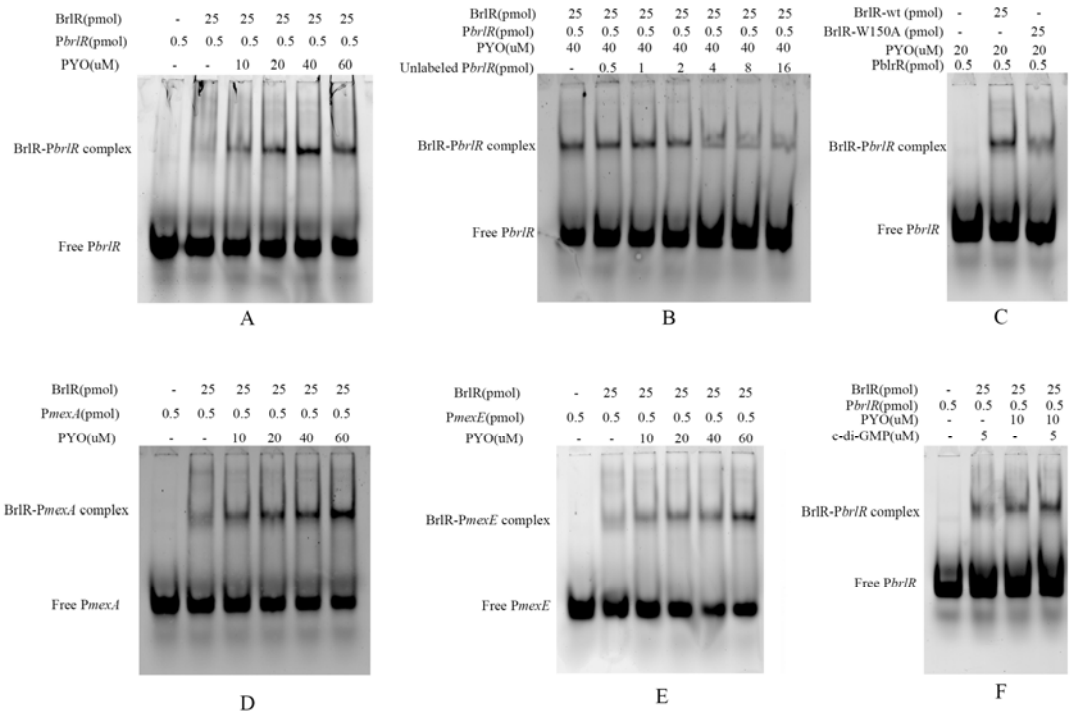

Figure 4. EMSA assays for BrlR with pyocyanin. (A) EMSA assays with P*brlR*. The basic DNA binding of apo BrlR is relatively weak and BrlR-P*brlR* complex can be clearly observed only after adding 10 uM pyocyanin. Our assays shows that increased pyocyanin concentration can enhance the DNA binding of BrlR. (B) EMSA assays with unlabeled P*brlR*. With the increase of unlabeled P*brlR*, the bound labeled P*brlR* continues to decrease. (C) EMSA assays for BrlR W150A mutation. The binding between W150A mutation and P*brlr* promoter can not be enhanced by pyocyanin. (D, E) EMSA assays with P*mexA* and P*mexE*. The effect of pyocyanin on BrlR binding towards these two fragments are similar with P*brlR*. (F) EMSA assays for BrlR with pyocyanin and c-di-GMP. pyocyanin and c-di-GMP can cooperatively enhance the binding ability of BrlR to DNA. All of the EMSA assays were performed at least three times.

Table 1. Data collection and refinement statistics

Wavelength (Å)	0.98
Space group	P 65
a, b, c (Å)	111.64 111.64 261.13
α, β, γ (°)	90.00 90.00 120.00
Resolution range (Å)	35.00-3.30 (3.40-3.30)*
Completeness (%)	100.0 (99.7)
Redundancy	8.7 (8.7)
⟨ I/σ(I)⟩	23.9 (5.76)
$R_{merge.}$	0.082 (0.424)
Overall B factor from Wilson plot (Å^2)	57.4
Final Rcryst /Final Rfree	0.2683/0.2836
No. of non-H atoms	
Protein	8290
R.m.s. deviations	
Bonds (Å)	0.007
Angles (°)	1.106
Average B factors (Å^2)	
Protein	40.445
Ramachandran plot	
Most favoured (%)	90.90
Allowed (%)	8.90
Outliers (%)	0.20

*Values in parentheses correspond to highest resolution shell.