FEBS Letters

FEBS PRESS science publishing by scientists

The structure of BrlR reveals a potential pyocyanin binding site

Harikiran Raju, Rukmini Sundararajan and Rohan Sharma

Department of Biophysics, Molecular Biology and Bioinformatics, University of Calcutta, Kolkata, West Bengal, India

Correspondence
R. Sharma, Department of Biophysics, Molecular Biology and Bioinformatics, University of Calcutta, Kolkata 700009, West Bengal, India
Tel: +91 033 2350 8386
E-mail: rohan_vsharma@yahoo.com

(Received 1 October 2017, revised 5 December 2017, accepted 10 December 2017, available online 3 January 2018)

doi:10.1002/1873-3468.12950

Edited by Stuart Ferguson

The transcriptional regulator BrlR from *Pseudomonas aeruginosa* is a member of the MerR family of multidrug transport activators. Studies have shown that BrlR plays an important role in the drug tolerance of *P. aeruginosa* in biofilms. The tolerance to drugs can be enhanced by 3′,5′-cyclic diguanylic acid (c-di-GMP). In the present study, we analyze the apo structure of BrlR and the direct binding between GyrI-like domain of BrlR and *P. aeruginosa* toxin pyocyanin. Furthermore, we show that pyocyanin can enhance the binding between BrlR and DNA *in vitro*. These findings suggest that BrlR can serve as the binding partner for both c-di-GMP and pyocyanin.

Keywords: BrlR; cyclic diguanylic acid; *Pseudomonas aeruginosa*; pyocyanin

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

The *P. aeruginosa* toxin pyocyanin is a blue redox-active secondary metabolite that can easily penetrate biological membranes, resulting in damage [2,3]. It is synthesized from chorismate via a series of complex steps mediated by gene products encoded by two phzABCDEFG operons and its precursors are modified into the tricyclic compound by the genes for phzH, phzM and phzS [4,5]. The synthesis of pyocyanin is regulated by quorum sensing systems, although the details are not clear.

Pyocyanin was reported to kill fungi and *Caenorhabditis elegans* [6,7]. Recent studies demonstrated that pyocyanin is required for lung infection in mice [8,9]. Moreover, large quantities of pyocyanin can be readily recovered from the sputum of patients with cystic fibrosis who have been infected by *P. aeruginosa* [10]. It has been reported that pyocyanin interferes with multiple cellular functions, resulting in oxidative stress and the inactivation of V-ATPase, as well as a decrease in mitochondrial and cytoplasmic aconitase activity and ATP levels [2,11].

Many studies investigating pyocyanin focus on its role in pathogenesis, whereas the regulation and signal transduction pathways have long been ignored. To date, only few pyocyanin receptors have been reported. The human aryl hydrocarbon receptor is a highly conserved ligand-dependent transcription factor that can sense pyocyanin, induce detoxifying enzymes and modulate immune cell differentiation and responses [12–14]. RmcA from *P. aeruginosa* comprises a

Abbreviations
c-di-GMP, 3′,5′-cyclic diguanylic acid; Cy5, cyanine 5; EMSA, electrophoretic mobility shift assay; PDB, Protein Data Bank.

multiple domain protein with both phosphodiesterase and diguanylate cyclase domains, for which activity is modulated by pyocyanin via its PAS domain [15].

BrlR protein is a transcription regulator in *P. aeruginosa*. It can only be detected in biofilm cells and is activated by bacterial secondary messenger 3′,5′-cyclic diguanylic acid (c-di-GMP) [16]. The activated BrlR can upregulate the expression of multidrug efflux pumps, such as the MexAB-OprM and MexEF-OprN efflux pumps. Knockout of BrlR leads to the susceptibility of the biofilm when the bacteria are 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 the N-terminal HTH_MerR domain and the GyrI-like domain at the C-terminus. Members of the MerR protein family have been demonstrated to comprise multidrug resistance proteins and some of them can bind potential multidrug molecules directly. In a previous study, we determined the complex structure of BrlR-c-di-GMP and identified one conserved drug-binding pocket. However, so far, there are no studies available in the literature reporting on which small molecule can bind into this pocket and what is the common link between biofilm and multidrug resistance in *P. aeruginosa*. In the present study, we demonstrate that BrlR from *P. aeruginosa* is a binding partner for pyocyanin and identify the GyrI-like domain as a potential binding site for pyocyanin.

Materials and methods

Protein expression and purification

Expression and purification of full-length *brlR* gene and 119-end fragment (GyrI-like domain) were each performed in accordance with a previous protocol [18]. Briefly, the genes encoding the corresponding sequences were amplified by PCR and inserted into the pET-28a vector (Novagen, Madison, WI, USA). The *Escherichia coli* BL21 (DE3) cells were used as a host for expression. The proteins were obtained by a 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 throughout the purification was used for western blotting. The protein was then concentrated in gel filtration buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 5% glycerol). Protein purity was checked using Coomassie brilliant blue-stained SDS/PAGE. The mutagenesis of BrlR (W150A) was performed by overlap-extension PCR and both the expression and purification of the mutant protein used the same method as that employed for the wild-type.

Crystallization, data collection, structure determination and refinement

Purified BrlR proteins were concentrated to ~ 10 mg·mL^{-1} in the gel filtration buffer and screened for crystallization using commercially available kits (Molecular Dimensions Ltd, Newmarket, UK). A sitting-drop vapor diffusion method was used in a 24-well Itelli-plate to optimize the crystals at 20 °C (10% v/v 1,4-dioxane, 0.1 M Mes/NaOH, pH 6.5, 1.6 M ammonium sulfate; Structure Screen 1 & 2; Molecular Dimensions Ltd). The grown crystals were dehydrated using 1.8 M lithium sulfate to improve the resolution.

The diffraction data were collected on beamline BM14 at the European Synchrotron Radiation Facility (ESRF, Grenoble, France). Macromolecular crystal annealing was performed. Briefly, before data collection, the cold nitrogen stream was blocked for ~ 3 s, on three occasions, at intervals of 5 s [19]. The data were indexed, integrated and scaled with HKL2000 (HKL Research Inc., Charlottesville, VA, USA) [20]. PHASER (PHENIX) was used to perform the molecular replacement [21]. The BrlR coordinate from the c-di-GMP complex structure [Protein Data Bank (PDB) code: 5XQL] was used as the search model. COOT was used to modify the result model and PHENIX.REFINE was used for refinement [21,22]. The structure of BrlR was deposited in RCSB under PDB code: 5YC9. Crystallographic statistics are summarized

Table 1. Data collection and refinement statistics.

Characteristic	Value
Data collection	
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)	35.00–2.90 (2.95–2.90)[a]
Completeness (%)	99.7 (100.0)
Redundancy	7.9 (7.9)
$\langle I/\sigma(I)\rangle$	18.71 (1.39)
R_{pim}	0.037 (0.606)
CC1/2	0.564
Overall B factor from Wilson plot (Å^2)	43.16
Refinement	
Number of reflections	60 294
Final R_{cryst}/Final R_{free}	0.2693/0.2969
Number of non-H atoms	
Protein	8290
rmsd	
Bond lengths (Å)	0.006
Bond angles (°)	1.154
Average B factors (Å^2)	
Protein	65.84
Ramachandran plot	
Most favored (%)	91.21
Allowed (%)	8.59
Outliers (%)	0.20

[a] Values in parentheses correspond to the highest resolution shell.

in Table 1. All of the illustrations of the structures were prepared with PYMOL (http://www.pymol.org).

Electrophoretic mobility shift assay (EMSA)

In accordance with previous studies, three DNA probes were designed and labeled with cyanine 5 (Cy5) fluorescent dye (Thermo Fisher Scientific Inc., Waltham, MA, USA; Table S1) [17,23]. The DNA probes (0.5 pmol), BrlR proteins (25 pmol) and different concentration of pyocyanin (Everon Life Sciences, New Delhi, India) 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 dithiothreitol 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, Lincoln, NE, USA).

Ligand trapping assay

To check the binding between BrlR and pyocyanin, the ligand trapping assay were performed with a 5-kDa cut-off centrifugal filter. BrlR proteins were incubated with its ligand in a 1 : 2 ratio for 20 min at room temperature. Then, BrlR proteins with ligand were concentrated using a centrifugal filter at 4 °C for 30 min. The flow-through was collected to measure the concentration of untrapped ligand at a wavelength of 310 nm and the buffer with ligand was used as a control experiment. Every binding assay was repeated at least three times.

Binding affinity measurement

The T_m value of BrlR was determined by thermal-denaturation assay with increments of 2 °C from 50 to 68 °C. Briefly, 20 μg of 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 denaturation curve. The BrlR-pyocyanin binding affinity was measured using a ligand concentration-dependent thermal-denaturation assay. The 0.05 μM BrlR protein was incubated with various concentrations of pyocyanin (0.5, 1, 5, 10 and 20 μM) and denatured at 57.5 °C for 3 min. The supernatants were collected for western blotting. The protein was detected using primary mouse anti-His antibody (dilution 1 : 5000; Clontech, Palo Alto, CA, USA) and secondary horseradish peroxidase conjugated rabbit anti-mouse antibody (dilution 1 : 2000; Cell Signaling Technology, Beverly, MA, USA). The image was analyzed using IMAGEJ (NIH, Bethesda, MD, USA) and dissociation constant (K_d) was calculated with a fitted curve.

Results

Tetrameric apo BrlR structure

The crystal structure of apo BrlR was determined at 2.9 Å, with four BrlR molecules in the asymmetry unit. The density is poor around two regions (amino acid residues 27–40 and 137–144) in each protomer because these regions are flexible (Fig. 1A). According to a previous BrlR-c-di-GMP complex structure, amino acid residues

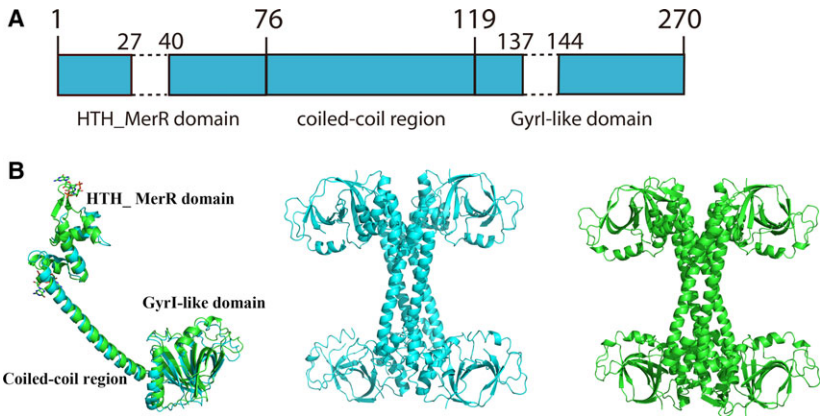

Fig. 1. Overall structure of apo BrlR structure. (A) Domain structure of BrlR. The dashed line indicates the disordered regions in the apo BrlR structure. (B) Left: superposition of cartoon presentation of apo BrlR monomer (in cyan) with BrlR monomer from c-di-GMP complex (in green) (PDB: 5XQL). No obvious conformation change is observed between the two forms. Apo BrlR monomer can be clearly divided into three parts: (a) N-terminal HTH_ MerR domain (amino acids 1–76); (b) middle coiled-coil region (amino acids 77–119); and (c) C-terminal GyrI-like domain (amino acids 120–270). Middle and right: cartoon presentation of tetramer of apo BrlR (in cyan) and BrlR tetramer from the c-di-GMP bound form (in green). Similar to 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, whereas the relative position of these four chains has only a minor difference.

27–40 take part in c-di-GMP binding and amino acid residues 137–144 are located near the drug-binding pocket [18]. Therefore, both areas show a binding potential for small molecules as a result of their flexibility.

The overall structure of apo BrlR is very 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 a result of the crystal packing artifact (Fig. 1B). Similar to the c-di-GMP bound form, the apo BrlR structure can be divided into three parts: (a) N-terminal HTH_ MerR domain; (b) middle coiled-coil region; and (c) C-terminal GyrI-like domain (Fig. 1). The apo BrlR exists as a tetramer in solution as revealed in a previous gel filtration experiment [18].

The c-di-GMP binding did not change the overall conformation and oligomerization state of BrlR (Fig. 1B). Therefore, the mechanisn by which the c-di-GMP stimulates the BrlR remains elusive.

Pyocyanin binds BrlR in the GyrI-like domain

The similarity and conservation of the GyrI-like domain between BrlR and other multidrug resistance proteins discussed in the previous study [18] indicates that BrlR can bind flat shape molecules (Fig. S1). For detailed analysis, we compared the GyrI-like domain of BrlR with SAV2435 (PDB code: 5KAU). The GyrI-like domains of BrlR and SAV2435 align well, with an rmsd value of 2.3 Å over 165 residues (Fig. 2A). Similar to SAV2435, the drug-binding pocket of the BrlR GyrI-like domain is deep and hydrophobic, indicating that this site may have the same preference for small molecules with aromatic rings. Unlike BmrR and

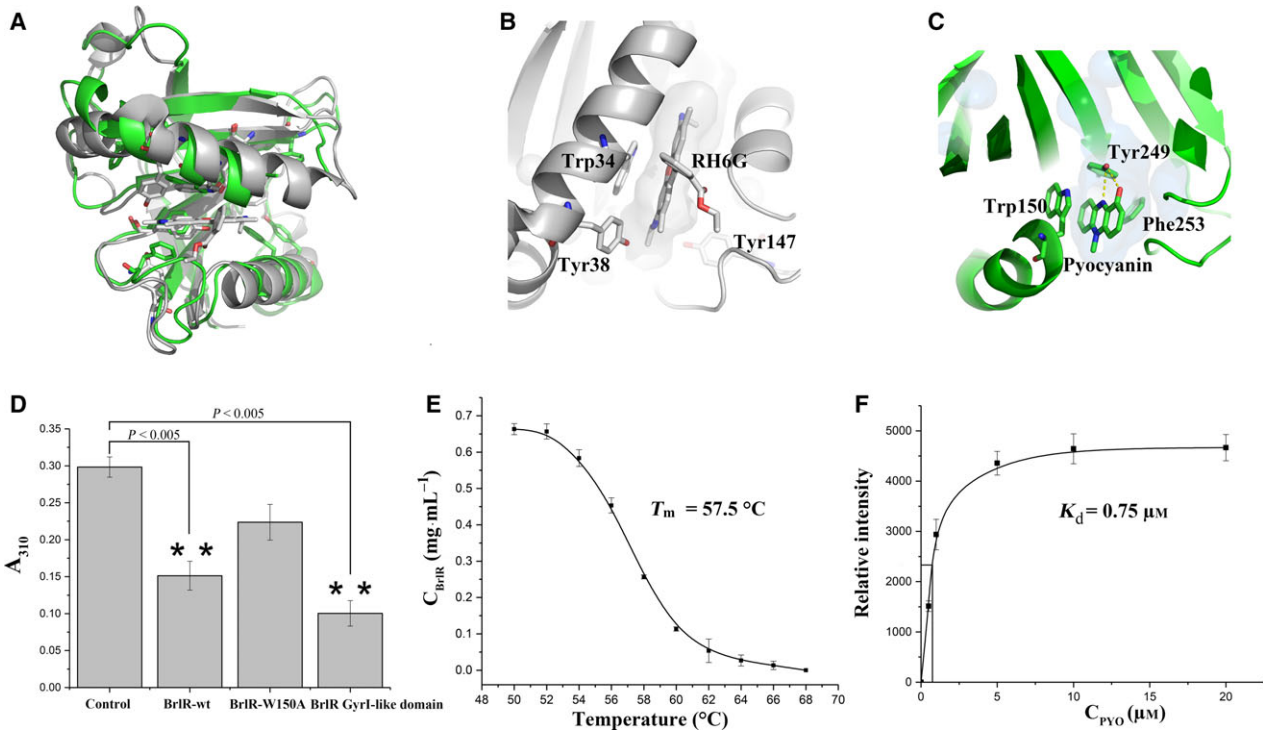

Fig. 2. Pyocyanin binds BrlR in the GyrI-like domain. (A) Superposition of cartoon presentation of the GyrI-like domain of BrlR with RH6G bound SAV2435 (PDB: 5KAU). BrlR is shown in cyan and SAV2435 is shown in grey. Aromatic residues around the drug-binding pocket and RH6G molecule are rendered in stick form. (B) RH6G binding site in SAV2435. The binding cavity is presented in surface mode; key residues and RH6G are rendered in stick form. (C) Predicted pyocyanin binding site in the BrlR GyrI-like domain. The potential binding cavity is presented in surface mode; predicted key residues and pyocyanin are rendered in stick form. The yellow dashed lines indicate the possible interaction for pyocyanin Tyr249, which may play a role in binding specificity. (D) Ligand trapping assay for BrlR and pyocyanin. Although the GyrI-like domain of BrlR can even bind pyocyanin tighter, the full-length W150A mutation binds less pyocyanin. At least three experiments were performed. Error bars indicate the SD. Significant difference from control: $^{**}P < 0.005$. *P*-values were calculated using Student's *t*-test (two-tailed). (E) Denaturation curve of BrlR. Error bars indicate the SD. (F) Ligand concentration-dependent thermal-denaturation assay for BrlR and pyocyanin. Data are calculated for the relative intensity of bands detected by western blotting and analysis using IMAGEJ. Error bars indicate the SD.

SAV2435, which bind many molecules, no small molecules are reported to bind the GyrI-like domain of BrlR so far. We noted that the central xanthene ring of rhodamine 6G (RH6G) in theSAV2435 complex structure is very similar to pyocyanin, which exists in large quantities in *P. aeruginosa*.

The addition of exogenous pyocyanin can affect the expression of dozens of genes [24]. This may be a result of pyocyanin serving as a redox-active molecule or its direct binding to some transcription factors that cause this regulation. However, to date, no transcription regulators have been identified as pyocyanin receptors. We noted that eight of the upregulated genes 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 by 2.3-fold. This regulation pattern is somewhat similar to that of the BrlR gene regulation by c-di-GMP. In addition, BrlR will activate genes encoding the MexAB-OprM and MexEF-OprN multidrug efflux pumps after stimulation by c-di-GMP [17]. Under such conditions, we hypothesize that BrlR can bind pyocyanin and trigger its gene regulatory function.

To better characterize the binding property of BrlR towards pyocyanin, we modelled the pyocyanin into the surface cavity of the GyrI-like domain in silicon according to the binding mode of RH6G in SAV2435 (Fig. 2B,C). The results obtained show that both RH6G and pyocyanin are buried in the cavity of the drug-binding pocket, with several aromatic residues around them. Similar to Trp34 in SAV2435, Trp150 forms stacking interactions with pyocyanin in this model. At the same time, Tyr249 forms hydrogen bonds with pyocyanin, which plays a role in its binding specificity (Fig. 2C). To confirm this hypothesis, we tested the binding between BrlR protein and pyocyanin using a ligand trapping assay. Figure 2D clearly shows that BrlR can bind pyocyanin and reduce its chance to flow through the filter membrane. The binding also changes the color of the solution from blue to green, especially under more concentrated

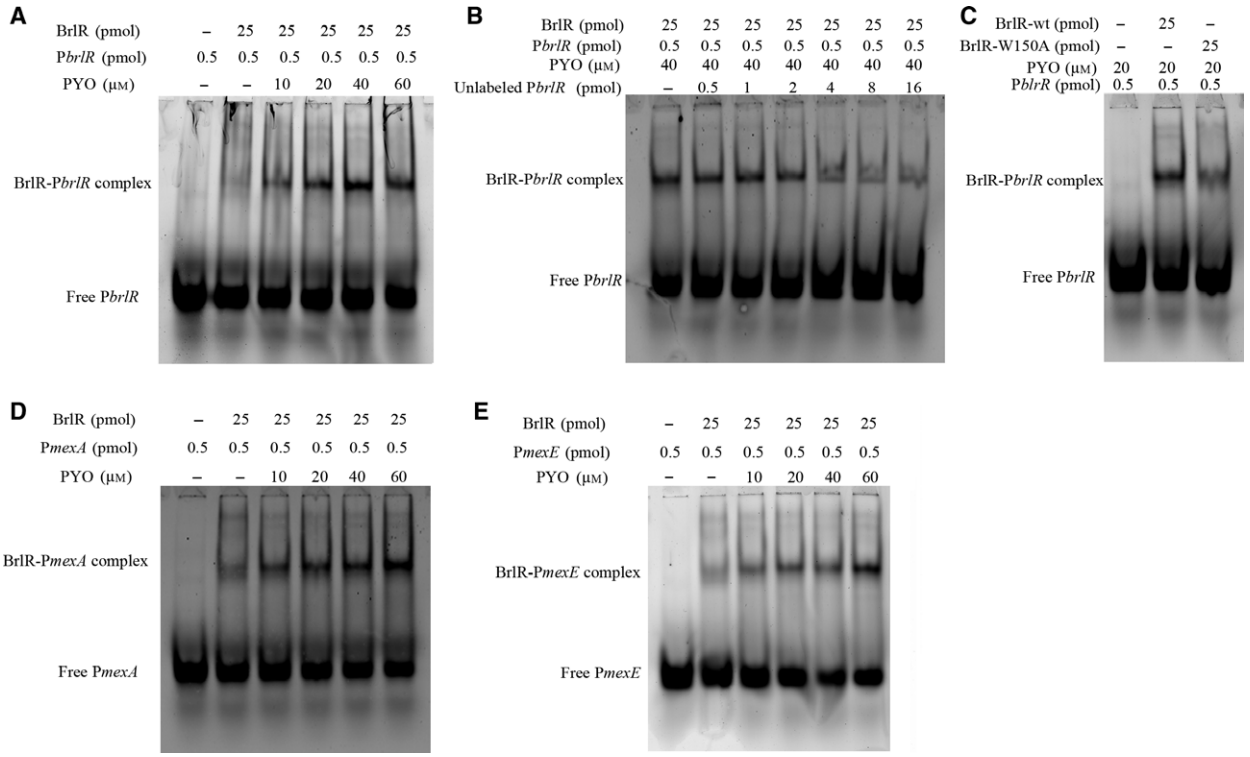

Fig. 3. EMSA assays for BrlR with pyocyanin. (A) EMSA assays with P*brlR*. The basic DNA binding of apo BrlR is relatively weak and the BrlR-P*brlR* complex can be clearly observed only after adding 10 μM pyocyanin. The assays show that an increased pyocyanin concentration can enhance the DNA binding 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 cannot be enhanced by pyocyanin. (D,E) EMSA assays with P*mexA* and P*mexE*. The effects of pyocyanin on BrlR binding towards these two fragments are similar with P*brlR*. All of the EMSA assays were performed at least three times.

conditions (Fig. S2). The T_m value of BrlR is ~ 57.5 °C and 20 μM pyocyanin can increase this to 61.5 °C (Figs 2E and S2). To test whether pyocyanin binding pocket is located in our predicted site in the C-terminal GyrI-like domain, we used the C-terminal part of BrlR for this binding assay and found that it binds pyocyanin much better than the full-length protein, indicating that the full-length protein may be in a more regulated conformation (Fig. 3D). The binding affinity between BrlR and pyocyanin is ~ 0.75 μM (Figs 2F and S2), which is comparable to the binding affinity of c-di-GMP (2.2 μM) [16]. However, the binding between the W150A mutation protein and pyocyanin is decreased, as shown by the ligand trapping assay (Fig. 2D), which suggests that the predicted binding site is correct.

Pyocyanin can enhance the DNA binding of BrlR

BrlR can bind to its own promoter (P*brlR*) and the promoter of *mexA* (P*mexA*) and *mexE* (P*mexE*) [17]. Although 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 electrophoretic mobility shift assay (EMSA) assays of BrlR with Cy5 labeled P*brlR*, P*mexA* and P*mexE* using different concentrations of pyocyanin (Fig. 3). Because of chemical activity, we checked the effect of pyocyanin on Cy5 labeled DNA (Fig. S3). It appears that pyocyanin can quench the Cy5 fluorescent signals, especially at a high concentration. Although the DNA binding of apo BrlR is weak, an increased pyocyanin concentration can enhance the binding of BrlR towards P*brlR*, P*mexA* and P*mexE*. Because BrlR can nonspecifically bind many dyes (data not shown), we use unlabeled P*brlR*, P*mexA* and P*mexE* to demonstrate the specificity (Figs 3B and S4). In our assays, a high concentration of unlabeled P*brlR*, P*mexA* and P*mexE* can successfully compete the BrlR protein, which indicates that the labelling dye does not disturb our EMSA assays. A failure to obtain the complex structure of BrlR and pyocyanin means that the activation mechanism remains unknown. In our size-exclusion chromatography experiments, the apo and pyocyanin bound forms of BrlR share the same elution time, which suggests that pyocyanin does not change the oligomeric sate of BrlR, similar to the c-di-GMP (Fig. S5). The W150A mutation that lost pyocyanin binding shows a compromised binding ability to P*brlR*, P*mexA* and P*mexE* DNA in the presence of pyocyanin (Figs 3C and S4). In sum, these data show that pyocyanin can bind and regulate the transcription factor BrlR.

Discussion

The second messenger c-di-GMP can promote the biofilm formation in *P. aeruginosa* and the BrlR protein can only be detected after biofilm formation. The binding of pyocyanin or c-di-GMP by BrlR leads to the expression of the *BrlR* gene and BrlR regulated multidrug efflux pump genes [17]. It appears that both of these small molecules contribute to the multidrug resistance of *P. aeruginosa*. As a result of the unknown BrlR-ligand-DNA ternary complex structure, the molecular mechanism responsible for BrlR stimulation by c-di-GMP or pyocyanin remains elusive.

In summary, our data suggest that BrlR is a binding partner for both c-di-GMP and pyocyanin. As an unusual transcription regulator, BrlR has two separate binding sites with respect to c-di-GMP and pyocyanin. Future studies aim to focus on the physiological significance of the binding between BrlR and pyocyanin. BrlR appears to represent a promising drug target for the cure of intractable *P. aeruginosa* infections.

Acknowledgements

We thank the staff of BM14 beamline at the European Synchrotron Rediation Facility (ESRF) for their support with the data collection. We also acknowledge the financial support received from the University of Calcutta.

Author contributions

HR and RSu performed the experiments, analyzed the data and wrote the draft. RSh designed the experiments, analyzed the data and wrote the draft.

References

1 Lyczak JB, Cannon CL and Pier GB (2000) Establishment of *Pseudomonas aeruginosa* infection: lessons from a versatile opportunist. *Microbes Infect* **2**, 1051–1060.
2 Ran H, Hassett DJ and Lau GW (2003) Human targets of *Pseudomonas aeruginosa* pyocyanin. *Proc Natl Acad Sci USA* **100**, 14315–14320.
3 Lau GW, Hassett DJ, Ran H and Kong F (2004) The role of pyocyanin in *Pseudomonas aeruginosa* infection. *Trends Mol Med* **10**, 599–606.
4 Mavrodi DV, Bonsall RF, Delaney SM, Soule MJ, Phillips G and Thomashow LS (2001) Functional analysis of genes for biosynthesis of pyocyanin and phenazine-1-carboxamide from *Pseudomonas aeruginosa* PAO1. *J Bacteriol* **183**, 6454–6465.
5 Gallagher LA, McKnight SL, Kuznetsova MS, Pesci EC and Manoil C (2002) Functions required for

extracellular quinolone signaling by *Pseudomonas aeruginosa*. *J Bacteriol* **184**, 6472–6480.
6 Mahajan-Miklos S, Tan MW, Rahme LG and Ausubel FM (1999) Molecular mechanisms of bacterial virulence elucidated using a *Pseudomonas aeruginosa Caenorhabditis elegans* pathogenesis model. *Cell* **96**, 47–56.
7 Kerr JR, Taylor GW, Rutman A, Hoiby N, Cole PJ and Wilson R (1999) *Pseudomonas aeruginosa* pyocyanin and 1-hydroxyphenazine inhibit fungal growth. *J Clin Pathol* **52**, 385–387.
8 Lau GW, Ran H, Kong F, Hassett DJ and Mavrodi D (2004) *Pseudomonas aeruginosa* pyocyanin is critical for lung infection in mice. *Infect Immun* **72**, 4275–4278.
9 Allen L, Dockrell DH, Pattery T, Lee DG, Cornelis P, Hellewell PG and Whyte MK (2005) Pyocyanin production by Pseudomonas aeruginosa induces neutrophil apoptosis and impairs neutrophil-mediated host defenses *in vivo*. *J Immunol (Baltimore, Md: 1950)*, **174**, 3643–3649.
10 Wilson R, Sykes DA, Watson D, Rutman A, Taylor GW and Cole PJ (1988) Measurement of *Pseudomonas aeruginosa* phenazine pigments in sputum and assessment of their contribution to sputum sol toxicity for respiratory epithelium. *Infect Immun* **56**, 2515–2517.
11 O'Malley YQ, Abdalla MY, McCormick ML, Reszka KJ, Denning GM and Britigan BE (2003) Subcellular localization of *Pseudomonas* pyocyanin cytotoxicity in human lung epithelial cells. *Am J Physiol Lung Cell Mol Physiol* **284**, L420–L430.
12 Nguyen LP and Bradfield CA (2008) The search for endogenous activators of the aryl hydrocarbon receptor. *Chem Res Toxicol* **21**, 102–116.
13 Schrenk D (1998) Impact of dioxin-type induction of drug-metabolizing enzymes on the metabolism of endo- and xenobiotics. *Biochem Pharmacol* **55**, 1155–1162.
14 Moura-Alves P, Fae K, Houthuys E, Dorhoi A, Kreuchwig A, Furkert J, Barison N, Diehl A, Munder A, Constant P *et al*. (2014) AhR sensing of bacterial pigments regulates antibacterial defence. *Nature* **512**, 387–392.
15 Okegbe C, Fields BL, Cole SJ, Beierschmitt C, Morgan CJ, Price-Whelan A, Stewart RC, Lee VT and Dietrich LEP (2017) Electron-shuttling antibiotics structure bacterial communities by modulating cellular levels of c-di-GMP. *Proc Natl Acad Sci USA* **114**, E5236–E5245.
16 Chambers JR, Liao J, Schurr MJ and Sauer K (2014) BrlR from *Pseudomonas aeruginosa* is a c-di-GMP-responsive transcription factor. *Mol Microbiol* **92**, 471–487.
17 Liao J, Schurr MJ and Sauer K (2013) The MerR-like regulator BrlR confers biofilm tolerance by activating multidrug efflux pumps in *Pseudomonas aeruginosa* biofilms. *J Bacteriol* **195**, 3352–3363.
18 Raju H and Sharma R (2017) Crystal structure of BrlR with c-di-GMP. *Biochem Biophys Res Comm* **490**, 260–264.
19 Yeh JI and Hol WG (1998) A flash-annealing technique to improve diffraction limits and lower mosaicity in crystals of glycerol kinase. *Acta Crystallogr D Biol Crystallogr* **54**, 479–480.
20 Otwinowski Z and Minor W (1997) Processing of X-ray diffraction data collected in oscillation mode. In Methods in Enzymology (Carter CW Jr, ed), pp. 307–326, Academic Press, New York.
21 Adams PD, Afonine PV, Bunkoczi G, Chen VB, Davis IW, Echols N, Headd JJ, Hung LW, Kapral GJ, Grosse-Kunstleve RW *et al*. (2010) PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr D Biol Crystallogr* **66**, 213–221.
22 Emsley P, Lohkamp B, Scott WG and Cowtan K (2010) Features and development of Coot. *Acta Crystallogr D Biol Crystallogr* **66**, 486–501.
23 Liao J and Sauer K (2012) The MerR-like transcriptional regulator BrlR contributes to *Pseudomonas aeruginosa* biofilm tolerance. *J Bacteriol* **194**, 4823–4836.
24 Dietrich LE, Price-Whelan A, Petersen A, Whiteley M and Newman DK (2006) The phenazine pyocyanin is a terminal signalling factor in the quorum sensing network of *Pseudomonas aeruginosa*. *Mol Microbiol* **61**, 1308–1321.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Multiple sequence alignment result of BrlR GyrI-like domain with BmrR and SAV2435.

Fig. S2. Pyocyanin can bind to BrlR.

Fig. S3. The control experiment with the increased pyocyanin concentration alone on the migration of BrlR promoter.

Fig. S4. EMSA assays for BrlR with pyocyanin.

Fig. S5. SEC result of apo BrlR and BrlR with pyocyanin.

Table S1. Oligonucleotide sequences used.