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BrIR from *Pseudomonas aeruginosa* is a c-di-GMP-responsive transcription factor

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Abstract

The transcriptional regulator BrIR is a member of the MerR family of multidrug transport activators that contributes to the high-level drug tolerance of *Pseudomonas aeruginosa* biofilms. While MerR regulators are known to activate both the expression of multidrug efflux pump genes and their own transcription upon inducer-binding, little is known about BrIR activation. We demonstrate using promoter reporter strains, *in vivo* and *in vitro* DNA-binding assays combined with 5'RACE, that BrIR binds to its own promoter, likely via a MerR-like palindromic sequence. Unlike known MerR multidrug transport activators, BrIR and *brlR* expression are not activated by multidrug transporter substrates. Instead, BrIR-DNA binding was enhanced by the secondary messenger c-di-GMP. In addition to enhanced BrIR-DNA binding, c-di-GMP levels contributed to *PbrlR* promoter activity in initial attached cells with elevated c-di-GMP levels correlating with increased expression of *brlR*. While not harboring amino acid motifs resembling previously defined c-di-GMP-binding domains, BrIR was found to bind c-di-GMP *in vitro* at a ratio of one c-di-GMP per two BrIR. Crosslinking assays confirmed dimer formation to be enhanced in the presence of elevated c-di-GMP levels. Our findings demonstrate BrIR to be an unusual MerR-family member in that BrIR function and expression require the secondary messenger c-di-GMP.

INTRODUCTION

Biofilms are composed of surface-attached microbial communities. A hallmark of biofilms is their profound tolerance to antimicrobial agents (Costerton *et al.*, 1999). Several reasons have been suggested to explain biofilm tolerance including slow growth and starvation-induced growth arrest, increased presence of persister cells that neither grow nor die in the presence of bactericidal antibiotics as well as diffusion limitation of antimicrobials into biofilms (Shah *et al.*, 2006) (Campanac *et al.*, 2002, Picioreanu *et al.*, 2001, Anderl *et al.*, 2000, Lewis, 2001, Stewart & Costerton, 2001, Stewart, 1996, Drenkard, 2003, Nguyen *et al.*, 2011). Recent reports further suggest that bacteria within these microbial communities are physiologically distinct from planktonic bacteria, expressing specific protective factors such as multidrug (MDR) efflux pumps and stress response regulons (Anwar *et al.*, 1992, Sauer & Camper, 2001, Sauer *et al.*, 2002, Stoodley *et al.*, 2002, Donlan & Costerton, 2002,

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Gilbert *et al.*, 2002, Mah & O'Toole, 2001, Mah *et al.*, 2003, Stewart & Costerton, 2001). In addition, quorum sensing (QS) is required for the formation of the biofilm architecture (Davies *et al.*, 1998), and has been shown to play a role in resistance (Bjarnsholt *et al.*, 2005). Biofilm and planktonic cells further differ in the level of the secondary messenger molecule c-di-GMP, with high concentrations of this molecule correlating with an aggregative or sessile lifestyle (e.g. biofilm formation), while its absence favors motility (e.g. twitching, swarming) and the free swimming lifestyle (D'Argenio & Miller, 2004). While c-di-GMP has not been directly linked to resistance, c-di-GMP contributes to the production of extracellular polysaccharides which has been suggested to offer protection from environmental factors, antimicrobials, and the immune response, likely by limiting diffusion or sequestration (Merritt *et al.*, 2007, Romling *et al.*, 2005, Irie *et al.*, 2012, Mishra *et al.*, 2012). Moreover, low c-di-GMP levels have recently been linked to the expression of proteins required for antimicrobial peptide resistance in *Pseudomonas aeruginosa* (Chua *et al.*, 2013). The findings indicated that biofilms themselves are not simply a diffusion barrier to these antibiotics, but rather that bacteria within these microbial communities employ distinct mechanisms to resist the action of antimicrobial agents. This was further supported by the finding of the transcriptional regulator BrIR (Biofilm resistance locus Regulator) contributing to the high-level tolerance of biofilms formed by the human pathogen *P. aeruginosa* (Liao & Sauer, 2012). In particular, BrIR was found to contribute to the tolerance of *P. aeruginosa* biofilms to hydrogen peroxide and five different classes of antibiotics by affecting MIC and recalcitrance of biofilms to killing by bactericidal antimicrobial agents (Liao & Sauer, 2012), suggesting BrIR may play a role in the activation of genes involved in resistance to antimicrobial agents. The mechanism by which BrIR, a MerR-like regulator, confers tolerance relies on BrIR binding promoter regions and activating at least two multidrug efflux pumps encoded by *mexAB-oprM* and *mexEF-oprN* (Liao *et al.*, 2013).

Activation of multidrug transporter genes is a common feature of members of the MerR family of multidrug efflux pump gene activators. However, while MerR activators induce expression of multidrug transporter genes, their induction requires binding of a specific 'coactivator' molecule, typically the transporter substrate (Heldwein & Brennan, 2001, Zheleznova *et al.*, 1999, Newberry & Brennan, 2004). For instance, BItR is induced by binding of rhodamine to the C-terminal domain, resulting in activation of transcription of multidrug transporters, TipA is activated by binding thioestrepton, and BmrR by binding both rhodamine and tetraphenylphosphonium (Ahmed *et al.*, 1994, Neyfakh, 2001, Vazquez-Laslop *et al.*, 2000, Brown *et al.*, 2003, Baranova *et al.*, 1999, Godsey *et al.*, 2002, Zheleznova *et al.*, 1999, Holmes *et al.*, 1993). Inducers for the global activator MtaN are not known (Godsey *et al.*, 2001, Godsey *et al.*, 2002). Expression of the *Bacillus subtilis* multidrug transporter Bmr was shown to be inducible at the level of transcription by two of its diverse substrates, rhodamine 6G and thioestrepton, and this induction is mediated by the regulatory protein BmrR encoded immediately downstream of the *bmr* gene. Structural analysis of BmrR indicated inducer binding to the C-terminal domain of BmrR (Heldwein & Brennan, 2001, Zheleznova *et al.*, 1999). In addition to activating gene expression of multidrug efflux pump genes, members of this MerR family activate their own transcription upon inducer binding (Summers, 1992, Holmes *et al.*, 1993). Activation of transcription

coincides with MerR regulators binding to palindromic promoters in which the spacing between the -35 and -10 elements recognized by the sigma factor is greater than the optimal 17+/-1 bp, usually 19 bp (Heldwein & Brennan, 2001, Godsey et al., 2001, Newberry & Brennan, 2004). Binding to suboptimal 19-bp spacers requires DNA distortion, a DNA-binding mechanism that is common to the entire MerR family (Newberry & Brennan, 2004). Despite MerR family members sharing homologous DNA binding domains and recognizing palindromic promoters with suboptimal spacing, the MerR-DNA binding sites are diverse (Brown et al., 2003).

BrlR harbors two domains, an N-terminal Helix-Turn-Helix DNA binding HTH_BmrR domain, and a C-terminal modulation or “coactivator” GyrI-like binding domain (Liao & Sauer, 2012), (Fig. S1). While BrlR shares similarities to MerR activators including domain organization, sequence homology and activation of multidrug transporter genes (Liao & Sauer, 2012, Liao et al., 2013), little is known about the BrlR-DNA binding sites, the inducer(s) to which BrlR respond in order to activate BrlR-dependent drug tolerance in biofilms, nor its mechanism of activation contributing to the biofilm-specific expression of *brlR* (Liao & Sauer, 2012, Liao et al., 2013). To begin elucidating the mechanism by which BrlR is activated and *brlR* is expressed in a biofilm-specific manner, we first mapped the *brlR* promoter region and investigated the DNA binding properties of BrlR. Promoter reporter strains were then used to probe for cues activating *brlR* expression. In contrast to known MerR family members, BrlR and *brlR* expression is not activated by multidrug transporter substrates. Instead, induction of *brlR* expression coincides with increased levels of the intracellular signaling molecule c-di-GMP, conditions that have previously been linked to attachment and surface attached growth. We further demonstrate that BrlR binds to a palindromic sequence, with BrlR-DNA binding and *brlR* promoter reporter activity being enhanced by the secondary messenger c-di-GMP. Consistent with BrlR being a c-di-GMP responsive transcription factor, BrlR was found to bind c-di-GMP.

RESULTS

Mapping of the *brlR* promoter region

To begin elucidating the mechanism by which BrlR is activated and *brlR* is expressed in a biofilm-specific manner, we first mapped the approximate promoter region as well as the transcriptional start site of *brlR*. Using 5'RACE, the transcriptional start site was found to be 23 bp upstream of the *brlR* open reading frame (Figs. 1A, S2). Our transcriptional start site is located within 2 bp of the start site recently identified by Wurtzel et al. (Wurtzel *et al.*, 2012) using RNA-Seq in conjunction with mapping of the ends of untranslated regions (UTR). The 23 bp long UTR harbors a putative Shine-Dalgarno sequence (Fig. 1A). Moreover, a predicted promoter sequence upstream of the transcriptional start site was identified as a σ^{70} -like -10 box centered approximately 4 bp upstream of the transcriptional start site, followed by a -35 box (Fig. 1A). The predicted promoter sequence is located in the intergenic region of *brlR* and PA4877. The gene PA4877 encoding a hypothetical protein is located 147 bp upstream of the BrlR ATG start codon and is transcribed in the reverse orientation (Fig. 1B). To locate the *brlR* promoter region, DNA sequences located 120, 250, and 500 bp upstream from the BrlR start codon (Fig. 1B) were PCR amplified and cloned

into the pMiniCTX-lacZ vector, yielding three transcriptional promoter fusions (*PbrlR-120-lacZ*, *PbrlR-250-lacZ*, *PbrlR-500-lacZ*). DNA sequences located 23 bp upstream from the BrlR start codon (*PbrlR-23-lacZ*) were used as control. Under biofilm conditions, no difference in promoter activity was detected for the *PbrlR-23-lacZ* reporter construct and the vector control (Fig. 1C). However, significantly increased β -galactosidase activities compared to the vector control were detected for the promoter reporter strains harboring DNA located 120, 250, and 500 bp upstream of the *brlR* start codon (Fig. 1C). In contrast, no difference in β -galactosidase activity was noted under planktonic growth conditions, with β -galactosidase activity levels being comparable to those observed for the vector control (Fig. 1C). These data confirmed previous findings of *brlR* expression being biofilm-specific (Liao & Sauer, 2012). Moreover, our observations suggested that the *brlR* promoter was located within 23-120 bp upstream of the BrlR ATG start codon, in the intergenic region between *brlR* and PA4877 (Fig. 1B). The respective region harbored the putative σ^{70} -like -10 and -35 promoter elements (Fig. 1A).

BrIR binds to its own promoter

MerR proteins, including TipA and BmrR, have been shown to bind to their own promoters (Baranova et al., 1999, Newberry & Brennan, 2004, Neyfakh, 2001). To determine whether BrIR acts in a similar manner, we purified BrIR with a C-terminal His₆V5-tag (BrIR–His₆V5; Fig. 2A) to perform electrophoretic mobility shift assays using promoter fragments spanning the region located 443-543 bp (region A), 223-459 bp (region B), 91-240 bp (region C) and 7-108 bp (*PbrlR*) upstream of the *brlR* translational start codon. The tagged BrIR construct has been previously shown to complement a *brlR* mutant *in vivo* (Liao & Sauer, 2012). BrIR did not bind to radiolabeled DNA probes spanning the DNA sequences located 91-543 bp upstream of the *brlR* start codon (representing regions A-C, Fig. 2B). In contrast, a specific complex was formed when BrIR was added to the radiolabeled DNA probe *PbrlR*, at a molar ratio of 20:1 (Fig. 2C). The *PbrlR* sequence is located 7-108 bp upstream of the *brlR* start codon (Fig. 2C). BrIR-DNA binding was specific as no binding to the labeled non-specific promoter fragment (*PpscE-F*) was observed, and dependent on both the BrIR protein and *brlR* promoter DNA concentration. When the molar concentrations of BrIR were increased, binding to *PbrlR* increased. When increasing amounts of unlabeled *PbrlR* fragment (Fig. 2C) were added to the gel mobility shift assay, decreased BrIR binding to the labeled fragment was observed.

While BrIR binding to the *PbrlR* promoter appeared to be specific, binding of BrIR was observed at high BrIR:DNA molar ratios. To further confirm BrIR binding to the respective promoter region, we probed for *in vivo* BrIR-DNA binding using chromatin immunoprecipitation (ChIP) assays and *P. aeruginosa* biofilms expressing a C-terminally His₆V5-tagged BrIR construct (BrIR–His₆V5). A *P. aeruginosa* strain overexpressing the untagged protein was used as control. DNA isolated via anti-V5 antibody immunoprecipitation from PAO1/pJN-*brlR* and PAO1/pMJT-*brlR*-His₆V5 biofilm samples was subjected to qPCR using primers spanning the promoter region located 7-108 bp upstream of the *brlR* ATG start codon. On average, the promoter region of *brlR* was enriched 14-fold, while no enrichment was detected for the non-specific promoter region of

PpscE-F (Fig. 2D). The finding suggested BrIR binding to the 7-108 bp region upstream of the BrIR start codon both *in vitro* and *in vivo*.

BrIR binds to a palindromic sequence located between the putative -35 and -10-boxes

Binding to palindromic sequences is common among MerR regulators including the MerR regulator BmrR which has been shown using DNA footprint analysis to bind to a palindromic sequence located between the -35 and -10-boxes of the *bmrR* promoter (Ahmed et al., 1994, Brown et al., 2003). Analysis of the DNA sequence comprising the putative -35 and -10-boxes of the *brlR* promoter using the software “Palindrome” indicated the presence of a perfect inverted repeat located between the -10 and -35 elements (Fig. 3A). The sequence shared similarity to the symmetrical spacer regions of MerR-like regulated promoter regions including TipA, BmrR, and SoxR as well as the metal-dependent promoters of MerR, CoaR, and CueR (Fig. 3B). The spacer regions have been shown to be the regulator binding sites (Brown et al., 2003). DNA gel mobility shift assays using short DNA sequences lacking either the putative -10 or the -35 promoter elements (prom-35, prom-10, respectively), or a 19bp-long DNA sequence located between the -10 and -35 elements (see “prom-19bp”) demonstrated BrIR-binding to such short DNA sequence (Fig. 3C). Mutating one half of the palindromic sequence by substituting the G’s with T’s impaired BrIR-binding (see “prom-mut1”), suggesting BrIR-binding to occur in-between the putative -35 and -10-boxes in a sequence-specific manner (Fig. 3A, C). Lack of BrIR-binding to a chimeric 19bp long sequence (see “prom-mut2”) composed in part of a portion of the 19 bp spacer region and a DNA sequence located 119-128 bp upstream of the ATG start codon, further supported BrIR-binding to be sequence-specific rather than DNA length-specific (Fig. 3A, C). Interestingly, the 19bp-long DNA sequence is the site of the perfect inverted repeat as determined using the palindrome software analysis tool (Fig. 3A-B).

To determine whether the palindromic BrIR-DNA binding sequence shown in Fig. 3A-B is also present in the promoter DNA of *mexAB-oprM* and *mexEF-oprN*, which were previously demonstrated to be BrIR-targets (Liao et al., 2013), we made use of MEME to search for common DNA binding motifs (Bailey *et al.*, 2009). One motif common to all sequences was identified as [TC][GTA][ATC][GCT][TC][TC][TC][CGA][CG]X[CG][CT][CA]GG[GAT][GA][CG]A[GA][GCT]C[GCT][TC][ATC][GC][AT], with p-values ranging from $5.3e^{-9}$ for the *mexAB-oprM* promoter region to $4.4e^{-11}$ for the *mexEF-oprN* promoter region (Fig. 3D). The motifs identified by MEME, while not perfect inverted repeats, were found to be located 38-65 bp and 39-66 bp upstream of the transcriptional start sites of *mexA* and *mexE*, respectively (Fig. 3D). Moreover, the location of the MEME-derived BrIR-DNA binding motif in the upstream region of the multi-drug efflux pump operons overlapped with the DNA sequences previously used to demonstrate BrIR-binding the promoter DNA of the *mexAB-oprM* and *mexEF-oprN* operons (Liao et al., 2013).

brlR expression is enhanced by c-di-GMP *in vivo*

Our findings suggested BrIR binds to DNA in a manner similar to known MerR family regulators. Considering that MerR multidrug transport activators have been demonstrated to respond to, and to be activated by, the substrates of the multidrug transporters that they are regulating (Ahmed et al., 1994, Neyfakh, 2001, Vazquez-Laslop et al., 2000, Brown et al.,

2003, Baranova et al., 1999, Godsey et al., 2002, Zheleznova et al., 1999, Holmes et al., 1993, Heldwein & Brennan, 2001, Summers, 1992), we hypothesized that BrIR may likewise require an inducer. We further hypothesized that such an inducer is not likely to be an antimicrobial agent as *brlR* expression was found to be biofilm-specific (Liao & Sauer, 2012) (Fig. 1C). To gain a better understanding of the nature of such an inducer, we first determined when *brlR* expression is induced over the course of initial biofilm formation. This was performed using a *gfp* promoter reporter and monitoring *gfp* expression and thus, *brlR*, by confocal microscopy. No *gfp* expression was noted in *P. aeruginosa* cells grown planktonically (not shown) and in cells that were attached via their poles, indicating that *brlR* expression is not induced in initially or reversibly attached cells (Fig. 4A). The earliest time point at which *brlR* expression was detected was 6 hr post initial attachment, primarily in cells attached longitudinal to the glass surface. However, only a small percentage of the population expressed *gfp*. Expression of *gfp* significantly increased upon continued attachment, with *gfp* expression, indicative of *brlR* expression, being maximal following 24 hr of growth (Fig. 4A). *brlR* expression remained detectable at later biofilm stages (not shown). Under the conditions tested, no *gfp* expression was detectable when using a vector control (Fig. 4A). Increased *brlR* expression following 6 hr post-attachment compared to 4 hr post-attachment and vector control was supported by β -galactosidase activity determinations using the *PbrlR-120-lacZ* promoter reporter strains (Fig. 4D).

Given the link between initial attachment and induction of *brlR* expression, we next asked whether factors or conditions associated with surface-attached growth may contribute to the expression of *brlR* in a biofilm-specific manner. Conditions that have been ascribed to the biofilm mode of growth include slow growth, oxygen and nutrient limitation, presence of oxidative stress, as well as increased viscosity and cell density. However, none of these conditions induced *brlR* expression (Fig. S3). We likewise excluded a role of multidrug efflux pump substrates tobramycin, norfloxacin, trimethoprim, or a mixture of the three antimicrobial agents as likely inducers of *brlR* expression (Fig. S4). Biofilms further differ from planktonic cells in the level of the intracellular secondary messenger molecule cyclic di-GMP (c-di-GMP). C-di-GMP has been associated with controlling the transition between a motile and biofilm lifestyle, with high concentrations of this molecule correlating with a sessile lifestyle (e.g. biofilm formation), while its absence favors motility (e.g. twitching, swarming) and the free-swimming lifestyle (Basu Roy *et al.*, 2012, Barraud *et al.*, 2009, D'Argenio & Miller, 2004). We therefore asked whether the secondary messenger c-di-GMP plays a role in *brlR* expression by determining c-di-GMP levels of cells attached for 4, 6, and 24 hr. Cells attached for 4 hr were characterized by 30 ± 0.95 pmol c-di-GMP per mg biofilm biomass (Fig. 4B). Upon continued attachment, c-di-GMP levels increased significantly to 43 pmol/mg following 6 hr, and 79 pmol c-di-GMP per mg biofilm biomass following 24 hr of attachment under flowing conditions (Fig. 4B). Interestingly, the c-di-GMP levels of cells attached for 4 hr were comparable to levels previously described for planktonic cells while the c-di-GMP level detected in 24 hr-attached cells was comparable to c-di-GMP levels previously described to be present in 4-6 day old biofilms (Basu Roy *et al.*, 2012, Barraud *et al.*, 2009).

The finding suggested induction of *brlR* expression to correlate with elevated c-di-GMP levels. To further confirm a link between *brlR* expression and c-di-GMP levels, we introduced the c-di-GMP producing diguanylate cyclase PA4843 under the control of the arabinose inducible P_{BAD} promoter into the *PbrlR-gfp* promoter reporter to monitor the effect of elevated c-di-GMP levels on *brlR* expression in *P. aeruginosa* during attachment. PA4843 was chosen as it was previously demonstrated to be an active diguanylate cyclase (Petrova & Sauer, 2012a). Under the conditions tested, no *PbrlR-gfp* promoter activity was detected at the 5 hr time point in the absence of arabinose (due to lack of PA4843 expression, PA4843 is under control of P_{BAD} promoter, Fig. 4C). Expression of *gfp* and thus, expression of *brlR*, however, was significantly increased upon induction of PA4843 expression via addition of arabinose, as evident by nearly 100% of the attached population expressing *gfp* (Fig. 4C). β -galactosidase activity assays using a *PbrlR-lacZ* promoter reporter construct confirmed increased *PbrlR* promoter activity upon multicopy expression of the cyclase PA4843 (Fig. 4D). The β -galactosidase activity in attached cells overexpressing the cyclase PA4843 at the 6 hr timepoint was similar to the activity detected in the *PbrlR-gfp* cells attached for 24 hr (Fig. 4D). It is of interest to note that multicopy expression of the cyclase PA4843 correlated with a significant increase in c-di-GMP levels compared to vector control at the 6 hr time point (Fig. 4B). In fact, c-di-GMP levels in attached cells overexpressing cyclase PA4843 were similar to those detected in cells attached for 24 hr (Fig. 4B). However, no significant difference in the number of attached cells was noted upon multicopy expression of the cyclase PA4843 indicating that the difference in *brlR* expression is likely due to elevated c-di-GMP levels rather than increased biofilm biomass accumulation (Fig. 4E). Moreover, at the 6 hr time point, overexpression of PA4843 correlated with a significant, 7.4 ± 1.3 -fold increase in the previously described BrlR-target *mexE* encoding a component of the MexEF-OprN multidrug efflux pump (Liao et al., 2013), compared to attached cells not overexpressing PA4843. This observation suggested that overexpression of the cyclase PA4843 and subsequent formation of the secondary messenger c-di-GMP enhances *brlR* promoter reporter activity, and thus, *brlR* expression as well as expression of BrlR target genes in *P. aeruginosa*. Moreover, our findings suggested that unlike the known MerR regulators BmrR and TipA, *brlR* expression is linked to attachment and/or biofilm developmental conditions rather than induced upon exposure to antimicrobial agents.

BrlR is a c-di-GMP responsive transcriptional regulator

To determine whether enhanced *brlR* expression in the presence of c-di-GMP correlated with enhanced BrlR activity and thus, BrlR-DNA binding, electrophoretic mobility shift assays were performed in the absence or presence of c-di-GMP. While BrlR-DNA binding was detected in the absence of c-di-GMP, addition of c-di-GMP resulted in increased BrlR-DNA binding (Fig. 5A). While c-di-GMP enhanced DNA binding by BrlR, no additional shift was noted (Fig. 5A). To further explore the role of c-di-GMP in BrlR-DNA binding, DNA binding assays using streptavidin magnetic beads were performed in the absence and presence of increasing c-di-GMP concentrations, using the biotinylated *PbrlR* DNA. BrlR-DNA binding was detected using anti-V5 antibodies. While BrlR-DNA binding was detected in the absence of c-di-GMP, addition of c-di-GMP resulted in increased BrlR-DNA binding in a concentration-dependent manner (Fig. 5B). When increasing amounts of

unlabeled *PbrlR* fragment (Fig. 5C) were added to the streptavidin DNA binding assay, decreased BrlR binding to the labeled fragment was observed. Under the conditions tested, no BrlR binding to the beads alone was detected. Likewise, no DNA binding was observed using cell extracts obtained from *P. aeruginosa brlR* (Fig. 5B-C). Enhanced DNA binding was not limited to *PbrlR* as BrlR-binding to its target promoter *PmexE* was likewise enhanced by the presence of c-di-GMP (Fig. 5D).

BrIR binds to c-di-GMP *in vitro*

While lacking predicted secondary structure resembling those of known c-di-GMP binding regions, our findings not only indicated that BrlR-DNA binding was enhanced by c-di-GMP but also that BrlR may be a c-di-GMP responsive transcriptional regulator. To determine whether BrlR binds c-di-GMP *in vitro*, we made use of a pulldown assay using biotinylated c-di-GMP, with BrlR binding to c-di-GMP being detected by immunoblot analysis using anti-V5 antibodies. V5-tagged BdlA which has been reported to bind heme but not c-di-GMP (Petrova & Sauer, 2012b), as well as the c-di-GMP-responsive transcriptional regulator FleQ that binds c-di-GMP half-maximal at a concentration of 15–25 μ M (Hickman & Harwood, 2008) were used as controls. While no c-di-GMP binding was noted for BdlA, FleQ bound c-di-GMP as expected in a concentration-dependent manner (Fig. 6A). Likewise, BrlR bound c-di-GMP in a concentration-dependent manner with c-di-GMP binding detectable at as little as 1.25 μ g BrlR (Fig. 6A). c-di-GMP binding to BrlR was characteristic with first order binding kinetics (Fig. 6B). Under the conditions tested, half-maximal binding of c-di-GMP occurred at 1.5 μ g BrlR which is equivalent to a concentration of 2.2 μ M (Fig. 6B). No c-di-GMP binding was observed to a no-protein control or to cell extracts obtained from *P. aeruginosa brlR* (Fig. 6A). BrlR binding of biotinylated c-di-GMP was outcompeted by non-biotinylated c-di-GMP (Fig. 6C) but not by GTP or cyclic AMP (cAMP, Fig. 6C) indicating a specificity of BrlR to c-di-GMP.

In addition, detection of direct binding of c-di-GMP to BrlR was done using pulldowns, in conjunction with HPLC-based detection of c-di-GMP. To do so, *P. aeruginosa*-derived BrlR was first immunoprecipitated using anti-V5 antibodies. BrlR-bound c-di-GMP was subsequently extracted from the pulled down protein, and the resulting extracts were subjected to HPLC-based detection of c-di-GMP. Under the conditions tested, extracts obtained from pulldowns using His₆V5-tagged BrlR protein in the presence of anti-V5 antibodies were found to contain c-di-GMP (Fig. 6D) based on the identical elution time with commercially available c-di-GMP (Fig. S5). Overall, up to 14 pmol c-di-GMP were detected per 1 μ g of BrlR suggesting the presence of 1 pmol c-di-GMP per 2 pmol BrlR. In contrast, no c-di-GMP was detected in pulldown controls lacking anti-V5 antibodies (Fig. 6D). Detection of c-di-GMP by HPLC correlated with the presence of His₆V5-tagged BrlR in pulldown eluates as determined by immunoblot analysis (Fig. 6E).

The presence of 1:2 ratio of c-di-GMP:BrlR suggested BrlR dimerization. To confirm dimer formation of BrlR in the presence of c-di-GMP, we made use of *in vivo* crosslinking of planktonic PAO1/pMJT-*brlR*-His6V5 either overexpressing the cyclase PA4843 or the phosphodiesterase PA2123. PAO1/pMJT-*brlR*-His6V5 harboring the empty plasmid pJN105 was used as control. Following cell lysis, the resulting cell extracts were separated

by SDS/PAGE, blotted onto PVDF, and probed for BrIR using anti-V5 antibodies. While BrIR in its monomeric form (apparent mass of 33 kDa) was detectable under all conditions tested, a faint band the apparent mass of a BrIR-dimer was detected in of PAO1/pMJT-*brlR*-His6V5 harboring the empty plasmid pJN105 (Fig. 6F). The band increased in intensity upon overexpression of the cyclase PA4843 but disappeared upon overexpression of the phosphodiesterase PA2123 (Fig. 6F). The finding suggested c-di-GMP to enhance dimerization of BrIR.

DISCUSSION

The MerR family is a group of transcriptional activators with similar N-terminal helix-turn-helix DNA binding regions but diverse C-terminal effector binding regions that are specific to the effector recognized. The signature of the family is amino acid similarity in the first 100 amino acids, including a helix-turn-helix motif followed by a coiled-coil region. The MerR family of transcriptional regulators have further in common that they act on their own promoter to not only regulate their own expression but also induce the expression of their respective regulons. In the present study, we likewise demonstrated that BrIR is capable of binding to its own promoter, likely located within a spacer region between the putative -10 and -35 regions located 5-35 bp upstream of the *brlR* transcriptional start site. The respective sequence was found to be 19 bp long and characterized by the presence of a perfect inverted repeat, with similar palindromic sequences being present in the previously identified BrIR-dependent *mexAB-oprM* and *mexEF-oprN* promoters. The presence of a 19 bp-long spacer region is not uncommon. All MerR family promoter so far identified have an elongated spacer region between the -10 and -35 sequences including all metal-dependent promoters such as the *merR*, *cueR*, and *coaR* promoters (Brown et al., 2003, Ahmed et al., 1994). Moreover, the palindromic spacer region has been shown in many cases to be the regulator binding site (Brown et al., 2003). Likewise, BmrR binds to a dyad symmetrical sequence in the 19 bp spacer region of the *bmr* promoter and affinity of BmrR for this binding site is increased by addition of the co-activators, rhodamine or tetraphenylphosphonium (TPP) (Newberry & Brennan, 2004, Grkovic *et al.*, 2002). Enhanced DNA binding via a co-activator or inducer is not limited to BmrR as the majority of regulators in the MerR family respond to environmental stimuli, such as oxidative stress, heavy metals or antibiotics (Brown et al., 2003, Ahmed et al., 1994, Heldwein & Brennan, 2001). Similar to other known MerR proteins, DNA binding of BrIR is enhanced by an inducer. However, instead of an environmental stimulus prevalent under biofilm conditions or a multidrug transporter substrate(s) acting as an inducer, here we demonstrate BrIR to be a c-di-GMP responsive regulator, with elevated levels of c-di-GMP not only enhancing BrIR-DNA binding *in vitro* but also resulting in increased *brlR* expression for initially attached cells *in vivo*. Considering that high concentrations of c-di-GMP have been correlated with the sessile lifestyle (e.g. biofilm formation) while its absence or low levels favor motility (e.g. twitching, swarming) and the free living lifestyle (D'Argenio & Miller, 2004), the role of c-di-GMP in BrIR-DNA binding and *brlR* expression are in agreement with the observed timing of induction of *brlR* expression upon the transition to surface-associated growth (Liao & Sauer, 2012). Such growth mode dependent expression is unique among members of the MerR family. What makes BrIR stand out even further among members of the MerR

family is its c-di-GMP binding capability, with c-di-GMP acting as an inducer. Elevated levels of c-di-GMP were found to correlate with enhanced BrlR dimer formation. While it is unclear whether dimer formation by BrlR is a direct consequence of c-di-GMP binding, other MerR-like regulators have been demonstrated to be dimeric DNA-binding proteins including MerR from *B. subtilis* and SoxR from *E. coli* (Helmann *et al.*, 1990, Shewchuk *et al.*, 1989, Watanabe *et al.*, 2008). Moreover, high c-di-GMP levels correlated with increased BrlR binding to *PmexE* and increased expression of *mexE*, indicating c-di-GMP also contributes to the expression of BrlR-target genes.

The finding of *brlR* expression following attachment and BrlR activation being c-di-GMP dependent is in agreement with recent findings of *P. aeruginosa* biofilms gaining their extraordinary tolerance soon after surface attachment, a stage requiring the action of the two-component hybrid SagSs and with attached cells being characterized by elevated c-di-GMP levels (Petrova & Sauer, 2009, Petrova & Sauer, 2011, Gupta *et al.*, 2013). BrlR has been demonstrated to contribute to *Pseudomonas aeruginosa* biofilm drug tolerance to antimicrobial agents known as multidrug efflux pumps substrates by activating multidrug-efflux pumps (Liao *et al.*, 2013) but to suppress colistin resistance by repressing of *phoPQ* expression (Chambers & Sauer, 2013). Our results are in agreement with findings by Chua *et al.* (Chua *et al.*, 2013) demonstrating that reduced c-di-GMP levels present in dispersed cells correlated with increased production of proteins involved in antimicrobial peptide resistance and increased resistance towards colistin compared to biofilm cells. Together, these observations may explain why colistin, unlike many other antibiotics, kills *P. aeruginosa* cells in the center rather than the periphery of the biofilm (Pamp *et al.*, 2008)

To our knowledge, no other transcriptional regulator of the MerR family has been described to bind c-di-GMP. Our findings suggest BrlR to be a member of a new and growing class of c-di-GMP binding proteins that are transcriptional regulators and only the second c-di-GMP-responsive transcriptional regulator to be described in *P. aeruginosa*. C-di-GMP responsive transcriptional regulators include the *P. aeruginosa* flagella biosynthesis gene activator FleQ (Hickman & Harwood, 2008), the *Mycobacterium smegmatis* LtmA that broadly regulates the expression of lipid transport and metabolism genes (Li & He, 2012), the *Xanthomonas campestris* global regulator Clp (Tao *et al.*, 2010), the *Burkholderia cenocepacia* biofilm formation and virulence regulator Bcam1349 (Fazli *et al.*, 2011), and VpsT responsible for regulating matrix production and motility in *Vibrio cholerae* (Krasteva *et al.*, 2010). LtmA was found to have a strong binding affinity to c-di-GMP as characterized by a K_d for the specific interaction between LtmA and c-di-GMP of 0.83 μ M (Li & He, 2012), FleQ appears to more weakly bind to c-di-GMP as indicated by a K_d of 15-20 μ M (Hickman & Harwood, 2008). In comparison, BrlR was characterized by a K_d of 2.2 μ M indicating BrlR to bind c-di-GMP stronger than FleQ but weaker than LtmA. The findings suggest BrlR activation and thus, activation of BrlR target genes in *P. aeruginosa* to occur at lower c-di-GMP levels or at earlier stages of biofilm development than FleQ. While capable of c-di-GMP binding, BrlR, FleQ, and LtmA lack regions that have a predicted secondary structure resembling those of known c-di-GMP binding regions, such as the *P. aeruginosa* PelD protein, PilZ domains or the I-sites of diguanylate cyclases (Amikam & Galperin, 2006, Chan *et al.*, 2004, Wassmann *et al.*, 2007, De *et al.*, 2008). Likewise, BrlR does not contain any regions with

similarity to known c-di-GMP binding regions. However, the *in vitro* system that we have established for examining BrIR binding to DNA and/or c-di-GMP provides an opportunity to examine in detail the mechanism by which c-di-GMP controls the biochemical activity of a transcriptional regulator as well as reveal alternative determinants of c-di-GMP binding.

Taken together, our results indicate BrIR to be a c-di-GMP responsive transcriptional regulator, with BrIR-DNA binding and *brlR* expression being enhanced by the secondary messenger c-di-GMP. Considering biofilms having been correlated with elevated levels of c-di-GMP, and that BrIR contributes to the drug tolerance of *P. aeruginosa* biofilms, with elevated c-di-GMP resulting in increased *brlR* expression and activation, our findings strongly suggest a contribution of c-di-GMP to biofilm tolerance.

Material and Methods

Bacterial strains, plasmids, media, and culture conditions

All bacterial strains and plasmids used in this study are listed in Table 1. *P. aeruginosa* strain PAO1 was used as parental strain. All planktonic strains were grown in Lennox Broth (LB, BD Biosciences) in flasks at 220 rpm in the absence or presence of 0.1-1.0% arabinose. *Escherichia coli* cultures were grown in LB in the absence or presence of 1mM Isopropyl β -D-1-thiogalactopyranoside (IPTG). Antibiotics were used at the following concentrations: 50-75 μ g/mL gentamicin, 200-250 μ g/mL carbenicillin, and 60-75 μ g/mL tetracycline for *P. aeruginosa*; and 20 μ g/mL gentamicin, 50 μ g/mL ampicillin and 20 μ g/mL tetracycline for *E. coli*.

Strain Construction

Reporter constructs harboring putative promoter sequences of *brlR* were generated by introducing the respective sequences into pMini-CTX-*lacZ* or pMini-CTX-*gfp*. C-terminal V5-tagging of FleQ was accomplished introducing the V5-tag via PCR (using the sequence ggtaagcctatccctaaccctctcctcggtctcgattctacg). The tagged construct was introduced into pMJT-1. The identity of vector inserts was confirmed by sequencing and proper integration of the pMini-CTX vectors confirmed by PCR using Pser-up/-down PCR primers. Plasmids were introduced into *P. aeruginosa* via conjugation or electroporation. Primers used are listed in Table S1.

Biofilm formation

For reporter studies, biofilms were grown in a continuous flow tube reactor system (1 m long size 13 silicone tubing, Masterflex, Cole Parmer, Inc.) with an inner surface area of 25 cm² at a flow rate of 0.1 ml/min and in flow cell reactors (BioSurface Technologies) which also allowed for the analysis of biofilm architecture as previously described (Sauer et al., 2002, Sauer *et al.*, 2004, Petrova & Sauer, 2009). Biofilms were grown at 22°C in 20-fold diluted LB medium for up to 4 days. The same growth conditions were used to cultivate biofilms to obtain RNA.

Transcriptional analysis

Microscopic analyses of *brlR* expression during surface-attached growth were accomplished by allowing the respective reporter strain (PAO1::P*brlR*-*gfp*) to attach to microscope slides under flowing conditions in flow cells, as previously described (Sauer et al., 2002). Expression of *gfp* was monitored by confocal scanning laser microscopy using a Leica TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany). To quantitate *brlR* expression, P*brlR*-*lacZ* fusions were used. β -galactosidase activity of strains harboring the P*brlR*-*lacZ* promoter reporters was determined using the Miller assay (Miller, 1972) with the following modification: instead of using total cells, specific β -galactosidase activity was determined using protein extracts (Petrova & Sauer, 2010, Petrova *et al.*, 2011). Cells were lysed by sonication as previously described (Petrova & Sauer, 2009), and the resulting lysate centrifuged for 2 min at $21,200 \times g$ to pellet unbroken cells and cell debris. Protein determination was done using the modified method of Lowry (Peterson, 1977). A total of 5-10 μ g of cell extract was used per assay. An extinction coefficient for *o*-nitrophenyl- β -galactoside cleavage at 420 nm of 4500 nl/nmol*cm was used.

Transcriptional start site mapping

The transcriptional start site of *brlR* was determined using Invitrogen's 5' RACE system for rapid amplification of cDNA ends (version 2.0) as recommended by the manufacturer (Invitrogen). RNA was isolated from 4-day-old *P. aeruginosa* wild type biofilms as described above and used as the template. Oligonucleotides used for reverse transcription and PCR are listed in Table S1. Products were separated by agarose gel electrophoresis to assess purity and product size. If necessary, products were excised and gel eluted (Qiagen). The 5' RACE products were sequenced by the Cornell University Life Sciences Core Laboratories Center (CLC). The sequencing results were interpreted by pairwise alignments of the 5' RACE product sequence with the *P. aeruginosa* PAO1 genomic sequence.

Chromatin immunoprecipitation (ChIP) analysis

In order to determine whether BrlR binds to its own promoter region *in vivo*, 24-hr-old biofilms of *P. aeruginosa* PAO1/pMJT-*brlR*-V5/His₆, bearing the His₆V5-tagged BrlR, were subjected to chromatin immunoprecipitation (ChIP) analysis as previously described (Petrova *et al.*, 2011). *P. aeruginosa* PAO1 expressing untagged *brlR* was used as control. Briefly, *in vivo* DNA-protein crosslinking using 1% formaldehyde for 10 min at 37°C and immunoprecipitation using anti-V5 antibodies (Invitrogen Corp.) were done essentially as previously described (Chiu & Thomas, 2004, Solomon & Varshavsky, 1985, Leech *et al.*, 2008, Liao *et al.*, 2013). Following immunoprecipitation, DNA was liberated by reversing the crosslinking via incubation with 0.5 M NaCl in TE at 65°C for 4 hr. Purified DNA from PAO1/pJN-*brlR* and PAO1/pMJT-*brlR*-V5/His₆ samples was subjected to qPCR using primers listed in Table S1. Relative transcript quantitation was accomplished using the *ep realplex* software (Eppendorf AG) by first normalizing transcript abundance (based on Ct value) to *mreB* followed by determining transcript abundance ratios for P*brlR* and P*pscE-F* using primers listed in Table S1. Melting curve analyses were employed to verify specific single product amplification.

Purification of His-tagged BrlR

For electrophoretic mobility shift assay (EMSA), His₆V5-tagged BrlR protein was purified from *E. coli* or *P. aeruginosa*. For *in vitro* c-di-GMP binding assays, His₆V5-tagged BrlR was purified from *P. aeruginosa*. Briefly, supernatants following sonication of planktonic cells, and centrifugation at 21,200 × g were loaded onto nickel-nitrilotriacetic acid (Ni-NTA) affinity resin (Qiagen), washed with buffer, and eluted with an imidazole gradient according to the manufacturer's instructions for native protein purification. Protein preparations were examined for purity by SDS-PAGE, and fractions containing His₆V5-tagged BrlR were pooled and desalted using VivaSpin centrifugal concentrator columns (10 kDa cut-off, Sartorius).

Electrophoretic mobility shift assay (EMSA)

BrlR binding to the region upstream of *brlR* start codon was performed as described previously (Fuchs *et al.*, 2010, Jones *et al.*, 2010, Liao *et al.*, 2013) using purified His₆V5-tagged BrlR. BrlR purification was accomplished by Ni-NTA affinity chromatography (QIAGEN) following the manufacturer's instructions (see Fig. 3D). The DNA promoter probe was generated by PCR using the indicated oligonucleotides (*PbrlR*, Table S1) and end-labeled using 10 μCi of [γ-³²P]-ATP (GE Healthcare) and 10 U of T4 polynucleotide kinase (New England Biolabs). EMSAs were performed as previously described (Fuchs *et al.*, 2010, Jones *et al.*, 2010). Briefly, probes (0.25 nM each) were incubated in binding buffer (10 mM Tris [pH 7.5], 50 mM KCl, 1 mM EDTA, 1 mM DTT, 5% glycerol and 100 μg/ml bovine serum albumin) containing 50 ng/ml poly (2'-deoxyinosinic-2'-deoxycytidylic acid) [poly(dI-dC)] (Sigma) for 5 min at 25°C. BrlR protein was then added at indicated concentrations for a final reaction volume of 20 μl and incubated for an additional 15 min at 25°C. Samples were subjected to electrophoresis on a 6% polyacrylamide glycine gel (10 mM Tris [pH 7.5], 380 mM glycine, 1 mM EDTA) at 4°C. Imaging and data analyses were performed using a Molecular Imager FX phosphorimager (BioRad) and Imagequant software. Moreover, we made use of biotinylated rather than radiolabeled DNA fragments. To do so, we made use of the LightShift Chemiluminescent EMSA kit (Thermo Scientific) as previously described (Petrova & Sauer, 2010) for determining BrlR-DNA binding to *brlR* promoter DNA (*PbrlR*), DNA sequences spanning the 7-108 and the 5-37 bp region located upstream of the transcriptional start site. The respective regions were amplified using primers listed in Table S1. His₆V5-tagged BrlR obtained from planktonic cells (5-20 pmol) was used. Samples were separated as described above, blotted onto a Hybond nylon membrane and probed using anti-biotin antibodies. Bands were visualized using the LightShift Chemiluminescent EMSA kit (Thermo Scientific).

Streptavidin magnetic bead DNA binding assay

BrlR binding to the putative *brlR* promoter DNA was confirmed using the streptavidin magnetic bead DNA binding assay as previously described (Petrova *et al.*, 2011). Briefly, biotinylated target DNA fragment *PbrlR* was amplified using the primer pair *PbrlR*-7-108F/R (*PbrlR*, spanning the 7-108 bp upstream of start codon, Table S1). A total of 0.5 pmol of target DNA was incubated for 30 min at room temperature with cell extract containing 25 pmol of His₆V5-tagged BrlR (as indicated by immunoblot analysis using

purified His₆V5-tagged BrlR) in 25 mM Tris-Cl, pH 8, 5 mM MgCl₂, 0.5 mM dithiothreitol, 1 mM EDTA, and 50 ng/uL poly(dI-dC) as nonspecific competitor DNA. For specific competition, non-biotinylated *PbrlR* target DNA (0-50 pmol) was used. c-di-GMP was added where indicated. Briefly, streptavidin magnetic beads (Thermo Scientific, 100 µg) were used to capture biotinylated DNA. Following three washes, the proteins co-purified with the biotinylated DNA were separated by 10% SDS/PAGE and assessed by immunoblot analysis for the presence of BrlR using anti-V5 antibodies (Invitrogen). An aliquot prior to addition of streptavidin magnetic beads was used to determine total BrlR present in each DNA binding assay (loading control). Bands were visualized by chemiluminescence.

Bioinformatics

Putative BrlR-DNA binding sequences were identified by aligning the upstream DNA region of *brlR* to the BmrR-DNA binding sequence. The putative BrlR-DNA binding sequences were analyzed for inverted repeats using the palindrome program (<http://emboss.bioinformatics.nl/cgi-bin/emboss/palindrome>) and default settings.

Detection of and quantitation of BrlR-c-di-GMP binding

To assess the interaction between c-di-GMP and BrlR, the binding was analyzed by pulldown assays using streptavidin magnetic beads (Thermo Scientific, 100 µg) to capture biotinylated c-di-GMP. Following three washes, the beads harboring biotinylated c-di-GMP were incubated with increasing concentration of cell extract (0-10 µg) containing His₆V5-tagged BrlR. Cell extracts inactivated in *brlR* (*brlR*) were used as a negative control. Following three washes, proteins co-purified with the biotinylated c-di-GMP were separated by 10% SDS/PAGE and assessed by immunoblot analysis for the presence of BrlR using anti-V5 antibodies (Invitrogen). Bands were visualized by chemiluminescence. Pulldowns were repeated in the presence of non-biotinylated c-di-GMP (0-1000 pmol), GTP (0-1000 pmol), and cAMP (0-1000 pmol).

To directly detect c-di-GMP binding to BrlR and subsequently quantitate bound c-di-GMP, pulldowns were used as previously described (Petrova & Sauer, 2011, Petrova et al., 2011) with some modifications. Briefly, pulldowns were carried out in the presence of 200 pmol c-di-GMP using whole-cell lysates obtained from PAO1/pMJT-*brlR*-His₆V5 grown planktonically to exponential phase and protein A/G agarose beads (Cell Signaling) linked with anti-V5 antibodies (Invitrogen). Controls were done in the absence of anti-V5 antibodies. Following immunoprecipitation using immobilized anti-V5 antibodies, the resulting precipitate was subjected to heat and ethanol precipitation (Morgan *et al.*, 2006) to extract c-di-GMP. C-di-GMP was subsequently extracted in duplicate from pulldowns in the presence and absence of anti-V5 antibodies and quantitated essentially as previously described (Petrova & Sauer, 2011, Basu Roy et al., 2012, Basu Roy *et al.*, 2013). Briefly, c-di-GMP was extracted in duplicate using heat and ethanol precipitation followed by centrifugation. Supernatants were combined, dried using a Speed-Vac and resuspended in 10 mM ammonium acetate. Samples (20 µl) were analyzed using an Agilent 1100 HPLC equipped with an autosampler, degasser, and detector set to 253 nm, and separated using a reverse-phase C₁₈ Targa column (2.1 × 40 mm; 5 µm) at a flow rate of 0.2 ml/min with the following gradient: 0 to 9 min, 1% B; 9 to 14 min, 15% B; 14 to 19 min, 25% B; 19 to 26

min, 90% B; 26 to 40 min, 1% B (buffer A, 10 mM ammonium acetate; buffer B, methanol plus 10 mM ammonium acetate). Commercially available cyclic di-GMP (Biolog) was used as a reference for the identification and quantification of cyclic di-GMP in cell extracts. An aliquot of the pulldown reaction mixture prior to and following immunoprecipitation was retained and subsequently analyzed by immunoblot analysis as described above using anti-V5 antibodies to assess the presence of V5/His-tagged BrlR but the absence of V5/His-tagged BrlR in controls.

Detection of BrlR dimers by protein crosslinking

Dimerization of BrlR was determined via reversible *in vivo* crosslinking with dithiobis(succinimidyl propionate) (DSP) as previously described (Petrova & Sauer, 2012a). Whole cells of PAO1/pMJT-*brlR*-His₆V5 harboring the empty plasmid pJN105, or PAO1/pMJT-*brlR*-His₆V5 either overexpressing the cyclase PA4843 or the phosphodiesterase PA2123, were used. Briefly, following incubation for 9 min with 2mM DSP (stock solution prepared at 15mM in DMSO), thus treated cells containing V5-tagged BrlR were centrifuged for 1 min at 16000×g, for a total crosslinking time of 10 min. The crosslinking reactions were stopped by resuspending and lysing the cells via sonication directly into TE buffer (10 mM Tris/HCl pH 8.0, 1 mM EDTA, plus 0.3 µg/ml PMSF). Crosslinking was reversed using β-mercaptoethanol (β-ME), while crosslinked samples were treated using a non-reducing SDS-PAGE loading buffer. The resulting cell extracts were separated by SDS/PAGE, and assessed by immunoblot analysis for the presence of V5/His-tagged BrlR using anti-V5-HRP antibodies (0.1 µg/ml, Invitrogen Corp.).

Statistical analysis

A Student's *t*-test was performed for pair-wise comparisons of groups, and multivariate analyses were performed using a 1-Way ANOVA followed by a posteriori test using Sigma Stat software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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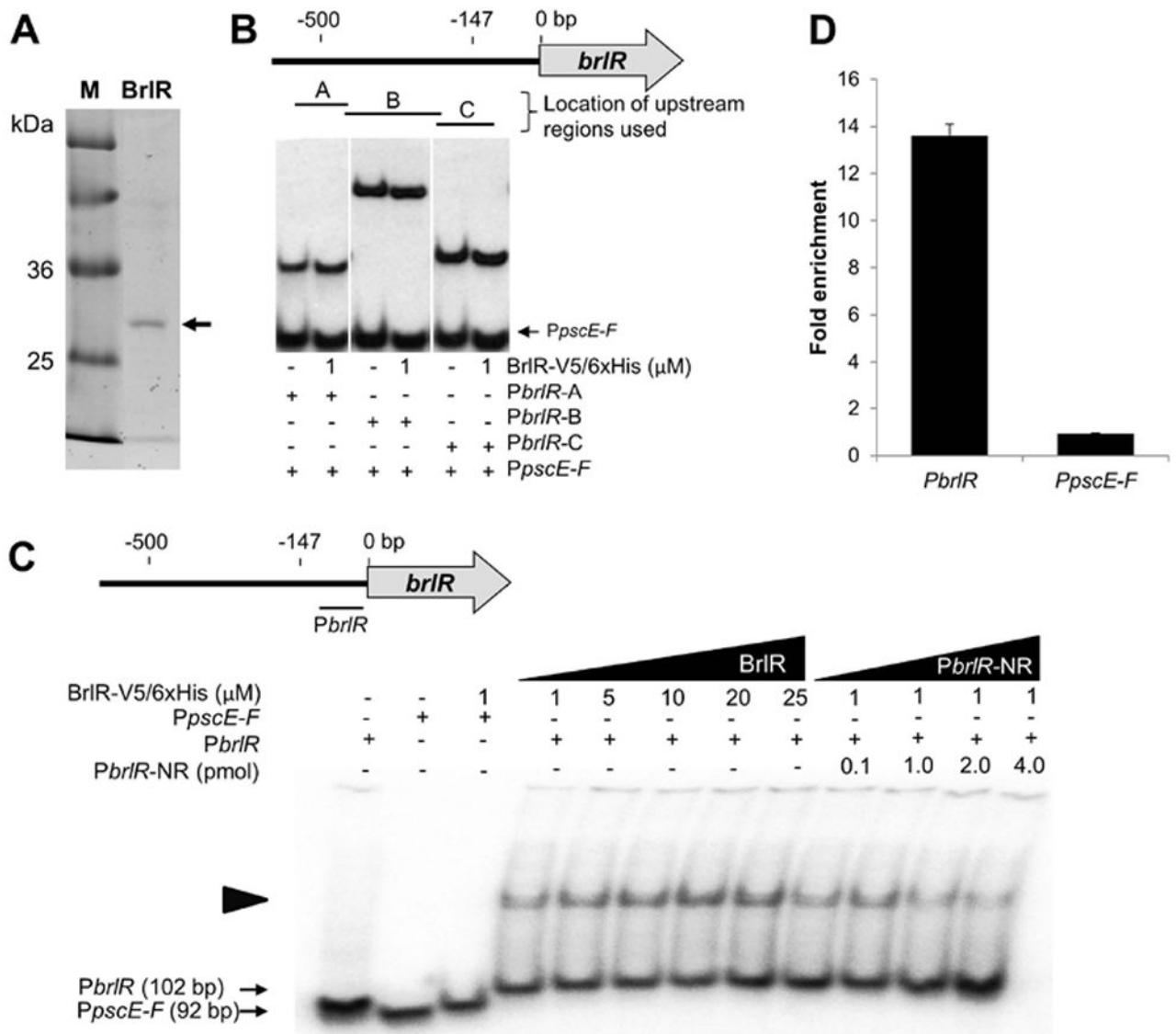


Figure 2. BrIR binds to its own promoter

(A) His₆V5-Tagged BrIR has an apparent molecular weight of approximately 33 kDa (see arrow). BrIR was purified using Ni-NTA affinity chromatography. M, protein ladder. (B) DNA gel mobility shift assays using purified BrIR and DNA fragments A-C located 443-543, 223-459, and 91-240 bp upstream of the ATG start codon, respectively. The schematic diagram indicates the location and approximate length of the three DNA fragments A-C used in DNA binding assays. A total of 0.1 pmol was used for DNA regions A-C. (C) DNA gel mobility shift assays indicating BrIR binding to a DNA fragment located 7-108 bp upstream of the ATG start codon, demonstrating specificity of BrIR binding. Both the concentration of purified BrIR (1-25 μM BrIR) and the concentration of non-labeled competitor DNA (*PbrIR*-NR, 0.1-4 pmol) were varied. The PCR amplified DNA fragment *PpscE-F* (0.1 pmol) was used as a non-specific radiolabeled control. The schematic diagram indicates the location and length of the *PbrIR* fragment relative to *brlR*. Experiments were carried out in triplicate and representative images are shown. The schematic diagram indicates the location and length of the *PbrIR* DNA fragment (7-108 bp) used in DNA binding assays. Numbers above indicate approximate distances to the *brlR* start codon. (D) Fold enrichment of the promoter sequences of *brlR* and *psscE-F* in

BrIR-V5/His₆ ChIP sample compared to control (ChIP carried out in the presence of untagged BrIR), as determined by qPCR. Experiments were carried out at in triplicate. Error bars indicate standard deviation.

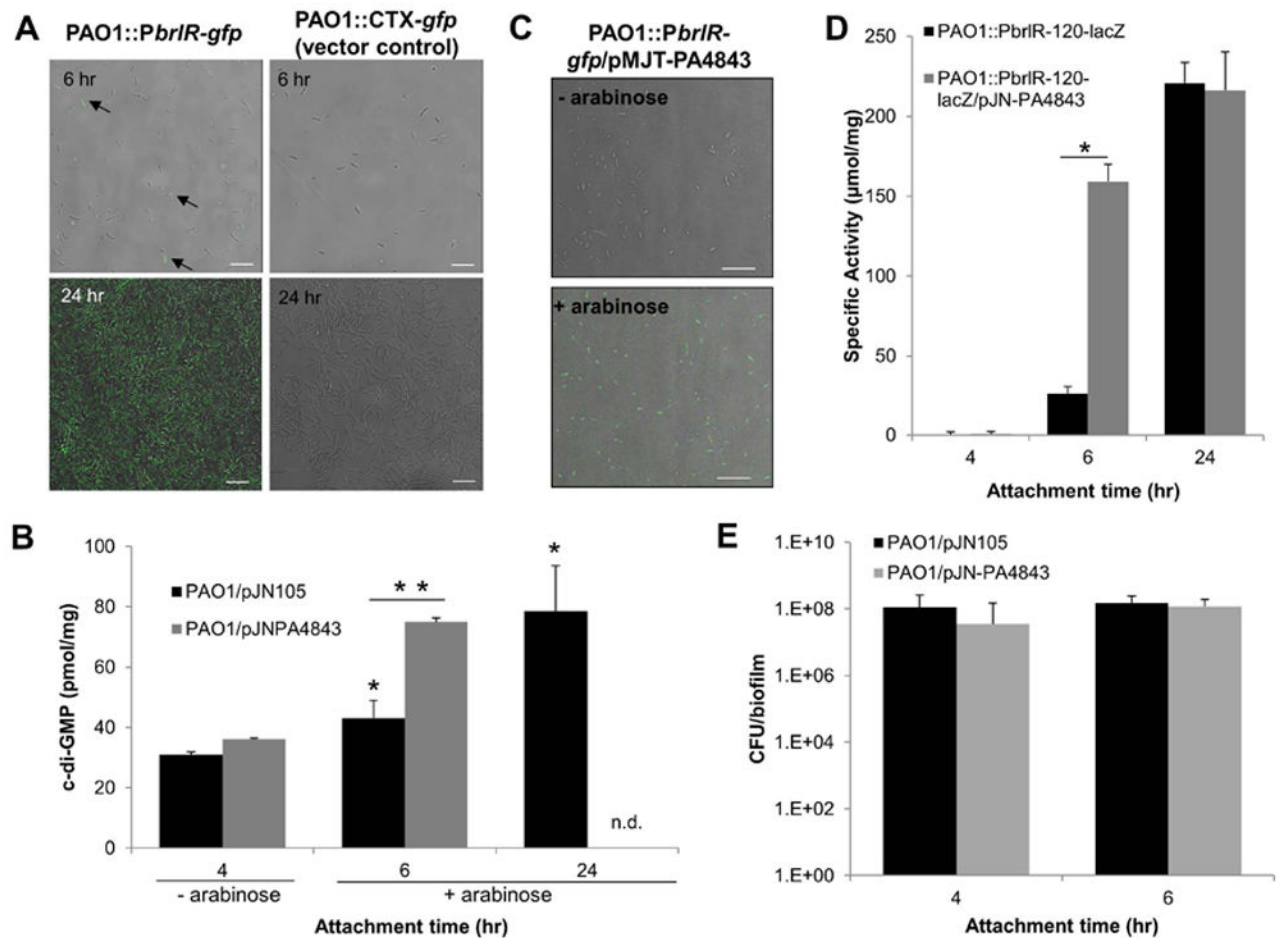


Figure 4. *brlR* expression and BrIR-DNA binding are enhanced by the secondary messenger c-di-GMP

(A) Induction of *brlR* expression over the course of biofilm formation was monitored by confocal microscopy using a *P. aeruginosa* PAO1 carrying chromosomal *gfp* fusions to the upstream region of *brlR*. Total cells were visualized by brightfield microscopy. Overlays acquired 6 hr and 24 hr post initial attachment are shown. *brlR* expression was detected as early as 6 hr following initial attachment. Arrows indicate *gfp*-expressing cells. *P. aeruginosa* harboring an empty pMini-CTX-*gfp* vector was used as control. White bar = 10 µm. (B) c-di-GMP levels present in *P. aeruginosa* harboring an empty pJN105 vector or overexpressing PA4843 (PAO1/pJN-PA4843). The strains were grown attached in tube reactors under flowing conditions for 4, 6, and 24 hr. – arabinose, no arabinose was added to the growth medium. + arabinose, arabinose was added to the growth medium 4 hr post initial attachment to induce PA4843 expression. n.d., not determined. *, significantly different from PAO1/pJN105 at the 4-hr time point; $p < 0.05$. **, significantly different from PAO1/pJN105 at the 6-hr time point; $p < 0.01$. (C) Expression of the diguanylate cyclase PA4843 results in enhanced *gfp* expression indicative of *brlR* expression, compared to untreated controls. – arabinose, no arabinose was added to the growth medium. + arabinose, arabinose was added to the growth medium 4 hr post initial attachment to induce PA4843 expression. (D) *brlR* transcriptional reporter activity during surface-attached growth is enhanced upon overexpression of the cyclase PA4843. Strains harboring the *brlR-lacZ* reporter construct in the absence or presence of the plasmid pJN-PA4843 were grown under continuous flowing conditions in flow cells for 4, 6 hr, and 24 hr. Expression of PA4843 was induced by the addition of arabinose to the growth medium 4 hr post initial attachment. β -galactosidase activity has been corrected by the activity observed for the vector control strain. *, significantly different from PAO1/pJN105 at the 6-hr time point; $p < 0.05$. (E) Number of viable cells of PAO1/pJN105 and PAO1/pJN-PA4843 attached to

glass following 4 and 6 hr post initial attachment under flowing conditions, expressed as CFU/biofilm. All experiments were carried out in triplicate. Error bars indicate standard deviations.

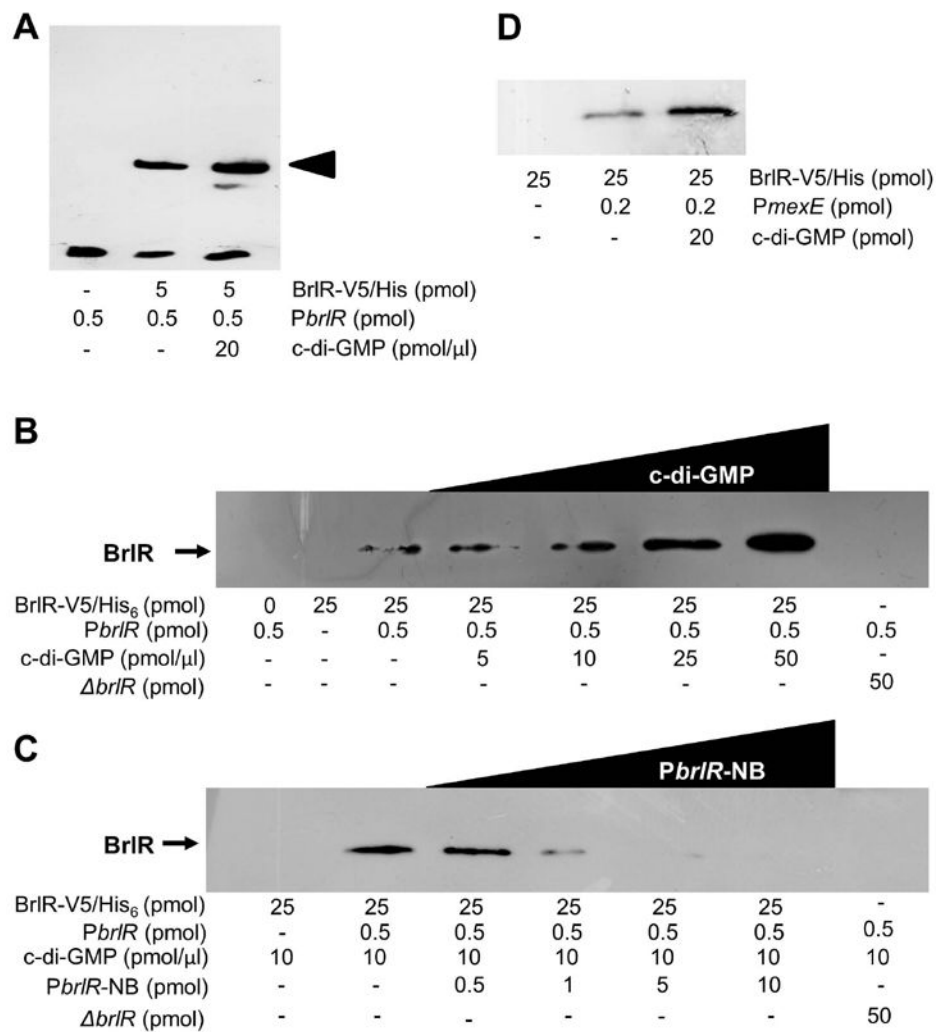


Figure 5. BrIR is a c-di-GMP responsive DNA binding protein

(A) BrIR-DNA gel mobility shift assays using (A) biotinylated *PbrIR* DNA (0.5 pmol) in the absence and presence c-di-GMP. BrIR-DNA binding was detected by immunoblot analysis using anti-biotin antibodies. Arrowhead denotes shift. (B) Streptavidin BrIR-DNA binding assays using biotinylated *PbrIR* DNA (0.5 pmol) in the absence and presence of increasing concentrations of c-di-GMP. A total of 25 pmol of purified His₆V5-tagged BrIR was used. BrIR-DNA binding was detected by immunoblot analysis using anti-V5 antibodies. (C) BrIR binding to *brlR* promoter DNA can be outcompeted by increasing concentrations of non-biotinylated *PbrIR* DNA (*PbrIR*-NB). (D) BrIR binding to biotinylated *PmexE* (0.2 pmol) is enhanced by the presence of c-di-GMP as indicated using streptavidin binding assays. Experiments were carried out in triplicate and representative images are shown.

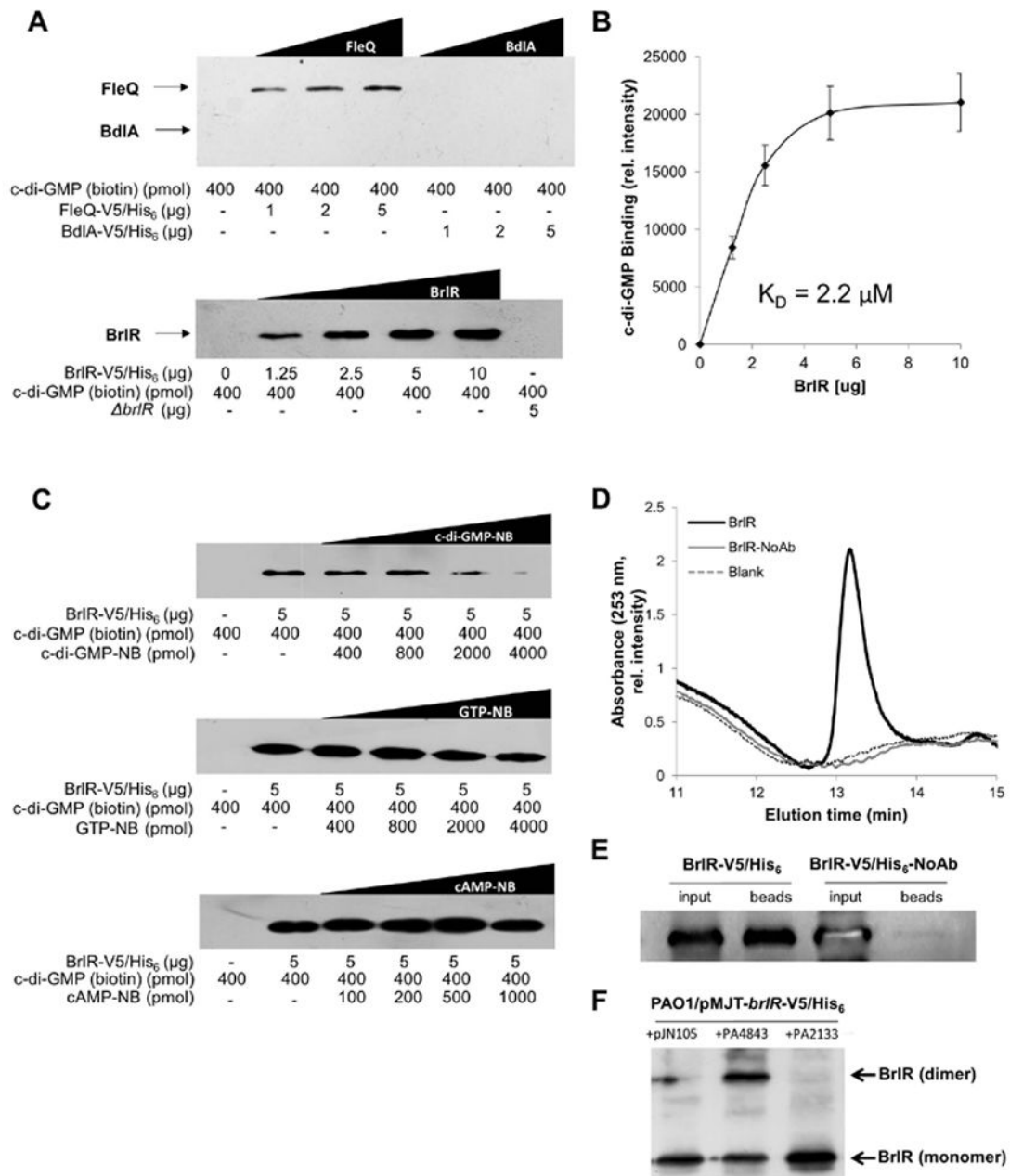


Figure 6. BrIR binds c-di-GMP *in vitro*

(A) Pull-downs using biotinylated c-di-GMP immobilized to streptavidin magnetic beads demonstrating that BrIR binds c-di-GMP. Increasing concentrations of V5-tagged BrIR were used (1.25–10 μg). A no-protein sample (0 μg) and cell extracts obtained from *P. aeruginosa* inactivated in *brlR* (*brlR*) were used as controls. BrIR-c-di-GMP binding was detected by immunoblot analysis using anti-V5 antibodies. V5-tagged FleQ and BdlA, having apparent mass of 58.3 and 49.9 kDa, respectively, were used as controls. (B) Concentration-dependent binding of biotinylated c-di-GMP by BrIR. Data are based on relative intensity of bands detectable following probing with anti-biotin antibodies and subsequent analysis using ImageJ. K_D , dissociation constant for c-di-GMP binding to BrIR. (C) BrIR-c-di-GMP binding assays in the presence of increasing concentrations of non-biotinylated c-di-GMP (c-di-GMP-NB), GTP (GTP-NB) and cyclic AMP (cAMP-NB). BrIR-c-di-GMP binding was detected by immunoblot analysis using anti-V5 antibodies. (D) Detection of BrIR-bound c-di-GMP by HPLC

analysis. Detection was based on absorbance at 253 nm. C-di-GMP was extracted from BrlR-pulldowns in the absence and presence of anti-V5 antibodies by heat and ethanol extraction. A total of 2 mg of cell extract obtained from *P. aeruginosa* PAO1/pMJT-*brlR*-His₆V5 was used per pulldown. (E) Immunoblot analysis demonstrating absence/presence of BrlR in pulldown samples prior to (input) and following immunoprecipitation (beads). The respective immunoprecipitation eluates (from beads) were used for c-di-GMP extraction and subsequent HPLC analysis. Immunoblots were probed using anti-V5 antibodies. (F) BrlR oligomerization as determined using immunoblot analysis and crosslinking. Increased dimerization of BrlR is detectable upon multicopy expression of the cyclase PA4843, while only BrlR monomers are detectable upon overexpression of the phosphodiesterase PA2133 as indicated using *in vivo* crosslinking with dithiobis(succinimidyl propionate) (DSP) and probing with anti-V5 antibodies. Experiments were carried out at least in duplicate and representative data are shown. Error bars represent the standard deviations between replicates

Table 1

Strains and plasmids used.

Strains/Plasmids	Relevant genotype or description	Source
Strains		
<i>Escherichia coli</i>		
DH5 α	<i>F</i> : ϕ 80 <i>lacZ</i> <i>M15</i> (<i>lacZYA-argF</i>)/ <i>U169</i> <i>recA1</i> <i>endA1</i> <i>hsdR17</i> (<i>rk</i> , <i>mk</i> ⁺) <i>phoA</i> <i>supE44</i> <i>thi-1</i> <i>gyrA96</i> <i>relA1</i> <i>tonA</i>	Invitrogen Corp.
BL21	<i>F</i> : <i>ompT</i> <i>hsdS_B</i> (18 mB) <i>gal</i> <i>dcn</i> <i>rne131</i> (DE3)	Invitrogen Corp
<i>Pseudomonas aeruginosa</i> PAO1		
PAO1	Wild type strain PAO1	B.H. Holloway
<i>brlR</i>	PAO1, <i>brlR</i> (PA4878)	(Liao & Sauer, 2012)
Plasmids		
pET101D	Vector for directional cloning and His ₆ V5 fusion protein expression, Amp ^R	Invitrogen Corp
pRK2013	Helper plasmid for triparental mating; <i>mob</i> ; <i>tra</i> ; Km ^R	(Figurski & Helinski, 1979)
pJN105	Arabinose-inducible gene expression vector; pBRR-1 MCS; <i>araC</i> -P _{BAD} ; Gm ^R	(Newman & Fuqua, 1999)
pMJT1	<i>araC</i> -P _{BAD} cassette of pJN105 cloned into pUCP18, Amp ^R (Carb ^R)	(Kaneko <i>et al.</i> , 2007)
pJN-PA4843	PA4843 cloned into pJN105; Gm ^R	(Petrova & Sauer, 2012a)
pJN- <i>brlR</i>	<i>brlR</i> cloned into pJN105; Gm ^R	(Liao & Sauer, 2012)
pJN- <i>bdIIA</i> -His ₆ V5	C-terminal V5/6xHis-tagged <i>bdIIA</i> cloned into pJN105 at <i>EcoRI</i> / <i>SpeI</i>	(Petrova & Sauer, 2012b)
pMJT- <i>brlR</i> -His ₆ V5	<i>brlR</i> -His ₆ V5 cloned into pMJT1	(Liao & Sauer, 2012)
pMJT- <i>fleQ</i> -V5	<i>fleQ</i> -V5 cloned into pMJT1	This study
pMini-CTX- <i>lacZ</i>	<i>attP</i> site-specific integration vector, Tet ^R	(Becher & Schweizer, 2000)
p <i>brlR</i> -23- <i>lacZ</i>	pMini-CTX- <i>lacZ</i> with 1-23 bp upstream of <i>brlR</i> , Tet ^R	This study
p <i>brlR</i> -120- <i>lacZ</i>	pMini-CTX- <i>lacZ</i> with 1-120 bp upstream of <i>brlR</i> , Tet ^R	(Liao & Sauer, 2012)
p <i>brlR</i> -250- <i>lacZ</i>	pMini-CTX- <i>lacZ</i> with 1-250 bp upstream of <i>brlR</i> , Tet ^R	This study
p <i>brlR</i> -500- <i>lacZ</i>	pMini-CTX- <i>lacZ</i> with 1-500 bp upstream of <i>brlR</i> , Tet ^R	This study
pMini-CTX- <i>gfp</i>	<i>attP</i> site-specific integration vector, Tet ^R	(Becher & Schweizer, 2000)
p <i>brlR</i> - <i>gfp</i>	pMini-CTX- <i>gfp</i> with 1-750 bp upstream of <i>brlR</i> , Tet ^R	This study