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The structure of the biofilm-controlling response regulator BfmR from *Acinetobacter baumannii* reveals details of its DNA-binding mechanism

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Abstract

The rise of drug-resistant bacterial infections coupled with decreasing antibiotic efficacy poses a significant challenge to global healthcare. *Acinetobacter baumannii* is an insidious, emerging bacterial pathogen responsible for severe nosocomial infections aided by its ability to form biofilms. The response regulator BfmR, from the BfmR/S two-component system, is the master regulator of biofilm initiation in *A. baumannii* and is a tractable therapeutic target. Here we present the structure of *A. baumannii* BfmR using a hybrid approach combining X-ray crystallography, nuclear magnetic resonance spectroscopy, chemical crosslinking mass spectrometry, and molecular modeling. We also show that BfmR binds the previously proposed *bfmRS* promoter sequence with moderate affinity. While BfmR shares many traits with other OmpR/PhoB family response regulators, some unusual properties were observed. Most importantly, we observe that when phosphorylated, BfmR binds this promoter sequence with a lower affinity than when not phosphorylated. All other OmpR/PhoB family members studied to

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Accession numbers. The structures of BfmRN and BfmRC have been deposited to the Protein Data Bank with PDB entry IDs 5HM6 (BfmRN), 6BR7 (BeF₃⁻-BfmRN), and 2NAZ (BfmRC).

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date show an increase in DNA binding affinity upon phosphorylation. Understanding the structural and biochemical mechanisms of BfmR will aid in the development of new antimicrobial therapies.

Graphical abstract

Keywords

Biofilm; BfmR; *Acinetobacter baumannii*; response regulator; two-component system

Introduction

The bacterium *Acinetobacter baumannii* is a Gram-negative opportunistic human pathogen notorious for causing severe nosocomial infections. Its prevalence in hospitals is often associated with patients in intensive care units and it is responsible for causing urinary tract infections, blood-stream bacteremia, and acute pneumonia [1]. *A. baumannii* shows extensive multidrug-resistance to an array of traditional antibiotics, making it a formidable adversary in clinical settings [2,3]. The ability to readily form robust biofilms is central to the antibiotic resistance capacity of *A. baumannii*, and is frequently accompanied by other cellular protection strategies such as virulence [4–8].

Biofilm initiation and the implementation of drug-resistance mechanisms are often regulated using protein two-component systems [9]. This makes these signal transduction modules very attractive targets for drug intervention [10]. A typical two-component system utilizes a membrane-associated histidine sensor kinase that transmits signals to downstream response regulators which dictate the cellular response to environmental stimuli [11,12]. In *A. baumannii*, the BfmRS two-component system controls both biofilm development and virulence [13,14].

The biofilm master regulator, BfmR, is important for control of the *A. baumannii* *csu* operon genes *csuC* and *csuE*. These genes are primarily involved in facilitating attachment of biofilms to abiotic surfaces both in nature and in the clinic [13,15]. Additionally, BfmR regulates the chaperone-usher assembly system (*csuA/BABCDE*), which controls pilin production and cell motility machinery [13]. Recent work also suggests that BfmR plays a critical role in *A. baumannii* growth/survival in human ascites fluid and serum by mediating capsule-independent resistance to the antimicrobials meropenem and colistin [16]. These pivotal regulatory roles make BfmR an appealing therapeutic target, and reinforce the significance of studies suggesting BfmR is a specific target for 2-aminoimidazole based anti-biofilm agents [10].

BfmR is classified as an OmpR/PhoB-like, two-domain response regulator. The N-terminal receiver domain contains a well-conserved site of phosphorylation/activation by a cognate histidine kinase, while the C-terminal effector domain contains a putative DNA-binding domain. Structural information for BfmR is incomplete. The highly conserved N-terminal receiver domain structure was recently determined using X-ray crystallography [16]; however, no structural information exists for the full-length protein or the more variable C-terminal DNA-binding effector domain. Two-domain response-regulators have proven to be particularly difficult to characterize structurally as full-length proteins due to their innate flexibility [17]. Consequently, only a few full-length response-regulator structures are available. More often, structures of the two separate domains are characterized independently. In those cases, alternative methods must be employed in order to correctly orient the two domains with respect to one another to build a single full-length model.

Here we provide a high-resolution structural model of BfmR from *A. baumannii* strain 19606 determined using an approach combining X-ray crystallography, solution nuclear magnetic resonance (NMR) spectroscopy, chemical crosslinking, and molecular modeling. In the past, this method was successfully employed to build structures of challenging full-length multi-domain proteins [18,19]. We also provide direct evidence of BfmR interacting with an inverted-repeat sequence located in the putative *bfmRS* promoter site [13] and characterize details of its DNA-binding mechanism *in vitro*. We show that BfmR is capable of binding DNA sequences within the *bfmRS* promoter in both active and inactive states. Inactive BfmR binds the DNA substrate with low to moderate affinity with 2:1 stoichiometry, consistent with the successful formation of a stable dimer on DNA. We also observed that the BfmR DNA binding affinity decreases upon activation. Combined, these results provide the first look at the structure of BfmR in its entirety, and characterization of BfmR:DNA binding to the only known BfmR binding sequence. Finally, this work provides insight into the similarities and differences of DNA binding and activation properties of BfmR and other OmpR/PhoB family response regulators.

Results

Oligomerization of BfmR

The multimeric state of BfmR was investigated using sedimentation velocity analytical ultracentrifugation (AUC). BfmR sedimented as a single monomeric species in solution with a homogenous 2.6 S distribution (Fig. 1a) with an estimated mass of an approximately 34 kDa protein. This agrees with the theoretical mass of ~31.8 kDa (including purification tag). Size exclusion chromatography (SEC) experiments using ultra performance liquid chromatography (UPLC) confirmed that monomeric BfmR elutes as an apparent 26.6 kDa protein (Fig. 1b). A wide range of protein concentrations were explored to determine if dimerization was concentration dependent. At 900 μ M, BfmR remains a monomer in solution.

Since activation is known to promote dimerization, BfmR was incubated with the phosphomimic beryllium trifluoride (BeF_3^-) [20]. Treatment with BeF_3^- resulted in the significant enrichment of a species with an apparent molecular weight of 61 kDa, consistent with the theoretical molecular weight of a BfmR dimer (no tag) of ~55.8 kDa (Fig. 1c). Less

than 10% of the population remains in a monomeric state. Approximately 20% of the population formed high order oligomers eluting as a broad distribution centering around ~200 kDa. These species are likely a result of the high concentrations of BeF_3^- -BfmR (900 μM) used in the experiment and are not physiologically relevant.

Additional sizing experiments were conducted to assess the multimeric states of the N- and C-terminal domains of BfmR (termed BfmRN and BfmRC, respectively). The domains eluted with apparent molecular weights of 15.5 kDa and 17.9 kDa, respectively. Both values are consistent with monomers in solution (Fig. S1). At a concentration of over 1.5 mM, a very small species consistent with a BfmRN dimer was observed but could not be further enriched due to concentration limitations.

Crystal structure of the N-terminal receiver

The BfmRN structure was solved using the N-terminal domain of *M. tuberculosis* MtrA as a search model (PDB ID 3NHZ [17]) for a molecular replacement solution. The structure was refined at 2.0 Å resolution to a crystallographic R_{work} of 0.202 and R_{free} of 0.227 (Table S1). The protein crystallized in the same P6₅ space group as the previously solved BfmRN structure (PDB ID 5E3J [16]), but under different crystallization conditions [16]. There are two molecules in the asymmetric unit, with each molecule adopting an alternating α/β -fold consisting of 5-helices and 5-strands (Fig. 2a). This folding motif is highly conserved in the OmpR/PhoB-like family of response regulators [21].

The structure of BeF_3^- -BfmRN was solved to 1.86 Å and refined to a crystallographic R_{work} of 0.149 and R_{free} of 0.178. A conserved aspartate residue (Asp58) clearly coordinates the BeF_3^- molecule acting as the phosphomimic (Fig. 2b). An active site Mg^{2+} is coordinated by a fluoride, Asp15, Asp58, the carboxyl group of Met60, and two water molecules. The structure of BeF_3^- -BfmRN aligns nearly perfectly with the structure of BfmRN, with an Ca RMSD of 0.315 across 227 atoms. The largest discrepancy between the two structures involves side chain orientation within the active site. In the absence of BeF_3^- , many of the sidechains are shifted slightly from the active site. The addition of BeF_3^- and Mg^{2+} pulls the side chains into position within the active site. There are no significant disturbances in the backbone or side chain conformations of the rest of the structure. Most importantly, the “switch” threonine (Thr87) which defines the active versus inactive conformation of the receiver domain [22] aligns perfectly between the two BfmR structures, confirming an active state conformation. These observations provide solid support that, even in the absence of BeF_3^- , the crystal structure of BfmRN is representative of the active dimer conformation.

The interface between BfmRN molecules involves interactions between the $\alpha 4$ - $\beta 5$ - $\alpha 5$ segment of each molecule (Fig. 2c). A net buried surface area of 977.6 Å² per monomer resulting in 15.0% of each monomer excluded from exposed solvent is consistent with this interface being physiologically relevant. Subsequent analysis of the residues at the interface revealed three major salt bridges between Glu98, Arg112, and Arg119; Asp102 and Arg124; and Asp103 and Arg117. Additional hydrogen bonds and hydrophobic interactions further facilitate the dimer interface. Additionally, a high degree of sequence homology was found among residues mediating salt bridges and/or making contacts at the $\alpha 4$ - $\beta 5$ - $\alpha 5$ homodimer

interface (Fig. S2). Furthermore, this dimer interface has been observed for other response regulators and is considered the physiologically relevant conformation (Fig. S3a,b) [23–25]). A previously solved structure of BfmRN (PDB ID 5E3J [16]) presented an additional dimer interface involving the $\alpha 1$ - $\beta 2$ - $\alpha 2$ surface [16]. The Dali structural similarity server [26] was utilized to assess the potential of this secondary interface. No conservation for the $\alpha 1$ - $\beta 2$ - $\alpha 2$ interface was observed among the over 100 structures scored (Fig. S3c). This suggests that the previously observed secondary interface is likely a crystallization artifact.

Solution NMR structure of the C-terminal DNA-binding domain

The BfmRC protein structure was characterized using restraints derived from Phi/Psi dihedral angles, nuclear Overhauser effects (NOEs), hydrogen bonds, and amide backbone ^1H - ^{15}N residual dipolar couplings (RDCs) (Table S2). The final ensemble of the 10 lowest energy conformers has a low C α RMSD of backbone (0.19 ± 0.10 Å) and heavy (0.61 ± 0.05 Å) atoms for all ordered residues (Fig. 3a). The structural ensemble also has no distance restraint violations >0.2 Å, no dihedral violations >5 degrees, and no close contacts <1.6 Å for hydrogens or <2.2 Å for heavy atoms. The structure of BfmRC adopts the canonical winged helix-turn-helix (wHTH) motif present in the effector domain of many OmpR/PhoB-like RRs. Residues R198 and R206, in L1 and $\alpha 3$ respectively, correspond to the major contributors to DNA binding in similar response regulator wHTH motifs (Fig. 3b). The C-terminal sheet (sheet-2; composed of $\beta 4$, $\beta 5$, and $\beta 6$) includes several electropositive residues (K219, R220, K222, R225, K227, and K233) and two aromatic residues (Y229 and F231). This relatively high local concentration of basic amino acid residues creates a large electropositive surface patch on the L1- $\alpha 3$ -(sheet-2) face of BfmRC (Fig. 3b). This electropositive surface likely accommodates a negatively-charged DNA substrate.

BfmRC is most structurally similar to the DNA-bound crystal structure of RstA (PDB ID 4NHJ [23]) from *Klebsiella pneumoniae* (*kpRstA*), with a Dali Z-score of 13.8 and C α RMSD of 2.3 Å (Fig. 3c, grey) [23]. Superimposition of BfmRC with *kpRstA* shows that the solution structure of BfmRC maintains a fold that is similar to the DNA-bound *kpRstA* effector domain (Fig. 3c).

There are minor differences in the position of $\alpha 2$ and adjacent L1, which non-specifically interact with the DNA substrate. Sequence and structural alignments between the two proteins revealed strong identity in the L1- $\alpha 3$ -(sheet-2) face including several residues critical for DNA-binding. Residues R199 and R207 in *kpRstA* are in the DNA recognition helix $\alpha 3$, and have been identified as responsible for making base-specific intercalations with the DNA substrate [23]. The homologous residues in BfmR, R198 and R206, are positioned to fulfill the same function (Fig. 3b, green spheres). Additionally, residues R181, R189, and R208 from $\alpha 3$ and R225 and Y229 of BfmRC (Fig. 3b, blue spheres) are homologous to *kpRstA* residues R182, R190, R209, R226, and Y230, respectively. In *kpRstA*, these residues make nonspecific contacts with the DNA backbone [23]. Several additional residues which exhibit non-specific backbone interactions with DNA in *kpRstA* are conserved in BfmRC (A164, S200, V203, S206, T224, which are homologous to A163, S199, V202, S205, and T223 in BfmRC, respectively).

Chemical crosslinking and mass spectrometry of the BfmR full-length

The dynamic nature of BfmR rendered attempts to solve the complete full-length structure by both NMR and X-ray crystallography unsuccessful. Chemical crosslinking studies were performed to orient the individual domain structures of BfmR using the reagents BS3, bis(sulfosuccinimidyl)suberate, and BS(PEG)9, PEGylated bis(sulfosuccinimidyl)suberate. BS3 and BS(PEG)9 are water-soluble, homobifunctional, amine-reactive molecules which interact with proximal lysine side-chains and the N-terminal amines of polypeptides with linker lengths of 11.4 Å and 35.8 Å and maximum lysyl crosslinking distances of 24.1 Å and 48.5 Å, respectively. Fig. S4 shows the successfully crosslinked complexes analyzed by 10% SDS-PAGE. Bands with molecular masses above ~54 kDa (BfmR dimer) were considered aggregation artifacts and not analyzed further. Monomeric BfmR (~27 kDa) was the dominant species observed in each crosslinking reaction. A second species corresponding to a ~54 kDa BfmR dimer was enriched as a product of the crosslinking reaction, particularly with addition of BS3. BS3 and BS(PEG)9 produced similar crosslinking results as observed by gel.

Dimeric and monomeric bands were excised and independently subjected to in-gel digestion for subsequent analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS). The monomeric species provides only intramolecular crosslinking information between lysine residues within the N- and C-terminal domains while the dimeric species provides intermolecular crosslinking information. Each band was positively identified as the protein BfmR based on the NCBI Acinetobacter database. Each crosslinking reagent modified the majority of available lysine residues indicating appropriate reaction conditions. The MassMatrix [27] search engine was used to identify, assess, and score crosslinked lysine residues.

Orientation of BfmR Domains from Observed Crosslinks

A structural model of the full-length BfmR homodimer was generated with the molecular docking program HADDOCK [28] using the crystal structure of the BfmRN dimer and solution NMR structure of the BfmRC monomer (Fig. 4a). BfmR domains were oriented using the *in vitro*-derived inter- and intra-molecular crosslinking restraints. Unambiguous C α - C α restraints were generated for the following residues based on chemical crosslinking MS data: K127 to K222, K227, and K233 for both monomeric domains (Fig. 4b). BS(PEG)9 identified identical cross-links to BS3, so only BS3 distances were used in modeling studies (Fig. S5a). 11.6 Å was added to the BS3 spacer arm length to account for the length of each lysine side chain. The resulting distance yielded a range of 12.0–23.0 Å. Intramolecular C α - C α distances within respective N- and C-terminal domains were calculated and utilized as restraints to ensure retention of globular protein shapes. The final ensemble of 200 structural poses satisfied unambiguous distance restraints, and displays remarkable inter-domain mobility where BfmRC exhibits a rocking motion with respect to BfmRN, resulting in a 10.1 Å deflection of the proposed DNA-recognition helix (Fig. 4c).

Characterization of a BfmR recognition sequence within the bfmRS promoter

A 45 bp blunt-ended duplex DNA substrate that spans the putative promoter was designed based on previous work describing a putative *bfmRS* promoter sequence [13]. Centrally

positioned within this substrate is a 14 bp perfect inverted repeat sequence separated by a single base pair spacer (Fig. 5a). Electrophoretic mobility shift assays (EMSA) were performed to evaluate the DNA binding ability of full-length BfmR. Using a fixed concentration of DNA at 2 μ M, a moderately-shifted diffuse band was observed at a 10:1 molar ratio of protein to DNA (Fig. 5b). A complete shift was achieved at a 50:1 molar excess of BfmR. This result is consistent with observations of other low-affinity protein-DNA complexes where complete dissociation of the complex is discouraged by the gel matrix during migration [29].

Since phosphorylation often influences DNA binding in response regulators, the phosphomimetic BeF_3^- was used to generate the active conformation of BfmR. BeF_3^- -BfmR showed similar relative abundances of both free and shifted DNA (Fig. 5c) when compared to native BfmR (Fig. 5b). However, a higher concentration of BeF_3^- -BfmR, 100:1 molar ratio, was required to observe a complete shift of the DNA substrate. A non-phosphorylatable BfmR construct (BfmR^{D58A}) was generated by mutating the conserved phosphorylation site, Asp58, to alanine. The mutant was used to confirm that the observed DNA binding by untreated BfmR was not an artifact of the purification process producing phosphorylated BfmR. Diffuse bands were also observed in the BfmR^{D58A}:DNA interaction at similar molar ratio as wild-type BfmR (Fig. 5d). This suggests that treatment with BeF_3^- is required to generate an activated BfmR construct. The C-terminal DNA-binding domain alone was also tested for DNA binding potential. Assays with BfmRC resulted in a nearly undetectable DNA-binding affinity by EMSA (Fig. 5e). This strongly suggests that the N-terminal dimerization domain is needed to facilitate the binding of BfmR to DNA.

Isothermal titration calorimetry (ITC) was performed to further assess the BfmR:45-mer *bfmRS* promoter substrate interaction, and to evaluate the energetics of the interaction. ITC titrations resulted in a highly reproducible signal with negative changes in both enthalpy and entropy (Fig. 5f). Stoichiometries of $n \sim 0.5$ correlate with one DNA binding two copies of BfmR, *i.e.* one homodimer bound per DNA duplex. To confirm this observation, untreated BfmR was incubated with DNA and analyzed by size exclusion chromatography. The resulting complex was consistent with the molecular weight of a single BfmR dimer bound to DNA (data not shown). ITC data were fit using a single-site specific-binding model and resulted in an observed K_d of $13.16 \pm 0.13 \mu\text{M}$. The interaction was found to be exothermic ($G_{obs} = -6.56 \pm 0.06 \text{ kcal/mol}$) and enthalpy-driven based on a large change in enthalpy ($H_{obs} = -5.35 \pm 0.05 \text{ kcal/mol}$) relative to entropy ($T S_{obs} = 1.21 \pm 0.01 \text{ kcal/mol}$). Thus, even when not activated BfmR is able to form a dimer on DNA. Although this affinity is modest compared to the nanomolar affinities observed for other response regulators, such as a K_d of $10.1 \pm 2.5 \text{ nM}$ for PhoP from *M. tuberculosis* (He and Wang, 2014), it is comparable with the binding of *kpRstA* for DNA which has a K_d in the low μM range [23].

The inverted repeat of the *bfmRS* promoter sequence spans the central region of the originally identified 45-mer sequence. Variable length promoter constructs were generated to evaluate BfmR binding potential (Fig. S6a). BfmR bound substrates from 21 to 45 bp in length as observed through EMSAs using 50-fold excess BfmR to DNA (Fig. S6b). BfmR did not bind a substrate that was composed solely of the core 15 bp inverted repeat (data not shown). This suggests that the three additional base pairs flanking the inverted repeat in the

21-mer substrate are important for BfmR DNA binding. The importance of an inverted repeat sequence as opposed to a tandem repeat was studied by testing binding interactions to the substrate TR-26mer (Fig. S6a). No binding interactions were detected up to 50-fold excess BfmR. An additional substrate with two base pairs separating the inverted repeat, 27mer-2S (Fig. S6a) was made to determine if BfmR could tolerate variations in binding site spacing. At up to a nearly 50-fold excess of BfmR to DNA, no binding activity was observed by EMSA (Fig. S6c). These results indicate that, when using the *bfmRS* promoter sequence, BfmR requires at least 21 bp substrate to bind the DNA, that BfmR likely prefers to bind an inverted repeat sequence, and that spacing of the recognition sequence is important for binding.

Often, activation of a response regulator increases the DNA binding affinity. Since no significant differences were observed between BfmR and BeF_3^- -BfmR using EMSAs, fluorescence polarization assays were performed to quantify and directly compare differences in DNA-binding due to activation. Untreated BfmR binds DNA with a K_d of $3.78 \pm 0.73 \mu\text{M}$ (Fig. 5g), comparable to the K_d determined by ITC. On the other hand, BeF_3^- -BfmR binds DNA with a nearly 15-fold lower binding affinity, K_d of $44.14 \pm 5.09 \mu\text{M}$ (Fig. 5g). Fitting the untreated BfmR data to a non-linear regression resulted in a Hill coefficient of 1.22 ± 0.09 , corresponding to positive cooperative binding. One monomer bound to the DNA increases the affinity for the second monomer. This observation is in line with dimerization being promoted by DNA binding. On the other hand, BeF_3^- -BfmR has a Hill coefficient of 1.04 ± 0.09 , corresponding to noncooperative binding. Since the activated protein would be binding as a preformed dimer, there is only one binding event and no cooperative should be observed. Thus, binding of an untreated BfmR monomer increases the binding affinity of a second monomer, forming a stable dimer, while BeF_3^- -BfmR binds the DNA as a dimer. More importantly, BfmR binds the *bfmRS* promoter based substrate with a significantly higher affinity when untreated than treated with BeF_3^- .

Discussion

The combined evidence from sedimentation analysis and gel-filtration chromatography strongly suggests that BfmR is a monomer in solution, as are the individual domains of BfmRC and BfmRN. Activation by the phosphomimic BeF_3^- results in nearly complete dimer formation in solution. These findings are consistent with previous characterizations of response regulators where proteins predominantly exist as monomers prior to phosphorylation, upon which dimerization occurs [24,30–33]. A widely accepted hypothesis is that response regulators sample inactive and active conformations at equilibrium [22,34,35]. This is observed by the presence of a small transient subpopulation of inactive BfmR dimer (Fig. 1b). The fact that untreated BfmR forms an active conformation dimer in crystals is a well-known phenomenon for response regulators [21,36–38]. For other OmpR/PhoP family response regulators, phosphorylation strongly stabilizes the active conformation and acts as an “energetic gateway” [24]. Through the stabilization of the activated state, equilibrium shifts in favor of dimer formation. The same shift in equilibrium is observed when BfmR is activated with BeF_3^- (Fig. 1c).

By coupling X-ray crystallography, solution NMR, chemical crosslinking, and mass spectrometry, a model of full-length BfmR was generated. This combination approach to structure solutions has been successful in other systems [18,39,40]. By building a full-length model from structures of individual domains, structures of highly flexible proteins like response regulators become obtainable. The model reveals that BfmR takes on the canonical OmpR/PhoP family morphology with the N-terminal receiver domains forming the dimer interface and the individual C-terminal DNA binding domains tethered via a flexible linker region. This arrangement allows the DNA binding domains to have considerable flexibility relative to the rest of the protein. This flexibility is vital for recognition of multiple DNA binding sequences [41,42].

This work presents direct evidence that BfmR binds a perfect inverted-repeat within the *bfmRS* promoter near the predicted -35 promoter sequence previously identified by Actis et al. [13]. We show that both unphosphorylated and BeF_3^- -BfmR can successfully bind DNA substrates (Fig. 5b,c,g). In the case of unphosphorylated BfmR, this observation suggests that the dimer formation may be enhanced by exposure to a DNA substrate. Curiously, BfmRC showed little to no affinity for the *bfmRS* promoter sequence (Fig. 5e). This behavior contrasts other OmpR/PhoB family response regulators where the DNA-binding domain binds DNA in the absence of the N-terminal domain [23,43,44]. While the N-terminal domain of other response regulators significantly enhance DNA binding, it appears the N-terminal domain is required for stabilizing a BfmR:DNA complex. This idea is further supported by the fact that BfmRC is clearly a monomer by both SEC (Fig. S1) and NMR (Fig. 3). These data in combination with ITC results (Fig. 5f) strongly support a mechanism where BfmR recognizes DNA as a dimer as observed with OmpR [24].

Further evaluation of the binding properties of BfmR to the *bfmRS* promoter provided insight into the mechanism of BfmR DNA binding. The base pairs flanking the inverted-repeat appear to be necessary for DNA binding. Docking BfmR to DNA reveals the potential binding interaction (Fig. 6). The recognition helices align to interact with each inverted-repeat. Key residues identified in other response regulators are in position to facilitate binding [23,43–45]. This suggests that BfmR utilizes a very similar mode of DNA binding as other OmpR/PhoB family members.

An interesting observation is that upon activation with BeF_3^- , BfmR binds the *bfmRS* promoter with a significantly lower binding affinity than when inactivated. Frequently, phosphorylation leads to dimerization which increases/facilitates DNA binding [22–24]. As noted, response regulators are constantly sampling active and inactive states. Stock and co-workers have determined that with OmpR, phosphorylation greatly stabilizes the active conformation. This shifts the equilibrium in favor of dimer formation. This shift towards the dimeric state is the sole driving force towards enhancing DNA binding, as opposed to phosphorylation directly enhancing binding [24]. BfmR significantly increases in the dimer population upon phosphorylation (Fig. 1c) and binds DNA as a dimer as confirmed by ITC (Fig. 5f) as expected based on homology to OmpR. Unexpectedly, BfmR exhibits the opposite behavior in regard to activation and DNA-binding affinities. These observations may be attributed to the nature of the *bfmRS* promoter sequence, and could be indicative of a potential self-regulatory strategy within the cell. It is possible that during normal cellular

activity a baseline of BfmR is being produced. When an external signal triggers a BfmR activation event, BfmR would favor binding to its downstream targets over production of more BfmR. The fact that BfmR regulates its own promoter as a function of internal protein concentration may be physiologically relevant. This is similar to the concentration dependent multiple modes of binding seen previously in Spo0A [46,47]. Unfortunately, the *bfmRS* promoter is the only known BfmR binding sequence to date. The identification of additional recognition sites will be needed to further evaluate this phenomenon.

In conclusion, we present the crystal structures of the active and inactive BfmR N-terminal receiver domain and the solution NMR structure of the C-terminal DNA-binding domain. Using a combination of several biophysical approaches, we provide the first structural model for full length BfmR from the bacterial pathogen *A. baumannii*. We show that it binds to a DNA substrate derived from its own *bfmRS* promoter. Furthermore, we find that BfmR preferentially binds the *bfmRS* promoter in the inactivated state over the activated state. While this behavior is not common for other response regulators, it presents intriguing potential for a self-regulation strategy. Structural information is, of course, highly valuable for drug design. While many OmpR/PhoB family members often share similar structural traits, understanding the structural differences between response regulators will aid in drug development. We believe that using a multi-pronged structural approach, such as that described above, to investigate other key response regulators will greatly assist in developing future antimicrobial compounds.

Methods

Cloning, expression, and purification

BfmR from *A. baumannii* strain ATCC 19606T (UniProt: Q2VSW6) was expressed using either a pET200 or pET28a expression vector containing an amino-terminal His₆ affinity tag. BfmRN, residues 1–130, was expressed using pET28a with a C-terminal His₆ affinity tag, and BfmRC, residues 130–238, was expressed using pET21b with a C-terminal His₆ affinity tag. The BfmR^{D58A} mutant was generated with the QuikChange II site-directed mutagenesis kit (Agilent). All BfmR plasmid constructs were transformed into *Escherichia coli* BL21(DE3) pLysS expression cells. Samples of BfmR, BfmR^{D58A}, BfmRN, and BfmRC were prepared from 1 L cultures grown at 37 °C in LB, then shifted to 30 °C for 4-hour induction with 1 mM IPTG at 140 rpm prior to harvest. Cell pellets were resuspended in lysis buffer containing 20 mM Tris pH 7.9, 500 mM NaCl, 5 mM imidazole, 0.05% sodium azide. Protein was purified based manufacturer's protocol (Qiagen). Protein was eluted with an imidazole gradient from 0–250 mM. pET28a constructs were cleaved for 2 h with 100 U of thrombin at room temperature, reaction quenched with 0.1 mM AEBSF. Protein was dialyzed into appropriate buffer. Uniformly ¹³C/¹⁵N-labeled samples of BfmRC were prepared as described previously [48].

Analytical ultracentrifugation

AUC samples were dialyzed into buffer containing 20 mM HEPES (pH 6.5), 100 mM NaCl, 5 mM MgCl₂, 0.5 mM DTT, and 0.02% NaN₃ and concentrated to 70 μM. Samples were loaded into cell assemblies containing Epon charcoal-filled centerpieces and quartz

windows, followed by centrifugation at 45,000 rpm in an An60 Ti rotor. All sedimentation velocity experiments were collected on an Optima XL-A analytical ultracentrifuge (Beckman Coulter) at 20 °C while monitoring absorbance at 280 nm. Scans were recorded every 8 min with 0.003 cm spacing. Sedimentation profiles were analyzed with SedFit (NIH) using buffer corrections determined from SednTerp (sednterp.unh.edu; University of New Hampshire). Plots of the resultant $s_{20,w}$ versus the normalized $c(s)$ for each velocity dataset were calculated using Kaleidagraph, version 4.52 (Synergy Software).

Ultra performance size exclusion liquid chromatography

A Waters ACQUITY UPLC H-Class system was used for all SEC experiments. BfmR, BeF₃⁻-BfmR, BfmRN, and BfmRC were equilibrated into mobile phase (20mM Tris pH 7.5, 300 mM NaCl, 0.3 mM DTT, 5 mM MgCl₂, and 0.02% NaN₃) prior to concentration. A 150 mm BEH125 SEC (Waters Corp.) column was equilibrated with 10 column volumes of mobile phase prior to each initial injection. 10 µL of each sample were injected onto the column at a flow rate of 0.3 mL/min. All experiments were performed at ambient temperature with single wavelength UV monitoring at 280 nm. An SEC filtration standard (BioRad) was used to generate a standard curve under identical conditions. Plots of the raw data and chromatograms were made in Kaleidagraph, version 4.52 (Synergy Software).

Chemical activation with beryllium fluoride

Samples were activated as previously described [20,49,50]. A final concentration of 7 mM MgCl₂ was added to a 1 mg/mL solution of BfmR in 20 mM Tris, 200 mM NaCl. BeCl₂ was added to the sample at a final concentration of 5 mM. NaF was then quickly added to a final concentration of 20 mM. The solution was mixed by gentle pipetting and incubated undisturbed at room temperature for at least 1 h.

X-ray crystallography

Purified BfmRN was concentrated to 8 mg mL⁻¹ in buffer containing 20 mM Tris pH 7.5, 500 mM NaCl, 1mM DTT, 0.02% NaN₃, and 125 mM imidazole. Crystals were harvested from wells containing 0.1 M Bis-Tris propane (pH 8.0) and 20% (v/v) MeOH and cryoprotected in mother liquor containing 22% (v/v) glycerol prior to flash-freezing in liquid nitrogen. 120° of diffraction data in 1° increments were collected at 100 K on the SER-CAT BM22 beamline of the Advanced Photon Source (APS) and processed using *HKL2000* [51]. The BfmRN structure was determined by molecular replacement with PHASER [52] using the coordinates of the N-terminal domain of *M. tuberculosis* MtrA as the search model (PDB ID: 3NHZ [17]). The structure was then refined with *REFMAC5* [53], alternating with manual fitting using COOT [54]. Water molecules with appropriate hydrogen bond distances were included in later rounds of refinement and alternate conformations were modeled for some sidechains.

BeF₃⁻-BfmRN was dialyzed into 20 mM Tris pH 7.9, 200 mM NaCl, and 1 mM DTT. Crystals were grown through sitting-drop vapor diffusion against 1.6 M ammonium sulfate, 0.1 M MES pH 6.5, and 10% w/v 1,4-Dioxane at room temperature. Crystals grew at 7 mg mL⁻¹ with a protein to cocktail ratio of 1:1. Crystals were harvested by flash freezing in liquid nitrogen. Data was collected at a wavelength of 1.033 Å in 0.5° increments at 100 K

on APS beamline 23-ID-D (GM/CA). Diffraction was indexed, merged, and scaled using *HKL2000* [51]. Molecular replacement was carried out in Phaser-MR within the PHENIX suite [52,55] using our BfmRN structure (PDB ID 5HM6) as a starting model. The resulting solution was further refined using COOT [54] and phenix.refine [55]. Statistics for data collection and refinement are located in Table S1. All structure figures were produced using PyMOL (The PyMOL Molecular Graphics System, Delano W.L., 2002).

Solution NMR spectroscopy and structure calculations

BfmRC samples for NMR experiments were dialyzed into 25 mM Tris pH 6.5, 50 mM KCl, 1 mM DTT, 1 mM EDTA, and 0.02% sodium azide and concentrated to 1 mM. Standard NMR protocols were used to determine backbone, sidechain, and aromatic chemical shifts. All experiments were collected on uniform $^{13}\text{C}/^{15}\text{N}$ -labeled BfmRC samples at 298 K on Varian Inova 600 MHz and Bruker Avance 700 MHz spectrometers equipped with cryoprobes. Double- and triple-resonance experiments were collected, processed, and analyzed as described previously [48]. Generation of preliminary Phi and Psi dihedral angle restraints was performed with Talos+ [56]. Distance restraints were obtained from a 3D ^1H - ^{15}N -NOSEY with a 120 ms mixing time. Generation of preliminary NOE restraints and initial structure calculations was performed with CYANA 3.0 [57]. Conformers with the lowest CYANA target function, RMSD, and violations were selected for further refinement using restrained energy minimization in implicit solvent in AMBER [58]. Restraints were used and evaluated in further refinement steps. Residual dipolar couplings were measured on a 0.8 mM uniform ^{15}N -labeled BfmRC sample prepared in a 6.0 mm to 4.2 mm radially-compressed 6% polyacrylamide gel prior to data collection on a Bruker Avance 700 MHz spectrometer using IPAP-HSQC experiments, and analyzed with REDCAT [59]. Quality of structures during refinement were assessed with the Protein Structure Validation Software suite (PSVS version 1.5) [60], and analyzed visually with *PyMOL*.

Chemical crosslinking and LC-MS/MS mass spectrometry

BfmR samples for BS3 treatment were dialyzed into conjugation buffer containing 20 mM HEPES pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, and 0.02% NaN_3 . Solutions of 63 μM BfmR were incubated with 38- to 144-fold excess of BS3 (Thermo Fisher) and allowed to react for 30 or 60 min at room temperature before quenching with 50 mM Tris-HCl pH 7.5. Samples to be modified with BS(PEG)9 (Thermo Fisher) were dialyzed into conjugation buffer containing 50 mM sodium phosphate pH 7.2, 150 mM NaCl, and 0.5 mM DTT. Freshly prepared stocks of BS(PEG)9 were prepared at 25 mM in anhydrous DMSO from a master stock at 250 mM. Solutions of 79 μM BfmR were incubated with 10-, 20-, and 50-fold excess BS(peg)9 and allowed to react for 30 or 60 min at room temperature before quenching the reaction with 50 mM Tris-HCl pH 7.5. All crosslinked BfmR samples were size-fractionated by SDS-PAGE using 10% tricine-polyacrylamide gels and stained with Coomassie Brilliant Blue. Bands corresponding to the desired molecular weight, monomer or dimer, (Fig. S4) were extracted from the gel. Excised protein bands were suspended in milliQ water and submitted to the Duke Center for Genomic and Computational Biology at Duke University Medical Center for LC-MS/MS analysis. Following data acquisition, all spectra were searched against the NCBI *Acinetobacter* database with BS(PEG)9 and BS3 as a variable modification. Mass Matrix

(www.massmatrix.com) was used to search, identify and assign confidence scores to successfully crosslinked peptides (Fig. S5).

Full-length BfmR molecular modeling

Generation of the full-length BfmR model was performed with HADDOCK v2.1 [28,61]. An initial full-length BfmR starting model was created using two BfmRC monomers visually oriented to dimeric BfmRN using PyMOL. BfmRN residues missing due to a lack of electron density were manually inserted and joined to their corresponding C-terminal domains by creating a peptide bond within each linker region in PyMOL. Default HADDOCK parameters were used throughout the docking procedure [28] with the following exceptions: non-crystallographic and C2 symmetry restraints were used for the dimerization of respective N-terminal domains, non-crystallographic symmetry for each of the two monomeric C-terminal domains, and non-crystallographic symmetry for the overall full-length BfmR dimer. From the initial model, two PDBs were generated for each monomeric unit of full-length BfmR (residues 1–238 and 1'-238' with prime denoting residues of the other monomeric subunit). Successfully identified crosslinks were used as intramolecular restraints to orient a single C-terminal monomer with respect to its corresponding N-terminal domain. Unambiguous C α to C α restraints were generated for the following residues based on chemical crosslinking MS data: K127 to K222, K227, and K233 for both monomers. Sidechain length was incorporated within each distance restraint (approximate distance from C α to terminal N ζ is 5.8 Å) and 11.6 Å was added to the BS3 spacer arm length (11.4 Å) to yield a final range of 12.0–23.0 Å. BS(PEG)9 were not incorporated due to observation of identical cross-links. Intramolecular C α - C α distances for each domain were calculated and utilized as restraints to ensure retention of globular protein shapes. Interactions within the BfmRN X-ray crystal structure were used to bring the two full-length monomeric subunits together in the final docking process. Ambiguous restraints and definition of passive or active residues were not used.

One thousand structures were generated for the first iteration (rigid docking), 200 were generated for the second iteration (semi-flexible docking), and 200 lowest energy structures were water refined. The C α RMSD values of the complexes were calculated using ProFit (Martin ACR. ProFit V3.1 edit. SciTech Software; 1992). A cluster analysis was then performed on the final docking solutions using a minimum cluster size of four. The RMSD cutoff for clustering was manually determined to be 7.5 Å. The RMSD matrix was then calculated over the backbone atoms of the interface residues of the proteins. The lowest energy structure from the molecular docking within the highest populated cluster was further analyzed by visually inspecting the resulting atomic coordinates file to ensure no interaction between C-terminal domains. PSVS was used to confirm stereochemical quality of the final protein structure [60].

DNA bound BfmR model generation

A model of the *bfmRS* promoter sequence was generated using 3D-DART [62]. In *PyMOL*, the DNA model was aligned to the DNA from the RstA crystal structure (PDB ID 4NHJ) such that the inverted regions lined up. The C-terminal domains of the full-length model of BfmR was then aligned to the crystal structure of RstA. One DNA-binding domain of BfmR

was manually rotated to match the orientation of RstA. Alignment of the two DNA recognition helix $\alpha 3$ was confirmed to ensure proper positioning of the DNA binding domains.

Electrophoretic mobility shift assays

DNA substrates were designed from the *bfmRS* promoter region described previously [13]. Oligonucleotides used for binding studies: 5'-TGAACGCCTACTTGTTTACAATGTAAACGTGTGTATATTGCAATG were synthesized and purified by high performance liquid chromatography (IDT DNA Technologies). DNA duplexes were annealed by suspending equimolar concentrations of both strands together in annealing buffer (20 mM HEPES pH 6.5, 100 mM NaCl, 2 mM MgCl₂, and 10% v/v glycerol) and heating them at 95 °C for 10 min before slow cooling to room temperature. Samples of BfmR were dialyzed into binding buffer containing 20 mM HEPES pH 6.5, 50 mM NaCl, 5 mM MgCl₂, 0.5 mM DTT, and 0.02% NaN₃. BfmR at a final concentration of 2, 20, 40, 100, and 200 μ M (corresponding to 1x, 10x, 20x, 50x, and 100x, respectively the concentration of DNA) was incubated with 2 μ M DNA for 10 minutes, and then loaded onto 4 °C pre-chilled 8% native TBE-acrylamide gels. Gels were electrophoresed for approximately 90 minutes at 83 V with 4 °C pre-chilled 1X TBE running buffer then stained with ethidium bromide for 10 min. Gels were scanned with a Quantity One imager (Bio Rad) or Gel Doc EZ System (Bio Rad) and the image files were processed with ImageJ (NIH).

Isothermal titration calorimetry

ITC experiments were performed on a low volume NanoITC (TA Instruments) at the North Carolina State University Biological Core Facility. Samples of BfmR and DNA were extensively dialyzed into binding buffer containing 20 mM HEPES pH 6.5, 50 mM NaCl, 5 mM MgCl₂, 10% v/v glycerol, and 1 mM β -mercaptoethanol. Titrations were performed with 24 injections of 2 μ L and a 0.5 μ L initial injection separated by 300 sec intervals with continuous 250 rpm stirring at 20 °C. The syringe titrant contained 100 μ M DNA and the cell titrate contained 20 μ M BfmR. Reference titrations to account for the heat of dilution of the DNA into reaction buffer were subtracted from the experimental titrations. Isotherms were processed with Nano Analyze (TA Instruments) and extracted to Prism (GraphPad Software)) for simulated curve fitting of a “one-site specific-binding” model to derive experimental binding constants and thermodynamic parameters. Errors are reported based on the goodness-of-fit ($R^2 = 0.9976$) to an averaged binding isotherm generated from two separate representative titrations. Plots of the raw data and simulated isotherms were made in Kaleidagraph, version 4.52 (Synergy Software).

Fluorescence Polarization DNA Binding Assay

The DNA binding affinities of BfmR and BeF₃⁻ - BfmR were determined using a fluorescence polarization assay performed on a Synergy H1 Hybrid Multi-Mode microplate reader (BioTek). All assays were performed at ambient temperature in 20 mM Tris pH 7.9, 100 mM NaCl, 2 mM MgCl₂, 1 mM DTT, and 0.1 mg ml⁻¹ BSA with constant 20 nM fluorescein labeled DNA substrate. The DNA substrate was based on the initial BfmR 45-mer with the following sequence; 5'- 6-FAM-CTACTTGTTTACAATGTAAACGTGTG-3'

(the predicted inverted repeat DNA binding site is centrally positioned and underlined). Experiments were performed with 120 μ M BfmR and 240 μ M BfmR-BeF₃⁻ followed by a series of seven 2-fold serial dilutions. The reactions were initiated by the sequential addition of protein to the DNA 26-mer in a final reaction volume of 100 μ L in a black/opaque 96-well microplate (BrandTech). Fluorescent substrates were excited at 482 nm and polarization data were collected at 528 nm. Data were analyzed and K_d values were determined using DynaFit software (BioKin Ltd.). Hill coefficients were determined using non-linear regression analysis in SigmaPlot.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- BfmR is the master regulator of biofilm formation in *Acinetobacter baumannii*
- Full-length structure of BfmR by coupling biophysical techniques
- Activated BfmR binds DNA with a lower affinity than inactivated BfmR

Figure 1. Oligomerization analysis of BfmR

(a) Sedimentation coefficient distribution of BfmR collected at 20 °C as a function of $s_{20,w}$. (b) Size exclusion chromatogram of BfmR (black line). (c) Size exclusion chromatograph of BeF_3^- -BfmR (black line). Both BfmR and BeF_3^- -BfmR were injected at a concentration of 900 μM . Standards peaks (grey line) from left to right correspond to thyroglobulin (670 kDa), γ -globulin (158 kDa), ovalbumin (44 kDa) and myoglobin (17 kDa), respectively and are displayed for reference.

Figure 2. X-ray crystal structure of BfmRN

(a) Cartoon structure of BfmRN showing each molecule in the dimeric asymmetric unit colored individually (blue and green), with secondary structural elements labeled. (b) BeF₃⁻ (light green and white) and Mg²⁺ (lime green) in the active site of BfmRN. Coordinating water molecules are in red. Hydrogen bonding interactions are in yellow. (c) Residues at the BfmRN dimer interface involved in intermolecular contacts via salt bridges (interactions in yellow).

Figure 3. Solution NMR structure of BfmRC

(a) Cartoon structure of the BfmRC monomeric ensemble with secondary structural elements labeled. (b) ABPS electrostatic surface potential of BfmRC in the same 120°-rotated position from (a) showing electropositive charge in blue, electronegative charge in red, and neutral charge in white. Conserved residues involved in DNA intercalation are highlighted using green spheres with passively interacting residues highlighted using blue spheres. (c) BfmRC (blue) overlaid with DNA-bound RstA (PDB ID 4NHJ [23], light grey) from *K. pneumoniae* to show interaction with major groove of a potential DNA substrate.

Figure 4. Full-length BfmR shows orientation of receiver and effector domains from chemical crosslinks

(a) Cartoon structure of BfmR full-length dimer with the N-terminal dimer colored in green and blue with corresponding C-terminal effector domains colored in pale green and pale blue. (b) The observed crosslinks and their respective positions with regard to intra- or inter-molecular distance. (c) The C-terminus exhibits inherent model flexibility while still satisfying the MS cross-linking data. The C-terminal domain shows considerable flexibility, 10.1 Å deflection, relative to the N-terminal domain with conserved DNA recognition helix highlighted in red.

Figure 5. Identification of a BfmR recognition sequence from the *bfmRS* promoter

(a) 45 bp DNA duplex sequence derived from the putative *bfmRS* promoter [13]. Inverted repeat elements are underlined. (b) EMSA of BfmR with 45-mer. (c) BeF₃⁻-BfmR, (d) a non-phosphorylatable BfmR^{D58A} mutant, and (e) BfmRC, all incubated with 2 μM of 45-mer DNA substrate. 1x, 10x, 20x, 50x, and 100x correspond to 2, 20, 40, 100, and 200 μM BfmR, respectively. (f) Plots of the raw rate of change in heat and the normalized heat change for a representative titration of DNA injected into BfmR (g) Fluorescence polarization was used to determine DNA binding affinities between 6-FAM 5'-labelled DNA 26-mer with untreated BfmR (solid squares) and BeF₃⁻-BfmR (open circles).

Figure 6. Model of BfmR DNA binding mechanism

Cartoon structure of full-length BfmR dimer is visually docked to DNA with the inverted repeat sequence highlighted (orange). The DNA sequence is derived from the *bfmRS* promoter sequence used in the biochemical studies. The blowout shows key DNA binding residues (Fig. 3b) are in proximity to form interactions with the DNA.