The Response Regulator BqsR/CarR Controls Ferrous Iron (Fe^{2+}) Acquisition in *Pseudomonas aeruginosa*

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Abstract

Pseudomonas aeruginosa is a ubiquitous, Gram-negative bacterium that forms biofilms and is responsible for antibiotic-resistant nosocomial infections in humans. The *P. aeruginosa* BqsRS two-component system (TCS) regulates biofilm formation and dispersal by sensing extracytoplasmic Fe^{2+}, but the mechanistic details of this process are poorly understood. In this work, we report the crystal and solution structures of the *Pa*BqsR response regulator (RR) receiver domain (RD), comprising a $(\beta\alpha)_5$ response regulator assembly, and DNA-binding domain (DBD), comprising a winged helix-turn-helix motif. Consistent with its cognate stimulus being Fe^{2+}, we show that *Pa*BqsR binds directly to the promoter region of the *feo* operon that encodes the bacterial Fe^{2+} transport system Feo. Transcriptional studies show that *Pa*BqsR is a global regulator that negatively controls the *feo* operon, and deletion of *bqsR* provides a growth advantage of PAO1 in the presence of Fe^{2+} as its only iron source. Surprisingly, X-ray absorption spectroscopy (XAS) shows that recombinant *Pa*BqsR binds a single Fe^{2+} ion in a His-rich site, divergent from other members of the OmpR-like family. These exciting results reveal that the BqsRS system controls Fe^{2+} acquisition in *P. aeruginosa* and suggest that this system may be an attractive future therapeutic target to treat nosocomial infections.

Keywords

biofilms; two-component system; signal transduction; iron; Feo; transcriptional regulation; response regulator

Abbreviations

CV, column volumes; DBD, DNA-binding domain of *Pa*BqsR; EMSA, electrophoretic mobility shift assays; EXAFS, extended X-ray absorption fine structure; FI, fit index; FUR, ferric uptake regulator; IMAC, immobilized metal affinity chromatography; ICP-MS, inductively couple plasma mass spectrometry; IPTG, isopropyl β-D-1-thiogalactopyranoside; LB, Luria broth; HK, membrane sensor histidine kinase; MWCO, molecular weight cutoff; RD, N-terminal receiver domain; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser effect spectroscopy; OD, optical density; PMSF, phenylmethylsulfonyl fluoride; RR, response regulator; SEC, size-exclusion chromatography; TEV, tobacco etch virus; TCS, two-component signal transduction system; UV, ultraviolet; WT, wildtype; XANES, X-ray absorption near-edge structure (XANES); XAS, X-ray absorption spectroscopy.

INTRODUCTION

Bacteria are robust microorganisms that are capable of surviving in numerous, diverse conditions due to their abilities to sense, to respond, and to adapt to dynamic environments.[1–5] Most bacteria utilize two-component signal transduction systems (TCSs) to mediate communication between extracellular and intracellular milieus in order to facilitate this adaptation.[6,7] TCSs are widespread among bacteria and regulate critical cellular processes like motility, chemotaxis, the production of virulence factors, and the acquisition of essential nutrients such as transition metals.[1-8] The most common type of TCSs are membrane-bound and canonically

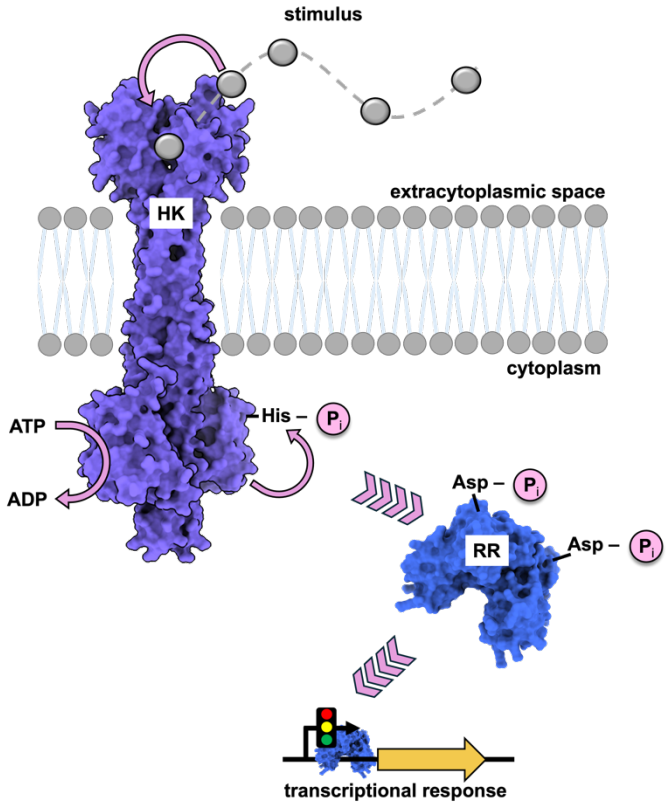

Figure 1. The signal transduction pathway of a canonical two-component system (TCS). Membrane-bound His kinases (HK) generally function as homodimers; when activated by an extracytoplasmic signal, the cytosolic kinase catalytic core of one HK monomer phosphorylates the same subunit of the neighboring dimer by transferring the γ-phosphate of ATP to the conserved His residue located in the dimerization and His phosphotransfer (DHp) domain. Upon the completion of this autophosphorylation event, the linked cytosolic response regulator (RR) accepts the transfer of the phosphate group to a conserved Asp within its N-terminal regulatory domain. As a result, the phosphorylated RR undergoes dynamical and conformational changes (generally dimerization) that modify the affinity of the RR to particular genes, ultimately leading to an altered transcriptional response.

combine two proteins to serve three functions: environmental recognition, signal transduction, and (most commonly) transcriptional regulation (Fig. 1). The two typical proteins comprising a TCS are the transmembrane sensor His kinase (HK) and the cytoplasmic response regulator (RR) (Fig. 1). The membrane-bound sensor HK receives the environmental stimulus by interacting directly with a signaling ligand, thus beginning the signal transduction process. Stimulus binding then induces HK autophosphorylation (HK-P_i),[9] after which the signal may be readily transferred to a conserved residue on the RR (RR-P_i) (Fig. 1).[9-11] Phosphorylation of the RR occurs at the N-terminal regulatory domain (site of a conserved Asp residue), triggering conformational and dynamical changes in the protein, altering the affinity of the RR to DNA and affecting transcriptional regulation (Fig. 1).[9, 12] Stimulus-specific HKs are generally paired with a cognate RR that activates or (less frequently) inhibits transcription of DNA when bound, thus regulating bacterial responses to external inputs.[9, 13-14] Since these systems facilitate the ability of bacteria to adapt to diverse environments, TCSs also function as critical virulence factors that enhance the infections of pathogens within hosts.[15-18]

One increasingly problematic pathogen that makes extensive use of TCSs to adapt to the mammalian host is *Pseudomonas aeruginosa*, a ubiquitous and opportunistic Gram-negative bacterium that is present within virtually all environments and is most notably associated with nosocomial infections.[2,12] As one of the six multidrug-resistant ESKAPE pathogens, *P. aeruginosa* continues to be a global health threat and is well-known for infecting human patients such as those with burn wounds, neutropenia, and/or those suffering from cystic fibrosis.[12] As part of its outstanding adaptability, *P. aeruginosa* can form biofilms, protective and intricate three-dimensional aggregates composed of polysaccharides, proteins, lipids, and DNA known as biofilms.[13–15] These complex structures enhance the ability of *P. aeruginosa* to cause infections

and significantly decrease the immune response of the host while increasing antibiotic resistance of the pathogen (≥1000-fold).[13,14] Moreover, *P. aeruginosa* may also employ numerous additional virulence factors that allow this infectious prokaryote to adapt to the environment of the host and to evade the immune system. Importantly, upon colonization, *P. aeruginosa* undertakes a transcriptional reprograming in order to transition to a biofilm- and virulence factor-producing mode. This process is accomplished chiefly by TCSs that function to control the transcription of genes responsible for the formation and dispersal of biofilms as well as several essential virulence factors.[16–19]

One such critical TCS in *P. aeruginosa* is BqsRS, first discovered as a regulator of biofilm dispersal in mPAO1,[20] then later found to sense extracellular Fe^{2+} and to promote resistance to cationic stress in PA14.[21] Also known as CarRS, the *Pa*BqsRS/CarRS TCS has also been reported to be highly induced by Ca^{2+}, to function as a regulator of Ca^{2+} homoeostasis, and to control Ca^{2+}-dependent pyocyanin production and tobramycin resistance.[22] *Pa*BqsRS is a canonical two-component system composed of a membrane-bound HK, BqsS, and its cognate RR, BqsR. Several lines of evidence have shown that the membrane HK *Pa*BqsS recognizes Fe^{2+} at μM metal concentrations as well as Ca^{2+} at mM concentrations, both of which are physiologically-relevant concentrations of metal cations within the lungs of cystic fibrosis patients.[23–28] Importantly, *P. aeruginosa* deletion mutants of *bqsS* and/or *bqsR* are defective in the production of short chain quorum sensing signals and rhamnolipid production, a glycolipid biosurfactant necessary to maintain biofilm architecture.[20] This BqsRS-mediated alteration in biofilm maintenance ultimately leads to biofilm dispersal that is commonly associated with the spread of infection, indicating that the BqsRS likely plays a critical regulatory role in *P. aeruginosa* virulence.[29] Furthermore, studies using the motif search program MEME[30] coupled with electrophoretic mobility shift assays

(EMSAs) discovered that *Pa*BqsR binds within a tandem repeat DNA sequence 5'-TTAAG$(N)_6$-TTAAG-3' (also known as the "BqsR box").[21] This sequence is upstream of over 100 genes in *P. aeruginosa*, including those involved in metal transport, lipopolysaccharide modulation, polyamine synthesis and transport, and even antibiotic resistance, suggesting that this TCS could be a potential drug target and emphasizing the importance of investigating this system's structural properties, its regulated genes, and its mechanism of action.[21,31,32]

We have recently characterized intact and truncated *Pa*BqsS to understand its function and its mechanism of metal selectivity.[33] Specifically, we showed that intact, detergent-solubilized *Pa*BqsS functions as an oligomer and binds a single Fe^{2+} ion within a dimeric interface of the *Pa*BqsS periplasmic domain. X-ray absorption spectroscopy (XAS) and site-directed mutagenesis demonstrated that this Fe^{2+} ion was bound in a unique octahedral geometry, coordinated by 4 Asp and 2 Asn residues (part of a conserved RExxEN motif), supporting previous transcriptomics data.[21] We then characterized the ATP hydrolysis of intact *Pa*BqsS and its truncated cytoplasmic domain, and we showed that intact *Pa*BqsS was selectively and exclusively stimulated by the binding of Fe^{2+} in its periplasmic sensor domain. This study represented the first to characterize an intact HK bound to its cognate metal. However, many questions remained regarding the structure, the function, and the DNA-binding mechanism of the linked RR of this system, *Pa*BqsR.

In this study, we have determined the structural and the functional properties of the intact, activated, and truncated forms of the *P. aeruginosa* response regulator BqsR. We have solved the X-ray crystal structure of the N-terminal *Pa*BqsR receiver domain to 1.3 Å resolution, we have determined the structure of the C-terminal *Pa*BqsR DNA-binding domain (DBD) using nuclear magnetic resonance (NMR), and we have modeled the *Pa*BqsR-DNA interactions using AlphaFold. Excitingly, we have found a *Pa*BqsR DNA-binding site upstream of the *feo* operon

that encodes for the primary bacterial Fe^{2+} transporter machinery in *P. aeruginosa*. Using *in vitro* DNA-binding assays, we show that *Pa*BqsR can bind upstream of *feo* and within a location that overlaps the FUR box that regulates this operon in iron-replete conditions. Correlating with these *in vitro* observations, transcriptomic changes in Δ*bqsR* PAO1 show that *feo* is among the most differentially regulated genes by *Pa*BqsR, making *feo* one of the primary targets of this TCS. Interestingly, we also show that *Pa*BqsR binds Fe^{2+} itself, which has not been observed in other OmpR-like response regulators and could represent an additional layer of regulation in this system. Taken together, these data elucidate the structure and the DNA-binding properties of *Pa*BqsR for the first time, define its transcriptional regulon, and reveal an important and novel function for BqsRS in controlling *P. aeruginosa* Fe^{2+} acquisition.

RESULTS

The crystal structure of the N-terminal domain of PaBqsR reveals an OmpR/PhoB-like phospho-acceptor domain.

To determine the structural and biophysical properties of *P. aeruginosa* BqsR (*Pa*BqsR), we cloned, expressed, and purified the intact RR to homogeneity (Fig. S1a). At low concentrations (<< 1 mM), *Pa*BqsR migrated via gel filtration as a monomer, although at high concentrations (*ca.* 1 mM) this oligomerization shifted to a dimeric species (Fig. S1b), suggesting this protein is in dynamic equilibrium between monomer and dimer. Using both species of protein, we set up an extensive series of crystallization screens, optimized the obtained crystals, and tested for diffraction. All crystals indexed to the same space group, and our highest resolution was resolved to 1.3 Å (Table S1). However, a Matthew's analysis of the lattice parameters was inconsistent with the presence of intact *Pa*BqsR. Guided by sequence conservation amongst other similar RRs, we generated independent AlphaFold models of the N-terminal receiver and C-terminal DNA-binding domains (DBD) of *Pa*BqsR, and we used those as search models for molecular replacement (MR). The N-terminal receiver domain alone produced a definitive MR solution, whereas the C-terminal DBD did not. Subsequent refinement converged to a final model (R_w = 0.158, R_f = 0.175) in which only residues 1-123 (chiefly comprising the N-terminal receiver domain) are present (Table S1; PDB ID 8GC6). Despite exhaustive attempts to crystallize the intact, wildtype (WT) *Pa*BqsR protein, we were unable to do so, and all conditions produced crystals of the proteolyzed N-terminal receiver domain alone, likely due to the high dynamics in the linker region connecting this domain to the C-terminal DBD.[34]

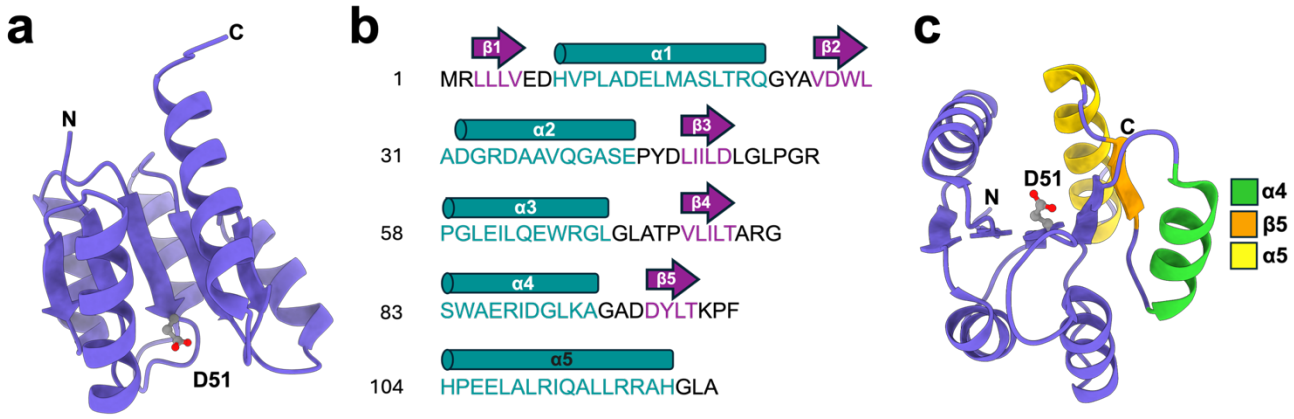

Figure 2. The crystal structure of the *Pa*BqsR N-terminal receiver domain (residues 1-123) resembles a phopho-acceptor domain of the OmpR/PhoB-like family. **a**. Crystal structure of the *Pa*BqsR N-terminal receiver domain reveals a canonical $(\beta\alpha)_5$ response regulator assembly with a central five-stranded parallel β-sheet surrounded by five α-helices. **b**. Cartoon topology of the *Pa*BqsR N-terminal receiver domain. **c**. Dimer formation for OmpR/PhoB response regulators is generally mediated by the α4–β5–α5 interface, highlighted in green, orange, and yellow, respectively. In all cases, 'N' and 'C' represent the N- and C-termini in the structure, respectively.

The N-terminal *Pa*BqsR receiver domain has strong structural homology to phosphor-acceptor domains of the family of OmpR/PhoB-like RRs (Fig. 2). In particular, our new structure reveals that *Pa*BqsR possesses a canonical $(\beta\alpha)_5$ response regulator assembly that consists of a central five-stranded parallel β-sheet surrounded by five α-helices (Fig. 2a,c). This topology is very common for the OmpR/PhoB-like family, and superpositioning reveals strong structural homology (Cα RMSDs < 1.0 Å) to other receiver domains found in bacterial RRs such as PhoP, ArlR, and KdpE (Fig. S2). One end of the receiver domain is highly negative in its electrostatic surface (likely driving interactions with its partner HK *Pa*BqsS), and the key, conserved phospho-accepting residue Asp^{51} stretches out to the surface of this part of the protein, likely to facilitate reaction with the phosphorylated HK to catalyze phospho-relay (Fig. 2a,c). In contrast, the dimerization of *Pa*BqsR is facilitated through a conserved dimerization domain comprising the α4–β5–α5 region (Fig. 2c) that is of mixed electrostatic polarity and ultimately links to the C-terminal DBD through a flexible linker. Unfortunately, in WT *Pa*BqsR, the dynamics of the flexible linker make this protein prone to spontaneous proteolysis over time in the crystallization

drop, which could not be avoided despite numerous efforts. Thus, a different approach was needed to characterize the C-terminal DBD.

The C-terminal DNA-binding domain (DBD) of PaBqsR bears a helix-turn-helix (HTH) topology.

As we failed to crystallize the intact structure of *Pa*BqsR, we cloned, expressed, and purified to homogeneity a construct that consisted only of the C-terminal *Pa*BqsR DBD (Fig. S1a). Gel filtration experiments demonstrate that the DBD maintains a monomeric oligomerization at both low and high concentrations (Fig. S1b), in contrast with the WT protein but consistent with

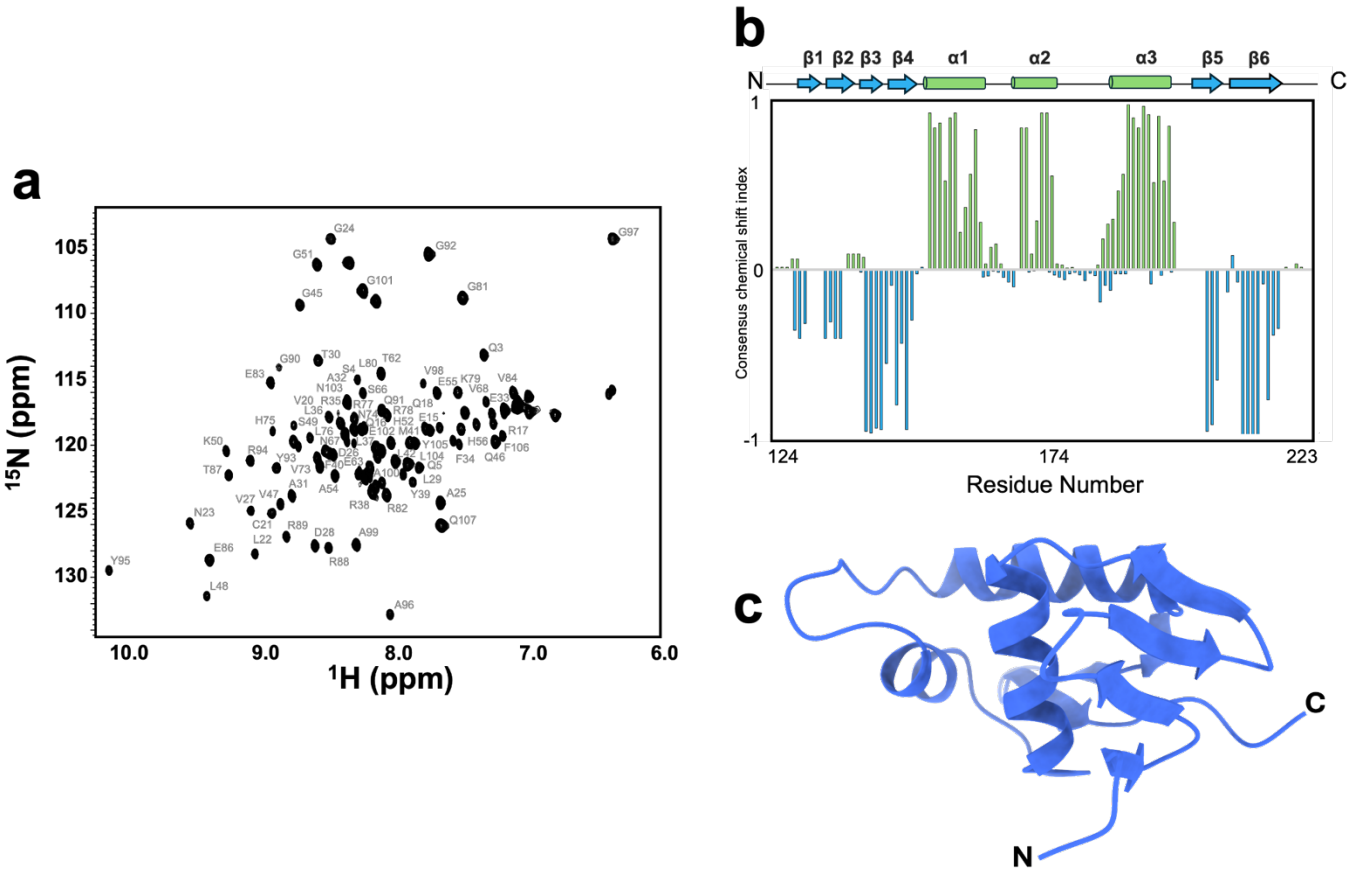

Figure 3. NMR and AlphaFold modeling reveal that the structure of the *Pa*BqsR DBD resembles a helix-turn-helix (HTH) topology common to the OmpR/PhoB-like family. **a**. Assigned 2D ^{1}H-^{15}N HSQC NMR spectrum of *Pa*BqsR DBD (500 µM at 298 K in 50 mM sodium phosphate, pH 5.5, 5 mM NaCl, 10 % (v/v) D_2O). **b**. Consensus chemical shift index (CSI) (Hα, Cα, and Cβ and C0) of *Pa*BqsR DBD, calculated using the program CSI 3.0.[35] Values nearing +1 indicate α-helical structure, values near 0 indicate unstructures regions/random coil, and values nearing -1 indicate β-strand structure.The cartoon at the top indicates the predicted toplogy of the *Pa*BqsR DBD based on CSI values derived from NMR experiments. **c**. AlphaFold model of *Pa*BqsR DBD corroborated with the 2D, 3D, and 4D HSQC and NOE NMR data. 'N' and 'C' represent the N- and C-termini in the structure, respectively.

the dimerization domain being located in the *Pa*BqsR N-terminal domain. Despite exhaustive attempts, we were unable to produce crystals with this protein. Therefore, we instead turned to nuclear magnetic resonance (NMR) coupled with AlphaFold modeling to determine the structure of the DBD. We first collected high-quality 2D ^{1}H-^{15}N heteronuclear single quantum coherence (HSQC) spectra of the *Pa*BqsR DBD (Fig. 3a) that indicated folded protein based on its well-dispersed amide signals. We then tested several pH ranges to optimize the intensity of the ^{1}H and ^{15}N chemical shifts (pH 5.5 was found to be optimal), we confirmed that the protein was stable for several weeks at this pH, and we used 2D NMR data collected at this pH for backbone amide assignments. We could ultimately assign 88 % of the non-proline backbone amide signals this way (Fig. 3a).

Cα chemical shift indices (CSI) combined with 3D ^{15}N-edited nuclear Overhauser effect (NOE) spectra, 4D ^{15}N/^{13}C-, and ^{13}C/^{13}C-edited NOE NMR spectra, and an AlphaFold model reveal that the *Pa*BqsR C-terminal DBD has a helix-turn-helix (HTH) DNA-binding topology common to the OmpR/PhoB RR family. First, the NMR-derived CSIs[35] (Fig. 3b) indicate that the secondary structure of the DBD is composed of a N-terminal set of 4 β-strands (β1, Leu^{128}–Ala^{130}; β2, Leu^{133}–Asp^{136}; β3, Lys^{141}–Leu^{144}; β4, Ala^{147}–Asp^{150}) followed by three α-helices (α1, Ala^{153}–Leu^{164}; α2, Lys^{172}–Leu^{179}; α3, Ser^{188}–Leu^{202}), wholly consistent with the AlphaFold model (Fig. 3c). The N-terminal β-sheet interacts with helix α1 through hydrophobic interactions while π-stacking interactions between the aromatic rings of Phe^{162} and Tyr^{215} bring the N-terminal β-sheet in proximity to the C-terminus. The three α-helices (α1-α3) form the trihelical motif of the HTH, with α3 representing the longest and most polar helix that interacts with the DNA major groove (*vide infra*). The DBD then terminates in an important β-hairpin (β5, Leu^{207}–Arg^{210}; β6, Gly^{214}–Tyr^{217}) (*vide infra*). This overall HTH fold is common to the DBDs amongst this family of RR,

and structural superpositioning reveals strong structural homology (Cα RMSDs < 1.0 Å) to other DBDs found in bacterial RRs such as PhoP, ArlR, and KdpE (Fig. S3).[36–40] However, subtle differences in the DNA-binding helices generally drive specificity and differentiate the gene targets of these RRs, which we then explored.

In vitro DNA-binding assays reveal that PaBqsR binds upstream of the feo operon in PAO1.

As we previously demonstrated that the sensor HK of the BqsRS system, *Pa*BqsS, binds and forms a stable interaction with Fe^{2+},[33] we hypothesized that one (or several) of the gene targets of this TCS would be involved in iron homeostasis, an essential component for bacterial survival and a critical factor in biofilm formation and virulence of *P. aeruginosa*.[29,41,42] Prior work defined the "BqsR box" sequence as 5'-TTAAG(N)$_6$-TTAAG-3'[21], so we searched the PAO1 genome for

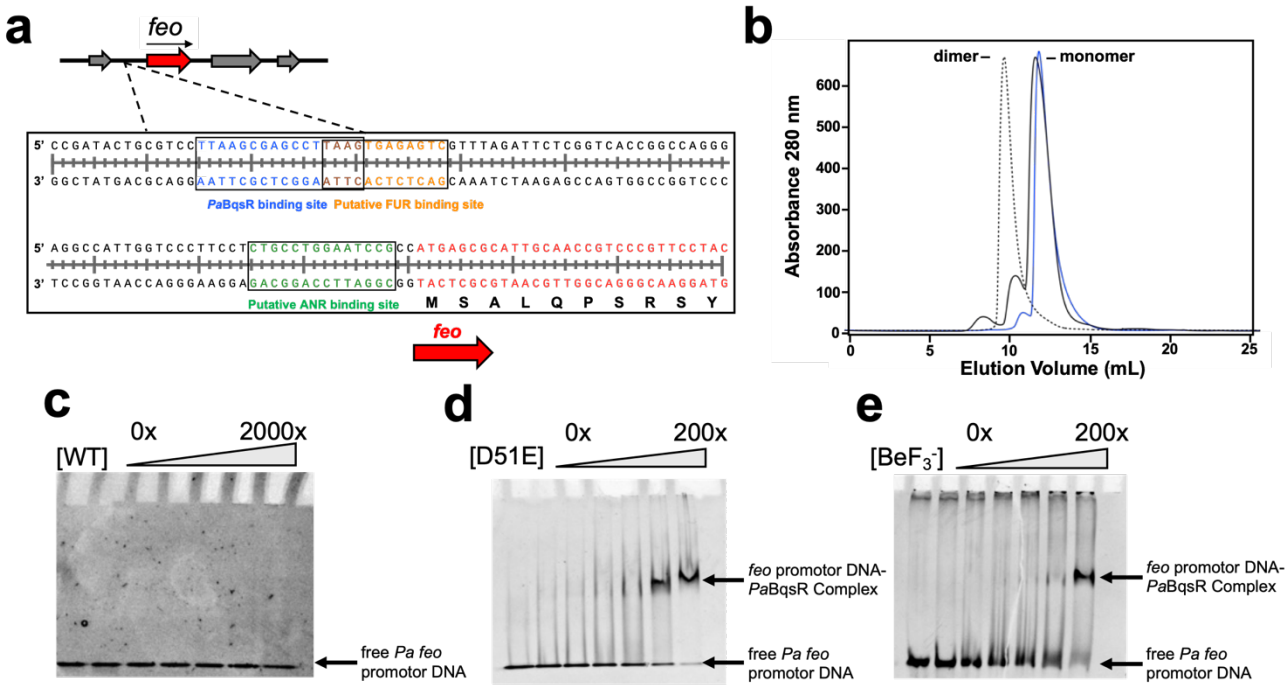

Figure 4. *Pa*BqsR binds upstream of the *feo* operon in PAO1. **a**. A putative "BqsR box" sequence (5'-TTAAG(N)$_6$-TTAAG-3'; blue) is present in PAO1 upstream of *feo*, overlaps with a putative ferric uptake regulator (FUR) box (orange), and is upstream of the anaerobic transcriptional regulator (ANR) box (green), adjacent to the start site of the *feo* operon (red). **b**. Size exclusion chromatography (SEC) profiles of WT *Pa*BqsR (black), D51E *Pa*BqsR (blue), and BeF_3^--activated *Pa*BqsR (dashed). BeF_3^--activated *Pa*BqsR is a dimer, while WT and D51E *Pa*BqsR are both monomers at low concentrations (<< 1 mM). **c**. EMSAs with WT *Pa*BqsR and the *feo* duplex. **d**. EMSAs with D51E *Pa*BqsR and the *feo* duplex. **e**. EMSAs with the BeF_3^--activated *Pa*BqsR and the *feo* duplex.

this DNA sequence and found it upstream of several genes of interest related to iron homeostasis and iron metabolism, such as those involved in spermidine synthesis,[43] Fe^{3+} reduction,[44,45] and Fe^{2+} acquisition[46] (Table S2). Given that *Pa*BqsR can sense Fe^{2+} directly, we were particularly interested in whether *Pa*BqsR could target the *feo* operon that encodes for the primary Fe^{2+} transport machinery in PAO1. Interestingly, we observe a putative "BqsR box" upstream of the PAO1 *feoA* start site, overlapping with a putative ferric uptake regulator (FUR) box (a global, negative iron regulator), but distal from, and independent of, a putative anaerobic transcriptional regulator (ANR) box (a global regulator of anaerobic genes) (Fig. 4a). These results suggested that *Pa*BqsR could regulate *feo* expression in PAO1, which we then tested *in vitro*.

To determine whether *Pa*BqsR could bind to the "BqsR box" upstream of the *feo* operon, we used *in vitro* electrophoretic mobility shift assays (EMSAs). To do so, we tested binding using a 21 base-pair (bp) DNA duplex corresponding to minimal region needed for protein binding (Table S3). Intact, monomeric, WT *Pa*BqsR (Fig. 4b) failed to bind to this duplex when tested across a range of concentrations from 0-2000 mol. eq. protein:DNA (Fig. 4c). This behavior was expected, as the canonical TCS paradigm indicates that the RR must either be phosphorylated or pseudo-phosphorylated to facilitate DNA binding.[7] As RR pseudo-phosphorylation can be achieved by producing an Asp→Glu variant in which alteration in length of the phosphorylatable Asp residue results in RR activation, or by reaction with BeF_3^- that binds to the phosphorylatable Asp residue in the RR and mimics the presence of a phosphate group, we tested both approaches. We first used mutagenesis to create a *Pa*BqsR D51E variant that we expressed and purified to homogeneity (Figs. 4b, S1b). Interestingly, while this protein remained monomeric in solution (Figs. 4b, S1b), the D51E variant showed clear and distinct binding to the *feo* duplex (Fig. 4d), demonstrating that protein dimerization in the absence of DNA is not a pre-requisite for intact

*Pa*BqsR to bind to DNA, and that pseudo-phosphorylation enhances the *Pa*BqsR-DNA interaction. We then generated the pseudo-phosphorylated, WT BeF_3^--activated form of *Pa*BqsR that was clearly dimeric in solution (Figs. 4b, S1b). The BeF_3^--activated phospho-mimetic of *Pa*BqsR also bound to the *feo* duplex with an apparent affinity nearly identical to the D51E variant (Fig. 4e). Control EMSAs using bovine serum albumin (BSA) in place of *Pa*BqsR failed to bind to the *feo* duplex, demonstrating this interaction to be *Pa*BqsR-specific (Fig. S4d). Finally, we tested whether the monomeric *Pa*BqsR C-terminal DBD alone could bind to DNA. Indeed, we observed binding to the *feo* duplex with only the DBD (Fig. S4), but the apparent binding was 2-3 fold weaker than that of either D51E or the BeF_3^--activated form of full-length *Pa*BqsR (Fig. S4), indicating that the presence of the N-terminal receiver domain contributes to the binding affinity of this RR to its DNA sequence. Taken together, these results demonstrate that *Pa*BqsR may target the *feo* upstream region *in vitro*, suggesting that this TCS is a regulator of a critical iron uptake system frequently used by *P. aeruginosa* at the host-pathogen interface.[47]

Modeling of PaBqsR and the PAO1 feo operon illustrates a unique mode of DNA binding.

We next sought to use structural approaches to understand how *Pa*BqsR binds to the upstream *feo* region. To do so, we first tried to crystallize both *Pa*BqsR D51E and the BeF_3^--activated forms of *Pa*BqsR in the presence of the *feo* upstream region duplex modified to include an additional G-C overhang, which has been shown to increase crystallization propensity of other DNA-protein complexes.[48] While we were able to generate crystals of both complexes, these crystals only diffracted weakly and anisotropically despite extensive optimization. We then turned to AlphaFold to model the *Pa*BqsR-*feo* interaction (Fig. 5a), as we already possessed experimentally-determined structural information on both domains of *Pa*BqsR. Surprisingly, there

is little consensus on how exactly OmpR/PhoB-like RRs recognize and bind to their target DNA sequences. For example, the DBDs of PhoB, PmrA, or RstA bind DNA in a head-to-tail orientation,[36,38,40] whereas the DBD of OmpR binds to various DNA sequences in either a head-to-head or head-to-tail orientation,[37,49,50] further underscoring the importance of investigating the *Pa*BqsR DBD. Consistent with the behavior of the BeF_3^--activated form of WT *Pa*BqsR, AlphaFold predicts binding of *Pa*BqsR to the *feo* upstream region as a dimer (Fig. 5a), as no binding is predicted when only a single copy of *Pa*BqsR is provided as an input. Intriguingly, modeling predicts that dimerization at the N-terminal phospho-acceptor domain occurs in a head-to-tail manner, and this dimerization orientation may be facilitated by the phosphorylation of Asp[51] (Fig. 5a). Importantly, as predicted based on NMR data (Fig. 3), α3 of the trihelical HTH motif of

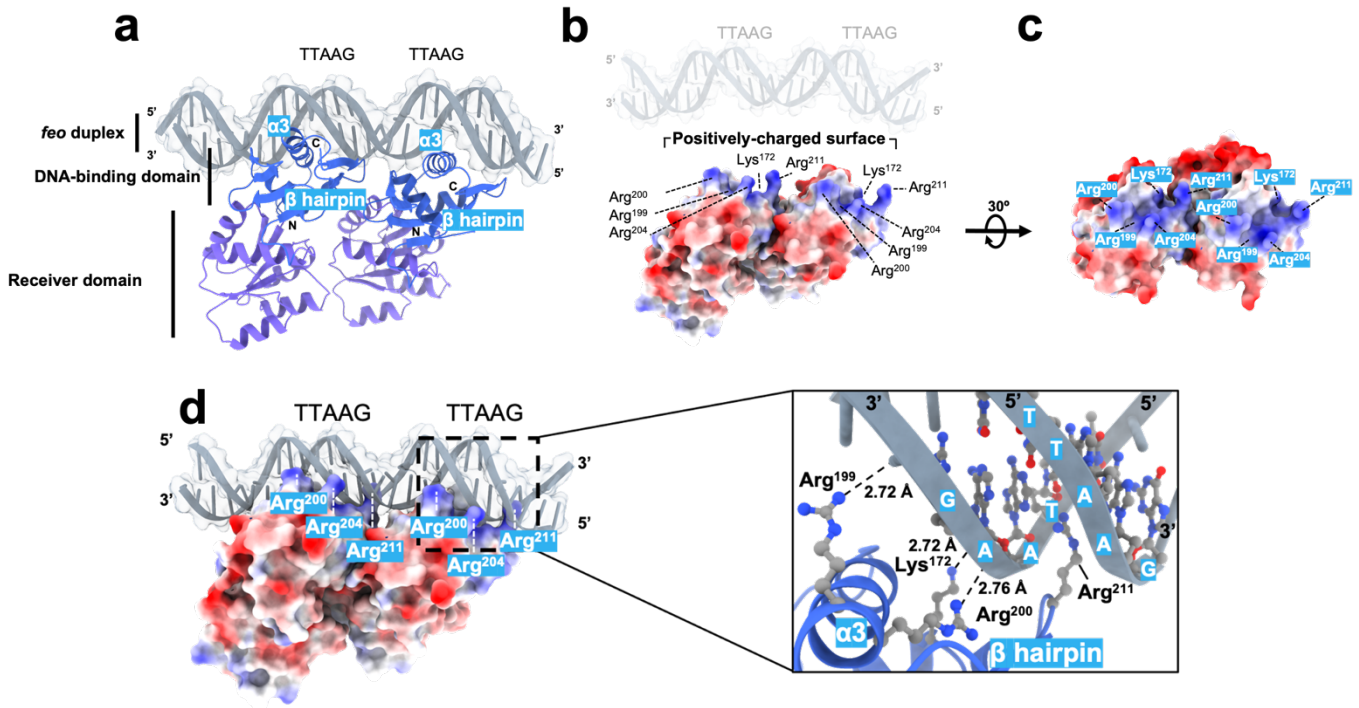

Figure 5. Modeling of *Pa*BqsR binding to the *feo* duplex. **a**. AlphaFold model of WT, intact *Pa*BqsR bound to the *feo* upstream duplex. The region in purple is the N-terminal receiver domain, while the region in blue is the C-terminal DNA-binding domain (DBD). **b,c**. An electrostatic surface comprising Lys^{172}, His^{194}, His^{197}, Arg^{199}, Arg^{200}, and Arg^{204} extends across the dimer and utilizes the exposed α3 of each *Pa*BqsR monomer to bind within the DNA major groove. **d**. Lys^{172}, Arg^{199}, Arg^{200}, and Arg^{211} directly interact with the "BqsR box," with residue Arg^{211} forming an anchor that π-stacks into each half of the tandem repeat, providing specificity. In all cases, 'N' and 'C' represent the N- and C-termini in the structure, respectively.

the C-terminal DBD binds within the major groove of DNA (Fig. 5a) but within the variable nucleotide region and upstream (towards the 5' end of the sense strand) of the conserved 5'-TTAAG-3' sequence (Fig. 5a). Basic residues of *Pa*BqsR such as Lys^{172}, His^{194}, His^{197}, Arg^{199}, Arg^{200}, and Arg^{204} create a positively-charged surface that includes the exposed face of α3 on each *Pa*BqsR protomer and extends across the dimer (Fig. 5a-c). This important region contacts the DNA backbone and helps place α3 into the major groove of the DNA (Fig. 5b-d) with the sidechains of Lys^{172}, Arg^{199}, and Arg^{200} making contact tightly with the DNA phosphates (with the closest sidechain-phosphate distances of 2.72 Å, 2.72 Å, and 2.76 Å, respectively) (Fig. 5d, inset). Additionally, the terminating β-hairpin of each dimer contains Arg^{211}, an anchoring Arg residue that makes direct contacts with the "BqsR box" through three specific nucleobases of the tandem repeat: Arg^{211} on the first protomer hydrogen bonds with T^{33} and A^{34} on the sense strand and A^{24} on the antisense strand, while Arg^{211} on the second protomer hydrogen bonds with A^{36} on the sense strand and T^{22} and A^{23} on the antisense strand (Fig. 5d, inset). Thus, modeling suggests that at least 7 different amino acids in the DBD facilitate critical contacts between each *Pa*BqsR and DNA, with Lys^{172}, Arg^{199}, Arg^{200}, and Arg^{211} likely playing the most important roles in recognizing the "BqsR box". These data confirm the observations made by our EMSAs (Fig. 4) and suggest how *Pa*BqsR interacts with the previously defined "BqsR box" for the first time.

Transcriptomic data reveal that BqsR controls Fe^{2+} acquisition in PAO1.

To identify the regulon of BqsR, a genome-wide RNAseq-based transcriptomics analysis was performed for Δ*bqsR* and its parent strain, WT PAO1. Approximately 44% of the PAO1 genome was differentially expressed in Δ*bqsR* compared to the WT, with 1311 genes significantly upregulated ($\log_2FC > 1$, $p < 0.05$) and 1128 genes significantly downregulated ($\log_2FC < -1$, $p <$

0.05), demonstrating that *Pa*BqsR functions as both a positive and negative regulator (Fig 6). This vast array of regulation has been corroborated by previous results[21,51] and indicates that *bqsR* is a global regulator in *P. aeruginosa.* The ability to act as both a positive and negative transcriptional regulator is not unique to *Pa*BqsR, as several other RRs have been reported to have similar

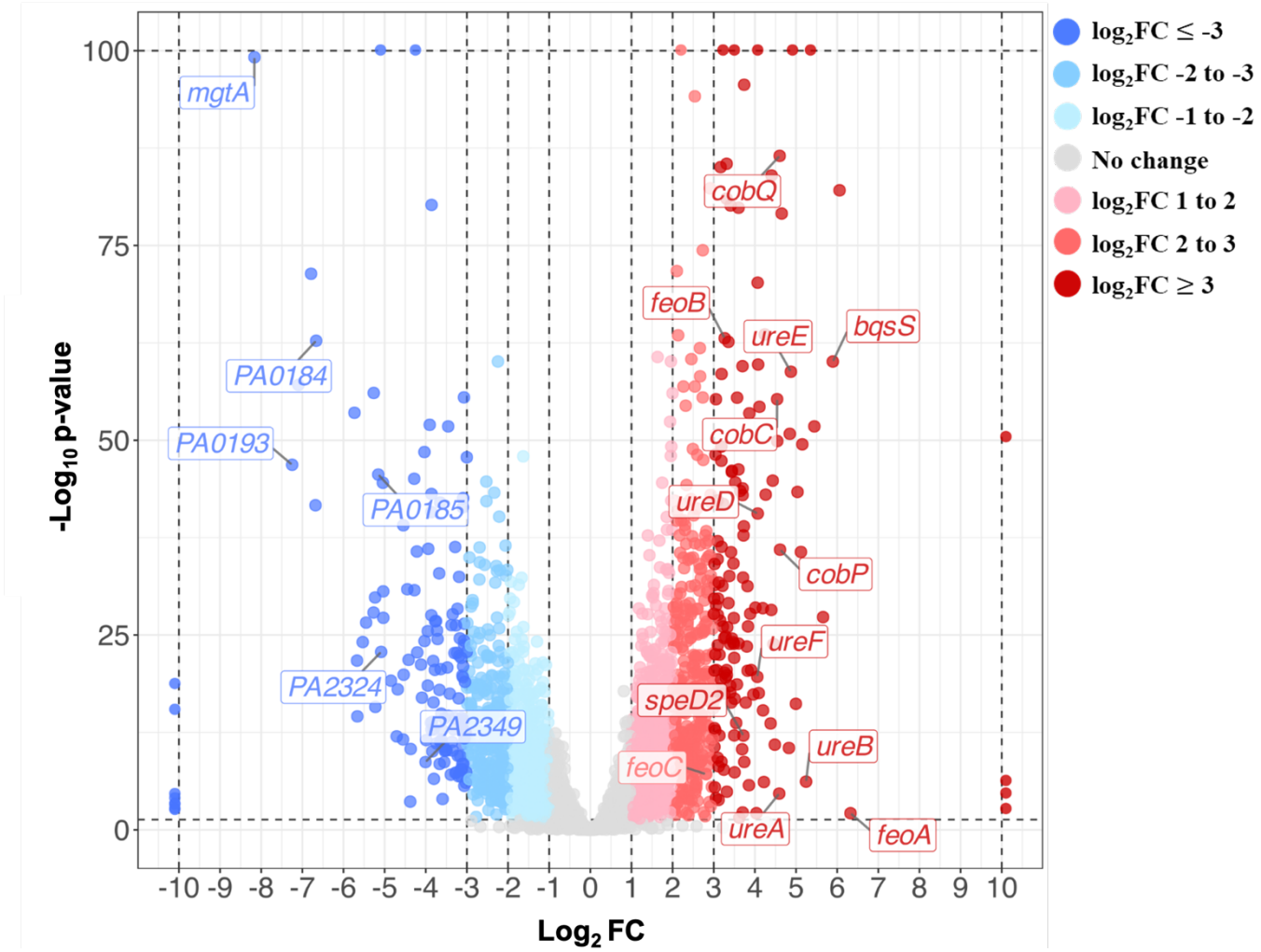

Figure 6. RNA-seq based genome-wide transcriptional response in *P. aeruginosa* PAO1 to the deletion of *bqsR*. Differently expressed genes ($\log_2$FC > |1|, $p < 0.05$) were stratified based on their $\log_2$FC into three categories: low response with the expression changed by $\log_2$FC |1-2| (light red/blue); moderate response with $\log_2$FC |2-3| (medium red/blue); and top responders with $\log_2$FC ≥ |3| (dark red/blue). Genes that showed no change in expression are shown in gray. False discovery rate (*p*-value) is plotted as $-\log_{10}$ *p*-value with a cutoff of 1.3 ($p = 0.05$) indicated by a horizontal dashed line. Genes with $\log_2$FCs ≥ 10 and/or $-\log_{10}$ *p*-values ≥ 10 are depicted along the outermost dashed lines for a clear visual. Genes encoding the most significantly altered pathways are labeled with their gene name or PA number (as annotated in pseudomonas.com). The graph was generated in RStudio.

dynamic function.[18,52–54] However, previous research has implied that *Pa*BqsR acts as a classical transcriptional activator when phosphorylated.[21] To better understand the response, we stratified

the differentially regulated genes into three categories: a low "tuning" response with the expression changed by |1| < log2FC < |2| that includes 1527 genes; a moderate response with |2| < log2FC < |3| that includes 616 genes; and 296 top responders with log2FC > |3|. It is possible that the moderate and top responders are the targets of direct regulation by *Pa*BqsR, whereas the low "tuning" response is the result of indirect secondary regulation. To determine the biological significance of the regulation and identify the *Pa*BqsR regulated processes, the moderate and top responders were sorted into functional categories based on curated gene annotations. Among them, we detected vitamin B_{12} biosynthesis, urea metabolism and transport, sulfur metabolism, antibiotic resistance, and magnesium transport (Fig 6). As predicted, one of the top responding pathways was Fe^{2+} acquisition encoded by the *feo* operon with *feoA*, *feoB*, and *feoC* expression increased by a log_2FC of 6.3, 3.3, and 2.8, respectively (Fig. 6). The observed increased expression of *feoABC* in the mutant compared to WT indicates that *Pa*BqsR plays a negative role in *feo* expression. Further, we observed that the deletion of *bqsR* increased the expression of its cognate sensor, *bqsS*,

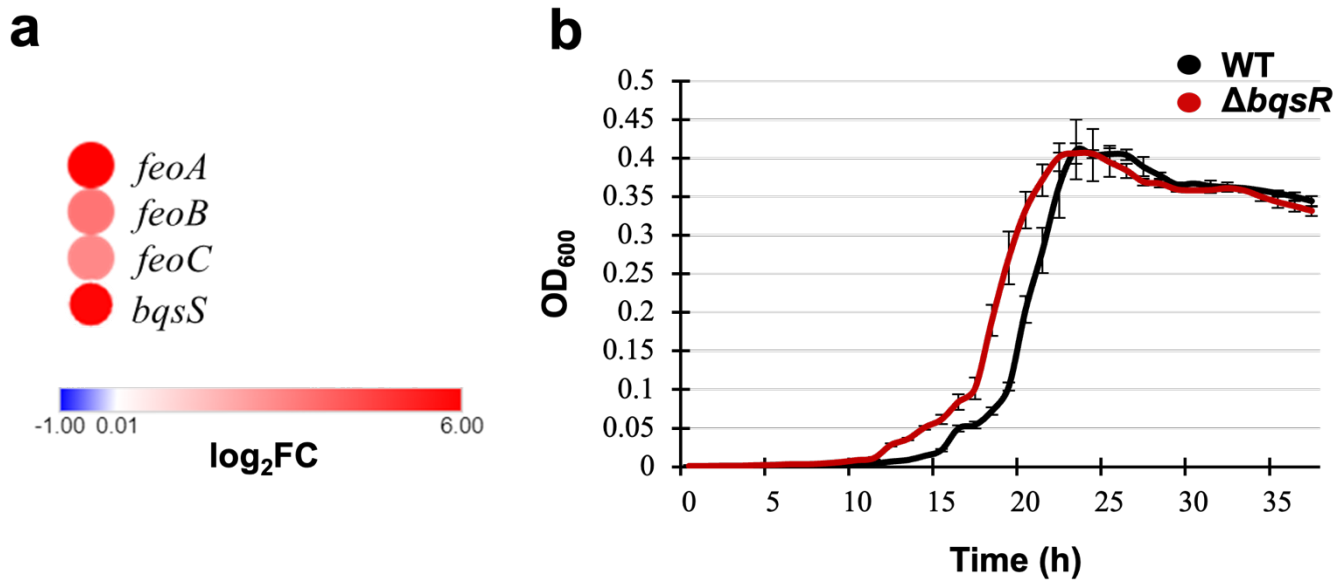

Figure 7. *Pa*BqsR regulates itself and Fe^{2+} uptake. **a**. The RNA-seq based heat map showing log_2FC values of expression comparisons of the *feo* operon and *bqsS* in Δ*bqsR* vs WT PAO1. **b**. The deletion of *bqsR* elicits an earlier transition to the log phase when grown statically in BMM8.5 in the presence of 3.6 μM Fe^{2+} compared to WT PAO1.

by a $\log_2$FC of 5.8 (Fig. 6). This observation corroborates previous data, as *Pa*BqsRS has been identified to autoregulate its expression.[31] Importantly, these findings identify a relatively large regulon of *Pa*BqsR and indicate that this TCS has a major role in controlling Fe^{2+} acquisition, likely by sensing the increasing levels of extracytoplasmic Fe^{2+} and negatively regulating *feoABC* expression, potentially as a protective mechanism.

To test the impact of BqsR regulation on *P. aeruginosa* Fe^{2+} uptake, we performed growth studies for WT and Δ*bqsR* PAO1. Compared to the WT, Δ*bqsR* showed a positive growth impact, as indicated by a shorter lag phase when grown in the presence of Fe^{2+} as the sole source of iron (Fig. 7). These data suggest that the positive expression of the *feo* genes in the absence of *bqsR* provides the capacity for the mutant to take up more Fe^{2+}, in turn, conferring a positive effect on growth. These findings are consistent with the negative regulatory role of *Pa*BqsR on *feo* expression revealed in the RNA-seq analysis (*vide supra*).

PaBqsR binds to Fe^{2+}, revealing a novel mode of regulation in this TCS.

Finally, in our structural characterization of the various domains of *Pa*BqsR, we noted two regions of the protein conspicuously rich in potential iron-binding residues (a CxC motif in the DBD and a His-rich motif at the domain-domain interface; Fig. 8a), so we tested whether intact, WT *Pa*BqsR could bind Fe^{2+} like that of its HK counterpart, *Pa*BqsS.[33] To our surprise, we observed stable binding of a single Fe^{2+} ion to WT *Pa*BqsR after anoxic loading and copious buffer exchanges, as confirmed by ferrozine assays and ICP-MS (Fig. S5). To characterize the identity and local structure of the Fe^{2+} ion bound to WT *Pa*BqsR, we then used X-ray absorption spectroscopy (XAS). The energy of the inflection of the X-ray absorption near-edge structure (XANES) spectrum of Fe^{2+}-bound WT *Pa*BqsR as well as its lack of pre-edge features are

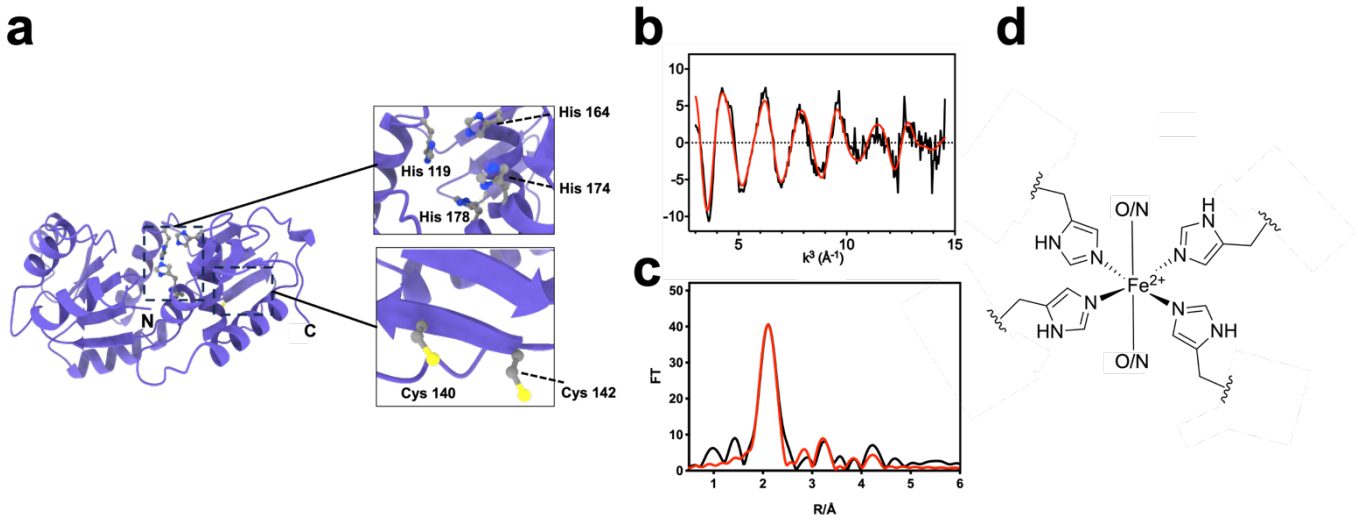

Figure 8. Intact, WT *Pa*BqsR binds Fe^{2+}. **a**. AlphaFold model of intact, WT *Pa*BqsR. Highlighted are the His-rich region at the interface of the two domains (*top right*) and the Cys-rich region (CxC motif; *bottom right*) in the DBD. **b**,**c**. EXAFS of *Pa*BqsR bound to Fe^{2+} under anoxic conditions. The processed EXAFS (**b**) and the resulting Fourier transformed data (**c**) are consistent with the presence of Fe^{2+} in a His-rich, octahedral geometry. The black traces represent the experimental data, while the red traces represent the EXCURVE-fitted data. **d**. Cartoon representation of the proposed Fe^{2+} coordination of *Pa*BqsR. 'N' and 'C' represent the N- and C-termini in the structure, respectively.

consistent with an octahedrally-coordinated Fe^{2+} ion (Fig. S6). Simulations of the extended X-ray absorption fine structure (EXAFS) data of *Pa*BqsR (Fig. 8b,c) reveal only N/O-based environments surrounding the Fe^{2+} ion (Fig. 8d), with 4 ligands best fitted to His residues at 2.09 Å from the Fe^{2+} ion, while 2 ligands (perhaps water) are coordinated more weakly and are observed at 2.17 Å from the Fe^{2+} ion (Table S4). These data definitively rule out coordination of Fe^{2+} at the CxC motif in the C-terminal binding domain and strongly suggest that the His-rich region at the interface of the two domains in *Pa*BqsR (comprising His^{119}, His^{164}, His^{174}, His^{178}) binds the Fe^{2+} ion. Consistent with these notions, anoxic Fe^{2+} loading with just the C-terminal DBD of *Pa*BqsR failed to show any metal binding (data not shown). The effects of Fe^{2+}-binding on the ability of *Pa*BqsR to interact with DNA remain currently unknown, but this observation suggests that the concentration of intracellular labile Fe^{2+} in *P. aeruginosa* may further regulate *Pa*BqsR function. To our knowledge, *Pa*BqsRS thus represents the first example in which a cytosolic RR of a TCS binds and responds to the same signal/stimulus that is sensed extracytoplasmically by its cognate

HK, and these data demonstrate an important functional divergence in this RR compared to other members of the OmpR-like family.

DISCUSSION

TCSs are known to play a key role in the production of virulence factors by infectious bacteria,[5,55] allowing for these pathogens to adapt and to establish infection within diverse environments of host organisms. As just a couple of examples, the *E. coli* PhoPQ TCS alters the ability of *E. coli* to survive when challenged to antibiotics,[56,57] while the *F. tularensis* QseBC TCS alters bacterial motility for downstream infection.[58] Germane to this work, the *P. aeruginosa* BqsRS TCS regulates the production of the virulence factor pyocyanin,[22] the expression of genes involved in biofilm formation,[20] and the expression of genes involved in antibiotic resistance.[21] Despite this critical link between TCS function and the establishment of infection,[20] our understanding of how a membrane HK selects for its cognate stimulus, and how its cognate RR is selective for target genes both remain understudied, particularly for the Fe^{2+}-sensing *Pa*BqsRS system.

While *Pa*BqsS was recently characterized at the protein level,[33] its cognate partner, *Pa*BqsR, had not been. Based on sequence homology, *Pa*BqsR was predicted to be an OmpR/PhoB-like RR,[21] the most common family of RR present in Gram-negative bacteria.[59] Consistent with this notion, our X-ray crystal structure of the *Pa*BqsR receiver domain reveals a $(\beta\alpha)_5$ response regulator assembly consisting of a central five-stranded parallel β-sheet surrounded by five α-helices with an overall strong structural homology to members of the OmpR-like family. Stretching to the surface of the domain is the Asp residue (Asp^{51}) conserved across the OmpR-like family that receives the phosphate moiety from the HK to set into motion the downstream DNA-binding process, mediated by the *Pa*BqsR DBD. Our NMR structural results show that the *Pa*BqsR DBD comprises a trihelical helix-turn-helix (HTH) fold that is common for a number of DNA-binding proteins.[36–40] However, the DNA-binding domains of RRs serve as the bases for promoter

sequence recognition within an organism,[49] and we explored which genes in PAO1 may be the functional targets of BqsR and how this RR interacts with its DNA targets.

As our previous work demonstrated that the membrane HK of the BqsRS system, *Pa*BqsS, was selectively activated by Fe^{2+},[33] we considered whether the DBD of the *Pa*BqsR might target one or several genes related to Fe^{2+} homeostasis. RRs of the OmpR-like class are well-known to bind tandem repeat DNA sequences,[36,60] and a previous study demonstrated that *Pa*BqsR binds to a DNA sequence comprising 5'-TTAAG$(N)_6$-TTAAG-3' (also known as the "BqsR box").[21] A genome-wide search of *P. aeruginosa* found this sequence upstream of over 100 genes, including those involved in metal transport, lipopolysaccharide modulation, polyamine synthesis and transport, and antibiotic resistance.[21,31,32] Of particular importance, we have shown that the "BqsR box" is found in the promoter region of *feo*, an operon that encodes for the Feo system, the most widespread prokaryotic Fe^{2+} transport system.[46,61] *In vitro* EMSAs with recombinantly-produced, pseudo-activated *Pa*BqsR confirmed its ability to bind to the upstream region of *feo*. Moreover, the ability of *Pa*BqsR to bind directly to Fe^{2+} is, to our knowledge, unique amongst OmpR-like response regulators, although its functional ramifications require further investigation.

In order to determine whether *feo* is under the regulation of BqsR *in vivo*, transcriptional analyses and subsequent growth studies utilizing PAO1 were performed. The genome-wide RNA-seq analysis identified a relatively large *Pa*BqsR regulon comprising *ca*. 44 % of the PAO1 genome, 37 % of which belong to moderate and top responders that are likely to represent the direct targets of *Pa*BqsR. Consistent with our EMSAs, the *feo* operon was one of the top set of genes regulated, as well as those encoding for Mg^{2+} transport, antibiotic resistance, polyamine biosynthesis, vitamin B_{12} biosynthesis, urea metabolism/transport, and even sulfur metabolism. Interestingly, both vitamin B_{12} biosynthesis and urea metabolism/transport genes were

upregulated, whereas genes encoding Feo, sulfur metabolism, and Mg^{2+} transport were downregulated in the deletion mutant, suggesting that *Pa*BqsR functions as both a positive and negative global regulator, consistent with prior results.[18,52–54] Supporting the negative regulation of the *feo* operon, we observed a growth advantage in the mutant strain in the presence of Fe^{2+}. Moreover, the expression of *bqsS* was also significantly increased in the absence of *bqsR*, corroborating previous results and underscoring the regulatory feedback controlling the expression of this system.[31] Together with our previous findings,[33] this work indicates that *Pa*BqsRS senses both the cytoplasmic and extracytoplasmic Fe^{2+} concentrations, and, in response, regulates Fe^{2+} acquisition in a balancing act between its nutritional necessity and its toxicity.

This work now shows that the BqsRS system plays an important role in general iron homeostasis (Fig. 9) as well as general metal homeostasis in PAO1. Based on current and previous data, we propose that *Pa*BqsS senses extracytoplasmic Fe^{2+} selectively, upregulating its ATP-related kinase activity,[33] thus causing *Pa*BqsR dimerization and DNA binding. Activated *Pa*BqsR then binds upstream of *feo* and downregulates its expression, therefore acting as a selective negative regulator for the Feo system (before deactivation by FUR) in order to attenuate Fe^{2+} uptake and to prevent Fe^{2+} toxicity. This control of the *feo* operon may be linked to the bacterial biofilm lifecycle, another function known to be regulated by *Pa*BqsR.[20] Specficially, in *P. aeruginosa* too little iron prevents biofilm formation while excess iron is linked to biofilm dispersal.[62–64] Thus, a finely-tuned ability to sense environmental iron via BqsRS may allow *P. aeruginosa* to select its mode of growth most apt for survival, making this TCS an important virulence factor. Interestingly, and in contrast to Fe^{2+} transport, Mg^{2+} transport appears to be positively regulated by *Pa*BqsR, as the expression of Mg^{2+} transport genes (*mgtA*, *mgtC*, and *mgtE*) are significantly downregulated in the Δ*bqsR* PAO1 strain. Considering the previously

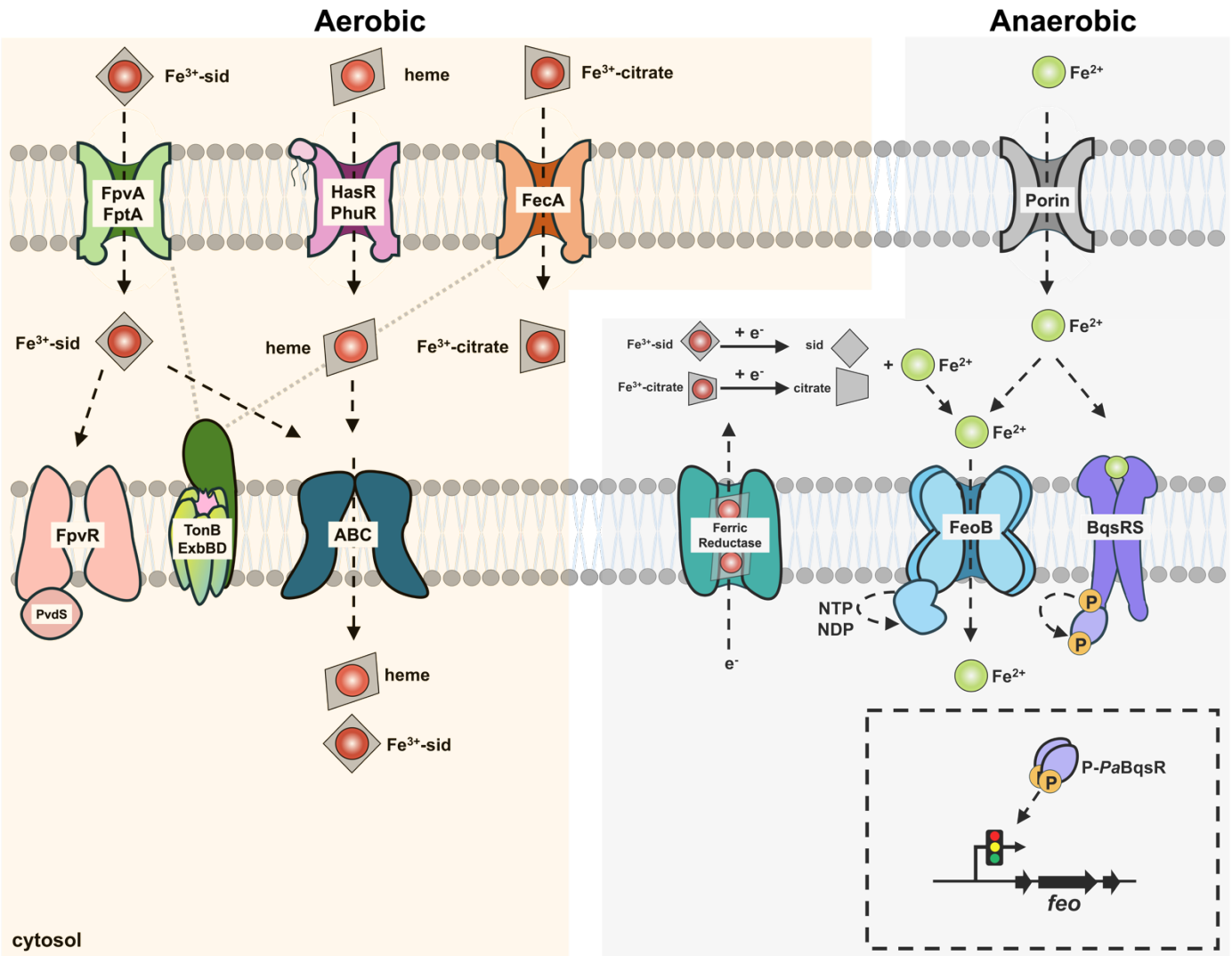

Figure 9. Cartoon of the primary iron homeostasis pathways in *P. aeruginosa* and their relationships to BqsRS. Under oxic conditions, chelated Fe^{3+}-siderophore complexes such as pyoverdine are shuttled into the periplasm by outer membrane proteins such as FpvA and FptA. Fe^{3+}-citrate uptake is accomplished by FecA. Iron protoporphyrin IX (heme *b*) is sensed and imported by the HasR/PhuR system. These chelated iron complexes are typically brought into the cytosol via ABC transporters, although other mechanism may strip the chelates of the iron itself. Under anoxic conditions, Fe^{2+} is thought to diffuse into the periplasm through outer membrane porins or may be generated in the periplasmic space by ferric reductases. The Feo system then transports Fe^{2+} into the cytosol. The Fe^{2+}-specific TCS BqsRS directly senses Fe^{2+} and transcriptionally regulates *feo* (among other genes), which is shown here for the first time.

characterized responsiveness of BqsRS (CarRS) to Ca^{2+} and its role in maintaining intracellular Ca^{2+} homeostasis,[22] these data expand the role of BqsRS as a general regulator of metal homeostasis, highlighting its multifaceted contributions to *P. aeruginosa* adaptive physiology. When taken together, our findings reveal that the BqsRS TCS directly regulates *feo* expression while also sensing extra- and intracellular signals in order to regulate the transcription of numerous

genes across the metabolic landscape of PAO1. Given this importance, the BqsRS system may represent a very attractive future therapeutic target to treat *P. aeruginosa* infections.

Acknowledgements

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Author contributions

A.P., H.S., M.H., J.B.B., K.N.C., M.A.P., and A.T.S. designed the research; A.P., H.S., M.H., D.G., A.J.N., J.B.B., and K.N.C. performed the research; A.P, H.S., M.H., K.N.C., M.A.P., and A.T.S. analyzed the data; A.P., H.S., M.H., K.N.C., M.A.P., and A.T.S. wrote and edited the paper.

Additional information

Supplementary information. Supplementary information accompanies this paper that includes supplementary figures S1-S6, supplementary tables S1-S4, as well as any additional experimental details, materials, and methods.

Competing interests. The authors declare no competing interests.

METHODS

Materials. All codon-optimized genes used in this study were synthesized and verified by GenScript. Materials used for buffer preparation, protein expression, and protein purification were purchased from standard commercial suppliers and were used as received. Isotopically-enriched ammonium chloride ($^{15}NH_4Cl$) and glucose (globally $^{13}C_6$-labeled) were purchased from Cambridge Isotope Laboratories and used as received. All DNA duplexes encoding for the upstream region of the *feo* operon in *Pseudomonas aeruginosa* strain PAO1 were synthesized and verified by Integrated DNA Technologies. D_2O was purchased from MilliporeSigma and used as received.

Bacterial strains and media. *P. aeruginosa* strains PAO1 and Δ*bqsR*[22] were grown in biofilm minimal media 8 (BMM8.5) containing 9.0 mM sodium glutamate, 5 mM glycerol, 0.08 mM $MgSO_4$, 0.15 mM NaH_2PO_4, 0.34 mM K_2HPO_4, 145 mM NaCl, 200 µL trace metals, and 1 mL vitamin solution (per L of medium). The trace-metals solution was prepared with 5.0 g $CuSO_4 \cdot 5H_2O$, 5.0 g $ZnSO_4 \cdot 7H_2O$, and 2.0 g $MnCl_2 \cdot 4H_2O$ per liter of 0.83 M hydrochloric acid. The vitamin solution was prepared by dissolving 0.5 g thiamine and 1 mg biotin (per L of the final medium). Filter sterilized trace metals, $MgSO_4$, and the vitamin solution were added aseptically to autoclaved medium and adjusted to a pH of 7. This prepared BMM contains no iron. To prepare BMM8.5 with added iron, $FeSO_4$ was prepared as 100x stocks containing 360 µM iron. To minimize the oxidation of Fe^{2+}, ascorbic acid (1 mM final) was added to the water prior to mixing in the iron sources. The stocks were then filter sterilized and added to the "no-iron" BMM8.5 for a final concentration of 3.6 µM of $FeSO_4$.

Growth studies. *P. aeruginosa* strains were grown in "no iron" BMM8.5 at 37 °C until mid-log phase (12 h), after which they were normalized to an OD_{600} of 0.3 and inoculated 1:100 into fresh medium containing 3.6 µM $FeSO_4/FeCl_3$. Cultures were grown statically (to minimize oxidation of Fe^{2+}) in clear 96-well plates at 37 °C for 36 hr in a BioTek SynergyMx. The absorbance at 600 nm was measured every hr.

Protein expression and purification. Codon-optimized DNA blocks encoding for *Pseudomonas aeruginosa* strain PAO1 BqsR (Uniprot identifier Q9I0I1) with either intact (amino acids 1-223, WT *Pa*BqsR) or the C-terminal DNA-binding domain (amino acids 123-223, *Pa*BqsR DBD) modified to encode for an additional C-terminal tobacco etch virus (TEV) protease site (ENLYFQS) were commercially synthesized by Genscript. These DNA sequences were then subcloned into the pET-21a(+) expression vector such that the resulting expression construct was read in-frame with a terminating C-terminal $(His)_6$ tag. Generation of the intact D51E variant of *Pa*BqsR was accomplished using site-directed mutagenesis. Mutations to the WT *Pa*BqsR expression plasmid was accomplished by the QuikChange Lightning Multi Site-Directed Mutagenesis kit (Agilent). The following primers were used to introduce the mutated bases (underlined): (D51E forward: 5'-CCCGGCAGACCCAGTTCCAGAATGATCAG-3'; D51E reverse: 5'-CTGATCATTCTGGAACTGGGTCTGCCGGG-3'). The presence of the appropriate mutation was verified by nanopore DNA sequencing. Each expression plasmid was electroporated into electrocompetent *Escherichia coli* BL21 (DE3) cells, spread onto Luria Broth (LB) agar plates supplemented with 0.1 mg/mL ampicillin (final), and grown overnight at 37 °C. Large-scale expression of all natural-abundance *Pa*BqsR constructs (intact WT, D51E, or the DBD) in rich media was accomplished in 6 baffled flasks each containing 1 L sterile LB supplemented with 0.1

mg/mL ampicillin (final) and inoculated with a pre-culture derived from a single bacterial colony. Cells were shaken at 200 RPM until the optical density (OD) at 600 nm (OD_{600}) reached *ca*. 0.6-0.8. Cell cultures were then cold-shocked for approximately 2 hours at 4 ºC before being induced with 1 mM (final) isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubated at 18 ºC overnight with continued shaking. Cells were then harvested by centrifugation for 12 min at 5000×*g* and 4 ºC and resuspended with a cellular resuspension buffer (50 mM Tris, pH 7.5, 300 mM NaCl, 10 % (v/v) glycerol, 1 mM TCEP). Immediately prior to cell lysis, solid phenylmethylsulfonyl fluoride (PMSF, *ca.* 50-100 mg) was added to the cellular suspension. Cells were then kept cold in an ice bath while being disrupted via sonication (80 % maximal amplitude, 30 s on pulse, 30 s off pulse, 12 min total time on). The resulting lysate was clarified by ultracentrifugation at 160,000×*g*, 4 ºC for 1 hr. The clarified lysate was then purified via immobilized metal affinity chromatography (IMAC) and size-exclusion chromatography (SEC). Briefly, the lysate was injected onto a prepacked 5 mL HisTrap HP column (Cytiva). The column was then washed with 5 column volumes (CVs) of wash buffer (50 mM Tris, pH 7.5, 300 mM NaCl, 10 % (v/v) glycerol, 1 mM TCEP) and eluted stepwise with 7.0 %, 16.7 %, 50.0 %, and 100.0 % (all v/v) respective concentrations of elution buffer (the same composition of the wash buffer plus 300 mM imidazole). Fractions containing *Pa*BqsR were pooled and concentrated using a 10 kDa molecular weight cutoff (MWCO) Amicon spin concentrator. The protein was then buffer exchanged into a TEV cleavage buffer (50 mM Tris, pH 8.0, 200 mM NaCl, 5 % (v/v) glycerol, 1 mM TCEP, 0.5 mM EDTA) by repeated concentration and dilution in the same spin concentrator. A mixture was then made comprising *ca*. 20 µg TEV protease per *ca*. 1 mg protein and allowed to rock at 4 ºC overnight. Following tag cleavage, the protein sample was applied to a HiLoad 10/300 GL Superdex 75 preparative SEC column (Cytiva) pre-equilibrated with SEC buffer (50 mM Tris, pH

7.5, 100 mM NaCl, 5 % (v/v) glycerol, 1 mM TCEP) and eluted isocratically. Fractions containing monomeric protein were collected, quantified, and analyzed using 15 % SDS-PAGE and Western blotting. The phosphomimetic *Pa*BqsR-BeF_3^- complex was generated by incubating WT *Pa*BqsR with 7 mM $MgCl_2$, 5.3 mM $BeSO_4$, and 35 mM NaF (all final concentrations) prior to further experimentation.

Crystallization and data collection. Initial crystals of *Pa*BqsR were obtained using sparse-matrix screening applying the sitting-drop vapor-diffusion method. Crystals were subsequently optimized by sitting-drop vapor-diffusion using MiTeGen-XtalQuest Plates with a 1:1 (v:v) of *Pa*BqsR (*ca.* 10 mg/mL) and reservoir solution mixture at room temperature. The precipitant solution consisted of 1.0 M sodium/potassium phosphate at pH 8.2. Medium, three-dimensional, colorless octahedral crystals appeared within 72 hr and reached their maximal sized within 7 days (on average). Crystals were transferred into cryoprotectant, looped, flash-frozen, and stored in liquid N_2. Data sets were collected on beamlines LS-CAT 21-ID-F at the Advanced Photon Source, Argonne National Laboratory, using a Marmosaic 300 mm CCD detector. Data were processed automatically with Xia2.[65] Phases were determined via molecular replacement using Phaser in Phenix[66] with an input of a truncated *Pa*BqsR AlphaFold model (AF-Q9I0I1-F1-v4). Extended model building and refinement cycles were performed in Coot[67] and Phenix Refine[66] respectively, and final validations were performed using Phenix Validate.[66] The final model consists of the N-terminal receiver domain of *Pa*BqsR (amino acids 1-123) and has been deposited in the Protein Data Bank (PDB ID 8GC6).

Nuclear magnetic resonance (NMR) spectroscopy. In order to isotopically-label *Pa*BqsR (intact WT, D51E, or the DBD), the cellular growth and protein production processes were both slightly modified. Briefly, *E. coli* BL21 (DE3) cells containing each expression plasmid grown in minimal media (1x M9 minimal salts, 1 mM $MgSO_4$, 1x trace elements, 0.1 mM $ZnCl_2$, 0.1 mM $CaCl_2$, and 1x MEM vitamin solution) supplemented with $^{15}NH_4Cl$, $^{13}C_6$-glucose, or both for global nitrogen and carbon labeling, respectively. Cells were then grown with shaking, induced, harvested, resuspended, and lysed in the same manner as cells grown in rich media (*vide supra*). Isotopically-labeled *Pa*BqsR proteins were purified in the same way as their natural-abundance isotopologues (*vide supra*). Once purified, isotopically-labeled *Pa*BqsR proteins (intact WT, D51E, or the DBD) were then buffer exchanged into an NMR-compatible buffer (50 mM sodium phosphate, pH 5.5, 5 mM NaCl, 1 mM DTT, and 10 % (v/v) D_2O). All NMR spectra were acquired at 25 °C on a Bruker 800 MHz spectrometer equipped with a cryogenic probe. Protein backbone resonance assignments of the *Pa*BqsR DBD were achieved by standard triple resonance experiments, including HNCA, HBHA(CO)NH, HNCACB, CBCA(CO)NH, and HNCO.[68–72] Consensus chemical shift index (CSI) (Hα, Cα, and Cβ and C0) of the *Pa*BqsR DBD were calculated using the program CSI 3.0.[35] Both ^{13}C- and ^{15}N-labeled four-dimensional nuclear Overhauser effect spectroscopy (NOESY) data were collected in order to calculate the lowest-energy tertiary fold conformers of the *Pa*BqsR DBD.

Electrophoretic mobility shift assays. Electrophoretic mobility shift assays (EMSAs) were performed in order to test the DNA binding capabilities of *Pa*BqsR to the upstream region of the PAO1 *feo* operon. Increasing concentrations of either intact WT, D51E, or BeF_3^--activated *Pa*BqsR were incubated for 30 minutes at room temperature in SEC buffer with a constant

concentration of double stranded *feo* DNA containing the "BqsR box" (5'-CCTTAAGCGAGCCTTAAGTGA-3' and 5'-TCACTTAAGGCTCGCTTAAGG-3'). Analyses were then performed at 4 °C using a 5 % acrylamide gel (0.5 % TB, 29:1 acrylamide:bis-acrylamide). DNA was stained using ethidium bromide and visualized with an ultraviolet (UV) transilluminator.

Structural modeling. All AlphaFold models were generated from the predicted AlphaFold Protein Structure Database[73] or modeled *de novo* using the AlphaFold3 structure prediction server.[74] To generate the intact WT and D51E *Pa*BqsR models bound to the PAO1 *feo* uptstream region, two copies of the respective protein sequences were used as input into the AlphaFold3 structure prediction server along with the following DNA duplex sequence: 5'-TCCACTCCGATACTGCGTCCTTAAGCGAGCCTTAAGTGAGAGTCGTTTAGATTCTC-3' and 5'-GAGAATCTAAACGACTCTCACTTAAGGCTCGCTTAAGGACGCAGTATCGGAGTGGA-3'. AlphaFold3 structural predictions were carried out without any modifications of the input parameters.

RNA sequencing. Total RNA was collected from mid-log phase WT PAO1 and Δ*bqsR* cells grown in BMM using the RNeasy Mini Kit (Qiagen). Purified RNA was treated with Turbo DNase (Invitrogen) and contaminating DNA was assessed using PCR with primers specific for the 16S rRNA gene (forward 5'-GTGCCAGCAGCCGCGGTAA-3'; reverse 5'-AGGGTTGCGCTCGTTG-3'). RNA concentrations were determined using a NanoDrop spectrophotometer (Thermo Scientific) and the RNA quality was analyzed using an RNA 6000

Nano Chip on a Bioanalyzer 2100 (Agilent) and by 1.2% agarose gel electrophoresis. RNA samples with A_{260}/A_{280} ratio of 1.8-2 and an RIN value ≥ 7.0 were selected for sequencing analysis. Ribosomal RNA was removed using the Ribo-Zero rRNA Depletion Kit (Illumina). For sequencing, 250-300 bp insert size, strand-specific libraries were prepared and sequenced by Novogene Co., Ltd using paired-end sequencing (2 x 150 bp) on a Nova Seq 6000 platform.

Analysis of RNA-seq reads. All raw RNA sequences were further analyzed using Kbase (https://www.kbase.us/). Three biological replicates were used for each stain. Reads were checked for quality using FastQC-v0.11.5 and trimmed using Trimmomatic-v0.36. The trimmed reads were aligned to the PAO1 genome using HISAT2-v2.1.0 and assembled using Cufflinks-v2.2.1. A differential expression matrix was generated using DESeq2-v1.20.0, and the resulting raw gene count matrix was uploaded to iDEP2.01 (http://bioinformatics.sdstate.edu/idep/) to determine $\log_2$ of fold change (FC) in gene expression in Δ*bqsR* compared to WT along with their false discovery rate (adjusted *p*-values). A volcano plot depicting differentially expressed genes ($\log_2 FC > |1|$) with significant -log10 *p*-values (> 1.3 for $p < 0.05$) was generated using Rstudio. The differentially expressed genes were sorted into functional categories using annotations from Gene Ontology (https://www.geneontology.org/), Kyoto Encyclopedia of Genes and Genomes (KEGG) (https://www.kegg.jp/kegg/pathway.html), and the Pseudomonas Community Annotation Project (PseudoCAP) (https://pseudomonas.com/). Heatmaps of selected pathways were generated using MORPHEUS (https://software.broadinstitute.org/morpheus/).

Metal-binding assays. All $(His)_6$-cleaved *Pa*BqsR constructs (intact WT, D51E, or DBD) were tested for their ability to bind Fe^{2+}. Briefly, each construct was degassed and brought into an anoxic

chamber containing an N_2/H_2 atmosphere and operating at < 5 ppm O_2. After overnight equilibration with the N_2/H_2 atmosphere, each protein solution was incubated with 1-5 mol eq. of Fe^{2+} to assess stability of the protein in the presence of metal. The intact protein only tolerated a maximal loading of 1.2 mol eq. of Fe^{2+}, so all metal-loading experiments were performed with this slight excess of metal cation in solution. After the addition of metal and incubation for 10 min at *ca.* 6 °C, the protein-metal solution was buffer exchanged into SEC buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 5 % (v/v) glycerol, 1 mM TCEP) through extensive rounds of concentration and dilution using a 0.5 mL 10 kDa MWCO spin concentrator. A modified version of the ferrozine assay[75,76] was performed as previously described.[77] All metal loading was then further confirmed by measuring metal content using a PerkinElmer NexION 300D mass spectrometer connected to inductively couple plasma mass spectrometry (ICP-MS).

X-ray absorption spectroscopy (XAS). Intact WT *Pa*BqsR samples were metal-loaded anoxically and concentrated to *ca.* 2 mM Fe^{2+} (final concentration), mixed anoxically with ethylene glycol (20% (v/v) final), and were aliquoted anoxically into Lucite cells wrapped with Mylar tape, flash-frozen in liquid N_2 and stored at -80 ºC until data collection. X-ray absorption spectroscopy (XAS) was performed on beamlines 7-3 and 9-3 at the Stanford Synchrotron Radiation Lightsource (Menlo Park, CA), and samples in replicates were collected when possible. Extended X-ray absorption fine structure (EXAFS) of Fe (7210 eV) was measured using a Si 220 monochromator with crystal orientation $\varphi = 90°$. Samples were measured as frozen aqueous glasses at 15 K, and the X-ray absorbance was detected as Kα fluorescence using either a 100-element (beamline 9-3) or 30-element (beamline 7-3) Ge array detector. A Z-1 metal oxide filter (Mn) and slit assembly were placed in front of the detector to attenuate the elastic scatter peak. A

sample-appropriate number of scans of a buffer blank were measured at the absorption edge and subtracted from the raw data to produce a flat pre-edge and eliminate residual Mn Kβ fluorescence of the metal oxide filter. Energy calibration was achieved by placing a Fe metal foil between the second and third ionization chamber. Data reduction and background subtraction were performed using EXAFSPAK (Microsoft Windows version).[78] The data from each detector channel were inspected for drop outs and glitches before being included into the final average. EXAFS simulation was carried out using the program EXCURVE (version 9.2) as previously described.[78–80] The quality of the fits was determined using the least-squares fitting parameter, *F*, which referred to as the fit index (FI) and is defined as:

$$F^2 = \frac{1}{N}\sum_{i=0}^{N} k^6(\chi_i^{theory} - \chi_i^{exp})^2 \qquad (1)$$

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