The *Pseudomonas aeruginosa* Membrane Histidine Kinase BqsS/CarS Directly Senses Environmental Ferrous Iron (Fe^{2+})

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Abstract

Canonical prokaryotic two-component signal transduction systems (TCSs) are widely utilized by bacteria to respond to their environment and are typically composed of a transmembrane sensor His kinase (HK) and a cytosolic DNA-binding response regulator (RR) that work together to respond to environmental stimuli. An important TCS that regulates the expression of genes involved in biofilm formation and antibiotic resistance in many pathogens is the BqsRS/CarRS system, originally identified in *Pseudomonas aeruginosa*. Transcriptomics data suggested that the cognate *Pa*BqsRS stimulus is Fe^{2+}, but *Pa*BqsS has not been characterized at the protein level, and a direct interaction between Fe^{2+} and *Pa*BqsS has not been demonstrated. In this work, we biochemically and functionally characterize intact *Pa*BqsS, an iron-sensing membrane HK, for the first time. Using bioinformatics, protein modeling, metal analyses, site-directed mutagenesis, and X-ray absorption spectroscopy (XAS), we show that *Pa*BqsS binds a single Fe^{2+} ion per protein dimer within the periplasmic sensor domain by using a unique ligation motif comprising Glu^{48} and Asn^{49}. Using activity assays, we show that both intact *Pa*BqsS and its truncated cytosolic domain have competent ATPase activity, consistent with predicted function. Importantly, we show that the ATP hydrolysis of intact *Pa*BqsS is stimulated exclusively by Fe^{2+}, revealing metal-based activation of a functional, intact membrane HK for the first time. Taken together, this work uncovers important structural and biochemical properties of an intact, metal-sensing membrane HK that could be leveraged to target the BqsRS system for future therapeutic developments.

Keywords

biofilms; two-component system; signal transduction; metalloproteins; iron; kinase

Abbreviations

CA, catalytic ATP-binding domain; CMC, critical micellular concentration; CV, column volume; DHp, dimerization and His phosphotransfer domain; EXAFS, extended X-ray absorption fine structure; FI, fit index; GDN, glyco-diosgenin; IMAC, immobilized metal affinity chromatography; IPTG, isopropyl β-D-1-thiogalactopyranoside; LB, Luria broth; HK, membrane sensor His kinase; MWCO, molecular weight cutoff; OD, optical density; PMSF, phenylmethylsulfonyl fluoride; RNS, reactive nitrogen species; ROS, reactive oxygen species; RR, response regulator; SEC, size-exclusion chromatography; TB, terrific broth; TEV, tobacco etch virus; TCS, two-component signal transduction system; XANES, X-ray absorption near-edge structure; XAS, X-ray absorption spectroscopy.

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative, opportunistic, virulent bacterium that can survive in many conditions and is highly adaptable, making it a pressing threat to human health.[1] As part of its adaptability, *P. aeruginosa* can grow either as a plankton (*i.e.*, freely floating in solution such as stagnate water) or within a biofilm (*i.e.*, stationary within a protective, complex, three-dimensional network composed of polysaccharides, proteins, lipids, and DNA).[1–4] Due to its widespread nature, its adaptability, and its virulence, *P. aeruginosa* is one of the most common causes of nosocomial infections, especially for patients suffering with pneumonia, neutropenia, and/or cystic fibrosis.[1,5,6 7] Moreover, infections of *P. aeruginosa* are notoriously difficult to treat, as this bacterium continues to evolve multidrug-resistance, which is exacerbated by its ability to form protective biofilms and to employ numerous virulence factors that allow this pathogen to colonize and to establish itself within a mammalian host such as a human.[8]

An important factor that governs the infectivity of many pathogens, such as *P. aeruginosa*, is the ability of these bacteria to acquire key nutrients from their hosts, including essential transition metal ions. Elements such as Mn, Fe, Cu, and Zn are generally necessary for the normal metabolic functions of virtually all bacteria, and their limited presence can often control microbial virulence (Fig. 1).[9–12] However, these crucial metal ions can also be toxic to cells at high concentrations, as they can disrupt cellular functions through protein and/or nucleic acid misfolding and via the generation of reactive oxygen and nitrogen species (ROS and RNS). Due to their dual nature, bacteria have developed complex mechanisms to sense the presence of transition metals and to regulate their influx and efflux, especially at the host-pathogen interface.[10,12–16]

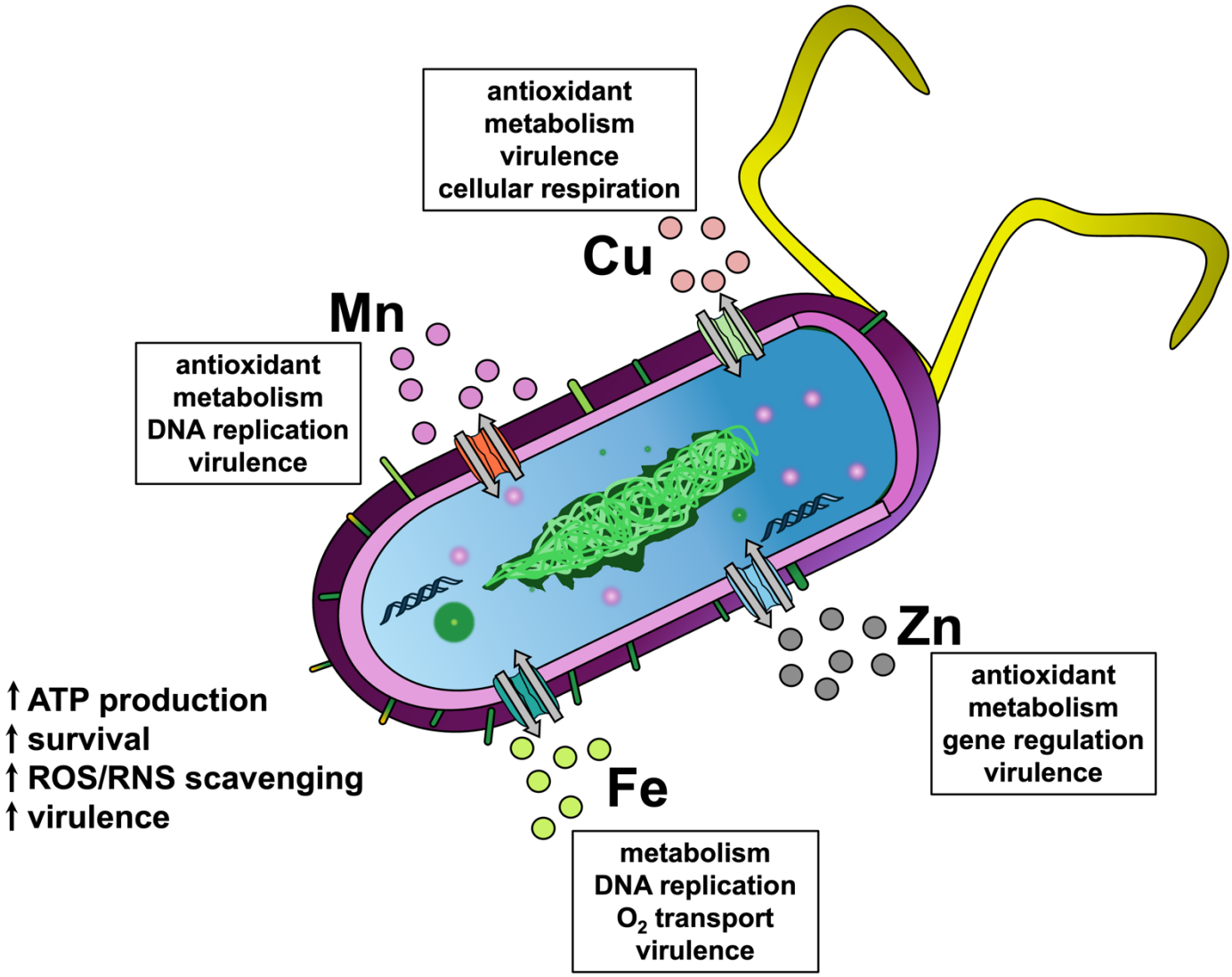

Figure 1. Transition metals such as Mn, Fe, Cu, and Zn are critical for normal bacterial cellular function and may be leveraged by pathogenic strains. Transition metals are involved in several critical metabolic roles within prokaryotes, such as electron transport, DNA replication, and even transcriptional regulation. Importantly, transition metals are also a part of microbial virulence, as these micronutrients can help increase ATP production, can be leveraged to defend against ROS/RNS, and can even be involved in biofilm formation and dispersion.

Two-component signal transduction systems (TCSs) are common mechanisms used by bacteria to sense the presence of environmental nutrients, such as transition metals, and these systems function to regulate intracellular concentrations of metals, allowing bacteria to adapt to a wide array of conditions found within the human host.[5,9,17–19] A bacterial TCS is canonically composed of two separate proteins: a transmembrane sensor His kinase (HK) and a cytosolic DNA-binding response regulator (RR).[20,21] The extracytoplasmic domain of the HK senses the

external nutrient/stimulus, leading to an autophosphorylation event that occurs at the HK cytoplasmic domain. This domain consists of two sub-domains spatially adjacent to one another: the catalytic ATP-binding (CA) domain that catalyzes the transfer of the γ phosphate from ATP to the HK, and the dimerization and His phosphotransfer (DHp) domain that houses a conserved phosphate-accepting His residue. Once phosphorylated, this labile moiety is then transferred from the cytoplasmic domain of the HK to a conserved Asp residue on the cytoplasmic RR. Phosphorylation of the RR leads to dynamical changes, often accompanied by protein dimerization, that increase the affinity of the RR towards its specific DNA sequence(s), ultimately resulting in the transcriptional regulation of target genes.[20,21] The types of stimuli that may be sensed are often specific (*e.g.*, H^+, transition metals, small molecules), while the genes targeted by the RR can be varied and often remodel the metabolic architecture of the cell, frequently increasing the infectivity of pathogenic bacteria through the expression of numerous virulence factors.[20–28]

BqsRS (also known as CarRS) is an important but understudied metal-sensing TCS first discovered in *P. aeruginosa* that regulates biofilm formation and decay.[29,30] Like most canonical TCSs, BqsRS is composed of a membrane-bound HK, BqsS, and its cognate RR, BqsR.[29,30] Initial transcriptional profiling showed that the membrane HK BqsS specifically recognizes Fe^{2+} at μM concentrations,[30] while subsequent studies have shown that BqsRS may also respond to mM concentrations of Ca^{2+}.[31] These metal concentrations are similar to levels present in the cystic fibrosis lung environment,[32–37] and *bqsS* transcripts have been detected in cystic fibrosis sputum samples.[38] Further underscoring its importance, functional studies in *P. aeruginosa* have linked the presence of BqsRS to the regulation of genes involved in biofilm formation and antibiotic resistance, such as those responsible for rhamnolipid production and the formation of short chain quorum sensing signals like *N*-butyryl-*L*-homoserine lactone.[29] *Pa*BqsS has been proposed to

sense Fe^{2+} via a conserved RExxE motif present in the BqsS extracytoplasmic domain, but no metal-protein interaction in this system has been directly observed. In fact, to our knowledge no previous work has shown a stable interaction between any transition metal and any intact membrane HK, and how this important system selects for Fe^{2+} vs Ca^{2+} is unclear, prohibiting a targeted approach of this TCS to treat *P. aeruginosa* infections.

In this work, we biochemically and biophysically characterize the metal-binding and functional properties of *P. aeruginosa* membrane HK BqsS in its intact and truncated forms. Recombinantly expressed, detergent-solubilized *Pa*BqsS is purified to homogeneity and is consistent with a dimeric state, similar to other canonical HKs. Importantly, we quantitate and characterize a stable interaction between Fe^{2+} and intact *Pa*BqsS for the first time. Using X-ray absorption spectroscopy (XAS) combined with site-directed mutagenesis, we show that Fe^{2+} binds to this intact membrane HK in an octahedral geometry, coordinated by 4 Asp residues and, surprisingly, 2 Asn residues. Using enzymatic assays, we characterize the ATP hydrolysis of intact *Pa*BqsS in the presence and absence of Fe^{2+} as an external stimulus, and we benchmark this rate against the truncated, *Pa*BqsS cytoplasmic domain (cytoBqsS). To our knowledge, this study represents the first characterization of an intact HK bound to, and stimulated by, its cognate metal. Results from this work illustrate the unique coordination chemistry used by *Pa*BqsS to sense extracytoplasmic Fe^{2+}, which may be targeted in the future to disrupt this essential TCS that regulates biofilm formation and antibiotic resistance.

RESULTS

Expression and purification of intact, homogenous PaBqsS

To explore the biophysical properties of intact *Pa*BqsS, we first cloned, expressed in *Escherichia coli*, isolated, solubilized, and purified intact *Pa*BqsS to homogeneity. Surprisingly, very few intact HKs have been purified, and no intact metal-sensing HK has been biophysically characterized in the presence of its cognate metal stimulus, likely due to the difficulty of producing and purifying the full-length membrane protein.[39–42] After cloning intact *Pa*BqsS comprising its periplasmic sensor domain, two transmembrane helices, and a soluble domain CA domain linked to a DHp domain (Fig. 2a), we verified its overexpression and optimized its membrane isolation conditions. We then tested multiple different non-denaturing detergents for their ability to

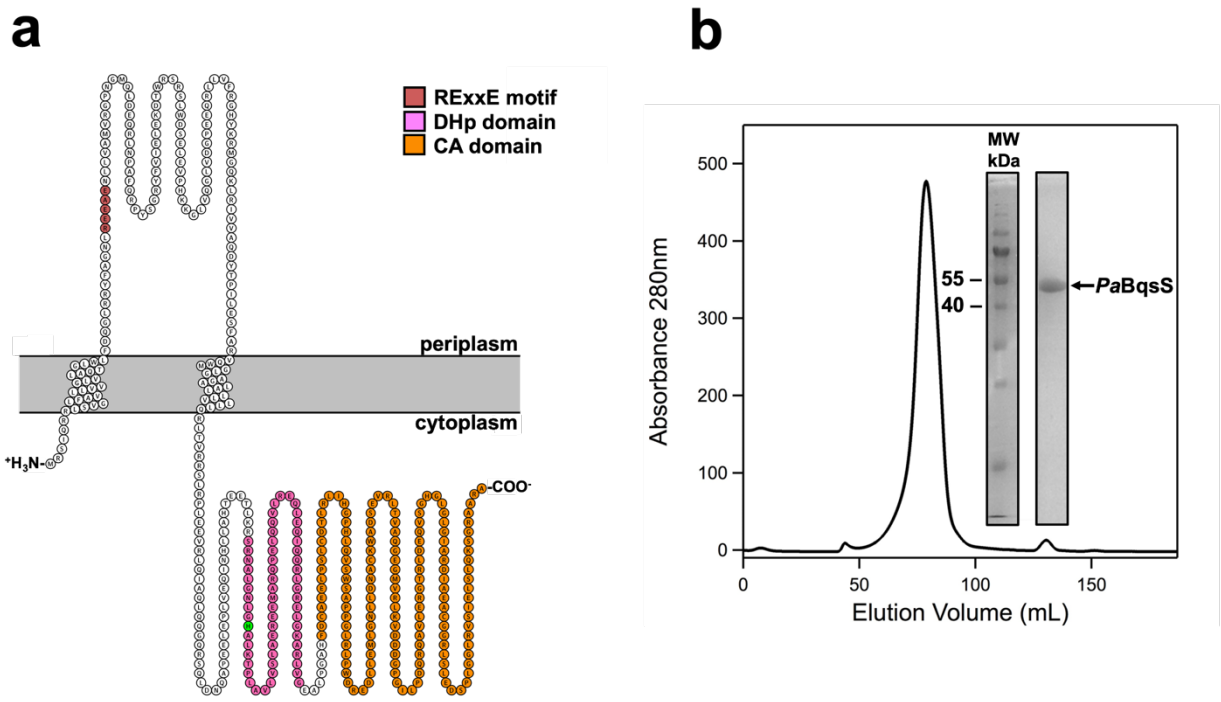

Figure 1. Topological modeling and purification to homogeneity of intact *Pa*BqsS. **a**. A cartoon image of the predicted topology of *Pa*BqsS based on the TMHMM server.[81,82] The predicted topology of intact *Pa*BqsS includes two transmembrane helices, a periplasmic domain with a putative iron-sensing RExxE motif, and a long C-terminal domain composed of CA and DHp sub-domains. Image partially created using Protter.[83] **b**. The size-exclusion chromatogram (SEC) and SDS-PAGE analysis (*inset*) of cleaved, purified *Pa*BqsS in Fos-Choline-14 demonstrates good homogeneity and purity when recombinantly produced in *E. coli*.

solubilize *Pa*BqsS stably (Fig. S1). Based on Western blotting and downstream characterization, we settled on the use of *n*-tetradecylphosphocholine (Fos-Chol-14) as the detergent for large-scale purification, tag cleavage, and gel filtration studies, as other detergents such as *n*-dodecyl-β-D-maltoside (DDM) and lauryldimethylamine-*N*-oxide (LDAO) caused irreversible protein aggregation. Our *Pa*BqsS isolated in Fos-Chol-14 was highly pure and homogeneous (Fig. 2b). Size-exclusion chromatography indicates that *Pa*BqsS migrates as an oligomer in Fos-Choline-14, with calibrations suggesting that *Pa*BqsS make exist either as a tetramer with a single micelle or a dimer of dimers, with each dimer possessing a single micelle (Fig. 2b). While gel filtration alone cannot rule out either possibility, we believe the latter is operative for several reasons. First, most canonical membrane HKs exists as homodimers.[43,44] Second, Fos-Chol-14-purified *Pa*BqsS buffer exchanged into a detergent with a lower critical micellular concentration (CMC) such as glyco-diosgenin (GDN) and then analyzed using gel filtration is most consistent with dimeric *Pa*BqsS (Fig. S2). Finally, the interaction of *Pa*BqsS with its cognate stimulating ligand (Fe^{2+}) also suggests an operative dimer is in solution (*vide supra*). Thus, we were able to isolate and to purify intact *Pa*BqsS for the first time for downstream biophysical and enzymatic characterizations.

PaBqsS uses a unique ligation motif to bind Fe^{2+} at a dimeric interface

A previous *in vivo* study proposed that *Pa*BqsS binds Fe^{2+} by utilizing a RExxE motif in its HK periplasmic domain,[45] but the atomic level details and stoichiometry of this process were not defined. We first wondered whether studies on other iron homeostasis proteins would shed light onto how *Pa*BqsS binds iron. A more general ExxE motif has been implicated in iron transport both in bacteria (*e.g.*, FeoB)[46] and yeast (*e.g.*, *Saccharomyces cerevisiae* and *Candida albicans*

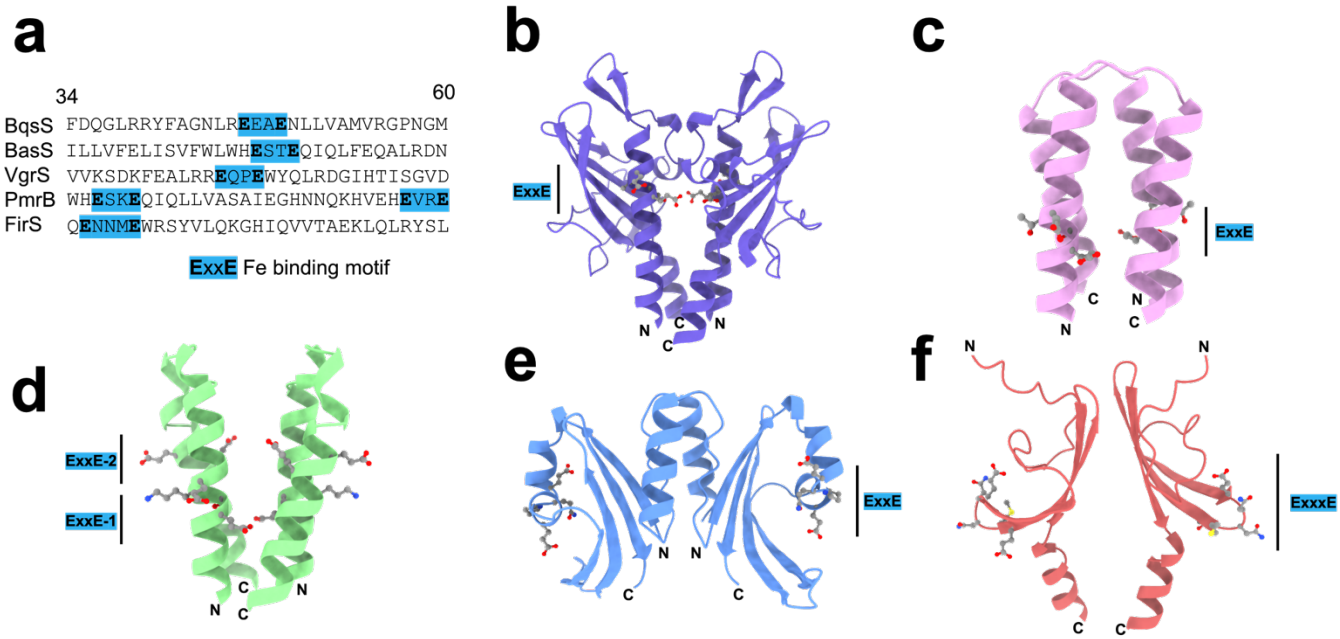

Figure 3. A periplasmic Exx(x)E motif is conserved across dimeric, iron-sensing HKs. **a**. Partial periplasmic amino acid sequences illustrate the presence of the Exx(x)E motif in several iron-sensing HKs. While the general Exx(x)E motif is present in all iron-sensing HKs, its internal and flanking residues differ, as does its predicted structural positioning relative to the dimer interface. The numbers at the top of the sequences indicate the positioning relative to *Pa*BqsS. **b**. The AlphaFold prediction of the periplasmic domain *Pa*BqsS suggests an ExxE motif is present at the dimeric interface. **c**. The AlphaFold prediction of the periplasmic domain of *E. coli* BasS, an iron-sensing HK, suggests an ExxE motif is present at the dimeric interface. **d**. The AlphaFold prediction of the periplasmic domain of *Xanthomonas campestris* VgrS, an iron-sensing HK, suggests an ExxE motif is present but is distal to the dimeric interface. **e**. The AlphaFold prediction of the periplasmic domain of *Klebsiella pneumoniae* PmrB, and iron-sensing HK, suggests two ExxE motifs are present at the dimeric interface. **f**. The AlphaFold prediction of the periplasmic domain of *Haemophilus influenzae* FirS, an iron-sensing HK, suggests a longer ExxxE motif is present but is distal to the dimeric interface.

FTR proteins)[47 48], as well as in iron storage (*e.g.*, ferritin)[49], although the selectivity of this motif for Fe^{3+} *versus* Fe^{2+} is unclear. We then considered if this motif were conserved amongst putative iron-sensing HKs in general. We compiled the sequences of the few iron-sensing HKs that have been characterized at either the *in vitro* or the *in vivo* levels (*Pa*BqsS, *Ec*BasS, *Xanthomonas campestris* VgrS, *Klebsiella pneumoniae* PmrB, and *Haemophilus influenzae* FirS),[30,50–54] we used TMHMM to predict the extent of the periplasmic domains in each, and we searched these domain sequences for the presence of an ExxE motif (Fig. 3a). In almost all cases, an ExxE motif was predicted to be present in the HK periplasmic domain and to be preceded by a basic residue (Arg or His), with the exception of *Hi*FirS that has a slightly longer ExxxE motif preceded by a Gln

residue (Fig. 3a). We then used AlphaFold modeling to predict the dimeric structures of these domains, with the hypothesis that these structural predictions would provide insight into coordination and stoichiometry. Interestingly, several of the AlphaFold periplasmic domain models such as *Pa*BqsS, *Ec*BasS, and *Kp*PmrB appear to place these ExxE motifs at the dimer interface (common to the binding interface for many HKs),[43,44] whereas the AlphaFold periplasmic domain models of *Xc*VgrS and *Hi*FirS place the Exx(x)E motifs distal to the dimeric interface (Fig. 3b-f). Whether these motifs exclusively bind Fe^{2+}, Fe^{3+}, or both is unclear, but the flanking residues could be involved in this selectivity. Moreover, whether the ExxE motif is clustered at, or distant from, the dimeric interface may implicate different binding stoichiometries.

We then tested whether *Pa*BqsS would bind to Fe^{2+} in a stable manner, and we biophysically characterized this binding interaction and compared it to our AlphaFold model. We focused specifically on Fe^{2+} and not Fe^{3+} as previous research has shown that *Pa*BqsS has no transcriptional response to Fe^{3+} compared to Fe^{2+};[45] moreover, free Fe^{3+} is highly insoluble and is thus bound tightly to small molecules and proteins *in vivo*, making uncomplexed Fe^{3+} an unlikely signal for an iron-responsive HK. We then loaded purified, intact *Pa*BqsS with excess Fe^{2+} under anoxic conditions, buffer exchanged copiously, and measured *ca.* 0.5 mol. eq. of Fe^{2+} (0.50 ± 0.06) bound per polypeptide (Fig. 4a). As sensor HKs largely function as homodimers[43,44], as our gel filtration data suggest that *Pa*BqsS is either a dimer or a dimer of dimers (dependent on detergent; Fig. 2b), and as our modeled metal-binding site appears to be at the homodimer interface (Fig. 3b), we initially hypothesized that a single Fe^{2+} ion binds per *Pa*BqsS dimer with each $R^{44}E^{45}xxE^{48}$ motif contributing to binding. To test this hypothesis, we used site-directed mutagenesis and created three *Pa*BqsS variants that we attempted to express and purify: $R^{44}E^{45}xxA^{48}$, $R^{44}A^{45}xxE^{48}$, and $R^{44}A^{45}xxA^{48}$. While we could purify the first two variants (Fig. S3), the third variant (the

double modification) failed to express and to purify, suggesting that the protein may have been misfolded when both Glu residues are lost. Surprisingly, only one of the two Glu residues (Glu^{48}), appeared to be critical for Fe^{2+} binding, as loss of Glu^{45} did not impact metal loading (Fig. 4a). To gain greater insight into the identity of the Fe^{2+} ligands and the structure of the Fe^{2+} binding site, we used X-ray absorption spectroscopy (XAS) (Fig. 4b,c). The energy of the inflection of the X-ray absorption near-edge structure (XANES) spectrum of Fe^{2+}-bound WT *Pa*BqsS as well as its lack of pre-edge features are consistent with an octahedrally-coordinated Fe^{2+} ion (Fig. S4). Simulations of the extended X-ray absorption fine structure (EXAFS) data of *Pa*BqsS (Fig. 4b,c) reveal an N/O-based primary shell environment surrounding the Fe^{2+} ion, with 2 N/O scatterers at 2.00 Å from the Fe^{2+} ion, 4 N/O scatterers at 2.14 Å from the Fe^{2+} ion (Table 1), and strong carbon secondary shell contributions from the C-bonded N/O ligands. As we could not model a fully symmetric ligand set about the Fe^{2+} ion, and as only loss of Glu^{48} (providing 2 O ligands each from the sidechain carboxylate) impacted metal binding, we considered what other residues may

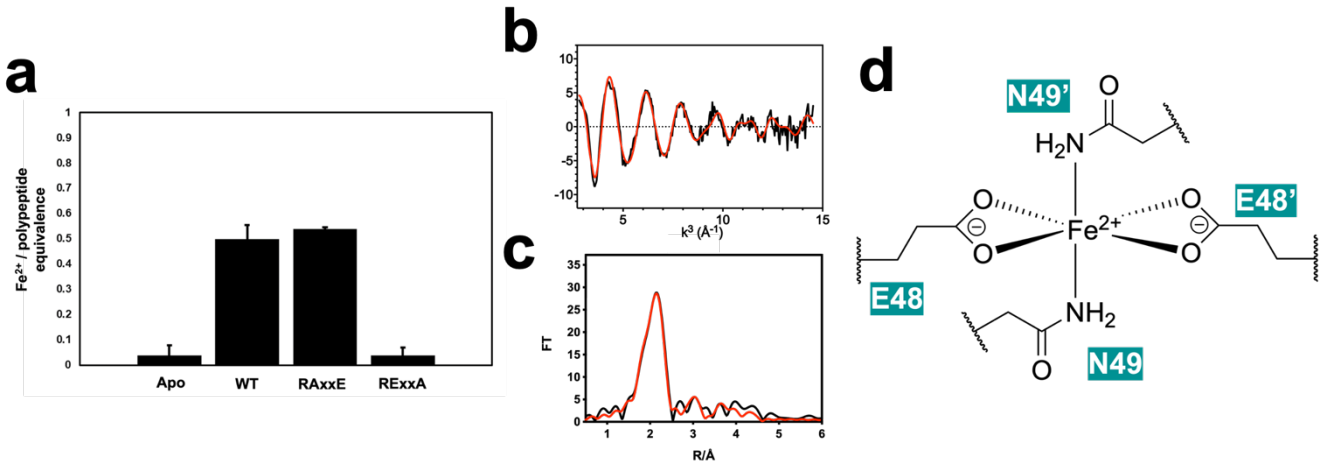

Figure 4. Intact *Pa*BqsS binds Fe^{2+} at its dimeric interface using a unique octahedral motif comprising Glu^{48} and Asn^{49}. **a**. WT, intact *Pa*BqsS binds approximately one Fe^{2+} ion per dimer, and loss of Glu^{48} but not Glu^{45} disrupts Fe^{2+} binding. **b,c**. EXAFS of *Pa*BqsS bound to Fe^{2+} under anoxic conditions. The processed EXAFS (**b**) and resulting Fourier transformed data (**c**) are consistent with the presence of Fe^{2+} in a six-ligand geometry composed of an O/N-rich environment that can be best fitted using 2 carboxylates of Glu^{48} and the amide nitrogen of Asn^{49}, as supported by XAS data of small molecule model compounds.[55] 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 within the periplasmic domain of *Pa*BqsS.

coordinate the Fe^{2+} ion. Based on our AlphaFold model (Fig. 3b), the only additional N/O ligand in proximity of the proposed binding site was that of the amide sidechain of Asn^{59}. Based on well-established small-molecule complexes[55], we then modeled the EXAFS data with Asn^{59} interacting with the Fe^{2+} ion through the amide nitrogen, which converged to a very good fit (Fig. 4b,c; Table 1). Thus, we propose that *Pa*BqsS senses its single Fe^{2+} ion at a dimeric interface in which Glu^{48} and Asn^{49} (the final two residues in the $R^{44}E^{45}xxE^{48}N^{49}$ sequence) coordinate that Fe^{2+} ion (Fig. 4d). To our knowledge, this discovery represents the first time that Fe^{2+} bound to an intact, iron-sensing HK has been characterized.

		Fe-O/N (non His)			Fe-C (non His)			E_o[e] (eV)
Sample	**Fit index[a]**	**No[b]**	**R[c] (Å)**	**DW[d] (Å²)**	**No**	**R (Å)**	**DW (Å²)**	
WT *Pa*BqsS + Fe^{2+}	0.58	2.0	2.00	0.004	2.0	3.04	0.009	-3.73
		4.0	2.14	0.005	2.0	3.75	0.002	
					2.0	4.01	0.011	
					2.0	4.30	0.005	

[a]The least-squares fitting parameter (see *Methods*) [b]Coordination number [c]Bond length [d]Debye-Waller factor [e]Photoelectron energy threshold

Table 1. Fits obtained for the Fe K-EXAFS of WT *Pa*BqsS bound to Fe^{2+}. Fits were determined by curve fitting using the program EXCURVE (version 9.2).

Binding of Fe^{2+} to the PaBqsS HK specifically and significantly stimulates ATPase activity

The AlphaFold model of intact *Pa*BqsS (Fig. 5a) suggests that binding Fe^{2+} in the periplasmic sensor domain of this HK may have downstream functional consequences. Specifically, as we have shown that a single Fe^{2+} ion binds within the periplasmic sensor domain utilizing Glu^{48} and Asn^{49}, we considered how this binding event could alter *Pa*BqsS function. The entire stretch of the $R^{44}E^{45}xxE^{48}N^{49}$ sequence is predicted confidently to be located along an α-helix, with Glu^{48} and Asn^{49} from each protomer suggested to point towards the dimeric interface (Fig. 5a, *inset*). However, each of these sets of ligands on each protomer are modeled to be oriented 180° relative to the other set (Fig. 5a, *inset*), which would necessitate a large conformational change in order to facilitate binding of Fe^{2+} at the dimeric interface of the HK. Such a conformational change may seem extreme, but research has shown that a structural reorientation is important for sensor domains of TCSs,[56] and such movements has even been structurally observed for other stimulus-sensing membrane HKs.[42]

Alterations along these α-helices that span across the membrane (*i.e.*, from extracytoplasmic to intracytoplasmic) are hypothesized to propagate to the intracellular DHp and CA domains to alter HK function and ATP hydrolysis, which we then tested. First, to separate any potential effects of the presence of Fe^{2+} on ATP hydrolysis catalyzed by just the intracellular, cytosolic DHp and CA domains, we cloned, expressed, and purified a construct corresponding to only this region (cytoBqsS; amino acids 210-438) (Fig. S5). Because sensor HKs all contain a CA domain that catalyzes ATP-dependent protein kinase activity,[21] we used a modified malachite green assay[57] to measure the ATPase activity of *Pa*BqsS. Indeed, isolated cytoBqsS is a competent ATPase with a maximal velocity (V_{max}) of 8.82 ± 0.42 μmol ATP×min^{-1} (Fig. 5b), a Michaelis constant (K_M) of 244.34 ± 17.28 μM (Fig. 5b), and a specific activity of 3.64 ± 0.64 μmol ATP×mg

protein^{-1}×min^{-1} (Fig. 5c). While cytoBqsS also bears an ExxE motif, it failed to bind Fe^{2+} and,

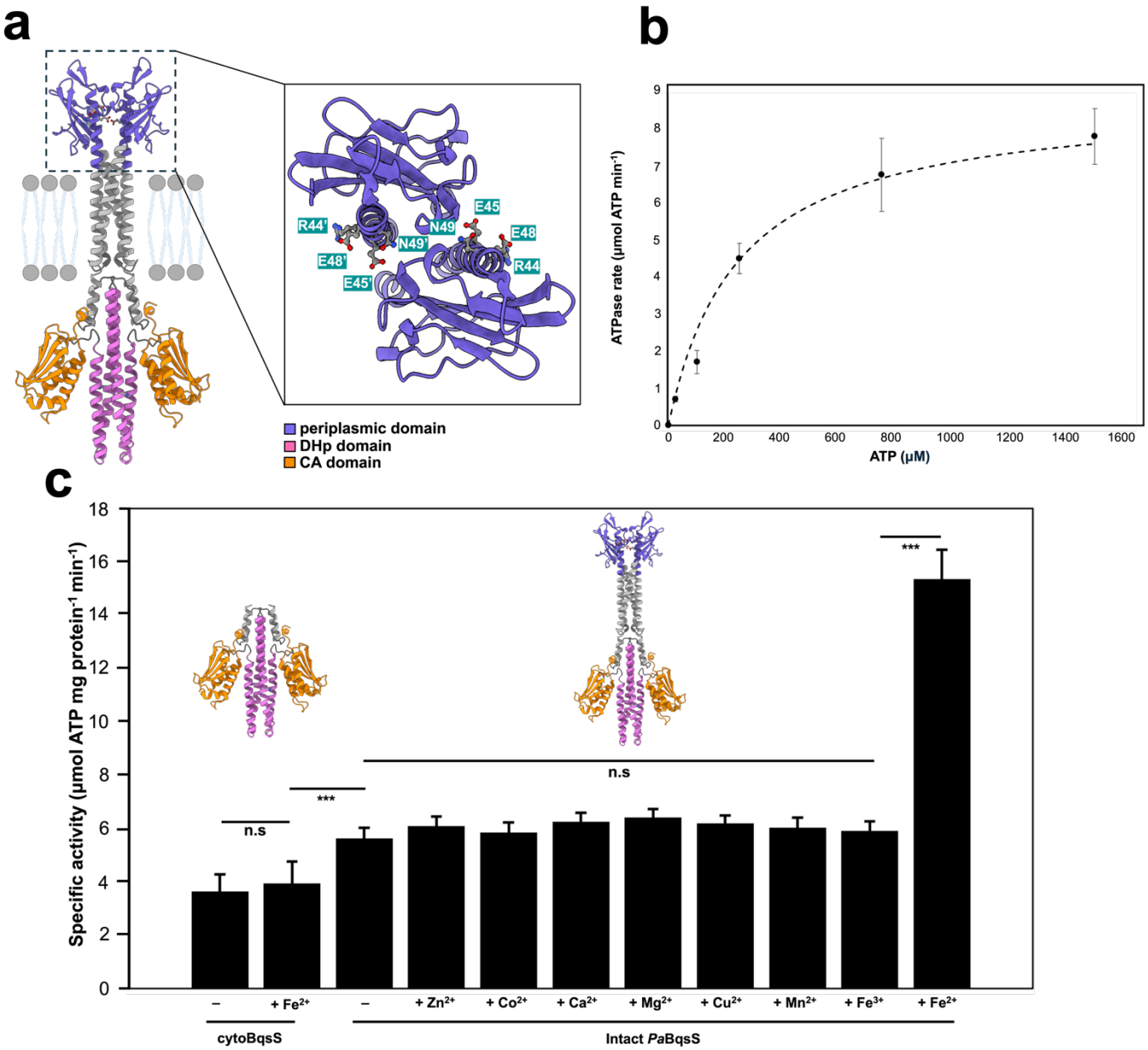

Figure 5. The binding of Fe^{2+} in the periplasmic sensor domain of *Pa*BqsS is transmitted through the transmembrane region to the DHp and CA domains of *Pa*BqsS leading to an alteration in ATP hydrolysis rates. **a**. The AlphaFold model of intact *Pa*BqsS with the various domains (periplasmic, DHp, CA) color-coded. *Inset*. A conformational change in the periplasmic sensor domain may be needed to coordinate Fe^{2+}, which could be transmitted through the transmembrane region to the intracytoplasmic domains. The amino acids comprising the $R^{44}E^{45}xxE^{48}N^{49}$ sequence in the periplasmic sensor domain are highlighted. **b**. The Michaelis-Menten profile of cytoBqsS-catalyzed ATP hydrolysis demonstrates a maximum velocity (V_{max}) of 8.82 ± 0.42 µmol ATP×min^{-1} and a Michaelis constant (K_M) of 244.34 ± 17.28 µM. **c**. The specific ATPase activity of cytoBqsS and intact *Pa*BqsS both with and without the presence of divalent metals. There is no difference in ATPase activity in the presence of metals for cytoBqsS, while intact *Pa*BqsS has an Fe^{2+}-dependent increase in ATPase activity. n.s. indicates not statistically significant ($P > 0.05$) whereas *** indicates $P < 0.001$ based on the student's t-test. $N = 3$ for all samples.

consistent with this notion, addition of Fe^{2+} to cytoBqsS showed no effect on specific activity (Fig. 5c; 3.93 ± 0.82 μmol ATP×mg protein^{-1}×min^{-1}, $P > 0.05$). We then tested the ATPase activity of intact *Pa*BqsS purified in Fos-Chol-14 and showed that it was indeed active as well, with specific activity of 5.64 ± 0.38 μmol ATP×mg protein^{-1}×min^{-1}, modestly higher than cytoBqsS alone (Fig. 5c). Importantly, and in contrast to cytoBqsS alone, the binding of Fe^{2+} to intact *Pa*BqsS substantially increased its basal ATPase activity with strong significance (Fig. 5c; 15.35 ± 1.10 μmol ATP×mg protein^{-1}×min^{-1}; $P < 0.001$). Dramatically, the presence of other transition metals (including Fe^{3+}) as well as Ca^{2+} all failed to elicit this effect (Fig. 5c). These observations show that although *Pa*BqsS has some inherent ATPase activity in the absence of an external stimulus, the presence of Fe^{2+} (and only Fe^{2+}) dramatically and significantly increases this activity, corroborating previous transcriptomic data.[30] These results are wholly consistent with our hypothesis that the binding of Fe^{2+} within the extracytoplasmic domain is communicated across the lipid bilayer to affect HK function and downstream signaling, and such a result is shown here with an intact, metal-sensing HK for the first time.

DISCUSSION

The sensing of transition metals is important for the survival and the virulence of infectious prokaryotes, as these micronutrients are needed for normal bacterial function and are also leveraged to establish infection, especially within the mammalian host.[11,12,13] A tight control of transition metal concentration is critical to pathogens, as too little metal disrupts general metabolic function, while too much metal can be toxic to the cell.[9,58,59] In order to control this balance, bacteria employ TCSs that are able to respond to extracytosolic concentrations of numerous cations, including the most important transition metal ions (Mn^{2+}, $Fe^{2+/3+}$, Cu^{+}/Ag^{+}, Zn^{2+}). Once sensed, the commonly-accepted paradigm posits that a signal is passed through the HK, across the

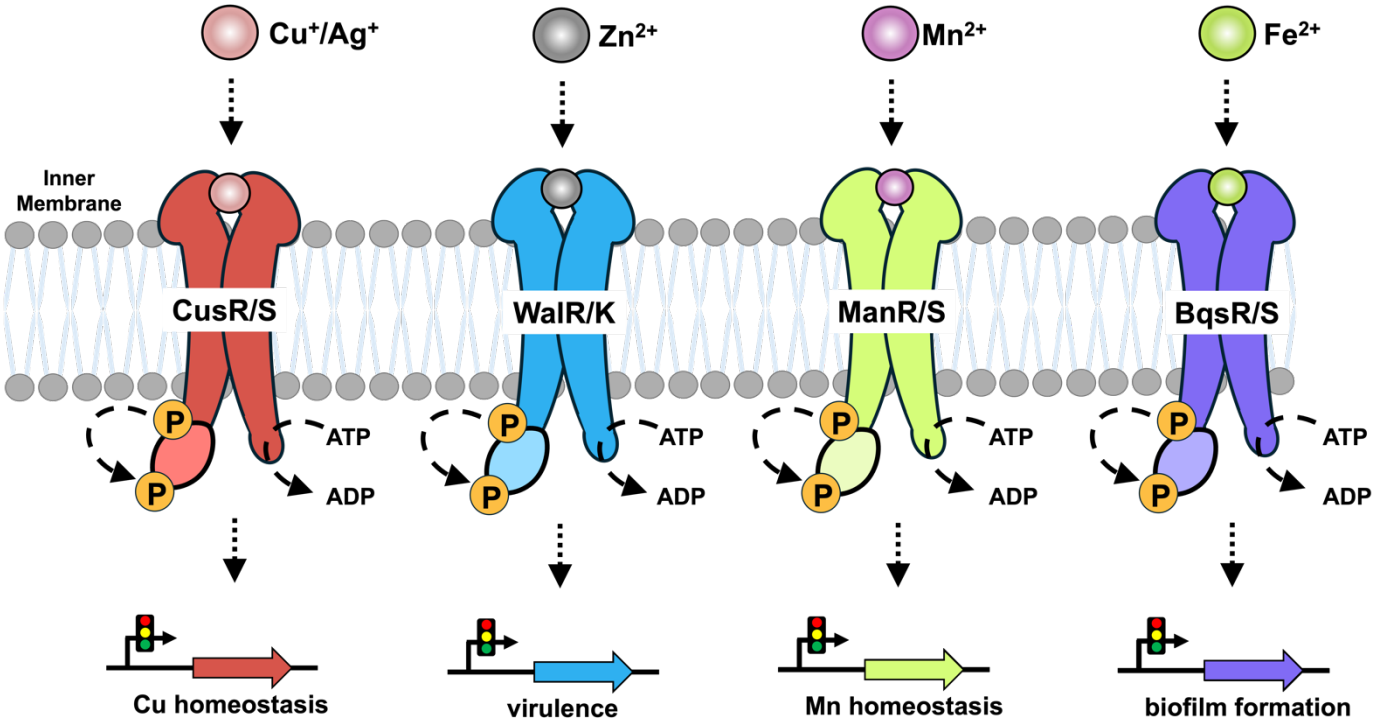

Figure 6. BqsRS complements the functions of other bacterial metal-sensing two-component signal transduction systems (TCSs). Bacterial metal-sensing TCSs that have been partially characterize include: CusRS (red) that senses Cu^{+}/Ag^{+} and regulates the expression of Cu efflux transporters and redox enzymes that are responsible for alleviating Cu toxicity through its response regulator (RR); WalRK (blue) that senses Zn^{2+} and regulates the expression of various genes important for virulence, biofilm formation, and cell viability through its RR; and ManRS (green) that senses Mn^{2+} and regulates the expression of genes involved in Mn homeostatsis through its RR. Previous work has shown that BqsRS (purple) regulates the expression of genes involved in biofilm formation and antibiotic resistance, and this work demonstrates that BqsS directly binds and senses Fe^{2+} selectively and responds to the presence of Fe^{2+} by upregulating ATPase activity, representing the first time an intact membrane HK has been show to make a stable and functional interaction with its cognate metal *in vitro*.

membrane, and ultimately to a response regulator (RR) that is phosphorylated and then targets genes involved in homeostasis and virulence (among others) for transcriptional regulation (Fig. 6).[9] In pathogenic prokaryotes, the ability to sense and to respond to extracytoplasmic iron is particularly important due to its intimate involvement in critical bacterial metabolic processes[46,60–62], its low abundance in a mammalian host due to nutritional immunity,[63,64] and its high toxicity if misregulated.[63,65,66] Moreover, for many pathogens such as *P. aeruginosa*, the success of infection and survival both depend on iron, as this transition metal also promotes formation of biofilms and exacerbates antibiotic resistance.[67–71]

To gain a better understanding of how a pathogenic bacterium uses at TCS to sense extracytoplasmic Fe, we expressed, solubilized, purified, and characterized the intact membrane sensor HK of the BqsRS system, an important biofilm-regulating TCS originally identified in *P. aeruginosa*. We first established that *Pa*BqsS binds a single Fe^{2+} ion at a dimeric interface, and using mutagenic and biophysical approaches, we characterized the composition of this metal-binding site. While we initially hypothesized that an ExxE motif would be involved in coordination of Fe^{2+} due to its conservation among other iron sensing, transport, and storage proteins,[46–49] we surprisingly found that only the latter Glu residue within the ExxE motif (Glu^{48}) and part of its flanking motif (Asn^{59}) within the periplasmic domain dimer were required for the binding of a single Fe^{2+} ion in an octahedral geometry. These results represent the first time that an intact, transition-metal sensing membrane HK has been characterized in the presence of its cognate metal, and these findings have interesting implications for the coordination chemistry utilized by other putative Fe-sensing two-component systems that may bind Fe^{2+}, Fe^{3+}, or both.[50–54] Moreover, we demonstrated that the binding of Fe^{2+} to *Pa*BqsS has functional ramifications. Specifically, when tested against the absence of metal, intact *Pa*BqsS showed a dramatic and significant increase in

ATP hydrolysis in the presence of Fe^{2+} that was not observed when just the cytosolic DHp and CA domains of *Pa*BqsS were tested. This consequential observation demonstrates for the first time that the binding of metal within the periplasmic domain of a metal-sensing membrane HK functions as a signal that is transmitted to the cytosolic domain of the HK that hydrolyzes ATP and ultimately transmits the phosphate moiety to the RR. Finally, when tested against a panel of other metal cations (including both Fe^{3+} and Ca^{2+}), this increase in ATP hydrolysis was only observed in the presence of Fe^{2+}, demonstrating specificity of both metal ion identity as well as metal ion oxidation state. When taken together, these results significantly advance our understanding of the structure-function paradigm of a bacterial metal-sensing membrane HK.

These results have important implications for the function of BqsRS in *P. aeruginosa* as well as in other pathogenic bacteria. Interestingly, previous results have indicated that the BqsRS system may sense Ca^{2+} in addition to Fe^{2+}, and that Ca^{2+} sensing via BqsRS may lead to differential downstream gene regulation (thus resulting in BqsRS also being known as CarRS).[31] Our results here show that Ca^{2+} has no effect on the ATPase activity of *Pa*BqsS directly and that *Pa*BqsS is sensitive to Fe^{2+} alone. Thus, the previously observed Ca^{2+}-related effects on the BqsRS/CarRS system[31] are unlikely to be related to the ability of BqsS to bind and to sense Ca^{2+} itself; however, a protein within the larger *bqsPQRS* operon (locus PA2656-PA2659 in PAO1) or elsewhere within *P. aeruginosa* may instead function to sense Ca^{2+} in this manner.[30] The intertwined relationship between *bqs* expression and the presence of both Fe^{2+} and Ca^{2+} is perhaps unsurprising, as both of these metals are present at elevated concentrations in the cystic fibrosis lung environment, a common habitat for *P. aeruginosa*, and *bqsS* transcripts have been detected in cystic fibrosis sputum samples.[32–38] As the BqsRS system regulates rhamnolipid production (an important component of biofilms in *P. aeruginosa*),[72] the presence of Fe^{2+} could target BqsS directly to

regulate biofilm formation via BqsR, while Ca^{2+} may play a role in biofilm dispersal by controlling the *bqs* operon via a separate regulator. As other bacteria (e.g., *Vibrio cholerae, Enterobacter clocae*) also contain BqsRS/CarRS homologs, understanding the intricate but enigmatic crosstalk between Fe^{2+}, Ca^{2+}, biofilm formation, and biofilm dispersal mediated by this TCS warrants further investigation if we are to target this system for future therapeutic developments.

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Author contributions

A.P., K.N.C., and A.T.S. designed the research; A.P., C.I., and K.N.C. performed the research; A.P., K.N.C., and A.T.S. analyzed the data; and A.P., K.N.C., and A.T.S. wrote and edited the paper.

Additional information

Supplementary information. Supplementary information accompanies this paper that includes supplementary figures S1-S5, supplementary table S1, 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. Detergents and Na_2ATP were purchased from Anatrace, MilliporeSigma, and/or RPI, stored at -20 °C, and used as received. Primers for all site-directed mutagenesis experiments were synthesized and verified by Integrated DNA Technologies.

Protein expression and purification. Codon-optimized DNA blocks encoding for *Pseudomonas aeruginosa* strain PAO1 BqsS (Uniprot identifier Q9I0I2) either intact (amino acids 1-445, *Pa*BqsS), the RAxxA periplasmic variant ($R^{44}A^{45}xxA^{48}$), or the cytosolic domain containing both the catalytic ATP-binding and dimerization-His phosphotransfer domains (amino acids 210-440, cyto-*Pa*BqsS), all 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. The 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 intact *Pa*BqsS and variant constructs (E44A, E48A, and E44A/E48A; *vide infra*) was accomplished in 6 baffled flasks each containing 1 L sterile terrific broth (TB) media supplemented with 0.1 mg/mL ampicillin (final) and inoculated with a pre-culture derived from a single bacterial colony. Cells were grown at 37 °C with shaking of 200 RPM until the optical density (OD) at 600 nm (OD_{600}) reached *ca.* 1.2-1.5, at which point cell

cultures were cold-shocked for 2 hr at 4 °C. After cold shocking, another bolus of ampicillin was added to each flask, and protein production was then induced with 1 mM (final) isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were shaken overnight at 200 RPM and 18 °C. After 16-20 hr incubation, cells were harvested by centrifugation at 5000 ×*g* for 12 min at 4 °C, and cell pellets were resuspended in a cellular resuspension buffer (25 mM Tris, pH 8.0, 100 mM sucrose). Immediately prior to cell lysis, phenylmethylsulfonyl fluoride (PMSF, *ca.* 50-100 mg) was added. 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). Cellular debris was cleared by centrifugation at 10,000 ×*g* and 4 °C for 1 hr. The supernatant was decanted and used to prepare crude cellular membranes by ultracentrifugation at 160,000 ×*g* and 4°C for 1 hr. These crude membranes were washed with resuspension buffer, homogenized with a Dounce homogenizer, and ultracentrifuged again at 160,000 ×*g* and 4°C for 1 hr to pellet purified membranes. Purified membranes were then diluted with resuspension buffer and homogenized one final time with a Dounce homogenizer. Solubilization of intact *Pa*BqsS and variants thereof was accomplished by dropwise addition of a stock solution of 10% (w/v) detergent in 4M NaCl until a final concentration of 1% (w/v) detergent and of 500 mM NaCl was reached. During detergent and salt addition, vigorous stirring occurred at room temperature (RT), and the solubilization solution was allowed to continue for *ca.* 3 h at RT. The solution was then clarified by ultracentrifugation at 160,000 ×*g* and 4°C for 1 hr, and the supernatant was then purified via immobilized metal affinity chromatography (IMAC). Briefly, the supernatant containing the Fos-Chol-14-solubilized *Pa*BqsS was applied to 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 8.0, 300 mM NaCl, 1 mM TCEP, 10 % (v/v) glycerol, and 0.05% (w/v) Fos-Chol-14) and protein was then eluted stepwise with 7.0%,

16.7%, 50.0%, and 100.0% (all v/v) respective linear gradients of elution buffer (the same composition of the wash buffer plus 300 mM imidazole). Fractions containing purified *Pa*BqsS were pooled and concentrated using a 100 kDa molecular weight cutoff (MWCO) Amicon spin concentrator. The protein was then combined with *ca.* 20 µg of TEV protease per *ca.* 1 mg *Pa*BqsS and allowed to rock overnight at 4°C in order to cleave the $(His)_6$ tag. Following tag cleavage, another IMAC run was performed and the now cleaved *Pa*BqsS was collected from the flow-through and concentrated using a 100 kDa MWCO Amicon spin concentrator. Cleaved *Pa*BqsS was then applied to a HiLoad 16/600 Superose 6 pg preparative SEC column (Cytiva) pre-equilibrated with SEC buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 1 mM TCEP, 5 % (v/v) glycerol, and 0.02% (w/v) Fos-Chol-14) and eluted isocratically. Fractions containing homogenous protein were collected, quantified, and analyzed using 15% SDS-PAGE and Western blotting.

Expression and purification of cyto*Pa*BqsS was accomplished differently than the intact membrane protein. Large-scale expression of cyto*Pa*BqsS 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 OD_{600} reached *ca*. 0.6-0.8. Cell cultures were then cold-shocked for 1.5-2 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, phenylmethylsulfonyl fluoride (PMSF, *ca.* 50-100 mg) was added. 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 containing 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 step-wise with 7.0 %, 16.7 %, 50.0 %, and 100.0 % (all v/v) respective linear gradients of elution buffer (the same composition of the wash buffer plus 300 mM imidazole). Fractions containing intact cyto*Pa*BqsS were pooled and concentrated using a 10 kDa 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 concentrations and dilutions 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 homogenous protein were collected, quantified, and analyzed using 15 % SDS-PAGE and Western blotting.

Site-directed mutagenesis. Mutations to the intact *Pa*BqsS expression plasmid were accomplished by the QuikChange Lightning Multi Site-Directed Mutagenesis kit (Agilent) in order to encode for the following protein variants with modifications in the periplasmic RExxEN metal-binding motif: E44A, E48A, and E44A/E48A. The following primers were used to introduce the mutated bases (underlined): RAxxE; forward 5'-GTTCTCCGCTTCC<u>G</u>CACGCAGGTTGCC-3' reverse: 5'-GGCAACCTGCGTG<u>C</u>GGAAGCGGAGAAC-3'; RExxA; forward: 5'-CAACCAGCAGGTTC<u>G</u>CCGCTTCCTCACGC-3' reverse: 5'-

GCGTGAGGAAGCGGCGAACCTGCTGGTTG-3'. The presence of each mutation was verified by nanopore DNA sequencing.

Metal-binding assays. All $(His)_6$-cleaved *Pa*BqsS constructs and its variants 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 at 6 °C for 15 min with excess (10 mol eq.) of Fe^{2+}. After the incubation period, the protein-metal solution was buffer exchanged into SEC buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 1 mM TCEP, 5 % (v/v) glycerol, and 0.02% (w/v) Fos-Chol-14) through extensive rounds of concentration and dilution using a 0.5 mL 100 kDa MWCO spin concentrator. To quantitate the amount of Fe^{2+} bound, a modified version of the ferrozine assay[73,74] as previously described.[75]

X-ray absorption spectroscopy (XAS). Samples containing *Pa*BqsS and its variants were metal-loaded anoxically, were concentrated to *ca.* 1-2 mM Fe^{2+} (final concentration), were 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).[76] 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.[76–78] 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:

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

Structural modeling. All AlphaFold models were acquired from the predicted AlphaFold Protein Structure Database[79] or modeled *de novo* using the AlphaFold3 structure prediction server.[80] In order to obtain the predicted structures as well as any aligned error, the following AlphaFold model IDs or UniProt IDs were used, respectively: AF-Q9I0I2-F1-v4 and Q9I0I2.

ATP hydrolysis assays. The rate of ATP hydrolysis as a proxy for kinase activity was determined using a modified form of the malachite green assay that has been adapted for membrane proteins in the presence of detergents.[57] Briefly, stocks of WT *Pa*BqsS were diluted to 0.80-3.0 mg/mL (final) using SEC buffer. Either anoxic stock solutions of $(NH_4)_2Fe(SO_4)_2$ (added to anoxic WT *Pa*BqsS or cytoBqsS) or oxic stock solutions of $ZnCl_2$, $CoCl_2$, $CaCl_2$, $MgCl_2$, $CuCl_2$, $MnCl_2$, and $FeCl_3$ ranging from 0.5-100 mM (final concentration) were added to *Pa*BqsS or cytoBqsS to

stimulate activity. Stock solutions of substrate (Na_2ATP, 0-1.60 mM final concentration) and Mg^{2+} (5.0 mM final concentration) were added to initiate the reaction. Anoxic assays were carried out within an anoxic chamber (*vide supra*) and all reactions were run at 37 °C with 300 RPM shaking for 0-30 min, depending on the construct. The reaction was then immediately halted by addition of a 3:1 (v:v) ratio of working solution (1.05 % (w/v) ammonium molybdate tetrahydrate, 0.0338 % (w/v) malachite green carbinol, 1.0 M HCl) to the protein assay solution. After addition of 100 µL 34 % (w/v) sodium citrate, the absorbance at 660 nm of the enzyme-containing solution was measured using Cary 60 UV-Vis spectrophotometer (Agilent), and [P_i] was interpolated using a standard curve. Specific activity data are corrected against background hydrolysis of Na_2ATP in the absence of enzyme ± metal.

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