The opportunistic pathogen *Pseudomonas aeruginosa* causes antibiotic-resistant, nosocomial infections in immuno-compromised individuals and is a high priority for antimicrobial development. Key to pathogenicity in *P. aeruginosa* are biofilm formation and virulence factor production. Both traits are controlled by the cell-to-cell communication process called quorum sensing (QS). QS involves the synthesis, release, and population-wide detection of signal molecules called autoinducers. We previously reported that the activity of the RhlR QS transcription factor depends on a protein–protein interaction with the hydrolase, PqsE, and PqsE catalytic activity is dispensable for this interaction. Nonetheless, the PqsE–RhlR interaction could be disrupted by the substitution of an active site glutamate residue with tryptophan ([PqsE(E182W)]). Here, we show that disruption of the PqsE–RhlR interaction via either the E182W change or alteration of PqsE surface residues that are essential for the interaction with RhlR attenuates *P. aeruginosa* infection in a murine host. We use crystallography to characterize the conformational changes induced by the PqsE(E182W) substitution to define the mechanism underlying disruption of the PqsE–RhlR interaction. A loop rearrangement that repositions the E280 residue in PqsE(E182W) is responsible for the loss of interaction. We verify the implications garnered from the PqsE(E182W) structure using mutagenic, biochemical, and additional structural analyses. We present the next generation of molecules targeting the PqsE active site, including a structure of the tightest binding of these compounds, BB584, in complex with PqsE. The findings presented here provide insights into drug discovery against *P. aeruginosa* with PqsE as the target.
We recently showed that the ability of PqsE to interact with RhlR can be weakened by the introduction of an “inhibitor mimetic” mutation in which the glutamate residue at position 247 residues to alanine completely abolishes the PqsE–RhlR interaction.25 When introduced into P. aeruginosa, neither PqsE(E182W) nor PqsE(R243A/R246A/R247A) [the latter called PqsE(NI) for “non-interacting”] can promote PqsE–RhlR-dependent virulence phenotypes, including the production of the pyocyanin toxin. The effects of the PqsE E182W and PqsE NI substitutions on pyocyanin production are not through impairment of PqsE catalytic function, as the catalytically inactive variant, PqsE(D73A), remains fully capable of interacting with RhlR and driving pyocyanin production. These results indicate that PqsE has two independent functions, catalysis and interaction with RhlR, and it is interaction with RhlR, not catalysis, that is required for virulence.

From a drug discovery perspective, it is particularly promising that the PqsE active site E182W mutation weakens the distal PqsE–RhlR interaction, the consequence of which is the suppression of virulence phenotypes. We assert this because a PqsE active site-targeting molecule would likely be able to bind with high affinity and be more amenable to medicinal chemistry than a molecule targeting the interaction site on the surface of the protein. Protein–protein interactions, which typically occur over large, shallow protein surfaces, have proven notoriously difficult to target with small molecules.25 Molecules that can bind in protein–protein interaction domains typically do so with weak affinity, are large and structurally complicated, and are difficult to optimize through medicinal chemistry efforts.27 With this notion in mind, in this study, we aimed to determine the mechanism by which the PqsE E182W mutation disrupts the PqsE–RhlR interaction, and whether it is possible to achieve a similar effect with a small molecule inhibitor that binds in the PqsE active site. In a proof of principle experiment, we use our PqsE variants to demonstrate that disrupting the PqsE–RhlR interaction indeed attenuates in vivo P. aeruginosa virulence in a mouse lung infection model. We employ crystallography to characterize the structure of the PqsE(E182W) protein. We probe the functions of the PqsE active site through mutagenesis. Finally, we present the next generation of PqsE active site-targeting small molecules for further synthetic optimization. Our results can inform the discovery and/or design of effective antimicrobial agents to treat P. aeruginosa infections.

**RESULTS**

**PqsE Variants that Cannot Interact with RhlR Display Attenuated Infection Phenotypes in Cell Assays and in a Mouse Lung Infection Model.** We have shown previously that, unlike the overexpression of wildtype (WT) pqsE, the overexpression of pqsE mutants encoding proteins that cannot interact with RhlR in vitro impairs pyocyanin production in ΔpqsE P. aeruginosa PA14.24,25 To verify that the PqsE variants of interest display similar defects in virulence phenotypes when the genes encoding them are expressed from the native locus, we constructed P. aeruginosa PA14 strains harboring pqsE-(D73A), pqsE(E182W), and pqsE(NI) on the chromosome. Western blots showed that the WT and variant PqsE proteins were produced at the same levels and exhibited similar stabilities (Figure S1). WT P. aeruginosa PA14 made pyocyanin while the ΔpqsE strain did not (4% compared to WT) (Figure 1b). When the PqsE variant proteins were produced from the chromosomally encoded genes, the results were entirely consistent with our previous findings for each PqsE variant produced from a plasmid. Specifically, the catalytically inactive
The PqsE(E182W) variant made nearly WT levels of pyocyanin (96%), the PqsE(E182W) inhibitor mimetic variant was severely impaired (17%), and the PqsE(NI) variant was incapable of driving pyocyanin production (4%) (Figure 1b). Previously, we showed that the P. aeruginosa PA14 ΔrhlI strain forms smooth colonies, whereas the ΔrhlI ΔrhlR and ΔrhlI ΔpqsE double mutants form biofilms with hyper-rugose morphologies. Thus, both PqsE and RhlR are required to suppress hyper-rugose biofilm formation in the absence of the C4-HSL autoinducer. To determine which specific function of PqsE, catalysis and/or interaction with RhlR, is linked to the control of biofilm morphology, we tested our PqsE variants. Each pqsE mutant was incorporated at the native chromosomal locus in the ΔrhlI strain and biofilm morphology was assessed (Figure 1c). Both the ΔrhlI pqsE(E182W) and ΔrhlI pqsE(NI) mutants formed hyper-rugose biofilms similar to those of the ΔrhlI ΔrhlR and ΔrhlI ΔpqsE mutants. Only the ΔrhlII strain harboring the pqsE(D73A) mutation exhibited the smooth biofilm morphology of the parent ΔrhlII strain. This result demonstrates that the PqsE−RhlR interaction controls biofilm morphology and that PqsE catalytic activity is dispensable for this trait.

To explore the individual roles of PqsE catalysis and PqsE−RhlR interaction during host infection, we assessed the relative pathogenicity of WT P. aeruginosa PA14, ΔpqsE, pqsE(D73A), pqsE(E182W), and the pqsE(NI) strains in a murine model of acute pneumonia. Mice were infected intratracheally with equal strain inoculum levels (3 × 10⁶ cfu/mouse) and monitored over 48 h of infection. At 24 h, mice from all infection groups exhibited mild to moderate symptoms in response to infection, primarily displaying decreased mobility and increased breathing. Consistent with these symptoms, comparable levels of lung colonization were observed among all groups (Figure 1d). At 48 h, however, mice infected with either WT P. aeruginosa PA14 or the pqsE(D73A) mutant became more lethargic and appeared to progressively manifest additional symptoms, including increasingly labored breathing, hunched posture, and decreased response to stimuli. Moreover, those mice demonstrated >2 log increase in the bacterial burden compared to 24 h. In stark contrast, mice infected with either ΔpqsE, pqsE(E182W), or pqsE(NI) strains continued to display mild clinical symptoms with almost no change in their lung bacterial burden (Figure 1d). Together, the above findings demonstrate that the PqsE−RhlR interaction, and not PqsE catalytic activity, is responsible for shaping the pathogenicity of P. aeruginosa PA14 in vitro and in vivo.

PqsE E182W Substitution Induces a Loop Rearrangement near the Active Site.

Figure 2. PqsE E182W substitution induces a loop rearrangement that repositions residue E280. (a) Structure of PqsE(E182W) (blue) overlaid with that of WT PqsE (PDB: 2Q0I, gray). The active site iron atoms are shown in orange. The red arrow indicates repositioning of E280 in PqsE(E182W) relative to its position in WT PqsE. (b) Structure of PqsE(E182W/E280A) (maroon) overlaid with that of WT PqsE (as in a). In panels a and b, residue E280 is shown in stick representation. (c) Binding of WT and variant PqsE proteins to the active site fluorescent probe BB562. K_{app} was determined in two independent experiments performed in triplicate. (d) Hydrolysis of 4-methylumbelliferyl butyrate (MU-butyrate) by the designated purified PqsE proteins. Values are represented as % activity of WT PqsE protein. Results are the average of two independent experiments performed in triplicate. Error bars represent standard deviations. (e) First derivative plots (dF/dT is defined as the change in SYPRO Orange fluorescence divided by change in temperature) of melting curves for the designated PqsE proteins. The peak of each curve is defined as the T_m of that protein.
experiment demonstrated that weakening the ability of PqsE to interact with RhlR by mutating an active site residue [PqsE(E182W)] causes a similarly severe reduction in infectivity to that caused by the complete elimination of the PqsE−RhlR interaction [PqsE(N1)]. To understand, at an atomic level, what conformational changes the E182W alteration induced in PqsE to affect its ability to interact with RhlR, we determined the structure of PqsE(E182W) (Figure 2a). Although the PqsE(E182W) crystals grow in the same P3_1_21 crystal form as WT PqsE, there is a significant structural rearrangement in the active site. In WT PqsE, the sidechain of E182 lies at the edge of the ligand binding site and makes hydrogen bonds with R191 and Q272. These interactions do not occur in PqsE(E182W), and rather, new interactions are made between the sidechain of the introduced W182 residue and F276, L277, and P278. The E182W change induces the rearrangement of the G270-L281 loop between helices 6 and 7, with a portion of that loop becoming disordered. Examination of electron density within the active site indicated a surprising consequence—with the sidechain of E280 relocating by 12 Å and becoming Fe bound in the center of the active site. Indeed, the repositioned E280 sidechain directly binds both Fe atoms, acting as a bridging ligand, and thus is most likely responsible for the stabilizing effect the E182W alteration has on PqsE, which we have reported previously.\(^{24}\)

To test whether repositioning of the PqsE E280 residue underpinned the inhibitor mimetic characteristics of the PqsE(E182W) protein, we engineered the E280A substitution into PqsE(E182W) to make PqsE(E182W/E280A). We determined the crystal structure of PqsE(E182W/E280A) revealing further changes in the PqsE active site. In this case, the presence of an alanine at residue 280 eliminated interaction with the iron atoms, and the loop consisting of residues D275-L281 was disordered (Figure 2b). The remainder of the loop was ordered and its structure reverted to a conformation that was more native-like than in PqsE(E182W). Consequently, the removal of the glutamate−Fe interaction via the E280A substitution apparently unlocked the active site of PqsE, partially restoring WT activity. Indeed, whereas PqsE(E182W) exhibits reduced binding affinity for the active site fluorescent probe, BB562, PqsE(E182W/E280A) displays WT binding affinity for the probe (Figure 2c). Furthermore, PqsE(E182W/E280A) has reduced lytic capacity for a synthetic ester substrate, with only 7% activity relative to WT PqsE. The catalytic function of the PqsE(E182W/E280A) variant was greatly improved by the “unlocking” of the active site (34% compared to WT PqsE, Figure 2d). Consistent with the repositioning of E280 into the PqsE active site contributing to the increased stability of the PqsE(E182W) protein relative to WT PqsE, introduction of the E280A substitution reduced the Tm of the PqsE(E182W/E280A) protein to nearly that of WT PqsE (Figure 2e).

Introduction of the E280A alteration into PqsE(E182W) partially corrects the defects in small molecule binding and hydrolysis. Nonetheless, the PqsE(E182W/E280A) variant remains incapable of enhancing RhlR transcription factor activity in an Escherichia coli reporter assay and it does not drive pyocyanin production in P. aeruginosa PA14 (Figure 3a,b, respectively). Likewise, PqsE(E182W/E280A) is not improved for interaction with RhlR in vitro (Figure 4a). These findings further demonstrate the independence of the PqsE catalytic and virulence functions. Curiously, neither the PqsE(E182W) nor the PqsE(E182W/E280A) structure showed any changes in the region of the protein required for interaction with RhlR (R243, R246, and R247 on helix 5, Figure 2a,b, respectively). This result preliminarily suggests that either PqsE possesses an additional region of interaction with RhlR, perhaps employing the G270-L281 loop that is rearranged in PqsE(E182W) or partially disordered in PqsE(E182W/E280A), or alternatively, that a particular conformation of the G270-L281 loop is required to allosterically promote the interaction with RhlR. Irrespective of the underlying mechanism, the above structural and biochemical analyses characterizing PqsE(E182W) and PqsE(E182W/E280A) show that it is possible to disrupt the PqsE−RhlR interaction by manipulating the integrity of the G270-L281 loop that forms one face of the PqsE active site.

**PqsE Variants Harboring Combinations of Substitutions Decouple Catalytic Activity from RhlR Interaction.** Our previous and above work comparing the inhibitor mimetic and RhlR non-interacting PqsE variants to the catalytically inactive PqsE(D73A) protein strongly suggest that the ability of PqsE to interact with RhlR and, in turn, drive virulence phenotypes is independent of PqsE enzyme activity. To confirm this notion, and, additionally, to explore the potential for influence of one activity on the other, we constructed PqsE variants containing combinations of amino acid substitutions underlying defects in enzyme activity, RhlR interaction, or both functions. Purity of these PqsE variant proteins was verified by SDS-PAGE analysis (Figure S2). We assessed the phenotypes of this set of variants in vitro, in recombinant E. coli, and in P. aeruginosa PA14. Specifically, we measured interaction of the purified PqsE variant proteins with RhlR by our pull-down assay (Figure 4a), intrinsic stability by Tm measurements (differential scanning fluorimetry, DSF), catalytic function by hydrolysis of the synthetic ester substrate MU-butyrate, and active site accessibility via binding of the
and loaded into the lanes of the gel. PqsE appears as a ∼34 kDa band and RhlR as a ∼28 kDa band. For the variant combinations, “X” denotes that the protein in that lane contains the designated substitutions listed to the right. (b) Measured activities of PqsE variants in the designated assays.

Figure 4. PqsE enzymatic activity is dispensable for RhlR interaction, RhlR transcriptional activity, and pyocyanin production. (a) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of protein complexes formed in in vitro pull-down assays. 6xHis-PqsE proteins were immobilized on Ni resin and exposed to lysate containing RhlR. The resulting protein complexes were washed and eluted from the Ni resin (Figure 3). This result confirms that the inability of select PqsE variants to activate RhlR and drive pyocyanin production stems from loss of the PqsE—RhlR interaction, and is not the result of decreased PqsE protein production or stability.

One potential complication in the above analyses is that PqsE proteins harboring the "NI" triple arginine substitutions are less stable than WT and other of our variant PqsE proteins when overproduced in cells from a plasmid. Therefore, all PqsE proteins harboring the "NI" triple arginine substitutions were detected at lower levels compared to the other PqsE variant proteins in both E. coli and P. aeruginosa PA14 cell lysates (Figures S5 and S6). This feature could potentially have been the source of apparent reduced activities in the RhlR transcriptional reporter assay and the pyocyanin assay. By contrast, enzymatic capability did not correlate with the ability to form a complex with RhlR, activate RhlR transcription, or to produce pyocyanin.

One potential complication in the above analyses is that PqsE proteins harboring the "NI" triple arginine substitutions are less stable than WT and other of our variant PqsE proteins when overproduced in cells from a plasmid. Therefore, all variant proteins containing the NI substitutions were detected at lower levels compared to the other PqsE variant proteins in both E. coli and P. aeruginosa lysates (Figures S5 and S6). This feature could potentially have been the source of apparent reduced activities in the RhlR transcriptional reporter assay and the pyocyanin assay. Our results with the PqsE(E182W/NI) variant show, however, that this is not the case. Due to the stabilizing effect of the E182W alteration (Figures 2 and S3), the PqsE(E182W/NI) protein was produced and detected at WT levels in both E. coli and P. aeruginosa lysates (Figures S5 and S6). Nonetheless, PqsE(E182W/NI) was completely inactive in both the pyocyanin production and RhlR transcriptional activity cell-based assays (Figure 3). This result confirms that the inability of select PqsE variants to activate RhlR and drive pyocyanin production stems from loss of the PqsE—RhlR interaction, and is not the result of decreased PqsE protein production or stability.

**Synthetic Optimization of an Active Site-targeting Small Molecule Scaffold.** The results of our structural and mutagenic analyses suggest that manipulation of the PqsE active site, such that the G270-L281 loop becomes rearranged or disordered will result in decreased P. aeruginosa virulence due to inhibition of the PqsE—RhlR interaction. Thus, it is of interest to develop molecules that bind in the PqsE active site and, in so doing, inhibit the PqsE—RhlR interaction. We previously characterized two active site-targeting PqsE inhibitors, BB391 and BB393. Crystallographic analyses of each compound bound to PqsE revealed their respective binding poses and interactions in the active site. Each inhibitor exhibited mid-nanomolar competition with the BB562 active site probe for binding to PqsE.

Guided by our crystal structures of BB391 and BB393 bound to PqsE, we designed a series of BB391−BB393 hybrid derivatives to probe the contributions of each moiety to binding affinity (Figure 5a). BB580 and BB581 were designed to test the enhancement of PqsE(E182W/NI) titration curve, which occurred when the binding curve was too shallow or did not achieve saturation.
\(\pi\)-stacking with alternative aromatic groups at this core position (see position “2” in Figure 5a). None of these three derivatives, featuring thiazole, pyrazole, or pyridine rings, respectively, bound as tightly as BB580, and therefore among the molecules tested, a phenyl ring was deemed ideal at this core position (Figure 5b,c). Finally, derivatives BB582, BB583, BB584, BB587, and BB588 were designed to assess the importance of a hydrophobic moiety on the BB393 molecule (see position “3” in Figure 5a), which in the crystal structure, nestles into a hydrophobic groove near the solvent-exposed entrance to the PqsE active site. The results with BB587 and BB588 show that elimination of either the methyl or ethyl groups, respectively, leads to slightly decreased binding affinity (EC\(_{50}\) = 273 and 290 nM, respectively). However, if the morpholine ring is removed and either a phenyl (BB583) or tert-butyl (BB584) group is installed, a modest increase in binding affinity is achieved (EC\(_{50}\) = 71 and 34 nM, respectively) (Figure 5b,c). Such improvement was not observed for BB582, featuring a pyrazine ring, suggesting that increased hydrophobicity of this portion of the molecule tracks with the increased engagement of the hydrophobic groove in PqsE. None of the derivatives described here inhibit the PqsE–RhlR interaction. Within that context, BB584 displayed the tightest binding to PqsE and provides a starting scaffold for the design of new derivatives.

We determined the crystal structure of BB584 bound to PqsE, which showed a similar orientation in the active site and similar ligand–protein interactions to those observed in the crystal structures of PqsE with BB391 and BB393 bound in the active site (Figure 5d). Specifically, the nitrogen on the indazole ring of BB584 bonds with the Fe2 atom, displacing a water molecule that normally resides at this position. Two additional hydrogen bonds exist between BB584 and PqsE sidechains: the BB584 amide oxygen with the hydroxyl group of the S285 sidechain, and the BB584 urea oxygen with the backbone amide N–H of residue Y72. As predicted, the tert-butyl group tucks into the hydrophobic groove that sits between the \(\alpha\)-helix consisting of PqsE residues S104-L116 and the backbone of the conserved \(^6HXHXDH^7\) motif. Analogous to the structures of BB391 and BB393 bound to PqsE, no significant conformational changes in the protein were induced by binding of BB584 (rmsd of 0.24 Å for 293 Cα atoms), possibly explaining why none of the three compounds disrupt the PqsE–RhlR interaction. Nonetheless, the structures of these ligands bound to WT PqsE along with the structures of PqsE(E182W) and PqsE(E182W/E280A) can be

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**Figure 5.** Optimization of new PqsE active site-targeting small molecules. (a) Structures of the precursor molecules BB391 and BB393 and the hybrid derivative BB580. Positions denoted 1, 2, and 3 were derivatized to explore the binding affinity for the PqsE active site. (b) Fluorescence polarization competition curves for select BB391–BB393 hybrid derivatives competing with the BB562 active site probe for binding to PqsE. Polarization value for PqsE-BB562 in the absence of a competitor is defined as 100% probe bound. All polarization values were background-subtracted by the reading for the probe in the absence of PqsE (background fluorescence). (c) Competitive fluorescence polarization EC\(_{50}\) values calculated for all BB391–BB393 derivatives, determined from one experiment performed in triplicate. (d) Structure of BB584 bound to PqsE. Both the BB584 molecule and key PqsE amino acid sidechains are shown as sticks. Amino acid sidechain carbons are depicted in gray and BB584 carbons are shown in tan. Iron atoms and water molecules are shown as orange and green spheres, respectively. Oxygen and nitrogen atoms are in red and blue, respectively. Hydrogen bonds are shown as dotted yellow lines.
used as guides in the design of new derivatives with the potential to disrupt the interaction between PqsE and RhlR.

■ MATERIALS/EXPERIMENTAL DETAILS

Strains, Media, and Molecular Procedures. The P. aeruginosa UCBPP-PA14 strain was used as the parental strain for all experiments involving P. aeruginosa. All strains were grown in Luria–Bertani broth, unless otherwise stated, and antibiotics were used at the following concentrations: ampicillin (200 μg/mL), kanamycin (100 μg/mL), tetracycline (10 μg/mL), carbenicillin (400 μg/mL), gentamicin (30 μg/mL), and irgasan (100 μg/mL). Plasmids were constructed following a previously reported site-directed mutagenesis protocol and were transformed into P. aeruginosa PA14 strains as described. Deletion of and point mutations in pqsE were generated by a previously reported method, with some modifications. Briefly, pqsE variants were cloned onto the pEXG2 vector. E. coli SM10λpir carrying each pEXG2-pqsE-containing plasmid was mated with P. aeruginosa PA14 or the ΔrhlI strain and exconjugants were selected on LB agar containing gentamycin and irgasan. Colonies were grown in LB medium at 37 °C for 1–2 h and plated on LB agar containing 5% sucrose to force the elimination of the sacB gene on the plasmid. Resulting colonies were patched onto LB agar plates and onto plates containing gentamycin, and pqsE variants in Gent6 colonies were confirmed by sequencing. Strains used in this study are listed in Supporting Information, Table S1.

General Methods. 6xHis-PqsE proteins were purified for biochemical assays (tagged) and crystallography (tag removed), as described previously. Enzyme activities of purified 6xHis-PqsE variants were measured as previously reported with MU-buturate as the substrate. Fluorescence polarization assays with the BB562 active site probe were performed as described. The melting temperature (Tm) of each purified 6xHis-PqsE variant was measured using DSF as described previously. Interaction between PqsE and RhlR proteins in vitro was measured as described previously using pull-down assays. Pyocyanin production by P. aeruginosa PA14 strains carrying pqsE variants on the pUCP18 plasmid was measured as described. The ability of PqsE variants to increase RhlR transcription factor activity in an E. coli reporter assay was assessed as previously described. All small molecule syntheses and characterization are described in the Supporting Information.

Colony Biofilm Morphology Assay. Cultures were grown overnight in LB broth with shaking at 37 °C, and 1 μL of culture was spotted onto a 60 × 15 mm Petri plate containing 10 mL of biofilm medium (1% Tryptone, 1% agar, 40 mg/L Congo Red, 20 mg/L Coomassie Brilliant Blue). Biofilms were grown at 25 °C for several days and imaged throughout their development on a Leica stereomicroscope M125 mounted with a Leica MC170 HD camera at 7.78× magnification.

Mouse Lung Infection Studies. For all mouse experiments, P. aeruginosa strains were grown on Pseudomonas Isolation Agar (PIA) for 16–18 h at 37 °C and suspended in PBS to an OD600 of 0.5, corresponding to ~109 cfu/mL. These samples were adjusted spectrophotometrically and then diluted to the appropriate OD600 in phosphate-buffered saline (PBS). Eight-to-ten-week-old female BALB/c mice (Jackson Laboratories, Bar Harbor, ME) were anesthetized by the intraperitoneal injection of 0.2 mL of a mixture of ketamine (25 mg/mL) and xylazine (12 mg/mL). Mice were infected by noninvasive intratracheal instillation of 50 μL of ~3 × 106 cfu of P. aeruginosa WT or isogenic mutants. Mice were euthanized at 24 and 48 h post-infection and whole lungs were collected aseptically, weighed, and homogenized in 1 mL of PBS. Bacterial loads in tissue homogenates were enumerated by serial dilution and plating on PIA. Comparison and analyses of the numbers of viable bacteria obtained in lung homogenates were performed using GraphPad Prism version 7 software. Results were analyzed using one-way analysis of variance (ANOVA) and were compared using the Kruskal–Wallis test for comparison of three groups or the Mann–Whitney U test for the analysis of two groups.

Protein Crystallography. Purified proteins (~10 mg/mL) and protein-compound complexes were crystallized by hanging drop vapor diffusion at 22 °C following mixing at a 1:1 ratio with the well buffer. Crystallization buffers used were as follows: PqsE(E182W) (0.1 M HEPES pH 7.5, 0.2 M MgCl2, 15% (w/v) PEG 400) cryoprotected with 20% (v/v) glycerol prior to freezing, PqsE(E182W/E280A) (0.1 M HEPES pH 7.5, 0.2 M MgCl2, 27% (w/v) PEG 400) cryoprotected with 10% (v/v) ethylene glycol prior to freezing, and PqsE-BB584 (0.1 M HEPES pH 7.5, 0.2 M MgCl2, 15% (v/v) 2-propanol) cryoprotected with 30% (v/v) ethylene glycol with additional BBS54 (50 μM) in the cryoprotectant solution. Crystals typically formed within 48 h, but in some cases, took up to 5 days to form. PqsE(E182W), PqsE(E182W/E280A), and PqsE-BB584 crystals each grew in the same trigonal space group as had been previously observed for WT PqsE, PqsE-BB391, and PqsE-BB393 (P321, a = b = 60 Å c = 146 Å α = β = 90°, γ = 120°) with one molecule in the asymmetric unit. Data were collected on either the 17-ID-1 beamline of the NSLS-II synchrotron [PqsE(E182W) and PqsE(E182W/E280A)], or on a Rigaku MicroMax 007HF rotating anode source (PqsE-BB584) (Table S2). Data were processed either with XDS and AIMLESS or with DENZO and SCALEPACK. The starting model for each refinement was a prior ligand-bound structure (7KGX) with the ligand and water molecules removed but the iron atoms retained, subjected to rigid-body refinement, followed by conventional refinement using Phenix.refine. The structures were iteratively rebuilt using Coot and refined with Phenix.refine. Both PqsE variant proteins exhibited significant conformational changes in a loop region (G270-L281) relative to the starting model, which remodeled the active site. In the case of the BB584 ligand, an atomic model of the ligand was fit to the difference electron density observed in the active site of PqsE and refined with partial occupancy (0.93). Final refinement statistics are shown in Table S2 including the identifiers for the structures’ depositions in the Protein Data Bank [PqsE- (E182W) PDB ID: 7TZA, PqsE(E182W/E280A): 7U6G, and PqsE-BB584 PDB ID: 7TZA].

■ CONCLUSIONS

PqsE has two biochemical activities, a protein–protein interaction with RhlR, which increases RhlR transcriptional activity at target promoters, and an esterase activity. The substrate and product of PqsE catalysis are currently unknown and ongoing research aims to identify them. Here, we showed that the PqsE–RhlR interaction is separable from the PqsE catalytic activity. Moreover, we engineered a set of PqsE variant proteins that possess every combination of the two activities (catalytic/interaction, catalytic/interaction, catalytic/interaction, catalytic/interaction). Analysis of
these proteins, coupled with previous work, shows that the PqsE−RhlR interaction is linked to virulence factor production in *P. aeruginosa* PA14. Here, we demonstrated that the PqsE−RhlR interaction also controls the development of *P. aeruginosa* PA14 biofilm morphology and is crucial for establishing an infection in a host animal. Remarkably, PqsE catalytic function is dispensable for the regulation of RhlR-transcriptional activity, biofilm morphology, virulence factor production, and animal infectivity. As the PqsE variant phenotypes are consistent between all assays performed, the set of cell-based assays we employ here can effectively be used in lieu of infectivity assays in animals to probe and predict the potential of small molecules as *P. aeruginosa* antibiotics that target PqsE.

Our finding that the PqsE−RhlR interaction was disrupted following the introduction of the E182W substitution in the PqsE active site was surprising given that this residue is distal to the RhlR interaction site. The crystal structure of PqsE(E182W) revealed that a loop rearrangement inserts the E280 residue into the active site to coordinate both of the iron atoms at this site. It is the repositioning of E280 in the PqsE(E182W) variant—blocking substrate access to the catalytic iron atoms—that is responsible for the “inhibitor mimic” nature of this variant. Catalytic activity is restored in the PqsE(E182W/E280A) variant because the alanine substitution clears the active site, thus enabling substrate binding; however, PqsE(E182W/E280A) remains incapable of interacting with RhlR (Figures 3 and 4). Analysis of the PqsE(E182W/E280A) structure, and the surprisingly modest conformational changes that are apparent compared to the structures of WT PqsE and PqsE(E182W) (Figure 2a,b), suggest that the structural integrity of the G270-L281 loop is essential for PqsE to interact with RhlR. These results confirm that, although the RhlR-interaction is independent of PqsE catalytic activity, structural changes induced through the active site of PqsE can disrupt interaction with RhlR.

All prior crystal structures of WT PqsE showed that the E280 residue resides on the surface with the glutamate sidechain directed outward into the solvent. Our data show that single substitution of this glutamate with alanine [i.e., PqsE(E280A)] increased accessibility of the PqsE active site (Figure 2c). This finding potentially points to naturally occurring PqsE conformational dynamics in which the position of the E280 sidechain alternates between facing the solvent and being inserted into the active site. While not proven, it is possible that E280 serves as a dynamic gate-keeper residue for the active site. Perhaps, its position determines whether PqsE will undergo catalysis and/or will interact with RhlR. Such a mechanism would make considerations of the PqsE E280 sidechain position key for future drug discovery efforts. We recognize that our proposed ideas for how positioning of the PqsE E280 residue influences interaction with RhlR are speculative. Additional experimental investigation is required to reveal whether PqsE undergoes natural conformational dynamics that influence its interactions with RhlR and whether structural flexibility plays any role in its putative enzyme function.

Figure 5 presents a new series of molecules, inspired by the previous PqsE inhibitors BB391 and BB393, all of which bind in the PqsE active site. Our competitive binding assay allowed us to rank derivatives by their relative affinities providing preliminary structure–activity relationships (SAR). The molecules presented here were derivatized at positions that do not contact the regions of the PqsE active site at which the E182W substitution perturbs the structure. Thus, it is not surprising that the compounds do not affect the PqsE−RhlR interaction. Rather, the SAR explored in the current compound series was designed to probe interactions of the small molecule scaffolds with two regions of the active site: the iron-binding site and the hydrophobic groove near the solvent-exposed face of the active site. This compound series enabled identification of high-affinity PqsE active site binders that can be further derivatized, potentially at the indazole ring, to allosterically inhibit the interaction with RhlR. Among this set, compound BB854 binds most tightly in the PqsE active site (EC₅₀ = 34 nM). The crystal structure of the PqsE-BB854 complex solved here (Figure 5c) provides needed information for launching the structure-guided design of the next generation of molecules targeting the PqsE active site. An alternative strategy afforded by these molecules would be their use in proteolysis targeting chimera (PROTAC) design with PqsE as the target. Indeed, recent developments focused on using PROTACs for the targeted degradation of bacterial proteins encourage the use of such high-affinity ligands for this purpose. Our PqsE variant analyses and companion structures should propel the design of high affinity active site-targeting compounds that do disrupt the PqsE−RhlR interaction via an allosteric mechanism, presumably involving the movement of the G270-L281 loop.

**ETHICS STATEMENT**

All mouse procedures were performed in accordance with the established guidelines of the Emory University Institutional Animal Care and Use Committee (IACUC) under protocol number DAR-201700441. This study was carried out in strict accordance with established guidelines and policies at Emory University School of Medicine, the recommendations in the Guide for Care and Use of Laboratory Animals, as well as local, state, and federal laws.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biochem.2c00334.

Western blot characterization of PqsE proteins from cell lysates, SDS-PAGE analysis and biochemical characterization of purified PqsE variants, in vivo characterization of PqsE variants, SAR for BB391-BB393 hybrid derivatives, strains used in this study, crystallographic data collection and refinement statistics, and detailed synthetic methods (PDF)

**Accession Codes**

PqsE: A0A0H2Z6F6. RhlR: A0A0H2ZEG8.

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I.R.T., P.D.J., and D.A.M. conducted experiments; I.R.T., P.D.J., D.A.M., J.B.G., and B.L.B. designed experiments and prepared the manuscript.

Notes
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