Two-Component Signaling Systems Regulate Diverse Virulence-Associated Traits in *Pseudomonas aeruginosa*

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ABSTRACT *Pseudomonas aeruginosa* is an opportunistic pathogen that can cause problematic infections at different sites throughout the human body. *P. aeruginosa* encodes a large suite of over 60 two-component signaling systems that enable cells to rapidly sense and respond to external signals. Previous work has shown that some of these sensory systems contribute to *P. aeruginosa* pathogenesis, but the virulence-associated processes and phenotypic traits that each of these systems controls are still largely unclear. To aid investigations of these sensory systems, we have generated deletion strains for each of 64 genes encoding histidine kinases and one histidine phosphotransferase in *P. aeruginosa* PA14. We carried out initial phenotypic characterizations of this collection by assaying these mutants for over a dozen virulence-associated traits, and we found that each of these phenotypes is regulated by multiple sensory systems. Our work highlights the usefulness of this collection for further studies of *P. aeruginosa* two-component signaling systems and provides insight into how these systems may contribute to *P. aeruginosa* infection.

IMPORTANCE *Pseudomonas aeruginosa* can grow and survive under a wide range of conditions, including as a human pathogen. As such, *P. aeruginosa* must be able to sense and respond to diverse signals and cues in its environment. This sensory capability is endowed in part by the hundreds of two-component signaling proteins encoded in the *P. aeruginosa* genome, but the precise roles of each remain poorly defined. To facilitate systematic study of the signaling repertoire of *P. aeruginosa* PA14, we generated a library of deletion strains, each lacking one of the 64 histidine kinases. By subjecting these strains to a battery of phenotypic assays, we confirmed the functions of many and unveiled roles for dozens of previously uncharacterized histidine kinases in controlling various traits, many of which are associated with *P. aeruginosa* virulence. Thus, this work provides new insight into the functions of two-component signaling proteins and provides a resource for future investigations.

KEYWORDS two-component signaling systems, histidine kinase, virulence, *Pseudomonas aeruginosa*, signal transduction

Bacteria must constantly sense and respond to changes in their environments to survive and proliferate. One of the most common sensory mechanisms in bacteria involves two-component signaling pathways, typically composed of a receptor histidine kinase and a cognate response regulator (1). Upon stimulation by an external signal, the histidine kinase will autophosphorylate and then transfer a phosphoryl group to a cognate response regulator that can then trigger a cellular response, often by changing patterns of gene expression (Fig. 1A). Many pathogens also rely on complex histidine kinases harboring multiple phosphoacceptor and phosphodonor sites or on noncanonical pathways that include additional components, such as histidine-

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FIG 1 Summary of two-component signaling protein domain architectures and growth phenotypes. (A) Schematic of a typical two-component signaling system. The histidine kinase (HK) is anchored in the inner membrane and senses a signal in the periplasm. Upon activation, the HK autophosphorylates and then transfers a phosphoryl group to its cognate response regulator (RR) in the cytoplasm, which can then trigger changes in gene expression, as shown, or other cellular processes. OM, outer membrane; IM, inner membrane. (B) Schematic of different two-component signaling system architectures. Each domain type has a unique color. (C) Schematic of all 65 two-component signaling proteins examined in this study, color coded as in panel B. The predicted locations of these proteins in the cell (membrane or cytoplasmic) are shown. (D) Representative growth curves of mutants exhibiting a growth defect relative to the wild-type in ABTGC medium. (E) Doubling times for wild-type and slower-growing mutants.

containing phosphotransfer proteins (Hpt) (1) (Fig. 1B). Although a wide variety of two-component signaling proteins have been identified in many different pathogens (2), the roles that they play during infection are still largely unexplored.

The opportunistic pathogen *Pseudomonas aeruginosa* inhabits a wide range of environments, being free-living in habitats like soil and water (3) and also found in host-associated environments, such as the diseased lungs of persons with cystic fibrosis (CF) (4). To facilitate survival in these diverse niches, *P. aeruginosa* encodes a large suite of over 60 two-component signaling systems that likely allow it to rapidly sense and respond to diverse external cues (5, 6) (Fig. 1C). Genetic screens using various animal models (7, 8), as well as the sequencing of common mutations in CF clinical isolates

(9–11), have identified dozens of two-component signaling systems that likely contribute to *P. aeruginosa* pathogenicity. Perhaps the best-characterized of these systems is the GacS-GacA pathway (12), which controls the expression of virulence-associated traits such as biofilm formation and type VI secretion. However, the functions and phenotypic traits regulated by many of the other sensory systems in this microbe are still unclear.

A few recent studies have attempted to address this gap by examining a collection of two-component signaling mutants in various phenotypic assays, including an *in vivo* zebrafish model of infection (13), killing of cultured human lung cells (14), and swarming and biofilm formation under laboratory conditions (15). However, each of these studies used transposon mutants from the available *P. aeruginosa* transposon library (16), which can produce unwanted artifacts, such as polar effects on downstream genes and the generation of truncated gene products. Further, these studies assayed mutants for a relatively limited set of phenotypes.

Here, we constructed a library of in-frame deletion strains for 65 two-component signaling genes in the *P. aeruginosa* PA14 strain, including 64 sensor histidine kinases and one histidine phosphotransferase. To highlight the value of this collection, we determined initial phenotypic characterizations of this library by systematically assaying these mutants for over a dozen virulence-associated phenotypes, including growth, swarming motility, swimming motility, twitching motility, biofilm formation, secreted virulence factors, and antibiotic resistance. We found that multiple two-component signaling systems regulate each virulence phenotype, and we identified nearly two dozen regulators that control at least three of these virulence-associated processes. Overall, our results shed light on the multitude of functions regulated by two-component signaling proteins in *P. aeruginosa* and provide a foundation for further mechanistic studies of sensory systems in this microbe.

RESULTS

Two-component signaling proteins regulate growth and motility. *P. aeruginosa* PA14 encodes over 60 two-component signaling systems, but the cellular processes that these sensory systems control have not yet been fully characterized. To address this gap, we generated in-frame deletion constructs of 64 histidine kinases and one histidine-containing phosphotransfer protein (HptB) (see Tables S1 and S2 in the supplemental material) (5, 15, 17). Although an ordered transposon mutant library exists for *P. aeruginosa* PA14 (16), we chose to generate in-frame markerless deletions, as transposons can result in the production of truncated portions of a targeted gene or polar effects on downstream genes, each of which complicates the interpretation of any resulting phenotypes.

First, to determine if any of these two-component signaling proteins affect cell growth, we continuously measured the optical density at 600 nm (OD_{600}) of each mutant during growth in ABTGC (see Materials and Methods), a defined medium that is commonly used for *in vitro* studies of virulence pathways in *P. aeruginosa* (18–21). In this AB-based medium, glucose is a carbon source, while Casamino Acids serve as both a carbon and nitrogen source, with ammonium sulfate as an additional nitrogen source. Five mutants were identified that exhibited doubling times at least 10 min longer than that of the wild type (Fig. 1D and E; also, see Fig. S1A), including the $\Delta retS$ strain, which may exhibit a growth defect due to hyperproduction of the type VI secretion system and biofilm polysaccharides (12), and the $\Delta cbrA$ strain, which is less efficient at utilizing glucose and certain amino acids as carbon sources (22), both of which are present in ABTGC medium (23).

We next assayed the mutants for defects in various forms of motility, including swarming, swimming, and twitching. Swarming is a coordinated movement that occurs on a semisolid surface (24) and involves the use of biosurfactants and flagella, as well as the type IV pilus (24). This type of movement may occur in relatively viscous surfaces within a human host, such as in mucus (25). To assay for swarming, we used a well-

FIG 2 Swarming, swimming, and twitching motility phenotypes. (A) Representative images of wild-type PA14 versus mutants that have a swarming defect. (B) Quantification of swarm diameters of mutants, relative to the wild type. Bars show means $\pm$ standard errors of the means (SEM), with individual measurements shown ($n=3$ or 4 replicates). (C) Representative images of wild-type PA14 versus mutants that have a swimming defect. Dashed circles in mutant panels indicate the size of the wild-type swim area, for comparison. (D) Quantification of swim diameters of mutants, relative to the wild type. Bars show means $\pm$ SEM, with individual measurements shown ($n=3$ or 4 replicates). (E) Representative images of wild-type PA14 versus mutants that have a twitching defect. Dashed circles in mutant panels indicate the size of the wild-type twitch halo area, for comparison. (F) Quantification of twitch halo diameters of mutants, relative to the wild type. Bars show means $\pm$ SEM, with individual measurements shown ($n=3$ or 4 replicates).

established protocol in which *P. aeruginosa* PA14 forms tendrils across an M8 plate containing 0.5% agar (26). Wild-type cells exhibit characteristic swarm tendrils across an entire plate within 24 h (Fig. 2A). We identified 9 mutants that exhibited defects in swarming (>2 -fold decrease in swarm diameter relative to the wild type) (Fig. 2A and B; Fig. S1B). These include well-characterized swarming mutants such as the chemosensory $\Delta cheA$ mutant (25), the flagellar $\Delta fleS$ mutant (27), the $\Delta retS$ mutant (28), and the $\Delta hptB$ mutant (29), as well as five mutants that have not been as well studied in regard to swarming. In comparison to a recent study that identified 44 two-component signaling proteins that may affect swarming (15), our work identified far fewer mutants. However, the previous work used six different media to screen for swarming, including M8, which may suggest that different sensory systems regulate swarming under different environmental conditions. In addition, 7 of the 9 swarming mutants identified here were also reported to have a swarming defect on M8 plates in this previous study, suggesting that most of these swarming defects are reproducible.

To test our library for defects in swimming motility, which is another type of flagellum-dependent movement that occurs in low-viscosity or liquid settings (30), we used M63 plates supplemented with 0.3% agar (31). When inoculated at the center of a plate, wild-type *P. aeruginosa* swims outwards in a radial motion (32). We identified 5 mutants that displayed a defect in swimming motility, 4 of which were also identified as swarming mutants, namely, the $\Delta cheA$, $\Delta fleS$, $\Delta retS$, and $\Delta hptB$ mutants (Fig. 2C and D; Fig. S1C). This overlap may reflect the fact that both swarming and swimming are flagellum-dependent movements. The remaining mutant, a $\Delta pilS$ strain, has also been shown to indirectly regulate swimming motility (33).

Unlike swarming and swimming, twitching motility in *P. aeruginosa* is driven by the type IV pili, which use a grappling-hook-like motion to move cells across more solid surfaces (34). To test for twitching motility, we used an M63 plate supplemented with 1.5% agar (35). In these assays, the black interior of each panel is the bacterial colony, while the halo surrounding the black interior indicates the twitching area for each mutant. As there was some variability in the size of the bacterial colonies that grew on M63 plates, twitching diameters were calculated by subtracting the diameter of each interior colony. Through this approach, we identified 10 mutants that displayed defects in twitching (>2 -fold reduced twitching diameter relative to the wild-type), including the $\Delta chpA$ (36), $\Delta pilS$ (33), and $\Delta algZ$ (37) mutants, which have been well characterized in regard to twitching, as well as 7 additional mutants that have not been as well studied in regard to twitching (Fig. 2E and F; Fig. S1D). Because the *P. aeruginosa* type IV pilus is a common phage receptor (38), these identified mutants may also have various levels of susceptibility to phage infection. Overall, our results indicate that 15 of 65 assayed two-component signaling proteins ($\sim 23\%$) regulate at least one type of motility under the conditions we tested.

Two-component signaling proteins regulate biofilm formation. A hallmark of chronic *P. aeruginosa* infection is biofilm formation, in which a community of cells assembles into higher-order aggregates and surrounds itself with an extracellular matrix. One qualitative way to assay for the production of extracellular polymeric substances (EPS) is by Congo red staining, in which the Congo red dye binds to different kinds of biomass, including Pel, a biofilm polysaccharide produced by *P. aeruginosa* (39). In this assay, mutants that secrete more EPS are both redder and usually more wrinkled than the wild type (40). In contrast, mutants that are unable to produce EPS are typically less red and/or less wrinkled than the wild type. To determine which two-component signaling systems regulate biofilm formation, we grew each deletion mutant on a Congo red agar plate and incubated it for 7 days at room temperature to promote biofilm formation (Fig. S2A). Under these conditions, the wild-type PA14 colonies were red and wrinkled (Fig. 3A). We identified 14 mutants that were less red and/or less wrinkled than the wild-type strain, suggesting that their capacity to produce EPS was reduced (Fig. 3A). Some of these mutants have been previously shown to modulate biofilm formation, including the $\Delta phoQ$ (41), $\Delta gacS$ (42), and $\Delta kinB$ (13) strains, but the mechanistic basis for how many of these other mutants of interest regulate biofilm formation has not been fully elucidated. The experimental conditions of this assay were not ideal to identify hyper-biofilm formers due to the long incubation time (7 days) chosen to ensure that the wild type formed robust biofilms. Only one mutant, the $\Delta cbrA$ mutant, was identified in this category, which has been previously been shown to hyperproduce EPS (44).

To assay biofilm formation in a more quantitative way, we tested the ability of our mutants to form biofilms using crystal violet assays in a 96-well-plate format (43). In this assay, *P. aeruginosa* forms biofilms at the air-liquid interface of each well, which can be quantified by crystal violet binding, a dye that stains biomass, including biofilms. Using this assay, we found 6 mutants that decreased biomass formation >2 -fold relative to the wild type and 12 mutants that produced more biomass after overnight growth (Fig. 3B; Fig. S2B). Mutants that produced more biomass included the $\Delta cbrA$ (44), $\Delta retS$ (12), and $\Delta hptB$ (17) strains, which are known hyper-biofilm formers.

FIG 3 Biofilm phenotypes. (A) Representative images of Congo red-stained colonies of the wild type and selected mutants. (B) Quantification of crystal violet biofilms of mutants, relative to the wild type. Bars show means $\pm$ SEM ($n = 3$ or 4 replicates).

Further, of the 6 poor biomass formers detected in the crystal violet assay, 4 also made less biofilm, as judged by Congo red plates. However, there were also mutants in which biofilm defects could be detected only on Congo red plates or in liquid crystal violet assays. These differences could reflect the different media used for these experiments or result from measuring secreted polysaccharides in the Congo red assay but attached cells in the crystal violet assay. Previous work has also suggested that biofilm and swarming are often inversely regulated (45). In support of this model, we found that 5 of the 9 swarming-defective mutants we identified simultaneously exhibited increased biofilm formation, namely, the Δ sagS, Δ PA14_36420, Δ cbrA, Δ retS, and Δ hptB mutants.

Overall, these results indicate that 25 of the 65 two-component signaling genes ($\sim 38\%$) in our mutant library influence the production of biofilm, suggesting that biofilm formation is a complex process in *P. aeruginosa* that is influenced by many pathways and multiple signals.

Two-component signaling proteins regulate the production of virulence factors. To establish an infection, *P. aeruginosa* encodes a plethora of virulence factors that harm both host cells and other microbes. However, the upstream sensory pathways that regulate the production of many of these virulence factors remain poorly characterized. To address this gap, we screened our deletion library for the production of four well-studied virulence factors: pyocyanin, rhamnolipid, elastase, and lipase.

Pyocyanin is a redox-active small molecule that plays an important role in microbial respiration (46), and can also serve as a virulence factor (47). This blue-green molecule gives *P. aeruginosa* its characteristic color. To identify two-component signaling systems that regulate pyocyanin production, we grew our deletion library on ABTGC agar plates and visually identified 9 mutants that reproducibly appeared bluer than the wild

type and 3 mutants that consistently produced less blue pigment (Fig. 4A; Fig. S3A). We also extracted pyocyanin from these agar plates to quantify differences in pyocyanin production (Fig. 4B). Mutants that produced more pyocyanin included the $\Delta retS$ mutant, which is a known hyperproducer of pyocyanin, whereas mutants that produced less pyocyanin included the $\Delta gacS$ mutant, in which pyocyanin production is known to be ablated (48). However, the mechanisms by which many of these other mutants regulate pyocyanin production is not currently clear.

We next tested the ability of each two-component signaling system to regulate rhamnolipid production. Rhamnolipids are a class of glycolipids that promote swarming motility (24) and may also contribute directly to pathogenesis by inducing host cell damage (49). To measure rhamnolipid production, we grew each mutant on a cetyltrimethylammonium bromide (CTAB)-methylene blue plate (26). CTAB is a cationic surfactant that forms an insoluble precipitate when it reacts with rhamnolipids, which can then be stained with methylene blue (50). In turn, this stained precipitate can be seen as a dark blue/purple ring around a colony, which we observed with wild-type cells (Fig. 4C). In contrast, we detected 3 mutants that did not produce any rhamnolipids under these conditions, including the $\Delta bqsS$ strain, which has previously been shown to produce less rhamnolipids (31) (Fig. 4C and D). We also identified 10 other mutants that were able to produce rhamnolipids but reproducibly exhibited a lighter halo than wild-type cells (Fig. S3B and C). Furthermore, rhamnolipids are thought to contribute to swarming motility (26), and accordingly, we found that 5 of the 9 swarming mutants we identified also exhibited defects in rhamnolipid production, namely, the $\Delta cheA$, $\Delta PA14_36420$, $\Delta phoQ$, $\Delta PA14_57170$, and $\Delta hptB$ mutants. The ability of the other 4 swarming mutants to still produce rhamnolipids may reflect differences in conditions in these two phenotypic assays, or it may indicate that the ability of these mutants to swarm does not solely rely on rhamnolipid production (51).

Elastase is another well-studied virulence factor that can degrade elastin-containing host tissue (52). To determine which two-component signaling systems regulate elastase production, we grew our library on agar plates supplemented with 0.5% elastin (53) (Fig. 4E and F; Fig. S4A). As elastin is insoluble, the degradation of elastin by elastase is directly proportional to the zone of clearing around each colony. Whereas wild-type cells were able to form a robust halo, we identified 3 mutants that had decreased elastase activity, including the $\Delta phoQ$ (54) and $\Delta kinB$ (13) strains, which have previously been investigated in this regard.

P. aeruginosa infection is also characterized by the production of lipases, which can damage host tissue (55). To measure the ability of each strain to produce lipase, we grew the deletion library on agar plates supplemented with Tween 80 and calcium (56). In this assay, lipase-induced degradation of Tween 80 generates shorter fatty acids which are insoluble in the presence of calcium, thereby causing a zone of precipitate to form around each colony. Using this assay, we identified 13 mutants with defective lipase production, including known lipase regulators such as the $\Delta phoQ$ (54) and $\Delta retS$ mutants (48) (Fig. 4G and H; Fig. S4B and C). This relatively large number of lipase-regulating mutants suggests that the production of lipase may be influenced by many different environmental factors.

Overall, we identified 27 mutants (~40% of the library) that were defective in the production of at least one of the four secreted virulence factors that we assayed for, which suggests that *P. aeruginosa* senses and responds to a diverse set of cues to regulate its virulence program accordingly during the course of infection.

Two-component signaling proteins play a role in antibiotic resistance. Microbes have evolved numerous strategies to cope with external stresses. For pathogens like *P. aeruginosa*, this includes threats like antibiotics, which are commonly administered to treat *P. aeruginosa* infections. To determine if any two-component signaling systems affect the ability of *P. aeruginosa* to survive antibiotic treatment, we grew our deletion library in the presence of four antibiotics that kill cells in different ways: gentamicin, colistin, ciprofloxacin, and ceftazidime.

FIG 4 Pyocyanin, elastase, lipase, and rhamnolipid production phenotypes. (A) Representative images of wild-type and mutant strains that produced either more or less pyocyanin on ABTCG plates than the wild type. (B) Quantification of extracted pyocyanin from mutants grown on ABTCG plates, relative to the wild type. Bars show means $\pm$ SEM, with individual measurements shown ($n=3$ or 4 replicates). (C) Representative images of wild-type and mutant strains on CTAB-methylene blue plates for rhamnolipid detection. The location of the purple halo of rhamnolipids is shown with an arrow. (D) Quantification of rhamnolipid halo diameters in mutants, relative to the wild type. Bars show means $\pm$ SEM, with individual measurements shown ($n=3$ or 4 replicates). (E) Representative images of wild-type and mutant strains on elastin plates. Dashed circles represent the size of the elastase-clearing area in the wild type for comparison. (F) Quantification of elastase diameters in mutants, relative to the wild type. Bars show means $\pm$ SEM, with individual measurements shown ($n=3$ or 4 replicates). (G) Representative images of wild-type and mutant strains on Tween 80 and calcium plates. (H) Quantification of lipase-clearing diameter in mutants, relative to the wild type. Bars show means $\pm$ SEM, with individual measurements shown ($n=3$ or 4 replicates).

Gentamicin is an aminoglycoside that targets the 30S subunit of the ribosome, in turn preventing translation. We first grew wild-type PA14 on LB agar plates supplemented with different concentrations of gentamicin and found that cells were no longer viable on LB agar containing 10 $\mu\text{g/ml}$ of gentamicin. We then used a 96-well pin replicator to spot the entire library onto LB agar plates with 10 $\mu\text{g/ml}$ of gentamicin (Fig. S5A and B). Through this approach, we identified 4 mutants that could survive at this concentration of gentamicin, indicating that they are more resistant to this antibiotic than the wild type (Fig. 5A). These include the ΔcpxA mutant (10), which has been previously studied in regard to aminoglycoside resistance. Although the mechanisms that mediate gentamicin resistance in these mutants are unclear, they may impact cell permeability through the effects of downstream target genes on cell envelope integrity; alternatively, these mutants may affect the expression of efflux systems, which has also been suggested to impact aminoglycoside tolerance in *P. aeruginosa* (57). We also sought to identify mutants that were more susceptible to gentamicin than the wild type. To do this, we used a 96-well pin replicator to spot cells on LB agar containing 2 $\mu\text{g/ml}$ gentamicin, a concentration at which the wild type is viable (Fig. S5C). We identified two mutants that were no longer able to grow at this concentration of antibiotic, including the ΔamgS mutant, which has previously been implicated as a critical regulator of aminoglycoside resistance in *P. aeruginosa* (Fig. 5B) (58).

We next grew our deletion library in medium containing colistin (polymyxin C), a polycationic peptide that displaces divalent cations in the lipopolysaccharide (LPS) layer of the outer membrane, in turn damaging the cell membrane and eventually leading to death. We found that the wild-type strain was no longer viable on LB agar plates supplemented with 10 $\mu\text{g/ml}$ of colistin. When growing the entire library at this concentration of colistin (Fig. S5D), we identified 2 mutants that were reproducibly able to grow, namely, the ΔcreC and ΔphoQ strains (59) (Fig. 5C). Although it is unknown why the ΔcreC mutant is more resistant to this antibiotic, resistance of the ΔphoQ mutant to colistin is known to be mediated by changes to LPS structure (60). We then screened our library for increased susceptibility to colistin by using the 96-well pin replicator to plate the mutants on LB agar plates containing 2.25 $\mu\text{g/ml}$ colistin (Fig. S5E). Through this approach, we identified four mutants that were more susceptible to colistin than the wild type, including the ΔcolS strain, whose mutation is known to contribute to colistin resistance in this microbe (61) (Fig. 5D).

We also assayed the deletion library against ciprofloxacin, a fluoroquinolone that interferes with DNA replication by targeting DNA gyrase. We found that a relatively low concentration of 0.6 $\mu\text{g/ml}$ of ciprofloxacin was sufficient to kill wild-type PA14. When plating all mutants on LB-agar plates containing this lethal concentration of antibiotic (Fig. S5F), we identified 7 mutants that were able to survive (Fig. 5E). Interestingly, 6 of the 7 mutants with increased resistance to ciprofloxacin also displayed increased production of pyocyanin (Fig. 4A), which raises the intriguing possibility that phenazine production may be tied to ciprofloxacin resistance. Indeed, this observation was reported previously in the literature (62, 63), but the mechanisms that underlie this phenomenon are still unclear. To identify mutants hypersusceptible to ciprofloxacin, we plated the library on LB agar containing 0.05 $\mu\text{g/ml}$ of ciprofloxacin, at which the wild type is able to grow (Fig. S5G). However, there were five mutants that were not viable on these plates (Fig. 5F). Of these, both the ΔphoQ and ΔpmrB mutants are known to modify LPS structure (61, 64), which may indicate that alterations to LPS indirectly affect the susceptibility of *P. aeruginosa* to fluoroquinolones, as has been previously suggested (65). Inactivation of PhoQ was also recently shown to increase the susceptibility of *Salmonella enterica* serovar Typhi to ciprofloxacin (66), but the mechanistic basis for this effect is not known.

The final antibiotic we tested was ceftazidime, a third-generation cephalosporin that disrupts peptidoglycan synthesis, in turn disturbing the bacterial cell wall and leading to cell death. We found that 10 $\mu\text{g/ml}$ of ceftazidime was sufficient to kill wild-type PA14. When plating the entire library on LB agar plates supplemented with

FIG 5 Antibiotic resistance phenotypes. Representative images of wild-type and mutant strains on LB agar plates supplemented with 10 $\mu\text{g/mL}$ gentamicin (A), 2 $\mu\text{g/mL}$ gentamicin (B), 10 $\mu\text{g/mL}$ colistin (C), 2.25 $\mu\text{g/mL}$ colistin (D), 0.6 $\mu\text{g/mL}$ ciprofloxacin (E), 0.05 $\mu\text{g/mL}$ ciprofloxacin (F), 10 $\mu\text{g/mL}$ ceftazidime (G), or 1.25 $\mu\text{g/mL}$ ceftazidime (H).

10 $\mu\text{g/mL}$ of ceftazidime (Fig. S5H), we identified 2 mutants that could still reproducibly grow at this concentration of antibiotic (Fig. 5G), namely, the ΔparS and ΔtctE strains. The mechanistic basis for why the increased resistance to ceftazidime in these mutants is unclear, although a deletion of *parS* also increases the resistance of *P. aeruginosa* to

similar antibiotics that target the cell wall (67). We also plated our library on LB agar containing 1.25 μ g/ml ceftazidime to identify hypersusceptible mutants (Fig. S5I) and found only one mutant ($\Delta cbrA$) that had a moderate growth defect on these plates relative to the wild type (Fig. 5H).

Overall, these results indicate that 23 of the 65 two-component signaling proteins in our library alter the resistance of *P. aeruginosa* to at least one of the four antibiotics tested here, suggesting that a number of these sensory systems regulate the expression of downstream genes or cellular processes involved in mediating antibiotic tolerance or resistance under our experimental conditions.

Complementation of deletion mutants. The deletion library we generated involved markerless, in-frame deletions to minimize potential polar effects. Nevertheless, these mutants may still produce polar effects, complicating interpretation of the phenotypes identified. To address this possibility, we first identified those histidine kinases that have downstream, co-operonic genes, finding that 33 of the 65 genes in our library are located at the beginning or middle of an operon (Table S3). We then generated complementation strains for four mutants that had multiple phenotypes—the $\Delta PA14_11630$, $\Delta gacS$, $\Delta PA14_32570$, and $\Delta pprA$ mutants—with each gene expressed from a plasmid in the relevant mutant background. These plasmid-based complementation constructs generally rescued each of the phenotypes seen for these different mutants. For example, the $\Delta PA14_11630$ mutant exhibited increased pyocyanin production (Fig. 4A) and altered Congo red colony morphology (Fig. 2A), whereas the complemented $\Delta PA14_11630$ strain no longer hyperproduced pyocyanin in overnight cultures, as evidenced by its lack of blue coloring (Fig. S6A), and exhibited Congo red colony morphology more similar to that of the wild type than to that of the deletion strain (Fig. S6B). Similarly, the $\Delta gacS$ mutant exhibited phenotypes in pyocyanin production (Fig. 4A) and in the Congo red assay (Fig. 2A), which were largely reversed in the complementation strain (Fig. S6C and D). For the $\Delta PA14_32570$ mutant, the complementation construct partially rescued the lipase and Congo red defects seen in the original $\Delta PA14_32570$ mutant (Fig. S6E and G) but failed to restore rhamnolipid production to wild-type levels (Fig. S6F). Thus, the rhamnolipid defect seen with this mutant could be due to polar effects, or it may indicate that overexpressing this kinase from a high-copy-number plasmid interferes with rhamnolipid production. Finally, the $\Delta pprA$ complementation strain showed partial or full rescue each of the phenotypes documented in the original $\Delta pprA$ mutant, namely, lipase production (Fig. S6H), rhamnolipid production (Fig. S6I), twitching motility (Fig. S6J), and Congo red colony morphology (Fig. S6K). Taken together, these results indicate that the in-frame deletions generated in this study likely do not have substantial polar effects, but future studies of individual histidine kinases will require full characterization of the appropriate complementation strain.

Phylogenetic analysis of two-component signaling systems. We also sought to investigate the evolutionary history of each two-component signaling gene in PA14 to see how conservation relates to the phenotypes we documented. To this end, we generated maximum-likelihood trees (68) for the histidine kinases in our deletion library and then plotted the phenotypic changes of each mutant directly under the corresponding node of the tree (Fig. 6). Through this analysis, we found that some closely related kinases regulated the same phenotypes. For example, rhamnolipid production is affected by the related kinases CheA and PA14_45590, while elastase production is regulated by the related kinases PhoQ and BqsS. In general, however, the proteins that regulated each phenotype were generally broadly distributed across the tree. Indeed, for some phenotypes, such as swimming and gentamicin resistance, none of the observed mutants of interest were closely related to any other mutants of interest (Fig. 6). This result is consistent with a model in which *P. aeruginosa* has evolved independent pathways to regulate each of these virulence-associated traits. We also examined whether the chromosomal locations of these signaling proteins are correlated with their phenotypic outputs. In particular, we asked whether these genes tended to appear in genomic islands across the chromosome. However, our bioinformatic analysis revealed that only 2 of the 65 genes in our collection (*rscC* and *pvrS*) are found in a

FIG 6 Summary of histidine kinase mutant phenotypes. (Top) Maximum-likelihood tree of all histidine kinases in our deletion library. HptB is not included in the tree, as it is not a histidine kinase, and is shown on the far left. Due to its unusual sequence, AlgZ is also excluded from the tree and is also shown on the far left. Numbers on branches indicate support values calculated using aBayes. (Bottom) Summary heat map of the mutant phenotypes; blue indicates that the mutant had a deficit relative to the wild type, whereas red indicates that the mutant value exceeded that of the wild type.

genomic island, suggesting that most of these signaling proteins are part of the core genome of *P. aeruginosa* (Table S4).

Overall, our analysis indicates that each of the phenotypes that we screened is regulated by multiple two-component signaling proteins (Fig. 6). In particular, 23 of the 65 proteins in our library (~34%) regulate at least three of these phenotypes, suggesting that they may play critical roles during infection. This list includes GacS (3 phenotypes) and RetS (6 phenotypes) (12), which are two of the best-characterized and most important regulators of virulence in *P. aeruginosa*. We have also identified other two-component signaling proteins previously implicated in virulence, including PhoQ (8

phenotypes) (41), HptB (5 phenotypes) (29), KinB (6 phenotypes) (13), CbrA (6 phenotypes) (44), and AlgZ (3 phenotypes) (69). Beyond these relatively well-studied two-component signaling proteins, our results indicate that at least a dozen other histidine kinases, including some yet to be named, also regulate multiple virulence-associated traits. Overall, of the 23 two-component signaling proteins we identified as impacting at least three phenotypes, 22 have been previously identified as essential for successful infection in various animal models (7, 8). Although the precise contributions and the cellular processes impacted by many of these sensory systems were not previously known, the reported phenotypes collected here provide insight into why these particular two-component signaling systems may be broadly important during infection.

DISCUSSION

P. aeruginosa is a remarkably versatile microbe that thrives in a wide range of environments, from non-host-associated niches like soil and water to the mucous environments of CF lungs. To enable survival in these diverse niches, *P. aeruginosa* encodes >60 two-component signaling systems to detect and react to external cues. In particular, many of these two-component systems are likely important during infection, but the actual processes that these sensory systems control have remained largely unclear. Here, we generated a new toolbox for studies of *P. aeruginosa* sensory systems by constructing deletions of 64 sensor histidine kinases and 1 histidine phosphotransferase in this microbe. We performed initial phenotypic characterizations of these mutants and found that the mutations influence a wide range of virulence phenotypes, including different kinds of motility (swarming, swimming, and twitching), biofilm formation, virulence factor production (pyocyanin, elastase, lipase, and rhamnolipids), and resistance to diverse antibiotics (gentamicin, colistin, ciprofloxacin, and ceftazidime). Through this approach, we identified both known and novel regulators of each of these phenotypes. Further, we identified nearly two dozen mutants that regulate at least three of the above-mentioned virulence-associated traits and thus may play particularly central roles in infection and that warrant further investigation.

Previous characterization of two-component signaling systems in *P. aeruginosa* has primarily relied on transposon-based mutants (13–15), which may not represent straightforward null alleles. Thus, we anticipate that the in-frame deletion library of two-component signaling genes generated here will be broadly useful in studies of *P. aeruginosa* sensory systems. In particular, the initial phenotypic characterizations of the mutants reported here should provide a foundation for future studies that elucidate the mechanisms by which each sensory system regulates each respective downstream virulence-associated pathway. These results are also reminiscent of a previous study in which a clean deletion library in *P. aeruginosa* was generated for all genes involved in the production of c-di-GMP (70), a secondary metabolite that plays important roles during pathogenesis. The combined use of both of these collections may yield further insights into how *P. aeruginosa* regulates its virulence program. Intriguingly, certain two-component signaling proteins such as HptB (17) and GacS (71) modulate the production of c-di-GMP, suggesting that the levels of this secondary metabolite may be regulated by external signals during infection.

Our study highlights the importance of systematically assaying mutants in parallel, under the same conditions. For example, we found that some biofilm mutants were detectable only in crystal violet or Congo red assays (Fig. 3), which has also been previously observed in the deletion library of c-di-GMP mutants (70). This finding indicates that different two-component signaling systems may mediate biofilm formation under different conditions. Previous discrepancies in reported phenotypes among two-component signaling mutants may reflect differences in how the assays were performed or general variability in laboratory conditions. For example, one previous paper reported that the $\Delta gacS$ mutant is impaired in swarming motility and exhibits increased biofilm formation (15), while our results here, as well as other previously published results (28), suggest that the $\Delta gacS$ mutant is proficient at swarming but defective in biofilm

formation. Our library of markerless, in-frame deletion strains should enable the parallel testing of desired two-component mutants and decrease experimental variability under any specific condition.

P. aeruginosa encodes an unequal number of response regulators (72) and histidine kinases (64), raising the possibility that some kinases may have more than one direct phosphorylation target. Indeed, previous work has shown that the RocS1 histidine kinase can interact with both the RocA1 and RocR response regulators (72). When examining the genomic locations of each of these signaling proteins in PA14, we found that 39 of these histidine kinases are in the same operon as their cognate response regulators, while 25 are not (Table S3). Thus, it is possible that these 25 non-co-operonic histidine kinases may phosphorylate multiple response regulators, which could complicate the phenotypic readouts observed here. The construction of an additional library of response regulator deletions could further clarify the phenotypic effects of each of the two-component signaling proteins in *P. aeruginosa*.

Although we have characterized many of the virulence-associated processes these kinases may control, the signals that most of these kinases respond to remain unclear. For example, RetS is one of the best-characterized sensor kinases in *P. aeruginosa*, but, despite decades of searching, only an unidentified signal from *P. aeruginosa* lysate (73) and a subset of mucin glycans (74) have been identified as RetS-activating signals thus far. In turn, one important area of future research is the elucidation of signals for these >60 two-component systems in *P. aeruginosa*, which will provide further insight into the physiological role of these sensory pathways.

Beyond its well-studied role as a human pathogen, *P. aeruginosa* can also infect a variety of other species, including other mammals (8), fish (13), plants (75), worms (76), and flies (77). *P. aeruginosa* is also a commonly encountered environmental microbe that can be found in various non-host-associated niches, including in the soil and in water (78, 79). The remarkable ability of this microbe to survive and thrive in these diverse niches may be in part mediated by its large collection of two-component signaling pathways. Although our results here focus on understanding how these sensory pathways may mediate virulence-associated traits, which may be important during infection, the sensory systems that allow *P. aeruginosa* to grow and proliferate in external environments like the soil have not yet been studied. Interestingly, our results indicate that 24 of the 65 two-component signaling proteins in our library (~37%) did not regulate any of the virulence-associated phenotypes tested here, which raises the possibility that they play important roles in noninfectious settings. Ultimately, further characterization of the two-component signaling systems in *P. aeruginosa* will yield exciting and novel insights into how microbes perceive the world around them.

MATERIALS AND METHODS

General growth and cloning procedures. *P. aeruginosa* and *E. coli* strains were both grown overnight in LB at 37°C under shaking conditions. For general cloning procedures, antibiotics were added at the following concentrations: for *E. coli*, 20 µg/ml gentamicin, and for *P. aeruginosa*, 75 µg/ml gentamicin. Strains and primers used are listed in Tables S1 and S2.

For growth curve experiments, overnight cultures were diluted 1:100 into 1 ml of ABTGC medium [15.1 mM ammonium sulfate, 33.7 mM sodium phosphate dibasic, 22 mM potassium dihydrogen phosphate, 0.05 mM sodium chloride, 1 mM magnesium chloride, 100 µM calcium chloride, 1 µM iron(III) chloride, 0.2% glucose, and 0.2% Casamino Acids]. Cultures were grown in a 24-well format on a Biotek Synergy H4 plate reader at 37°C, with reads at 600 nm every 15 min. Plates were shaken at 750 rpm.

Generation of deletion mutants. A list of two-component signaling proteins in *P. aeruginosa* was curated from the Microbial Signal Transduction Database (MiST) (80), and their gene sequences were collected from pseudomonas.com. In-frame deletions of each two-component signaling protein were made by allelic exchange using the suicide vector pMQ30, as previously described (81). Primers were designed such that each gene would be deleted in frame, to minimize any potential polar effects. Briefly, regions corresponding to ~500 to 1,000 bases immediately upstream and downstream of each gene were amplified from PA14 genomic DNA (gDNA). PCR fragments were then assembled into the pMQ30 vector using Gibson Assembly (NEB). Sequence-verified plasmids were then transformed into S17 on LB agar supplemented with 20 µg/ml gentamicin. The next morning, colonies were scraped from the plate and grown in LB medium with 20 µg/ml gentamicin until reaching an OD₆₀₀ of ~0.5 to 0.7. At the same time, an overnight of PA14 was incubated at 42°C for at least 2 h. PA14 (0.5 ml) was then mixed with 1.5 ml of S17 and centrifuged at 13,000 × *g* for 2 min. The supernatant was discarded, and the pellet was resuspended in 100 µl of PBS. This cell suspension

was then plated on a LB agar plate overnight at 30°C for conjugation to occur. The next morning, the patch of cells was scraped off, resuspended in 1 ml of PBS, and then plated on *Pseudomonas* isolation agar (PIA) supplemented with 75 µg/ml gentamicin. Colonies were then streaked out onto LB agar supplemented with 15% sucrose, and candidate deletions were verified using PCR. Correct deletions were verified by Sanger sequencing.

Complementation of deletion mutants. Complementation was done by expressing a given histidine kinase from the pPSV38 vector, in each respective mutant background, under the control of its native promoter. Briefly, primers were designed so that the histidine kinase of interest, as well as ~400 to 500 bp of upstream and downstream regions, could be amplified from PA14 genomic DNA. PCR fragments were then assembled in the pPSV38 vector using Gibson Assembly (NEB), and sequence-verified plasmids were transformed into S17 *Escherichia coli* cells. The above steps were then taken to conjugate these complementation plasmids into each respective PA14 deletion mutant. Successful complementations were verified using colony PCR and by streaking on LB agar plates containing gentamicin.

Motility assays. Swarming assays were performed on M8 medium supplemented with 0.2% glucose, 0.5% Casamino Acids, 1 mM MgSO₄, and 0.5% agar. Swarm agar was made fresh each day and allowed to dry at room temperature for a few hours. Fresh colonies from each mutant were then used to inoculate the center of each swarm plate, which was then incubated at 37°C for 24 h. Colonies were inoculated directly onto the surface of the agar, as previously described (26). Preliminary screening was done on a large square petri plate (245 mm by 245 mm), with 9 strains per plate (8 mutants and 1 wild-type control). Potential mutants of interest were then inoculated on separate swarm agar plates. Swarming diameter was measured on ImageJ.

Swimming assays were performed on M63 medium supplemented with 0.2% glucose, 0.5% Casamino Acids, 1 mM MgSO₄, and 0.3% agar. Swim agar was made fresh each day and allowed to dry at room temperature for a few hours. Fresh colonies from each mutant were then used to inoculate the center of each swim plate, which was then incubated at 37°C for 24 h. Colonies were inoculated into the agar, but not all the way to the bottom of the petri plate, as previously described (30). Preliminary screening was done on a large square petri plate (245 mm by 245 mm), with 9 strains per plate (8 mutants and 1 wild-type control). Potential mutants of interest were then inoculated on separate swim agar plates. Swimming diameter was measured on ImageJ.

Twitching assays were performed on M63 medium supplemented with 0.2% glucose, 0.5% Casamino Acids, 1 mM MgSO₄, and 1.5% agar. Fresh colonies from each mutant were used to inoculate the center of each twitch plate, which was then incubated at 37°C for 24 h. Twitch agar plates were then incubated at room temperature for 3 days for twitch halo development. Colonies were stabbed all the way to the bottom of the petri plate for these assays, as previously described (34). Twitching diameter was measured on ImageJ, with the diameter of the internal bacterial colony subtracted.

Biofilm assays. Congo red assays were done on M63 agar supplemented with 0.2% glucose, 0.5% Casamino Acids, 1 mM MgSO₄, 1.5% agar, and 2 ml of a 50× Congo red-Coomassie blue solution (2 mg/ml Congo red and 1 mg/ml Coomassie blue) per 100 ml of medium. Portions (3 µl) of overnight cultures of each mutant were used to inoculate the center of each Congo red plate, which were then incubated at 37°C for 24 h. Congo red agar plates were then incubated at room temperature for 7 days so that biofilms could form.

Crystal violet assays were performed as previously described (43). Briefly, overnight cultures of each strain were inoculated 1:100 into fresh ABTGC medium, and 125 µl of each strain was then grown overnight in a 96-well format. The next morning, the plate was rinsed twice in distilled water to remove non-attached cells. The remaining biofilm was then stained using 150 µl of 1% crystal violet for 20 min. Nonattached crystal violet was then removed by washing with distilled water four times. The remaining crystal violet was then solubilized using 150 µl of 30% acetic acid for 20 min. The resulting absorbance was measured using a BioTek Synergy H4 plate reader at 550 nm.

Measurement of secreted virulence factors. To visualize pyocyanin production, each strain was streaked out on ABTGC plates supplemented with 1.5% agar. The clear color of this agar allowed pigmentation to be easily seen. Pyocyanin production of mutants that appeared bluer or less blue than the wild-type were then quantified by first scraping cells off the agar surface and then extracting pyocyanin from the agar in the plates, as described previously (82). Preliminary assays were done in ABTGC agar solidified in 24-well plates (1 ml agar per well) for rapid visualization. Potential mutants of interest were then further examined by streaking on individual ABTGC agar plates.

Rhamnolipids were visualized on CTAB-methylene blue agar, as previously described (26). Briefly, 5 µl of overnight cultures from each strain was spotted onto M8 agar supplemented with 0.2% glucose, 0.5% Casamino Acids, 1 mM MgSO₄, 0.02% cetyltrimethylammonium bromide (CTAB), 0.001% methylene blue, and 1.5% agar. Samples were incubated at 37°C for 24 h and then at 4°C for 48 h to allow color to better develop. Rhamnolipid production was quantified by measuring the diameter of CTAB precipitation on ImageJ, which is seen as a purple ring around each colony, with the bacterial colony diameter subtracted.

Elastase activity was measured on LB agar plates supplemented with 0.5% elastin, as previously described (83). Portions (2.5 µl) of overnight cultures from each strain were inoculated on elastin agar plates. Colonies were grown at 37°C for 24 h and then at room temperature for 2 days so that halos of elastin degradation could be observed. The diameter of each clearing zone was then measured on ImageJ, with the diameter of the bacterial colony subtracted.

Lipase activity was measured on 1.5% agar supplemented with 10 g peptone, 5 g NaCl per, 1 mM CaCl₂, and 10 ml of Tween 80 per liter of medium, as previously described (84). Portions (2.5 µl) of overnight cultures from each strain were inoculated on these plates with agar medium and incubated at 37°C for 24 h, so that halos of lipase activity could be observed. The diameter of each lipase zone was then measured on ImageJ, with the diameter of the bacterial colony subtracted.

Antibiotic resistance assays. To test the library for antibiotic resistance and susceptibility, LB medium with 1.5% agar was supplemented with gentamicin (10 μ g/ml or 2 μ g/ml), colistin (10 μ g/ml or 2.25 μ g/ml), ciprofloxacin (0.6 μ g/ml or 0.05 μ g/ml), or ceftazidime (10 μ g/ml or 1.25 μ g/ml) and poured into large petri plates (245 $\times$ 245 mm). The deletion library was grown overnight in a 96-deep-well format (2 ml per culture), and a 96-well pin replicator was then used to spot $\sim$ 0.2 μ l of each strain onto each antibiotic plate. The samples were then incubated at 37°C for 36 h to test for antibiotic resistance or for 14 h to test for antibiotic susceptibility. Mutants that were found to grow were then validated by further streaking on the respective antibiotic plates.

Phylogenetic analysis. Protein sequences of each kinase were obtained from pseudomonas.com, compiled into a text file in FASTA format, and uploaded to ngphylogeny.fr (68) to align the sequences using MUSCLE. Sensory domains were then manually trimmed from these aligned sequences to reduce uncertainty in the tree. Sequences were then realigned using MUSCLE, and alignment positions with gaps in >80% of sequences were removed. Maximum-likelihood trees were then built using PhyML with LG model and support evaluated using aBayes. Trees were visualized using iTOL (85).

Statistics and computational methods. Analysis of variance (ANOVA) was performed for each of the quantified phenotypes to account for multiple comparisons (Table S5), and Dunnett's test was used to correct for multiple comparisons. All statistical analysis was done on GraphPad Prism. Operons were annotated on pseudomonas.com using the DOOR database (86). Genomic islands were identified using Islandviewer 4.0 (87).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.9 MB.

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B.X.W., K.R., and M.T.L. designed the experiments. K.C.C. performed preliminary experiments and constructed strains. G.O.C. provided high-resolution Congo red images. B.X.W. performed all experiments and analyses. B.X.W., K.R., and M.T.L. wrote the paper.

We declare no competing interests.

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