The two-component sensor kinase KinB acts as a non-canonical switch between acute and chronic infection

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**Pseudomonas aeruginosa** is an opportunistic pathogen that occupies diverse environmental niches and is capable of causing a range of infections in humans. This versatility suggests that it has sophisticated mechanisms to sense and respond to the surrounding micro-environment. Two-component sensors are commonly used by bacteria to sense and respond to environmental stimuli, and **P. aeruginosa** has one of the largest sets of two-component sensors known in bacteria. We took advantage of a non-redundant transposon library and a recently characterized vertebrate model host, *Danio rerio*, that is amenable to higher throughput analysis than mammalian models, to systematically test the role of 60 two-component sensors that are required for **P. aeruginosa** virulence in acute infection. We found that the sensor kinase KinB is required for acute infection in zebrafish embryos and regulates a number of virulence-related phenotypes in a manner independent of its kinase activity and its known response regulator, AlgB. Thus, the regulation of virulence by KinB highlights the increasing recognition of non-canonical two-component signaling mechanisms.

**Pseudomonas aeruginosa** is a Gram-negative opportunistic pathogen that occupies diverse environmental niches including soil and water. In humans **P. aeruginosa** can cause acute infections such as bacteraemia and sepsis in immunocompromised individuals, urinary tract infections, ulcerative keratitis and skin infections in trauma/burn victims. **P. aeruginosa** is also a cause of chronic pulmonary infections in patients with cystic fibrosis (CF). In the laboratory, **P. aeruginosa** can infect a wide variety of model hosts including amoeba,2 plants (**Arabidopsis thaliana**),3 lettuce leaves4 and alfalfa seedlings5, insects (**Drosophila melanogaster** and *Galleria mellonella*), nematodes (*Caenorhabditis elegans*), zebrafish (*Danio rerio*9-10), mice,11 guinea pigs12 and rats.13 This versatility is likely driven by finely tuned gene regulatory mechanisms.

Several screens have identified **P. aeruginosa** mutants that display attenuated virulence in multiple different host model systems, suggesting that **P. aeruginosa** likely has a core set of virulence factors that contribute to infection. However, mutants that are attenuated in only one or a subset of host systems have also been identified.14 Given that **P. aeruginosa** is able to cause such a wide variety of infections it is reasonable to expect that while certain virulence factors may play a role only under a specific set of host micro-environmental conditions, others are required more generally for infection in multiple hosts.

Two-component signal transduction systems are widely used by bacteria to sense stimuli and respond to the environment.15 The name ‘two-component’ system derives from the observation that the canonical system comprises of a membrane bound sensor histidine kinase that senses an environmental cue and then phosphorylates a cognate response regulator (Fig. 1A). The canonical sensor kinase consists of a variable N-terminal input...
domain, which senses a stimulus (e.g., a small molecule or a change in osmolality) and a C-terminal kinase domain that auto-phosphorylates a conserved histidine residue in response to a stimulus detected by the input domain. This phosphoryl group is then transmitted to a conserved aspartate residue on the N-terminal domain of the response regulator. The response regulator mediates a response to the stimulus through its C-terminal output domain. Variations on this theme exist and some two-component sensors are cytoplasmic, while other sensors have a more complex domain architecture.

*P. aeruginosa* has one of the largest sets of two-component systems known in bacteria, with approximately 120 sensor kinases and response regulators encoded in its genome.19 Several of these two-component systems have been shown to play roles in the regulation of virulence by this pathogen in different host systems. For example, a *P. aeruginosa* phoQ sensor kinase mutant is attenuated for virulence in the lettuce leaf and chronic rat lung infection models20 and is involved in mediating resistance to anti-microbial peptides and aminoglycosides.18 PhoQ mutants are also impaired in cytotoxicity and twitching motility.20

A complex dynamic exists between the sensor kinases GacS and RetS, which together with LadS play a key role in coordinating the transition between acute and chronic infections.16,21,22 In response to a currently unknown signal, the GacS sensor kinase phosphorylates its cognate response regulator GacA, which controls the expression of the small RNAs *rsmZ* and *rsmY*.23 These small RNAs modulate the activity of the RNA-binding protein, RsmA, which regulates the expression of several genes, many of which encode virulence factors.24 The RetS sensor kinase interacts with GacS to inhibit the phosphorylation of GacA (Fig. 1B) and thus, a retS deletion mutant shows increased *rsmZ* transcription.16 In contrast, the LadS sensor kinase is believed to promote GacA phosphorylation as deleting ladS results in decreased *rsmZ* transcription.22 Hence, these sensor kinases act as a switch wherein LadS and GacS promote phenotypes important for chronic infection, while a RetS interaction with GacS promotes phenotypes important for acute infection.18 However, the role of the majority of *P. aeruginosa* two-component sensor kinases remains unknown.

Taking advantage of a non-redundant transposon mutant library25 and a recently characterized *Danio rerio* host model that allows higher throughput analyses than rodent models of infection while possessing an immune system more similar to mammals than plants and invertebrate models such as *C. elegans*,9,10 we conducted a systematic screen to identify *P. aeruginosa* two-component sensors that are required for full virulence in acute infection.26

We identified both known and novel two-component sensors, which are required for full virulence in embryos. Previously identified regulators of virulence included GacS and RetS. Notably, we found that deletion of the sensor kinase KinB, which had not previously been implicated in playing a role in acute infection, resulted in significantly increased survival in infected embryos. However, KinB has been implicated in the repression of chronic infection phenotypes such as alginate production through its cognate response regulator AlgB.27 Recently, it was reported that inactivation of KinB in *P. aeruginosa* strain PA01 results in alginate overproduction, through AlgB, but in a phosphorylation-independent manner.27 We found that PA14ΔkinB is also more mucoid than wild-type PA14. However, it is worth noting that PA14ΔkinB is

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**Figure 1.** Some of the known mechanisms by which two-component systems regulate cellular responses. (A) The canonical mechanism: A histidine sensor kinase (HK) phosphorylates its cognate response regulator (RR) in response to an environmental stimulus. Phosphorylation of the response regulator changes its conformation and leads to a cellular response. (B) A histidine sensor kinase can directly associate with another sensor kinase in the membrane and prevent it from phosphorylating its cognate response regulator.16 (C) A histidine sensor kinase can act as a phosphatase and de-phosphorylate a response regulator in response to an environmental stimulus. (D) An auxiliary regulator (AR) can interact with a sensor kinase through a variety of different mechanisms, some of which are unknown to regulate its activity.27 This regulation may promote phosphorylation of the response regulator and the default output or inhibit it. This figure is based on models proposed in the literature.16-18
not as mucoid as a PA14ΔmucD strain (Fig. 2). In a follow-up study, Damron et al. reported that expression of an outer membrane protein, LptF, was upregulated in a PAO1ΔkinB mutant and that LptF is also upregulated in *P. aeruginosa* isolates from CF patients. Thus, the authors suggest that LptF might also be important for *P. aeruginosa* survival in the CF patient lung. This study further highlights the role of KinB in regulating chronic infection phenotypes. Interestingly, we found that while the PA14ΔkinB mutant was far less virulent than wild-type PA14 in zebrafish embryos, the PA14ΔalgB mutant was not attenuated, suggesting that KinB’s role in virulence may be AlgB-independent.

Further, we found that KinB plays a role in regulating a number of virulence-associated phenotypes associated with acute infection. The PA14ΔkinB mutant produced significantly lower amounts of quorum-sensing regulated toxins such as pyocyanin (a blue-green phenazine pigment secreted by *P. aeruginosa*) and elastase than wild-type PA14. We also found that the PA14ΔkinB mutant had modest defects in swimming motility and biofilm formation. However, the PA14ΔkinB mutant was not defective for type three secretion and does not appear to directly control the production of homoserine lactone autoinducers. This role for KinB is in contrast to AlgB, since the PA14ΔalgB mutant produced wild-type levels of pyocyanin and elastase and did not have swimming motility or biofilm formation defects.

Despite KinB’s regulation of acute virulence phenotypes being independent of its corresponding response regulator AlgB, KinB could act by phosphorylating alternative response regulators, since sensor histidine kinases have been reported to phosphorylate multiple response regulators. In order to determine whether KinB’s kinase activity is required for its acute virulence phenotype, we engineered an allele in which His-385 (which is required for its kinase activity) was mutated to alanine and introduced this allele episomally into the PA14ΔkinB mutant. To our surprise, this allele was able to restore wild-type levels of pyocyanin and elastase, although it partially complemented the defects in swimming motility and biofilm formation. Furthermore, this allele was able to restore full virulence to the deletion mutant in the zebrafish embryo infection model. Hence, KinB’s ability to signal independent of its cognate response regulator and its kinase activity places it into a non-canonical model for two-component sensors.

It is becoming increasingly clear that the current view of two-component sensors acting merely as signal-recognition and phosphotransfer modules needs to be refined. Several recent papers support an emerging view that the activity of two-component signaling systems is often influenced by auxiliary factors and thus a canonical two-component system can be regulated in a complex manner. For instance, a two-component sensor such as KinB may possess phosphatase activity in addition to its kinase activity, which does not require the His-385 residue (Fig. 1C). Since phosphorylation may serve as an ‘on-off’ switch for response regulators, KinB could regulate a response regulator by dephosphorylation rather than the classic phosphorylation event, therein acting as a phosphatase. On dephosphorylation, a response regulator would change its conformation, thus changing its activity. For example, a response regulator that binds DNA and acts as a transcriptional activator in its phosphorylated form would no longer bind DNA on being dephosphorylated. Indeed, it has been proposed that the sensor kinase PhoQ dephosphorylates its cognate response regulator PhoP under certain conditions.

As noted earlier, a non-canonical regulatory mechanism has also been proposed for the GacS/GacA two-component system. Our studies suggest that KinB phenocopies GacA in several virulence phenotypes including pyocyanin production and biofilm formation without a significant effect on autoinducer production. Moreover, KinB and GacS are both required for full virulence in zebrafish embryos and *C. elegans* (Ausubel FM, Massachusetts General Hospital, personal communication). Given the similarity in phenotypes between PA14ΔgacA and PA14ΔkinB we wondered whether there was a link between KinB and the GacS/RetS/LadS signaling system, wherein KinB could act like RetS to inhibit the activity of GacS and promote acute infection. Since GacA regulates production of virulence factors through the small RNAs *rmsY* and *rmsZ* levels. Using *rmsY* and *rmsZ* promoter-*lacZ* fusions (kindly provided by Steve Lory, Harvard Medical School) in wild-type PA14 and PA14ΔkinB backgrounds, we find that KinB in fact does not modulate either *rmsY* or *rmsZ* promoter activity,

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**Figure 2.** *P. aeruginosa* strains grown on PAI agar at 37°C for 3 d and at room temperature for 24 h. The PA14ΔkinB strain is slightly more mucoid than wild-type PA14 but less mucoid than PA14ΔmucD.
context might its kinase activity actually be required given its dispensability in both acute virulence phenotypes and chronic virulence phenotypes such as alginate production. KinB could be regulating acute virulence phenotypes, independent of its kinase activity in a number of ways. It may possess phosphatase activity, dephosphorylating an unknown response regulator and changing its output. Alternatively, it may form heterodimers with another sensor kinase, inhibiting its activity, in a manner analogous to RetS and GacS. Lastly, KinB may interact with another regulatory protein through a novel mechanism (Fig. 1D). It would be of interest to determine the intermediate signaling proteins that facilitate signal transduction from KinB to downstream expression of virulence factors. These principles may apply generally to other histidine kinases, thus forming the basis for a variation in models by which two-component sensors may regulate the activity of downstream proteins (summarized in Fig. 1).

In conclusion, two-component signal transduction and its role in the regulation of virulence in P. aeruginosa is likely more complex than previously appreciated. P. aeruginosa makes extensive use of dynamic and intertwined two-component systems to finely tune its virulence. It uses several sensors to regulate the switch between acute and chronic infection. Apart from the GacS/RetS/LadS signaling system, the AlgR/FimS and the SadA/R/S systems have also been implicated in regulating this switch. Given that KinB is required in acute infection, PA14ΔkinB is slightly more mucoid than wild-type PA14 (Fig. 2) and a PAO1ΔkinB mutant overproduces alginate relative to wild-type PAO1, demonstrates that KinB appears to play a role in upregulating phenotypes involved in acute infection while repressing phenotypes involved in chronic infection. P. aeruginosa strains that are able to persist in the CF lung and cause chronic infections must adjust to hostile conditions within the host lung and undergo several changes that adapt their physiology to the particular milieu, distinct from that required in the environment or during acute infection. It is likely that two-component systems play a key role in

Figure 3. Activity of promoter-lacZ fusions in various P. aeruginosa mutant backgrounds. (A) The activity of the rsmY-lacZ fusion in various P. aeruginosa mutant backgrounds. β-galactosidase levels were measured in cultures of P. aeruginosa PA14, PA14ΔkinB, PA14 gacS and PA14 retS transposon mutants. (B) The activity of the rsmZ-lacZ fusion in various P. aeruginosa mutant backgrounds. β-galactosidase levels were measured in cultures of P. aeruginosa PA14, PA14ΔkinB, PA14 gacS and PA14 retS transposon mutants. The data are representative of two biological replicates. A break in the scale has been introduced in the Y-axes due to the high activity of the retS mutant.
allowing \( P. \) aeruginosa to adapt to such conditions. Thus, future work may identify additional two-component systems that are involved in mediating the transition between acute and chronic infection.

Given the important role that two-component signaling networks appear to play in different disease states and their absence in humans, targeting these networks as an antimicrobial approach should be considered. In order to do so, we will need an increased understanding of these networks of two-component sensors including how they signal, the signaling pathways that emerge from each sensor, the degree of crosstalk involved, and levels of redundancy. One key insight that could aid in the development of drugs targeting two-component sensors and a major outstanding question is the nature of the signals they sense, since these are still unknown for the majority of known two-component systems, not only in \( P. \) aeruginosa but also in most species of bacteria. Determining the nature of these signals will provide significant insight into the strategies that bacteria utilize as they adapt to their ever-changing environment.

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