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JOURNAL OF BACTERIOLOGY, June 2011, p. 2989–2999 Vol. 193, No. 12
0021-9193/11/$12.00 doi:10.1128/JB.01546-10

The Sensor Kinase KinB Regulates Virulence in Acute *Pseudomonas aeruginosa* Infection▽

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Received 22 December 2010/Accepted 6 April 2011

Two-component sensors are widely used by bacteria to sense and respond to the environment. *Pseudomonas aeruginosa* has one of the largest sets of two-component sensors known in bacteria, which likely contributes to its unique ability to adapt to multiple environments, including the human host. Several of these two-component sensors, such as GacS and RetS, have been shown to play roles in virulence in rodent infection models. However, the role and function of the majority of these two-component sensors remain unknown. *Danio rerio* is a recently characterized model host for pathogenesis-related studies that is amenable to higher-throughput analysis than mammalian models. Using zebrafish embryos as a model host, we have systematically tested the role of 60 two-component sensors and identified 6 sensors that are required for *P. aeruginosa* virulence. We found that KinB is required for acute infection in zebrafish embryos and regulates a number of virulence-associated phenotypes, including quorum sensing, biofilm formation, and motility. Its regulation of these phenotypes is independent of its kinase activity and its known response regulator AlgB, suggesting that it does not fit the canonical two-component sensor-response regulator model.

Two-component signal transduction systems (TCSs) are found in bacteria (53), yeasts (36), and plants (7). They are widely used by bacteria to sense a variety of stimuli and adapt to diverse environments (53). The classical two-component system consists of a membrane-spanning histidine sensor kinase protein, which senses an environmental cue and then phosphorylates its cognate response regulator. A canonical sensor kinase contains two domains, a variable N-terminal input domain that senses the signal and a conserved C-terminal domain that catalyzes phosphorylation. In response to a stimulus detected by the input domain, the kinase domain autophosphorylates on a conserved histidine residue. Subsequently, this phosphoryl group is transferred to a conserved aspartate residue on the N-terminal receiver domain of the cognate response regulator. Phosphorylation of the N-terminal receiver domain of the response regulator then activates its C-terminal output domain, which generates an appropriate response to the stimulus (5).

The number of TCSs encoded within the *Pseudomonas aeruginosa* genome represents one of the largest sets of TCSs known in bacteria, which may contribute to *P. aeruginosa*'s ability to occupy a wide range of environmental niches, including the human host. Around 60 putative sensor kinases and putative response regulators have been annotated in the genome of the opportunistic pathogen *P. aeruginosa*, though the exact number of TCSs can vary between different *P. aeruginosa* strains and depending on the annotation. For instance, in comparison to *P. aeruginosa* strain PAO1, for which 64 sensors and 59 regulators have been annotated (8, 17, 49), strain PA14 encodes two additional TCSs on a unique pathogenicity island (23) while lacking one TCS, PA4101/PA4102, for which there are no orthologs (58). These TCSs may play an important role in the pathogenesis of *P. aeruginosa*, which can cause a broad range of disease, including acute infection in immunocompromised patients, patients on ventilators, and burn victims as well as chronic infection in cystic fibrosis patients (56).

P. aeruginosa elaborates a vast number of virulence factors such as pyocyanin, elastase, exotoxin A, type III secretion (T3S) effectors, and rhamnolipids, the production of which is tightly regulated, depending on the lifestyle adopted by the pathogen. The large number of TCSs encoded in the *P. aeruginosa* genome likely enables it to regulate the production of these virulence factors in different environments. Perhaps the best-characterized TCS in *P. aeruginosa* is the GacS sensor kinase and its cognate response regulator, GacA. The GacA/GacS TCS is thought to be a master regulator of virulence in *P. aeruginosa* (55), as it controls a number of virulence-related phenotypes, including biofilm formation and the production of several virulence factors, including pyocyanin (38, 47). GacA and GacS are required for virulence in plant (*Arabidopsis thaliana*), nematode (*Caenorhabditis elegans*), insect (*Drosophila melanogaster* and *Galleria mellonella*), and mouse models of acute and chronic infection (10, 26, 42, 54).

Given the large number of TCSs present in the genome and the variety of environmental niches that *P. aeruginosa* inhabits, the interplay between multiple TCSs is likely to be highly complex and dynamic. For example, it was recently shown that the two-component sensor RetS, which is required for acute infection in rodents (18), modulates the phosphorylation state

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▽ Published ahead of print on 12 April 2011.

of GacS independent of its own kinase activity by forming a direct, physical interaction with the GacS protein. This physical association with GacS, rather than its kinase activity, ultimately allows RetS to control the switch between expression of virulence factors required for acute and chronic infection (19). Thus, TCS regulation of virulence phenotypes can be far more complex than the simple sum of phosphorylation events between a sensor kinase and its cognate response regulator.

Here we report the results of a systematic screen that we conducted to identify TCSs that are required for *P. aeruginosa* acute infection in a vertebrate host. We utilized a recently characterized host model of zebrafish embryos that offers a number of advantages, including the ability to conduct higher-throughput analyses than conventional rodent models while involving an immune system that is more similar to mammals than other invertebrate models such as *C. elegans.* Moreover, *P. aeruginosa* infection in zebrafish embryos resembles rodent models of acute infection in that both quorum sensing and T3S are required for full virulence in zebrafish embryos (6, 9).

In this work, we identify a number of two-component sensor kinases required for full virulence in zebrafish embryos, including KinB. We find that KinB, which has been shown to play a role in alginate biosynthesis *in vitro* in *P. aeruginosa* (12, 45), is required for acute infection in zebrafish embryos and regulates a number of virulence-associated phenotypes, including quorum sensing, biofilm formation, and motility. Interestingly, we find that KinB's *in vivo* and *in vitro* virulence-associated phenotypes are independent of its known cognate response regulator, AlgB (31), and do not require its kinase activity, underscoring the highly complex nature of TCS sensing and signal transduction in *P. aeruginosa*.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. All strains and plasmids used in this study are listed in Table 1. The MAR2XT7 transposon mutants used in this study were obtained from a nonredundant PA14 mariner transposon insertion library (30). Strains were initially streaked from frozen glycerol stocks onto LB agar plates supplemented with gentamicin and grown for 18 h at 37°C. The transposon insertion site was confirmed for each strain using arbitrary PCR, followed by sequencing of the PCR product as described previously (30). *P. aeruginosa* strains were grown at 37°C in LB broth or on LB agar, supplemented with an antibiotic where appropriate. Antibiotics used were Irgasan, 15 μg/ml; gentamicin, 15 μg/ml; and carbenicillin, 150 μg/ml. *Escherichia coli* strains were grown in LB supplemented with 50 μg/ml ampicillin. The transposon mutants used are listed below. Since some mutants are represented more than once in the library, the locus ID, followed by the transposon library mutant ID, is indicated (http://ausubellab.mgh.harvard.edu/cgi-bin/pa14/home.cgi). The mutants were PA14_06060 (40341), PA14_52240 (32799), PA14_50200 (42610), PA14_49420 (28960), PA14_49170 (33095), PA14_31950 (30263), PA14_29360 (48566), PA14_27800 (33682), PA14_22960 (29729), PA14_22730 (27958), PA14_13740 (42530), PA14_55780 (45812), PA14_60250 (34757), PA14_62530 (57211), PA14_65540 (37031), PA14_69480 (31696), PA14_70760 (40314), PA14_72390 (35639), PA14_07820 (38961), PA14_54500 (37875), PA14_46980 (28210), PA14_41270 (28523), PA14_43350 (31640), PA14_30840 (31994), PA14_29740 (41224), PA14_26810 (56008), PA14_24340 (48348), PA14_11630 (40995), PA14_56940 (37990), PA14_58320 (41430), PA14_65860 (29855), PA14_67670 (52857), PA14_68680 (54624), PA14_72740 (43067), PA14_52260 (47013), PA14_24720 (48172), PA14_12820 (41405), PA14_12420 (31421, 38371), PA14_10770 (37655), PA14_64230 (gift from F. Ausubel, MGH), PA14_65860 (29855), PA14_10700 (28567), PA14_48160 (40171), PA14_09680 (45377), PA14_48160 (40171), PA14_30700 (55936), PA14_27550 (38969), PA14_21700 (38780), PA14_19340 (29763), PA14_36420 (39759), PA14_59800 (24601), PA14_57980 (32292), PA14_38970 (1242), PA14_16470 (25859), PA14_02250 (28227), PA14_45590 (34047), PA14_05390 (35863), PA14_63160 (35538), PA14_45870 (24217), PA14_43670 (36736), PA14_50360 (56704), PA14_16250 (gift from F. Ausubel, MGH), and PA14_24480 (26187).

Mutant strain construction. To construct in-frame deletions, we made a Gateway-compatible vector, pEXG2-attP. Utilizing the homology between the *attP1* and *attP2* sites, we were able to PCR amplify the *attP* Gateway cassette from pDONR221 (Invitrogen) with a single *attP* primer (attP1). The attP1 primer contains SphI restriction sites that enabled us to clone the PCR product into the SphI site in pEX18Gm (25) to create pEX18Gm-attP. The *attP* cassette was then excised from pEX18Gm-attP using SphI and cloned into the SphI site on pEXG2-M13, a derivative of pEXG2 (48) that was derived by digesting with HindIII and NdeI and ligating the annealed oligonucleotides M13-1 and M13-2, which contain an SphI site flanked by M13 primer binding sites, to create pEXG2-attP. All vectors were sequence verified.

For in-frame deletion of specific genes, the upstream and downstream sequence fragments (500 to 1,000 bp) flanking the target gene were PCR amplified using primers PA14_723905p left, PA14_723905p right, PA14_723903p left, and PA14_723903p right (for deletion of *kinB*) and PA14_723805p left, PA14_723805p right, PA14_723803p left, and PA14_723803p right (for deletion of *algB*). The flanks were fused together using the crossover PCR method with primers attB1 and attB2. Primers were designed such that a PacI restriction site would be introduced at the site of the gene being deleted. The PCR products with the in-frame deletion of the target gene were cloned into our modified vector using Gateway BP Clonase (Invitrogen, Carlsbad, CA). A two-step allelic exchange procedure was employed with the constructs for in-frame deletion. The single-crossover merodiploid exconjugants were selected based on gentamicin and Irgasan resistance. After overnight incubation of the merodiploids in LB broth at 37°C, the double-crossover recombinants were isolated on LB agar with 10% sucrose to force the removal of vector DNA containing the *sacB* gene. The in-frame deletion of the target gene was confirmed by PCR amplification of the flanking region of the target gene with multiple sets of primers and amplicon sequencing.

The *kinB* gene was amplified from wild-type PA14 using primers NSC09 and NSC10. Primers were designed using the published PA14 genome sequence (29). The gene was cloned into the shuttle vector pHERD20T (41) for complementation with gene expression driven by the P_{BAD} arabinose-inducible promoter. The sequences of primers used are listed in Table 2. All constructs were sequence verified.

To construct a plasmid expressing the KinB protein with a substitution at the conserved histidine residue (H385A), plasmid pKinB was mutagenized using primers NSC11 and NSC12 to obtain plasmid pNSC4 using the Phusion site-directed mutagenesis kit per the manufacturer's protocol. The mutation was confirmed by sequence analysis.

Screening methodology. AB line zebrafish embryos were inoculated with either a *P. aeruginosa* sensor kinase transposon mutant or the wild-type PA14 strain into the yolk circulation valley 50 h postfertilization (hpf) as previously described (9). In every experiment 20 to 30 zebrafish embryos from a single clutch were infected with a mutant or wild-type PA14 as a positive control. In addition 4 to 10 embryos were infected with heat-killed PA14 as a negative control from the same clutch. A mutant was defined as attenuated if the Kaplan-Meier survival curve generated for the mutant infections was statistically different ($P < 0.05$) from the infection with wild-type PA14 by the log-rank test. If a mutant appeared attenuated in the initial screen, the mutant was then retested. The phenotype for every mutant was confirmed at least 2 times.

Bacterial enumeration from infected embryos. Zebrafish embryos were infected with either wild-type PA14 or PA14Δ*kinB*. At the stated time points for each condition, 8 individual embryos were sacrificed and bacterial load enumerated as previously described (9).

Imaging infection. Zebrafish embryos were infected with either wild-type PA14(+pSMC21) or PA14Δ*kinB*(+pSMC21). Microscopy was performed with a Zeiss Discovery V12 stereomicroscope as previously described (9). Embryos were monitored for fluorescence at several different time points over the course of infection.

Growth curves. Growth of PA14Δ*kinB* was quantified in standard culture flasks. An overnight culture was diluted 1:1,000 in 50 ml LB in a 250-ml culture flask and incubated at 37°C with shaking (250 rpm). The optical density at 600 nm (OD_{600}) was determined at several different time points over a time period of 18 h.

Pyocyanin quantitation assay. Pyocyanin was extracted from the supernatant fraction of *P. aeruginosa* isolates grown in LB medium for 22 h and quantified as previously described (51). For strains carrying the pHERD20T plasmid or its derivatives, the medium was supplemented with 150 μg/ml carbenicillin.

TABLE 1. *P. aeruginosa* strains, plasmids, and oligonucleotides used in this study

Strain, plasmid, or oligonucleotide	Characteristic(s) or sequence	Source and/or reference
Bacterial strains		
E. coli		
DH5α	*fhuA2* Δ(*argF-lacZ*)*U169 phoA glnV44* ϕ80 Δ(*lacZ*)M15 *gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	Lab strain
SM10	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km λ*pir*	Lab strain
XL1-Blue(pECP61.5)	*E. coli* bioassay strain to detect C_4-HSL; *endA1 gyrA96*(*nalR*) *thi-1 recA1 relA1 lac glnV44* F′[::Tn*10 proAB*$^+$ *lacI*^{q} Δ(*lacZ*)M15] *hsdR17*(r_K^- m_K^+) +pECP61.5	39
P. aeruginosa		
PA14	Virulent burn wound isolate of *P. aeruginosa*	43
Transposon mutants	From nonredundant library, Mar2XT7 transposon	30
PA14Δ*kinB*	PA14Δ*kinB* (1.7-kb in-frame deletion of *kinB)*	This study
PA14Δ*algB*	PA14Δ*algB* (1.3-kb in-frame deletion of *algB)*	This study
PA14Δ*gacA*	PA14Δ*gacA*	Ausubel lab; 44
PA14Δ*lasR*	PA14Δ*lasR*	9
PA14Δ*kinB*(+pKinB)	PA14Δ*kinB* (+pKinB)	This study
PA14Δ*kinB*(+pHERD0T)	PA14Δ*kinB*(+pHERD20T)	This study
PA14Δ*kinB*(+pKinBH385A)	PA14Δ*kinB*(+pKinBH385A)	This study
PA14(+pSMC21)	PA14(+pSMC21)	This study
PA14Δ*kinB*(+pSMC21)	PA14Δ*kinB*(+pSMC21)	This study
PA14Δ*exoU*Δ*exoT*Δ*exoY*	PA14Δ*exoU* Δ*exoT* Δ*exoY*	34
PA14Δ*phz1/2*	PA14 with deletions of operons *phzA1-G1* and *phzA2-G2*	Ausubel lab; 13
PA14Δ*algD*	PA14Δ*algD*	Ausubel lab; 59
A. tumefaciens KYC 55	*A. tumefaciens* bioassay strain to detect total levels of homoserine lactones	28
Plasmids		
pDONR221	Gateway donor vector; Kan^r	Invitrogen
pEX18Gm	Positive selection suicide vector, Gent^r	25
pEX18Gm-attP	pEX18Gm with an *attP* cassette	This study
pEXG2	Derivative of pEX18Gm	48
pEXG2-M13	pEXG2 with annealed oligonucleotides M13-1 and M13-2 cloned into the HindIII/NdeI site	This study
pEXG2-attP	pEXG2-M13 with *attP* cassette cloned from pEX18Gm-attP into the SphI site	This study
pNSC1	pEXG2-attP with *kinB* flanks from PA14 cloned in; flanks were amplified using primers PA14_723905p left, PA14_723905p right, PA14_723903p left, and PA14_723903p right	This study
pNSC2	pEXG2-attP with *algB* flanks from PA14 cloned in; flanks were amplified using primers PA14_723805p left, PA14_723805p right, PA14_723803p left, and PA14_723803p right	This study
pNSC3	pEXG2-attP with *kinB* flanks from PAO1 cloned in	This study
pHERD20T	pUCP20T Plac replaced by 1.3-kb AflII-EcoRI fragment of *araC*-P_{BAD} cassette (5,087 bp)	Lory lab; 41
pKinB	pHERD20T with PA14 *kinB* at the KpnI/HindIII site; the *kinB* gene was amplified using primers NSC09 and NSC10	This study
pKinBH385A	pHERD20T:*kinB* with H385A using primers NSC11 and NSC12 for site-directed mutagenesis	This study
pSMC21	GFP expression plasmid (GFP$^+$ Ap^r Kan^r)	4

Elastase activity assay. Elastolysis activity was quantified for each mutant as previously described (40). For strains carrying the pHERD20T plasmid or its derivatives, the medium was supplemented with 150 μg/ml carbenicillin.

Autoinducer activity assay. The total levels of homoserine lactones (HSLs) present in cell-free supernatants of wild-type PA14 and PA14Δ*kinB* were measured using an *Agrobacterium tumefaciens* bioassay strain as previously described (28) while the levels of C_4-HSL were determined using an *E. coli* bioassay strain as described previously with the following modifications (39). Filter-sterilized culture supernatants (1 ml) from *P. aeruginosa* were prepared and mixed with equal volumes of the *E. coli* reporter strain XL1-Blue(+pECP61.5) diluted to an OD_{600} of 0.1 in LB supplemented with 0.1 M IPTG (isopropyl-β-D-thiogalactopyranoside). After 6 h of incubation at 30°C, β-galactosidase assays were conducted as previously described (33).

Cytotoxicity assay. Cytotoxicity of *P. aeruginosa* strains was examined *in vitro* on A549 lung epithelial cells as previously described (1). Wild-type PA14 was used as a positive control, while PA14Δ*exoU*Δ*exoT*Δ*exoY* was used as a negative control.

Biofilm formation assay. Each *P. aeruginosa* strain to be analyzed was grown overnight and then inoculated into LB medium at an estimated OD_{600} of 0.0025. Biofilm formation was assayed as previously described (37), with minor modifications. Diluted cultures (100 μl) were added to a 96-well microtiter dish, such that there were eight replicates for each strain. The microtiter dish was incubated at 30°C for 24 h. The wells were thoroughly rinsed with water and stained for 30 min at 25°C with a 0.1% solution of crystal violet. The plates were then washed, and the crystal violet staining was solubilized with 33% acetic acid. The resulting solution was diluted 1:4 in water in a fresh polystyrene microtiter dish, and A_{590} was measured on a spectrophotometer.

Motility assays. (i) Swimming. Swim plates were prepared as described previously (46) with agar (USB). Swim plates were inoculated with a single bacterial colony using a sterile toothpick. The plates were wrapped with Saran Wrap to prevent dehydration. Photographs were taken after incubating the plates for 18 h at 30°C. The diameter of the circular turbid zone was measured for each bacterial strain.

TABLE 2. Oligonucleotides used

Primer	Sequence	Source
attP1	5′-AGCTTGCATGCTCGGGCCCCAAATAAT-3′	This study
M13-1	5′-AGCTTTGTAAAACGACGGCCAGTGCATGCCATGGTCATAGCTGTTTCCTGCA-3′	This study
M13-2	5′-TATGCAGGAAACAGCTATGACCATGGCATGCACTGGCCGTCGTTTTACAA-3′	This study
attB1	5′-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3′	This study
attB2	5′-GGGGACCACTTTGTACAAGAAAGCTGGGT-3′	This study
PA14_723905p left	5′-TACAAAAAAGCAGGCTCTCAACGTGATCGTGCTCAAC-3′	This study
PA14_723905p right	5′-ACCGGTTAATTAAGGAGGAACAACCGGGTCCGGA-3′	This study
PA14_723903p left	5′-TCCTCCTTAATTAACCGGTGTGACCGGGGCCGCT-3′	This study
PA14_723903p right	5′-TACAAGAAAGCTGGGTCTGGTATCCGTTCAGCGAA-3′	This study
NSC09	5′-ATTAGGTACCTATGAGCATGCCGCTGCCGATGAAG-3′	This study
NSC10	5′-AATAAAAGCTTTCACACCGGCAGCAGCATGTAGAA-3′	This study
PA14_723805p left	5′-TACAAAAAAGCAGGCTCGAGAGATTCATCACCCAGTC-3′	This study
PA14_723805p right	5′-GCATCTTAATTAAGCATCGTTGCTTTTTATCCTC-3′	This study
PA14_723803p left	5′-CGATGCTTAATTAAGATGCCTCGACCCTGTACCG-3′	This study
PA14_723803p right	5′-TACAAGAAAGCTGGGTAGTATTTCGTCGATCCGCTTG-3′	This study
NSC11	5′-Phos-GCGCGCCTCCGCCGAACTGCGCACG-3′	This study
NSC12	5′-Phos-AGGACGAATTCGCTGCGGA-3′	This study

(ii) Twitching. Twitch plates were prepared as described previously (46) with 1% (wt/vol) USB granulated agar. Plates were dried and inoculated with a toothpick to the bottom of the petri dish from an overnight-grown LB agar (1.5%, wt/vol) plate. After incubation at 37°C for 24 h, the zone of motility at the agar/petri dish interface was measured.

Statistical methods. Prism 5 (Graphpad Inc.) was used for all statistical analyses. Kaplan-Meier survival curves were plotted, and significance was calculated using the log-rank test. To determine significance for the expansion analysis presented in Fig. 2B and the Miller assays presented in Fig. 3C and D, the two-tailed *P* value was calculated using the Mann-Whitney test. The data in Fig. 3A and B and Fig. 5 were compared using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test (set at 0.05).

RESULTS

Identification of two-component sensors required for full *P. aeruginosa* PA14 virulence in zebrafish embryos. Transposon mutants in 60 two-component sensors from a previously constructed, nonredundant PA14 transposon mutant library (30) were analyzed for their ability to infect zebrafish embryos. Transposon insertions in all strains were sequence verified prior to analysis.

We infected embryos 50 hpf with individual mutants by microinjecting bacteria directly into the zebrafish bloodstream in the yolk circulation valley (9). The ability of each mutant to establish a lethal infection in zebrafish embryos was measured by determining whether survival curves generated from embryos infected with the mutant were statistically different from survival curves generated from embryos infected with wild-type PA14 as determined by the log-rank test. Each mutant was tested at least twice for its ability to establish a lethal infection in zebrafish embryos. From the collection of 60 TCS sensors, 6 mutants resulted in a statistically significant increase in embryo survival (Fig. 1 and Table 3). The attenuated mutants identified included strains containing a transposon disruption of *gacS* (42) and *retS* (18), known regulators of virulence. In addition we identified several two-component sensors that have not previously been implicated in playing a role in acute infection, whose disruption resulted in an attenuated phenotype in acute vertebrate infection. These two-component sensors included PA14_70760 (*phoR*), known to regulate the production of rhamnolipids (27); PA14_27800 (*copS*), a copper ion sensor; PA14_29740 (*bqsS*), recently shown to play a role in biofilm formation (14); and PA14_72390 (*kinB*), which is reported to regulate alginate biosynthesis *in vitro* (12, 45). Since alginate is not thought to be an important virulence factor in acute infection models (59), we found the putative requirement for KinB during acute infection to be intriguing and thus selected it for further study.

A *kinB* deletion mutant shows reduced virulence in zebrafish embryos. Transposon disruption of *kinB* was identified in our screen to result in a significantly increased level of survival in infected embryos ($P < 0.02$). We confirmed the defect in virulence by infecting embryos with a clean, in-frame *kinB* deletion mutant (PA14Δ*kinB*) and a complemented mutant [PA14Δ*kinB*(pKinB)] in *P. aeruginosa* PA14 (Fig. 2A). We also constructed an in-frame deletion mutant in its cognate response regulator gene *algB* (PA14Δ*algB*), which lies adjacent to *kinB* in its operon and has been shown to be phosphorylated by KinB *in vitro* (31). We examined *in vitro* bacterial growth rates for both PA14Δ*kinB* and PA14Δ*algB* and found no significant differences in growth rates between wild-type PA14 and these mutants (data not shown). PA14Δ*kinB* was, however, markedly attenuated for infection in zebrafish embryos when infected at a dose similar to wild-type PA14 (Fig. 2A, $P < 0.0001$). In contrast, there was no significant difference in survival between embryos infected with wild-type PA14 and PA14Δ*algB* (Fig. 2A). These results suggest that the regulation of virulence by KinB occurs in an AlgB-independent manner.

We enumerated the bacterial load within zebrafish embryos when they were infected with wild-type PA14 or with PA14Δ*kinB* at 22 and 41 h postinfection (hpi). At both time points, we found a statistically significant difference between the bacterial load carried by embryos infected with wild-type PA14 and by embryos infected with PA14Δ*kinB* ($P = 0.04$ [22 hpi] and $P = 0.01$ [41 hpi] by the Mann-Whitney test) (Fig. 2B). Embryos infected with PA14Δ*kinB* had ~0.6- to 1-log CFU reduction in bacterial load at each time point. This modest difference correlated with a rather marked increase in survival. To determine whether PA14Δ*kinB* mutants localized to different anatomic regions of the embryo, we compared *in vivo* localization patterns of embryos infected with green fluorescent protein (GFP)-expressing PA14 and PA14Δ*kinB* strains.

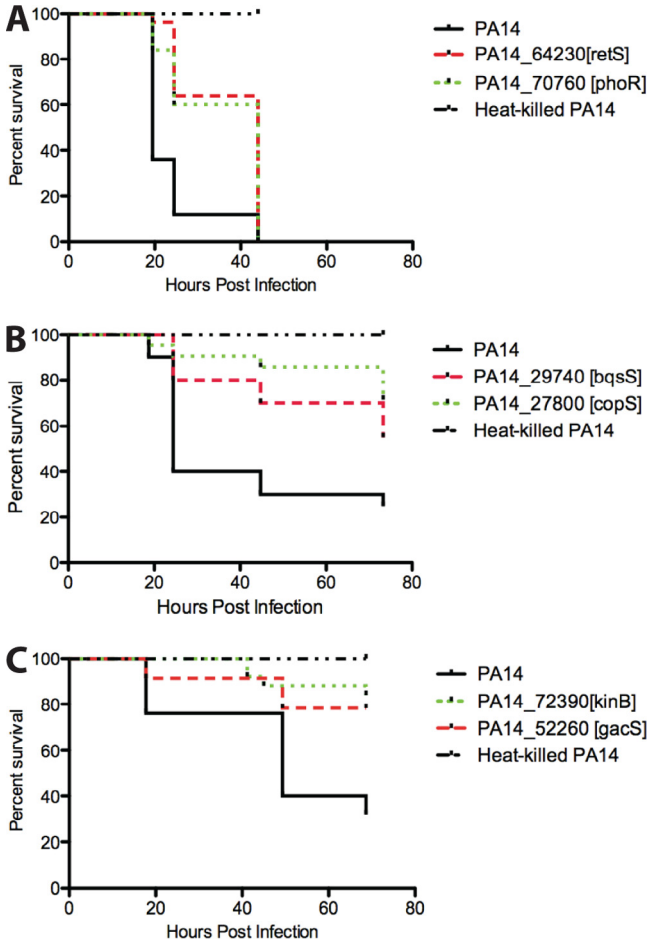

FIG. 1. Zebrafish embryo survival in embryos infected with different *P. aeruginosa* two-component sensor mutants. (A) Kaplan-Meier embryo survival curves following infection of 50-hpf embryos with PA14 (7,060 ± 1,185 CFU), heat-killed PA14 (0 CFU), the *retS* transposon mutant (6,565 ± 1,708 CFU), or the *phoR* transposon mutant (6,940 ± 1,423 CFU). (B) Kaplan-Meier embryo survival curves following infection of 50-hpf embryos with PA14 (4,200 ± 230 CFU), heat-killed PA14 (0 CFU), the *bqsS* transposon mutant (4,560 ± 673 CFU), or the *copS* transposon mutant (4,470 ± 486 CFU). (C) Kaplan-Meier embryo survival curves following infection of 50-hpf embryos with PA14 (4,920 ± 700 CFU), heat-killed PA14 (0 CFU), the *kinB* transposon mutant (6,760 ± 698 CFU), or the *gacS* transposon mutant (6,795 ± 613 CFU). Each panel is a separate experiment comparing the survival of embryos infected with either one of two transposon mutants to wild-type PA14. Since clutch size limits the number of mutants that can be examined in each experiment and the 100% lethal dose (LD_{100}) of *P. aeruginosa* can vary between different embryo clutches, whether a mutant is attenuated or not is determined with reference to infection with the wild-type PA14 strain within the same embryo clutch. Each mutant was compared individually to wild-type PA14 at least twice, with 20 to 30 embryos per condition per replicate.

Similar to previous work (9), we noticed that fluorescent bacteria in both PA14- and PA14Δ*kinB*-infected embryos were detectable only late in infection (just prior to death) under a fluorescent stereomicroscope. We were therefore not surprised to observe that embryos infected with PA14Δ*kinB* became fluorescent at slightly later time points compared to embryos infected with wild-type PA14, since PA14Δ*kinB*-infected embryos have a greater survival advantage and modest but significantly lower bacterial load. However, we did not observe any difference in the localization pattern of bacteria between PA14- and PA14Δ*kinB*-infected embryos at any time point during infection (data not shown).

TABLE 3. *P. aeruginosa* two-component sensors required for virulence in zebrafish embryos

PA14 no.	Gene/homolog name	Known role	*P* value (Fig. 1)
PA14_72390	*kinB*	Alginate biosynthesis	<0.02
PA14_70760	*phoR*	Rhamnolipid production	<0.0001
PA14_52260	*gacS*	Master regulator of virulence	<0.02
PA14_64230	*retS*	Master regulator of virulence	<0.0001
PA14_27800	*copS*	Copper sensing	<0.02
PA14_29740	*bqsS*	Biofilm formation	<0.02

KinB regulates pyocyanin and elastase production. It has previously been shown that both quorum sensing and T3S are required for *P. aeruginosa* infection of zebrafish embryos at 50 hpf, similar to rodent models of acute *P. aeruginosa* infection (6, 9). Thus, since PA14Δ*kinB* was attenuated in fish, we ex-

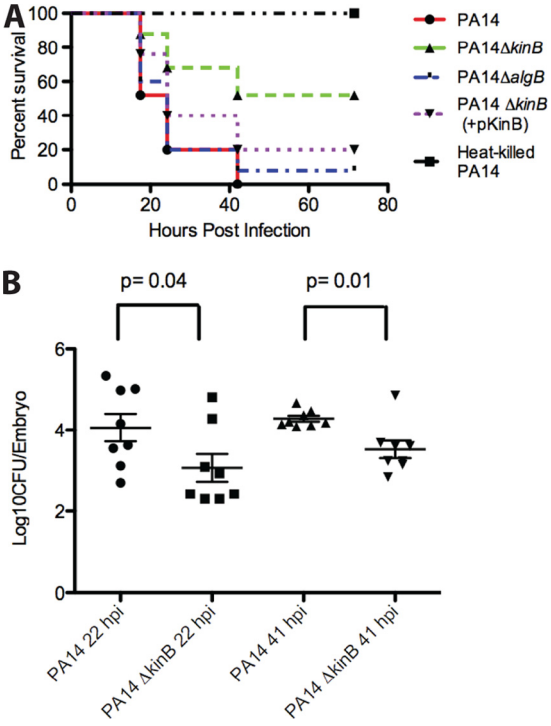

FIG. 2. Zebrafish embryo survival and bacterial load in embryos infected with different *P. aeruginosa* strains. (A) Kaplan-Meier embryo survival curves following infection of 50-hpf embryos with wild-type PA14 (5,150 ± 577 CFU), heat-killed PA14 (0 CFU), the PA14Δ*kinB* mutant (5,330 ± 644 CFU), the PA14Δ*algB* mutant (2,790 ± 534 CFU), or the PA14Δ*kinB* mutant complemented with pKinB (3,150 ± 605 CFU). The data are representative of three replicates with 25 embryos per condition per replicate. (B) Expansion of wild-type PA14 and the PA14Δ*kinB* mutant in zebrafish embryos over time. Bacterial enumeration from embryos infected at 50 hpf with wild-type PA14 (4,353 ± 351 CFU) or the PA14Δ*kinB* mutant (4,757 ± 593 CFU) at 22 hpi and 41 hpi. Two-tailed *P* values noted on the graph were calculated using the Mann-Whitney test. The data are representative of two replicates.

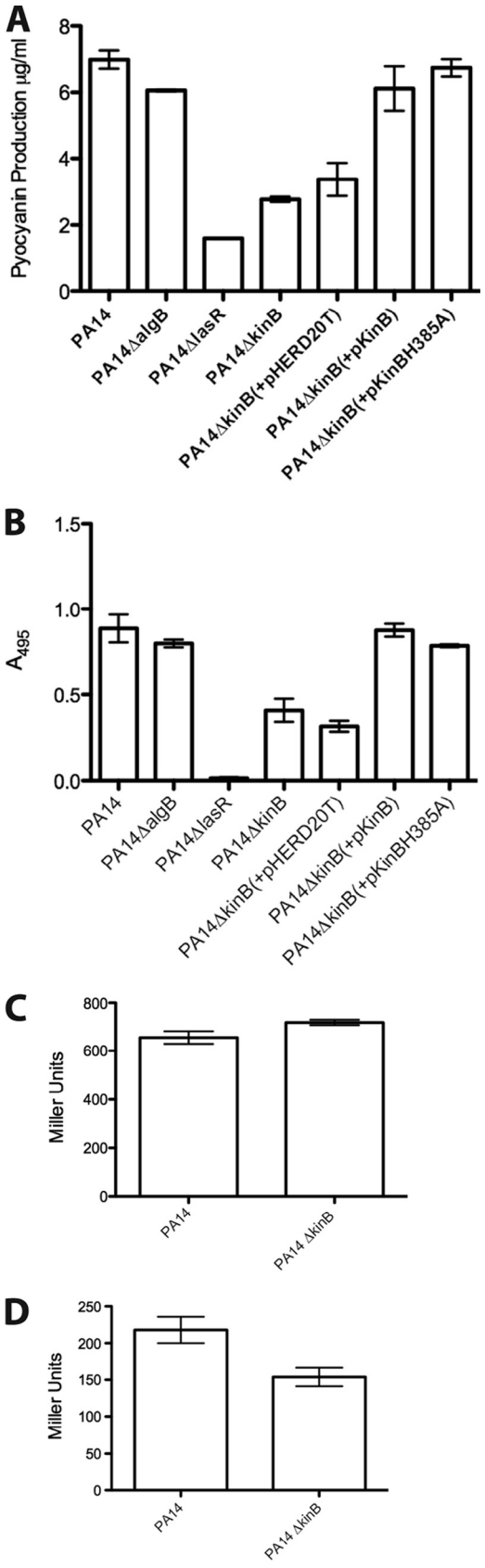

FIG. 3. The PA14Δ*kinB* mutant is impaired in the production of pyocyanin and elastase but not in the production of autoinducers.

amined whether KinB also regulates the production of toxins typically regulated by quorum sensing (pyocyanin and elastase), the production of quorum-sensing-regulated molecules themselves, and whether T3S was defective in PA14Δ*kinB*.

Pyocyanin is a redox-active phenazine secreted by *P. aeruginosa* whose production is regulated by quorum sensing. It has toxic effects on other prokaryotes and eukaryotic cells (13). The amount of pyocyanin in the supernatants of PA14Δ*kinB* was 3-fold less compared to wild-type PA14 and complemented PA14Δ*kinB*(pKinB) (Fig. 3A; analysis using one-way ANOVA followed by Bonferroni's multiple comparison test indicated that there was a statistically significant difference [$P < 0.05$] between the amount of pyocyanin produced by wild-type PA14 and the PA14Δ*kinB* mutant), similar to a *lasR* deletion mutant, which is a key transcriptional regulator of quorum sensing (56). Meanwhile, PA14Δ*algB* did not show reduced pyocyanin production.

Elastase, a protease that is capable of causing corneal ulcers and necrotic lesions (35), is also a virulence factor produced by *P. aeruginosa* in a quorum-sensing-regulated manner. The elastin-Congo red (ECR) assay, commonly used to estimate elastase levels produced by a *P. aeruginosa* strain (40), revealed that PA14Δ*kinB* was also impaired in the ability to produce elastase (analysis using one-way ANOVA followed by Bonferroni's multiple comparison test indicated that there was a statistically significant difference [$P < 0.05$] between the amount of elastase produced by wild-type PA14 and the PA14Δ*kinB* mutant). Elastase production was restored to wild-type levels in the complemented PA14Δ*kinB*(pKinB). Likewise, the PA14Δ*algB* mutant did not show any defect in elastase production (Fig. 3B). Thus, KinB appears to regulate the production of the quorum-sensing-regulated virulence factors elastase and pyocyanin in a manner independent of AlgB.

The role of KinB in quorum sensing. Since KinB regulates the production of virulence factors known to be under the control of quorum sensing, we speculated that KinB might do so by affecting the production of the two homoserine lactone autoinducers produced by *P. aeruginosa*, 3-oxododecanoyl homoserine lactone (3-oxo-C_{12}-HSL) and butanoyl homoserine lactone (C_4-HSL). However, we found no significant difference between wild-type PA14 and PA14Δ*kinB* stimulation

(A) Pyocyanin produced by different *P. aeruginosa* strains after 22 h of growth at 37°C, 250 rpm. The data are representative of three biological replicates. Error bars indicate standard errors of the means computed from two technical replicates. (B) Elastase activities from culture fluids from 21-h cultures in PTSB medium (5% peptone, 0.25% tryptic soy broth [pH 7]) (40) were measured in elastin-Congo red assays. The data are representative of three biological replicates. Error bars indicate standard errors of the means computed from two technical replicates. (C) The total levels of homoserine lactones present in cell-free supernatants of wild-type PA14 and PA14Δ*kinB* were measured using an *Agrobacterium tumefaciens* bioassay strain. The data are representative of three biological replicates. Error bars indicate standard errors of the means computed from three technical replicates. (D) The levels of C_4-homoserine lactones present in cell-free supernatants of wild-type PA14 and PA14Δ*kinB* grown to an OD_{600} of 2.0 were measured using an *E. coli* bioassay strain. The data are representative of three biological replicates. Error bars indicate standard errors of the means computed from three technical replicates.

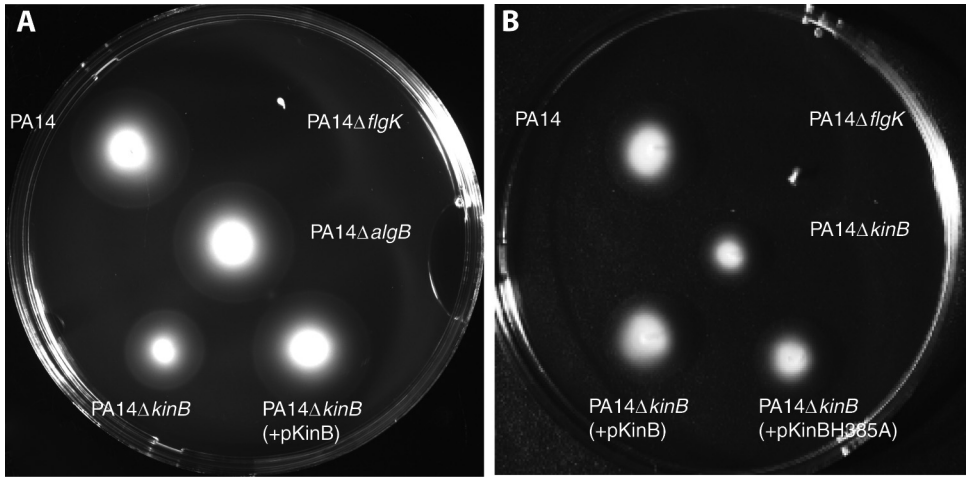

FIG. 4. The PA14Δ*kinB* mutant is less motile than wild-type *P. aeruginosa* PA14. (A) Swimming motility of *P. aeruginosa* PA14, the PA14Δ*kinB* mutant, an *flgK* transposon mutant, and the complemented *kinB* mutant on a semisolid agarose plate. (B) Swimming motility of *P. aeruginosa* PA14, the PA14Δ*kinB* mutant, a *flgK* transposon mutant, the complemented *kinB* mutant [PA14Δ*kinB*(pKinB)], and the deletion mutant complemented with a site-directed mutant (H385A) of the KinB protein [PA14Δ*kinB*(pKinB$_{H385A}$)] on a semisolid agarose plate. Swim plates were photographed after 17 h of incubation at 30°C.

of reporter activity of an *A. tumefaciens* bioassay strain (28) (Fig. 3C; the two-tailed *P* value calculated using the Mann-Whitney test did not indicate a significant difference between the two conditions [$P > 0.05$]). This result suggests that there is no difference between the total levels of homoserine lactones produced by these two strains. Since this assay more sensitively detects 3-oxo-C_{12}-HSL, we also determined whether there was any difference in the level of C_4-HSL produced by the PA14Δ*kinB* mutant using an *E. coli* bioassay strain (39). We found that β-galactosidase activity was slightly but not significantly reduced when the *E. coli* bioassay strain was incubated with supernatants from the PA14Δ*kinB* mutant compared to the wild-type strain (Fig. 3D; the two-tailed *P* value calculated using the Mann-Whitney test did not indicate a significant difference between the two conditions [$P > 0.05$]). This suggests that the level of C_4-HSL produced by the PA14Δ*kinB* mutant is slightly but not significantly less than the levels produced by wild-type PA14. We also observed no significant difference in reporter activity of bioassay strains when comparing wild-type PA14 and PA14Δ*kinB* at OD_{600}s of 1.0 and 1.5 (data not shown). Thus, KinB appears to modulate the production of the quorum-sensing-regulated virulence factors pyocyanin and elastase without significantly affecting the production of the quorum sensing autoinducer molecules themselves. This phenotype is very similar to a PA14Δ*gacA* mutant phenotype where the production of quorum-sensing-regulated virulence factors is also impaired with minimal alteration in autoinducer production (38, 44). However, it is worth noting that the *in vitro* HSL levels measured here may not be accurately reflective of HSL production *in vivo* and that PA14Δ*kinB* could produce lower levels of HSLs *in vivo*.

T3S is unaffected in a *kinB* deletion mutant. In addition to quorum sensing, T3S is also an important determinant of *P. aeruginosa* virulence in the zebrafish infection model, similar to rodent models of acute infection (9). Since translocation of T3S effectors into host cells plays a key role in *P. aeruginosa*-mediated cytotoxicity (16), we examined whether KinB was involved in T3S-mediated cytotoxicity in cultured human epithelial cells. Following infection of A549 cells with either wild-type PA14, PA14Δ*exoU*Δ*exoT*Δ*exoY* (a negative-control strain, which does not express T3S effectors), or PA14Δ*kinB*, we found no difference in cytotoxicity, as measured by lactate dehydrogenase (LDH) release (1) 4 h postinfection, between wild-type PA14 and PA14Δ*kinB* (data not shown), indicating that KinB does not play a role in the modulation of T3S-dependent cytotoxicity.

KinB regulates swimming but not twitching motility. In addition to other virulence factors, motility also plays a critical role in pathogenesis (2). Specifically, swimming motility may play a role in the dissemination of *P. aeruginosa* within the circulation of zebrafish embryos. We determined whether PA14Δ*kinB* had a swimming motility defect using a plate assay. As shown in Fig. 4, PA14Δ*kinB* is impaired in swimming motility in semisolid agarose plates. This swimming defect is less pronounced than in a completely nonmotile strain containing a transposon mutation in *flgK*, a flagellar biosynthesis gene (37). The motility defect is fully complemented in PA14Δ*kinB*(pKinB). Further, PA14Δ*algB* is not impaired in swimming motility (Fig. 4A), suggesting, once again, that the regulation of acute virulence phenotypes, like swimming motility by KinB, occurs in a manner independent of AlgB.

In addition to swimming motility, *P. aeruginosa* also exhibits type IV pilus-mediated twitching motility (24). Mutants with nonfunctional pili show reduced virulence in acute *P. aeruginosa* infection models (11). Thus, we determined whether twitching motility was also affected in PA14Δ*kinB*. This was examined using a stab assay and measuring the interstitial twitch zone formed at the agar-plastic interface on a petri dish. We found no difference in twitching motility between wild-type PA14 and PA14Δ*kinB* (data not shown).

KinB regulates biofilm formation. Flagellar motility is necessary for *P. aeruginosa* biofilm development (37). Since PA14Δ*kinB* displayed a motility defect, we hypothesized that the strain might also have a biofilm attachment defect.

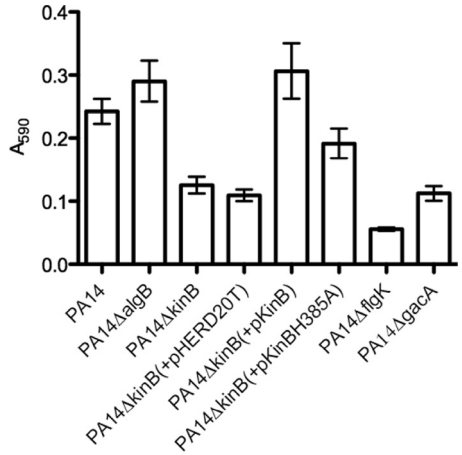

FIG. 5. Deletion of the *kinB* gene results in reduced biofilm formation. Biofilm formation was assayed by crystal violet staining and measuring absorbance at 590 nm 24 h after seeding the plate. A *flgK* transposon mutant was used as a control. All other strains are in-frame deletion mutants. The data are representative of three biological replicates. Error bars indicate standard errors of the means computed from eight technical replicates.

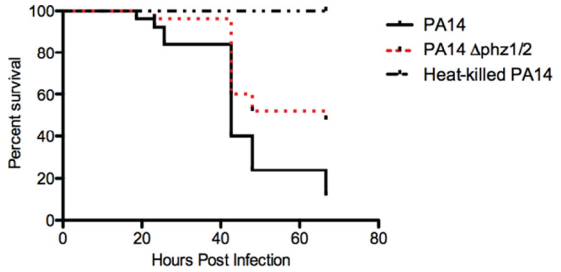

FIG. 6. Phenazine biosynthesis is a critical virulence factor in zebrafish embryos. Kaplan-Meier embryo survival curves following infection of 50-hpf embryos with PA14 (7,815 ± 430 CFU), heat-killed PA14 (0 CFU), or the PA14Δ*phz1/2* mutant (9,625 ± 2,374 CFU), which contains deletions in both phenazine operons. The data are representative of 3 replicates with 20 to 30 embryos per condition per replicate.

PA14Δ*kinB* produced less biofilm than wild-type PA14, although biofilm formation was not completely abolished, compared to the *flgK* transposon mutant (Fig. 5; analysis using one-way ANOVA followed by Bonferroni's multiple comparison test indicated that there was a statistically significant difference [$P < 0.05$] between the amounts of biofilm produced by wild-type PA14 and the PA14Δ*kinB* mutant). PA14Δ*kinB* adheres to polyvinyl chloride (PVC) at levels comparable to PA14Δ*gacA*, which also produces lower levels of biofilm (38) (Fig. 5), highlighting once again the similarity between PA14Δ*kinB* and PA14Δ*gacA* mutant phenotypes and underscoring the important role that KinB plays in the regulation of multiple aspects of *P. aeruginosa* virulence.

The role of canonical *P. aeruginosa* virulence factors in zebrafish embryo infection. Since the PA14Δ*kinB* mutant was attenuated for virulence in zebrafish embryos and impaired in the production of multiple virulence factors such as pyocyanin, we examined whether these virulence factors played important roles in the infection of zebrafish embryos by *P. aeruginosa.* To determine whether this was indeed the case, we infected zebrafish embryos with strains containing mutations in genes required for motility or required for the production of pyocyanin, elastase, alginate, or exopolysaccharide. This set included the PA14Δ*phz1/2* mutant, which contains deletions in both phenazine operons (13); a strain with a transposon mutation in *lasB*, which is required for the production of elastase (40); a strain with an in-frame deletion in *algD* (59), which codes for GDP-mannose dehydrogenase, an enzyme essential for alginate synthesis (50); a strain with a transposon mutation in *flgK*, required for motility (37); and a strain with a transposon mutation in *pelA*, required for pellicle formation (15). Strikingly, only the PA14Δ*phz1/2* mutant was attenuated for virulence in the zebrafish embryo infection model, suggesting that phenazine biosynthesis is a critical virulence factor in zebrafish embryos ($P < 0.02$) (Fig. 6). In contrast, the PA14Δ*algD* and *pelA* transposon mutants were not attenuated for acute infection in zebrafish embryos, which is perhaps not surprising since both biofilm formation (19) and alginate production are associated with chronic rather than acute infection; alginate production has been shown to be dispensable in acute infection models (59). We were surprised to find that the *flgK* mutant was as virulent as wild-type PA14 in this infection model, since it has previously been shown that flagellar motility plays an important role in an acute mouse burn infection model (2). It is possible that the *flgK* mutant is fully virulent in zebrafish embryos because bacteria were inoculated intravenously, and thus, the dissemination of nonmotile *P. aeruginosa* throughout the embryo was likely facilitated by the embryo's circulation itself. Based on these observations, the reduction in phenazine production could be solely responsible for the attenuation of the PA14Δ*kinB* mutant. However, because the mutant is not completely defective in phenazine production, we suggest that downregulation of an as-yet-unidentified virulence determinant or simultaneous repression of multiple virulence factors in the absence of *kinB* may more likely be responsible for PA14Δ*kinB*'s loss of virulence.

The role of KinB's kinase activity in regulating virulence phenotypes. It has been shown that KinB signaling through AlgB to regulate alginate production does not require AlgB phosphorylation (12). Based on this observation, we sought to ask whether KinB uses a conventional, kinase-dependent or noncanonical, kinase-independent TCS mechanism to regulate virulence. Because KinB modulates acute virulence phenotypes in an AlgB-independent manner, KinB could phosphorylate a different response regulator or function independently of its kinase activity altogether. A recent example of a sensor kinase that acts in the latter manner is RetS, which regulates the phosphorylation state of the GacS sensor kinase by forming a direct, physical association with GacS (19).

To begin to understand the mechanism by which KinB might regulate acute virulence phenotypes, we tested whether KinB's kinase activity was required for regulating the production of quorum-sensing-regulated virulence factors, motility, biofilm formation, and virulence *in vivo*. We engineered an allele of *kinB* in which the histidine required for kinase activity (31) was replaced with alanine ($kinB_{H385A}$) and introduced it episomally into PA14Δ*kinB.* Episomal introduction of this catalytically inactive kinase allele (p$kinB_{H385A}$) into PA14Δ*kinB* restored

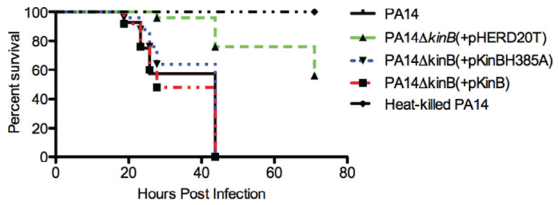

FIG. 7. The catalytically inactive KinB protein can restore virulence in a PA14Δ*kinB* mutant. Kaplan-Meier embryo survival curves following infection of 50-hpf embryos with wild-type PA14 (6,080 ± 841 CFU), heat-killed PA14 (0 CFU), the PA14Δ*kinB* mutant complemented with empty vector (pHERD20T) (4,255 ± 908 CFU; $P < 0.0001$), the PA14Δ*kinB* mutant complemented with wild-type protein (pKinB) (5,375 ± 916 CFU), or the PA14Δ*kinB* mutant complemented with the catalytically inactive protein (pKinBH385A) (7,465 ± 265 CFU). The data are representative of two replicates with 20 to 30 embryos per condition per replicate.

wild-type levels of pyocyanin (Fig. 3A) and elastase (Fig. 3B) production. The defect in swimming motility and biofilm formation was only partially complemented by p*kinB*$_{H385A}$ in PA14Δ*kinB* (Fig. 4B and 5). Notably, p*kinB*$_{H385A}$ restored full virulence to PA14Δ*kinB* in the zebrafish embryo infection model (Fig. 7). Thus, it appears that KinB's kinase activity is not required for the regulation of quorum-sensing-regulated virulence factor production nor for *in vivo* virulence, similar to its regulation of alginate production, which likewise does not require its kinase activity (12). These results suggest that KinB is not, in fact, phosphorylating an alternate response regulator in order to modulate these virulence phenotypes but must regulate these processes by an alternate mechanism, possibly in a manner similar to RetS.

DISCUSSION

In this study we undertook a systematic screen to identify *P. aeruginosa* two-component sensors that are required for virulence in a vertebrate acute infection model. It has been suggested that two-component sensors are attractive drug targets, since few histidine kinases are found in eukaryotes (20). We took advantage of zebrafish embryos, a recently characterized host model that allows higher-throughput analysis than mammalian model hosts, and access to a comprehensive, arrayed, nonredundant transposon library of mutants in PA14 (30). Using this model, we identified several two-component sensors (GacS and RetS) that are known to be required for virulence in other vertebrate models of acute infection. Using the rigorous cutoff of a survival curve being statistically different from wild type by the log-rank test to define a mutant as attenuated, we also identified several sensors that were previously not known to be involved in pathogenesis. While we did not identify any mutants as hypervirulent, it is possible that other TCS sensor transposon mutants in this collection have more subtle effects on pathogenesis, resulting in either modest attenuated or hypervirulent phenotypes that could not be detected in our screen because they do not significantly alter the kinetics of embryo survival, as measured by the log-rank test, during infection.

Transposon insertions in *phoR*, *copS*, *bqsS*, and *kinB* resulted in reduced virulence in this model. We do not yet know why CopS is critical during zebrafish infection; however, we note that it has previously been shown that CopR expression is downregulated during mouse burn infections and that CopR participates in the regulation of type III secretion through control of *ptrA* (21, 22). It is possible that CopS might be sensing copper or other divalent cations within the host that may be important for pathogenicity. BqsS has been shown to play important roles in the regulation of biofilm formation *in vitro*, with PAO1Δ*bqsS* having been reported to enhance biofilm formation, reduce pyocyanin levels, and reduce levels of C_4-HSL (14). Interestingly, these phenotypes are more pronounced in PAO1Δ*bqsS* than in a deletion mutant of its corresponding response regulator, PAO1Δ*bqsR* (14), suggesting that regulation of these phenotypes by BqsS also may not fit into the canonical two-component sensor-response regulator model.

Finally, the two-component sensor KinB, which has not previously been implicated in pathogenesis, is required for *P. aeruginosa* virulence in zebrafish embryos. KinB is also required for full virulence in a slow-killing *C. elegans* assay (F. Ausubel, Massachusetts General Hospital, personal communication). It has previously been shown that the sensor KinB phosphorylates its cognate response regulator AlgB *in vitro* (31), though this phosphorylation event is not required for its regulation of alginate production (12). Here we find that PA14Δ*kinB* is defective in the production of the quorum-sensing-regulated virulence factors elastase and pyocyanin. However, reporter assays suggest that the levels of homoserine lactone autoinducers produced by PA14Δ*kinB* are not significantly lower than the levels of autoinducers produced by wild-type PA14. These phenotypes are reminiscent of PA14Δ*gacA*, which also produces lower levels of pyocyanin (44) and is similarly impaired in biofilm formation but produces homoserine lactones at levels that are not significantly lower than wild-type PA14 (38). Furthermore, both the PA14Δ*kinB* and the PA14*gacS* transposon mutant are attenuated for infection in zebrafish embryos and *C. elegans* (43). In contrast to PA14Δ*gacA*, however, PA14Δ*kinB* also has a flagellar motility defect, suggesting that it may modulate the production of biofilms through a different mechanism (38). Thus, KinB appears to play an important role within the hierarchy of virulence regulation in *P. aeruginosa* PA14 by controlling numerous phenotypes in a manner that is reminiscent of yet distinct from GacA.

PA14Δ*kinB* might be attenuated *in vivo* as a result of its decreased ability to survive and expand in the host, the direct impact of impaired production of virulence factors, or a combination of the two phenomena, as impaired virulence may indirectly impact *in vivo* survival. It is challenging to distinguish which of these three mechanisms truly underlies an attenuated phenotype in a given host model. We did observe that infecting embryos with an inoculum of PA14Δ*kinB* that is double the inoculum required to achieve complete lethality with wild-type PA14 resulted in complete lethality (data not shown). While this observation might be suggestive that PA14Δ*kinB*'s decreased ability to expand in zebrafish embryos may be contributing to its attenuated phenotype, we note that infecting embryos with mutants in known virulence factors (*lasR* and *pscD*) also results in complete lethality when inoculum sizes are sufficiently large (data not shown). These observations suggest

that at sufficiently high bacterial loads, mutants defective in virulence may nevertheless overwhelm a host organism's ability to immunologically control infection.

Since KinB was required for virulence in zebrafish embryos, we hypothesized that AlgB might also play a role in the regulation of virulence based on the canonical pairing of histidine kinase sensors and their cognate response regulators. However, we found that PA14Δ*algB* was not attenuated in the studied host model, nor was it impaired in pyocyanin production, elastase production, biofilm formation, or swimming motility. Furthermore, we found that PA14Δ*kinB* could be complemented in *trans* not only by episomal expression of a wild-type *kinB* allele but also by a mutant allele in which His-385 in the H-box (required for phosphorylation activity [31]) was mutated to alanine. This suggests that KinB does not require its kinase activity for the regulation of acute virulence-related phenotypes such as the production of quorum-sensing-regulated virulence factors. Such precedent exists in the dispensability of KinB's kinase activity for modulation of AlgB function (12) and RetS's kinase-independent modulation of the phosphorylation state of GacS through a direct and specific physical interaction (19). In light of this and our result that KinB also regulates virulence in a kinase activity-independent manner, by analogy to RetS, KinB could modulate the phosphorylation state of a second sensor kinase, through a direct, physical interaction. This hypothesis remains to be explored.

P. aeruginosa likely uses different two-component sensors to regulate the shift from an environmental reservoir to a host and in mediating the shift between acute and chronic infection lifestyles. The sensor kinases GacS, RetS, AlgZ, and LadS have been shown to play a role in coordinating the transition between acute and chronic infections (17–19, 57) and in regulating phenotypes such as motility and T3S, which appear to be important during acute but not chronic infection (19). However, AlgZ and LadS were not identified as attenuated in zebrafish embryos, based on our screen. Here we find that the two-component sensor KinB is also required for full virulence in acute infections. Interestingly, KinB has been identified as negatively regulating alginate (12), which is important in chronic infection, suggesting that KinB may play a role in suppressing *P. aeruginosa* functions that are important to chronic infection while promoting functions that are important to acute infection. Future efforts will be required to elucidate how KinB might work in concert with other TCSs such as GacS, RetS, AlgZ, and LadS to sense and transduce a stimulus into virulence factor modulation in the transition between acute and chronic infection.

Our results, in combination with those of others (17, 19), suggest that two-component signaling in *P. aeruginosa* is extremely complex and interwoven. Elucidation of these systems is appealing because TCSs are not found in humans, suggesting that they could serve as novel targets for antibacterial drug development (3, 32, 52). Indeed, several inhibitors of TCSs, such as walkmycin B, have already been discovered (20). However, in order to fully exploit *P. aeruginosa* TCSs as therapeutic targets, a highly detailed understanding of potential redundancies and cross talk among sensors and regulators as well as a precise molecular understanding of the mechanism of TCS signal transduction (i.e., whether it be a conventional, kinase-dependent or noncanonical, kinase-independent mechanism) is needed.

ACKNOWLEDGMENTS

We thank Erik Hett and Pratik Shah for technical assistance and Rhonda Feinbaum and Fred Ausubel (MGH) for reading the manuscript and useful discussions. We thank Jun Zhu (University of Pennsylvania) for the *A. tumefaciens* KYC55 strain, Steve Lory (Harvard Medical School) for the pHERD20T plasmid, and E. Peter Greenberg (University of Washington) for the *E. coli* bioassay strain.

This work was supported by the Pew Scholars Program in Biomedical Sciences (D.T.H.).

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