Redundant phenazine operons in *Pseudomonas aeruginosa* exhibit environment-dependent expression and differential roles in pathogenicity

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Evolutionary biologists have postulated that several fitness advantages may be conferred by the maintenance of duplicate genes, including environmental adaptation resulting from differential regulation. We examined the expression and physiological contributions of two redundant operons in the adaptable bacterium *Pseudomonas aeruginosa* PA14. These operons, *phzA1-G1* (*phz1*) and *phzA2-G2* (*phz2*), encode nearly identical sets of proteins that catalyze the synthesis of phenazine-1-carboxylic acid, the precursor for several phenazine derivatives. Phenazines perform diverse roles in *P. aeruginosa* physiology and act as virulence factors during opportunistic infections of plant and animal hosts. Although reports have indicated that *phz1* is regulated by the *Pseudomonas* quinolone signal, factors controlling *phz2* expression have not been identified, and the relative contributions of these redundant operons to phenazine biosynthesis have not been evaluated. We found that in liquid cultures, *phz1* was expressed at higher levels than *phz2*, although *phz2* showed a greater contribution to phenazine production. In colony biofilms, *phz2* was expressed at high levels, whereas *phz1* expression was not detectable, and *phz2* was responsible for virtually all phenazine production. Analysis of mutants defective in quinolone signal synthesis revealed a critical role for 4-hydroxy-2-heptylquinoline in *phz2* induction. Finally, deletion of *phz2*, but not of *phz1*, decreased lung colonization in a murine model of infection. These results suggest that differential regulation of the redundant *phz* operons allows *P. aeruginosa* to adapt to diverse environments.

Gene duplications give rise to genetic redundancy, an unstable condition that would not be expected to persist over time (1). This property is exhibited by diverse genomes (2–4), however, and evolutionary theorists have proposed several mechanisms whereby duplicate genes might provide selective advantages (5); for example, redundancy may be favored when spatial or temporal differences in expression enable tissue-specific variation or survival under varying environmental conditions (6, 7). Products of duplicated genes are involved in crucial cellular processes, including signal transduction, development, and metabolism (8).

The genome of the bacterium *Pseudomonas aeruginosa*, an opportunistic pathogen that thrives in both soil and host environments, contains two redundant seven-gene operons termed *phzA1-G1* (*phz1*) and *phzA2-G2* (*phz2*). These operons are nearly identical (~98% similarity at the DNA level), and each encodes the biosynthetic enzymes for phenazine-1-carboxylic acid (PCA) (9). Downstream modifications derivatize this precursor, generating other phenazines (*SI Appendix*, Fig. S1*A*). Pseudomonad phenazines are toxic to many other organisms and cell types due to their inherent redox activity (10, 11). Studies conducted in various plant and animal models of infection have implicated phenazines in colonization and pathogenicity (12, 13). In addition, recent studies have elucidated beneficial roles for phenazines in the physiology of *P. aeruginosa*, which is not negatively affected by the redox toxicity of phenazines (14). These roles are thought to contribute to the dramatic effects of phenazines on biofilm development, where their production induces a switch between wrinkled (rugose) and smooth morphotypes (15).

Advantages conferred by phenazines may support conservation of the *phz* operon in the more than 57 currently identified phenazine-producing species (16). However, although *phz* operons display a broad phylogenetic distribution, genomes containing more than one *phz* operon are rare; of the bacterial genomes sequenced to date, only those belonging to *P. aeruginosa* and *Streptomyces cinnamonensis* contain a second, redundant *phz* operon (16). In *P. aeruginosa*, the regions surrounding *phz1* and *phz2* are highly divergent. *phz1* is flanked by *phzM* and *phzS*, which encode enzymes that convert PCA to pyocyanin (PYO) (*SI Appendix*, Fig. S1*B*). *phz2* lies ~2.6 MB away from *phz1* and is not flanked by phenazine-modifying enzymes. A third phenazine-modifying enzyme, PhzH, is encoded at a distinct site and converts PCA to phenazine-1-carboxamide. Additional phenazines that have been detected in *P. aeruginosa* cultures are intermediates and by-products arising from PhzM and PhzS activity or are produced by enzymes with unknown coding genes (17, 18).

The duplicate *P. aeruginosa phz* operons are preceded by distinct promoter regions (9). The *phz1* promoter contains a *las* box 390 bp upstream of the *phzA1* translational start site (19). Located upstream of many genes regulated by quorum sensing, *las* box motifs recruit the transcriptional regulators LasR and/or RhlR (20, 21). The *las* box, LasR, and RhlR are required for complete induction of *phzA1* (20). The *phz1* operon has been shown to be regulated by *P. aeruginosa* quinolones, and binding of the *Pseudomonas* quinolone signal (PQS) to the transcriptional regulator PqsR (also known as MvfR) is required for WT PYO production (22, 23). Furthermore, the PQS dependence of *phz* gene expression has been reported in several RNA array studies and is often attributed to the quinolone signal control of *phz1*; however, it is unlikely that microarray probes can distinguish between highly similar *phz1* and *phz2*. Thus, the quinolone dependence of *phz2* expression has remained an open question. Finally, the GacA-dependent orphan repressor QscR, encoded upstream of *phz2*, has a negative effect on the expression of both *phz* operons through an unknown mechanism (24, 25).

In this study, we generated mutants lacking genes involved in phenazine and quinolone biosynthesis and evaluated phenazine production, colony morphogenesis, and pathogenicity. We used promoter–GFP fusions to examine the relative expression levels of *phz1* and *phz2* during growth in liquid batch cultures and colony development. We tested the contributions of *phz1* and

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phz2 to *P. aeruginosa* pathogenicity using a murine model of lung infection. Our results demonstrate a major role for *phz2* during planktonic growth, and an almost exclusive role for *phz2* in *P. aeruginosa* phenazine production during biofilm development and host infection.

Results

Both *phz1* and *phz2* Contribute to Phenazine Production During Planktonic Growth. *P. aeruginosa* liquid batch cultures begin to produce phenazines in early stationary phase. To investigate the relative contributions of the two *phz* operons to phenazine production in planktonic cultures, we deleted each individual operon in *P. aeruginosa* PA14. We quantified phenazines in culture supernatants from the *phz1* deletion mutant (Δ*phz1*), the *phz2* deletion mutant (Δ*phz2*), and a mutant in which both phenazine operons had been deleted (Δ*phz*). Both Δ*phz1* and Δ*phz2* produced significantly less PCA compared with WT (Fig. 1*A*, *Left*). Δ*phz1* produced more PCA than Δ*phz2*, but the combined total PCA produced by the two mutants did not reach the WT level. Δ*phz1* produced slightly less PYO than WT, whereas Δ*phz2* produced approximately one-third of WT (Fig. 1*A*, *Right*). The levels of other phenazines were below the detection limit of this study.

PCA modification might have prevented us from accurately quantifying the total PCA produced by each operon. Thus, we created a triple-deletion mutant (Δ*phzHMS*) that lacks *phzH*, *phzM*, and *phzS* and in this background deleted each *phz* operon. Δ*phzHMS* produced more PCA than WT (Fig. 1*B*); this represents the total PCA production from both operons. This amount was higher than the combined total PCA produced by Δ*phz1* and Δ*phz2*, confirming that the conversion of PCA to other phenazines prevented accurate quantification of total PCA. Deleting *phz1* in this background decreased PCA production slightly, whereas deleting *phz2* drastically reduced PCA production, indicating that *phz2* is responsible for the majority of the PCA produced by planktonic cultures.

To address the possibility that the *phz* operons are expressed at different levels, we created fluorescent reporter constructs containing the 500-bp promoter regions upstream of each operon fused to *gfp*. These reporters, P*phzA1*GFP and P*phzA2*GFP, were integrated into the chromosome at a neutral site in WT and Δ*phz*. We found higher expression of P*phzA1*GFP than of P*phzA2*GFP in the WT and Δ*phz* backgrounds (Fig. 1*C*).

***phz2* Is Sufficient for WT Phenazine Production in Colony Biofilms.** Phenazine production has been best characterized using planktonic cultures, but phenazines also have been shown to affect the morphology of different types of biofilms (15, 26). We compared the development of Δ*phz*, Δ*phz1*, and Δ*phz2* with that of WT in a colony morphology assay. The Δ*phz* mutant exhibited a wrinkled morphology, as described previously (27). Strikingly, the presence of *phz2* alone was sufficient for maintenance of the WT phenotype, whereas deletion of *phz2* led to a hyper wrinkled morphology much like that of Δ*phz* (Fig. 2*A*). Biofilms formed by Δ*phz* and Δ*phz2* exhibited an almost twofold increase in agar surface coverage compared with those formed by the WT (Fig. 2*B*), whereas loss of *phz1* had no effect on colony surface coverage. These results suggest that *phz2*, but not *phz1*, is important for phenazine production in biofilms.

To confirm that the mutant biofilm phenotypes were consistent with their phenazine production profiles, we extracted and quantified phenazines from the agar on which the biofilms were grown. Δ*phz1* produced ~60% of the PCA produced by WT, but generated ~60% more PYO (Fig. 3*A*). The combined total of PYO and PCA produced by the Δ*phz1* was comparable to that produced by the WT biofilm (*SI Appendix*, Fig. S2). HPLC analysis detected no PCA or PYO from Δ*phz2* (*SI Appendix*, Fig. S3). Deletion of *phzH*, *phzM*, and *phzS* in the Δ*phz1* and Δ*phz2* backgrounds confirmed that all of the detectable phenazines

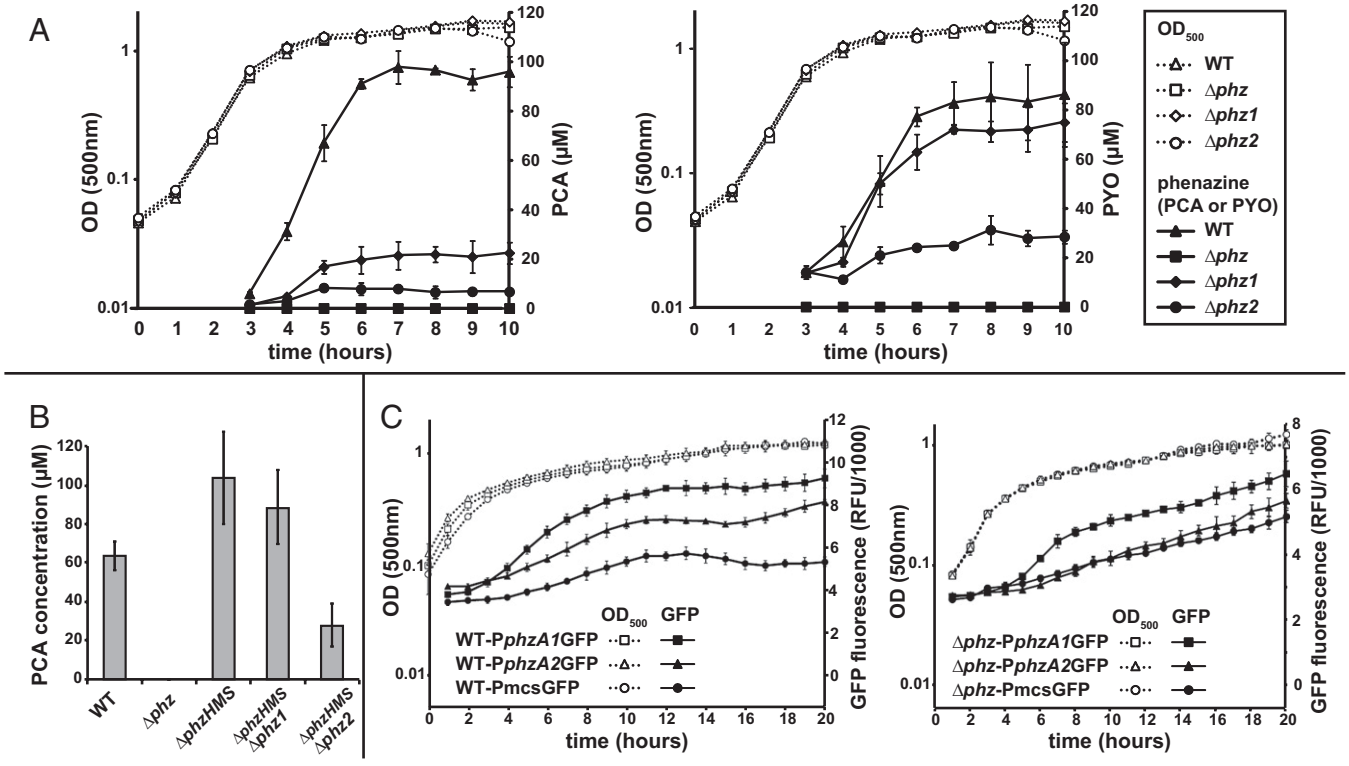

Fig. 1. *P. aeruginosa phz1* and *phz2* contribute to phenazine production in planktonic culture. (*A*) Growth of WT, Δ*phz*, Δ*phz1*, and Δ*phz2* with PCA (*Left*) and PYO (*Right*) quantification. (*B*) PCA in Δ*phzHMS*, Δ*phzHMS*Δ*phz1*, and Δ*phzHMS*Δ*phz2* culture supernatants measured after 16 h of growth. (*C*) Expression levels of GFP reporter constructs for *phz1* and *phz2* operons in WT (*Left*) and Δ*phz* (*Right*) backgrounds. Error bars indicate SD of biological triplicates.

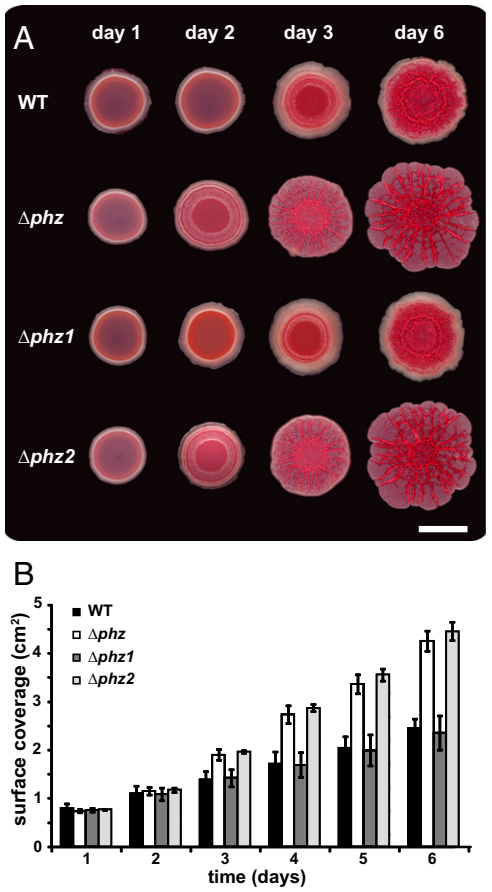

Fig. 2. *phz2* is sufficient for maintaining WT colony morphology. Colony development (*A*) and surface area quantification (*B*) over the course of 6 d. (Scale bar: 1 cm.) Error bars indicate SD of three independent experiments.

produced by the WT colony could be produced by *phz2* alone (Fig. 3*B*). Complementation with *phz2* restored phenazine production and the WT phenotype (*SI Appendix*, Fig. S4).

Recently, Huang et al. (28) reported temperature-dependent regulation of phenazine production in *P. aeruginosa* M18 and PAO1. We grew *P. aeruginosa* PA14 colonies at 25 °C and 37 °C to test whether phenazine production would be affected by temperature. Similar to what Huang et al. described for liquid cultures, we found increased PYO production at 37 °C for PA14 WT and Δ*phz1* colonies. The higher growth temperature did not significantly alter the phenazine production phenotypes that we observed, however; at both 25 °C and 37 °C, Δ*phz2* colonies produced no detectable amounts of phenazines (*SI Appendix*, Fig. S5).

We next evaluated *phz* operon expression in biofilms. We quantified fluorescence across the midsection of colonies grown from strains containing P*phzA1*GFP and P*phzA2*GFP. Fluorescence levels for P*phzA2*GFP were significantly higher than the background in a colony containing the GFP gene cloned without a promoter (PmcsGFP) (Fig. 4). Fluorescence for the P*phzA1*GFP colony was indistinguishable from background. Quantification of colony midsection fluorescence gave rise to a "Batman"-shaped plot, likely related to an increased cell concentration at the colony perimeter resulting from the "coffee ring" effect (29) when a cell suspension is first spotted onto an agar surface.

Quinolone-Dependent Regulation of the *phz2* Operon. Although signaling mechanisms controlling the expression of *phz1* have been identified (30), little is known about the regulation of *phz2*. We sought to test whether quinolones are required for *phz2* induction in biofilms. *P. aeruginosa* produces three major types of alkyl quinolones: PQS, 4-hydroxy-2-heptylquinoline (HHQ), and 4-hydroxy-2-heptylquinoline-*N*-oxide (HQNO) (31, 32). We generated a mutant lacking the genes *pqsABC* (Δ*pqsAC*), which is unable to produce quinolones (33). We also created individual deletions of the genes encoding PqsL and PqsH, which catalyze the formation of HQNO and PQS, respectively (32, 34). Δ*pqsAC* showed the most significant defect in PCA production, whereas Δ*pqsL* was unaffected (Fig. 5*A*). Δ*pqsHL* had PCA production similar to that of Δ*pqsH*, suggesting that HHQ, not HQNO, is responsible for the majority of PCA production. Of note, mutants lacking PqsR (MvfR) or the PQS response protein PqsE did not produce PCA, consistent with previous work indicating that these proteins are required for phenazine synthesis (35).

To investigate whether quinolones affect PCA production by modulating *phz2* expression, we deleted the *pqsABC* genes in the Δ*phzHMSΔphz1* and Δ*phzHMSΔphz2* backgrounds and assayed for phenazine production from biofilms. Δ*pqsACΔphzHMSΔphz1* showed a 90% reduction in PCA production compared with Δ*phzHMSΔphz1* (Fig. 5*B*), demonstrating quinolone-dependent regulation of *phz2*. Removing quinolones also abolished PCA production from *phz1* (i.e., the Δ*pqsACΔphzHMSΔphz2* mutant). Finally, to evaluate which quinolones are responsible for *phz2* induction, we generated the mutants Δ*pqsHΔphzHMSΔphz1* and Δ*pqsHΔpqsLΔphzHMSΔphz1*. PCA production in these mutants was half that in the Δ*phzHMSΔphz1* mutant, suggesting that both PQS and its precursor HHQ positively regulate phenazine production from *phz2* (Fig. 5*C*).

To further verify that quinolones affect *phz2* expression, we introduced P*phzA2*GFP into the Δ*pqsAC,* Δ*pqsR*, and Δ*pqsHL* mutants and compared fluorescence among the strains. *phz2* expression was significantly reduced in the Δ*pqsAC*-P*phzA2*GFP

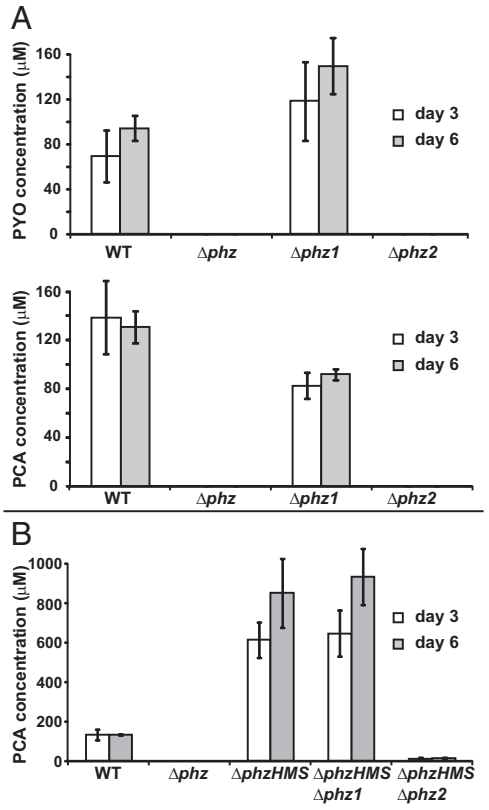

Fig. 3. The total PCA pool in biofilms is produced by *phz2*. (*A*) Quantification of PYO (*Upper*) and PCA (*Lower*) produced by WT, Δ*phz*, Δ*phz1*, and Δ*phz2* colonies grown on agar plates. (*B*) Quantification of PCA produced by Δ*phzHMS*, Δ*phzHMSΔphz1*, and Δ*phzHMSΔphz2* strains grown on agar plates. Error bars indicate SD of three independent experiments.

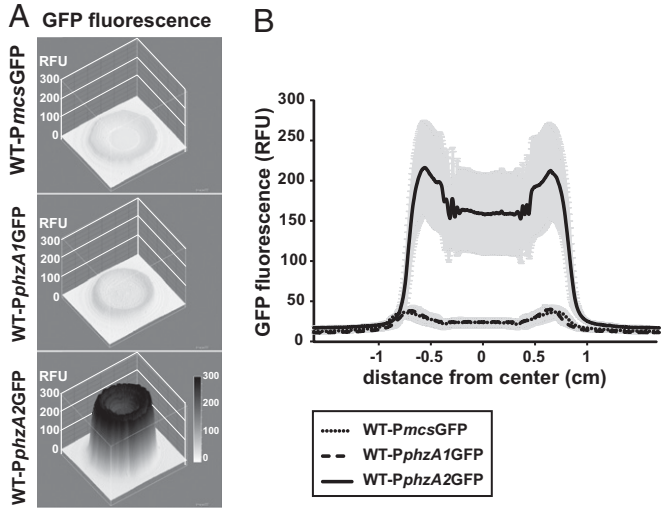

Fig. 4. *phz2* is expressed at higher levels than *phz1* in colony biofilms. A 3D surface fluorescence intensity plot (*A*) and quantification of fluorescence (*B*) of GFP reporter constructs for *phz1* and *phz2* operons. Fluorescence quantification was performed using the surface plot analysis across the middle of the colony with ImageJ. Shading indicates the SD of biological triplicates.

and Δ*pqsR*-P*phzA2*GFP strains in biofilms (Fig. 5*D*), demonstrating that PqsR-mediated quinolone signaling regulates *phz2* expression; however, *phz2* expression in Δ*pqsHL*-P*phzA2*GFP was similar to that in WT (Fig. 5*E*). These observations were recapitulated in planktonic cultures (*SI Appendix*, Fig. S6). These results indicate that quinolones can positively regulate transcription of *phz2*, and that HHQ in particular plays a more significant role in induction of *phz2* than in induction of *phz1*.

PQS production requires molecular oxygen as a substrate for the monooxygenase PqsH, suggesting that expression of *phz1*, but not of *phz2*, is oxygen-dependent. We tested this by growing WT, Δ*phz*, Δ*phz1*, and Δ*phz2* colonies in an anaerobic chamber on a medium containing 40 mM nitrate (an alternate electron acceptor for *P. aeruginosa* respiration). Indeed, extracts from agar on which WT or Δ*phz1* colonies were grown contained comparable amounts of PCA, whereas extracts from media supporting Δ*phz* or Δ*phz2* colonies contained no phenazines (*SI Appendix*, Fig. S7).

***phz2* Operon Is Required for Lung Colonization in a Murine Model of Infection.** Phenazine production contributes to virulence in diverse infection models (13, 36, 37). Characterizations of the bacterial populations associated with infections have suggested that *P. aeruginosa* assumes a biofilm-like lifestyle during host colonization (38). Based on our observation that the *phz2* operon is required for phenazine production in biofilms, we tested whether *phz2* is required for respiratory infection in a murine model (37). Clearance of Δ*phz2* and Δ*phz* bacteria was significantly greater than that of WT bacteria (Fig. 6). Clearance was similar for Δ*phz1* and WT. These results suggest that the *phz2* operon is required for *P. aeruginosa* virulence.

Discussion

Differential expression of highly similar genes may confer selective advantages and increase complexity (5). We evaluated the expression and physiological roles of two nearly identical operons, *phz1* and *phz2*, encoded by the *P. aeruginosa* genome. We hypothesized that the nonhomologous promoter regions of these redundant operons allow for condition-dependent regulation of PCA biosynthesis in diverse environments. To test this, we generated mutants with deletions in each of these operons and created fluorescent reporters for monitoring operon expression. We also deleted genes required for downstream conversion of PCA to PYO and phenazine-1-carboxamide, enabling accurate assessment of PCA production from each operon.

We found that when *P. aeruginosa* was grown in liquid cultures, *phz1* was expressed at higher levels than *phz2* (Fig. 1*C*, *Left*). However, both operons contributed significantly to PCA production, with *phz2* making a greater contribution (Fig. 1*B*), suggesting that factors other than transcriptional regulation control the amount of PCA produced planktonically. In contrast, *phz2* was the sole operon expressed and responsible for all of the phenazine production in colonies (Figs. 3 and 4). *phz2* was also required for WT colony morphogenesis.

We then evaluated the quinolone dependence of *phz* operon expression and phenazine production, and found that a mutation

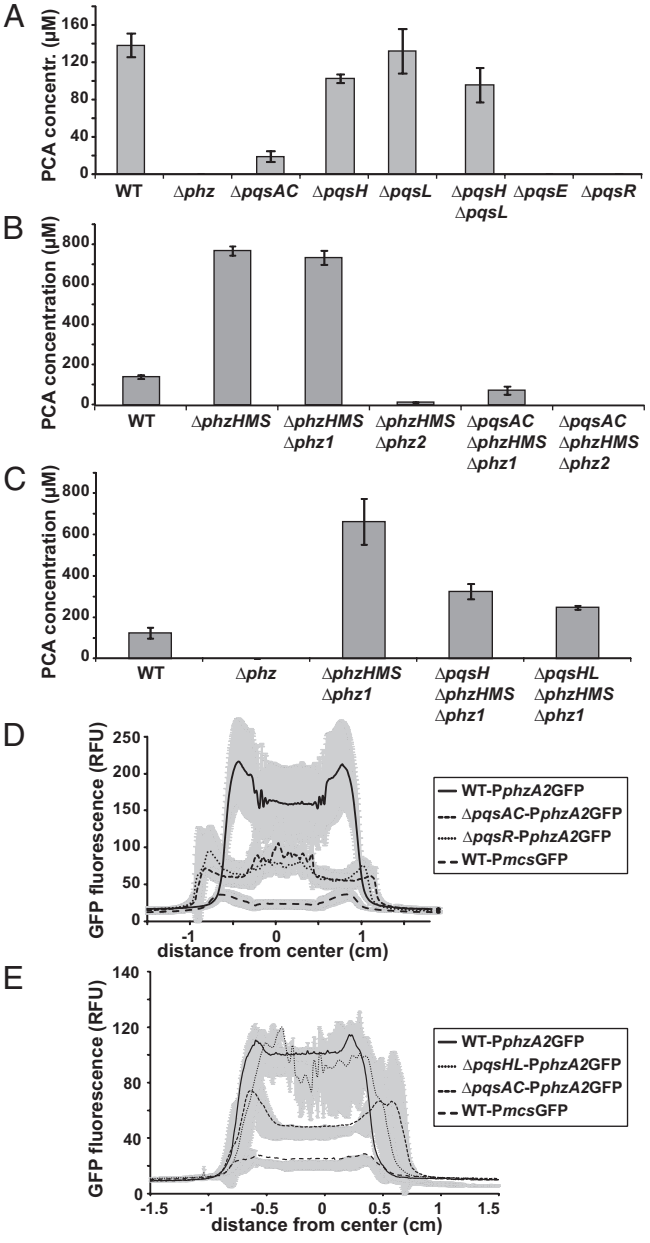

Fig. 5. Quinolones regulate the *phz2* operon in biofilms. (*A*–*C*) Quantification of PCA from strains containing deletions in various biosynthetic genes involved in H-alkyl-quinolone (HAQ) and phenazine production. (*D* and *E*) Expression levels of the *phz2* operon in strains containing deletions in HAQ and phenazine biosynthetic genes in representative experiments. Error bars in *A*–*C* and shading in *D* and *E* indicate SD of biological triplicates.

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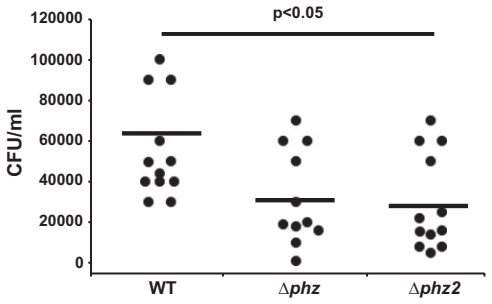

Fig. 6. The *phz2* operon is necessary for full virulence in a murine lung infection model. CFU counts of WT, Δ*phz*, and Δ*phz2* strains from mouse lungs. Mice were inoculated with 0.3–1 × 10^5 CFU of *P. aeruginosa* WT, Δ*phz1*, or Δ*phz2* and euthanized at 18 h after infection. CFU counts were performed by dilution and plating of whole lung homogenates. Straight lines within the data points indicate average CFU/mL. *P* values between data are shown.

that abolished all quinolone production led to a much greater reduction in PCA compared with a mutation that abolished production of PQS alone. Previous studies have demonstrated that *phz1* induction is highly dependent on PQS (22, 23). Our observations indicate that quinolone-dependent regulation of *phz2* differs from that of *phz1*, in that HHQ is the major signal controlling *phz2* expression. However, like *phz1* induction, *phz2* induction is PqsR- and PqsE-dependent (Fig. 5). Intriguingly, neither *phz* operon promoter contains an identifiable PqsR-binding motif, and additional regulators may be required for their induction (39). We also detected some quinolone-independent expression of *phz2*, given that removing quinolone biosynthesis genes did not abolish PCA production completely (Fig. 5*B*).

The differences in *phz1* and *phz2* expression with respect to quinolone dependence and growth mode (i.e., planktonic vs. biofilm) are consistent with a role for oxygen as a major environmental cue for *P. aeruginosa* lifestyle transitions. *P. aeruginosa* produces HHQ, but not PQS, under anaerobic conditions (40). Cells in the crowded interior of biofilms and aggregates formed during infection experience anoxia. The findings that HHQ is more important for *phz2* expression, and that *phz2* expression is the source of PCA in biofilms, suggest that *phz2* is regulated in response to oxygen indirectly via inhibition of PqsH. Accordingly, the dependence of *phz1* expression on PQS is consistent with the higher expression levels in aerobically grown liquid batch cultures. Another monooxygenase gene, *phzS*, is located adjacent to *phz1* in the genome, and both loci may be controlled by the same promoter; thus, oxygen availability may signal transitions between planktonic and biofilm lifestyles, allowing *P. aeruginosa* to fine-tune phenazine production in response (Fig. 7). Additional studies are needed to examine the microscale distribution of oxygen and its correlation with *phz* operon expression in biofilms.

We also observed that *phz2* is important for host colonization in a murine model of infection (Fig. 6). Although the contributions of phenazines to *P. aeruginosa* pathogenicity via host damage are well recognized (41, 42), we and others have recently reported beneficial roles for phenazines in *P. aeruginosa* physiology, including intercellular signaling, redox balancing, and enhanced iron acquisition (27, 43, 44). These diverse effects likely act together to support host colonization and infection. Furthermore, phenazines have dramatic effects on the morphological development of *P. aeruginosa* colonies. Whether biofilms form in our particular infection model is not known, but airway obstruction by bacterial aggregates has been reported in a similar model (37). The fact that *phz2* is the relevant operon for pathogenicity in an acute infection model suggests that *P. aeruginosa* experiences oxygen limitation and initiates aggregate/biofilm formation even in a short time period.

The maintenance of redundant genes is paradoxical, because the presence of a duplicate gene relaxes selection pressure, allowing rapid divergence (1). Redundant genes can persist when they retain overlapping activities, yet diverge sufficiently to give rise to individualized functions (2). Alternatively, changes in the regulation of duplicated genes, in the absence of major changes at the coding sequence level, also can give rise to unique "functions," in that the localization or timing of their expression can alter their physiological roles or ultimate effects (5, 8, 45, 46). Surveys examining genetic redundancy in yeast and bacterial genomes have identified duplicate genes that exhibit such subfunctionalization (3, 47, 48), but most of these gene pairs show relatively low sequence similarity, and their functional redundancy is usually inferred. The *P. aeruginosa phz1* and *phz2* operons are exceptional in their high degree of sequence similarity and redundancy. Here we have presented evidence that their specialization derives from their differential expression. Complementary regulation of the two *phz* operons appears to confer an advantage over regulation of a single operon, such that these nearly identical gene clusters are maintained (Fig. 7).

Materials and Methods

Bacterial Strains and Growth Conditions. For cloning and strain construction, bacterial cultures were routinely grown at 37 °C in LB broth. For all planktonic phenazine production and gene expression experiments, strains (listed in *SI Appendix*, Table S1) were grown in 1% tryptone at 37 °C. Colony biofilms were grown on 1% tryptone/1% agar plates at room temperature (22–25 °C) and >95% humidity. Coomassie blue (20 μg/mL) and Congo red (40 μg/mL) were added to the plates used for morphology assays (60 mL medium; 10-cm square plates). Biofilm phenazine quantification assays were performed with 40 mL of 1% tryptone/1% agar medium without dye in 10-cm round Petri dishs. The primers used in this study are listed in *SI Appendix*, Table S2, and protocols for the generation of mutants and fluorescent reporter constructs are described in *SI Appendix*.

Quantification of Phenazines from Biofilms and Liquid Cultures. For biofilm phenazine quantification, precultures were grown overnight in LB. Then 10 μL from these stationary-phase cultures was spotted on 1% tryptone/1% agar plates (described above) in technical replicates (10 per preculture). On day 3 or 6, five of these colonies were scraped from the plate, and half of the solid growth substrate was transferred to a 50-mL conical tube with 3 mL of water and nutated overnight to extract phenazines. Filtered extract was then loaded directly onto a Waters Symmetry C18 reverse-phase column (4.6 × 250 mm; 5 mm particle size) in a Beckman SystemGold high-performance liquid chromatograph with a photodiode array detector. Phenazines were separated following a protocol and under conditions described previously (27). Standards at known concentrations were used to calculate conversion factors for PYO and PCA (8 × 10^{-6} mM/AU and 9.5 × 10^{-6} mM/AU, respectively).

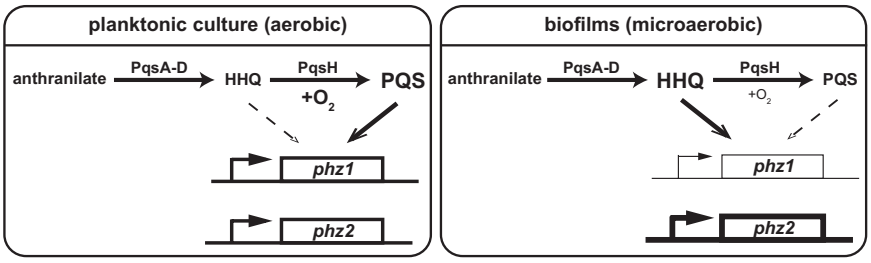

Fig. 7. Model for environment-dependent expression of *phz1* and *phz2*. (*Left*) In shaken planktonic cultures, the presence of oxygen leads to the production of PQS. PQS positively regulates the expression of *phz1* and *phz2*. (*Right*) In biofilms, oxygen-dependent production of PQS is decreased owing to the presence of micro aerobic and anaerobic environments. HHQ positively regulates the expression of *phz2* (as also suggested by ref. 49), and *phz2* is expressed at higher levels than *phz1*.

For liquid cultures grown in 1% tryptone, 200-μL samples were taken after ~16 h of growth. These were then filtered and analyzed by HPLC as described above.

GFP Fluorescence Quantification. For planktonic cultures, strains were grown in LB overnight, then diluted in 1% tryptone to an OD_{500} of 0.05 into 96-well plates (Costar; Corning). The plates were incubated at 37 °C with continuous shaking at the "low" speed in a BioTek Synergy 4 plate reader equipped with Gen5 data analysis software. The excitation wavelength was 480 nm, and emission was measured at 510 nm. For biofilms, strains were grown on 90 mL of solid media (1% tryptone, 1% agar in 10-cm square plates). Images were acquired using a Typhoon Trio variable mode scanner (GE Healthcare) after 3 d of growth. The excitation wavelength was 488 nm, and emission was measured at 520 nm. Fluorescence data were processed by surface plot analysis in ImageJ.

In Vivo Infection Model. *P. aeruginosa* strains were grown in LB liquid precultures at 37 °C. Lung infections of *P. aeruginosa* were performed using 8-wk-old C57BL/6J mice. Mice were anesthetized with 100 mg/kg of ketamine and 5 mg/kg of xylazine and then inoculated with 0.3–1 × 10^5 CFU of organism before being euthanized at 18 h after infection. Bacterial CFU were assayed by homogenizing the whole lung and plating dilutions of the resuspended tissue on LB agar. All mouse infections were performed in accordance with the guidelines of Columbia University's Institutional Animal Care and Use Committee.

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