Supporting Information

Text S1. Materials and methods

Construction of mutants

We generated unmarked deletions of the phenazine modifying genes *phzH*, *phzM*, *phzS*, the two redundant phenazine biosynthetic operons *phzA1-G1* (*phz1*) and *phzA2-G2* (*phz2*), and the quinolone biosynthetic genes *pqsABC*, *pqsH* and *pqsL* in various combinations in a panel of *P. aeruginosa* PA14 strain backgrounds (supporting table 1). The deletion plasmids for *phzA1-G1* and *phzA2-G2* have been described previously (1). All other deletion plasmids were generated using the yeast gap repair method (2). Here we describe the protocol for generating the unmarked deletion of *phzA2-G2*. All other deletion strains were generated according to a similar protocol. ~1 kb of the sequences flanking the gene(s) to be deleted were amplified using the primer pairs US-1 and US-2 (for the 5' flanking region) and DS-1 and DS-2 (3′ flanking region) (supporting table 2). These flanking DNA fragments were joined and integrated into the linearized plasmid pMQ30 by gap repair cloning using the yeast strain InvSc1 (2). The resulting deletion plasmid was transformed into *E. coli* BW29427 and mobilized into PA14 using biparental conjugation. PA14 single recombinants (merodiploid containing the intact allele(s) and the null/deleted allele(s)) were selected on LB agar containing 100 µg/ml gentamicin. Potential deletion mutants were generated by selecting for loss of the plasmid (formation of the double recombinant) by identifying strains that grew in the presence of 10% sucrose (these strains lost the *sacB*-containing plasmid because *sacB* is toxic in the presence of sucrose). Strains with properties of a double recombination were verified by PCR.

Construction of the GFP-reporter plasmids

Primers for the construction of these plasmids are listed in Table S2A. A SpeI site was engineered into the miniTn7(Gm)$P_{A1/04/03}$ *eyfp*-a plasmid in between the KpnI and SphI sites upstream of the *yfp* gene. Next, the multiple cloning site from pUC18-mini Tn7T-Gm-lacZ (3) was amplified with primers 1 and 2. The PCR product was digested with SpeI and SphI and ligated with SpeI/SphI-digested miniTn7(Gm)$P_{A1/04/03}$ *eyfp*-a to give pAKN69-MCS. The multiple cloning site from pAKN69-MCS was then amplified with primers 3 and 4. The PCR product was digested with SalI and MfeI and ligated with XhoI and EcoRI digested pYL122 (4). This step replaced the *rhlA* promoter in pYL122 with a multiple cloning site to give pSEK-GFP. The *phzA1* promoter was amplified from *P. aeruginosa* PA14 genomic DNA by PCR with primers 5 and 6. The *phzA2* promoter was amplified from *P. aeruginosa* PA14 genomic DNA by PCR with primers 7 and 8. These PCR products were digested with SpeI and XhoI and ligated with SpeI and XhoI digested pSEK-GFP to give pSEK-P*phzA1*GFP and pSEK-P*phzA2*GFP.

Genomic integration of the reporter fusions into *P. aeruginosa* PA14

P*phzA1*-GFP and P*phzA2*-GFP fusions were inserted as single-copies into the chromosomal *attB* site in the *P. aeruginosa* PA14 using a modified version of a previously described protocol (4). Briefly, pSEK-P*phzA1*GFP and pSEK-P*phzA2*GFP were transformed into chemically competent *E. coli* BW29427 cells for conjugation with *P. aeruginosa*. Merodiploids were selected with 200 µg/mL tetracycline, and Flp-catalyzed excision of the integrase and Tet^R cassette was carried out as previously described (5, 6).

Supporting Table S1.

Strains and plasmids used in this work

Strain	**Comments/Genotype**	**Source or Reference**
Pseudomonas aeruginosa		
PA14	Clinical Isolate, UCBPP-14	(7)
Δ*phz*	PA14 with deletions in the *phzA1-G1* and the *phzA2-G2* operons	(1)
Δ*phz1*	PA14 with deletion of the *phzA1-G1* operon	this study
Δ*phz2*	PA14 with deletions of the *phzA2-G2* operon	this study
ΔphzHMS	PA14 with deletions of the *phzM, phzH and phzS* genes	this study
ΔphzHMSΔphz1	PA14 with deletions of *phzM, phzH ,phzS genes* and *phzA1-G1* operon	this study
ΔphzHMSΔphz2	PA14 with deletions of *phzM, phzH ,phzS genes* and *phzA2-G2* operon	this study
Δ*pqsAC*	PA14 with deletions of the *pqsA-C genes*	this study
Δ*pqsR*	PA14 with deletion of the *pqsR gene*	Deborah Hogan, Hanover, NH
Δ*pqsH*	PA14 with deletion of the *pqsH gene*	Deborah Hogan Hanover, NH
Δ*pqsE*	PA14 with deletion of the *pqsE* gene	this study
Δ*pqsL*	PA14 with deletion of the *pqsL* gene	this study
Δ*pqsHL*	PA14 with deletions of the *pqsH* and *pqsL* genes	this study
*ΔpqsACΔphzHMS*Δ*phz1*	PA14 with deletions of the *pqsA-C genes* in the Δ*HMS1* background	this study
*ΔpqsACΔphzHMS*Δ*phz2*	PA14 with deletions of the *pqsA-C* genes in the Δ*HMS2* background	this study
*ΔpqsHΔphzHMS*Δ*phz1*	PA14 with deletions of the *pqsH gene* in the Δ*HMS1* background	this study
*ΔpqsLΔphzHMS*Δ*phz1*	PA14 with deletions of the *pqsL gene* in the Δ*HMS1* background	this study
*ΔpqsHLΔphzHMS*Δ*phz1*	PA14 with deletions of the *pqsH* and *pqsL* genes in the Δ*HMS1* background	this study
WT PmcsGFP	PA14 with GFP insert with no promoter in the multiple cloning site	this study
WT P*phzA1*GFP	PA14 with P*phzA1*GFP insert	this study
WT P*phzA2*GFP	PA14 with P*phzA2*GFP insert	this study
Δ*pqsAC* P*phzA2*GFP	Δ*pqsAC* with P*phzA2*GFP insert	this study
Δ*pqsR* P*phzA2*GFP	Δ*pqsR* with P*phzA2*GFP insert	this study
Δ*pqsHL* P*phzA2*GFP	Δ*pqsHL* with P*phzA2*GFP insert	this study

Δ*phz2* pUCP18	Δ*phz2* with pUCP18 plasmid inserted	this study
Δ*phz2* pUCP18-*phz2*	Δ*phz2* with pUCP18 plasmid with *phz2* insert	this study
Escherichia coli		
UQ950	*E. coli* DH5α λ(pir) host for cloning; F-Δ(*argF-lac)169 Φ80 dlacZ58*(ΔM15) *glnV44*(AS) *rfbD1 gyrA96*(Nal^R) *recA1 endA1 spoT1 thi-1 hsdR17 deoR λpir*+	D. Lies, Caltech
BW29427	Donor strain for conjugation: *thrB1*004 *pro thi rpsL hsdS lacZ* ΔM15RP4–1360 Δ(*araBAD*)*567* Δ*dapA*1341::[*erm pir*(wt)]	W. Metcalf, University of Illinois
Saccharomyces cerevisiae		
InvSc1	Used for yeast gap repair cloning	(2)

Plasmids	**Description**	**Source**
pUCP18	Multi-copy plasmid with ColEI ORI; Amp^R; *lacZ* α gene	(8)
pUCP18-*phz2*	pUCP18 plasmid with *phz2* operon inserted	this study
pUC18-mini Tn7T-Gm-lacZ	Broad host plasmid with mini Tn7T-Gm^R-lacZ	(3)
miniTn7(Gm)$P_{A1/04/03}$ *eyfp*-a	Integration vector containing *eyfp*, Gm^R	(9)
pAKN69-MCS	Integration vector containing multiple cloning site (MCS)	this study
pYL122	*rhlAB*::gfp transcriptional reporter on mini-CTX, Tet^R	(4)
pSEK-GFP	GFP reporter plasmid containing MCS	this study
pSEK-P*phzA1*GFP	GFP reporter plasmid with *phzA1* inserted	this study
pSEK-P*phzA2*GFP	GFP reporter plasmid with *phzA2* inserted	this study
pMQ30	Yeast-based allelic exchange vector, *sacB*,[a] CEN/ARSH, URA3$^+$, Gent^R	(2)
pLD338	*phzM* deletion fragments cloned into pMQ30	this study
pLD294	*phzS* deletion fragments cloned into pMQ30	this study
pLD741	*phzH* deletion fragments cloned into pMQ30	this study
pLD11	*phz1* deletion fragments cloned into pSMV10	(1)
pLD18	*phz2* deletion fragments cloned into pSMV10	(1)
pLD710	*pqsABC* deletion fragments cloned into pMQ30	this study
pLD1140	*pqsH* deletion fragments cloned into pMQ30	this study
pLD706	*pqsL* deletion fragments cloned into pMQ30	this study

Supporting Table S2.

A. Primers for fluorescent reporter constructs

Construction of pAKN69-MCS	
1. speI-MCS-FOR	TGCCCGAGGCATAGACTGTA
2. sphI-MCS-REV	ggatg*gcatgc*CTGTTTCCTGTGTGATAAAGAAAG
Construction of pSEK-GFP	
3. salI-MCS-FOR	tgag*gtcgac*TACCGCCACCTAACAATTCG
4. mfeI-MCS-REV	tcga*caattg*TACCGGGCCCAAGCTTCT
Construction of pSEK-PphzA1GFP	
5. speI-PphzA1-FOR	cgcc*actagt*TTCCTGCGTACCGAAAGAAT
6. xhoI-PphzA1-REV	cgag*ctcgag*CGAGAGGGCTCTCCAGGTAT
Construction of pSEK-PphzA2GFP	
7. speI-PphzA2-FOR	cgcc*actagt*GCCTGCTCAACTGAATCGAC
8. xhoI-PphzA2-REV	cgag*ctcgag*AGTTCGAATCGACTGGCATC

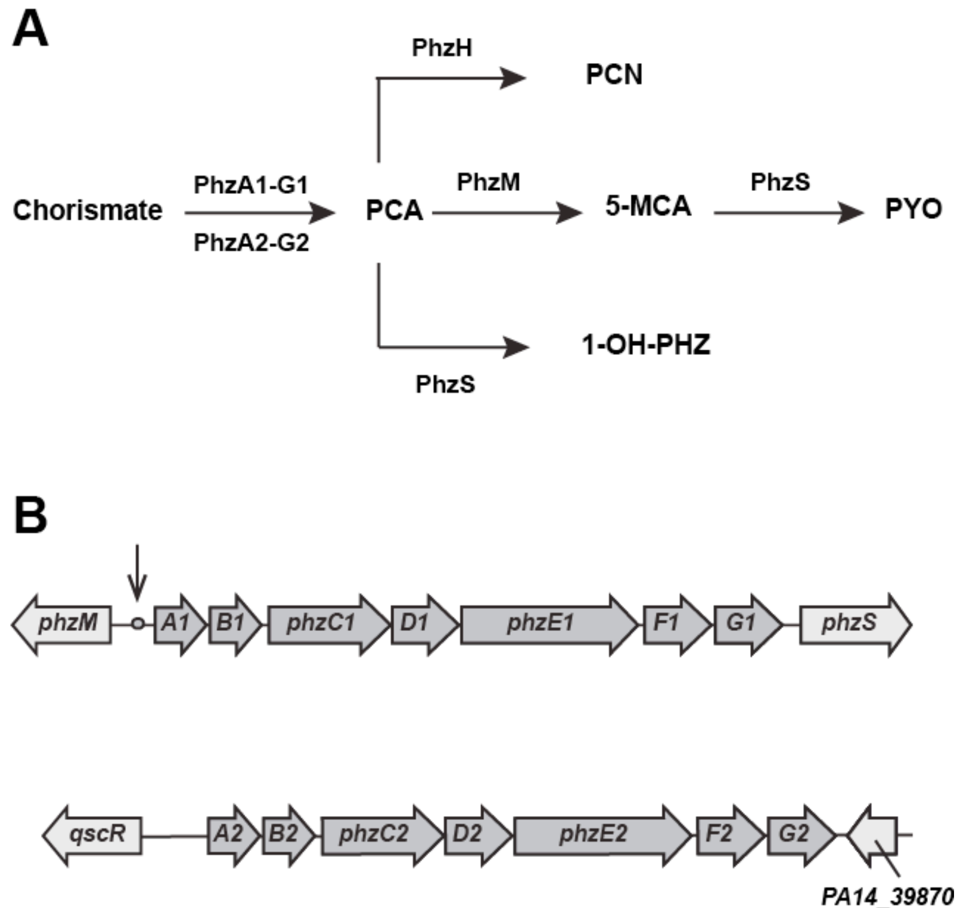
B. Primers for deletion strains

***pqsABC* deletion strains**	
pqsABC-US-1	ggaattgtgagcggataacaatttcacacaggaaacagctAGAGGCTCCGATCACCCTAT
pqsABC-US-2	ctcagcacaccagcacctcGTCTGGCCCCGATAGTGATA
pqsABC-DS-3	tatcactatcggggccagacGAGGTGCTGGTGTGCTGAG
pqsABC-DS-4	ccaggcaaattctgttttatcagaccgcttctgcgttCTGAACCGTAGGTCAGGACCAG
***pqsL* deletion strains**	
pqsL-US-1	ggaattgtgagcggataacaatttcacacaggaaacagctCGCCTGTTCCTCAAGTACG
pqsL-US-2	gctgataggaacgctcgcCCTGCTCCACTACCACCAC
pqsL-DS-3	gtggtggtagtggagcaggGCGAGCGTTCCTATCAGC
pqsL-DS-4	ccaggcaaattctgttttatcagaccgcttctgcgttCTCGAACAGGTGTTCCTCAATC
***pqsH* deletion strains**	
pqsH-US-1	ggaattgtgagcggataacaatttcacacaggaaacagctGATATCCACATCCACGGTGTC
pqsH-US-2	tattcctcagccagacgctcGATGCCTGCCTTGGTGAAT
pqsH-DS-3	attcaccaaggcaggcatcCTGAGGAATACCCTCGTTCG
pqsH-DS-4	ccaggcaaattctgttttatcagaccgcttctgcgttctgatGGAGATGCTCTGCACCTTGT
***pqsE* deletion strains**	
pqsE-US-1	ggaattgtgagcggataacaatttcacacaggaaacagctGCAATCATGACCTGGTAGGG

pqsE-US-2	atgctccccaggtgcagtCCAACAGGCACAGGTCATC
pqsE-DS-3	gatgacctgtgcctgttggACTGCACCTGGGGAGCAT
pqsE-DS-4	ccaggcaaattctgttttatcagaccgcttctgcgttCTGACAGGCACAACTGGCGATAG
***phzH* deletion strains**	
phzH-US-1	ggaattgtgagcggataacaatttcacacaggaaacagctGTTTCGACCAAGGAGGTCAG
phzH-US-2	gctcacctgggtgttgaagtGTATCGGTCATGGCGAAGAT
phzH-DS-3	atcttcgccatgaccgatacACTTCAACACCCAGGTGAGC
phzH-DS-4	ccaggcaaattctgttttatcagaccgcttctgcgttCTGATCGCTTCCTCGACTCCATC
***phz*M deletion strains**	
phzM-US-1	ggaattgtgagcggataacaatttcacacaggaaacagctCACTCGACCCAGAAGTGGTT
phzM-US-2	gttgagagttccggttcaggTATCAAATTACGCGCAGCAG
phzM-DS-3	ctgctgcgcgtaatttgataCCTGAACCGGAACTCTCAAC
phzM-DS-4	ccaggcaaattctgttttatcagaccgcttctgcgttctgatGCTGGTACGCCTGAGCAT
***phz*S deletion strains**	
phzS-US-1	ggaattgtgagcggataacaatttcacacaggaaacagctAAGGTCAACGCGGTACAGAT
phzS-US-2	ccatcgatatcctcattgccGCGACCGAAGACTGAGAAGA
phzS-DS-3	tcttctcagtcttcggtcgcGGCAATGAGGATATCGATGG
phzS-DS-4	ccaggcaaattctgttttatcagaccgcttctgcgttctgatACGCGAACATTTCCGAGTC

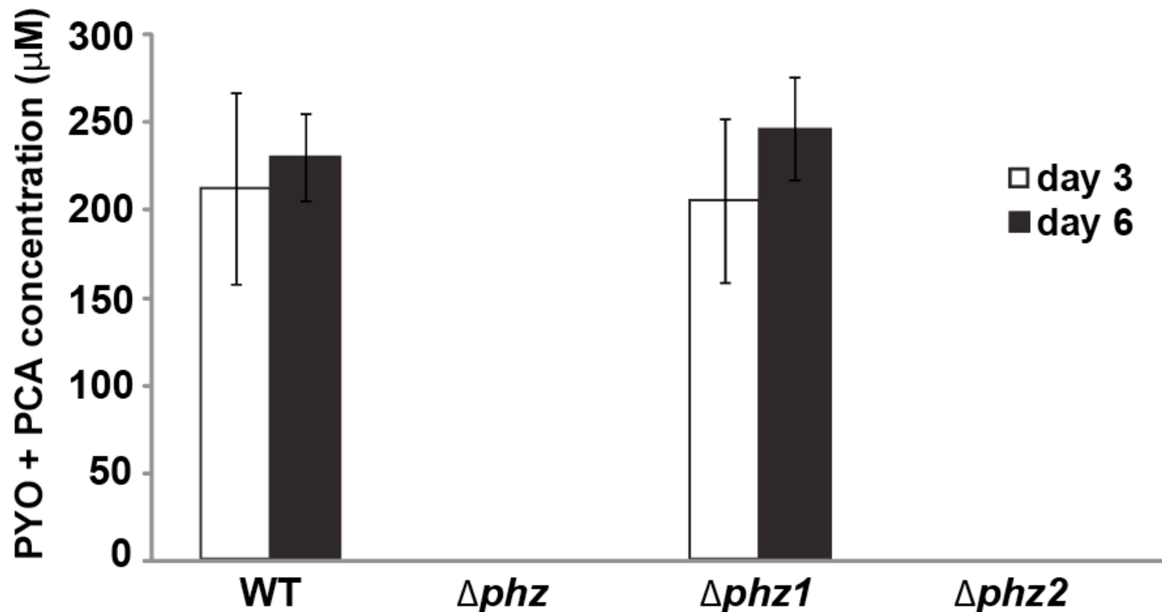
Capital letters indicate bases that anneal to the template. Restriction sites are in italic type and underlined.

Supporting Figure S1



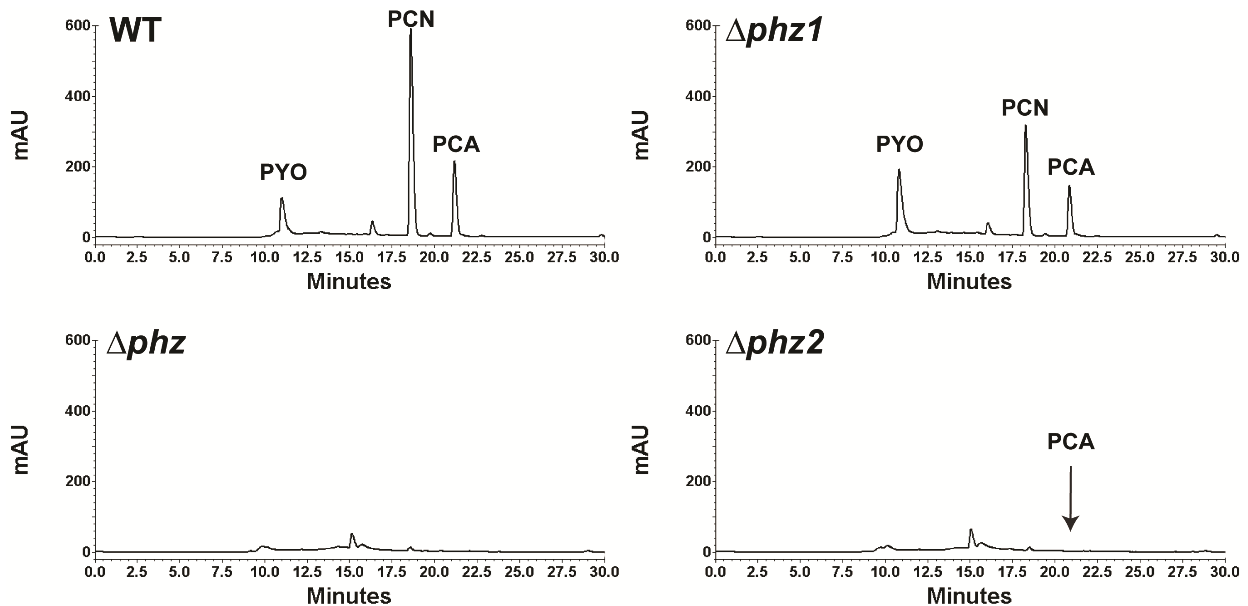
Supporting Figure 1 (Fig. S1). (A) Phenazine biosynthetic pathway. PCN, phenazine-1-carboxamide; 5-MCA, 5-methyl PCA; 1-OH-PHZ, 1-hydroxyphenazine. **(B) Genomic arrangement of the *phzA1-G1* (*phz1*) and *phzA2-G2* (*phz2*) operons and their surrounding regions.** *phz1* is flanked by *phzM* and *phzS,* which encode the enzymes that convert PCA to pyocyanin (PYO). The arrow indicates the location of the *las* box upstream of *phz1*. *phz2* is flanked by *qscR*, which encodes a transcription factor that senses acyl-homoserine lactones, and *PA14_39870*, which encodes a protein of unknown function that contains a HIT domain, characteristic of a superfamily of hydrolases and transferases.

Supporting Figure S2



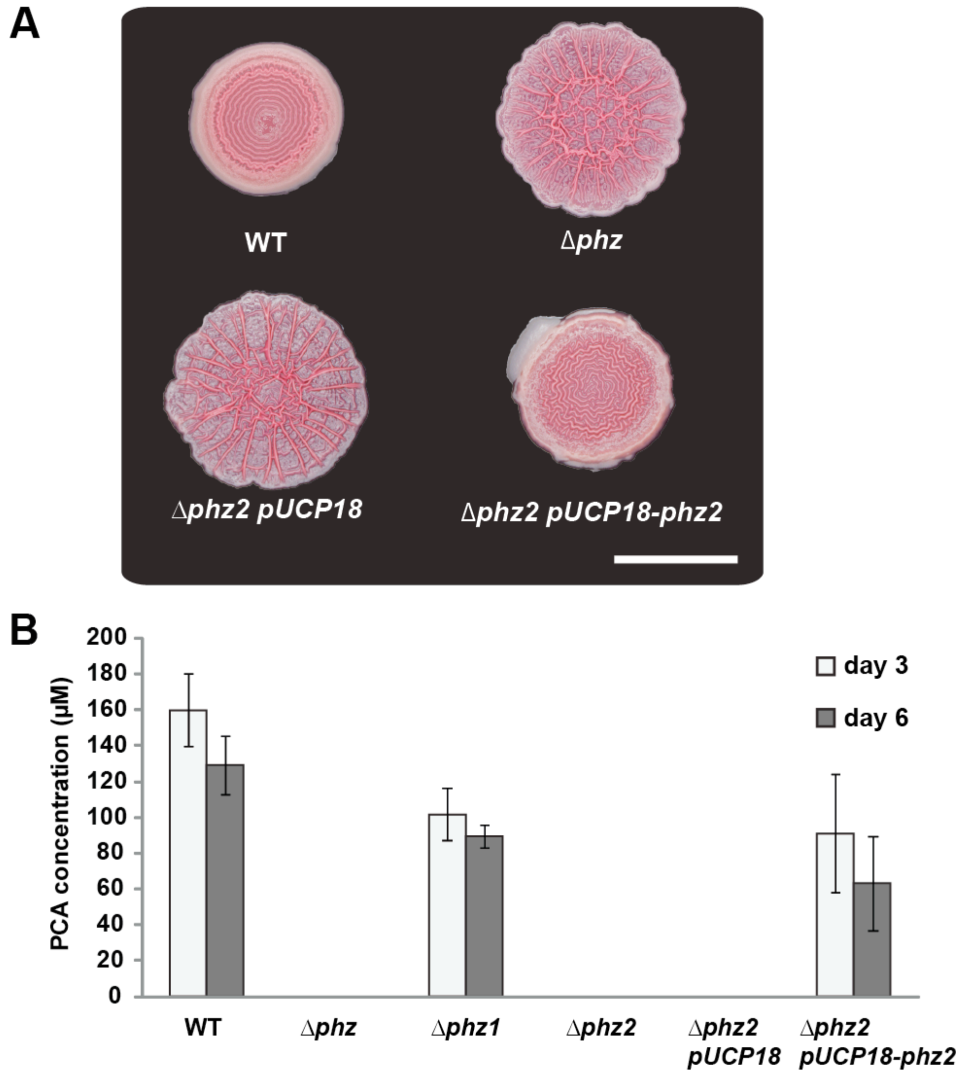
Supporting Figure 2 (Fig. S2). HPLC quantification of PYO and PCA from colonies grown on 1% tryptone/1% agar plates. Quantification of phenazines extracted from the agar on which biofilms were grown for three or six days. The PYO+PCA (combined concentration of PCA and PYO) from wild type are similar to that of the Δ*phz1* strain, indicating that the *phz2* operon is sufficient for production of wild-type levels of these phenazines. Error bars indicate standard deviation of three independent experiments.

Supporting Figure S3



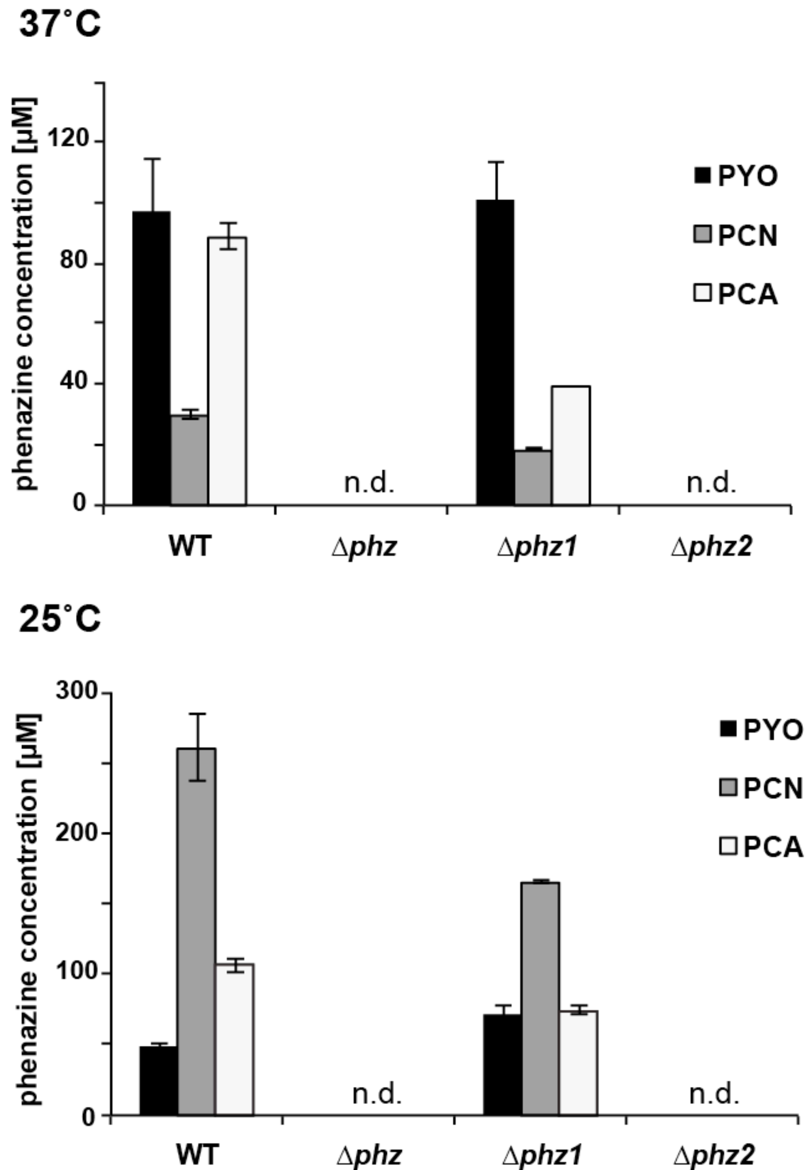
Supporting Figure 3 (Fig. S3). HPLC traces of phenazines extracted from colony biofilms after six days. Phenazines were extracted from agar and submitted to HPLC analysis for separation and quantification at a wavelength of 366 nm. Wild type produced the phenazines PYO, PCN and PCA. The Δ*phz* and Δ*phz2* strains did not produce detectable levels of any phenazines (arrow indicates where PCA peak would be expected in the Δ*phz2* strain). This suggests that *phz2* is necessary for phenazine production in biofilms. HPLC conditions and protocol were adapted from (1). Observed retention times for PYO and PCA agree with their results (~10 min and ~20 min respectively).

Supporting Figure S4



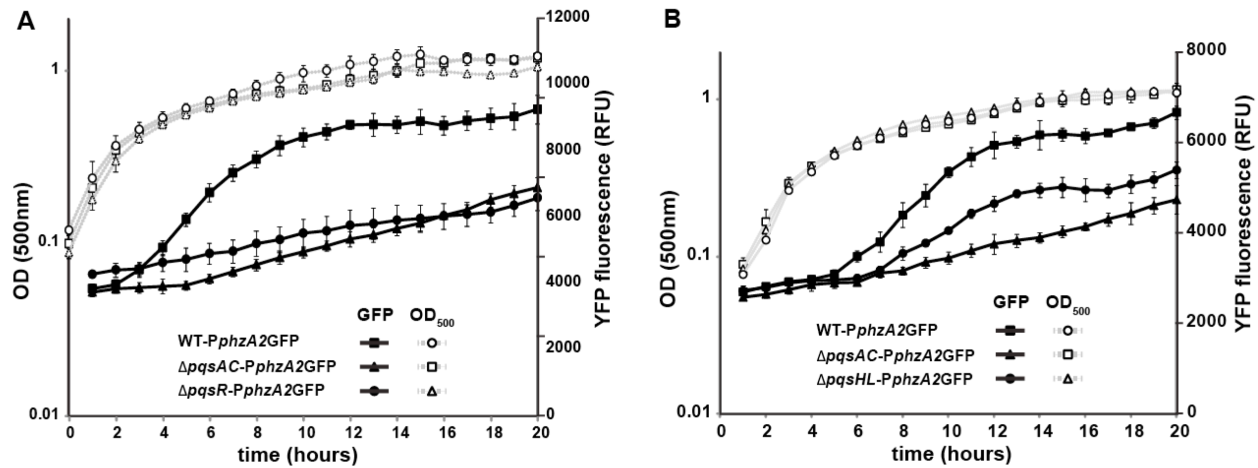
Supporting Figure 4 (Fig. S4). Complementation with *phz2* restores PCA production and restores wild type colony morphology. The *phz2* complementation strain was made by inserting a multi-copy plasmid containing the entire *phz2* operon into the Δ*phz2* mutant. (A) Colony morphology assay for wild type, Δ*phz*, control strain containing empty vector (Δ*phz2-pUCP18*), and complemented Δ*phz2*. Colonies were grown for three days. Scale bar is 1 cm. (B) Quantification of PCA production from deletion and complemented strains shows that complementation with *phz2* restores PCA production. Error bars indicate standard deviation of three independent experiments.

Supporting Figure S5



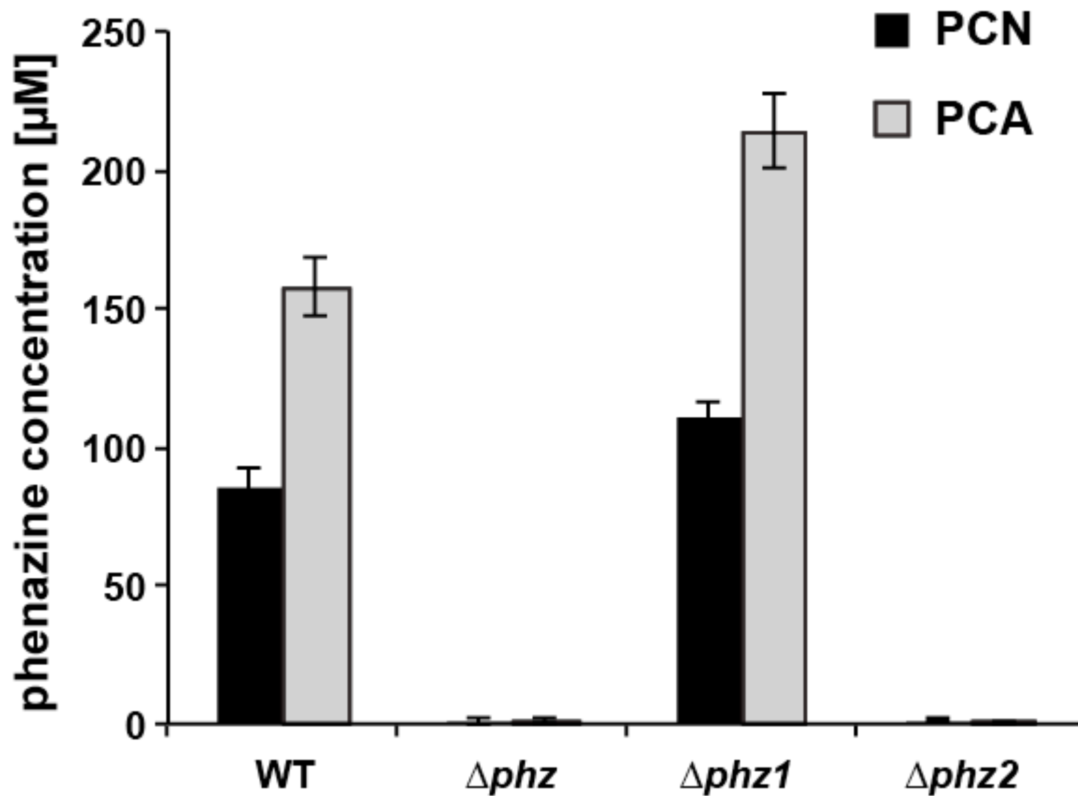
Supporting Figure 5 (Fig. S5). Effect of temperature on phenazine production. WT, Δ*phz*, Δ*phz1*, Δ*phz2* colonies were grown for 3 days on 1% tryptone, 1% agar at 25˚C and 37˚C. Phenazines were extracted from the agar into water and analyzed by HPLC. Phenazines could not be detected (n.d.) for Δ*phz* and Δ*phz2* colonies. Error bars indicate standard deviation of biological triplicates.

Supporting Figure S6



Supporting Figure 6 (Fig. S6). HHQ positively regulates the expression of *phz2* in planktonic cultures. We assayed for expression of the *phz2* operon using a GFP-reporter fusion containing the 500 bp region upstream of *phz2*. We integrated this reporter into the wild type, Δ*pqsAC* (no quinolones) and Δ*pqsHL* (HHQ only) strains and monitored growth and GFP expression in planktonic cultures for 20 hours. (A) Quinolone signaling is necessary for wild-type expression of *phz2* as Δ*pqsAC-PphzA2*GFP and Δ*pqsR-PphzA2*GFP exhibited a severe reduction in *phz2* expression. (B) Quinolone-dependent expression of *phz2* is achieved specifically through HHQ. The Δ*pqsHL-PphzA2*GFP strain produces HHQ (but no PQS or HQNO) and is able to induce expression of *phz2* although not to wild type levels. Error bars represent the standard deviation of one experiment performed in biological triplicates. Experiment was repeated three additional times with similar results.

Supporting Figure S7



Supporting Figure 7 (Fig. S7). *phz2* is expressed under anaerobic conditions. WT, Δ*phz*, Δ*phz1*, Δ*phz2* colonies were grown on 1% tryptone, 1% agar supplemented with 40 mM potassium nitrate in an anaerobic glove box filled with 80% N_2, 15% CO_2 and 5% H_2 (Coy) for five days. Phenazines were extracted from the agar into water and analyzed by HPLC. Error bars indicate standard deviation of biological triplicates.

References for Supporting Information

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