JOURNAL OF BACTERIOLOGY, Dec. 2011, p. 6770–6780 Vol. 193, No. 23
0021-9193/11/$12.00 doi:10.1128/JB.05929-11

2-Heptyl-4-Quinolone, a Precursor of the *Pseudomonas* Quinolone Signal Molecule, Modulates Swarming Motility in *Pseudomonas aeruginosa*▽

Dae-Gon Ha,[1] Judith H. Merritt,[1]§ Thomas H. Hampton,[1] James T. Hodgkinson,[2,3] Matej Janecek,[2] David R. Spring,[2] Martin Welch,[3] and George A. O'Toole[1]*

Dartmouth Medical School, Department of Microbiology and Immunology, Hanover, New Hampshire 03755[1]; Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, United Kingdom[2]; and Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, United Kingdom[3]

Received 2 August 2011/Accepted 20 September 2011

Pseudomonas aeruginosa **is an opportunistic pathogen capable of group behaviors, including biofilm formation and swarming motility. These group behaviors are regulated by both the intracellular signaling molecule c-di-GMP and acylhomoserine lactone quorum-sensing systems. Here, we show that the *Pseudomonas* quinolone signal (PQS) system also contributes to the regulation of swarming motility. Specifically, our data indicate that 2-heptyl-4-quinolone (HHQ), a precursor of PQS, likely induces the production of the phenazine-1-carboxylic acid (PCA), which in turn acts via an as-yet-unknown downstream mechanism to repress swarming motility. We show that this HHQ- and PCA-dependent swarming repression is apparently independent of changes in global levels of c-di-GMP, suggesting complex regulation of this group behavior.**

Pseudomonas aeruginosa is an opportunistic human pathogen capable of coordinated group behaviors, including swarming motility and biofilm formation. These group behaviors are regulated by both the intracellular signaling molecule c-di-GMP and the acylhomoserine lactone quorum-sensing (QS) systems (7, 19, 26, 32, 53).

P. aeruginosa swarming motility occurs on semisolid surfaces (i.e., on 0.5 to 0.7% agar) and is characterized by a fractal-like pattern of tendrils emanating from the point of inoculation (5, 24). Swarming motility requires a functional flagellum and the production of rhamnolipid biosurfactants, which are regulated by the acylhomoserine lactones 3-oxo-C12-HSL and 3-OH-C4-HSL (5, 35). Type IV pili, while not required for swarming, can impact swarm patterning (5).

Swarming motility and biofilm formation are inversely correlated in *P. aeruginosa* PA14, and this relationship is, in part, dependent on the intracellular level of c-di-GMP (26, 32, 33). We previously reported that a variety of amino acids could impact these group behaviors. In particular, we showed that arginine represses swarming and stimulates biofilm formation via an elevated intracellular pool of c-di-GMP (1). A Δ*sadC* Δ*roeA* double mutant results in reduced intracellular levels of this dinucleotide signal and thus relieves the arginine-mediated repression of swarming (1, 33).

Relevant to the human host, arginine appears to be a significant component of the cystic fibrosis patient (CF) lung (37). Recent data show that various regions of the CF lung are either low in oxygen or anoxic (45, 55). While *P. aeruginosa* can ferment arginine under such oxygen-limiting conditions (49), arginine in the CF lung is more likely assisting in redox balancing and cellular homeostasis under conditions promoting pyruvate fermentation and anaerobic respiration rather than promoting growth. Given the potential significance of arginine in the context of the CF lung and the arginine-dependent repression of swarming motility, we sought to identify molecular mechanism(s) of swarming regulation by arginine.

Here, we report the role of the signal molecule 2-heptyl-4-quinolone (HHQ) in the repression of swarm motility. We also show that HHQ, an intermediate in the synthesis of the *Pseudomonas* quinolone signal (PQS), controls swarming by positively regulating phenazine production. Of the four phenazines produced by *P. aeruginosa*, phenazine-1-carboxylic acid (PCA) modulates swarming motility via an unknown downstream mechanism. We present data to show that this HHQ/PCA-dependent pathway for swarm repression is c-di-GMP independent. Lastly, we present a model for the control of swarming motility that may be relevant in the context of the CF lung.

MATERIALS AND METHODS

Growth media. Strains, plasmids, and primers used in this study are listed in Table 1. *Pseudomonas aeruginosa* strain UCBPP-PA14 (abbreviated as *P. aeruginosa* PA14) was used in this study. *P. aeruginosa* PA14 and *Escherichia coli* were cultured in lysogeny broth (LB) at 37°C and, when appropriate, supplemented with antibiotics at the following concentrations: gentamicin (Gm), 10 μg ml^{-1} (*E. coli*) and 50 μg ml^{-1} (*P. aeruginosa*); carbenicillin (Cb), 50 μg ml^{-1} (*E. coli*) and 250 μg ml^{-1} (*P. aeruginosa*). M63 minimal medium supplemented with glucose (0.2%), arginine (0.4%), and $MgSO_4$ (1 mM) was used for c-di-GMP analysis. Swarming medium contained M8 salts (24), with glucose, arginine, and $MgSO_4$ at the same concentrations as those described for M63. When indicated, arabinose was added at 0.2%, and HHQ and PQS dissolved in dimethyl sulfoxide (DMSO) were added to the swarm agar medium (final concentration [C_f] = 0.5 μM), with equal volumes of DMSO in control plates. Phenazine-1-carboxylic acid (PCA), from Princeton Bio-Molecular Research (Princeton, NJ), was added to swarm medium from a stock of 500 mM in 5× M8, as indicated.

Molecular techniques. Plasmids constructed during the course of this study were prepared using homologous recombination in *Saccharomyces cerevisiae* (47). All restriction enzymes were obtained from New England BioLabs (Ips-

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Dartmouth Medical School, Room 505, Vail Building, Hanover, NH 03755. Phone: (603) 650-1248. Fax: (603) 650-1245. E-mail: georgeo@dartmouth.edu.
§ Present address: Glycobia Inc., Ithaca, NY 14850.
▽ Published ahead of print on 30 September 2011.

TABLE 1. Strains, plasmids, and primers used in this study

Strain, primer, or plasmid	Relevant genotype or primer sequence (5′→3′)	Source or reference
Strains		
S. cerevisiae InvSc1	*MAT***a**/*MAT*α *leu2/leu2 trp1-289/trp1-289 ura3-52/ura3-52 his3-*Δ*1/his3-*Δ*1*	Invitrogen
E. coli Top10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80 *lacZ*ΔM15 Δ*lacX74 recA1 ara*Δ*139* Δ(*ara leu*)*7697 galU galK rpsL* (St^r) *endA1 nupG*	Invitrogen
E. coli S17-1 (λpir)	*thi pro hsdR-hsdM*$^+$ Δ*recA* RP4-2::TcMu-Km::Tn*7*	48
SMC 232	Wild-type *P. aeruginosa* PA14	40
SMC 3809	SMC 232 Δ*sadC* Δ*roeA*	33
SMC 5013	SMC 232 Δ*pqsA*	Deb Hogan
SMC 5014	SMC 232 Δ*pqsB*	This study
SMC 5015	SMC 232 Δ*pqsD*	This study
SMC 5019	SMC 232 Δ*phnAB*	Deb Hogan
SMC 5018	SMC 232 Δ*pqsR*	10
SMC 5016	SMC 232 Δ*pqsE*	This study
SMC 5017	SMC 232 Δ*pqsH*	10
SMC 5021	SMC 232 Δ*lasR*	21
SMC 5022	SMC 232 *rhlR*::*tetR*	20
SMC 5023	SMC 232 Δ*lasR rhlR*::*tetR*	10
SMC 5020	SMC 232 Δ*phzA1-G1* Δ*phzA2-G2*	13
SMC 5127	SMC 232 Δ*phzH*	This study
SMC 5128	SMC 232 Δ*phzM*	This study
SMC 5129	SMC 232 Δ*phzHM*	This study
SMC 5123	SMC 232 SXO *phzS*	This study
SMC 5124	SMC 232 Δ*phzH* SXO *phzS*	This study
SMC 5125	SMC 232 Δ*phzM* SXO *phzS*	This study
SMC 5126	SMC 232 Δ*phzHM* SXO *phzS*	This study
Plasmids		
pMQ30	Suicide vector; Gm^r *sacB URA3 CEN6/ARSH4 lacZ*α	47
pKO pqsB	PA0997 (*pqsB*) knockout construct in pMQ30	This study
pKO pqsD	PA0999 (*pqsD*) knockout construct in pMQ30	This study
pKO pqsE	PA1000 (*pqsE*) knockout construct in pMQ30	This study
pDPM73	Cloning vector (derivative of pMQ70), Amp^r Cb^r *URA3* P_{BAD}-*araC CEN6/ARSH4*	47
pPqsA	PA0996 (*pqsA*); under the control of P_{BAD} promoter; Cb^r	This study
Primers		
pqsB dwst For	CTG TTT TAT CAG ACC GCT TCT GCG TTC TGA TGG ATT CTG TCG GGC GTT CGC TAC G	
pqsB dwst Rev	AGT TCA CAG GTG ATC GCT GCC AGT TTG ACC GCC CGT TCC TCC GGA AGG TTG TCG TGA	
pqsB upst For	TTA TCA CGA CAA CCT TCC GGA GGA ACG GGC GGT CAA ACT GGC AGC GAT CAC CTG TGA AC	
pqsB upst Rev	GCG GAT AAC AAT TTC ACA CAG GAA ACA GCT CGG CGA AAC CCC AGC CGG TGG C	
pqsD dwst For	TTG ACC GGG AGC CGA AAG CCG TAC AGC CCT CCT CGG ACA CCG TGG TTC	
pqsD dwst Rev	CTG TTT TAT CAG ACC GCT TCT GCG TTC TGA TAC CTC AGC GAG TCT TGG TGG CAA TTC TG	
pqsD upst For	GCG GAT AAC AAT TTC ACA CAG GAA ACA GCT CGC GAC GCT AGC GCG CAA C	
pqsD upst Rev	GTG TCC GAG GAG GGC TGT ACG GCT TTC GGC TCC CGG TCA ACT GGA T	
pqsE dwst For	CTG TTT TAT CAG ACC GCT TCT GCG TTC TGA TAA TCC GAT CCT GGC CGG GCT GGG TTT	
pqsE dwst Rev	GGC GGC GAT CGC CGC AAT GGA TGT CCC GCC GGC CGG TTC ACC TCC TCA GGT TTA CGG TAC	
pqsE upst For	GTA CCG TAA ACC TGA GGA GGT GAA CCG GCC GGC GGG ACA TCC ATT GCG GCG ATC	
pqsE upst Rev	GCG GAT AAC AAT TTC ACA CAG GAA ACA GCT GTA CGG GCT GGG GTT GCC CAG GCA C	
phzH dwst For	CTG TTT TAT CAG ACC GCT TCT GCG TTC TGA TAC GGA TCG TTG ATC GCT GTT TCG ACC AA	
phzH dwst Rev	CGC CAC GCC CCG CGT CAC GCA GGG AAA CTC CTC TAA TTG ATG TTT TAT CGG GAA ACT C	
phzH upst For	TCA ATT AGA GGA GTT TCC CTG CGT GAC GCG GGG CGT GGC	
phzH upst Rev	GCG GAT AAC AAT TTC ACA CAG GAA ACA GCT CAA GGC CAC TCG CAT GCC GCG	
phzM dwst For	CTG TTT TAT CAG ACC GCT TCT GCG TTC TGA TGT CGC ACT CGA CCC AGA AGT GGT TCG G	
phzM dwst Rev	CAG CCG TTG AGA GTT CCG GTC TTT TAT TCT CTC TCG TTA CAC ATT TCC GTA ACC CGA	
phzM upst For	GTA ACG AGA GAG AAT AAA AGA CCG GAA CTC TCA ACG GCT GGC CCC	
phzM upst Rev	GCG GAT AAC AAT TTC ACA CAG GAA ACA GCT CCG CGC CGA AGC GGC CGA C	

Continued on following page

TABLE 1—*Continued*

Strain, primer, or plasmid	Relevant genotype or primer sequence (5′→3′)	Source or reference
sxo phzS For	TGT TTT ATC AGA CCG CTT CTG CGT TCT GAT CAG TAC TCG ATC CAT CGC GGC GAA C	
sxo phzS Rev	GCG GAT AAC AAT TTC ACA CAG GAA ACA GCT GCG AAG AAC GGC AAC ACG TCT TCC AG	
PA14_51430 For	TTT TTT GGG CTA GCC CAA GGA AGC ACA ACC ATG TCC ACA TTG GCC AAC CTG ACC GAG GTT	
PA14_51430 Rev	AGA AGA TTT TCA GCC TGA TAC AGA TTA AAT TCA ACA TGC CCG TTC CTC CGG AAG GTT G	
pqsB For (confirm)	CCG AGC TGC GCC ACC TGG CC	
pqsB Rev (confirm)	CAG CGA ACA CCG GAT CGT CGT TTT CGT A	
pqsD For (confirm)	GTG TGC TGA GGC ATC GCC ATG TTG AAC C	
pqsD Rev (confirm)	CTG GAC GTC CCC CAA CAG GCA CAG GTC	
pqsE For (confirm)	GTT CCG GCG CGA CCT GGG GCG	
pqsE Rev (confirm)	TTT CCC CCA ATT GCG ACC GCT GCC	
phzH For (confirm)	GCG CCG CGA GCG GAC GG	
phzH Rev (confirm)	TCG AGA ACA ACG ACA AGA AGC GCT TCG	
phzM For (confirm)	CGA AGG AAT GGA TGT AGT GGT TCT CGC AAT AG	
phzM Rev (confirm)	TCG ACG CGC AGT GGG AAA TCG ACC	
phzA For (RT)	AAC GGT TAC AGC GGC ACA GCC TGT TC	
phzA Rev (RT)	CTC GAC CCA GAA GTG GTT CGG ATC CTC	
phzG For (RT)	TTT CCG AGT CCC TCA CCG GGA CCA TC	
phzG Rev (RT)	CGC GCT CGC CGA GTT CGG C	

wich, MA). Plasmids constructed in yeast were subsequently extracted by a modified "smash and grab" method (2) and electroporated into *E. coli* for confirmation by colony PCR (54), with minor modifications. Plasmids were propagated in *E. coli* Top 10 (Invitrogen, Carlsbad, CA) for complementation and in *E. coli* S17 for allelic exchange. Complementation constructs were extracted from bacteria using the Qiagen spin miniprep kit (Valencia, CA) and electroporated into *P. aeruginosa*, as previously reported (9). Allelic exchange constructs were conjugated into *P. aeruginosa*, as previously reported (25), and exconjugants were selected and counterselected by gentamicin and 5% sucrose, respectively. All resulting mutations were verified by PCR amplification of genomic DNA from mutants using primers flanking the deletion mutation and sequencing of the PCR products. Plasmid-harboring cells were maintained with necessary antibiotic selection on both LB liquid and agar. All transposon mutants were verified for correct transposon insertions via arbitrary primed PCR using nested primers to amplify genomic DNA, modified from published reports (3, 36), followed by sequencing of the PCR products.

RNA extraction and expression studies. Stationary-phase, LB-grown *P. aeruginosa* cultures were subcultured 1:1,000 into glucose-arginine M8 medium and then incubated at 37°C for 24 h. Total RNA was extracted from the glucose-arginine-grown cultures using the High Pure RNA isolation kit, and subsequent cDNA synthesis was performed with the Transcriptor first-strand cDNA synthesis kit (both kits are from Roche Applied Bioscience, Indianapolis, IN). Semi-quantitative reverse transcription-PCR (semi-qRT-PCR) was performed with NEB *Taq* DNA polymerase (Ipswich, MA).

To verify candidate genes from the microarray reanalysis, strains were scraped from glucose-arginine swarm motility plates following incubation at 37°C, and total RNA was extracted using the High Pure RNA isolation kit (Roche Applied Bioscience, Indianapolis, IN). cDNA was synthesized using the DyNAmo cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA), and subsequent qPCR studies were performed using SYBR green PCR master mix on a 7500 fast real-time PCR system (both are from Applied Biosystems, Bedford, MA).

Measurement of c-di-GMP levels. Nucleotide extraction from *P. aeruginosa* cultures were performed as previously reported (33, 34), with modifications. Briefly, a stationary-phase, LB-grown *P. aeruginosa* culture was subcultured 1:100 into glucose-arginine M63 medium to an optical density at 600 nm (OD_{600}) of 0.04. Cultures were harvested at an OD_{600} of 0.4 by centrifugation at 4°C for 10 min at 4,500 × *g*. Pellets were resuspended in 250 μl of extraction buffer (acetonitrile-methanol-water [40:40:20] plus 0.1 N formic acid) and incubated at −20°C for 30 min. Cell debris was pelleted for 5 min at 4°C, and the resulting supernatant was adjusted to a pH of ~7.5 by adding 15% $(NH_4)_2HCO_3$. Nucleotide extractions were analyzed via the Acquity Ultra Performance liquid chromatography (LC) system coupled to a Quattro Premier XE mass spectrometer (Waters Corporation, Milford, MA) (34). Each sample was compared to a standard curve of c-di-GMP resuspended in water to quantify the amount of nucleotide.

Microarray. The microarray data analyzed in this study was retrieved from the NCBI (GEO number GSE17147). To analyze these data, Affymetrix probe fluorescence values were first summarized and normalized using RMA (robust multichip average) (23) as implemented in Bioconductor. We then calculated the standard deviation for each probe and used Pearson distance to hierarchically cluster the 50 probes with the largest standard deviations.

RESULTS

Isolation and initial characterization of the PA14_36280::Tn and *pqsA*::Tn mutants. Two distinct group behaviors, swarming motility and biofilm formation, are inversely correlated in *Pseudomonas aeruginosa* PA14, and these phenotypes are regulated at least in part by the intracellular concentration of c-di-GMP (4). We recently identified two diguanylate cyclases (DGC), SadC and RoeA, and phosphodiesterase (PDE) BifA as responsible for regulating biofilm formation and swarming in *P. aeruginosa* PA14 (26, 32, 33). We also observed repression of swarming motility in the presence of arginine, and this effect is dependent upon the SadC and RoeA DGCs (Fig. 1A) (1).

To identify additional genes that may play a role in mediating the arginine-dependent repression of swarming motility in *P. aeruginosa* PA14, we performed a genetic screen to identify mutants that could swarm in the presence of this amino acid. This screen was prompted in part by the fact that deletion of the *sadC* and *roeA* DGCs could restore swarming on arginine (Fig. 1A), indicating that we could identify such a class of mutants.

We began by screening the *P. aeruginosa* PA14 nonredundant mutant library (29) for strains capable of swarming on arginine-containing medium. A total of 5,663 transposon mutants were screened on arginine medium, of which 75 showed a positive swarming phenotype in the initial screen. These 75 positive candidates were retested, and only 3 mutants demonstrated a reproducible swarm-positive phenotype on arginine

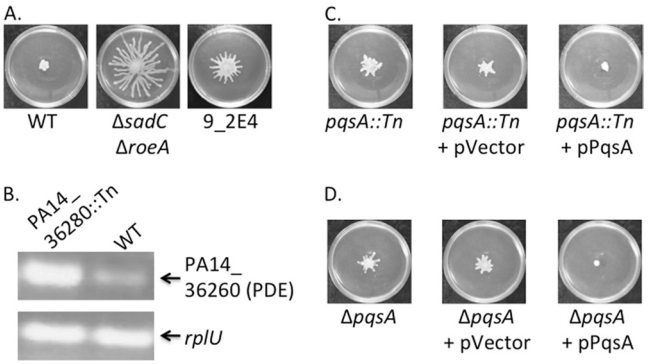

FIG. 1. Mutating *pqsA* relieves arginine-mediated repression of swarming motility. (A) Representative swarm phenotypes of the wild type (WT), Δ*sadC* Δ*roeA* mutant, and 9_2E4 mutant on swarm medium containing arginine. This and all subsequent swarm motility assays reported here are performed on swarm medium supplemented with 0.4% arginine. (B) Semi-qRT-PCR analysis comparing the expression levels of the PA14_36260 gene, a known phosphodiesterase (PDE) (27), in the PA14_36280::Tn mutant and wild type (WT). The expression of the *rplU* gene was used as a control for a gene not expected to differ between these strains. (C) Representative swarm phenotypes of the *pqsA*::Tn mutant, the *pqsA*::Tn strain carrying the vector control (+pVector), and the strain complemented with a wild-type copy of the *pqsA* gene (pPqsA). (D) Representative swarm phenotypes of the Δ*pqsA* mutant, the mutant carrying a vector control (+pVector), and the mutant complemented with a wild-type copy of the *pqsA* gene (+pPqsA).

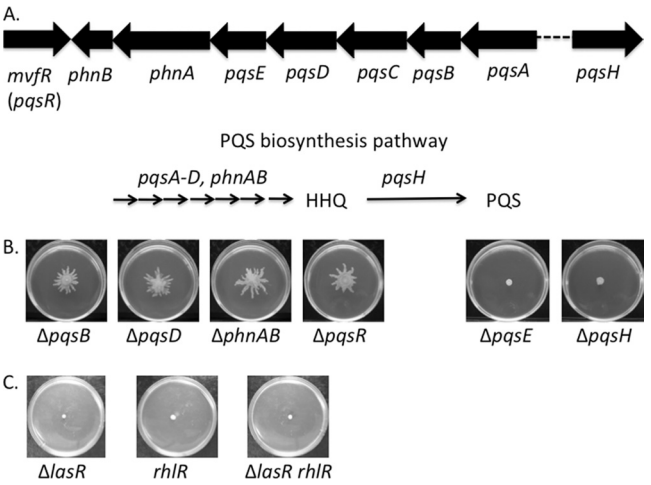

FIG. 2. HHQ mediates swarm repression by arginine. (A, top) Organization of the PQS operon (*pqsABCDE*, *phnAB*, *pqsR* [*mvfR*]) and the distant *pqsH* gene in *P. aeruginosa* PA14; (bottom) a simplified biosynthetic pathway of PQS. The genes involved in each portion of the pathway are indicated above the arrows. Also shown are the precursor, HHQ, and the final product, PQS. (B) Representative swarm phenotypes of the Δ*pqsB*, Δ*pqsD*, Δ*phnAB*, Δ*pqsR*, Δ*pqsE*, and Δ*pqsH* mutants. (C) Representative swarm phenotypes of other quorum-sensing (QS), Δ*lasR*, *rhlR*::*tetR*, and Δ*lasR rhlR*::*tetR* mutants.

medium. One of the mutants could not be complemented and was not examined further. The characterization of the other two mutants, with insertions in the PA14_36280 and *pqsA* genes, is presented here.

Consistent with the phenotypes observed in the Δ*sadC* Δ*roeA* mutant, we expected that candidates with transposon insertions in genes affecting c-di-GMP metabolism or signaling would emerge from the screen (i.e., with reduced c-di-GMP levels). The candidate mutant 9_2E4 (Fig. 1A) had a transposon insertion in the PA14_36280 gene, upstream of a gene encoding a documented PDE, PA14_36260, which corresponds to the PA2200 gene of *P. aeruginosa* PAO1 (27). Because the transposon used to generate this library has an outward-facing promoter, we predicted that the swarm phenotype of this mutant might have been due to the induction of expression of the PDE-encoding PA14_36260 gene. Consistent with this idea, semiquantitative RT-PCR analysis revealed a higher level of expression of the PDE-encoding PA14_36260 gene in the PA14_36280::Tn (9_2E4) mutant than in the wild type (WT) (Fig. 1B). This mutant helped serve to validate the utility of this screening approach.

We also isolated an additional candidate, designated 1_2A5, which demonstrated swarming motility on arginine swarm medium, albeit with a less striking phenotype than observed for the Δ*sadC* Δ*roeA* double mutant (compare Fig. 1A to C, left). The transposon insertion in the 1_2A5 mutant was mapped to the *pqsA* gene. The *pqsA* gene has no known DGCs or PDEs in close proximity. Introduction of a wild-type copy of the *pqsA* gene on a plasmid complemented the 1_2A5 mutant, that is, restored the swarming-repressed phenotype (Fig. 1C).

To confirm the finding above, a strain carrying a deletion of the *pqsA* gene was also tested on the arginine swarm medium; the Δ*pqsA* deletion strain, like the transposon insertion mutant, was capable of swarming on arginine-containing medium (Fig. 1D). Furthermore, introduction of the wild-type *pqsA* gene into the Δ*pqsA* mutant strain, but not the vector control, complemented the swarming phenotype on arginine medium (Fig. 1D).

Role of *pqs* genes in arginine-mediated swarm repression. The *pqsA* gene is the first gene in an operon that also includes the *pqsBCDE* genes. This operon and the bicistronic *phnAB* operon are similarly regulated (Fig. 2A) (8, 31). The *pqsABCD* and *phnAB* genes are required for the synthesis of HHQ, the immediate precursor of 2-heptyl-3-hydroxy-4-quinolone (PQS) (Fig. 2A) (for a review, see reference 22). HHQ is converted into PQS by the flavin-dependent monooxygenase encoded by the *pqsH* gene, which lies distant from the PQS operon (12, 18, 46). Although the *pqsE* gene is also in the *pqsABCDE* operon, it has no known biosynthetic function but rather is shown to be required for the expression of genes under the control of PQS (17, 42). Lastly, the LysR-type transcriptional regulator PqsR (also known as MvfR) positively regulates the expression of the PQS operons (*pqsABCDE* and *phnAB* genes) and HHQ production (50, 57).

Following the isolation of a mutation in the *pqsA* gene from the screen, we hypothesized that arginine's repressive effect on swarming motility was mediated via the PQS system and its quinolone molecule product(s). To test this hypothesis, we assayed strains that carry various mutations in the *pqsABCDE* and *phnAB* genes as well as the *pqsH* gene for their ability to swarm on the arginine medium. The Δ*pqsB*, Δ*pqsD*, Δ*phnAB*, and Δ*pqsR* mutants, similar to the Δ*pqsA* mutant, demonstrated swarming motility in the presence of arginine (Fig. 2B, left). However, under these same conditions, two mutants—the Δ*pqsE* and Δ*pqsH* mutants—failed to swarm (Fig. 2B, right).

TABLE 2. Swarm repression is mediated by a transferrable signal

Strain	Swarming phenotype[a]							
	WT	Δ*pqsA* mutant	Δ*pqsB* mutant	Δ*pqsD* mutant	Δ*pqsE* mutant	Δ*pqsH* mutant	Δ*pqsR* mutant	Δ*phnAB* mutant
WT	−	−	−	−	−	−	−	−
Δ*lasR* mutant	−	−	−	−	−	−	−	−
rhlR::*tetR* mutant	−	−	−	−	−	−	−	−
Δ*lasR rhlR*::*tetR* mutant	−	−	−	−	−	−	−	−
Δ*pqsA* mutant	−	+	+	+	−	−	+	+
Δ*pqsB* mutant	−	+	+	+	−	−	+	+
Δ*pqsD* mutant	−	+	+	+	−	−	+	+
Δ*pqsE* mutant	−	−	−	−	−	−	+	−
Δ*pqsH* mutant	−	−	−	−	−	−	+	+
Δ*pqsR* mutant	−	+	+	+	+	+	+	+
Δ*phnAB* mutant	−	+	+	+	−	−	+	+

[a] −, negative swarming phenotype; +, positive swarming phenotype. Phenotypes were scored based on observed tendril formation on at least six replicate plates.

Because the PQS system is one of three identified quorum-sensing (QS) systems in *P. aeruginosa*, we also tested strains with mutations in two other QS systems, Las and Rhl (16), for their potential role in swarm repression in the presence of arginine. Strains carrying mutations in the Las and Rhl systems, the Δ*lasR*, *rhlR*::*tetR*, and Δ*lasR rhlR*::*tetR* mutants, like the wild type, did not swarm on arginine-containing medium (Fig. 2C). Similarly, when inoculated on glucose medium that typically favors swarming motility by *P. aeruginosa*, none of these mutants were able to swarm (data not shown), indicating a general defect in swarming for these mutant strains, likely due to impaired rhamnolipid surfactant production (24, 44). Together, these data suggest that swarm repression mediated by arginine is dependent on the synthesis of HHQ but not PQS or the products of the Las or Rhl QS systems.

HHQ cross-complementation restores swarm repression by arginine. Our genetic data indicated a role for HHQ in repressing swarming motility on arginine. Previous work demonstrated that PQS can be transferred via outer membrane vesicles (OMV) (30) or secreted (6) by *P. aeruginosa*. HHQ has also been shown to be secreted (12). We exploited these facts to test the hypothesis that HHQ mediates swarm repression on arginine medium by performing cross-complementation assays. In these experiments, the two test strains were mixed in a 1:1 ratio prior to inoculation on the arginine-containing swarm plate.

In mixtures where HHQ is produced by at least one of the two strains (e.g., WT versus the Δ*pqsA* mutant, the Δ*pqsA* mutant versus the Δ*pqsH* mutant), swarming motility was repressed, but not when both lacked the ability to synthesize HHQ (e.g., the Δ*pqsA* mutant versus the Δ*pqsB* mutant, the Δ*pqsB* mutant versus the Δ*pqsD* mutant) (Table 2). As a control, we also "self-crossed" mutants and showed that strains lacking HHQ production (e.g., the Δ*pqsA* mutant versus the Δ*pqsA* mutant, the Δ*pqsR* mutant versus the Δ*pqsR* mutant) swarmed, while strains capable of HHQ production (e.g., WT versus WT, the Δ*pqsH* mutant versus the Δ*pqsH* mutant) did not swarm on arginine medium (Table 2). These results imply that HHQ can be transferred between cells to repress swarming motility, and furthermore, these findings are consistent with the model that HHQ rather than PQS represses swarming motility.

Exogenous HHQ, but not PQS, represses swarming motility on arginine. The combination of our genetic and cross-complementation data above suggested a role for HHQ, but not PQS, in mediating swarm repression on arginine medium. To explore this hypothesis further, we added exogenous, chemically synthesized and purified HHQ or PQS (both at 0.5 μM) to assess their effects on swarming motility. As a control, we added equal volumes of DMSO, which was used to solubilize these compounds. While HHQ was effective in repressing swarming motility by the Δ*pqs* mutants, neither PQS nor DMSO repressed swarming by these same mutants (Table 3). In contrast, the Δ*pqsR* mutant demonstrated swarming motility regardless of HHQ or PQS supplementation to the arginine medium (Table 3).

We also wanted to explore whether c-di-GMP- and HHQ-mediated repression of swarming motility comprise a single regulatory pathway. To begin to address this question, we performed swarming motility assays by mixing the WT and the Δ*sadC* Δ*roeA* double mutant; this mixture of strains was still capable of swarming (data not shown). Furthermore, contrary to the Δ*pqs* mutants' responses described above, the Δ*sadC* Δ*roeA* double mutant produced a robust swarm even when exogenous HHQ or PQS was added to the medium (Table 3).

Taken together, these results suggest that both HHQ and the PqsR protein contribute to the repression of swarming motility on arginine medium. Furthermore, our data suggest

TABLE 3. Swarming is repressed by the addition of exogenous HHQ

Strain	Swarming phenotype[a]		
	DMSO	HHQ	PQS
WT	−	−	−
Δ*sadC* Δ*roeA* mutant	++	++	++
Δ*pqsA* mutant	+	−	+
Δ*pqsB* mutant	+	−	+
Δ*pqsD* mutant	+	−	+
Δ*phnAB* mutant	+	−	+
Δ*pqsR* mutant	+	+	+

[a] −, negative swarming phenotype (e.g., no swarming); +, positive swarming phenotype; ++, hyperswarming phenotype. Phenotypes were scored based on observed tendril formation on at least five replicate plates.

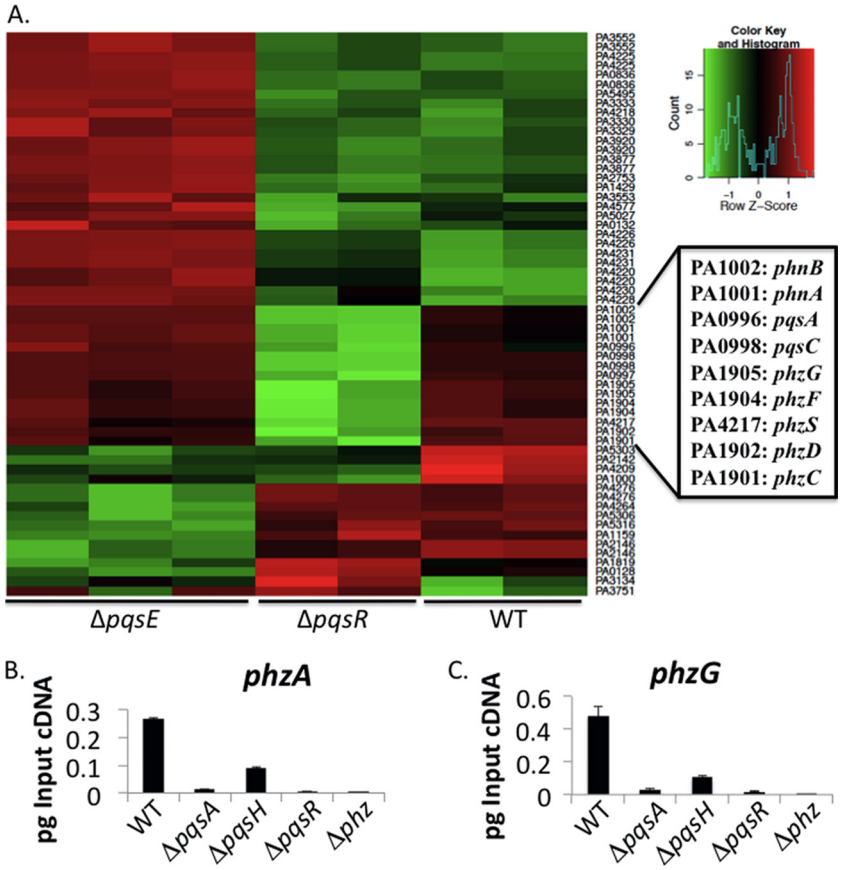

FIG. 3. HHQ-dependent candidate genes. (A) Reanalysis of a previous microarray data set (GEO number GSE17147) as a heat map comparing global gene expression patterns among the wild-type strain and the Δ*pqsE* and Δ*pqsR* mutants. Genes of interest are highlighted in the box to the right. See Materials and Methods for the statistical parameters. The key in the upper right of the panel shows the relative differential expression corresponding to the colors in the heat map. The gene names corresponding to the names from strain *P. aeruginosa* PAO1 are shown, because this is the strain studied in the original analysis (11, 28). (B) Relative expression of the *phzA* gene (±standard deviation [SD]; $n = 6$); (C) relative expression of the *phzG* gene (±SD). For the plots shown in panels B and C, picograms (pg) of input DNA are plotted versus the strain tested.

that HHQ- and c-di-GMP-mediated repression of swarming motility may be via distinct pathways, a point addressed in more detail below.

Phenazines are downstream effectors in HHQ-mediated, arginine-dependent swarm repression. The data presented here support a model wherein HHQ, jointly with the PqsR protein, represses swarming motility when arginine is present. We next sought to identify a candidate gene(s) regulated by HHQ that might contribute to arginine-mediated swarm repression. In particular, our emphasis was to distinguish HHQ-regulated genes from PQS-regulated genes, as HHQ but not PQS contributes to swarm repression under our experimental conditions. To identify HHQ-regulated gene(s), we reanalyzed a published set of microarray studies (11, 28) and compared the expression profiles of three strains—the wild type and the Δ*pqsR* and Δ*pqsE* mutants—to identify gene(s) whose expression is HHQ dependent.

Our rationale for choosing the three strains is as follows: the wild-type strain produces both PQS and HHQ molecules and is capable of responding to these signals. The Δ*pqsR* mutant, on the other hand, lacks the positive regulator of the PQS operons, which negatively impacts HHQ and PQS biosynthesis and which renders the Δ*pqsR* mutant unable to respond to these signals. Therefore, examination of the expression profiles of the wild type versus the Δ*pqsR* mutant should identify a combination of HHQ- and PQS-regulated genes. Lastly, the inclusion of the Δ*pqsE* mutant expression profile should assist in identifying specifically HHQ-dependent genes, as the Δ*pqsE* mutant produces both PQS and HHQ but lacks the PQS-dependent response mediated by PqsE (18). Thus, when comparing the three profiles, HHQ-dependent candidates will be similarly expressed in both the wild type and the Δ*pqsE* mutant while inversely expressed in the Δ*pqsR* mutant.

The microarray data comparing the wild-type strain to the Δ*pqsE* and Δ*pqsR* mutants were retrieved from the public GEO database (GEO number GSE17147) (28) and reanalyzed as described in Materials and Methods. The expression of the *pqs* genes served as an internal control for this data set; these genes were upregulated in both the wild-type strain and the Δ*pqsE* mutant, but they were downregulated in the Δ*pqsR* mutant (Fig. 3A). This finding is consistent with published reports wherein PqsR (MvfR), together with PQS or HHQ, can directly act as a positive regulator of the *pqsABCDE* and *phnAB* genes (50, 56). In addition to the *pqs*

genes, a set of phenazine biosynthesis genes (*phzG*, *phzF*, *phzD*, *phzC*, and *phzS*) emerged as candidate HHQ-dependent genes (Fig. 3A).

Prior to testing the downstream candidates (i.e., *phz* genes) for swarming phenotypes, the expression levels of these candidate genes were examined via qRT-PCR. Here, we used RNA extracted from strains grown on swarm motility plates containing arginine, thus using the growth conditions identical to those used for all the phenotypic assays in this report. For this set of experiments, we examined the expression levels of the *phzA* and *phzG* genes in the wild-type strain and the Δ*pqsA*, Δ*pqsH*, Δ*pqsR*, and Δ*phzA1-G1* Δ*phzA2-G2* (Δ*phz*) (13) mutants. Consistent with our microarray analysis, the wild-type strain, producing both PQS and HHQ molecules, demonstrated the highest level of transcripts for the *phzA* and *phzG* genes, while the Δ*pqsH* mutant consistently showed only ~25% of the wild-type expression levels (Fig. 3B and C), indicating that expression of *phzA* and *phzG* genes are at least partially PQS dependent under these conditions. However, the lack of swarming by the Δ*pqsH* mutant suggests that even this decreased *phz* gene expression is still sufficient to repress swarming.

We also examined the expression of *phzA* and *phzG* transcripts in both Δ*pqsA* and Δ*pqsR* mutants, which served as PQS/HHQ-deficient and PQS/HHQ-unresponsive controls, respectively. Expression of the *phzA* and *phzG* genes was reduced >93% under these conditions for both the Δ*pqsA* and Δ*pqsR* mutants, a finding consistent with the microarray data. Based on the swarm phenotype of these mutants, we suggest that this marked reduction in expression of the *phz* biosynthetic gene cluster is sufficient to relieve phenazine-dependent repression of swarming (see below). Finally, as expected, no *phzA* or *phzG* gene transcript was detected in the Δ*phz* mutant, which is deleted for both *phz* operons (13).

Thus, our microarray and qRT-PCR data support the HHQ-dependent induction of phenazine gene expression under these conditions and suggest that phenazines may mediate the ability of arginine to repress swarming motility.

Phenazines repress swarming on arginine medium. Based on our microarray and qRT-PCR data, we hypothesized that phenazines might be required for swarm repression on arginine medium. To test this hypothesis, we assessed the swarming motility of the phenazine-null Δ*phz* mutant (13) on arginine medium. The Δ*phz* mutant demonstrated swarming on arginine medium, which resembled the Δ*pqsA* mutant phenotype (Fig. 4A).

Proteins encoded by the phenazine biosynthesis operons (*phzA1-G1 phzA2-G2*) synthesize phenazine-1-carboxylate (PCA), which can be converted into terminal phenazines, such as phenazine-1-carboxamide (PCN), pyocyanin (PYO), or 1-hydroxyphenazine (1-OH-PHZ) (Fig. 4B). Because the Δ*phz* mutant lacks production of all phenazines, we sought to identify the specific phenazine(s) responsible for the repression of swarming motility on arginine medium. We tested strains with mutations in the genes responsible for converting PCA into the three terminal phenazines, *phzH*, *phzM*, and *phzS*, for their impacts on swarming motility. The corresponding transposon mutants (29) in any of these three genes failed to derepress swarming motility on arginine medium (Fig. 4C).

Consistent with the transposon mutant data, the Δ*phzH* and Δ*phzM* deletion strains and the single-crossover (SXO) *phzS* mutant were unable to swarm on the arginine medium (Fig. 4D). For reasons we do not understand, we were unable to delete the *phzS* gene via allelic exchange; hence, we have used the SXO mutant in all the studies described here. Collectively, these results suggest that PCA, but not the PCA derivatives PYO, 1-OH-PHZ, or PCN, is responsible for swarm repression. It is important to note that PYO is secreted at levels similar to PCA levels (13); thus, our observations are likely not due to different levels of the phenazines secreted. Alternatively, the terminal phenazines may have redundant function(s) in repressing swarming motility on arginine medium.

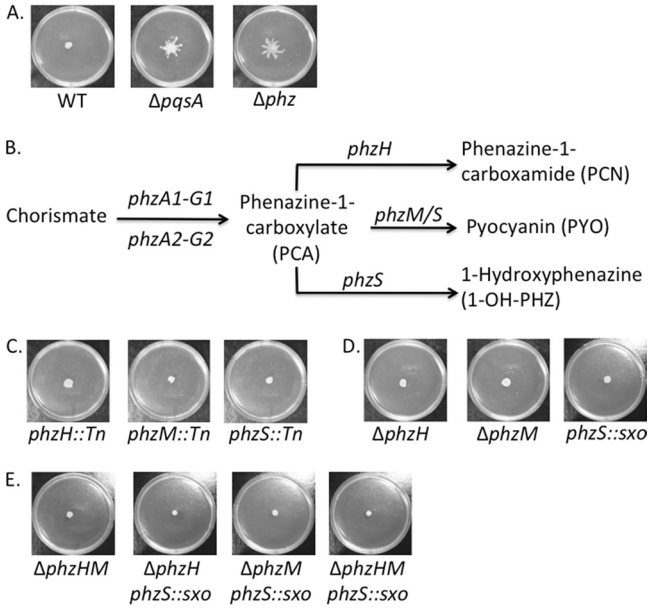

FIG. 4. Phenazine-1-carboxylate (PCA) represses swarming motility. (A) Representative swarm phenotypes of the wild type (WT) and the Δ*pqsA* and Δ*phzA1-G1* Δ*phzA2-G2* (Δ*phz*) mutants. (B) Simplified scheme of the phenazine biosynthesis in *P. aeruginosa* PA14. Genes involved are indicated above the arrows, and known phenazines produced by this organism are shown. (C) Representative swarm phenotypes of the *phzH*::Tn, *phzM*::Tn, and *phzS*::Tn mutants; (D) representative swarm phenotypes of the single mutants: Δ*phzH*, Δ*phzM*, and *phzS*::*sxo* mutants; (E) representative swarm phenotypes of double and triple mutants involved in the synthesis of terminal phenazines: Δ*phzHM*, Δ*phzH phzS*::*sxo*, Δ*phzM phzS*::*sxo*, and Δ*phzHM phzS*::*sxo* mutants.

To address these two hypotheses, double and triple mutants were created: the Δ*phzHM*, Δ*phzH phzS*::*sxo*, Δ*phzM phzS*::*sxo*, and Δ*phzHM phzS*::*sxo* mutants. As with the swarm phenotypes of the single mutants highlighted in Fig. 4D, double and triple mutants perturbed for terminal phenazine biosynthesis were all still repressed for swarming on arginine medium (Fig. 4E). In sum, the data presented in Fig. 4 support the hypothesis that PCA is responsible for repressing swarm motility on arginine medium.

Cross-complementation of phenazines restore swarm repression by arginine. Because the lack of phenazines derepresses swarming motility on arginine medium (Fig. 4A), the reintroduction of phenazines should repress swarming motility. Phenazines are redox-active compounds that are secreted by *P. aeruginosa* into the extracellular milieu (39, 51). Thus, we

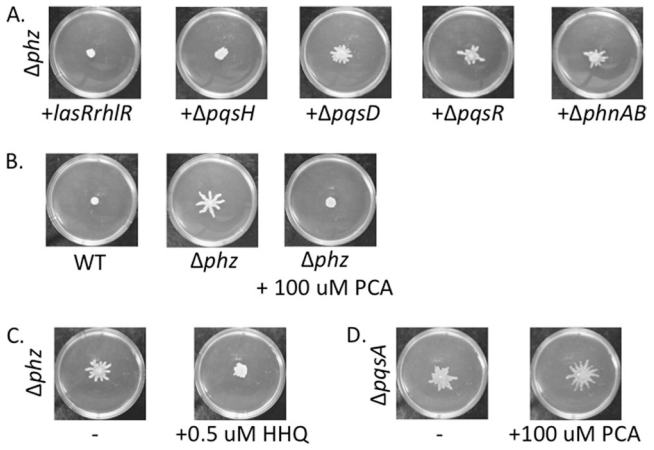

FIG. 5. PCA-dependent and -independent swarm repression. (A) Representative swarm phenotypes when the Δ*phzA1-G1* Δ*phzA2-G2* (Δ*phz*) mutant is coinoculated with other mutants (mutant genotype specified). Also see Table 4. (B) Representative swarm phenotypes of the wild-type strain and the Δ*phzA1-G1* Δ*phzA2-G2* (Δ*phz*) mutant with or without exogenous 100 μM PCA; (C) representative swarm phenotypes of the Δ*phz* mutant with or without exogenous 0.5 μM HHQ; (D) representative swarm phenotypes of the Δ*pqsA* mutant with or without exogenous 100 μM PCA.

asked if phenazines provided in *trans* from other strains could repress swarm motility in a Δ*phz* mutant, akin to the experiments described above wherein HHQ is provided in *trans* to the Δ*pqs* mutants.

We performed the previously described cross-complementation experiment wherein the Δ*phz* mutant was mixed with other strains in a 1:1 ratio. When the mixture lacked a phenazine-producing strain (e.g., Δ*pqsA*, *-B*, *-D*, and *-R* and Δ*phnAB* mutants), swarming motility was observed, whereas the inclusion of a phenazine-producing strain (e.g., the wild-type strain or the Δ*pqsH* mutant) repressed swarming motility on arginine medium (Fig. 5A and Table 4). Thus, it appears phenazines can be transferred between cells to repress swarming motility on arginine medium. Furthermore, the observation that the Δ*lasR rhlR*::*tetR* double mutant can repress swarming by the Δ*phz* strain (Fig. 5A) suggests that strains lacking the Las and Rhl quorum-sensing systems still produce at least some phenazines.

PCA represses swarming on arginine medium. Data presented in Fig. 4 and 5A suggest that PCA, and not terminal phenazines, may be important in repressing swarming motility on arginine medium. To directly address this hypothesis, chemically synthesized PCA (100 μM; Princeton BioSciences, Princeton, NJ) was added exogenously to the arginine swarm medium. The addition of PCA was sufficient to repress the swarming motility of the Δ*phz* mutant on arginine medium (Fig. 5B).

The data presented thus far are consistent with a simple model wherein HHQ is required for PCA production, and PCA in turn represses swarming motility. There are two predictions that grow from this simple model. First, the addition of HHQ to a mutant blocked in PCA production should no longer repress swarming motility. Second, the addition of PCA to a mutant lacking HHQ should still repress swarming motility. To our surprise, neither of these predictions was borne out. First, the addition of 0.5 μM HHQ to the Δ*phz* mutant still repressed swarming motility despite the inability of this mutant to produce PCA (Fig. 5C). Furthermore, the Δ*pqsA* mutant (which is unable to produce HHQ) still swarmed upon the addition of 100 μM PCA (Fig. 5D). Combined, these data suggest that there may be an HHQ-dependent, PCA-independent regulatory pathway for repressing swarming motility on arginine also operating in this microbe, indicating a complex mechanism of swarming regulation.

Mutations in the *pqsA* or *phz* genes do not alter pools of c-di-GMP. Our group has previously shown a role for c-di-GMP in regulating group behaviors, including swarming motility (25, 32, 33). It is possible that the swarming motility phenotype we observed in both the Δ*pqs* and Δ*phz* mutants on the arginine medium can be attributed to a reduction in their intracellular c-di-GMP levels. To address this issue, we quantified and compared the intracellular c-di-GMP concentrations among the wild-type strain and the Δ*pqsA* and Δ*phz* mutants via a previously described LC-tandem mass spectrometry (LC-MS/MS) method (33, 34).

Quantification of the intracellular c-di-GMP pool demonstrated comparable c-di-GMP levels in the three strains (Fig. 6), with a small but statistically significant increase in the c-di-GMP level in the Δ*pqsA* mutant compared to that in the wild-type strain. There was no significant difference between the wild-type strain and the Δ*phz* mutant. Therefore, our data suggest that derepression of swarming motility in the Δ*pqsA* and Δ*phz* mutants is independent of a decrease in the cellular level of c-di-GMP.

DISCUSSION

In this report, we sought to study the regulation of swarming motility in *Pseudomonas aeruginosa*. We exploited arginine-supplemented swarm medium, which represses swarming, to perform a transposon mutagenesis screen to identify mutants derepressed for swarming motility, and a candidate *pqsA*::Tn mutant was identified. Subsequent genetic analyses demonstrated a role for HHQ in repression of swarming motility in response to arginine. Combined microarray reanalysis, qRT-PCR, and mutational studies indicated that PCA is one can-

TABLE 4. Swarming by the Δ*phzA1-G1* Δ*phzA2-G2* mutant can be repressed by PCA-producing strains

Strain	Swarming phenotype by the Δ*phzA1-G1* Δ*phzA2-G2* mutant
WT	−
Δ*lasR* mutant	−
Δ*rhlR* mutant	−
Δ*lasR* Δ*rhlR* mutant	−
Δ*pqsA* mutant	+
Δ*pqsB* mutant	+
Δ*pqsD* mutant	+
Δ*pqsE* mutant	−
Δ*pqsH* mutant	−
Δ*pqsR* mutant	+
Δ*phnAB* mutant	+

[a] −, negative swarming phenotype (e.g., no swarming); +, positive swarming phenotype. Phenotypes were scored based on observed tendril formation on at least five replicate plates.

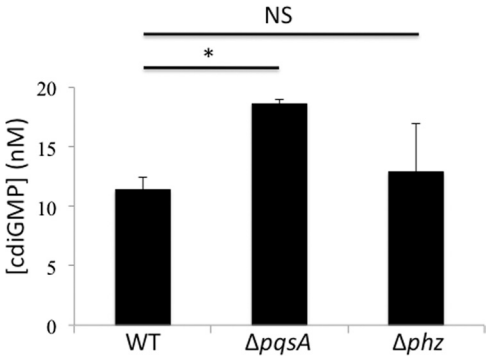

FIG. 6. Mutations in PQS and phenazine biosynthesis do not reduce c-di-GMP levels. Quantification of the global intracellular pool of c-di-GMP for the wild-type strain and the Δ*pqsA* and Δ*phz* mutants measured via liquid chromatography-tandem mass spectrometry (LC-MS/MS) (±SD; $n = 6$). *, $P < 0.03$; NS, not statistically significant.

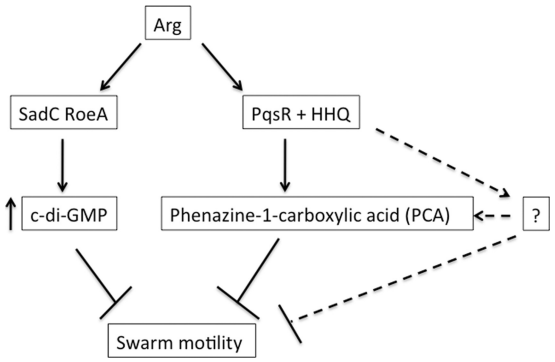

FIG. 7. Proposed model for swarming motility on arginine medium. The repression of swarming motility by arginine requires functional diguanylate cyclases (DGCs, SadC, and RoeA) as well as PqsR and HHQ (solid lines and arrows). A distinct HHQ-dependent but PCA-independent pathway is also predicted (dotted lines and arrows). Arrows and lines indicate genetic relationships only.

didate for an HHQ-dependent downstream regulator of swarm repression. Previously published studies support the possibility that HHQ, together with PqsR, likely directly positively regulates the expression of the phenazine biosynthetic genes (56). However, the mechanism by which PCA regulates swarming remains to be identified. Furthermore, our data also support the existence of an HHQ-dependent, PCA-independent pathway for repression of swarming motility. Finally, our data indicate that HHQ/PCA-mediated swarm repression is c-di-GMP independent. A model summarizing these findings is shown in Fig. 7.

Identification of HHQ-dependent downstream targets was achieved by reanalyzing a previous microarray experiment (GEO number GSE17147). We note that the strains used for this microarray experiment were cultured in LB medium, and their RNA was extracted at OD_{600s} of 2.5 (wild type and the Δ*pqsR* mutant) and 3.0 (the Δ*pqsE* mutant) (11, 28). These conditions are different from our swarm experimental conditions (M8 medium, incubated ~24 h). Therefore, we verified the expression of the microarray-derived candidate *phz* genes (*phzA* and *phzG*) using total RNA extracted from cells scraped from swarm agar plates. The Δ*pqsH* mutant, which is not capable of swarming motility on arginine medium, showed only 25% of the wild-type level for *phz* gene expression. Because the Δ*pqsH* mutant lacks the monooxygenase for HHQ-to-PQS conversion (12, 46), the remaining expression of the *phz* genes can be attributed to HHQ, and this residual level of *phz* gene expression is apparently sufficient to maintain repression of swarming motility under these conditions. Mutants that either fail to produce HHQ (the Δ*pqsA* mutant) or cannot respond to HHQ (the Δ*pqsR* mutant) showed very low relative expression of the *phz* genes compared to that of the wild type. Therefore, we propose that while both PQS and HHQ contribute to full expression of the *phz* genes, the HHQ-mediated expression of these genes is essential for the observed swarming phenotype. And although not explored here, we recognize that other quinolone molecules are produced via HHQ, such as 4Q-*N*-oxides (12, 15, 43, 52), which may also contribute to the expression of *phz* genes and/or repressing swarming motility on arginine. Deducing the effects of other quinolone molecules will be a topic for future investigation.

Importantly, we note that the genes in both *phz* operons share highly identical sequences (>97%) at the DNA level. Therefore, while our expression data likely reflect the sum of the expression of the two sets of *phz* genes (*phzA1-G1* and *phzA2-G2*), it is possible that the two *phz* operons differentially contribute to repression of swarming. For example, Gallagher et al. (18) showed differential expression between *phz1* and *phz2* expression, with *phz2* expressed independently of PQS and the PqsE protein, whereas *phz1* expression was dependent on both. Based on this published work and our studies, we hypothesize that PqsR/HHQ may regulate the *phz2* gene cluster.

Proteins encoded by the two *phz* operons synthesize the phenazine PCA. PCA is then converted to any of the three terminal phenazines (1-OH-PHZ, PCN, and PYO) by the activities of the PhzH, PhzM, and/or PhzS proteins (Fig. 4B). Similar to QS signals, phenazines are implicated in regulatory roles, such as impacting gene expression (13) and modulating group behaviors (14, 41). In fact, Ramos et al. (41) recently showed that the Δ*phz* mutant was a hyperswarmer on a minimal glucose medium and, furthermore, that the addition of 100 μM exogenous PCA could partially repress the hyperswarming phenotype of this mutant. Our work presented here is consistent with these findings. Interestingly, the Δ*phz* mutant showed a hyperswarming phenotype compared to the wild type on glucose medium (41) (data not shown) as well as on arginine medium (Fig. 4), indicating that phenazines like PCA may modulate swarming motility in a number of environments. We are currently focused on identifying the potential target(s) of PCA important for controlling swarming motility.

With regard to the Δ*sadC* Δ*roeA* mutant, we previously showed that this double mutant is a hyperswarmer with a decreased global pool of c-di-GMP (33); therefore, it was possible that the HHQ-mediated swarm repression was due to a reduction in intracellular c-di-GMP levels. However, our data suggest that HHQ-dependent regulation and c-di-GMP-dependent regulation of swarm motility define distinct pathways (Fig. 6 and 7), as global c-di-GMP pools of both Δ*pqsA* and Δ*phz* mutants show levels that are similar to or higher than those of the wild-type strain. Meanwhile, we see no consistent

or significant changes in *phz* gene expression in the wild type versus the Δ*sadC* Δ*roeA* double mutant (data not shown). In sum, we believe our study supplements the growing literature demonstrating signaling and regulatory properties of HHQ and PCA (12, 15, 43, 52).

This report originated from investigating the effects of arginine on swarming motility. Using arginine-supplemented swarm medium, we demonstrated that HHQ, which can be converted to PQS by the PqsH protein, is a modulator of swarm motility. Previous studies demonstrated an ~5-fold-higher PQS production level when *P. aeruginosa* PA14 was grown in a CF sputum specimen than in a standard laboratory (MOPS) medium (38). In the synthetic CF sputum medium (SCFM) described by Palmer and colleagues (37), *P. aeruginosa* PA14 also produced ~4-fold more PQS than in MOPS medium (37, 38). Thus, HHQ levels may be higher in the CF lung or CF-like environmental conditions, thereby contributing to the repression of swarm motility. Furthermore, phenazine levels were also shown to be elevated in *P. aeruginosa* grown in SCFM (37, 38), which, based on our findings, would likely repress swarm motility. Arginine is present in the CF lung at ~0.3 mM (37); therefore, the arginine-dependent, HHQ- and phenazine-mediated repression of swarming motility may in part explain why *P. aeruginosa* in the CF lung might favor biofilm formation over swarming motility.

ACKNOWLEDGMENTS

We thank D. Hogan and D. K. Newman for providing mutant strains and D. Hogan for advice and discussions in preparing the manuscript. We also thank the MSU Mass Spectrometry Facility for LC-MS/MS measurements.

This work was supported by NIH grant number R01A1003256 to G.A.O. J.T.H. is supported by an MRC strategic priority studentship awarded to M.W. and D.R.S. Work in the lab of M.W. is supported by the United Kingdom BBSRC.

REFERENCES

1. **Bernier, S. P., D. G. Ha, W. Khan, J. H. Merritt, and G. A. O'Toole.** 2011. Modulation of *Pseudomonas aeruginosa* surface-associated group behaviors by individual amino acids through c-di-GMP signaling. Res. Microbiol. **162:** 680–688.
2. **Burke, D., D. Dawson, and T. Stearns.** 2000. Methods in yeast genetics: a Cold Spring Harbor Laboratory course manual. Cold Spring Harbor Laboratory Press, Plainview, NY.
3. **Caetano-Anolles, G.** 1993. Amplifying DNA with arbitrary oligonucleotide primers. PCR Methods Appl. **3:**85–94.
4. **Caiazza, N. C., J. H. Merritt, K. M. Brothers, and G. A. O'Toole.** 2007. Inverse regulation of biofilm formation and swarming motility by *Pseudomonas aeruginosa* PA14. J. Bacteriol. **189:**3603–3612.
5. **Caiazza, N. C., R. M. Shanks, and G. A. O'Toole.** 2005. Rhamnolipids modulate swarming motility patterns of *Pseudomonas aeruginosa*. J. Bacteriol. **187:**7351–7361.
6. **Calfee, M. W., J. G. Shelton, J. A. McCubrey, and E. C. Pesci.** 2005. Solubility and bioactivity of the *Pseudomonas* quinolone signal are increased by a *Pseudomonas aeruginosa*-produced surfactant. Infect. Immun. **73:**878–882.
7. **Camilli, A., and B. L. Bassler.** 2006. Bacterial small-molecule signaling pathways. Science **311:**1113–1116.
8. **Cao, H., et al.** 2001. A quorum sensing-associated virulence gene of *Pseudomonas aeruginosa* encodes an LysR-like transcription regulator with a unique self-regulatory mechanism. Proc. Natl. Acad. Sci. U. S. A. **98:**14613–14618.
9. **Choi, K. H., et al.** 2005. A Tn*7*-based broad-range bacterial cloning and expression system. Nat. Methods **2:**443–448.
10. **Cugini, C., D. K. Morales, and D. A. Hogan.** 2010. *Candida albicans*-produced farnesol stimulates *Pseudomonas* quinolone signal production in LasR-defective *Pseudomonas aeruginosa* strains. Microbiology **156:**3096–3107.
11. **Deziel, E., et al.** 2005. The contribution of MvfR to *Pseudomonas aeruginosa* pathogenesis and quorum sensing circuitry regulation: multiple quorum sensing-regulated genes are modulated without affecting *lasRI*, *rhlRI* or the production of *N*-acyl-L-homoserine lactones. Mol. Microbiol. **55:**998–1014.
12. **Deziel, E., et al.** 2004. Analysis of *Pseudomonas aeruginosa* 4-hydroxy-2-alkylquinolines (HAQs) reveals a role for 4-hydroxy-2-heptylquinoline in cell-to-cell communication. Proc. Natl. Acad. Sci. U. S. A. **101:**1339–1344.
13. **Dietrich, L. E., A. Price-Whelan, A. Petersen, M. Whiteley, and D. K. Newman.** 2006. The phenazine pyocyanin is a terminal signalling factor in the quorum sensing network of *Pseudomonas aeruginosa*. Mol. Microbiol. **61:** 1308–1321.
14. **Dietrich, L. E., T. K. Teal, A. Price-Whelan, and D. K. Newman.** 2008. Redox-active antibiotics control gene expression and community behavior in divergent bacteria. Science **321:**1203–1206.
15. **Diggle, S. P., et al.** 2007. The *Pseudomonas aeruginosa* 4-quinolone signal molecules HHQ and PQS play multifunctional roles in quorum sensing and iron entrapment. Chem. Biol. **14:**87–96.
16. **Dubern, J. F., and S. P. Diggle.** 2008. Quorum sensing by 2-alkyl-4-quinolones in *Pseudomonas aeruginosa* and other bacterial species. Mol. Biosyst. **4:**882–888.
17. **Farrow, J. M., III, et al.** 2008. PqsE functions independently of PqsR-*Pseudomonas* quinolone signal and enhances the *rhl* quorum-sensing system. J. Bacteriol. **190:**7043–7051.
18. **Gallagher, L. A., S. L. McKnight, M. S. Kuznetsova, E. C. Pesci, and C. Manoil.** 2002. Functions required for extracellular quinolone signaling by *Pseudomonas aeruginosa*. J. Bacteriol. **184:**6472–6480.
19. **Harmsen, M., L. Yang, S. J. Pamp, and T. Tolker-Nielsen.** 2010. An update on *Pseudomonas aeruginosa* biofilm formation, tolerance, and dispersal. FEMS Immunol. Med. Microbiol. **59:**253–268.
20. **Hogan, D. A., and R. Kolter.** 2002. *Pseudomonas-Candida* interactions: an ecological role for virulence factors. Science **296:**2229–2232.
21. **Hogan, D. A., A. Vik, and R. Kolter.** 2004. A *Pseudomonas aeruginosa* quorum-sensing molecule influences *Candida albicans* morphology. Mol. Microbiol. **54:**1212–1223.
22. **Huse, H., and M. Whiteley.** 2011. 4-Quinolones: smart phones of the microbial world. Chem. Rev. **111:**152–159.
23. **Irizarry, R. A., et al.** 2003. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics **4:**249–264.
24. **Kohler, T., L. K. Curty, F. Barja, C. van Delden, and J. C. Pechere.** 2000. Swarming of *Pseudomonas aeruginosa* is dependent on cell-to-cell signaling and requires flagella and pili. J. Bacteriol. **182:**5990–5996.
25. **Kuchma, S. L., et al.** 2010. Cyclic-di-GMP-mediated repression of swarming motility by *Pseudomonas aeruginosa*: the *pilY1* gene and its impact on surface-associated behaviors. J. Bacteriol. **192:**2950–2964.
26. **Kuchma, S. L., et al.** 2007. BifA, a cyclic-di-GMP phosphodiesterase, inversely regulates biofilm formation and swarming motility by *Pseudomonas aeruginosa* PA14. J. Bacteriol. **189:**8165–8178.
27. **Kulasakara, H., et al.** 2006. Analysis of *Pseudomonas aeruginosa* diguanylate cyclases and phosphodiesterases reveals a role for *bis*-(3′-5′)-cyclic-GMP in virulence. Proc. Natl. Acad. Sci. U. S. A. **103:**2839–2844.
28. **Lesic, B., M. Starkey, J. He, R. Hazan, and L. G. Rahme.** 2009. Quorum sensing differentially regulates *Pseudomonas aeruginosa* type VI secretion locus I and homologous loci II and III, which are required for pathogenesis. Microbiology **155:**2845–2855.
29. **Liberati, N. T., et al.** 2006. An ordered, nonredundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants. Proc. Natl. Acad. Sci. U. S. A. **103:**2833–2838.
30. **Mashburn, L. M., and M. Whiteley.** 2005. Membrane vesicles traffic signals and facilitate group activities in a prokaryote. Nature **437:**422–425.
31. **McGrath, S., D. S. Wade, and E. C. Pesci.** 2004. Dueling quorum sensing systems in *Pseudomonas aeruginosa* control the production of the Pseudomonas quinolone signal (PQS). FEMS Microbiol. Lett. **230:**27–34.
32. **Merritt, J. H., K. M. Brothers, S. L. Kuchma, and G. A. O'Toole.** 2007. SadC reciprocally influences biofilm formation and swarming motility via modulation of exopolysaccharide production and flagellar function. J. Bacteriol. **189:**8154–8164.
33. **Merritt, J. H., et al.** 2010. Specific control of *Pseudomonas aeruginosa* surface-associated behaviors by two c-di-GMP diguanylate cyclases. mBio **1**(4)**:** e00183–10. doi:10.1128/mBio.00183-10.
34. **Newell, P. D., S. Yoshioka, K. L. Hvorecny, R. D. Monds, and G. A. O'Toole.** 2011. A systematic analysis of diguanylate cyclases that promote biofilm formation by *Pseudomonas fluorescens* Pf0-1. J. Bacteriol. **193:**4685–4698.
35. **Ochsner, U. A., J. Reiser, A. Fiechter, and B. Witholt.** 1995. Production of *Pseudomonas aeruginosa* rhamnolipid biosurfactants in heterologous hosts. Appl. Environ. Microbiol. **61:**3503–3506.
36. **O'Toole, G. A., and R. Kolter.** 1998. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Mol. Microbiol. **28:**449–461.
37. **Palmer, K. L., L. M. Aye, and M. Whiteley.** 2007. Nutritional cues control *Pseudomonas aeruginosa* multicellular behavior in cystic fibrosis sputum. J. Bacteriol. **189:**8079–8087.
38. **Palmer, K. L., L. M. Mashburn, P. K. Singh, and M. Whiteley.** 2005. Cystic fibrosis sputum supports growth and cues key aspects of *Pseudomonas aeruginosa* physiology. J. Bacteriol. **187:**5267–5277.
39. **Price-Whelan, A., L. E. Dietrich, and D. K. Newman.** 2007. Pyocyanin alters

redox homeostasis and carbon flux through central metabolic pathways in *Pseudomonas aeruginosa* PA14. J. Bacteriol. **189:**6372–6381.
40. **Rahme, L. G., et al.** 1995. Common virulence factors for bacterial pathogenicity in plants and animals. Science **268:**1899–1902.
41. **Ramos, I., L. E. Dietrich, A. Price-Whelan, and D. K. Newman.** 2010. Phenazines affect biofilm formation by *Pseudomonas aeruginosa* in similar ways at various scales. Res. Microbiol. **161:**187–191.
42. **Rampioni, G., et al.** 2010. Transcriptomic analysis reveals a global alkylquinolone-independent regulatory role for PqsE in facilitating the environmental adaptation of *Pseudomonas aeruginosa* to plant and animal hosts. Environ. Microbiol. **12:**1659–1673.
43. **Reen, F. J., et al.** 2011. The *Pseudomonas* quinolone signal (PQS), and its precursor HHQ, modulate interspecies and interkingdom behaviour. FEMS Microbiol. Ecol. **77:**413–428.
44. **Reimmann, C., et al.** 2002. Genetically programmed autoinducer destruction reduces virulence gene expression and swarming motility in *Pseudomonas aeruginosa* PAO1. Microbiology **148:**923–932.
45. **Sanderson, K., L. Wescombe, S. M. Kirov, A. Champion, and D. W. Reid.** 2008. Bacterial cyanogenesis occurs in the cystic fibrosis lung. Eur. Respir. J. **32:**329–333.
46. **Schertzer, J. W., S. A. Brown, and M. Whiteley.** 2010. Oxygen levels rapidly modulate *Pseudomonas aeruginosa* social behaviours via substrate limitation of PqsH. Mol. Microbiol. **77:**1527–1538.
47. **Shanks, R. M., N. C. Caiazza, S. M. Hinsa, C. M. Toutain, and G. A. O'Toole.** 2006. *Saccharomyces cerevisiae*-based molecular tool kit for manipulation of genes from Gram-negative bacteria. Appl. Environ. Microbiol. **72:**5027–5036.
48. **Simon, R., U. Priefer, and A. Puhler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. Nat. Biotechnol. **1:**784–791.
49. **Vander Wauven, C., A. Pierard, M. Kley-Raymann, and D. Haas.** 1984. *Pseudomonas aeruginosa* mutants affected in anaerobic growth on arginine: evidence for a four-gene cluster encoding the arginine deiminase pathway. J. Bacteriol. **160:**928–934.
50. **Wade, D. S., et al.** 2005. Regulation of *Pseudomonas* quinolone signal synthesis in *Pseudomonas aeruginosa*. J. Bacteriol. **187:**4372–4380.
51. **Wang, Y., S. E. Kern, and D. K. Newman.** 2010. Endogenous phenazine antibiotics promote anaerobic survival of *Pseudomonas aeruginosa* via extracellular electron transfer. J. Bacteriol. **192:**365–369.
52. **Wang, Y., et al.** 2011. Phenazine-1-carboxylic acid promotes bacterial biofilm development via ferrous iron acquisition. J. Bacteriol. **193:**3606–3617.
53. **Williams, P., and M. Camara.** 2009. Quorum sensing and environmental adaptation in *Pseudomonas aeruginosa*: a tale of regulatory networks and multifunctional signal molecules. Curr. Opin. Microbiol. **12:**182–191.
54. **Woodman, M. E.** 2008. Direct PCR of intact bacteria (colony PCR). Curr. Protoc. Microbiol. **9:**A.3D.1–A.3D.6.
55. **Worlitzsch, D., et al.** 2002. Effects of reduced mucus oxygen concentration in airway *Pseudomonas* infections of cystic fibrosis patients. J. Clin. Invest. **109:**317–325.
56. **Xiao, G., et al.** 2006. MvfR, a key *Pseudomonas aeruginosa* pathogenicity LTTR-class regulatory protein, has dual ligands. Mol. Microbiol. **62:**1689–1699.
57. **Xiao, G., J. He, and L. G. Rahme.** 2006. Mutation analysis of the *Pseudomonas aeruginosa mvfR* and *pqsABCDE* gene promoters demonstrates complex quorum-sensing circuitry. Microbiology **152:**1679–1686.