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The Stringent Response Modulates 4-Hydroxy-2-Alkylquinoline Biosynthesis and Quorum-Sensing Hierarchy in *Pseudomonas aeruginosa*

James Schafhauser,[a] Francois Lepine,[b] Geoffrey McKay,[c] Heather G. Ahlgren,[c] Malika Khakimova,[a] Dao Nguyen[a,c]

Department of Microbiology and Immunology, McGill University, Montreal, Canada[a]; INRS-Institut Armand Frappier, Laval, Canada[b]; McGill University Health Center Research Institute, Montreal, Canada[c]

As a ubiquitous environmental organism and an important human pathogen, *Pseudomonas aeruginosa* readily adapts and responds to a wide range of conditions and habitats. The intricate regulatory networks that link quorum sensing and other global regulators allow *P. aeruginosa* to coordinate its gene expression and cell signaling in response to different growth conditions and stressors. Upon nutrient transitions and starvation, as well as other environmental stresses, the stringent response is activated, mediated by the signal (p)ppGpp. *P. aeruginosa* produces a family of molecules called HAQ (4-hydroxy-2-alkylquinolines), some of which exhibit antibacterial and quorum-sensing signaling functions and regulate virulence genes. In this study, we report that (p)ppGpp negatively regulates HAQ biosynthesis: in a (p)ppGpp-null (ΔSR) mutant, HHQ (4-hydroxyl-2-heptylquinoline) and PQS (3,4-dihydroxy-2-heptylquinoline) levels are increased due to upregulated *pqsA* and *pqsR* expression and reduced repression by the *rhl* system. We also found that (p)ppGpp is required for full expression of both *rhl* and *las* AHL (acyl-homoserine lactone) quorum-sensing systems, since the ΔSR mutant has reduced *rhlI*, *rhlR*, *lasI*, and *lasR* expression, butanoyl-homoserine lactone (C_4-HSL) and 3-oxo-dodecanoyl-homoserine lactone (3-oxo-C_{12}-HSL) levels, and rhamnolipid and elastase production. Furthermore, (p)ppGpp significantly modulates the AHL and PQS quorum-sensing hierarchy, as the *las* system no longer has a dominant effect on HAQ biosynthesis when the stringent response is inactivated.

Pseudomonas aeruginosa is ubiquitous in the natural environment but is also an important opportunistic pathogen capable of causing severe human infections. This includes wound infections, hospital-acquired pneumonia, and chronic airway infections in individuals with the genetic disease cystic fibrosis. In order to survive in this wide range of habitats, from the soil to the human lung, *P. aeruginosa* must be capable of adapting to stress-inducing and changing environments. Key to its success are the large number of regulators encoded in its genome and an intricate regulatory network that allows *P. aeruginosa* to coordinate its gene expression and cell signaling in response to environmental conditions (1).

The stringent response is a conserved regulatory mechanism mediated by the alarmone (p)ppGpp (penta- and tetra-phosphorylated guanosine) that coordinates physiological adaptations to nutrient transitions and starvation (e.g., amino acid and carbon) and other environmental stresses. In Gram-negative bacteria, (p)ppGpp is synthesized by RelA (a ribosome-dependent synthetase) and SpoT (a bifunctional synthetase and hydrolase). (p)ppGpp primarily modulates gene transcription with widespread physiologic effects, including downregulation of the translational machinery, cell replication, modulation of metabolic processes, and induction of mechanisms required for stress survival (2, 3). To date, studies of the stringent response in *P. aeruginosa* have reported a role in virulence (4, 5), resistance to UV radiation (6), and regulation of oxidative defenses (7) and Usp universal stress proteins (8).

We recently demonstrated that the stringent response mediates antibiotic tolerance in nutrient-starved and biofilm *P. aeruginosa* (9). This led us to discover that the (p)ppGpp-null (ΔSR) mutant, in which *relA* and *spoT* are inactivated, accumulates high levels of HHQ (4-hydroxy-2-heptylquinoline) and PQS (3,4-dihydroxy-2-heptylquinoline, or pseudomonas quinolone signal), which are two HAQ (4-hydroxy-2-alkylquinoline) molecules. Interestingly, the ΔSR mutant is highly sensitive to killing by multiple classes of antibiotics, and abolishing HAQ biosynthesis restores antibiotic tolerance (9), suggesting that proper regulation of HAQ biosynthesis is key to the bacteria's survival under antibiotic stress.

HAQs are a family of secondary metabolites sharing a common quinoline ring. These molecules have diverse biological properties and have been implicated in interspecies interactions and competition (10–12), iron chelation (13, 14), colony morphology (15), biofilm formation (16), and virulence (17–19). PQS is the best-characterized HAQ molecule, which functions as the third quorum-sensing signal in *P. aeruginosa* cell-cell communication (20, 21). When bound to its cognate transcriptional regulator PqsR (also called MvfR), PQS controls the expression of nearly 200 genes involved in virulence, biofilm formation, and antimicrobial defenses (17, 22–24). HHQ, the precursor to PQS, can also bind to and activate PqsR, although with much lower affinity than PQS (25, 26). HQNO (4-hydroxy-2-heptylquinoline-*N*-oxide), has potent activity against Gram-positive bacteria (10, 20, 27). These three molecules constitute the dominant HAQ molecules pro-

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Address correspondence to Dao Nguyen, dao.nguyen@mcgill.ca.
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TABLE 1 Bacterial strains

Strain	Relevant characteristic(s)[a]	Source or reference
WT	Wild-type *P. aeruginosa* PAO1	B. Iglewski
ΔSR	Δ*relA* (Δ181–2019) Δ*spoT* (Δ200–1948) unmarked deletion mutant in PAO1 background	9
+SR	ΔSR mutant complemented for *relA* (attCTX::*relA*) and *spoT* (attTn*7*::*spoT*); Tc^r Gm^r	9
DN759	PAO1 *lasR* mutant with *lasR*-B10::IS*lacZ*/*hah* allele from PW3598; Tc^r	This study
DN760	ΔSR *lasR* mutant with *lasR*-B10 IS*lacZ*/*hah* allele from PW3598; Tc^r	This study
DN773	*lasI* unmarked deletion mutant (Δ90–572) in PAO1 background	This study
DN774	*lasI* unmarked deletion mutant (Δ90–572) in ΔSR background	This study
rhlA	PAO1 *rhlA* mutant with *rhlA*-A01::IS*phoA*/*hah* allele from PW6887; Tc^r	42

[a] Tc, tetracycline; Gm, gentamicin.

duced by *P. aeruginosa*, while many of the other HAQs are produced at low levels (28).

HAQ biosynthesis requires the biosynthetic genes *pqsA*, *pqsB*, *pqsC*, and *pqsD* (but not *pqsE*) located within the *pqsABCDE* operon (20, 29–31). *pqsH* and *pqsL*, located at separate chromosomal locations, are involved in the conversion of HHQ to PQS, and synthesis of HQNO, respectively (19, 28). PqsR (MvfR) is the primary positive regulator of the *pqsABCDE* operon: PqsR directly and strongly binds to the *pqsA* promoter in the presence of PQS, and to a lesser degree HHQ (14, 20, 22). HAQ biosynthesis is also regulated by the acyl-homoserine lactone (AHL) quorum-sensing systems *las* and *rhl*, each composed of a LuxI signal synthase (LasI, producing 3-oxo-C_{12}-HSL, and RhlI, producing C_4-HSL) and its cognate LuxR transcriptional regulator (LasR and RhlR) (21, 25, 32, 33). While earlier studies suggested that the *las* system is at the top of the quorum-sensing hierarchy, as it regulates both *rhl* and PQS systems (21, 34, 35), the interplay between these signaling networks is more complex, integrating a range of environmental and metabolic signals, as well as many other regulators (36–40). Both *las* and *rhl* signaling control HAQ biosynthesis at multiple levels, regulating *pqsA*, *pqsR*, and *pqsH* expression through a complex regulatory network.

Erickson et al. and Van Delden et al. previously reported a link between the stringent response and quorum sensing signaling (5, 41). While the studies by Van Delden et al. (41) showed that (p)ppGpp accumulation was sufficient to activate *las* and *rhl* quorum-sensing signaling independently of cell density, the studies by Erickson et al. (5) using a *relA* single mutant did not conclusively demonstrate the contribution of the stringent response. In light of our recent observations that HAQs are dysregulated in (p)ppGpp-deficient mutants, we set out to dissect how (p)ppGpp regulates HAQ biosynthesis and to define the relationship between the stringent response, PqsR, and the *las* and *rhl* quorum-sensing systems.

MATERIALS AND METHODS

Bacterial strains. The strains and plasmids used in this study are listed in Tables 1 and 2. The *pqsR* and *lasR* transposon mutants were obtained from the PAO1 two-allele transposon mutant library (42). To create the single mutants in the isogenic wild-type PAO1 parental strain and the corresponding ΔSR triple mutants, the transposon mutant alleles were moved by transformation with the respective transposon mutant genomic DNA as previously described (43). The mutations were selected using the tetracycline resistance marker and were confirmed by PCR as previously de-

TABLE 2 Plasmids

Plasmids	Relevant characteristics[a]	Source or reference
pTSL01	*pqsA-lacZ* transcriptional fusion containing a 716-bp fragment of the *pqsA* promoter region cloned into pUC18-miniTn*7*T-Gm-*lacZ*; Gm^r	E. Deziel
pGX1	*pqsR-lacZ* transcriptional fusion containing a 906-bp fragment of the *pqsR* promoter region cloned into pQF50; Cb^r	24
pML03	*pqsH-lacZ* transcriptional fusion containing a 512-bp fragment of the *pqsH* promoter region cloned into miniCTX2-*lacZ*; Tc^r	74
pLPRI	*rhlI-lacZ* transcriptional fusion containing 723 bp of the *rhlI* promoter region cloned into pLP170; Ap^r	75
pPCS223	*lasI-lacZ* transcriptional fusion containing 288 bp of the *lasI* promoter region cloned into pLP170; Ap^r	75
pPCS1001	*lasR-lacZ* transcriptional fusion containing 395 bp of the *lasR* promoter region cloned into pLP170; Ap^r	34
pPCS1002	*rhlR-lacZ* transcriptional fusion containing 500 bp of the *rhlR* promoter region cloned into pLP170; Ap^r	34
pDJMK3	*rhlR* overexpression construct containing *araC-pBAD-rhlR* cloned into mini-CTX2T2.1-GW vector; Tc^r	This study
pSC301	pEXG2 based suicide vector with unmarked deletion of *lasI* from +90 to 572 bp.	E.P. Greenberg
pJJH187	pDONR221P1P5r containing a 1,192-bp fragment encoding the *araC* gene and the *pBAD* promoter, Km^r	7
pDJRhlR1	pDONR221P5P2 containing a 726-bp fragment encoding a synthetic ribosomal binding site and PAO1 *rhlR* ORF, Km^r	This study
miniCTX2-promoterless-*lacZ*	Mini-CTX2 vector containing a promoterless *lacZ* reporter for chromosomal insertion at the *attB* site; Tc^r	45
pUC18-miniTn*7*T-Gm-*lacZ*	Mini-Tn*7*T-based promoterless *lacZ* transcriptional reporter for chromosomal insertion at the *glmS* site; Gm^r	46
pQF50	Replicative plasmid containing promoterless *lacZ* transcriptional reporter; Cb^r	76
pLP170	Replicative plasmid containing promoterless *lacZ* transcriptional reporter; Ap^r	77
pDONR221P1P5r	Multisite Gateway donor vector with attP1 and attP5r recombination sites; Cm^r Km^r	Invitrogen
pDONR221P5P2	Multisite Gateway donor vector with attP5 and attP2 recombination sites; Cm^r Km^r	Invitrogen
mini-CTX2T2.1-GW	Mini-CTX2 with a Gateway destination cloning site; *oriT1 int lacI ccdB cat aatR* attR2; Tc^r	7
pTNS2	Helper plasmid expressing *tnsABCD*; Ap^r	78

[a] Tc, tetracycline; Km, kanamycin; Gm, gentamicin; Cb, carbenicillin, Ap, ampicillin; Cm, chloramphenicol.

scribed (42). To create the *lasI* deletion strains by allelic exchange, the suicide plasmid pSC301 containing a mutant *lasI* allele construct was used as previously described (44). The pSC301 plasmid was mated into *P. aeruginosa* strains, and the transconjugants were selected on VBMM (Vogel-Bonner minimal medium) with gentamicin resistance. Double-recombinant *lasI* mutants were subsequently selected on LB agar with 10% sucrose and confirmed by PCR.

Plasmids and vector construction. Molecular biology procedures were carried out by standard methods, and all primers are listed in Table S1 in the supplemental material. To overexpress *rhlR*, we generated a chromosomally integrated construct with the mini-CTX2T2.1-GW vector (7) using the multisite Gateway technology (Invitrogen). The PAO1 *rhlR* ORF was amplified by PCR (using primers *rhlR*gatep3 and *rhlR*gatep2), and this was recombined into pDONR221P5P2 using BP clonase (Invitrogen), generating the entry vector pDJRhlR1. Subsequently, the entry vectors pDJRhlR1 and pJJH187 (containing the *araC-pBAD* promoter) were recombined with the destination vector mini-CTX2T2.1-GW using LR Clonase II Plus (Invitrogen) to generate the arabinose-inducible *rhlR* overexpression construct pDJMK3. Finally, the pDJMK3 construct was integrated into the *P. aeruginosa* chromosome at the *attB* sites by electroporation. Integrants were selected for tetracycline resistance and confirmed by PCR as previously described (45). Empty mini-CTX2T2.1-GW vectors were integrated as vector controls. M13 primers were used to sequence and confirm the pDJRhlR1 and pJJH187 plasmids. Transcriptional reporter fusions using the mini-Tn*7*-Gm-*lacZ* vectors were integrated into the *P. aeruginosa* chromosome by electroporation with the helper plasmid pTNS2. Integrants were selected for gentamicin resistance, and insertion of the vector at the *glmS* site was confirmed by PCR as previously described (46).

Bacterial growth conditions. All bacteria were grown in LB-Miller medium (Difco) at 37°C unless otherwise specified. Overnight planktonic cultures were diluted to an initial optical density at 600 nm (OD_{600}) of 0.05 and grown in 150-ml flasks filled to ~1/10 total volume in order to allow maximum aeration while shaking at 250 rpm. Antibiotics were used at the following concentrations for selection where appropriate: ampicillin at 100 μg/ml, kanamycin at 50 μg/ml, carbenicillin at 250 μg/ml, gentamicin at 10 μg/ml (*Escherichia coli*) or 50 μg/ml (*P. aeruginosa*), and tetracycline at 10 μg/ml (*E. coli*) or 90 μg/ml (*P. aeruginosa*). For *rhlR* overexpression, the inducer L-(+)-arabinose (1% [wt/vol]) (Sigma), C_4-HSL (final concentration, 10 μM) (Sigma), or dimethyl sulfoxide (DMSO; final concentration, 0.1%) was added as required.

LC-MS measurements. Bacteria were grown in LB medium as described above. For HAQ extraction, bacterial cultures were mixed with methanol (1:1) and then centrifuged for 15 min at 13,000 rpm. HAQ concentrations were measured by liquid chromatography-mass spectrometry (LC-MS) as previously described, with modifications (47). Briefly, 10 μl of HHQ-d4 (5,6,7,8,-tetradeutero-HHQ) and PQS-d4 (5,6,7,8,-tetradeutero-PQS) (500- to 1,000-μg/ml stock in methanol) were added to 500-μl samples as internal standards, and samples were stored at −20°C until analysis. A 10-μl portion of each sample was injected onto a 4.6- by 150-mm HP Eclipse XDB-C8 reverse-phase column in a Waters 2795 separation module (Waters Corporation) coupled to a Quattro Premier XE mass spectrometer (Micromass Technologies) in positive electrospray ionization mode. Tandem mass spectrometry (MS/MS) was performed for molecular confirmation of HAQs, and quantification of HAQs was performed in full scan mode using HHQ-d4 and PQS-d4 as internal standards. Results were analyzed using the MassLynx MS software v4.1 (Waters Corporation). HAQ concentrations were normalized to the bacterial protein concentration. All results are the means from at least three independent replicates.

For AHL extraction, 10-ml bacterial cultures were mixed with 2 ml of ethyl acetate and 2.4 μg HHQ-d4 as an internal standard and then centrifuged at 7,800 rpm for 25 min. The top organic layer was carefully removed and evaporated in a glass vial under continuous high airflow in a heat block at 37°C. The ethyl acetate extraction was repeated three times for each sample. After complete evaporation, samples were stored at −20°C until analysis. AHL concentrations were measured by LC-MS as previously described with modifications (47). Briefly, for analysis, samples were resuspended in 600 μl of acetonitrile, vortexed for 15 s, sonicated for 5 min, and vortexed again for 15 s. A 10-μl portion of each sample was injected onto a 4.6- by 100-mm Phenomenex Kinetex C_8 column in a Waters 2795 separation module (Waters Corporation) coupled to a Quattro Premier XE (Micromass Technologies) in positive electrospray ionization mode. A calibration curve was generated to obtain the response factor of C_4-HSL and 3-oxo-C_{12}-HSL relative to HHQ-d4. The AHL concentration was calculated based on the response factor and using the HHQ-d4 internal standard and was normalized to the bacterial protein concentration. All results are the means from at least three independent replicates.

Protein extraction and quantification. Bacteria were pelleted by centrifugation and resuspended in 2 N NaOH for alkali and heat lysis. The protein concentration was measured using the Bio-Rad protein assay (Bio-Rad) according to the manufacturer's instructions, with bovine serum albumin as a standard. All samples were measured in triplicates, and the average values were used.

β-Galactosidase activity. *P. aeruginosa* transcriptional reporter strains were grown overnight with the appropriate antibiotic and then subcultured from an OD_{600} of 0.05. Strains containing chromosomally integrated constructs were subsequently grown without antibiotic, while those containing plasmid-borne constructs were grown with the appropriate antibiotic. β-Galactosidase (LacZ) activity was assayed as previously described with modifications, and background activity (from a promoterless reporter control) was subtracted from all promoter reporter results (48). The β-galactosidase activity was normalized to the sample bacterial counts (in CFU/ml) rather than the OD_{600} and is reported as β-galactosidase activity per 10^8 CFU. This was done to ensure that viable cell counts were accurately measured in late-stationary-phase cultures. Viable CFU counts were measured by standard microdilution techniques. All results are the means from at least three independent replicates.

Rhamnolipid and elastase assays. Rhamnolipid production was assessed after bacterial growth on agar plates containing methylene blue and cetyl trimethylammonium bromide (CTAB) as previously described with minor modifications (49). After 10 μl of overnight culture was spotted on the agar surface, agar plates were incubated overnight at 37°C and then for 24 h at 30°C. Elastase activity was measured as previously described with minor modifications (50). Five hundred microliters of Elastin Congo red (ECR) buffer (100 mM Tris-Cl, 1 mM $CaCl_2$, pH 7.5) containing 5 mg/ml ECR (Sigma) was added to 100 μl of overnight cultures, and the mixture was incubated for 24 h at 37°C with shaking at 250 rpm. Samples were then centrifuged for 20 min at 4,680 rpm, and absorbance of the supernatant was measured at 492 nm.

Data analysis. Prism 5 (GraphPad Software) was used to calculate the means and standard deviations and perform statistical testing. Means were compared using the Student *t* tests.

RESULTS

HAQ biosynthesis is increased in the ΔSR mutant. The ΔSR mutant has an unusual colony morphology characterized by iridescence and autolysis (9), a phenotype previously associated with increased HHQ accumulation (51). In order to understand how the stringent response regulates HAQ biosynthesis, we first measured HAQs in the ΔSR mutant, which produces no (p)ppGpp due to inactivation of the (p)ppGpp synthases *relA* and *spoT*. For our analyses, we focused on the dominant molecules HHQ, PQS, and HQNO and measured HAQs by LC-MS following methanol extraction of whole planktonic cultures, thus including intracellular, extracellular, and membrane-associated HAQ molecules. In the wild-type strain, HHQ levels peak during stationary phase at 12 h, PQS levels peak at 24 h, and both gradually decrease in late

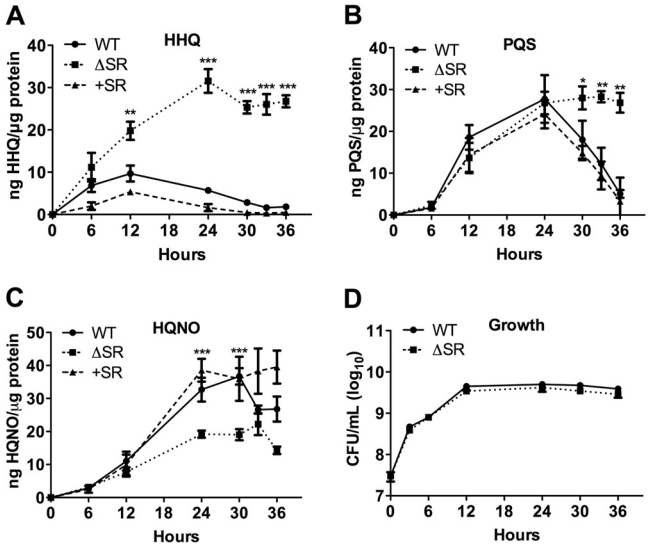

FIG 1 HAQ concentration and growth rate in the wild-type (WT), ΔSR mutant, and SR-complemented strains. (A to C) HHQ, PQS, and HQNO were measured by LC-MS in wild-type, ΔSR mutant, and SR-complemented planktonic cultures grown in LB. (D) Growth of the WT and ΔSR mutant strains in LB at 37°C with shaking at 250 rpm. Error bars represent standard deviations (SD). *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$, compared to the WT.

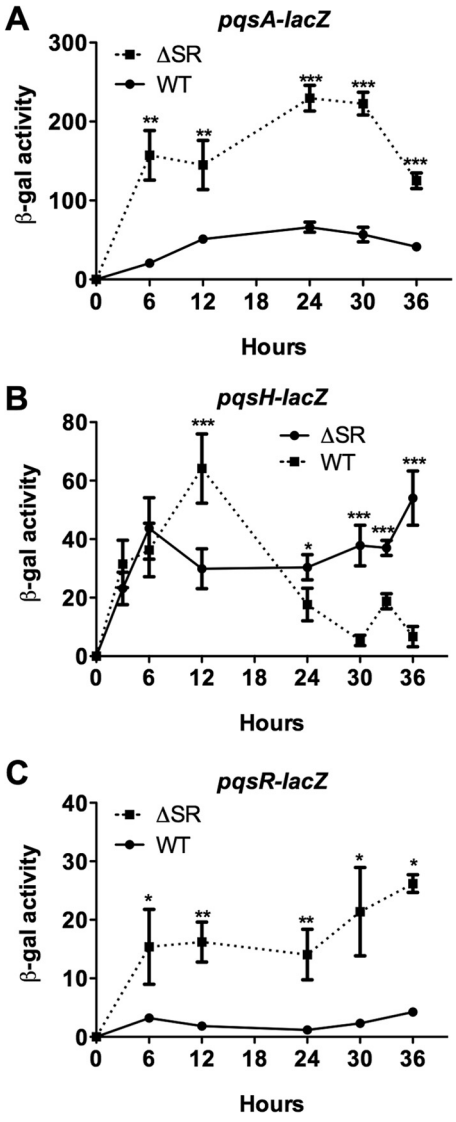

FIG 2 Expression of *pqsA*, *pqsH*, and *pqsR* are increased in the ΔSR mutant. *pqsA* (A), *pqsH* (B), and *pqsR* (C) expression in wild-type (WT) and ΔSR mutant planktonic cultures were measured using transcriptional *lacZ* reporters. β-Galactosidase activity is reported per 10^8 CFU. Error bars represent SD. *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$, compared to the WT.

stationary phase to very low values. In contrast, HHQ and PQS levels are 2- to 14-fold higher in the ΔSR mutant than in the wild type, and this difference is greatest in late stationary phase, where both HHQ and PQS are sustained at high levels (Fig. 1A and B). Interestingly, HQNO levels were slightly higher in the wild-type strain (Fig. 1C). It should also be noted that that the wild-type and ΔSR mutant strains grow at comparable rates and reach similar cell densities in LB medium (Fig. 1D). Complementation of the *relA* and *spoT* genes in the ΔSR mutant reduces HHQ and PQS to wild-type levels, thus confirming that deletion of the *relA* and *spoT* genes and absence of (p)ppGpp lead to excess HHQ and PQS accumulation.

HAQ biosynthesis is upregulated due to increased *pqsA*, *pqsH*, and *pqsR* expression in the ΔSR mutant. The *pqsABCD* genes, within the *pqsABCDE* operon, encode the primary HAQ-biosynthetic genes. PqsA is an anthranilate coenzyme A ligase that carries out the first and necessary step for HAQ biosynthesis, and HHQ synthesis requires PqsABCD (52, 79). To begin to understand how the stringent response regulates HAQ biosynthesis, we measured *pqsA* expression using a *pqsA-lacZ* promoter fusion that contains both the −339 and −71 transcriptional start sites of the *pqsABCDE* operon. During stationary phase, *pqsA-lacZ* activity is increased ~3- to 6-fold in the ΔSR mutant compared to the wild-type strain (Fig. 2A), and qRT-PCR measurements of *pqsA* transcripts confirm these observations (see Fig. S1 in the supplemental material). PQS is produced via hydroxylation of HHQ by PqsH, a NADH-dependent monooxygenase. Using a *pqsH-lacZ* promoter reporter, we found that *pqsH* expression was up to 8-fold higher during late stationary phase in the ΔSR mutant than in the wild type (Fig. 2B). The expression profiles of both *pqsA* and *pqsH* genes thus mirror HHQ and PQS levels, suggesting that increased HAQ biosynthesis in the ΔSR mutant is due to increased *pqsA* and *pqsH* transcription. Since PqsR is the primary positive regulator of *pqsA*, we also measured *pqsR* expression. As shown in Fig. 2C, highly increased *pqsR-lacZ* activity in the ΔSR mutant indicate that upregulated *pqsR* expression is an important contributor to the increased HAQ biosynthesis.

(p)ppGpp regulates HAQ biosynthesis through modulation of *rhl* quorum sensing. Previous studies have demonstrated that the *rhl* system represses *pqsA* and *pqsR* expression and HAQ biosynthesis (25, 32, 33). Given that *pqsA* expression is derepressed in the absence of (p)ppGpp, we hypothesized that this was caused by reduced *rhl* signaling. We first measured *rhlR* and *rhlI* expression using transcriptional *lacZ* reporters and found that *rhlR-lacZ* and *rhlI-lacZ* expression are reduced in the ΔSR mutant compared to the wild type (Fig. 3A and B). Furthermore, C_4-HSL levels and rhamnolipid production, an *rhl*-dependent phenotype, are decreased in the ΔSR mutant, consistent with reduced *rhl* signaling (Fig. 3C and D). We predicted that if increased *pqsA* expression is

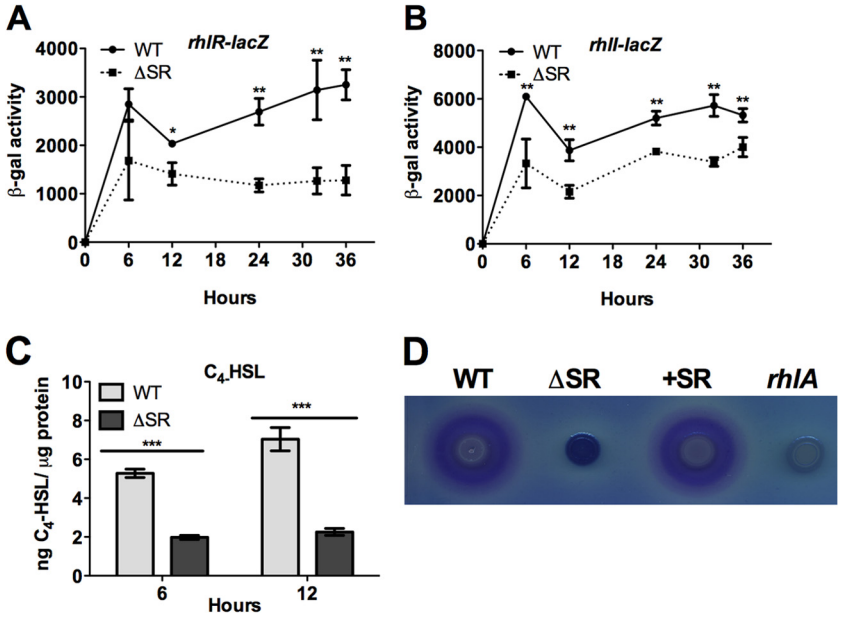

FIG 3 The *rhl* quorum sensing signaling is reduced in the ΔSR mutant. (A and B) *rhlR* and *rhlI* expression in wild-type (WT) and ΔSR mutant planktonic cultures was measured using transcriptional *lacZ* reporters. (C) Concentration of C_4-HSL in planktonic cultures was measured by LC-MS. (D) Rhamnolipid production was assessed by the blue halo surrounding colonies grown on methylene blue–CTAB plates. The *rhlA* mutant served as a negative control. β-Galactosidase activity is reported per 10^8 CFU. Error bars represent SD. *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$, compared to WT.

caused by reduced *rhl* signaling in the ΔSR mutant, then complementing the *rhl* system should restore *pqsA* expression to wild-type levels. To achieve this, we overexpressed *rhlR* under the control of an arabinose-inducible *pBAD* promoter and added exogenous C_4-HSL signal. Overexpression of the *rhl* system restores *pqsA* expression in the ΔSR mutant to wild-type levels, while *rhlR* overexpression or exogenous C_4-HSL addition alone was not sufficient (Fig. 4; also, see Fig. S2 in the supplemental material). Taken together, these results provide evidence that (p)ppGpp regulates HAQ biosynthesis by modulating the *rhl* system.

Full expression of the *las* quorum-sensing system also requires (p)ppGpp. Given that HAQ biosynthesis is under *las* quorum-sensing control, we also sought to determine whether (p)ppGpp regulates HAQ biosynthesis through *las* signaling. We first set out to measure *lasR* and *lasI* expression using transcriptional *lacZ* reporters and 3-oxo-C_{12}-HSL levels by LC-MS, and we found that expression of *lasR* and *lasI* and 3-oxo-C_{12}-HSL levels are reduced in the ΔSR mutant (Fig. 5). This is in keeping with our observations and previous reports that elastase (a LasR-regulated phenotype) is significantly reduced in (p)ppGpp-deficient mutants (Fig. 5D) (4). Given that LasR positively regulates *pqsH* and *pqsR*, reduced *las* signaling in the ΔSR mutant cannot explain its increased *pqsH* and *pqsR* expression.

(p)ppGpp modulates the AHL quorum-sensing hierarchy. The *las* signaling system is traditionally considered to be at the top of the quorum-sensing hierarchy. Most previous studies suggested that LasR positively regulates *pqsR* and that PQS synthesis is absent or significantly delayed in *lasR* mutants (23, 24, 34, 35, 53). Our observations that *pqsR* expression and HAQ biosynthesis are

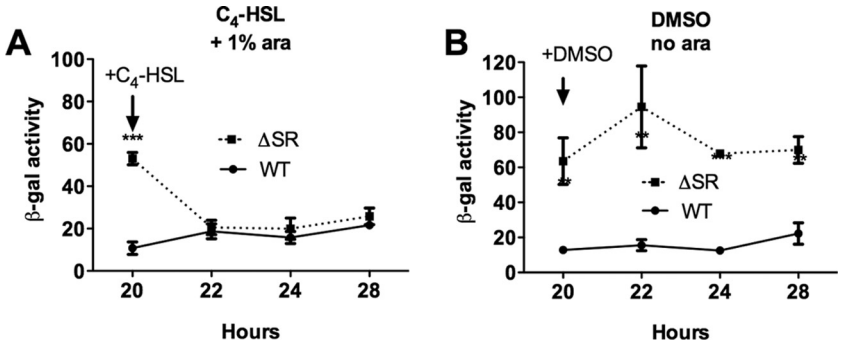

FIG 4 *pqsA* expression in the ΔSR mutant is restored to wild-type (WT) levels with *rhlR* overexpression and exogenous C_4-HSL. Expression of *pqsA* in wild-type (WT) and ΔSR mutant planktonic cultures was measured using a *pqsA-lacZ* transcriptional reporter. (A) *rhlR* was overexpressed using an arabinose-inducible *pBAD-rhlR* construct. Arabinose (1%) is added at 0 h, and exogenous C_4-HSL (final concentration, 10 μM) was added at 20 h. (B) Controls were grown without arabinose, and 0.1% DMSO (solvent control) was added at 20 h. β-Galactosidase activity is reported per 10^8 CFU/ml. Error bars represent SD. *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$, compared to WT.

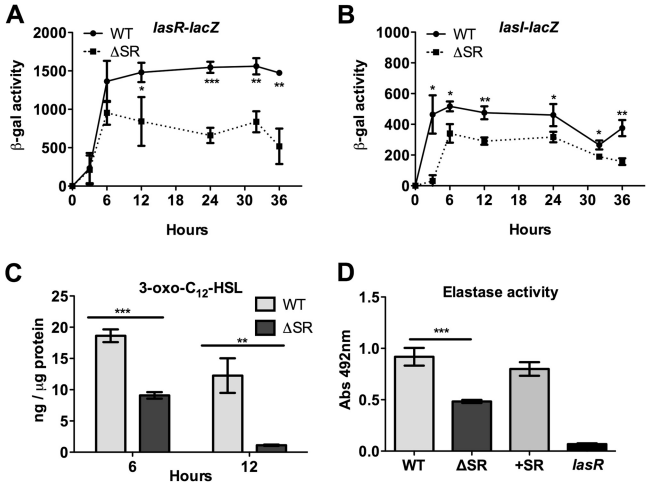

FIG 5 *las* quorum sensing is reduced in the ΔSR mutant. (A and B) *lasR* and *lasI* expression in wild-type (WT) and ΔSR mutant planktonic cultures was measured using *lasR-lacZ* and *lasI-lacZ* transcriptional reporters. (C) The concentration of 3-oxo-C_{12}-HSL in planktonic cultures was measured by LC-MS. (D) Elastase activity was measured in planktonic cultures grown in LB for 16 to 18 h. The *lasR* mutant was used as a negative control. β-Galactosidase activity is reported per 10^8 CFU. Error bars represent SD. *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$, compared to WT.

upregulated despite reduced *las* signaling in the ΔSR mutant (Fig. 2C) suggest instead that *las* signaling is conditional and not always dominant. To dissect the conditional contribution of *las* signaling, we generated *lasR* and *lasI* mutations in the wild-type and the ΔSR mutant backgrounds. HHQ levels were undetectable in the single *lasI* or *lasR* mutants but remained elevated in the ΔSR *lasR* and ΔSR *lasI* triple mutants (Fig. 6), indicating that *las* signaling is not required for HAQ biosynthesis in the ΔSR mutant and that (p)ppGpp levels modulate the AHL quorum-sensing hierarchy.

DISCUSSION

The cross talk between different regulatory networks can be extensive, allowing bacteria to fine-tune their physiology and gene expression in response to their environment (1, 54–56). Multiple layers of regulation coexist to integrate the signals, such as cell density, pH, and nutrient availability, and allow optimal bacterial survival in changing environments. (p)ppGpp, which mediates the stringent response, likely exerts its effect over a continuum, allowing bacteria to fine-tune transcription and regulatory networks to different levels of (p)ppGpp in response to nutrient shifts and environmental stress (57–59).

PQS and HHQ have cell-cell signaling functions, and HAQs are implicated in virulence, antibiotic tolerance, interspecies competition, and biofilm formation, among many roles. How *P. aeruginosa* responds to and integrates environmental signals to regulate HAQ biosynthesis has important implications for its success in microbial communities or within the host during infections. Based on our previous observation that HAQ biosynthesis is dysregulated in the absence of (p)ppGpp, we set out to understand how the stringent response regulates HAQ biosynthesis (9). In the ΔSR mutant, HAQ biosynthesis is highly upregulated during stationary phase, and this can be attributed to increased *pqsA* and *pqsH* transcription. This is consistent with the primary model that (p)ppGpp acts at the level of transcription (3) and suggests that (p)ppGpp negatively controls HAQ biosynthesis. We then investigated whether (p)ppGpp exerted its effects indirectly by modulating known HAQ regulators, namely, *pqsR* and AHL quorum sensing.

(p)ppGpp has been linked to AHL quorum-sensing systems in *P. aeruginosa*. Van Delden et al. reported that overexpression of *relA* led to premature activation of *lasR* and *rhlR* expression, and early induction of 3-oxo-C_{12}-HSL and C_4-HSL synthesis (41). Amino acid starvation with the serine analog serine hydroxamate also led to 3-oxo-C_{12}-HSL production, suggesting that the stringent response can prematurely activate both AHL quorum-sensing systems in a cell density-independent manner. Erickson et al. reported a slightly different link between the stringent response and quorum sensing, showing that the *relA* single mutant produced 3-oxo-C_{12}-HSL and C_4-HSL levels similar to those produced by the wild type but lower 3-oxo-C_{12}-HSL levels under low-magnesium conditions (5). In order to further dissect the link between the stringent response and AHL quorum sensing, we performed our studies in the ΔSR mutant, a (p)ppGpp-deficient strain in which both *relA* and *spoT* genes are inactivated. Our results demonstrated that (p)ppGpp is required for full AHL quorum-sensing signaling, since expression of both AHL autoinducer synthase genes (*lasI* and *rhlI*) and regulator genes (*lasR* and *rhlR*) is significantly reduced in the ΔSR mutant. Reduced AHL levels (C_4-HSL and 3-oxo-C_{12}-HSL), elastase activity (a LasR-dependent phenotype), and rhamnolipid production (a RhlR-dependent phenotype) in the ΔSR mutant further support these observations. Vogt et al. also reported that the *P. aeruginosa relA spoT*

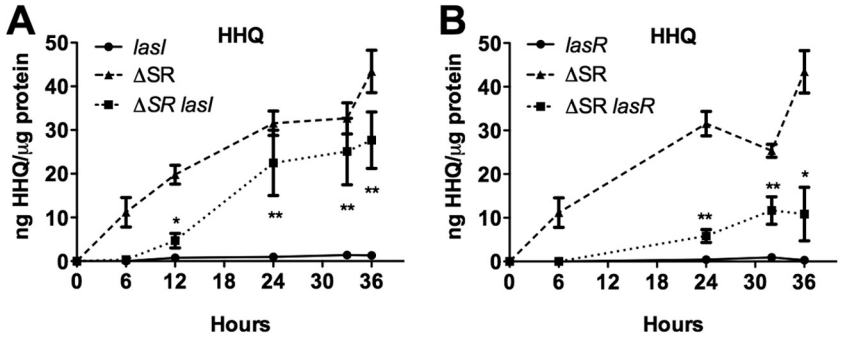

FIG 6 Role of *las* quorum sensing in HAQ biosynthesis. Concentration of HHQ in *lasI* mutants (A) and *lasR* mutants (B) was measured by LC-MS in planktonic cultures. Error bars represent SD. *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$, compared to the single Δ*lasR* or Δ*lasI* mutant.

mutant expressed low levels of LasR-controlled virulence factors (4). Taken together, these findings are consistent with the idea that (p)ppGpp is a master regulator sitting "above" the *las* and *rhl* hierarchy, as suggested by Schuster and Greenberg (60). (p)ppGpp exerts its effect through several direct and indirect pathways and regulates transcription in cooperation with DksA. It likely exerts a positive control of AHL through indirect mechanisms. The *lasR* promoter region contains a σ^{70} consensus sequence and a Vfr-binding sequence, where Vfr is a homolog of the *E. coli* cyclic AMP receptor protein (CRP) (61). *rhlR* is controlled by four promoters activated under different growth conditions, including a σ^{54} (RpoN)-responsive and a LasR-dependent promoter (62). (p)ppGpp may therefore enhance binding or competition of alternative σ factors (63), or even modulate Vfr/CRP (64), but the exact mechanisms remain unclear.

Both *las* and *rhl* quorum-sensing signaling systems regulate HAQ biosynthesis on several levels (20, 21, 25, 32, 33, 65, 66). The *rhl* system represses HAQ biosynthesis, as several studies showed that *pqsR* and *pqsA* expression and PQS levels are increased in *rhl* mutants (32, 33, 67). The *pqsA* promoter region contains a putative RhlR binding site located upstream of the *pqsA* −339 transcriptional start site, and deletion of this region increases *pqsA* transcription (25, 68). Based on our findings that *rhl* signaling is reduced in the ΔSR mutant and that overexpressing *rhlR* in conjunction with exogenous C_4-HSL in the ΔSR mutant is sufficient to restore wild-type *pqsA* expression, we can conclude that *rhl* signaling mediates (p)ppGpp's negative control of *pqsA* expression, and likely *pqsR* expression. This is consistent with the observations by Erickson et al. that the stringent response represses PQS production in a *relA*-dependent manner, when the stringent response was induced by amino acid starvation with the serine hydroxamate (5). The *las* system, on the other hand, positively regulates HAQ biosynthesis: LasR/3-oxo-C_{12}-HSL is sufficient to induce *pqsR* in *E. coli* (32), *pqsR* is induced by 3-oxo-C_{12}-HSL in a *lasI rhlI* mutant using microarrays (66), and *pqsR-lacZ* activity is also reduced in the *lasR* mutant (25, 32). Similarly, *pqsH* expression is under LasR positive control (66), and early studies showed that *lasR* mutants produce significant HAQs but delayed PQS (23, 24, 35). In contrast, our results suggest that under our (p)ppGpp-null conditions, the *las*-dependent regulation of HAQ biosynthesis is not dominant, since both *pqsR* and *pqsH* expression are high despite reduced *las* signaling. This supports the notion that *las*-dependent regulation of HAQ biosynthesis is conditional, an observation also made by others. For example, *lasR* mutants have undetectable PQS under some conditions (32) but not others (53).

In the traditional model of quorum-sensing hierarchy, the *las* system controls the *rhl* system, and PQS provides a regulatory link between the *las* and *rhl* quorum sensing systems, since exogenous PQS induces *lasB*, *rhlI*, and *rhlR* expression (21, 65). Our results suggest that this hierarchy breaks down in the absence of (p)ppGpp: the *rhl* and *las* systems are downregulated despite high PQS levels, *las*-mediated regulation is not dominant, and the repressive effects of *rhl* signaling on *pqsA* and *pqsR* expression are relieved (see Fig. 7 for a schematic diagram). Others have highlighted the idea that these relationships are likely condition dependent and modulated by environmental signals (53, 69). The regulatory network linking quorum sensing and HAQ biosynthesis is clearly plastic, and our results demonstrate that the (p)ppGpp can have a strong modulatory effect on this network, thus allowing environmental factors such as nutrient starvation and other stresses to be integrated into bacterial signaling.

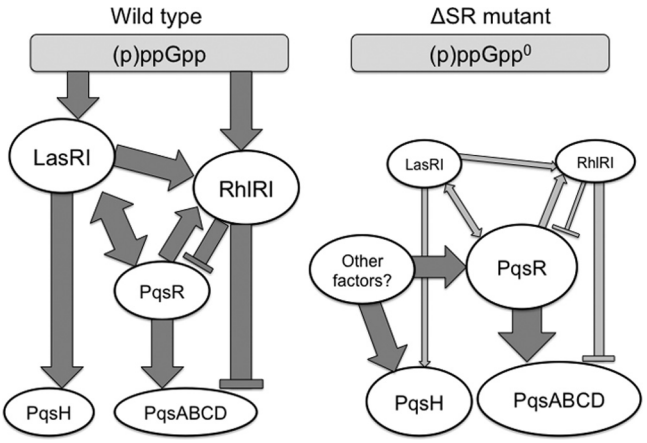

FIG 7 The relationship between AHL quorum sensing and HAQ biosynthesis is modulated by (p)ppGpp. A schematic organization of this regulatory network is presented to highlight the role of the stringent response. Dark gray connections indicate dominant relationships, and light gray connections represent minor relationships. Arrows indicate positive regulation, while blunt bars indicate negative regulation. These regulatory links may be direct or indirect, and other intermediate regulators are not represented here.

This study further elucidates how bacteria can sense their environment and adapt by modulating several signaling networks (36). Starvation and stress signals regulate cell-cell signaling in different bacterial species (70). Interestingly, Lee et al. (71) recently described IQS [2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde], a novel signal produced by the *ambBCDE* genes. IQS is produced under *las* signaling and PhoB-dependent phosphate limitation and can activate the PQS and *rhl* systems in the absence of *las* signaling (71). Lee et al. proposed that IQS plays a role in integrating the quorum sensing network with stress response mechanisms (in this case, phosphate starvation). PmpR (PA0964), a YebC family protein, is also reported to directly repress *pqsR* expression (72), and PqsR translation is enhanced by PhrS, a small RNA (sRNA) dependent on the oxygen-responsive regulator Anr (73). Whether IQS, PmpR, or PhrS mediates any (p)ppGpp control of the PQS and *rhl* systems has not been investigated. Finally, the alternate σ factor RpoS mediates some of the (p)ppGpp effect and likely adds another layer of regulation between the stringent response and quorum sensing (60).

In this study, we dissected how the stringent response modulates AHL quorum sensing and HAQ biosynthesis. The hierarchy and interplay between these regulatory networks are clearly shaped by environmental conditions, and (p)ppGpp is an important master regulator that allows bacteria to adapt to various stressors. Collectively, our results demonstrate that (p)ppGpp is required for curtailing excessive HAQ biosynthesis and also for full activation of the AHL signaling. Reduced *rhl* signaling, with its repressive effects on HAQ biosynthesis, is likely the major indirect mechanism for (p)ppGpp regulation of HAQ biosynthesis. Given that AHL quorum sensing controls expression of many virulence factors as well as protective defenses against host (e.g., catalases) and other bacterial species (e.g., hydrogen cyanide), this raises the possibility that environmental stress may signal early virulence and "self-defense" mechanisms through induction of the stringent response.

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