Candida albicans-produced farnesol stimulates *Pseudomonas* quinolone signal production in LasR-defective *Pseudomonas aeruginosa* strains

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Candida albicans has been previously shown to stimulate the production of *Pseudomonas aeruginosa* phenazine toxins in dual-species colony biofilms. Here, we report that *P. aeruginosa lasR* mutants, which lack the master quorum sensing system regulator, regain the ability to produce quorum-sensing-regulated phenazines when cultured with *C. albicans*. Farnesol, a signalling molecule produced by *C. albicans*, was sufficient to stimulate phenazine production in $LasR^{-}$ laboratory strains and clinical isolates. *P. aeruginosa* Δ*lasR* mutants are defective in production of the *Pseudomonas* quinolone signal (PQS) due to their inability to properly induce *pqsH*, which encodes the enzyme necessary for the last step in PQS biosynthesis. We show that expression of *pqsH* in a Δ*lasR* strain was sufficient to restore PQS production, and that farnesol restored *pqsH* expression in Δ*lasR* mutants. The farnesol-mediated increase in *pqsH* required RhlR, a transcriptional regulator downstream of LasR, and farnesol led to higher levels of *N*-butyryl-homoserine lactone, the small molecule activator of RhlR. Farnesol promotes the production of reactive oxygen species (ROS) in a variety of species. Because the antioxidant *N*-acetylcysteine suppressed farnesol-induced RhlR activity in $LasR^{-}$ strains, and hydrogen peroxide was sufficient to restore PQS production in *las* mutants, we propose that ROS are responsible for the activation of downstream portions of this quorum sensing pathway. LasR mutants frequently arise in the lungs of patients chronically infected with *P. aeruginosa*. The finding that *C. albicans*, farnesol or ROS stimulate virulence factor production in *lasR* strains provides new insight into the virulence potential of these strains.

INTRODUCTION

Cystic fibrosis (CF) is a genetic disease in which one of the pathologies is the inability to effectively clear potential pathogens from the airways, leading to chronic colonization of the lungs by a number of bacterial and fungal strains. Over 75 % of CF patients over 18 years of age are chronically colonized with *Pseudomonas aeruginosa*, which often persists throughout the life of the patient (Rajan & Saiman, 2002). While *P. aeruginosa* is the predominant pathogen in the CF lung, fungi such as *Candida albicans* are also frequently detected (Bakare *et al.*, 2003; Chotirmall *et al.*, 2010; Haase *et al.*, 1991; Navarro *et al.*, 2001). Bacterial–fungal co-infections may alter the host response (Allard *et al.*, 2009) or virulence factor expression in chronic biofilms (Cugini *et al.*, 2007; Gibson *et al.*, 2009; McAlester *et al.*, 2008).

The complexity of CF lung infections is further increased by phenotypic diversification of *P. aeruginosa* strains within these chronic infections (Ernst *et al.*, 2007; Li *et al.*, 2005; Mahenthiralingam *et al.*, 1994; Smith *et al.*, 2006). LasR, a transcriptional regulator that directly and indirectly controls a large set of virulence-related genes, is frequently defective in CF isolates (D'Argenio *et al.*, 2007; Hoffman *et al.*, 2009; Smith *et al.*, 2006; Tingpej *et al.*, 2007), and these mutations confer a variety of advantages in the CF lung environment (D'Argenio *et al.*, 2007). In a recent study by Hoffman *et al.* (2009), it was found that approximately 30 % of strains isolated from the airways of CF patients lacked LasR function, and that infection with $LasR^{-}$ strains correlates with decreased lung function and poor patient prognosis. While $LasR^{-}$ strains have a growth advantage in some environments and increased resistance to some antimicrobials (D'Argenio *et al.*, 2007; Hoffman *et al.*, 2010), the *in vivo* regulation of virulence factors in LasR-defective strains is not completely understood.

Abbreviations: 3OC12HSL, 3-oxo-C12-homoserine lactone; AHL, acyl homoserine lactone; C4HSL, *N*-butyryl-homoserine lactone; CF, cystic fibrosis; HHQ, 2-heptyl-4-quinolone; NAC, *N*-acetyl cysteine; PQS, *Pseudomonas* quinolone signal; RLU, relative luminescence units; ROS, reactive oxygen species; TLC, thin-layer chromatography; WT, wild-type.

Six supplementary figures are available with the online version of this paper.

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Evidence suggests that $LasR^-$ strains can exist as social cheaters by residing in the presence of $LasR^+$ counterparts that continue to produce quorum sensing signals and quorum-sensing-controlled virulence factors, which can cross-complement (Sandoz *et al.*, 2007; Wilder *et al.*, 2009). In addition, data indicate that alternate pathways for virulence factor production may exist in LasR loss-of-function mutants, as strains PA14Δ*lasR* and PAO1Δ*lasR* regain the ability to produce pyocyanin at late time points (Dekimpe & Deziel, 2009; Diggle *et al.*, 2003).

LasR is at the top of the *P. aeruginosa* quorum sensing regulatory cascade. Upon binding the acyl homoserine lactone (AHL) 3-oxo-C12-homoserine lactone (3OC12HSL), LasR activates the transcription of RhlR, another transcription factor, and stimulates the production of *N*-butyryl-homoserine lactone (C4HSL) (Pesci *et al.*, 1997). LasR also participates in the synthesis of the *Pseudomonas* quinolone signal (PQS; 2-heptyl-3-hydroxy-4-quinolone) (Pesci *et al.*, 1999; Xiao *et al.*, 2006b) by affecting the expression of PqsR (MvfR), which regulates the *pqsA–D* operon that encodes the enzymes to synthesize 2-heptyl-4-quinolone (HHQ), and by inducing the expression of *pqsH*, which catalyses the conversion of HHQ to PQS (Gallagher *et al.*, 2002). PqsE, which is induced by PQS through PqsR, in concert with RhlR, controls the expression of phenazine biosynthetic genes (Farrow *et al.*, 2008).

Production of PQS by *P. aeruginosa* is inhibited by farnesol, a *C. albicans* signalling molecule that participates in inter- and intraspecies interactions (Langford *et al.*, 2010; Peleg *et al.*, 2010; Shirtliff *et al.*, 2009b). Farnesol inhibits the PqsR-mediated transcription of the *pqsA–E* operon, probably due to direct interaction with PqsR, though the effects of farnesol on PqsR activity can be suppressed by higher levels of PQS (Cugini *et al.*, 2007). In *P. aeruginosa–C. albicans* biofilms, where *P. aeruginosa* and PQS concentrations are high, the presence of the fungus leads to increased production of phenazines through an uncharacterized pathway, suggesting that the effects of *C. albicans* on *P. aeruginosa* are complex (Gibson *et al.*, 2009). Here, we report that in colony biofilms formed by LasR-defective strains, the defects in PQS production that are normally associated with the Δ*lasR* genotype were no longer apparent in the presence of farnesol or farnesol-producing *C. albicans*. The increased PQS production translated into increased phenazine production. Farnesol led to an induction of *pqsH* transcription, and *pqsH* overexpression was sufficient to restore pyocyanin and PQS production in the absence of farnesol. The induction of *pqsH* by farnesol was dependent on RhlR, and we have demonstrated that farnesol stimulated RhlR activity and the production of C4HSL. Exogenous C4HSL was sufficient to stimulate PQS production in the Δ*lasR* strain. Clinical isolates of *P. aeruginosa* with LasR loss-of-function mutations also produced increased PQS in the presence of farnesol or farnesol-producing *C. albicans*. Because farnesol leads to the production of intracellular reactive oxygen species (ROS) in a variety of species (Machida *et al.*, 1998; Semighini *et al.*, 2006; Shirtliff *et al.*, 2009a), farnesol-induced PQS production in PA14Δ*lasR* is decreased upon the addition of the antioxidant *N*-acetyl cysteine (NAC), and hydrogen peroxide is sufficient to stimulate PQS production in *lasR* but not *lasRrhlR* mutants, we suggest that the activation of RhlR signalling in PA14Δ*lasR* may occur in response to oxidative stress. We propose that the stimulation of PQS production by the presence of *C. albicans* or other sources of oxidative stress, such as those present in association with inflammation, can lead to high level expression of downstream elements in the LasR-controlled quorum sensing pathway and the reactivation of quorum-sensing-controlled genes in LasR-defective strains.

METHODS

Bacterial strains, media and culture conditions. The bacterial and fungal strains used are listed in Table 1. Bacterial overnight cultures were grown in 5 ml LB at 37 °C on a roller drum and, when needed, antibiotics were provided at the following concentrations: gentamicin, 10 μg ml^{-1} for *Escherichia coli* and 75 μg ml^{-1} for *P. aeruginosa*; carbenicillin, 150 μg ml^{-1} for *E. coli* and 750 μg ml^{-1} for *P. aeruginosa*. *C. albicans* overnight cultures were grown in 5 ml YPD at 30 °C on a roller drum.

P. aeruginosa and *C. albicans* co-cultures were formed in either 96-well plate wells with 150 μl YPD agar or in 180 mm petri dishes. Overnight cultures (10 μl for wells and 500 μl for plates) of *C. albicans* SC5314 were top-spread. *C. albicans* lawns were grown for 24 h at 30 °C prior to inoculation with *P. aeruginosa*. Co-cultures in 96-well plates were inoculated with 5 μl *P. aeruginosa* from cultures grown to OD_{600} 0.2, and Petri plate co-cultures were spot-inoculated with 10 μl of LB-grown cultures.

For analysis of the effects of farnesol and hydrogen peroxide, *P. aeruginosa* was inoculated as described above onto LB agar containing DMSO (vehicle control), 250 μM farnesol, 5 or 10 mM NAC or 200 μM hydrogen peroxide, as specified, and visualized at time intervals described in the text. Farnesol (Sigma) was prepared as a 500 mM stock solution in DMSO prior to each experiment and added at a final concentration of 250 μM after autoclaving and cooling to pouring temperature; control plates received an equivalent amount of DMSO. C4HSL (Fluka) was prepared as a 1 mM stock solution in ethyl acetate and added at a final concentration of 25 μM to agar; the ethyl acetate solution was added to a glass bottle, and the solvent was allowed to evaporate prior to the addition of agar.

Construction of *P. aeruginosa* mutants. To construct the *pqsR* deletion plasmid (pPQSRdel), flanking sequences of the target gene were amplified by PCR from PA14 chromosomal DNA using primers corresponding to the PA14 genomic sequence. The deletion constructs were made by splicing by overlapping extension (SOEing) PCR generating a fusion of the 5′ and 3′ flanking regions of the target gene following two rounds of PCR (Horton *et al.*, 1989). PCR was performed using the high fidelity platinum *Taq* master mix kit (Invitrogen) with an additional 2.5 mM $MgCl_2$ and 5 % DMSO. The first round of PCR created two 1 kb products using the following primers: pqsR5′F (CCGAGCTCCATCTCCAGCGAATCGGATACGC) with pqsRSOE5′ (GGAGCGCCTTCGGGCCTGAGCGGCGCTGCACCGGATCCCGAA-CCGGAGGCGATGACCTGGAG), and pqsR3′R (GGAAGCTTGGC-GAACTGGATGCGCTGCATGC) with pqsRSOE3′ (ATGTTCCTC-CAGGTCATCGCCTCCGGTTCGGGATCCGGTGCAGCGCCGCTCA-GGCCCGAAGGCGCTCC). The second round of PCR used 20 pmol μl^{-1} of pqsR5′F/pqsR3′R primers and 1 μl of each of the 1 kb amplified

Table 1. Strains and plasmids

Strains	Lab DH no.	Description	Source
P. aeruginosa			
PA14	122	Wild-type	Rahme *et al.* (1995)
PA14Δ*lasR*	164	In-frame deletion of *lasR*	Hogan *et al.* (2004)
PA14Δ*lasI*	132	In-frame deletion of *lasI*	Hogan *et al.* (2004)
PA14 *rhlR*::TetR	6	Gene replacement of *rhlR*	Hogan & Kolter (2002)
PA14Δ*rhlI*	169	In-frame deletion of *rhlI*	Hogan *et al.* (2004)
PA14Δ*lasRrhlR*::TetR	237	In-frame deletion of *lasR* in DH6	This study
PA14Δ*lasR*Δ*rhlI*	238	In-frame deletion of *lasR* in DH169	This study
PA14Δ*pqsH*	1112	In-frame deletion of *pqsH*	This study
PA14Δ*lasR*Δ*pqsH*	1113	In-frame deletion of *lasR* in DH1112	This study
PA14Δ*pqsR*	1110	In-frame deletion of *pqsR*	This study
PA14Δ*lasR*Δ*pqsR*	1111	In-frame deletion of *lasR* in DH1110	This study
CIA467G	1097	LasR mutation, 467 nt mutated, A→G, nonsynonomous D→G	Smith *et al.* (2006)
CIG691C	1099	LasR mutation, 691 nt mutated, G→C, nonsynonomous A→P	Smith *et al.* (2006)
CIT341C	1100	LasR mutation, 341 nt mutated, T→C, nonsynonomous L→P	Smith *et al.* (2006)
CIG608A	1103	LasR mutation, 608 nt mutated, G→A, nonsynonomous C→Y	Smith *et al.* (2006)
CIG179A	1132	LasR mutation, 179 nt mutated, G→A, nonsynonomous W→STOP	Smith *et al.* (2006)
CIC181T	1134	LasR mutation, 181 nt mutated, C→T, nonsynonomous R→C	Smith *et al.* (2006)
CIG455-	1136	LasR mutation, 455 nt deleted, G→ –	Smith *et al.* (2006)
E. coli			
S17/λpir	71		Laboratory collection
DH5α	–	F′/*endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1* (*lacZYA-argF*) *U169 deoR* [*80 dlac* (*lacZ*)M15 *recA1*]	Laboratory collection
JM109	1406	F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q} Δ(*lacZ*)*M15*/Δ (*lac-proAB*) *glnV44 e14*$^-$ *gyrA96 recA1 relA1 endA1 thi hsdR17*	Laboratory collection
C. albicans			
SC5314	35	Wild-type	Gillum *et al.* (1984)
S. cerevisiae			
DC49-7.1C	–	*MATα, leu2-3, 112 trp1-289 ura3-52 arg4*$^-$ *Δ57, RV*	Shanks *et al.* (2006)
Plasmids			
pUCP22	160	Gm^R; *Escherichia–Pseudomonas* expression plasmid	West *et al.* (1994)
pEX18GM		Gm^R; *oriT sacB*, gene replacement vector with MCS from pUC18	Hoang *et al.* (1998)
pMQ30	962	Deletion plasmid for yeast cloning	Shanks *et al.* (2006)
pLASR	133	PA14Δ*lasR* deletion construct	Hogan *et al.* (2004)
pPQSRdel	949	PA14Δ*pqsR* deletion construct	This study
pPQSHdel	1109	PA14Δ*pqsH* deletion construct	This study
pCR2.1TOPO	–	Cloning vector	Invitrogen
pPQSHFLAG	1413	*pqsH* promoter region and gene in pUCP22	This study
pSB536	1407	*ahyR*$^+$ *ahyI*::*luxCDABE*; Amp^R *colE1* origin	Swift *et al.* (1997)

products from round 1 as template, to generate 5′ and 3′ flanking regions fusions. 5′ Primers were designed with *Sac*I sites at the 5′ terminus and 3′ primers were designed with *Hin*dIII, to allow for cloning into pGEX18Gm digested with *Sac*I and *Hin*dIII (New England Biolabs).

To construct the *pqsH* deletion plasmid (pPQSHdel), flanking sequences of the target gene were amplified by PCR from PA14 chromosomal DNA using primers corresponding to the PA14 genomic sequence containing tails that correspond to the plasmid sequence. PCR was performed using the high fidelity platinum *Taq* master mix kit (Invitrogen) with an additional 2.5 mM $MgCl_2$ and 5 % DMSO. The primers used were: pqsH/yeast primer 1 (CCAA-GCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCC**GCGCCA-GCCCGGCGACGCC**) pqsH/yeast primer 2 (ACCGACACCGGTG-AAAAGACGCTGGTGCAGGCGCCTGCGACggatcc**GCGCCAGCGC-CAGGCCGGCGATCCCGGCCCC**), pqsH/yeast primer 3 (GGGGC-CGGGATCGCCGGCCTGGCGCTGGCGCggatcc**GTCGCAGGCGC-CTGCACCAGCGTCTTTTCACCGGTGTCGGT**) with pqsH/yeast primer 4 (AACAGCTATGACCATGATTACGAATTCGAGCTCGG-TACCC**CGTGGCGGACGGCGAGCAGC**). Nucleotides in bold are those that overlap with either the PA14 sequence or the pMQ30 sequence. The plasmid pMQ30 (Shanks *et al.*, 2006) was digested with *Xba*I in order to linearize the plasmid for transformation in to *Saccharomyces cerevisiae* (strain DC49-7.1C). An overnight culture of *S. cerevisiae* (0.5 ml) grown in YPD was pelleted, the supernatant was removed, and the pellet was washed with TE. Transformation buffer (0.5 ml; 40 % polyethylene glycol, 0.1 M lithium acetate, 10 mM Tris/HCl, pH 7.5, and 1 mM EDTA), 20 μl 2 mg single stranded salmon sperm DNA ml^{-1}, digested plasmid DNA (10 μl) and 5′ and 3′ PCR products (20 μl each) were added to the pellet; this was mixed by vortexing for 1 min and incubated overnight at room temperature. Heat shock was applied to the cells by incubation for 10 min at 42 °C; cells were pelleted and washed with TE. The pellets were plated to SC drop-out medium that lacked uracil and were incubated at 30 °C. The resulting colonies were patched to a fresh plate and allowed to reach a high cell density. The plasmid was

liberated by a 'smash and grab' technique (Elble, 1992; Hoffman & Winston, 1987). All deletion and disruption constructs were electroporated into *E. coli* S17/λpir, mated into *P. aeruginosa* PA14 and single mutants, and resolved with gentamicin and sucrose selections as described previously (Hoang *et al.*, 1998). The *lasR* double mutants were constructed using the pLASR deletion construct described previously (Hogan *et al.*, 2004).

To construct the *pqsH* fragment for the overexpression plasmid (pPQSHFLAG) the following primers were used: pqsH5′ (ccgagctcTAGAAGGAGCAACGGATGACCG) and pqsH3′FLAG (cttaagcttCTACTTGTCGTCGTCGTCCTTGTAGTCCTGTGCGGCCATCTCACCGACACCGGTG). The 5′ and 3′ primers contain *Sac*I and *Hin*dIII sites, respectively, and the 3′ primer encodes a C-terminal FLAG tag. The *pqsH* gene was cloned into pUCP22, and transformed into PA14, PA14Δ*lasR*, PA14 *rhlR*::TetR, PA14Δ*lasRrhlR*::TetR, PA14Δ*pqsH* and PA14Δ*lasR*Δ*pqsH*.

Pyocyanin quantification. *P. aeruginosa* cells were grown as described above. Samples were obtained at the times indicated and were prepared as described previously with modifications (Gallagher *et al.*, 2002). At each time point, one agar plug was added to 750 μl chloroform, samples were mixed by vortexing for 2 min, then allowed to incubate for 2 h at 4 °C. The chloroform extracts were acidified by the addition of 750 μl 0.2 M HCl. The aqueous layer, containing pyocyanin, was removed and measured at 390 nm using a Spectra Max plate reader. Samples were collected at each time point in quadruplicate and each sample was assayed in triplicate for pyocyanin concentrations.

To determine pyocyanin production in the presence of hydrogen peroxide, *P. aeruginosa* strains were grown in 96-well plates as outlined above. After 48 h of growth, agar plugs were transferred to tubes containing chloroform (500 μl), mixed by vortexing for 2 min and then incubated for 2 h at 4 °C. The chloroform extracts were acidified by the addition of 500 μl 0.2 M HCl, and the aqueous layer, containing pyocyanin, was diluted 1 : 2 in 1 M Tris/HCl pH 8.0. Pyocyanin was measured by recording absorbance at 310 nm. For each strain under each condition, the pyocyanin levels were measured in five separate plugs. In all experiments, concentrations were determined using a standard curve of known concentrations of pyocyanin (Cayman Chemicals) dissolved in the appropriate buffer.

PQS quantification. *P. aeruginosa* was grown as described above. Samples were obtained at the times indicated and were prepared as described previously with modifications (Gallagher *et al.*, 2002). At each time point, one agar plug was added to 1 ml acidified ethyl acetate; samples were mixed by vortexing for 2 min, centrifuged for 2 min at 13 000 ***g***, and 750 μl was removed to a clean glass vial. The extraction was repeated, removing 1.5 ml total volume. The extracts were allowed to dry at 37 °C or under nitrogen. Extracts were resuspended in 200 μl 1 : 1 acidified ethyl acetate:acetonitrile by vortexing for 10 min at low speed. For thin-layer chromatography (TLC) analysis, aluminium TLC sheets (EMD Chemicals; silica gel 60 F_{254}) were activated in a 0.5 % KH_2PO_4 solution, air-dried, and baked at 100 °C for 1 h prior to use. Samples (1 or 2 μl) were applied to each plate using 1 μl glass capillaries. A mixture of 17 : 2:1 methylene chloride:acetonitrile:dioxane was used as a solvent, and plates were visualized using long-wave UV light. For each strain and treatment, PQS extracts for three biological replicates were each spotted in triplicate on a TLC plate. Spot intensities were determined with ImageJ software (Abramoff *et al.*, 2004). A dilution series of an authentic PQS standard was included on each plate in order to generate a standard curve for PQS quantification. An anthranilic acid standard was also included in some analyses for comparison.

Transcript analysis. To examine the timing of the effects of farnesol on *pqsH* expression, three biological replicates of *P. aeruginosa* cells grown on standard Petri plates were recovered from the agar surface at the times indicated and stored at −80 °C until RNA extractions were performed. To isolate *P. aeruginosa* RNA, 100 μl 3 mg ml^{-1} lysozyme solution was added to cells, and samples were allowed to stand at room temperature for 3 min. The RNeasy kit (Qiagen) was used for subsequent steps, with modifications. On-column DNase (Qiagen) and RQ1 (Promega) treatment was used to remove DNA. PCRs using the purified RNA as the template and *rplU* primer set (below) were carried out to ensure no DNA contamination of the RNA. The purified RNA was then used as a template to construct cDNA using SuperScript III (Invitrogen), according to the manufacturer's instructions, and random decamer primers [$(NS)_5$]. The resulting cDNA was used as a template in PCRs with the following primers: *rplU* forward (5′-GCAGCACAAAGTCACCGAAGG-3′)), *rplU* reverse (5′-CCGTGGGAAACCACTTCAGC-3′), *pqsH* forward (5′-ACGACCTCGAGGAGTTGG-3′) and *pqsH* reverse (5′-GAACAGGATCAGCGTCTCG-3′). The PCR cycles were 10 min at 94 °C; 30 cycles of 30 s at 94 °C, 30 s at 54 °C, 1 min at 72 °C; 15 min at 72 °C.

Real-time PCR was conducted with an Applied Biosystems 7500 cycler. Conditions were identical to those used in the previous PCRs, with the following exceptions: for *ppiD* we used *ppiD* forward (5′-CGGGCACCGGTTTCG-3′) and *ppiD* reverse (5′-AAGTCGCGGGTCTGCTTCT-3′) in place of *rplU* primers, 40 cycles were used instead of 30, and the addition of a dissociation curve cycling was added to the end of the 15 min at 72 °C hold. The Power SYBR Green PCR kit (ABI) was used in the reactions. Three technical replicates were analysed for each biological replicate.

C4HSL bioassay. *P. aeruginosa* cell extracts were obtained as described for PQS production at 10 or 14 h post-inoculation in biological triplicates as described in the text. Dried cell extracts were solubilized in 200 μl 1 : 1 acetonitrile:acidified ethyl acetate. Overnight cultures of *E. coli* JM109 harbouring pSB536 were grown overnight, diluted 1 : 100, then allowed to reach an OD_{600} of 0.3. C4HSL extracts (5 μl) were added in triplicate to each well of black-welled polypropylene plates (Costar), and the solvent was allowed to evaporate. An authentic C4HSL standard was included in each bioassay. One hundred microlitres of culture was added to each well, and the plate was statically incubated for 3 h at 37 °C. Luminescence was measured with a Victor^2 fluorometer (Perkin Elmer). Cells were transferred to a clear 96-well plate and the OD_{600} was measured to use to normalize luminescence readings.

Analysis of growth on agar plugs. *P. aeruginosa* microtitre dish cultures were grown as described above. Cells were grown at 37 °C for 6, 10 or 14 h, plugs were added to 1 ml PBS, mixed by vortexing, serially diluted, and plated in triplicate to enumerate colonies.

RESULTS

C. albicans-produced farnesol stimulates phenazine production in *P. aeruginosa* Δ*lasR* mutants

P. aeruginosa PA14 strains that lack *lasR* and *lasI* are defective in the production of quorum-sensing-controlled factors such as phenazines (Latifi *et al.*, 1995). When inoculated onto lawns of *C. albicans*, however, Δ*lasR* (Fig. 1a) and Δ*lasI* (not shown) exhibited greatly enhanced phenazine production, as indicated by the red and blue colours conferred by 5-methylphenazium-1-carboxylate and pyocyanin, respectively (Gibson *et al.*, 2009). *C. albicans* secretes farnesol, an auto-regulatory molecule,

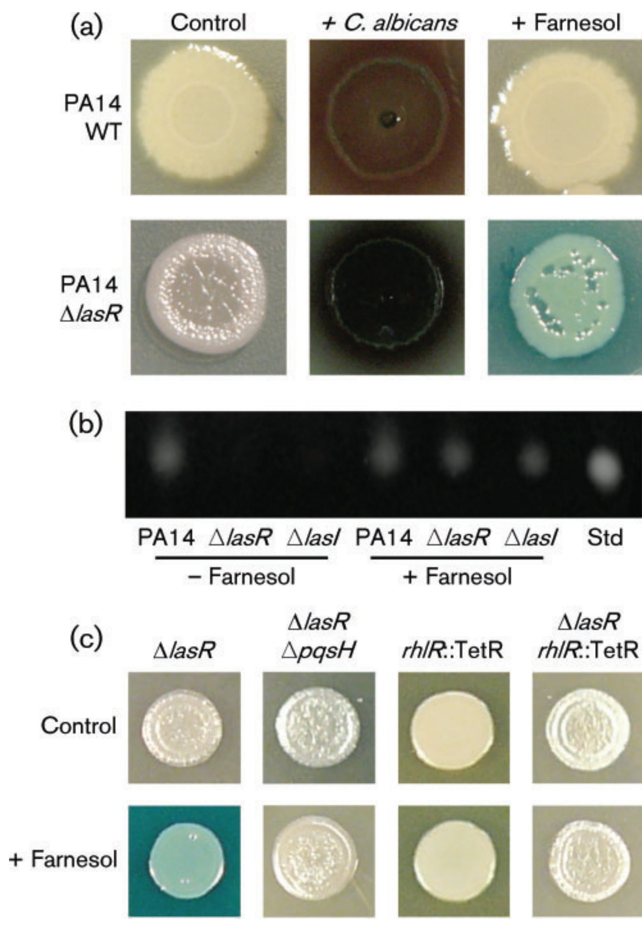

Fig. 1. *P. aeruginosa* colony appearance and PQS production in the presence of *C. albicans* or farnesol. (a) *P. aeruginosa* strain PA14 WT and Δ*lasR* colonies after 24 h of growth on LB (control), in the presence of *C. albicans* or on LB plates containing 250 μM farnesol. (b) PQS analysis by TLC from cultures of PA14 WT, PA14Δ*lasR* and PA14Δ*lasI* grown in the absence or presence of 250 μM farnesol for 14 h. An authentic PQS standard (25 ng) was included for comparison. (c) *P. aeruginosa* strains PA14Δ*lasR*, PA14Δ*lasR*Δ*pqsH*, PA14 *rhlR*::TetR and PA14Δ*lasRrhlR*::TetR grown in the absence (control) or presence of 250 μM farnesol. Colonies were photographed after 24 h of growth.

which participates in intraspecies (Hornby *et al.*, 2001; Langford *et al.*, 2009) and interspecies (Peleg *et al.*, 2010; Shirtliff *et al.*, 2009b) interactions. The addition of farnesol to the medium was sufficient to induce a striking increase in pyocyanin production in *lasR* and *lasI* mutant strains (Fig. 1a). Pyocyanin concentrations were more than threefold higher in PA14Δ*lasR* colonies grown with farnesol (0.22 μmol per plug) compared with control colonies (0.07 μmol per plug) (Supplementary Fig. S1, available with the online version of this paper). Farnesol induced only a modest increase in pyocyanin in PA14 wild-type (WT) colonies. In the presence of farnesol, the levels of pyocyanin in *lasR* colonies grown with farnesol were more than twofold higher than concentrations in WT colonies (Supplementary Fig. S1). Furthermore, pyocyanin appeared to be largely in its colourless reduced state in mature WT colonies, while the blue–green colour of oxidized pyocyanin was observed in *lasR* strains (Fig. 1a). In addition to the lack of pigmentation, PA14Δ*las* mutants appeared to accumulate HHQ, which gives colonies an iridescent sheen when grown on LB agar (Fig. 1a) (D'Argenio *et al.*, 2007). The presence of farnesol suppressed the apparent HHQ accumulation (Fig. 1a). The increased phenazine production and apparent decrease in the accumulation of the PQS precursor, HHQ, upon growth with *C. albicans* or farnesol suggest that farnesol stimulates PQS production in the *lasR* and *lasI* mutant strains.

Farnesol stimulates PQS production in Δ*lasR* and Δ*lasI* strains and LasR-defective clinical strains

To test the hypothesis that farnesol was stimulating PQS production in *lasR* and *lasI* mutants, colonies were grown on agar in a 96-well plate format in either the presence or absence of farnesol, followed by extraction and analysis of PQS from the entire agar plug at different time points. By 6 h, *P. aeruginosa* strain PA14 WT colonies produced detectable levels of PQS that continued to increase over 24 h. As expected, PQS levels were much lower in PA14Δ*lasR* and PA14Δ*lasI* colony cultures, with PQS at barely detectable levels at the 14 h time point (Fig. 1b). In contrast, on farnesol-containing medium, Δ*lasR*, Δ*lasI* and PA14 WT colonies all had comparable PQS levels at 14 h (Fig. 1b). We previously showed that farnesol significantly delays PQS production in *P. aeruginosa* PA14 WT cultures grown in liquid media (Cugini *et al.*, 2007), and we found that farnesol also inhibits PQS production in PA14 colonies at early time points (4 and 5 h) but this repression was no longer apparent once the population size reached approximately 10^9 c.f.u. per colony biofilm (data not shown). Farnesol did not cause any changes in growth rate in either WT or Δ*lasR* colonies (Supplementary Fig. S2, available with the online version of this paper).

Growth in co-culture with farnesol-producing *C. albicans* also abolished defects in PQS production in *lasR* and *lasI* mutant strains. In co-culture with the fungus, PA14 WT produced 71 ± 1 ng PQS per colony and PA14Δ*lasR* produced 135 ± 3 ng PQS per co-culture colony. The higher levels of PQS observed in Δ*lasR* colonies, relative to WT colonies, was consistent with the higher levels of pyocyanin in Δ*lasR* colonies grown with *C. albicans* or farnesol as described above (Fig. 1 and Supplementary Fig S1). To confirm that the fungus did not produce a compound that co-migrated with PQS, PA14Δ*pqsR* and PA14Δ*lasR*Δ*pqsR* strains were constructed and analysed using the TLC method. No PQS was detected in these backgrounds upon co-culture with *C. albicans* (Supplementary Fig. S3, available with the online version of this paper).

LasR-defective isolates are frequently isolated from the lungs of CF patients (Hoffman *et al.*, 2009; Smith *et al.*,

2006). To determine if LasR^{-} isolates from CF patients also showed enhanced PQS production upon growth with either *C. albicans* or farnesol, previously characterized *P. aeruginosa* strains with different loss-of-function mutations in *lasR* were examined. All of the seven LasR-defective clinical isolates tested produced little to no PQS on LB agar, and all but one strain showed a strong stimulation of PQS production on LB agar amended with farnesol (Supplementary Fig. S4a, available with the online version of this paper). In four of the six strains that produced PQS in the presence of farnesol, co-culture with *C. albicans* led to a strong increase in phenazine production (Supplementary Fig. S4b).

Farnesol promotes *pqsH* expression via the RhlR/C4HSL signalling pathway

Because farnesol caused a visual decrease in the apparent accumulation of HHQ (Fig. 1a) and stimulated the production of PQS (Fig. 1b), we hypothesized that farnesol was causing increased expression of *pqsH*. PqsH catalyses the conversion of HHQ to PQS, and in *P. aeruginosa* WT strains, LasR directly activates the transcription of *pqsH* (Gallagher *et al.*, 2002; Gilbert *et al.*, 2009). A Δ*lasR*Δ*pqsH* mutant was constructed, and its colony phenotype was comparable to that of the Δ*lasR* parent. However, while the *lasR* mutant lost its iridescent sheen and exhibited increased pyocyanin production upon growth with farnesol, the Δ*lasR*Δ*pqsH* mutant did not show any changes in colony morphology on farnesol-containing medium relative to control colonies (Fig. 1c). Consistent with published reports, deletion of *pqsH* in a WT strain abolished PQS production (Table 2) and led to the appearance of the iridescent sheen attributed to HHQ accumulation (data not shown) (Gallagher *et al.*, 2002). Furthermore, *pqsH* expression in Δ*lasR* strains was sufficient to restore the production of PQS and pyocyanin in the absence of *C. albicans* or farnesol. PA14Δ*lasR* colonies carrying *pqsH* on a plasmid produced 252 ± 7 ng PQS per plug at a time when no PQS was detected in colonies of Δ*lasR* with the empty vector (Table 2). Expression of *pqsH* also rescued PQS production in Δ*lasR*Δ*pqsH* and Δ*pqsH* strains (Table 2). Together, these data show that *pqsH* was necessary for farnesol-induced PQS production, and that the expression of *pqsH* was sufficient to induce robust PQS production in Δ*lasR* and Δ*lasI* strains.

Table 2. PQS production in *lasR* strains

ND, Not detected. Data shown are mean ± SD.

Strain	PQS (ng per plug)
PA14Δ*lasR* + pUCP22	ND
PA14Δ*lasR* + pPQSH	252 ± 7
PA14Δ*pqsH* + pUCP22	ND
PA14Δ*pqsH* + pPQSH	73 ± 3
PA14Δ*lasR*Δ*pqsH* + pUCP22	ND
PA14Δ*lasR*Δ*pqsH* + pPQSH	226 ± 8
PA14Δ*lasR*	ND
PA14Δ*lasR* + 25 μM C4HSL	106 ± 7
PA14Δ*lasRrhlR*::TetR	ND
PA14Δ*lasRrhlR*::TetR + 25 μM C4HSL	ND
PA14Δ*lasR*Δ*rhlI*	ND
PA14Δ*lasR*Δ*rhlI* + 25 μM C4HSL	11 ± 1

To determine whether farnesol led to increased *pqsH* transcript levels, Δ*lasR* colonies were grown in the presence and absence of farnesol, and RNA was extracted for analysis. PA14Δ*lasR* colonies grown in the presence of farnesol for 12 h had a significant increase in *pqsH* mRNA in comparison with those cells that received vehicle alone (Fig. 2a). As predicted by the model that PqsR positively regulates the expression of the *pqsA–E* operon in response to PQS, *pqsA* levels were several-fold higher in Δ*lasR* colonies that were grown in the presence of farnesol (data not shown).

In contrast with the stimulation of PQS production by farnesol in a *lasR* mutant, a Δ*lasRrhlR*::TetR double mutant still exhibited the HHQ-associated iridescent sheen, and pyocyanin was not observed in colonies grown on medium with farnesol (Fig. 1c), suggesting that RhlR may play a role in the farnesol response. Phenazine production was also not observed upon co-culture of the Δ*lasRrhlR*::TetR strain with the fungus (data not shown). Consistent with the phenotypic data, the increase in *pqsH* mRNA in *lasR* cells upon growth on farnesol was not seen in PA14Δ*lasRrhlR*::TetR cells (Fig. 2a). Even in control conditions, levels of *pqsH* mRNA were lower in Δ*lasRrhlR*::TetR compared with a Δ*lasR* mutant (Fig. 2a). Constitutive expression of *pqsH* restored PQS production in PA14Δ*lasRrhlR*::TetR (Supplementary Fig. S5a, available with the online version of this paper) and PA14Δ*lasR*Δ*rhlI* (data not shown). Evidence for RhlR-mediated regulation of *pqsH* expression in PA14Δ*lasR* in medium with farnesol is consistent with recently published data that show that RhlR is required for late (after 24 h) PQS production in the PA14Δ*lasR* liquid cultures (Dekimpe & Deziel, 2009).

Farnesol increases C4HSL production and C4HSL is sufficient to induce PQS production in Δ*lasR*

RhlR activity requires binding of C4HSL, which is synthesized by RhlI (Latifi *et al.*, 1995). To determine if farnesol stimulated RhlR activity in a *lasR* mutant background by acting as an alternative ligand to C4HSL for RhlR activation, we determined if RhlI was necessary for PQS production in *lasR* mutants grown on farnesol. A PA14Δ*lasR*Δ*rhlI* mutant was constructed, and found not to support PQS production on LB with farnesol, indicating that farnesol does not act as a surrogate for C4HSL and that it cannot stimulate RhlR activity in a C4HSL-independent manner. While RhlR and RhlI were required for PQS production in Δ*lasR* (Table 2 and Supplementary Fig. S5a), single *rhlR*::TetR or Δ*rhlI* mutants were not

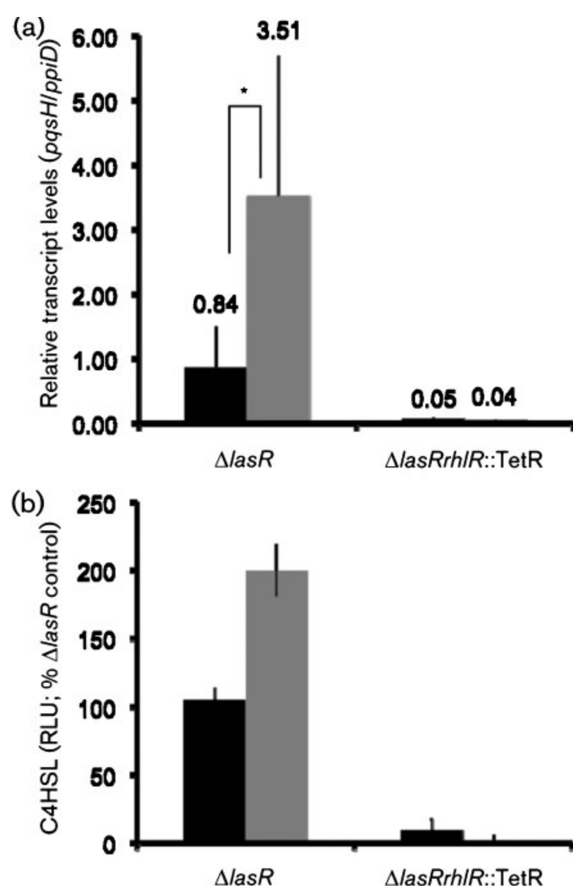

Fig. 2. Effects of farnesol on the levels of *pqsH* transcript and C4HSL. (a) RT-PCR analysis of strains PA14Δ*lasR* and PA14Δ*lasRrhlR*::TetR colonies grown in the absence (black bars) and presence (grey bars) of 250 μM farnesol. The levels of *pqsH* and *ppiD* were measured in control colonies and colonies grown in the presence of farnesol for 12 h. While there is variability in the biological replicates within an experiment, likely due to the fact that quorum-sensing-controlled genes are rapidly changing in colonies at these time points, statistically significant increases in *pqsH* in the presence of farnesol were observed in four independent experiments. Data presented represent three biological replicates from one independent experiment (mean ± SD). *$P<0.1$ based on Student's *t*-test. (b) C4HSL levels were measured from PA14Δ*lasR* and PA14Δ*lasRrhlR*::TetR colonies grown for 10 h in the absence (black bars) and presence (grey bars) of 250 μM farnesol. Levels were determined by luciferase activity generated by the reporter plasmid pSB536, which detects short-chain AHLs, verified by comparison with a titrated authentic C4HSL standard and reported as relative luminescence units (RLU) after normalization to levels in control cultures. Error bars, SD.

defective in PQS production and, in fact, produced higher levels of PQS relative to WT control strains (Supplementary Fig. S5b), as has been previously reported (McGrath *et al.*, 2004).

To determine if RhlR–C4HSL regulation was induced by farnesol, we determined if levels of C4HSL were higher in farnesol-grown cultures compared with controls. PA14Δ*lasR* grown with farnesol had a nearly twofold increase in C4HSL over Δ*lasR* control cultures as determined by the *E. coli* Lux-based biosensor assay that detects short chain AHLs (C4- and C6HSL) (Fig. 2b). As expected, PA14Δ*lasRrhlR*::TetR did not produce detectable levels of C4HSL in either the presence or absence of farnesol, further supporting the hypothesis that the increase in C4HSL induced by farnesol is dependent on the activity of RhlR.

Given the elevated C4HSL levels, we sought to determine if the addition of exogenous C4HSL was sufficient to restore RhlR-dependent production of PQS in the *lasR* mutant. In the presence of 25 μM C4HSL, PA14Δ*lasR* colonies produced 106 ± 7 ng PQS per plug at 14 h, whereas in control cultures without C4HSL, PQS was undetectable (Table 2). Furthermore, PA14Δ*lasR* colonies grown in the presence of C4HSL had a smooth colony morphology consistent with decreased accumulation of HHQ (data not shown). C4HSL addition to a PA14Δ*lasRrhlR*::TetR did not rescue PQS production (Table 2). As expected based on the results shown above, the Δ*lasR*Δ*rhlI* mutant regained the ability to produce PQS (Table 2) and pyocyanin (not shown) upon growth with C4HSL.

Farnesol-induced oxidative stress leads to increased C4HSL signalling

Previously, farnesol has been reported to induce oxidative stress in multiple microbes (Semighini *et al.*, 2006; Shirtliff *et al.*, 2009a), potentially through interactions with electron transfer reactions during respiration (Machida & Tanaka, 1999). To determine if oxidative stress induced by farnesol mediated the stimulation of RhlR–C4HSL signalling, we determined if hydrogen peroxide was sufficient to stimulate RhlR-dependent PQS and pyocyanin production in PA14Δ*lasR* strains. Hydrogen peroxide (200 μM) was added to the agar medium prior to inoculation, and the colonies were grown for 48 h. As shown in Fig. 3(a), hydrogen peroxide stimulated pyocyanin production in PA14Δ*lasR*, but not PA14Δ*lasRrhlR*::TetR strains. Quantification of pyocyanin levels in these cultures is presented in Supplementary Fig. S6(a). Addition of NAC, an antioxidant, to the medium abolished the stimulation of pyocyanin production by hydrogen peroxide in *lasR* mutant strains. NAC did not affect pyocyanin levels in aerated WT cultures, indicating that it does not have an independent effect on phenazine production. NAC also repressed the farnesol-mediated stimulation of pyocyanin (Fig. 3b) and PQS (Fig. 3c) in PA14Δ*lasR* strains suggesting that ROS were mediating farnesol's effects on quorum sensing. The number of c.f.u. in colonies grown with farnesol alone or farnesol with NAC were comparable (Supplementary Fig. S6b). C4HSL levels in Δ*lasR* colonies were also lower upon the addition of 10 mM NAC to medium with farnesol (Fig. 3d).

As has been reported previously, Δ*lasR* strains can produce PQS after prolonged incubation (Dekimpe & Deziel, 2009;

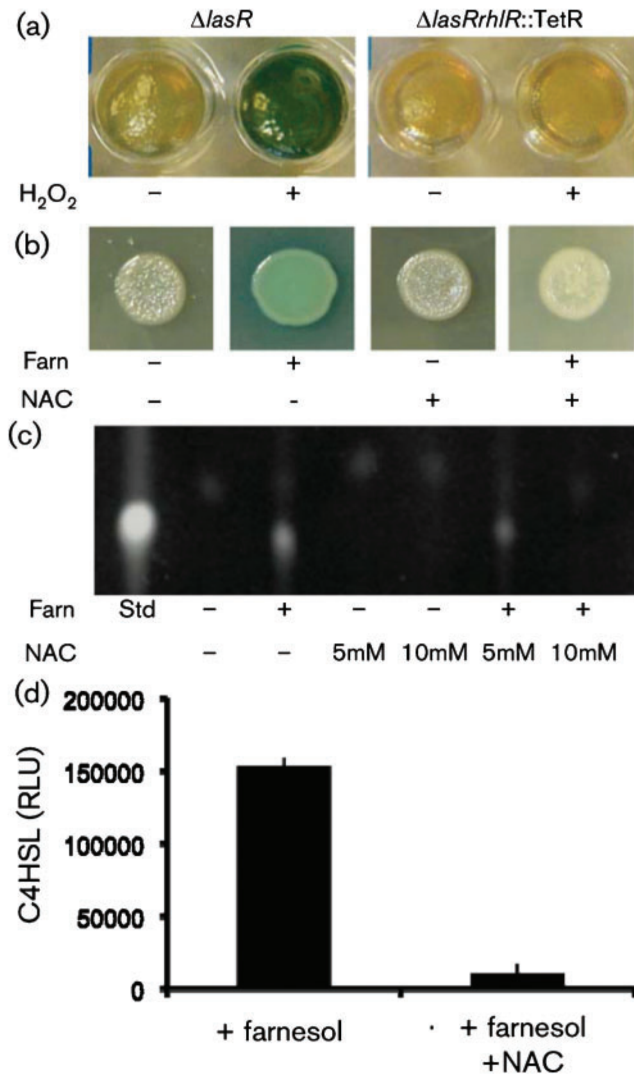

Fig. 3. *P. aeruginosa* colony appearance and PQS and C4HSL production by PA14Δ*lasR* grown in the presence of farnesol, hydrogen peroxide and the oxidative stress protectant NAC. (a) PA14 Δ*lasR* and Δ*lasRrhlR*::TetR colonies were grown in the absence or presence of hydrogen peroxide and photographed after 48 h. Colonies were grown on agar plugs in a 96-well microtitre dish. (b) PA14Δ*lasR* grown as spotted colonies on agar plates containing vehicle alone, 250 μM farnesol, 10 mM NAC or the combination of 10 mM NAC and 250 μM farnesol. (c) PQS production by Δ*lasR* colonies grown for 14 h in the absence and presence of 5 and 10 mM NAC, 250 μM farnesol or the combination of 5 and 10 mM NAC and 250 μM farnesol. PQS production was determined by TLC analysis and PQS was identified by comparison with an authentic PQS standard (25 ng). (d) C4HSL levels were measured from Δ*lasR* colonies grown for 14 h in the absence and presence of 250 μM farnesol or farnesol with 10 mM NAC. Levels were determined by luciferase activity (RLU) generated by the reporter plasmid pSB536, which detects short-chain AHLs. The assay was validated using a titrated authentic C4HSL standard.

Diggle *et al.*, 2003), and we observed low levels of PQS in Δ*lasR* colonies after entry into stationary phase. The slight production of PQS and pyocyanin by PA14Δ*lasR* in late stationary phase cultures or colonies was also suppressed by the addition of NAC to the medium, suggesting that late PQS production in $LasR^-$ strains is due to the effects of oxidative stress (data not shown). The PA14Δ*lasR*Δ*pqsH* and Δ*lasRrhlR*::TetR mutants, which no longer produced PQS on medium with farnesol (Fig. 1c), also lacked PQS production in cultures even when examined at very late time points (data not shown).

DISCUSSION

Numerous studies in multiple models of acute infection have demonstrated a marked decrease in virulence upon loss of LasR function, and this attenuation is likely due to the inability to induce important virulence factors (Lesprit *et al.*, 2003; Preston *et al.*, 1997; Rumbaugh *et al.*, 1999; Tang *et al.*, 1996). In contrast, the emergence of *lasR* mutants in chronic CF-related infections correlates with decreased lung function, suggesting that these strains may retain some virulence properties to complement their growth advantages in the CF lung environment (Hoffman *et al.*, 2009). Recent reports indicate that virulence factors, such as pyocyanin and elastase, which normally require LasR for production, are made by Δ*lasR* mutants in stationary phase cultures grown in TSB medium (Dekimpe & Deziel, 2009). The late production of these virulence factors is dependent on RhlR and C4HSL (Dekimpe & Deziel, 2009).

Here, we report that *C. albicans* and its secreted factor farnesol caused an increase in the levels of two quorum sensing molecules, PQS (Fig. 1b and Table 2) and C4HSL (Figs 2b and 3d), in the Δ*lasR* strain (see Fig. 4 for model). The increases in these signalling molecules translated into the increased production of the quorum-sensing-regulated phenazine virulence factors (Figs 1 and 3, and Supplementary Fig. S4). The increased production of PQS was dependent on *rhlR*, *rhlI* and *pqsH*, and RhlR was required for increased *pqsH* transcript levels in Δ*lasR* strains in the presence of farnesol (Fig. 2a). Similarly, the addition of C4HSL rescued PQS production in Δ*lasR* (Table 2) and Δ*lasI* strains (data not shown). While RhlR-dependent control of *pqsH* in a *lasR* mutant background was proposed previously (Dekimpe & Deziel, 2009), it had not previously been directly tested. We demonstrated that *pqsH* expression was sufficient to restore pyocyanin and PQS production in *lasR* and *lasI* mutants (Table 2 and data not shown). Farnesol induced C4HSL levels (Fig. 3b) and, as predicted by the positive feedback loop that controls *rhlR* and *rhlI* expression, *rhlR* and *rhlI* transcript levels increased by 11- and 7.3-fold, respectively, in the presence of farnesol (data not shown). Because RhlR is not required for the expression of *pqsH* in $LasR^+$ backgrounds, and, in fact, PQS levels are higher in *rhlI* and *rhlR* single mutants (Supplementary Fig. S5b) (McGrath *et al.*, 2004; Xiao *et al.*,

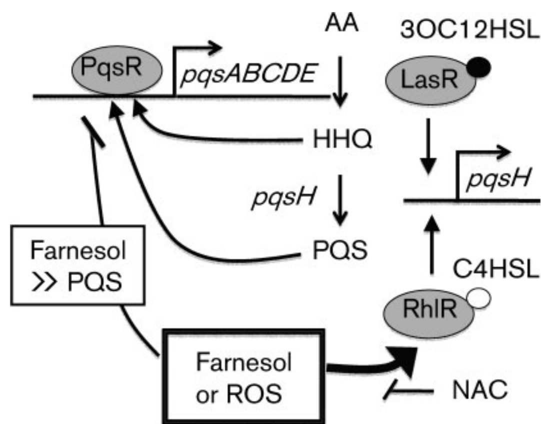

Fig. 4. Model for the effects of farnesol on PQS production in *P. aeruginosa*. Anthranilic acid (AA) is converted into HHQ by genes encoded by *pqsA–D*. HHQ is converted into PQS by PqsH. HHQ and PQS contribute to the positive feedback regulation of the *pqsA–D* genes via PqsR. Farnesol inhibits PqsR activity when PQS is at low levels. Inhibition of PqsR activity by farnesol is not complete, and as HHQ or PQS levels increase, farnesol no longer competes with natural PqsR ligands. Expression of *pqsH* is controlled by LasR when it is complexed with 3OC12HSL, and the RhlR–C4HSL complex is not needed for induction of *pqsH* in wild-type cells. In *lasR* or *lasI* mutants, RhlR–C4HSL can activate *pqsH*. While LasR–3OC12HSL is normally required for induction of *rhlR* and *rhlI*, farnesol or hydrogen peroxide can stimulate RhlR–C4HSL activity in the absence of LasR–3OC12HSL.

2006a), this activity of RhlR in LasR$^-$ strains represents an alternate mechanism for the regulation of the downstream portion of the quorum sensing regulatory pathway when the LasR function is absent.

Our data suggest that RhlR is activated in Δ*lasR* strains by ROS or oxidative stress. Farnesol perturbs electron transport in yeast mitochondria leading to the generation of ROS (Machida *et al.*, 1998) and promotes the generation of ROS in other fungi (Semighini *et al.*, 2006; Shirtliff *et al.*, 2009a). Research suggests that the same is true in bacteria (Gomes *et al.*, 2009). Because hydrogen peroxide is sufficient to induce PQS and pyocyanin production in Δ*lasR*, and NAC suppresses the induction of PQS production in Δ*lasR* strains grown with farnesol or hydrogen peroxide, we predict that farnesol-induced oxygen radicals are the key factor that triggers activation of the lower portion of the quorum sensing pathway (for a model see Fig. 4). While the mechanism by which ROS influence RhlR and RhlI remains the subject of future work, it is interesting to consider a potential role for the Lon protease. Lon degrades both short-lived regulators and damaged proteins (Van Melderen & Aertsen, 2009), and *P. aeruginosa* Lon has been shown previously to negatively regulate the RhlI synthase, which produces C4HSL (Takaya *et al.*, 2008). Perhaps ROS exposure somehow alters Lon-mediated degradation of RhlI allowing for the accumulation of higher levels of the C4HSL synthase, activation of the RhlR autoregulatory loop and, ultimately, *pqsH* expression. NAC also affects PQS production in PA14Δ*lasR* cultures in the absence of farnesol. The production of PQS and pyocyanin that is observed in late stationary phase Δ*lasR* and Δ*lasI* liquid cultures was suppressed by NAC (data not shown). It is not yet clear whether farnesol affects *P. aeruginosa* WT and Δ*las* strains differently, though there is a clear change in respiration upon the loss of LasR (Hoffman *et al.*, 2010), and these differences may impact how farnesol affects cells. It is interesting to note that NAC is currently in phase II clinical trials as a useful alternative therapy for treatment of CF (Cystic Fibrosis Foundation, 2006). In addition to the direct benefits of reducing ROS in the lungs, NAC might have the added benefit of decreasing *P. aeruginosa* virulence by preventing activation of this alternative regulatory pathway.

Published evidence supports the idea that there is a connection between oxidative stress and quorum sensing regulation. PQS not only induces genes involved in oxidative stress (Bredenbruch *et al.*, 2006; Häussler & Becker, 2008) but also confers the induction of a protective oxidative stress response (Häussler & Becker, 2008). Furthermore, an *oxyR* mutant, with decreased expression of genes that encode ROS scavenging enzymes, exhibits increased levels of pyocyanin (Vinckx *et al.*, 2010). While no differences in PQS levels were observed at 24 h, it would be interesting to determine whether the *oxyR* mutant has accelerated PQS production relative to WT strains. While it was not the focus of the work, we did observe slightly increased PQS when *P. aeruginosa* WT was grown on medium with farnesol or hydrogen peroxide (data not shown). Activation of PQS production by ROS may indicate a role for PQS in defence. In the presence of polymorphonuclear neutrophils, *P. aeruginosa* had elevated levels of RhlR-regulated rhamnolipids and increased expression of the PQS biosynthetic operon (Alhede *et al.*, 2009).

Farnesol has multiple effects on *P. aeruginosa* cells. Previously, we reported that *C. albicans* and farnesol led to decreased PQS and pyocyanin levels in *P. aeruginosa* by affecting the ability of the PqsR–PQS complex to activate *pqsA* transcription (Cugini *et al.*, 2007). We found that either PQS or farnesol promotes PqsR binding to the *pqsA* promoter, but only PQS leads to transcriptional activation of the *pqsA–E* promoter. The inhibition of PqsR activity by farnesol only occurred at lower cell densities due to the fact that PqsR appears to have a greater affinity for PQS than for farnesol by several orders of magnitude (see Fig. 4 for a model). As in liquid cultures, farnesol also led to a delay in early PQS production in PA14 WT colony biofilms, but this repression was not observed as colony density increased. These data indicate that farnesol affects *P. aeruginosa* quorum sensing through both interaction with specific targets (Cugini *et al.*, 2007) and the generation of ROS.

Both clinical and environmental strains commonly have mutations in *lasR*, and LasR loss-of-function mutants can be selected for under laboratory conditions (Cabrol *et al.*,

2003; Dénervaud *et al.*, 2004; Heurlier *et al.*, 2005; Hoffman *et al.*, 2009; Luján *et al.*, 2007; Smith *et al.*, 2006; Wilder *et al.*, 2009). Diffusible signals such as C4HSL and PQS can stimulate virulence factor production in *las* mutants (Déziel *et al.*, 2004; Sandoz *et al.*, 2007). Thus, the presence of LasR$^+$ *P. aeruginosa* strains may be sufficient to compensate for loss of LasR function (Sandoz *et al.*, 2007). Here, we demonstrate that other microbes, such as the fungus *C. albicans*, and the environmental conditions created through the secretion of small molecules, also induce virulence factor production in *lasR*-defective laboratory and clinical mutants (Fig. 1a and Supplementary Fig S4b).

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