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Intraspecies Signaling between Common Variants of *Pseudomonas aeruginosa* Increases Production of Quorum-Sensing-Controlled Virulence Factors

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ABSTRACT The opportunistic pathogen *Pseudomonas aeruginosa* damages hosts through the production of diverse secreted products, many of which are regulated by quorum sensing (QS). The *lasR* gene, which encodes a central QS regulator, is frequently mutated in clinical isolates from chronic infections, and loss of LasR function (LasR−) generally impairs the activity of downstream QS regulators RhlR and PqsR. We found that in cocultures containing LasR+ and LasR− strains, LasR− strains hyperproduce the RhlR/RhlI-regulated antagonistic factors pyocyanin and rhamnolipids in diverse models and media and in different strain backgrounds. Diffusible QS autoinducers produced by the wild type were not required for this effect. Using transcriptomics, genetics, and biochemical approaches, we uncovered a reciprocal interaction between wild-type and *lasR* mutant pairs wherein the iron-scavenging siderophore pyochelin produced by the *lasR* mutant induced citrate release and cross-feeding from the wild type. Citrate, a metabolite often secreted in low iron environments, stimulated RhlR signaling and RhlI levels in LasR−but not in LasR+ strains. These studies reveal the potential for complex interactions between recently diverged, genetically distinct isolates within populations from single chronic infections.

IMPORTANCE Coculture interactions between *lasR* loss-of-function and LasR+ *Pseudomonas aeruginosa* strains may explain the worse outcomes associated with the presence of LasR− strains. More broadly, this report illustrates how interactions within a genotypically diverse population, similar to those that frequently develop in natural settings, can promote unpredictably high virulence factor production.

KEYWORDS *Pseudomonas aeruginosa*, RhlR, citrate, intraspecies interactions, *lasR*, pyochelin, quorum sensing

In chronic infections and healthy microbiomes, genetic diversity frequently arises and persists within clonally derived microbial populations, and recent data highlight that heterogeneity within a population can pose challenges to clearance and treatment (1–4). Genotypic and phenotypic complexity is particularly well documented in the chronic lung infections associated with the genetic disease cystic fibrosis (CF), and studies have convincingly demonstrated that within a species, a common set of genes is under selection across strains and hosts (5–11).

Loss-of-function mutations in *Pseudomonas aeruginosa lasR* (LasR−) are commonly found in CF isolates and strains from acute infections and from environmental sources (12–16). LasR participates in the regulation of quorum sensing (QS) in conjunction with other transcription factors, including RhlR and PqsR (MvfR). These regulators have one or more autoinducer ligands: 3-oxo-C_{12}-homoserine lactone (3OC12HSL) for LasR, C_4-homoserine lactone (C4HSL) for RhlR, and hydroxy-alkyl-quinolones (pseudomonas quinolone signal [PQS] and hydroxy-heptyl quinolone [HHQ]) for PqsR (17). In the

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regulatory networks described in widely used *P. aeruginosa* model strains, LasR is an upstream regulator of RhlR and PqsR signaling, and together these regulators control the expression of a suite of genes associated with virulence, including redox-active small-molecule phenazines (18–20), cyanide (21), and rhamnolipid surfactants important for surface motility, biofilm dispersal, and host cell damage (22–24).

Although LasR positively regulates virulence factors, and *lasR* loss-of-function mutants have reduced virulence in infection models, LasR− strain culture positivity is correlated with worse disease outcomes in acute and chronic infections (12, 13). There are several possible explanations for this apparent contradiction. LasR− clinical isolates (CIs) are frequently found among strains with functional LasR (LasR+) where exoproducts can be shared or signal cross-feeding can occur (14), and some LasR− clinical isolates exhibit rewired QS regulation (25). Loss of LasR function also confers some fitness advantages, including altered catabolic profiles (26) and enhanced growth in low oxygen (27, 28), which may contribute to bacterial burden. Further, LasR− strains can activate QS in response to specific fungal products (29) or culture conditions (30, 31).

In addition to LasR status, iron acquisition strategies are often heterogeneous across *P. aeruginosa* isolates. *P. aeruginosa* procures iron through the use of siderophores, including pyochelin (32–34) and pyoverdine (35), from heme, or through a direct iron uptake system (36–38). Although it is common to encounter *P. aeruginosa* strains with loss-of-function mutations in genes required for biosynthesis of the high-affinity siderophore pyoverdine, genes associated with use of pyochelin, heme utilization, and ferrous iron import are generally intact (39–41). Iron limitation can deprioritize pathways that require abundant iron including the tricarboxylic acid (TCA) cycle (42), and consequently *Pseudomonas* spp. and other organisms release metabolic intermediates, such as citrate, that accumulate at iron-requiring steps (e.g., aconitase) (43, 44).

Here, we show that mixtures of *P. aeruginosa* LasR− and LasR+ strains had enhanced production of QS-controlled factors across media, culture conditions, and strain backgrounds. The unpredictably high levels of exoproducts in coculture were produced by LasR− strains due to activation of RhlR, likely through increased C4HSL synthase (RhlI) stability in LasR− strains. Our genetic, transcriptomic, and biochemical studies led us to uncover a set of interactions in which production of the siderophore pyochelin by Δ*lasR* cells induced citrate release by wild type (WT) but not by Δ*lasR* cells. We found that citrate led to increased RhlI protein levels and RhlR activity in Δ*lasR* cells but not in the WT. Together, these intraspecies interactions increased production of exoproducts known to cause host damage.

RESULTS

***P. aeruginosa* Δ*lasR* overproduces pyocyanin in coculture with the wild type.** We observed that mixtures of *P. aeruginosa* LasR+ and LasR− strains had high levels of total pyocyanin, a secreted, blue-pigmented phenazine. As shown in spot colonies of the PA14 wild-type (WT), Δ*lasR* strain, and WT and Δ*lasR* strain cocultures (here, WT/Δ*lasR* cocultures), the strain mixture showed increased blue pigmentation (Fig. 1A) and a significant 4-fold induction of pyocyanin above the background relative to the level with either strain alone (Fig. 1B). Phenazine-deficient derivatives, Δ*phz* (Δ*phzA1* Δ*phzB1* Δ*phzC1* Δ*phzD1* Δ*phzE1* Δ*phzF1* Δ*phzG1* Δ*phzA2* Δ*phzB2* Δ*phzC2* Δ*phzD2* Δ*phzE2* Δ*phzF2* Δ*phzG2*) and Δ*lasR* Δ*phz* (Δ*lasR* Δ*phzC1* Δ*phzC2*) strains, were also included, and as expected, Δ*phz* and Δ*lasR* Δ*phz* cells showed no blue colony pigmentation (Fig. 1A). The higher levels of pyocyanin in WT/Δ*lasR* cocultures relative to levels in single-strain cultures were also observed on artificial sputum medium (ASM) and on phosphate-buffered medium with or without amino acids, indicating that the phenomenon occurred under diverse conditions (see Fig. S1A in the supplemental material). Cocultures of clonally derived LasR+ and LasR− clinical isolates collected from single respiratory sputum samples from chronically infected individuals with cystic fibrosis (14) also had increased production of pyocyanin relative to monoculture levels when LasR+ and LasR− strains were grown together (Fig. S1B and C).

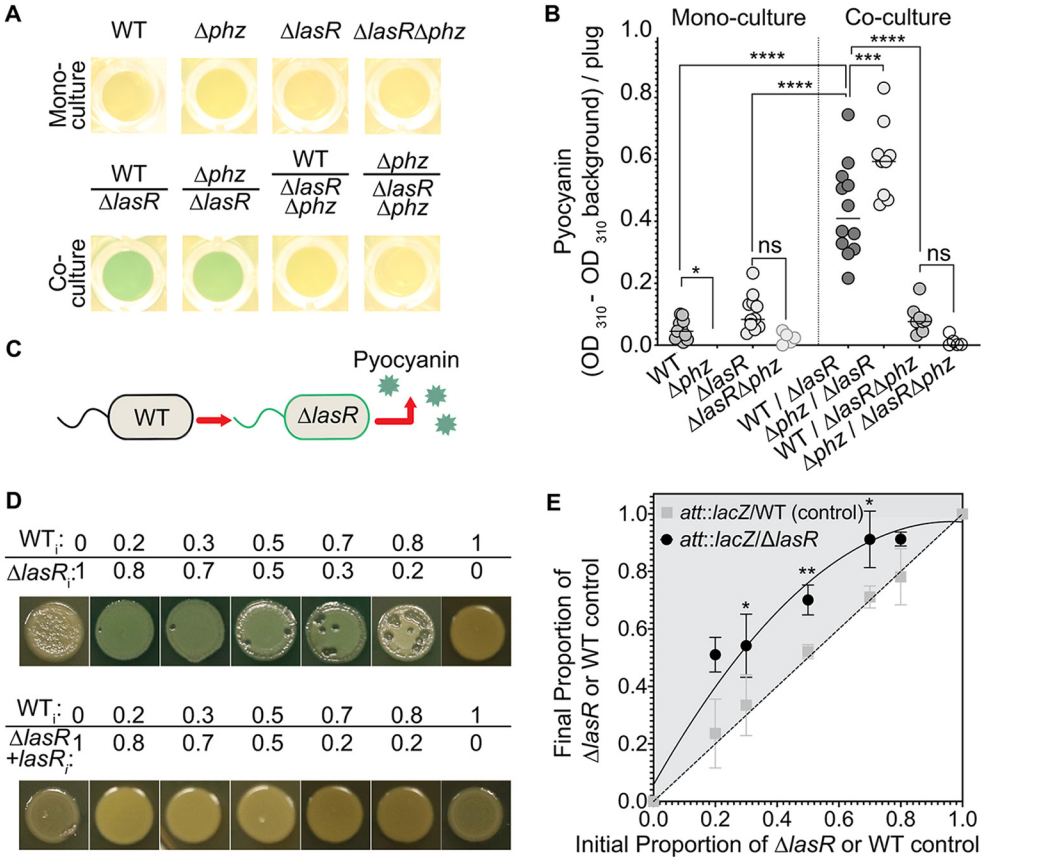

FIG 1 The Δ*lasR* strain produces pyocyanin in wild-type/Δ*lasR* cocultures beyond monoculture concentrations. (A) Representative images of the wild-type (WT) and Δ*lasR* strains and their phenazine-deficient derivatives (Δ*phz* strains) visualized from the bottom of a 96-well LB agar plate after 16 h growth as mono- and cocultures with 70:30 WT-to-Δ*lasR* cell initial ratio. (B) Pyocyanin levels above background, defined as the average signal for the Δ*lasR* Δ*phz* strain, quantified for cultures described in panel A. ns, not significant; *, $P < 0.05$; ***, $P < 0.0005$; ****, $P < 0.0001$, as determined by ordinary one-way analysis of variance with Tukey's multiple-comparison test for $n \geq 8$ replicates on three different days. (C) Model of pyocyanin production by the Δ*lasR* strain in coculture with the WT. (D) Representative pyocyanin production of the wild type cocultured with the Δ*lasR* strain or the Δ*lasR* strain complemented with the *lasR* gene at the native locus (Δ*lasR* + *lasR* strain) across several initial (designated by the subscript i) proportions on LB medium for 20 h. Three biological replicates were included in at least 3 independent experiments. (E) Average final proportion of 3 replicate colony biofilms quantified after 16 h growth for the WT strain and the Δ*lasR* strain cocultured with a WT strain tagged with *lacZ* in 3 independent experiments. Experimental setup was as described in panel D. *, $P < 0.05$; **, $P < 0.005$, as determined by two-tailed *t* tests of paired ratios between *att*::*lacZ*/WT (control) and *att*::*lacZ*/Δ*lasR* cocultures at each initial ratio. All results that reach significance are marked.

To assess individual strain contributions to increased pyocyanin in WT/Δ*lasR* cocultures, we replaced each strain with its phenazine-deficient derivative and measured pyocyanin in coculture. When the Δ*lasR* strain was cultured with the phenazine biosynthesis mutant Δ*phz* strain (Δ*phz*/Δ*lasR* coculture), we still observed increased blue pigmentation and total pyocyanin at a level above that of either monoculture (Fig. 1A and B). Surprisingly, pyocyanin production by Δ*phz*/Δ*lasR* cocultures was statistically higher than that of WT/Δ*lasR* coculture (Fig. 1B). In contrast, WT/Δ*lasR* Δ*phz* cocultures did not display the high-pyocyanin phenotype (Fig. 1A and B) and resembled Δ*phz*/Δ*lasR* Δ*phz* cocultures, where no pyocyanin was produced. Collectively, these data suggested that WT/Δ*lasR* cocultures produced more pyocyanin than either monoculture alone and that the Δ*lasR* strain contributed the pyocyanin in coculture (Fig. 1C).

That the levels of pyocyanin in WT/Δ*lasR* cocultures were higher than the level in each strain alone was not dependent on the initial ratios of WT to Δ*lasR* cells (Fig. 1D). We saw increased coculture colony pigmentation when the initial proportions of WT

cells were at 0.2, 0.3, 0.5, 0.7, and 0.8 of the initial inoculums, with the balance comprised of Δ*lasR* cells (Fig. 1D).

No increase in pyocyanin was observed at any ratio when the WT was cocultured with the Δ*lasR* complemented derivative strain (Δ*lasR* + *lasR*), indicating that the phenomenon was dependent on the *lasR* mutation (Fig. 1D). To assess the relative abundances of WT and Δ*lasR* cells in coculture, we competed each strain against a neutrally tagged WT strain (PA14 *att*::*lacZ*). We found that Δ*lasR* cells increased in proportion after 16 h of growth in colony biofilms regardless of the starting proportion whereas the proportions of untagged WT cells remained stable (Fig. 1E). We have previously shown that Anr activity is higher in Δ*lasR* strains and contributes to the competitive fitness of the Δ*lasR* strain against WT *P. aeruginosa* in colony biofilms (27, 45), but Anr was not required for coculture pyocyanin production (Fig. S2).

Pyocyanin is a product regulated by quorum sensing (QS) through the transcription factors LasR, RhlR, and PqsR (46–48), and because QS regulation is cell density dependent, it was important to assess the coculture population size relative to that of the monoculture. Total CFU counts did not increase in WT/Δ*lasR* mixed cultures relative to the level for either strain alone (Fig. S1D). Instead, we found that WT/Δ*lasR* cocultures had fewer CFU than WT monocultures on lysogeny broth (LB) medium (Fig. S1D). Taken together, these data suggested that altered behavior, rather than cell number, contributed to the increased phenazine profile of LasR− strains.

Independent of its ability to produce autoinducers, the WT promotes RhlR/I-dependent signaling in a Δ*lasR* strain. In the canonical QS pathway, LasR regulates both PqsR and RhlR, and mutants lacking either regulator in a WT background have impaired pyocyanin production (49, 50). Both *pqsR* and *rhlR* were required in Δ*lasR* cells for pyocyanin production in coculture with the WT (Fig. 2A). To determine if coculture increased RhlR- or PqsR-dependent signaling in Δ*lasR* strains, we fused *lacZ* to the promoters of *rhlI* and *pqsA* (P*rhlI* and P*pqsA*, respectively) which provide activity readouts of each respective regulator (17). We examined the interactions between WT and the Δ*lasR* strain in single-cell-derived colonies by spreading suspensions containing ~50 cells of WT with ~50 cells of either a Δ*lasR* P*rhlI-lacZ* or Δ*lasR* P*pqsA-lacZ* strain on LB agar containing the colorimetric β-galactosidase substrate 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-Gal). Intercolony distances and β-galactosidase activity in Δ*lasR* strains were measured. We found that the rise in P*rhlI-lacZ* activity was inversely correlated with the distance to a WT colony (Fig. 2B). Pearson correlation analyses showed that 54% of the variability in Δ*lasR* P*rhlI- lacZ* strain activity could be explained by changes in the distance to a WT colony (P value of $\leq$ 0.0001). The increased P*rhlI-lacZ* activity in the Δ*lasR* strain was not observed in the Δ*lasR* Δ*rhlR* strain, and close proximity to another Δ*lasR* P*rhlI-lacZ* colony did not affect promoter activity (Fig. 2B, inset). Because C4HSL (which is synthesized by RhlI) activates RhlR and because proximity to the WT stimulated Δ*lasR* P*rhlI-lacZ* strain activity, we examined the role of RhlI in the Δ*lasR* strain response. We observed that a Δ*lasR* Δ*rhlI* strain was greatly impaired in the induction of pyocyanin upon coculture with the WT (Fig. 2A), which suggests that WT production of C4HSL was insufficient to complement the Δ*lasR* Δ*rhlI* strain and further posits activation of RhlR and C4HSL synthesis in Δ*lasR* strains. Although PqsR was required in Δ*lasR* cells for coculture pyocyanin production, there was no significant correlation with proximity to the WT for Δ*lasR* P*pqsA-lacZ* strain activity (Fig. 2B). Collectively, these data indicated that a diffusible factor produced by the WT stimulated RhlR-dependent signaling in the Δ*lasR* strain to induce downstream production of RhlR- and PqsR-dependent factors.

Given differences in colony pigmentation between WT/Δ*lasR* Δ*rhlR* and WT/Δ*lasR* Δ*rhlI* (Fig. 2A) cocultures, C4HSL cross-feeding between the WT and Δ*lasR* strain likely occurred. Because C4HSL is diffusible and produced by WT cells, we tested the hypothesis that C4HSL or other acyl-homoserine lactones (AHLs) produced by the WT were necessary to induce RhlR-dependent activity in Δ*lasR* cells cocultured with the WT. To test this hypothesis, we cocultured the Δ*lasR* strain with Δ*rhlI* cells or Δ*lasI* Δ*rhlI* cells which lack both acyl-homoserine lactone synthases. Surprisingly, we found that like

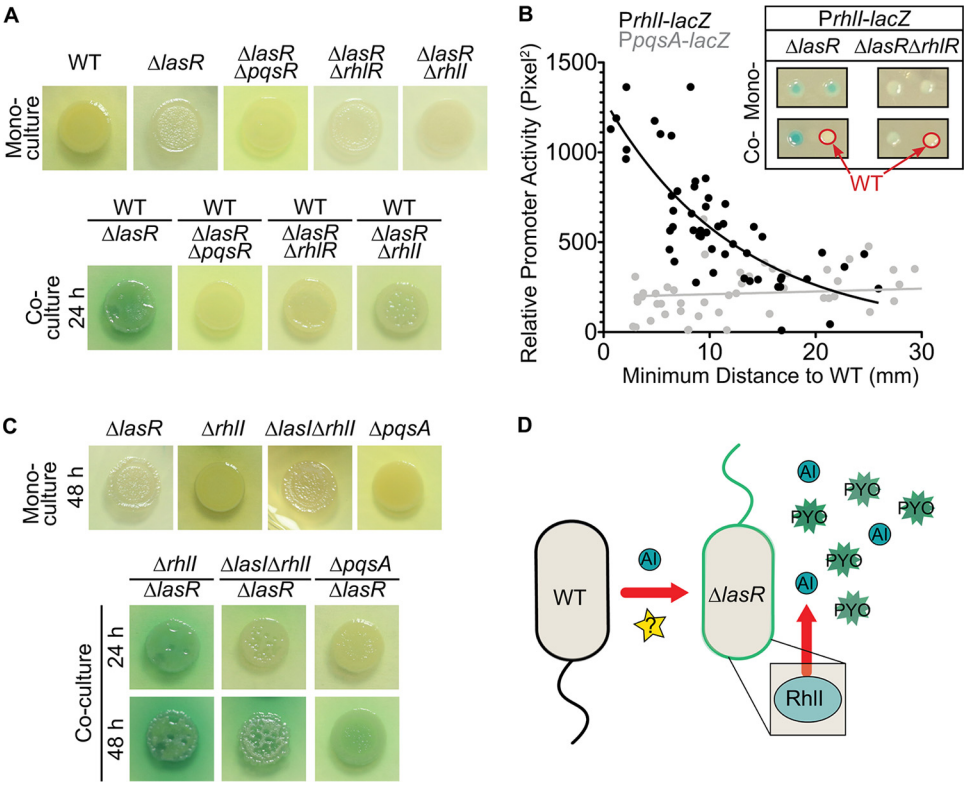

FIG 2 *P. aeruginosa* WT induces RhlR/I-dependent pyocyanin production in Δ*lasR* cells even in the absence of WT autoinducers. (A) Representative pyocyanin production by monocultures and WT cocultures (70% WT at time 0) of Δ*lasR* and Δ*lasR* strain derivatives that are deficient in PQS or RhlR/I-dependent quorum sensing on LB medium after 24 h growth. (B) Promoter activity of the Δ*lasR* P*pqsA-lacZ* (gray) and Δ*lasR* P*rhlI-lacZ* (black) strains, quantified by relative pixel intensity of single-cell-derived Δ*lasR* colonies grown near unmodified WT colonies. Solid best-fit nonlinear lines are for visualization. The inset shows representative colonies for RhlR-dependent Δ*lasR* P*rhlI-lacZ* strain activity in monoculture and coculture with the WT (red circles). The experiment was repeated with 2 replicates on at least 3 independent days. (C) Representative monoculture and Δ*lasR* coculture images for the Δ*pqsA*, Δ*rhlI*, and Δ*lasI* Δ*rhlI* mutants on LB medium after 24 h and 48 h. (30% Δ*lasR* cells at time 0). (D) Model of AHL-dependent and -independent induction of RhlR/I-dependent activity and phenazine production in Δ*lasR* cells grown in coculture with the WT.

WT/Δ*lasR* cocultures, Δ*rhlI*/Δ*lasR* cocultures had higher levels of pyocyanin than monocultures (Fig. 2C). Similarly, Δ*lasI* Δ*rhlI*/Δ*lasR* cocultures had higher levels of pyocyanin production than monocultures though the interaction was delayed by ~24 h relative to the interaction of the WT/Δ*lasR* cocultures (Fig. 2C). Consistent with the activity of the Δ*lasR* P*pqsA-lacZ* strain, which was not induced in coculture with WT, the PQS-deficient Δ*pqsA* strain supported high pyocyanin colony pigmentation in coculture with Δ*lasR* cells after 24 h of extended incubation (Fig. 2C). The AHL-independent activation observed in Δ*lasI* Δ*rhlI*/Δ*lasR* cocultures and the striking differences in pyocyanin production observed between the strongly stimulating Δ*rhlI*/Δ*lasR* cocultures and the weakly stimulating WT/Δ*lasR* Δ*rhlI* cocultures suggested that the Δ*lasR* strain may rely more heavily on production of its own autoinducer for activation in coculture. Consistent with this model, we found that the Δ*lasR* strain produces RhlR/RhlI (RhlR/I)-dependent AHLs in coculture with an AHL-sensing reporter strain (i.e., Δ*lasI* Δ*rhlI* strain with a *lacZ* promoter fusion to an AHL-responsive gene) (Fig. S3A and B). The dispensable contribution of WT-produced autoinducers implicated a novel signaling interaction in coculture-dependent activation of RhlR/I activity in the Δ*lasR* strain (Fig. 2D).

To assess whether WT-induced RhlR activity in the Δ*lasR* strain was sufficient to elicit other RhlR/I-controlled phenotypes in addition to pyocyanin production, we tested whether coculture with LasR+ strains enhanced swarming, a surface-associated motility which requires the production of RhlR-regulated rhamnolipid surfactants (51). While

the rhamnolipid-defective mutant Δ*rhlA*, Δ*lasR*, and Δ*lasR* Δ*rhlR* strains were unable to swarm, cocultures of the Δ*lasR* strain with the Δ*rhlA* strain swarmed considerably. The phenomenon was dependent on RhlR as the Δ*rhlA*/Δ*lasR* Δ*rhlR* cocultures did not swarm (Fig. S4). Altogether, these data implicated broad activation of RhlR-mediated QS in LasR− strains cocultured with LasR+ *P. aeruginosa*.

Pyochelin production by Δ*lasR* cells is required for coculture interactions. With evidence indicating that induction of RhlR activity in Δ*lasR* cells can occur in both mixed-strain spot colonies and adjacent colonies independent of autoinducer cross-feeding, we sought to gain further insight into the mechanisms that underlie the WT-Δ*lasR* cell interactions. We investigated the transcriptomes of the *lasR* mutant in coculture with either the WT or itself via RNA sequencing (RNA-seq). We grew Δ*lasR* colony biofilms on LB medium physically separated from a lawn of either the Δ*lasR* or WT strain by two filters with 0.22-μm pores to prevent mixing of genotypes while allowing for the passage of small molecules. In order to examine Δ*lasR* strain transcriptional profiles, RNA was extracted from cells within the Δ*lasR* colony biofilms grown on the topmost filter for 16 h (Fig. 3A). As expected, no *lasR* reads were detected in our sequencing data to suggest that the wild type was sufficiently excluded by filter separation. Expression levels of a total of 199 genes in the Δ*lasR* strain were higher, and those of 198 genes were lower by a $|\log_2(\text{fold change})|$ of ≥ 1 with a P value of <0.05, in coculture with the WT than levels in the Δ*lasR* strain alone (Table S1). Gene Ontology (GO) term analyses through PantherDB (52) indicated that the upregulated gene set was significantly overrepresented in two pathways related to siderophore biosynthesis: the pyoverdine biosynthetic process and salicylic acid biosynthetic process (an upstream precursor of pyochelin) with ~44- and ~77-fold enrichment, respectively (P values of <0.005). Twenty-eight out of the 33 genes in the pyochelin and pyoverdine siderophore biosynthesis- and acquisition-related GO families were significantly upregulated in Δ*lasR* cells upon coculture with the WT (Fig. 3B) (i.e.,$|\log_2(\text{fold change})|$ of ≥ 0 with a P value of <0.05). Other low-iron-responsive genes were differentially expressed, including the *has* genes involved in heme uptake and *antABC* genes (Table S1). While we observed stimulation of *rhlI* promoter activity and increased production of RhlR-regulated products, we did not see a broad transcriptional pattern indicative of RhlR activation at this early time point (Table S1), and this point is discussed below.

Given that siderophore biosynthesis genes were upregulated in Δ*lasR* cells cocultured with WT cells, we qualitatively examined production of fluorescent pyoverdine and pyochelin siderophores in monocultures and cocultures. To determine the contribution of both pyoverdine and pyochelin by the Δ*lasR* strain to fluorescence, genes required for pyoverdine biosynthesis (Δ*lasR* Δ*pvdA* strain), pyochelin biosynthesis (Δ*lasR* Δ*pchE* strain), or both pathways (Δ*lasR* Δ*pvdA* Δ*pchE* strain) were disrupted (Fig. 3C). Increased fluorescence attributable to both pyoverdine and pyochelin in coculture was due to siderophore production by Δ*lasR* strains, consistent with the RNA-seq data, as the increased fluorescence in WT/Δ*lasR* cocultures was lost in coculture when the Δ*lasR* strain was replaced with a Δ*lasR* Δ*pvdA*, Δ*lasR* Δ*pchE*, or Δ*lasR* Δ*pvdA* Δ*pchE* mutant. While cocultures of the WT and the pyoverdine-deficient derivative Δ*lasR* Δ*pvdA* strain (i.e., WT/Δ*lasR* Δ*pvdA* coculture) showed increased pyocyanin production relative to that of either monoculture, the Δ*lasR* Δ*pchE* and Δ*lasR* Δ*pvdA* Δ*pchE* strains did not promote pyocyanin production in coculture with the WT, as observed by colony pigmentation (Fig. 3D). The decrease in Δ*lasR* strain-derived pyocyanin was not due to decreased fitness as disruption of *pvdA* and *pchE* individually in Δ*lasR* cells had no effect on the final proportions; in contrast, the Δ*lasR* Δ*pvdA* Δ*pchE* strain had a significant defect in competitive fitness compared to the fitness of the Δ*lasR* parental strain (Fig. S5). These data suggested that pyochelin played a role in the coculture interaction. To test this model, we complemented the pyocyanin defect in the siderophore-deficient Δ*pvdA* Δ*pchE*/Δ*lasR* Δ*pchE* coculture with pyochelin-containing extracts from cultures of Δ*pvdA* cells which cannot produce pyoverdine or control extracts from

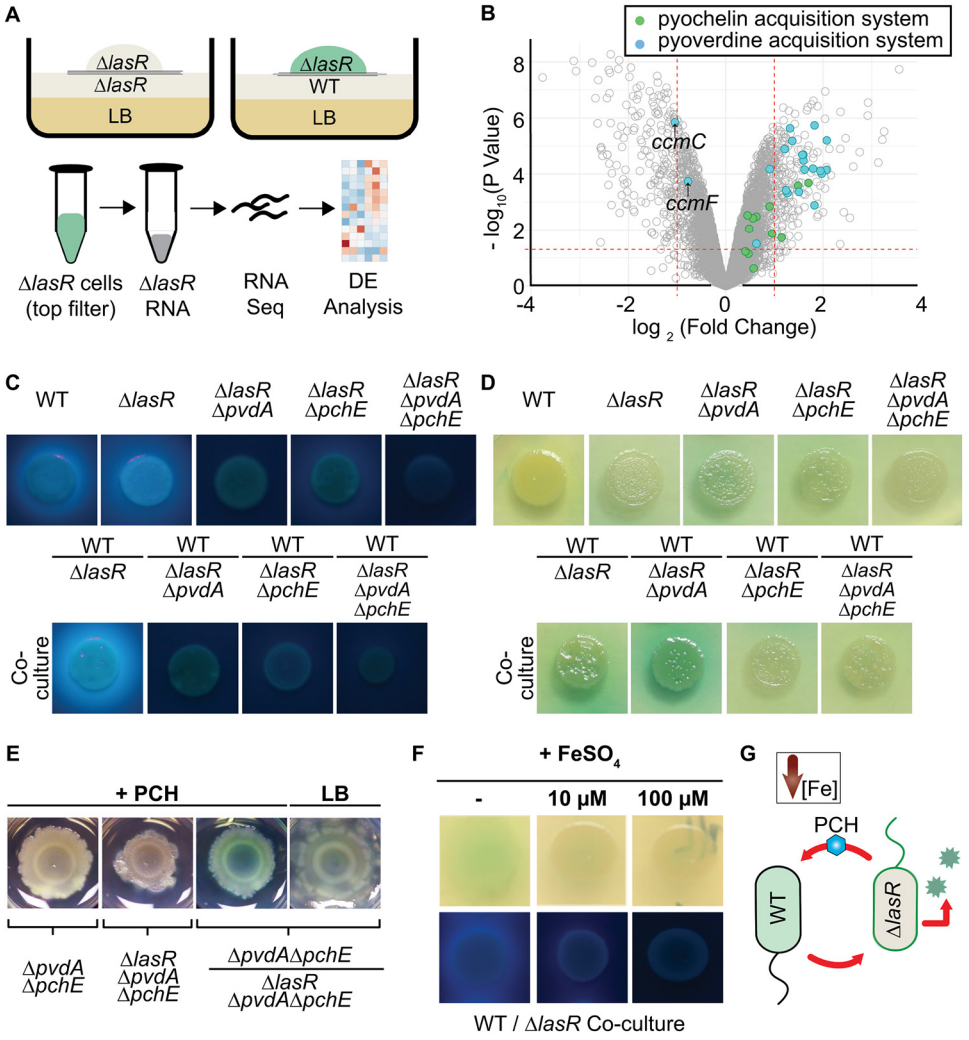

FIG 3 Biosynthesis of the coculture-induced iron scavenging siderophore pyochelin is required in the Δ*lasR* strain for pyocyanin production when it was cultured with the wild type (WT). (A) Scheme for the collection of RNA from Δ*lasR* colony biofilms grown above a lawn of Δ*lasR* or WT cells. DE, differential expression. (B) Volcano plot showing differential expression ($\log_2$) for Δ*lasR* cells grown over WT relative to Δ*lasR* cells grown over Δ*lasR* on the *x* axis; the *y* axis shows the $-\log_{10}$ *P* value for the difference between sample types. Genes involved in pyoverdine (blue) and pyochelin (green) iron acquisition systems are indicated. *ccmC* and *ccmF* (indicated with arrows) of the pyoverdine GO term are involved in *c*-type cytochrome biosynthesis, and strains with knockouts of these genes are reported to produce more pyochelin. (C) Monocultures and WT cocultured with Δ*lasR* strains deficient in pyoverdine (Δ*pvdA*) and/or pyochelin (Δ*pchE*) biosynthesis. Colonies were visualized under UV light in order to see fluorescent siderophores. Images are representative of at least 3 independent experiments. (D) Pyocyanin production visualized for the colonies shown in panel C. (E) Representative pyocyanin production by siderophore-deficient strains grown in mono- and coculture on LB medium or on LB medium with pyochelin-containing extract (+PCH). Colonies were grown in a 12-well plate with a 2-ml total volume and imaged after 24 h. (F) Mixed colony biofilms of wild-type and Δ*lasR* strains grown on LB medium (−) or LB medium supplemented with either 10 or 100 μM $FeSO_4$ visualized under ambient (top) and UV (bottom) light. (G) Model showing that pyochelin (PCH) production by the Δ*lasR* strain is required for WT/Δ*lasR* coculture phenazines.

siderophore-deficient Δ*pvdA* Δ*pchE* cultures (Fig. S6A gives supernatant absorption spectra). The two extracts were analyzed using a chrome azurol S (CAS) assay (53) to confirm that chelator activity was present in the Δ*pvdA* cell supernatant extracts but not in extracts from Δ*pvdA* Δ*pchE* cultures (Fig. S6B). Medium supplemented with pyochelin-containing extracts, but not siderophore-free extracts, restored pyocyanin production in Δ*pvdA* Δ*pchE*/Δ*lasR* Δ*pchE* cocultures (Fig. 3E), lending further support to the idea that pyochelin was required for coculture interactions. Consistent with this requirement, iron supplementation suppressed siderophore production, as expected, and diminished coculture pyocyanin in WT/Δ*lasR* cocultures (Fig. 3F) alongside a

decrease in Δ*lasR* strain RhlR/I-dependent AHL activity in coculture with the AHL-sensing reporter strain (Fig. S3C and D). Collectively, these data support a model in which pyochelin production by the Δ*lasR* strain is induced and required for pyocyanin-promoting interactions with the WT through initiation of a low-iron response (Fig. 3G).

In coculture with the WT, the Δ*lasR* strain responds to citrate, a pyochelin-inducible metabolite. Many of the upregulated genes in the Δ*lasR* strain upon coculture with the WT have annotations related to organic acids, such as anthranilate and citrate (Table S1 and Fig. S7A). Several lines of evidence suggest that anthranilate was not the factor that stimulated RhlR activity and pyocyanin production in coculture. First, anthranilate supplementation (up to ~15 mM) did not alter Δ*lasR* strain phenazine production (Fig. S7B). Further, cocultures of the Δ*lasR* mutant with the anthranilate synthase mutant Δ*phnAB* strain, with reduced extracellular anthranilate (Fig. S7C), or with the Δ*pqsA* mutant (Fig. 2C), which accumulates it (54), did not alter coculture phenazine production. Anthranilate is also generated from tryptophan catabolism through the kynurenine pathway (55). Given that coculture pyocyanin production could occur in the absence of exogenous amino acids (Fig. S1A), we concluded that the kynurenine pathway was likely not involved. Together, these data suggested that anthranilate was not a stimulating metabolite.

In light of the observation that 20% of the most strongly differentially expressed genes [$|\log_2$ (fold change)| of $\geq$2 with a *P* value of $<$0.05] were implicated in citrate sensing, transport, catabolism, and anabolism, as annotated by UniProt (56) and www.pseudomonas.com (57), we looked at all genes with annotations related to citrate to identify broad expression patterns (Fig. 4A). Among the genes induced in Δ*lasR*/WT cocultures [$|\log_2$ (fold change)| of $\geq$0 with a *P* value of $<$0.05] were genes annotated as citrate responsive or playing roles in citrate sensing and transport or metabolism, with the most strongly upregulated citrate genes involved in sensing, transport, and catabolism specifically (Fig. 4B).

We measured citrate in the supernatants of WT and Δ*lasR* LB cultures based on the following observations: (i) Δ*lasR* strains induced low-iron-responsive genes when grown near the WT but not itself; (ii) Δ*lasR* strain pyochelin production was necessary for coculture interactions that led to increased pyocyanin; (iii) citrate sensing and catabolism genes were induced in Δ*lasR* cells by the presence of WT cells; and (iv) numerous microbes, including *Pseudomonas putida*, were shown to secrete citrate and other organic acids when iron limited (44, 58–60). Citrate was detected in both WT and Δ*lasR* strain LB culture supernatants (Fig. 4C), and amendment with extracts containing 50 μM pyochelin increased extracellular citrate concentrations by ~2-fold in WT cultures compared to levels in cultures supplemented with extracts lacking pyoverdine and pyochelin, with a much smaller stimulation in Δ*lasR* cultures under the same conditions (Fig. 4C). This suggested that WT-produced citrate may be involved in WT/Δ*lasR* coculture interactions and that citrate release was enhanced by pyochelin produced by the Δ*lasR* strain.

Citrate induces RhlR-dependent activity and RhlI levels in a ClpX protease-dependent manner in Δ*lasR* cells. To determine if citrate was sufficient to stimulate RhlR activity in Δ*lasR* cells, we analyzed its effects on *rhlI* promoter fusion activity, colony morphology, and RhlI protein levels. We found that citrate increased *rhlI* promoter activity (P*rhlI*) in Δ*lasR* cells and that its effects were dependent on the presence of RhlR (Fig. 5A). Citrate was sufficient to promote increases in colony pigmentation and colony smoothness, previously characterized to be RhlR-mediated in Δ*lasR* cells (29) (Fig. 5A, inset). In contrast, citrate caused a small but significant reduction in WT P*rhlI* activity compared to that the LB control (Fig. 5A).

To determine if RhlI protein levels were influenced by citrate, we utilized an arabinose-inducible *rhlI*-hemagglutinin (HA) construct to assess RhlI protein levels and stability in the absence and presence of citrate independent of RhlR transcriptional control. RhlI-HA was functional as swarming defects of the Δ*rhlI* mutant were complemented upon expression of RhlI-HA but not by the empty vector (Fig. 5B, inset). RhlI-HA protein levels were 3-fold higher in the Δ*lasR* strain upon citrate supplementation than

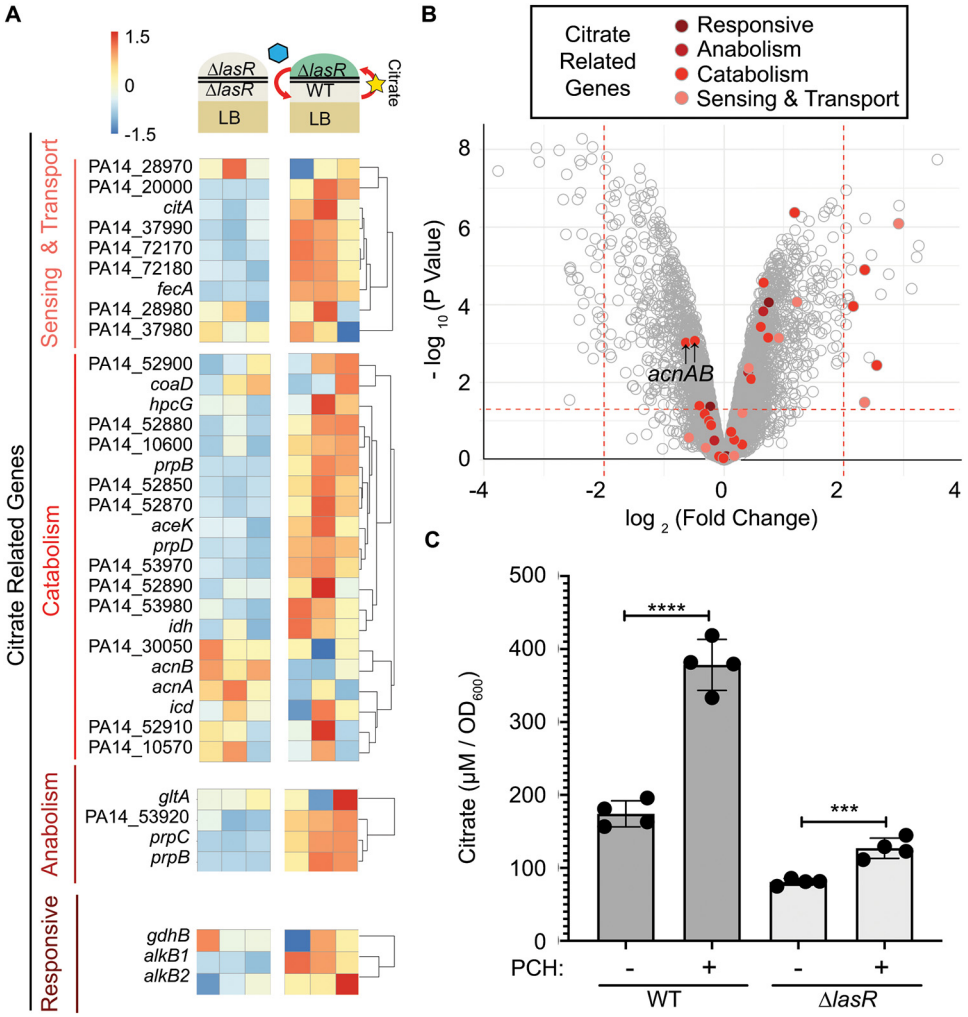

FIG 4 Citrate release by WT is induced by pyochelin exposure. (A) Subset of expression data in Δ*lasR* cocultures (Fig. 3A shows the setup) for genes annotated as being involved in citrate sensing, transport, catabolism, anabolism, and those shown to be responsive to citrate. (B) Volcano plot of expression data of Δ*lasR* cocultures with each point representing the $\log_2$(Δ*lasR* cells grown on WT/Δ*lasR* cells grown on Δ*lasR* cells) expression and $-\log_{10}$(*P* value) of a single gene. Genes shown in panel A are highlighted in color. (C) Citrate concentrations in supernatants from wild-type and Δ*lasR* stationary-phase cultures after growth in LB medium supplemented with extracts containing 50 μM pyochelin (PCH+) or an equal volume of control extracts (PCH−). A representative experiment with four biological replicates repeated 3 independent days is shown. ***, $P < 0.0005$; ****, $P \leq 0.0001$, as determined by two-tailed *t* tests of paired ratios.

in the controls (Fig. 5B). Consistent with the absence of an increase in *rhlI* promoter activity in WT strains (Fig. 5A), RhlI-HA protein levels were not higher with citrate in the Δ*lasR* complemented strain (Δ*lasR* + *lasR* strain) (Fig. 5B). The differential responses to citrate were also observed in LasR− and LasR+ pairs of clinical isolates (CIs). LasR− CIs from acute (strain 388D) and chronic (strain DH2415) infections had RhlI-HA levels 1.4- and 1.7-fold higher, respectively, in the presence of citrate (Fig. 5C), whereas alterations in RhlI-HA protein levels in LasR+ CIs from acute (strain 550A) or chronic (strain DH2417) infections were not observed (Fig. 5D). Through this work, we successfully identified citrate as a molecule in coculture that specifically promoted RhlI protein levels in LasR− strains, but not in LasR+ strains, by posttranscriptional control. In an attempt to identify transporters involved in the Δ*lasR* strain response to citrate and/or other coculture metabolites, we deleted two organic acid transporters: *dctA* (61) and PA14_51300 (62) in the Δ*lasR* strain background. We found that the Δ*lasR* Δ*dctA* strain showed induction of pyocyanin when it was cocultured with the WT and that induction was dependent on RhlR (Fig. S7D). Similar results were obtained with the Δ*lasR*

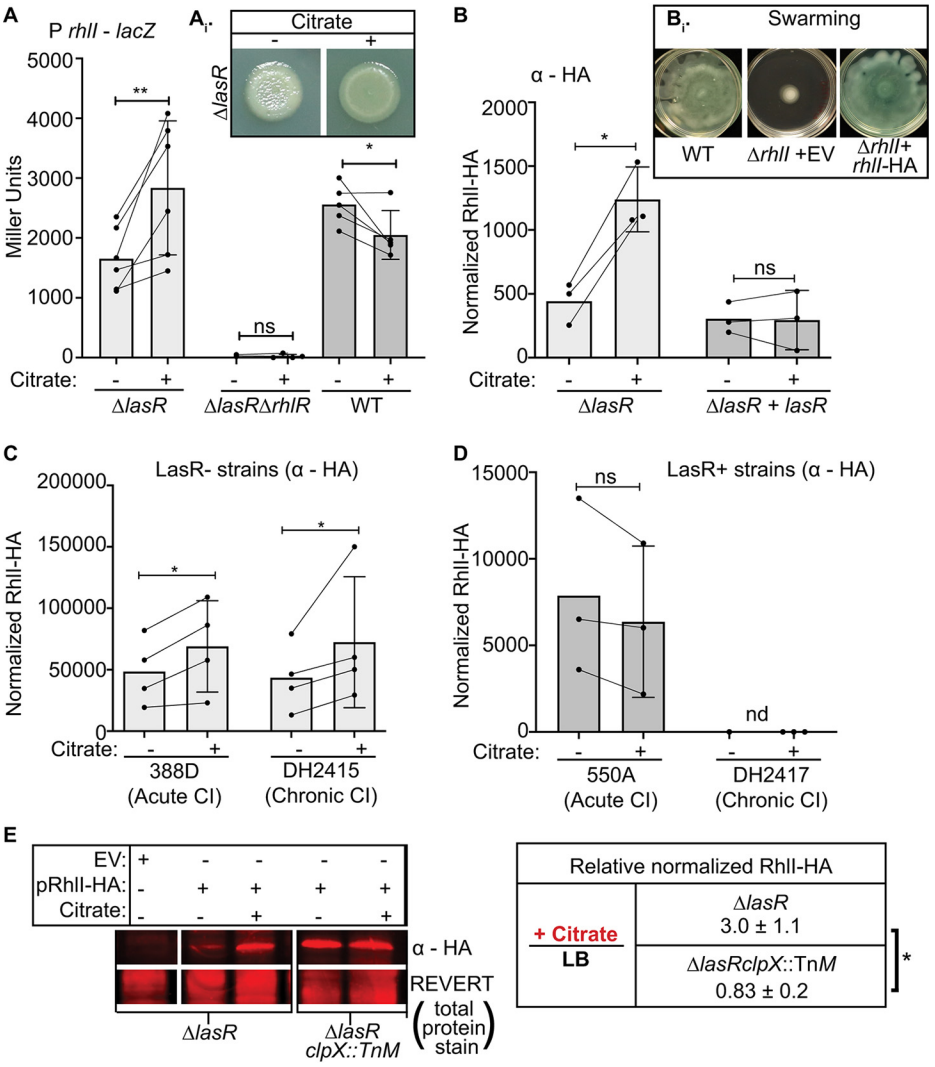

FIG 5 Citrate induced RhlR-dependent *rhlI* promoter activity and stabilized RhlI protein in LasR− strains in a ClpX protease-dependent manner. (A) β-Galactosidase activity for Δ*lasR*, Δ*lasR* Δ*rhlR*, and wild-type strains harboring *att*::P*rhlI-lacZ* on LB medium with or without 20 mM citrate at 24 h. Each point is the average of three biological replicates from 3 to 4 independent experiments. ns, not significant; *, $P < 0.05$; **, $P < 0.005$, as determined by one-way analysis of variance with Dunnett's multiple hypotheses correction of the indicated comparisons. The inset (A_i) shows a representative image of Δ*lasR* colony morphology on LB medium with and without 20 mM citrate at 24 h. (B) RhlI-HA protein signal normalized to Revert total protein stain (LiCor) on LB medium with and without 20 mM citrate for the Δ*lasR* strain and *lasR* complemented at the native locus (Δ*lasR* strain). ns, not significant; *, $P < 0.05$; **, $P < 0.005$, as determined by analysis of variance with Dunnett's multiple hypothesis correction for $n = 3$ biological replicates performed on three independent days. The inset (B_i) illustrates that plasmid-borne RhlI-HA, but not the empty vector (EV), complemented an Δ*rhlI* mutant for swarming. (C) RhlI-HA protein levels on LB medium with and without 20 mM citrate of LasR loss-of-function (LasR−) clinical isolates (CIs) from acute corneal (388D) and chronic CF (DH2415) Infections. *, $P < 0.05$, as determined by two-tailed *t* tests of paired ratios for $n = 3$ experiments for each isolate. (D) RhlI-HA protein levels on LB medium with and without 20 mM citrate of an LasR+ acute corneal CI (550A) of the same multilocus sequence type as 388D and LasR+ chronic CF CI (DH2417) from which DH2415 evolved. ns, not significant as determined by two-tailed *t* tests of paired ratios for $n = 3$ experiments for each isolate. nd, not detected. (E) Representative image and quantification of replicates for the anti-HA antibody analysis of Δ*lasR* and Δ*lasR clpX* Tn::*M* strains carrying a plasmid expressing RhlI-HA or an empty vector (pRhlI-HA or pEV, respectively) grown in LB medium with or without 20 mM citrate. *, $P < 0.05$. as determined by two-tailed *t* tests of paired ratios for $n = 3$ replicates from 3 independent days.

ΔPA14_51300 strain (Fig. S7E), suggesting that these transporters were not required for the interaction, perhaps due to redundant functions of other proteins or the involvement of other import mechanisms.

The temporal pattern of activation and the stimulation of RhlI-HA in the absence of RhlR control suggested that the RhlI protein may precede signal amplification via the

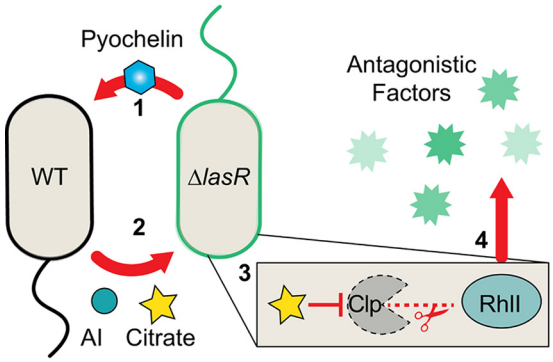

FIG 6 Model for wild-type and Δ*lasR* coculture interactions. Δ*lasR* strain-produced pyochelin promotes citrate release in the wild type (1). Citrate (and diffusible autoinducer) released by the wild type in coculture stimulates RhlR/I-dependent activity in a Δ*lasR* strain (2). Citrate stabilizes RhlI protein in Δ*lasR* cells potentially through a ClpXP protease-dependent mechanism (3), which ultimately promotes the production of antagonistic factors like pyocyanin toxin and rhamnolipid surfactant above monoculture levels (4).

QS transcriptional network. This would be consistent with a primary effect on post-transcriptional modulation of RhlI-mediated RhlR activity, as has been reported previously to occur through an RhlS small RNA (sRNA)-dependent mechanism (63); however, we found no apparent difference in RhlS expression levels in our RNA-seq reads in coculture. To begin to unravel the mechanisms by which citrate promoted RhlR/I-dependent signaling and RhlI stability in Δ*lasR* cells, we analyzed the role of two proteases previously found to target and degrade RhlI (i.e., Lon and ClpXP) (64). Given that knockouts of Lon protease have a less substantial rise in RhlR/I expression in Δ*lasR* strains than in the WT (65), we focused on the role of ClpXP in Δ*lasR* cells. We found that citrate induction of RhlI-HA protein levels in the Δ*lasR* strain relative to that in the LB control was dependent on functional ClpX protease (Fig. 5E). More specifically, when ClpX, a protease shown to degrade RhlI, is nonfunctional (i.e., Δ*lasR clpX*::Tn*M* strain), RhlI-HA levels did not increase on LB plus citrate relative to that of the LB control, unlike the level in the Δ*lasR* strain comparator (Fig. 5E). Under LB culture conditions, RhlI-HA levels were 3.20- ± 2.1-fold higher in the Δ*lasR clpX*::Tn*M* strain than in the Δ*lasR* strain, which mirrors the 3-fold induction observed for the Δ*lasR* strain on LB medium with or without citrate. Under citrate-supplemented conditions, no significant difference in RhlI-HA levels was observed for the Δ*lasR clpX*::Tn*M* relative to that in the Δ*lasR* strain (fold change of 1.01 ± 0.53). In other words, as previously noted for WT strains, ClpX may degrade RhlI in Δ*lasR* cells and play a role in the Δ*lasR* cell response to citrate. The distinct responses and mechanisms identified between LasR+ and LasR− strains under iron limitation and exposure to the low-iron-associated molecules, citrate and pyochelin, enabled increases in antagonistic factors beyond monoculture levels as an emergent property of *P. aeruginosa* intraspecies interactions.

DISCUSSION

In this study, we described an emergent outcome of coculturing LasR− and LasR+ strains of *P. aeruginosa* in which their interactions promoted the toxic exoproducts pyocyanin and rhamnolipids (Fig. 6 provides a model). We determined that, in coculture, the Δ*lasR* strain produces the siderophore pyochelin and that exogenous pyochelin induced citrate release more strongly in the WT than in Δ*lasR* strain. Citrate increased RhlI protein levels and induced RhlR-dependent activity only in Δ*lasR* cells and not WT cells (Fig. 6). Western blot analyses of RhlI-HA expressed from a regulated promoter led us to propose that the increase in RhlR-dependent signaling is due to decreased degradation of RhlI by ClpXP, a known negative regulator of RhlI (65, 66), or through other mechanisms of posttranscriptional regulation. The differences in siderophore production, citrate release, and RhlR/I-dependent activation between *P. aeruginosa* LasR+ and LasR− strains in coculture reflect the pronounced differences between

strains that drive QS reactivation. Previous studies have shown that LasR− strain colony morphology and phenazine production change in the presence of other species such as *Candida albicans* (29) and *Staphylococcus aureus* (see Fig. 3B in reference 67), and future work will determine if pyochelin and citrate also participate in these interspecies interactions as many microbial interactions have been shown to be influenced by iron availability (68–71). Furthermore, the induction of RhlR activity that can occur in late-stationary-phase Δ*lasR* cultures (30, 72) may relate to changes in iron or TCA cycle intermediates. While we found that WT-produced autoinducers, including 3OC12HSL, C4HSL, and PQS, were not required for coculture stimulation, they clearly contributed to the enhanced RhlR-dependent activity, which is consistent with intercolony QS interactions demonstrated previously (73).

The stimulatory relationship between LasR+ and LasR− strains was remarkably stable as it was observed when strains were mixed within single spot colonies (Fig. 1A) and when strains were separated by either filters (Fig. 3) or millimeter distances on an agar plate (Fig. 2B). The LasR−/LasR+ interactions occurred across distinct media (see Fig. S1A in the supplemental material), among genetically diverse LasR+ and LasR− clinical isolates (Fig. S1B), and over a wide range of relative proportions of each type (Fig. 1C). Of note, colonies inoculated at a 80:20 WT-to-Δ*lasR* cell ratio had more zones with the *lasR* mutant-associated phenotypes described as sheen and lysis than colonies with a 20:80 WT-to-Δ*lasR* cell ratio (Fig. 1C). At both ratios, Δ*lasR* cell numbers increased slightly relative to level of the wild type (Fig. 1D). We propose that the reduced appearance of sheen and lysis in mixed colonies inoculated with more Δ*lasR* cells reflects a requirement for a sufficient proportion of Δ*lasR* cells to initiate the WT-Δ*lasR* cell interactions that activate RhlR and restore a more WT-like phenotype to LasR− cells. Furthermore, if RhlR signaling is not fully activated in Δ*lasR* cells, there may be regions of increased Δ*lasR* cell killing via WT-produced factors such as cyanide (74).

The consequences of this intraspecies interaction may explain the worse outcomes exhibited by patients in which LasR− strains are detected (13), but future studies that include genotypes, monoculture and coculture phenotypes, and longitudinal outcome data will be required. RhlR plays other important roles in host interactions (75) which may benefit *P. aeruginosa* LasR− strains. The observation that *rhlR* mutants are rare in natural isolates and that LasR− strains with active RhlR are virulent (25, 76) underscores the relevance of this mechanism and highlights the importance of understanding how microbial interactions influence RhlR activity.

As studies of inter- and intraspecies interactions progress, it is becoming increasingly clear that the environment can dictate the outcome of microbial interactions (77). In fact, even the importance of QS regulation for fitness depends on nutrient sources and conditions (78, 79). As Δ*lasR* cell-produced pyochelin was a key component of the interaction and as pyochelin production is repressed under conditions of excess iron, it was not surprising that iron supplementation suppressed the interaction (Fig. 3F and Fig. S3C and D) without significantly altering the final colony CFU count or strain ratios relative to those of the LB control (Fig. S4). Siderophore-mediated iron uptake is often required *in vivo* (34, 80, 81) due to iron sequestration by host proteins (82–85); thus, *in vivo* settings may support these interactions.

Interestingly, pyoverdine, the higher-affinity siderophore, was not required for the coculture response, mirroring findings that genes for biosynthesis of pyoverdine, but not pyochelin, are commonly disrupted in isolates from chronic CF patients (39–41). In the absence of pyoverdine (i.e., the Δ*lasR* Δ*pvdA* strain), we observed more pyocyanin in coculture with the WT than with the Δ*lasR* strain (Fig. 3D), and we speculate that this is due to increased pyochelin production by Δ*pvdA* cells, but future studies will be required to test this model. It was interesting to find that in WT/Δ*lasR* coculture, heme-related proteins, *hasAP*, *hasS*, and *hasD*, were among the top eight most-upregulated genes by the Δ*lasR* strain because the presence of *lasR* mutants and heme content are both reported biomarkers of disease progression in CF patients (13, 86). Coculture-induced *lasR* mutant phenotypes may link these two correlative observations.

Citrate, a TCA intermediate released under iron limitation as a result of overflow metabolism (43, 44, 60, 87), can be used by *P. aeruginosa* and other microbes for iron acquisition due to its iron chelating properties (88). The increased siderophore production by Δ*lasR* cells in coculture likely reflects different metabolic strategies between genotypes. Ongoing work will investigate the mechanisms that drive differences in metabolism and iron requirements in order to determine how these differences shape microbial and host interactions. It is likely that Crc-mediated catabolite repression is involved in the response to citrate and the control of RhlI levels (64, 66). That a mechanism exists for the induction of RhlR-mediated QS in response to citrate secreted when iron is limiting dovetails with reports of increased expression of the *P. aeruginosa* QS regulon in low iron in LasR+ cells (89–91). This coordinate regulation may aid in iron acquisition as QS-controlled phenazines, such as pyocyanin, reduce poorly soluble Fe^{3+} to Fe^{2+} and facilitate its uptake via the Feo system (92). Additionally, rhamnolipids have been employed for iron remediation (93, 94), which suggests that their surfactant activity may increase *P. aeruginosa* substrate iron uptake in part through hydroxy-alkyl-quinolone-dependent mechanisms (95).

Given that anthranilate did not alter Δ*lasR* colony morphology or phenazine production, we did not further investigate anthranilate as a cross-fed metabolite involved in WT-Δ*lasR* cell interactions. We speculate that the increased expression of anthranilate catabolism genes in coculture may be more reflective of increases in RhlR activity than increased exposure to anthranilate given reports highlighting RhlR activation of *antABC* and *catABC* anthranilate catabolism genes (59).

As the presence of heterogeneous genotypes within single-species populations becomes increasingly appreciated, it is important to understand how commonly encountered genotypes interact to influence population-level behavior. Other work shows that cocultures can influence the survival of other genotypes (96, 97). Here, we show that intergenotype interactions lead to increased RhlR-dependent signaling in LasR− strains. It is likely that a wide array of such interactions has yet to be uncovered.

MATERIALS AND METHODS

Strains and growth conditions. Strains used in this study are listed in Table S2 in the supplemental material. Bacteria were maintained on LB (lysogeny broth) medium with 1.5% agar. *Saccharomyces cerevisiae* strains for cloning were maintained on yeast-peptone-dextrose (YPD) medium with 2% agar. With the exception of pyochelin complementation experiments, which were performed in 12-well dishes with a 2-ml total volume containing 50 μM pyochelin (or an equal-volume negative-control extract), colony biofilm assays were performed in 100-mm petri dishes with a 25-ml total volume. Where stated, a 20 mM concentration of the indicated metabolite was added to the medium (liquid or molten agar). Planktonic cultures were grown on roller drums at 37°C. Artificial sputum medium (ASM) was made as described previously (27).

Competition assays. Competition assays were performed to determine the relative fitness of *P. aeruginosa* mutants. Strains to be competed were grown overnight and adjusted to an optical density at 600 nm (OD_{600}) of 1. Competing strains were combined with the PA14 *att*::*lacZ* strain in a 1:1 ratio unless otherwise stated. Following a 15-s vortex, 5 μl of the combined suspension was spotted on LB agar. After 16 h, colony biofilms (and agar) were cored, placed in 1.5-ml tubes with 500 μl of LB, and agitated vigorously for 5 min using a Genie Disruptor (Zymo). This suspension was diluted, spread on LB plates supplemented with 150 μg/ml 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-Gal) using glass beads, and incubated at 37°C until blue colonies were visible (~24 h). The numbers of blue and white colonies per plate were counted, and the final proportions were recorded. Each competition was run in triplicate on 3 separate days.

Additional methods. See Text S1 in the supplemental material for methods describing plasmid construction, pyocyanin quantification, colony proximity image analysis, swarming, RNA collection and processing, pyochelin extraction and quantification, citrate quantification, β-galactosidase quantification, Western blotting, and acyl-homoserine lactone activity assays.

Data availability. Data for RNA-seq analysis of *P. aeruginosa* Δ*lasR* grown on the Δ*lasR* or WT strain in coculture has been uploaded to the Gene Expression Omnibus (GEO) repository (https://www.ncbi.nlm.nih.gov/geo/) under accession number GSE149385.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

TEXT S1, DOCX file, 0.03 MB.

FIG S1, PDF file, 0.3 MB.

FIG S2, PDF file, 0.1 MB.

FIG S3, PDF file, 2.4 MB.
FIG S4, PDF file, 0.3 MB.
FIG S5, PDF file, 0.1 MB.
FIG S6, PDF file, 0.1 MB.
FIG S7, PDF file, 0.5 MB.
TABLE S1, XLSX file, 0.02 MB.
TABLE S2, DOCX file, 0.1 MB.

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