Intra-species signaling between *Pseudomonas aeruginosa* genotypes increases production of quorum sensing controlled virulence factors

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Running title: Mixed genotype co-culture promotes virulence factor production

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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. The *lasR* gene, which encodes a central quorum-sensing regulator, is frequently mutated, and loss of LasR function impairs the activity of downstream regulators RhlR and PqsR. We found that in diverse models, the presence of *P. aeruginosa* wild type causes LasR loss-of-function strains to hyperproduce RhlR/I-regulated antagonistic factors, and autoinducer production by the wild type is not required for this effect. We uncovered a reciprocal interaction between isogenic wild type and *lasR* mutant pairs wherein the iron-scavenging siderophore pyochelin, specifically produced by the *lasR* mutant, induces citrate release and cross-feeding from wild type. Citrate stimulates RhlR signaling and RhlI levels in LasR- but not in LasR+ strains, and the interactions occur in diverse media. Co-culture interactions between strains that differ by the function of a single transcription factor may explain worse outcomes associated with mixtures of LasR+ and LasR loss-of-function strains. More broadly, this report illustrates how interactions within a genotypically diverse population, similar to those that frequently develop in natural settings, can promote net virulence factor production.
Introduction

Genetic diversity frequently arises and persists within clonally-derived bacterial and fungal populations in chronic infections and healthy microbiomes, and recent data highlight that this heterogeneity can pose challenges to clearance and treatment (1-3). Genotypic and phenotypic complexity has been particularly well-documented in the chronic lung infections associated with the genetic disease cystic fibrosis (CF), and these studies have convincingly demonstrated that within a species, a common set of genes is under selection across strains and hosts (4-10).

*P. aeruginosa* loss-of-function mutations in *lasR* (*LasR*-) are very commonly found in CF isolates, strains from acute infections, and from environmental sources (11-15). Although infection models often show that *lasR* loss-of-function mutants have reduced virulence compared to strains with functional LasR (*LasR*+) in several animal models (16, 17), the presence of LasR- strains is correlated with worse disease outcomes in acute and chronic infections (11, 12). There are several possible explanations for this apparent contradiction. Loss of *lasR* function confers some fitness advantages including altered catabolic profiles (18) and enhanced growth in low oxygen (19, 20). Further, some LasR- strains exhibit rewired regulation of quorum sensing (QS)-controlled exoproducts (21) and LasR- strains can activate QS signaling in response to products from other species (22) or in specific culture conditions (23, 24). LasR- strains are also frequently found among LasR+ *P. aeruginosa* strains where exoproducts can be shared or signal cross-feeding can occur (13).

*P. aeruginosa* LasR participates in the regulation of QS in conjunction with two transcription factors: RhlR and PqsR (MvfR). Each of these three regulators has one or
more autoinducer ligands: 3-oxo-C12-homoserine lactone (3OC12HSL) for LasR, C4-homoserine lactone (C4HSL) for RhlR, and hydroxyalkylquinolones (Pseudomonas Quinolone Signal (PQS) and hydroxy-heptyl quinolone (HHQ)) for PqsR (25). In the regulatory networks described in the 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 (26-28), cyanide (29), proteases (30-33), and rhamnolipid surfactants important for surface motility, biofilm dispersal, and host cell damage (34-36).

Other traits that are often heterogeneous across P. aeruginosa isolates relate to strategies for iron acquisition. P. aeruginosa procures iron through the use of siderophores, including pyochelin (37-39) and pyoverdine (40), from heme or through a direct iron uptake system (41-43). It is common to encounter P. aeruginosa strains with loss of function mutations in genes encoding pyoverdine, the high affinity siderophore, but genes associated with use of the pyochelin siderophore, heme utilization, and the direct iron uptake are generally intact (44-46). Iron limitation can reduce the function of pathways that require abundant iron including the TCA cycle (47), and when iron access is low, Pseudomonas spp. release partially oxidized metabolic intermediates that accumulate at iron requiring steps (48, 49). Many other species are known to release partially-oxidized metabolic intermediates upon iron limitation (48, 50-52).

Here, we show that mixtures of P. aeruginosa LasR- and LasR+ strains have enhanced production of QS-controlled factors across medium types, culture conditions, and strain backgrounds, and that this is due to activation of RhlR likely through
increased RhlI stability in LasR- strains. Our transcriptomic, genetic and biochemical studies led us to uncover a set of interactions in which ∆lasR production of the siderophore pyochelin was necessary for the activation of RhlR in ∆lasR when co-cultured with the LasR+ wild type (WT). Our studies suggested that ∆lasR responds to citrate in co-culture, and we found that pyochelin is sufficient to stimulate citrate release preferentially by WT cells. Citrate increased RhlI protein levels and RhlR activity in ∆lasR cells but not in the wild type. Together, these data highlight a set of complex, small molecule interactions between strains that differ by a single mutation and lead to increased production of exoproducts known to cause host damage.

RESULTS

*P. aeruginosa* ∆lasR over-produces pyocyanin in co-culture with 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 colony cultures of *P. aeruginosa* strain PA14 wild type (WT), ∆lasR, and WT / ∆lasR co-cultures, the strain mixture showed increased blue pigmentation (Fig. 1A) and a significant 2-fold induction of pyocyanin relative to either strain alone (Fig. 1B). Phenazine-deficient derivatives, ∆phz (∆phzA1-G1∆phzA2-G2) and ∆lasR∆phz (∆lasR∆phzC1C2) were also included, and as expected, ∆phz and ∆lasR∆phz, showed no blue colony pigmentation (Fig. 1A) and no pyocyanin signal was evident above background levels (Fig. 1B). The higher levels of pyocyanin in WT / ∆lasR co-cultures relative to single-strain cultures was also observed on pH-buffered M63 medium and on artificial sputum medium indicating that the phenomenon occurred in diverse conditions
(Fig. S1A). Co-cultures of clonally-derived LasR+ and LasR- clinical isolates collected from single respiratory sputum samples from chronically-infected individuals with cystic fibrosis also had increased production of a pyocyanin when LasR+ and LasR- strains were grown together relative to mono-culture levels (Fig. S1B, C).

To assess individual strain contributions to increased pyocyanin in WT / ΔlasR co-cultures, we measured pyocyanin levels in co-cultures wherein one strain was replaced with its phenazine-deficient derivative. When ΔlasR was cultured with the phenazine biosynthesis mutant Δphz (Δphz / ΔlasR), we still observed increased blue pigmentation (Fig. 1A) and total pyocyanin levels were higher relative to monocultures (Fig. 1B). Surprisingly, pyocyanin levels were statistically higher in Δphz / ΔlasR co-cultures relative to WT / ΔlasR (Fig. 1B). In contrast, WT / ΔlasRΔphz co-cultures did not display the high pyocyanin phenotype, and pyocyanin concentrations in these co-cultures were more similar to those in WT monocultures (Fig. 1A, B). Pyocyanin levels in the WT / ΔlasRΔphz co-cultures were below the limit of detection as there was no difference in pyocyanin levels when compared with Δphz / ΔlasRΔphz co-cultures or Δphz and ΔlasRΔphz monocultures. Collectively, these data suggested that WT / ΔlasR co-cultures produced more pyocyanin than either monoculture alone, and that pyocyanin was contributed by the ΔlasR strain.

The higher levels of pyocyanin in WT / ΔlasR co-cultures relative to each strain grown alone was not dependent on the initial ratio of WT to ΔlasR (Fig. 1C). We saw increased co-culture colony pigmentation when the initial proportion of the WT was at 0.2, 0.3, 0.5, 0.7, and 0.8 of the initial inoculum, with the balance comprised of ΔlasR (Fig. 1C). No increase in pyocyanin was observed at any ratio when WT was co-
cultured with the $\Delta$lasR complemented derivative ($\Delta$lasR + lasR) indicating that the phenomenon was dependent on the lasR mutation (Fig. 1C). To assess the relative abundances of WT and $\Delta$lasR in co-culture, we competed each strain against a neutrally-tagged WT strain (PA14 att::lacZ). We found that $\Delta$lasR increased in proportion after 16 h growth in colony biofilms regardless of the starting proportion whereas the proportions of tagged and untagged WT remained stable (Fig. 1D). We have previously shown that Anr activity is higher in $\Delta$lasR strains and contributes to $\Delta$lasR competitive fitness against WT P. aeruginosa in colony biofilms (53, 54), but Anr was not required for co-culture pyocyanin production by $\Delta$lasR (Fig. S2).

Pyocyanin is a product regulated by quorum sensing (QS) through the transcription factors LasR, RhlR, and PqsR (55-57), and because of the cell-density dependent quorum-sensing regulation, it was important to assess the population size in co-cultures relative to monocultures. Total CFUs did not increase in WT / $\Delta$lasR mixed cultures relative to either strain alone (Fig. S1D). Instead we found that WT / $\Delta$lasR co-cultures had fewer CFUs when compared to WT monocultures on LB (Fig. S1D). Taken together, these data suggested altered behavior, rather than cell number, contributed to the increased phenazine profile of LasR- strains.

P. aeruginosa WT induces $\Delta$lasR RhlR/I-dependent signaling independent of WT produced autoinducer.

In the canonical QS pathway, LasR regulates both PqsR and RhlR, and mutants lacking either of these regulators in a WT background have impaired pyocyanin production (58, 59). Both pqsR and rhlR were required for the production of pyocyanin
by $\Delta lasR$ in co-culture with WT (Fig. 2A). To determine if co-culture increased RhlR- or PqsR-dependent signaling in $\Delta lasR$ strains upon co-culture with the WT, we fused lacZ to the promoters of $rhlI$ and $pqsA$, which provide readouts of the activity of each respective regulator (25). To examine the effects of WT on $\Delta lasR$ QS, we examined the interactions between WT and $\Delta lasR$ in single-cell-derived colonies. To do so, suspensions containing ~50 cells of WT with ~50 cells of either $\Delta lasR P_{rhlI}–lacZ$ or $\Delta lasR P_{pqsA}–lacZ$ were spread on LB agar with the colorimetric $\beta$-galactosidase substrate X-gal. Intercolony distances and $\beta$-galactosidase activity in $\Delta lasR$ strains were measured. We found that $PrhlI–lacZ$ activity was inversely correlated with distance to a WT colony, with a significant increase in $\Delta lasR PrhlI–lacZ$ activity (Fig. 2B). Pearson correlation analyses showed that 54% of the variability in $\Delta lasR PrhlI–lacZ$ activity could be explained by changes in the distance to a WT colony (p-value $\leq$ 0.0001). The increased activity due to activation of $PrhlI–lacZ$ in $\Delta lasR$ was not observed in $\Delta lasR \Delta rhlR$, and close proximity to another $\Delta lasR PrhlI–lacZ$ colony did not affect promoter activity (Fig. 2B, inset). Because RhlR activity is canonically stimulated by C4HSL (which is synthesized by RhlI) and $PrhlI–lacZ$ is stimulated in $\Delta lasR$ by proximity to WT, we examined the role of RhlI in the $\Delta lasR$ response. We observed that a $\Delta lasR \Delta rhlI$ strain was greatly impaired in the induction of pyocyanin upon co-culture with the WT (Fig. 2A) which suggests activation of RhlR involved C4HSL synthesis within $\Delta lasR$ strains. Although PqsR was required in $\Delta lasR$ for co-culture pyocyanin production, there was no significant correlation with proximity to WT for $\Delta lasR P_{pqsA}–lacZ$ activity (Fig. 2B). Collectively, these data indicated that a diffusible factor produced
by WT stimulated RhlR-dependent signaling in $\triangle lasR$ to induce downstream production of RhlR and PqsR dependent factors.

Because C4HSL is readily diffusible and likely produced by WT cells, we tested the hypothesis that C4HSL or other acylhomoserine lactones produced by WT were necessary to induce RhlR-dependent increases in $rhlI$ promoter activity in $\triangle lasR$ co-cultured with WT. To test this hypothesis, we co-cultured $\triangle lasR$ with $\triangle rhil$ or $\triangle lasI\triangle rhil$, which lacks both acylhomoserine lactone synthases. Surprisingly, we found that like WT / $\triangle lasR$ co-cultures, $\triangle rhil$ / $\triangle lasR$ co-cultures had higher levels of pyocyanin production relative to mono-cultures (Fig. 2C). Likewise, $\triangle lasI\triangle rhil$ / $\triangle lasR$ had higher levels of pyocyanin production relative to mono-cultures though the interaction was delayed by ~24 h hours relative to the WT / $\triangle lasR$ co-cultures (Fig. 2C). Consistent with the data above which indicated that $pqsA$ was not induced upon co-culture with the WT, $\triangle lasR$ co-cultures of the PQS-deficient strain $\triangle pqsA$ had high pyocyanin colony pigmentation relative to monoculture levels after 24 h of extended incubation (Fig. 2C). The dispensability of WT-produced quorum sensing signals implicated a novel signaling interaction in co-culture-dependent activation of $\triangle lasR$ RhlR-dependent signaling.

To assess whether RhlR activity in $\triangle lasR$ strains grown in the presence of WT was sufficient to elicit other RhlR/I-controlled phenotypes in addition to pyocyanin production, we tested whether co-culture with the WT also enhanced other RhlR-regulated processes in $\triangle lasR$. RhlR regulates the production of rhamnolipid surfactants that are important for surface-associated motility known as swarming (60). While the rhamnolipid-defective mutant $\triangle rhilA$, $\triangle lasR$, and the $\triangle lasR\triangle rhilR$ strains were not able to swarm, we observed increased swarming in co-cultures of $\triangle lasR$ with $\triangle rhilA$. The
phenomenon was dependent on RhlR as the ΔlasRΔrhlR / ΔrhlA co-cultures did not swim (Fig. S3). Altogether, these data suggested broad RhlR-mediated quorum sensing activation in LasR- strains grown in co-culture with LasR+ P. aeruginosa.

**Pyochelin production by ΔlasR is required for co-culture interactions.**

With evidence indicating that the induction of RhlR activity in ΔlasR in both mixed strain spot colonies and adjacent colonies by WT occurred through a mechanism that does not require acylhomoserine lactone cross-feeding, we sought to gain further insight into the mechanisms that underlie the co-culture interactions. We investigated the transcriptomes of the lasR mutant when co-cultured with either WT or itself via RNA sequencing analysis. We grew ΔlasR colony biofilms on LB physically separated from a lawn of either ΔlasR or WT by two 0.22 µm filters to prevent mixing of genotypes while allowing for the passage of small molecules. RNA was extracted from cells within the ΔlasR colony biofilms grown on the topmost filter for 16 h in order to examine ΔlasR transcriptional profiles (Fig. 3A). One hundred and ninety-nine genes in ΔlasR were higher and 198 genes were lower by a $| \log_2 \text{fold change} | \geq 1$ with an FDR corrected p-value < 0.05 in co-culture with WT compared to ΔlasR alone (Supplemental Table 1). GO term analyses through PantherDB indicated that the upregulated gene set was significantly enriched in two pathways related to siderophore biosynthesis: pyoverdine biosynthetic process and salicylic acid biosynthetic process (an upstream precursor of pyochelin) with ~44 and ~77-fold enrichment, respectively (p-values < 0.005). Twenty-eight out of the 33 genes in the pyochelin and pyoverdine siderophore biosynthesis- and acquisition-related GO pathways were significantly upregulated in ΔlasR upon co-
culture with WT (Fig. 3B). Other genes regulated by the low iron response were also
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 pattern indicative of RhlR
activation at this early time point (Table S1), and this point is discussed in more detail
with respect to additional data shown below.

In light of the upregulation of siderophore biosynthesis genes in ΔlasR co-
cultured with WT but not with itself, we qualitatively examined production of fluorescent
pyoverdine and pyochelin siderophores in monocultures and co-cultures. To determine
the contribution of both pyoverdine and pyochelin to fluorescence, genes required for
pyoverdine biosynthesis (ΔpvdA), pyochelin biosynthesis (ΔpchE) or both pathways
(ΔpvdAΔpchE) were disrupted in the lasR mutant (Fig. 3C). Increased fluorescence
attributable to both pyoverdine and pyochelin in co-culture was due to siderophore
production by ΔlasR strains, consistent with the RNA-Seq data, as the increased
siderophore production in WT/ΔlasR co-cultures was lost for co-cultures in which the
ΔlasR strains were replaced with ΔlasRΔpvdA, ΔlasRΔpchE, or ΔlasRΔpvdAΔpchE.
While WT and the pyoverdine-deficient derivative ΔlasRΔpvdA (i.e. WT/ΔlasRΔpvdA)
showed increased pyocyanin production relative to either monoculture, ΔlasRΔpchE and
ΔlasRΔpvdAΔpchE did not support the overproduction of pyocyanin in co-culture with
WT (Fig. 3D). The decrease in ΔlasR-derived pyocyanin was not due to decreased
fitness as disruption of pvdA and pchE in ΔlasR individually had no effect on the final
proportions; in contrast, ΔlasRΔpvdAΔpchE had a significant defect in competitive
fitness compared to the ΔlasR parental strain (Fig. S4). These data suggested that
pyochelin played a role in the co-culture interaction. To test this model, we complemented the ΔlasRΔpchE in co-culture with supernatant extracts from cultures of ΔpvdA which only produced pyochelin, or ΔpvdAΔpchE which produced neither siderophore (Fig. S5A for supernatant absorption spectra). The two supernatants were analyzed using the chrome azurol S (CAS) assay (61) to confirm that chelator activity was present in the ΔpvdA supernatant extracts but not in extracts from ΔpvdAΔpchE cultures (Fig. S5B). The amendment of the medium with PCH-containing extracts, but not siderophore-free extracts, restored co-culture pyocyanin production in ΔpvdAΔpchE / ΔlasRΔpchE co-cultures (Fig. 3E) lending further support for the model that pyochelin was required for co-culture interactions. Consistent with the requirement of the siderophore for the stimulation of pyocyanin in WT / ΔlasR co-cultures, iron supplementation of the LB medium suppressed siderophore production and the stimulation of pyocyanin production (Fig. 3F).

Evidence for WT - ΔlasR interactions involving citrate secreted by the WT in response to pyochelin.

Many of the genes with higher transcript levels in ΔlasR upon co-culture with WT have annotations related to organic acids such as anthranilate and citrate (Supplemental Table 1, Fig. S6A). First, anthranilate was examined as a candidate molecule that was produced by the WT and that induced RhlR-dependent phenotypes in ΔlasR. Co-cultures of ΔlasR with anthranilate synthase mutant ΔphnAB did not alter high phenazine production compared to WT / ΔlasR (Fig. S6B). Additionally, anthranilate supplementation of the medium did not alter ΔlasR phenazine production at
any concentration tested up to ~15 mM (Fig. S6C). Furthermore, co-cultures of ΔlasR and the PQS deficient strain ΔpqsA, which accumulates anthranilate as an upstream precursor (62), did not show enhanced stimulation of RhlR-dependent phenotypes beyond WT / ΔlasR levels (Fig. 2C).

We then tested the ability of citrate to induce RhlR-dependent phenotypes in ΔlasR in light of the observation that twenty percent of the most strongly differentially expressed genes (| log₂ (fold change) | ≥ 2 with an FDR-corrected p-value < 0.05) were implicated with citrate sensing, transport, catabolism, and anabolism as annotated by UNIPROT and pseudomonas.com (Fig. 4A). Among the genes more highly induced in ΔlasR / WT co-cultures compared to ΔlasR cultured with itself were genes annotated as playing a role in citrate sensing or metabolism, with the most strongly upregulated genes were involved in citrate catabolism and sensing and transport (Fig. 4B).

In light of the findings that ΔlasR strains induced low iron responsive genes when grown near the WT but not itself, that ΔlasR pyochelin production was necessary for co-culture interactions that lead to increased pyocyanin and rhlI promoter activity, that citrate sensing and catabolism genes were induced in ΔlasR by the presence of the WT, and the knowledge that numerous microbes, including Pseudomonas putida, secrete citrate and other organic acids when iron limited (49, 50, 63, 64), we measured citrate in the supernatants of WT and ΔlasR LB cultures. Citrate concentrations were significantly higher in WT supernatants (Fig. 4C). Furthermore, amendment of the LB medium with extracts containing 50 µM pyochelin increased extracellular citrate concentrations by about 2-fold in WT cultures compared to control cultures containing extracts lacking pyoverdine and pyochelin, with a much smaller stimulation in ΔlasR cultures (Fig. 4C).
The difference in the effects of pyochelin on citrate levels in culture supernatants for WT and ∆lasR was significant using data from four independent experiments (p<0.05). This suggested WT-produced citrate was possibly involved in WT / ∆lasR co-culture interactions, and that its increased release was enhanced by ∆lasR-produced pyochelin.

Citrate induces RhlR activity in ∆lasR and RhlI levels in a ClpX-protease dependent manner.

To determine if citrate was sufficient to stimulate RhlR activity in ∆lasR, we analyzed its effects on both rhlI promoter fusion activity and RhlI protein levels. We found that citrate increased rhlI promoter activity in ∆lasR and that its effects were dependent on the presence of RhlR (Fig. 5A). In contrast, the inclusion of citrate in the medium caused a small but significant reduction in WT P_rhlI activity compared to LB control (Fig. 5A).

To determine if RhlI protein levels were influenced by citrate, we utilized an arabinose-inducible rhlI-HA construct to assess RhlI protein levels and stability of RhlI-HA in the absence and presence of citrate. RhlI-HA was functional as swarming defects of ∆rhlI 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 ∆lasR upon citrate supplementation relative to 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 ∆lasR complemented strain (ΔlasR + lasR) (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 (strains 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 (550A) or chronic (DH2417) infections was not observed (Fig. 5D). Through this work, we successfully identified citrate as a molecule in co-culture that specifically promoted RhlI protein levels in LasR- strains, but not LasR+ strains, by a mechanism other than transcriptional control. In order to identify transporters that could be involved in the ∆lasR response to citrate, we deleted two organic acid transporters: dctA (65) and PA14_51300 (66) in the ∆lasR background. The dctA gene was deleted in both a ∆lasR and ∆lasRΔrhlR mutant. We found that the ∆lasRΔdctA strain still showed induction of pyocyanin when co-cultured with the WT and that induction was dependent on RhlR (Fig S7A). Similar results were obtained with the ∆lasRΔPA14_51300 (Fig S7B) 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 suggested RhlI protein induction preceded signal amplification via the positive feedback loop of the quorum sensing transcriptional network. This would be consistent with a primary effect on post transcriptional modulation of RhlI-mediated RhlR activity. To begin to unravel the mechanisms by which citrate promoted RhlR/I-dependent signaling and RhlI stability in ∆lasR, we analyzed the role of two proteases previously found to target and degrade RhlI (i.e. Lon and ClpXP) (67). Given knockouts of Lon protease have a less substantial rise in RhlR/I expression in ∆lasR knockouts compared to WT (68), we focused on the role of ClpXP in ∆lasR. We found that citrate induction of RhlI-HA protein levels in ∆lasR relative to the LB control was dependent on the production of ClpX protease (Fig. 5E). More
specifically, in the absence of ClpX, a protease shown to degrade RhlI (i.e. \( \Delta lasRclpX::TnM \)), RhlI-HA levels did not increase on LB + citrate relative to LB control, unlike \( \Delta lasR \) comparator (Fig. 5E). In LB conditions, RhlI-HA levels were 3.20 ± 2.1 fold higher in \( \Delta lasRclpX::TnM \) compared to \( \Delta lasR \), which mirrors the 3-fold induction observed for \( \Delta lasR \) on LB + citrate. No significant difference in RhlI-HA were observed for \( \Delta lasRclpX::TnM \) relative to \( \Delta lasR \) in citrate supplemented conditions (fold change: 1.01 ± 0.53). In other words, as previously noted for WT strains, ClpX appeared to degrade RhlI in \( \Delta lasR \), and played a role in \( \Delta lasR \) 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 factor production beyond monoculture levels as an emergent property of \( P. aeruginosa \) intraspecies interactions.

In support of the model that induction in RhlR signaling in response to citrate was due to increased RhlI protein, we found that the induction of rhlI promoter activity (Fig. 5F) followed the increase in RhlI-HA levels (Fig. 5G) in response to citrate. The stimulation of rhlI promoter activity was greatest for citrate, but modest stimulation was observed for other organic acids including TCA cycle intermediates (succinate, fumarate and malate) and the common fermentation product acetate. In each case, the stimulation of rhlI-promoter activity was accompanied by higher RhlI-HA protein levels in \( \Delta lasR \), but not \( \Delta lasR + lasR \), aside from succinate (Fig. 5F,G). Together, these data may imply that organic acids, such as citrate, can serve as mediators of co-culture interactions that can activate RhlR activity in LasR- strains.
Discussion

In this study, we described an emergent outcome of co-culturing LasR- and LasR+ strains of *P. aeruginosa* in which their interactions promoted the increased production of toxic exoproducts including pyocyanin and rhamnolipids (see Fig. 6 for model). We determined that, in co-culture, the iron-binding siderophore pyochelin was largely contributed by ΔlasR, and that exogenous pyochelin induced secretion of citrate significantly more strongly in the WT than in ΔlasR. Citrate increased RhlI protein levels and activated RhlR activity only in ΔlasR, but not WT cells (Fig. 6). Western blot analysis of RhlI-HA expressed from a regulated promoter led us to propose that the increase in RhlR signaling is due to decreased degradation of RhlI by ClpXP, a known negative regulator of RhlI (68, 69). The differences in siderophore production, citrate release, and RhlR/I activation between *P. aeruginosa* LasR+ and LasR- strains in co-culture environments reflect the pronounced differences between strains that drive the reactivation of quorum sensing and enhanced production of secreted factors. Previous studies have shown that LasR- strains increase their production of phenazines in the presence of other species such as *Candida albicans* (22) and *Staphylococcus aureus* (see Fig. 3B in (70)) and future work will determine if pyochelin and citrate also participate in these interspecies interactions. Other microbial interactions have been shown to be influenced by iron availability (71-74). Furthermore, the activation of RhlR activity in ΔlasR strains that can occur in late stationary phase cultures (23, 75) may relate to changes in iron or TCA cycle intermediates. While we found that WT production of the diffusible autoinducers 3OC12HSL, C4HSL and PQS were not required for co-culture stimulation, they clearly contributed to the enhanced activation of
RhlR regulation which is consistent with intercolony QS interactions that have been demonstrated previously (76).

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 separated by either filters (Fig. 3) or mm distances on an agar plate (Fig. 2B). The LasR-/LasR+ interactions occurred across distinct media (Fig. S1A), among genetically diverse LasR+ and LasR- clinical isolates (Fig. S1B) and over a wide range of relative proportions of each type (Fig. 1C). The consequences of this intraspecies interaction between genotypes may explain the worse outcomes exhibited by patients in which LasR- strains are detected (12), but future studies with data that include genotypes, mono-culture and co-culture phenotypes, and longitudinal outcome data will be required. RhlR plays other important roles in host interactions (77) which may benefit P. aeruginosa LasR- strains. The observation that rhlR mutants are rare in natural P. aeruginosa isolates and that LasR- strains with active RhlR are virulent (21, 78) underscores the relevance of this mechanism and highlights the importance of understanding how microbial interactions activate RhlR.

As the study of inter- and intra-species interactions progresses, it is becoming increasingly clear that the environment can dictate the outcome of microbial interactions (79). In fact, even the importance of QS regulation for fitness depends on nutrient sources and conditions (80, 81). As ΔlasR-produced pyochelin was a key component of the interaction, and pyochelin production is repressed under conditions of excess iron availability, it was not surprising that the addition of iron to LB medium suppressed the interaction without significantly altering the final colony CFUs or strain ratios relative to
LB control (Fig. S4). Siderophore-mediated iron uptake is often required in vivo (39, 82, 83) due to iron sequestration by host proteins (84-87), thus the in vivo settings could support these interactions. Interestingly pyoverdine, the higher affinity siderophore, was not required for the co-culture response mirroring findings that genes for the biosynthesis of pyoverdine, but not pyochelin, are commonly disrupted in chronic CF clinical isolates (44-46). In the absence of pyoverdine (i.e. ΔlasRΔpvdA), we observed more pyocyanin in co-culture with WT than ΔlasR (Fig. 3D), and we speculate that this is due to increased pyochelin production by ΔpvdA but future studies will be required to test this model. If this is the case, it would be interesting to analyze the outcomes of interactions over gradients of iron and other nutrients. It was interesting to find that in WT / ΔlasR co-culture, heme-related proteins, hasAP, hasS, and hasD, were among the top eight most upregulated genes by ΔlasR because the presence of lasR mutants and heme utilization are both reported biomarkers of disease progression in CF patients (12, 88). Co-culture induced lasR mutant phenotypes may link these two correlative observations.

Citrate, a TCA intermediate, is released under iron limitation as a result of “overflow metabolism” (48-50, 52) and is also used by P. aeruginosa and other microbes for iron acquisition due to its iron chelating properties (89). The higher siderophore production by ΔlasR and stimulation of ΔlasR siderophore production in co-culture likely reflects different metabolic strategies between the two strains. 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 (67, 69). The existence of a mechanism for the induction of RhlR-mediated QS in response to citrate and other TCA cycle intermediates that are secreted when iron is limiting dovetails with reports of increased expression of the *P. aeruginosa* quorum sensing regulon in low iron in LasR+ cells (90-92). This coordinate regulation may aid in iron acquisition as quorum sensing-controlled phenazines, such as pyocyanin, reduce poorly soluble Fe$_3^+$ to Fe$_2^+$ and facilitate its uptake via the Feo system (93). Additionally, rhamnolipids have been employed for iron remediation (94, 95) which suggests their surfactant activity may increase *P. aeruginosa* substrate iron uptake in part through hydroxyalkylquinolone-dependent mechanisms (96).

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 the apparent behavior of the population. Here, we show that inter-genotype interactions lead to increased RhlR signaling in *lasR* strains; other work shows co-cultures can also influence the survival of other genotypes (97). It is likely that a wide array of such interactions have yet to be uncovered.
Methods

Strains and Growth Conditions. Bacterial strains used in this study are listed in Table S2. Bacteria were maintained on LB (lysogeny broth) with 1.5% agar. Yeast strains for cloning were maintained on YPD (yeast peptone dextrose) with 2% agar. Where stated, 20 mM of indicated metabolite was added to the medium (liquid or molten agar). Planktonic cultures were grown on roller drums at 37°C for *P. aeruginosa*.

Plasmid Construction

Plasmid constructs for making in-frame deletions, RhlI-HA expression, and for *pqxA* promoter fusions were constructed using a *Saccharomyces cerevisiae* recombination technique described previously (98). The RhlI-HA expression vector with the ampicillin cassette (pMQ70) was constructed by amplifying the *rhlI* gene with primers that added an HA-tag with sites for cloning into pMQ70 (AmpR). All plasmids were sequenced at the Molecular Biology Core at the Geisel School of Medicine at Dartmouth. In frame-deletions and integrated promoter fusions were introduced into *P. aeruginosa* by conjugation via S17/lambda pir *E. coli*. Merodiploids were selected by drug resistance and double recombinants were obtained using sucrose counter-selection and genotype screening by PCR. Both RhlI-HA expression vectors were introduced into *P. aeruginosa* by electroporation. The ∆*lasRclpX::TnM* was identified from a collection of transposon mutants in the ∆*lasR* and verified by sequencing.

Pyocyanin Quantification
P. aeruginosa strains were grown in a 96-well plate containing 200 µL LB agar per well by inoculation with 5 µL of overnight cultures adjusted to OD<sub>600</sub> = 1. After 16 h incubation at 37 °C, two agar plugs (with indicated P. aeruginosa mono- or co-cultures) were added to tubes containing chloroform (500 µL), mixed by vortexing for 30 s and then centrifuged for 2 min at 13,000 RPM. The lower chloroform layer (200 µL) was collected into new tubes, and the chloroform extraction was repeated with an additional 500 µL of chloroform. The chloroform extracts (400 µL) were acidified with 0.2 N HCl (500 µL) and vortexed for 30 s. The pink aqueous layer containing pyocyanin was diluted 1:2 in 200 mM Tris-HCl (pH 8.0). Relative pyocyanin was measured by reading absorbance at 310 nm relative to media blank extracts. Values were reported per plug. Each condition had at least eight replicates each for two independent experiments.

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 OD<sub>600</sub> = 1. Competing strains were combined with PA14att::lacZ strain in a 1:1 ratio, unless otherwise stated. Following 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 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 number of blue and white colonies per
plate were counted and the final proportions recorded. Each competition was run in triplicate on 3 separate days.

**Colony Proximity Image analysis**

Glass beads were used to spread 50 µL of a 1:1 mixture of untagged WT and ΔlasR possessing the indicated promoter fusion to lacZ onto LB plates (2% agar) supplemented with 150 µg/mL 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-Gal). After 16 h incubation at 37 °C, plates were placed at 4 °C for an additional 24 h to allow all ΔlasR colonies to develop blue coloration of various intensities. Plates were imaged on glass sheet to reduce glare using Canon EOS Rebel T6i digital camera. To process images, they were first cropped to remove background area surrounding each plate, converted to 8-bit for particle analysis in ImageJ, and the threshold was determined to count WT and ΔlasR CFU’s, separately. Colony parameters were collected for each individual CFU, including x, y coordinates and area for WT and ΔlasR CFU lists. A simple distance calculation was made between every WT and ΔlasR colony using the x,y coordinates and the minimum distance to a WT colony was plotted for each ΔlasR CFU against its area value, representative of the approximate lacZ intensity.

**Swarming Motility Assays**

Swarm assays were performed as previously described in (99), with a few modifications. Briefly, M8 medium with 0.5 % agar was poured into 60 x 15 mm plates and allowed to dry at room temperature for 4 h prior to inoculation. LB grown cultures (16 h at 37 °C) were diluted to OD_{600} = 1 in fresh LB, and co-cultures were mixed such
that ∆rhlA was at 0.7 proportion of the final cell suspension. Each plate was inoculated with 5 µL of the final cell suspensions and incubated upright for 24 h at 37 °C in an incubated chamber followed by 12 – 16 h at room temperature. Each strain was inoculated in four replicates and assessed on at least three separate days.

RNA Collection
A 200 µL aliquot of optical density normalized (OD$_{600}$ = 1) cultures of PA14 or PA14 ∆lasR from three independent overnights were spread onto LB plates with glass beads and briefly allowed to dry. Two isopore 0.2 µm PC membrane filters were stacked on the lawn (37 mm diameter filter directly on lawn then 25 mm diameter filter on top), and three 15 µL spots of normalized (OD$_{600}$ = 1) ∆lasR cultures were spotted on the top-most filter. After 16 h incubation at 37 °C, the top filter was collected and ∆lasR cells were resuspended in 1 mL LB by 5 min of vigorous shaking on the genie disruptor. Cells were pelleted for 10 min at 13,000 RPM and snap-frozen in ethanol and dry ice for RNA extraction. RNA was extracted according to manufacturer’s protocol with the QIAGEN RNeasy Mini kit and DNase treated twice with Invitrogen Turbo DNA-Free kit. DNase-treated samples were prepared for sequencing with ribodepletion and library preparation in accordance with Illumina protocols. Samples were barcoded and multiplexed in a NextSeq run by the Dartmouth Sequencing Core.

RNA-Seq Processing
Reads were processed using CLC Genomics Workbench wherein reads were trimmed and filtered for quality using default parameters. Reads were aligned to the $P$. 
aeruginosa UCPBB_PA14 genome from www.pseudomonas.com. Results were exported from CLC including total counts, CPM and TPM. EdgeR was used to process differential gene expression (100). Generalized linear models with mixed effect data design matrices were used to calculate fold-change, p-value and FDR. Volcano plots and heatmaps using EdgeR output (log fold-change and -log(p-value)) were made in R (ggplot2 and pheatmap respectively) (101-103). GO term pathway enrichment analysis was carried out using PantherDB (104).

Accession Number

Data for our RNA-Seq analysis of P. aeruginosa ΔlasR grown on ΔlasR or WT in co-culture has been uploaded to the GEO repository (https://www.ncbi.nlm.nih.gov/geo/) with the accession number GSE149385.

Pyochelin extraction, quantification, and validation

Pyochelin was extracted based on the methods of Cox et al. (37). Briefly 50 mL cultures of PA14 ΔpvdA and pyochelin biosynthesis deficient strain PA14 ΔpvdAΔpchE (negative control) were grown in Chelex-treated (i.e. media treated for > 2 h with 0.5 g Chelex resin per 10 mL media concentrate, followed by centrifugation and filtration with 0.22 µm filter unit) LB for 16 h. Cultures were pelleted for 15 min at > 5000 RPM, and the supernatant was passed through a 0.22 µm filter unit. The cell-free supernatant was acidified to pH 2 with 10 N HCl. For extraction, 5 mL ethyl acetate was added per 50 mL acidified solution in a separatory funnel. The top ethyl acetate layer was concentrated using a speedvac and quantified in 50 / 50 methanol:dH2O in a 1 mm quartz cuvette at 313 nm. Absorbance
was checked from 200 - 600 nm for expected peak profile. For some extractions, a 5 µL aliquot of \( \Delta pvdA \) extract in 50:50 methanol:dH2O was viewed under ultraviolet light for expected fluorescence relative to \( \Delta pvdA \Delta pchE \) extract that dissipated upon 10 µM FeSO4 supplementation. The concentration was determined using the molar extinction coefficient at 313 nm in 50 / 50 methanol:dH2O in a 1 mm quartz cuvette (37). Upon quantification, concentrated extract was lyophilized using rotovap to yellow resin, and used within 2 days of initial extraction by resuspension in LB for supplementation. As validation of biological activity, 10 µL of extract was spotted along with EDTA chelator as control on CAS agar prepared as described in.

**Citrate Quantification**

Citrate was quantified from cell-free supernatant of 5 mL LB-grown cultures inoculated from single colonies. Cultures were grown in quadruplicate and incubated on a roller drum for 16 h. OD \( 600 \text{ nm} \) was recorded, and cultures were pelleted for 15 min at > 5,000 RPM. The supernatant was passed through a 0.2 µm syringe filter unit. Citrate in the filtered supernatant was quantified according to manufacturer’s “manual assay” protocol (Megazyme) in \( \frac{1}{2} \) reactions. The extinction coefficient at 340 nm in a quartz cuvette was used to quantify concentration of citrate relative to OD \( 600 \) at the end of the 5 min enzymatic reaction. Citrate standard and blank media conditions were included in every assay. The average for 4 replicates for each experiment was reported across 3 - 4 independent days.

**Beta galactosidase assays**
Cells with a promoter fusion to *lacZ* - *GFP* integrated at the *att* locus were grown in 5 mL cultures of LB at 37°C for 16 h. The cultures were diluted to a starting OD 600 of 1 and 5 µL were spotted onto LB agar plates ± 20 mM pH 7 citrate (or other specified metabolite) in triplicate. After 24 h (or other indicated time) at 37 °C, colony biofilms were cored, resuspended in 500 µL by vigorous shaking on the Genie Disrupter for 5 min as previously described, and β-Gal activity was measured as described by Miller (105). The average for each experiment was reported across 3-4 independent days.

**Western Blot**

Strains were grown in LB broth under selection (60 µg / mL gentamycin or 60 µg / mL carbenicillin as appropriate) for 16 h at 37 °C on a roller drum, and 5 µL of culture was spotted onto LB plates under selection with 0.2% L-arabinose (v/v) and +/- 20 mM indicated carbon source. Inoculated plates were incubated at 37 °C for 16 h. Colony biofilms were resuspended in 325 µL of Laemmli buffer without reducing agent and heated at 100 °C for 15 min. Protein was quantified on 1:10 dilution of protein sample according to standard procedure via Thermo scientific BCA Protein Assay Kit. Reducing agent was added and samples were run on a 4 - 15% SDS gradient gel (Bio-Rad) at 60 V for 40 min followed by 110 V for 45 min. After SDS page electrophoresis, protein was transferred onto LF-PVDF (Bio-Rad) using the mixed molecular weight option on a turbo blot apparatus (Bio-Rad). After transfer the membrane was dried, rehydrated, and then a total protein stain was run according to manufacturer’s procedure (Li-Cor). Following protein quantification, the membrane was incubated in TBS blocking buffer (Li-Cor) for 1 h, and then purified anti-HA mouse monoclonal antibody (Biolegend) in TBS blocking
buffer (1:2,500 dilution) for 1 hr. Following primary antibody detection, the membrane was washed 4 times in TBST 0.1%. Secondary detection was done by incubation with goat anti-mouse in TBS blocking buffer (1:15000 dilution) for 1 hour in the dark. Following detection, the membrane was washed 3 times in TBST 0.1% and once in TBS. The membrane was then dried and imaged on the Li-Cor Odyssey CLx imager relative to REVERT total protein stain.

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Figure legends

**Figure 1.** Δ*lasR* produces pyocyanin in wild type / Δ*lasR* co-cultures beyond monoculture concentrations. **A.** Wild type (WT), Δ*lasR*, and their phenazine-deficient derivatives (Δ*phz*) visualized from the bottom of the 96 well LB agar plate after 16 h growth as mono- (top) and co-cultures (bottom). **B.** Pyocyanin levels quantified for cultures described in A; ****, p<0.001. **C.** Pyocyanin production for wild type co-cultures with Δ*lasR* or Δ*lasR* complemented with the *lasR* gene at the native locus (Δ*lasR* + *lasR*) across several initial (i) proportions on LB for 20 h. **D.** Final proportions quantified after 16 h growth for WT and Δ*lasR* co-cultured with a WT tagged with *lacZ*. Experimental setup as described in C.

**Figure 2.** *P. aeruginosa* WT induces RhlR/I dependent activity in Δ*lasR* even in the absence of WT AHLs. **A.** Pyocyanin production by monocultures and WT co-cultures of Δ*lasR* and Δ*lasR* derivatives that are deficient PQS or RhlR/I quorum sensing on LB after 24 h growth. **B.** Promoter activity, quantified by relative pixel intensity of single cell-derived colony forming units (CFU) in co-culture with untagged WT CFU for Δ*lasR* P* pqsA* - *lacZ* (grey) and Δ*lasR* P*rhlI* - *lacZ* (black). Inset shows representative CFUs for RhlR-dependent Δ*lasR* P*rhlI* – *lacZ* activity when in monoculture and co-culture with WT (red circles). **C.** Co-culture pyocyanin production by Δ*lasR* still occurs upon co-culture with Δ*pqsA*, Δ*rhlI*, and Δ*lasI*Δ*rhlI* on LB after 24 h. All monocultures are shown at 48 h time point for comparison.

**Figure 3.** Biosynthesis of the co-culture-induced iron scavenging siderophore pyochelin is required in Δ*lasR* for pyocyanin over-production when cultured with wild type (WT). **A.** Scheme for the collection of RNA from Δ*lasR* colony biofilms grown above a lawn of WT or Δ*lasR*. **B.** Volcano plot of Δ*lasR* expression data with each point representing the log₂(Δ*lasR* grown on WT / Δ*lasR* grown on Δ*lasR*) expression and −log₁₀(P Value) of a single gene. 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.** Mono- and co-cultures with Δ*lasR* strains deficient in pyoverdine (Δ*pvdA*) and/or pyochelin (Δ*pchE*). Colonies are visualized under ultraviolet light (UV) in order to see fluorescent siderophores. **D.** Pyocyanin production visualized for the colonies shown in panel C.
Pyocyanin production by siderophore deficient strains grown in mono- and co-culture on LB with (+PCH) or without (LB) pyochelin-containing extract. Colonies were grown in a 12 well plate and imaged after 48 h. F. Wild-type and ΔlasR mixed colony biofilms grown on LB (-) or LB supplemented with either 10 or 100 μM FeSO4 visualized under ambient (top) and UV (bottom) light.

Figure 4. Citrate release by WT is induced by pyochelin exposure. A. Subset of ΔlasR co-culture expression data (see Fig. 3A for set up) for genes annotated as being involved in citrate sensing, transport, catabolism, anabolism, and those shown to be responsive to citrate. B. Volcano plot of ΔlasR co-culture expression data with genes shown in panel A highlighted in red. C. Citrate concentrations in supernatants from wild type and ΔlasR stationary phase cultures after growth in LB supplemented with extracts containing 50 μM pyochelin (PCH +) or an equal volume of control extracts (PCH -). A representative experiment with four biological replicates is shown; ***, p≤ 0.001 and ****, p≤ 0.0001 by two-tailed t-test of paired ratios.

Figure 5. Citrate and related compounds induce RhlR-dependent rhlI promoter activity and stabilize RhlI protein in LasR- strains in a ClpX protease dependent manner. A. β-galactosidase activity for ΔlasR, ΔlasRΔrhlR, and wild type harboring att::PrhlI - lacZ on LB ± 20 mM citrate at 24 h. Each point is the average of three biological replicates from 3 - 4 independent experiments. Statistical analyses performed by one-way ANOVA with Dunnet’s multiple hypotheses correction. B. RhlI-HA protein signal normalized to REVERT total protein stain (Licor) on LB ± 20 mM citrate for ΔlasR and lasR complemented at the native locus (ΔlasR+lasR). n = 3 biological replicates performed on three independent days. Dunnet’s multiple comparison test with LB control. Inset illustrates that plasmid-borne RhlI-HA, but not the empty vector (EV) can complement an ΔrhlI mutant for swarming. C. RhlI-HA protein levels on LB and LB supplemented with 20 mM citrate of LasR LOF (LasR-) clinical isolates (CI) from acute corneal (388D) and chronic CF infections (DH2415). D. RhlI-HA protein levels on LB ± 20 mM citrate of LasR+ acute corneal CI (550A) of same MLST type as 388D and LasR+ chronic CF CI (DH2417) from which DH2415 evolved. E. Representative image and quantification of replicates for the anti-HA antibody analysis of ΔlasR and ΔlasRclpX::M‡ pRhlI-HA or pEV grown in LB and LB supplemented with 20 mM citrate. F. RhlR-dependent rhlI promoter activity of ΔlasR P rhlI - lacZ on LB supplemented with citrate, acetate, succinate, fumarate, and malate at 16 and 24 h relative to LB control. G. Normalized RhlI-HA protein levels ΔlasR and ΔlasR + lasR at 16 h of growth on citrate, acetate, and succinate. Inset shows representative blot of RhlI-HA protein under these same conditions. p-values: * (p<0.05), ** (p ≤ 0.05), *** (p≤ 0.001), and **** (p≤ 0.0001).

Figure 6. Model for wild type and ΔlasR co-culture interactions. (1.) ΔlasR produced pyochelin promotes citrate release in wild type. (2.) Citrate released by wild type in co-culture stimulates RhlR/I dependent activity (3.) by stabilizing RhlI protein in ClpXP protease dependent mechanism (4.) to promote the production of antagonistic factors like pyocyanin toxin and rhamnolipid surfactant.
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Figure S1. Increased pyocyanin production of LasR+ and LasR- strain co-cultures relative to either strain alone is stable across media and strains. A. Representative images from PA14 wild type (WT) and ΔlasR mono- and co-culture pyocyanin production on rich media (LB and Artificial Sputum Media-ASM) and buffered minimal medium (M63 pH 6.8 0.2% glucose 0.2% CAA). B. Pyocyanin production of mono- and co-cultures of strain PA14 WT and ΔlasR and clonally-derived clinical isolates DH2417 (LasR+) and DH2415 (LasR-). C. Pyocyanin concentrations of LasR+ and LasR- strains grown in mono- and co-culture on 96 well LB agar plugs for 16 h. Strain PA14 WT and ΔlasR, and previously characterized clinical isolate pairs DH2417 (LasR+) and DH2415 (LasR-) and DH1133 (LasR+) and DH1132 (LasR-), from two distinct CF patients, are also shown. Data are from two independent experiments with four biological replicates each. **** represents a significant difference from the co-culture, p<0.0001. D. Colony forming units (CFUs) in 16 h colony biofilms grown on LB. Each strain set is presented relative to WT or the LasR+ strain. PA14 WT and ΔlasR mono- and co-cultures data are the average from four independent experiments with at least three biological replicates. No significant (ns) differences found by ANOVA with Tukey multiple hypotheses correction for clinical isolate CFU counts. ** p-value < 0.005, *** p-value < 0.0005.
Figure S2. ∆lasR shows Anr independent increases in pyocyanin in co-culture with LasR+ P. aeruginosa. Pyocyanin production of WT, ∆anr, and ∆lasR∆anr in mono- and co-culture colony biofilms on LB after 24 h.
Figure S3. ΔlasR shows enhanced production of RhlR-regulated rhamnolipid surfactant in co-culture with LasR+ strain. RhlR-regulated swarming motility on soft agar of WT, rhamnolipid surfactant biosynthesis mutant (ΔrhlA), ΔlasR, and ΔlasRΔrhlR in mono- (top) and co-culture (bottom) after 36 h.
Figure S4. Fitness of ∆lasR lacking one or both major siderophore biosynthesis pathways. Final proportion of untagged colony forming units quantified after 16 h competition with att::lacZ wild type. Dotted line indicates 0.5 initial proportion (P_i). Final proportions (P_f) for ∆lasR, wild type (WT), and ∆lasR complemented with the lasR gene at the native locus (∆lasR + lasR) on LB (white background), for ∆lasR on LB supplemented with 10 µM FeSO4 (grey background), and siderophore deficient ∆lasR derivatives. Statistical analyses performed by one-way ANOVA for comparison to ∆lasR on LB with Sidak multiple hypotheses correction: a-b p-value < 0.0006, a-c p-value < 0.0001.
Figure S5. Pyochelin-containing extracts are biologically active. A. Absorbance spectra (230 to 600 nm) of extracts from supernatants of pyochelin-producing, Δpvda (solid line), and pyochelin-deficient, ΔpvdaΔpchE (dotted line) strains in a 50/50 MeOH/dH2O solution. Grey vertical lines indicate reported peaks for purified, iron-free pyochelin at 248 and 313 nm. B. Indicated extracts spotted on chrome azurol S (CAS) agar with ethylenediaminetetraacetic acid (EDTA) metal chelator as positive control wherein change from blue to yellow indicates iron chelating capacity. CAS activity for the extracts from Δpvda (+PCH) and ΔpvdaΔpchE (NEG) are shown.
Figure S6. Anthranilate (AA) did not stimulate ΔlasR pyocyanin production. A. Volcano plot indicating anthranilate metabolism gene expression (orange) in ΔlasR grown in co-culture with WT. B. RhlR dependent pyocyanin production of ΔlasR and anthranilate synthase mutant (ΔphnAB) mono- and co-cultures on LB after 18 h. C. Colony morphology and pyocyanin production are not different upon anthranilate supplementation across a gradient of concentrations.
Figure S7. Potential di- and tri-carboxylic acid transporters dctA and PA14_51300 were not required for ΔlasR pyocyanin production. A. Major succinate, fumarate, and malate transporter dctA was dispensable in ΔlasR background for RhlR-dependent pyocyanin production in co-culture with WT. B. Broad TCA cycle intermediate transporter PA14_51300 was not required in ΔlasR for pyocyanin production in co-culture with WT.