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Stationary Phase-Specific Virulence Factor Overproduction by a \textit{lasR} Mutant of \textit{Pseudomonas aeruginosa}

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Abstract

Secreted virulence factors of the human pathogen \textit{Pseudomonas aeruginosa} are often under quorum sensing control. Cells lacking the quorum-sensing regulator \textit{LasR} show reduced virulence factor production under typical laboratory conditions and are hypo-virulent in short-term animal infection models, yet \textit{lasR} mutants are frequently associated with long-term infection in cystic fibrosis patients. Here, I show that in stationary-phase or slow-growth conditions, \textit{lasR} cells continuously and strongly produce the important virulence factor pyocyanin while wild-type cells do not. Pyocyanin overproduction by \textit{lasR} cells is permitted by loss of repression by \textit{RsaL}, a \textit{LasR}-dependent negative regulator. \textit{lasR} cells also contribute pyocyanin in mixed cultures, even under “cheating” conditions where they depend on their wild-type neighbors for nutrients. Finally, some clinical \textit{P. aeruginosa} isolates with \textit{lasR} mutations can overproduce pyocyanin in the laboratory. These results imply that slow-growing clinical populations of \textit{lasR} cells in chronic infections may contribute to virulence by producing pyocyanin under conditions where \textit{lasR}+ cells do not.

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Introduction

\textit{Pseudomonas aeruginosa} is a common opportunistic bacterial pathogen that causes human infections in a variety of clinical situations [1] but is especially important in cystic fibrosis lung infections [2]. Many of the numerous virulence factors produced by \textit{P. aeruginosa} are under the control of quorum sensing, which uses diffusible autoinducer molecules as a way to monitor cell density [3]. Specific genes are thus activated when bacterial cell population density, and hence autoinducer concentration, exceeds a threshold. \textit{P. aeruginosa} has at least three quorum-sensing systems, with distinct autoinducer molecules and partially overlapping regulons, that are hierarchically arranged. The Las system is the first to become activated, and it in turn stimulates additional systems known as Rhl and PQS, which additionally regulate each other (Fig. 1) [3]. Finally, pyocyanin, a phenazine small molecule and virulence factor, acts as a terminal signaling factor in the quorum-sensing cascade [4]. Consistent with this hierarchy, inactivation of LasR, the regulatory protein of the Las quorum-sensing system, has been reported to severely attenuate quorum sensing, the production of quorum-regulated factors, and virulence in typical laboratory culture and in short-term animal models [5,6,7,8,9,10,11].

Decreased quorum sensing can permit \textit{lasR} mutants to become social cheaters that gain a growth advantage by utilizing quorum-regulated “public goods” (including virulence factors) produced by nearby wild-type cells rather than producing their own [12,13]. Cheating was thus proposed as one reason [13] why \textit{lasR} mutants are detected in highly chronic human infections such as those occurring in cystic fibrosis patients [14]. Consistent with this idea, \textit{lasR} mutant cells outcompeted co-infected wild-type cells and lowered overall virulence in a marine burn-infection model, consistent with their being non-producing cheaters [11]. However, recent work has revealed that cells lacking LasR function can in fact accomplish quorum sensing by employing the Rhl and PQS systems [15,16]. Without activation by LasR, the quorum response is substantially delayed, but it appears to resemble the wild-type response in terms of gene expression and virulence factor synthesis [15]. Such LasR-independent virulence factor production is another potential explanation for why \textit{lasR} mutants may not reduce overall virulence in long-term cystic fibrosis infections. In accord with this idea, the presence of \textit{lasR} mutant cells has been correlated with disease progression and declining lung function in cystic fibrosis patients [17].

Pyocyanin is one of the most important quorum-regulated virulence factors of \textit{P. aeruginosa}. It has numerous toxic effects on host tissues at such infection sites as the respiratory epithelium, where its toxicity is thought to be related to the generation of reactive oxygen species when pyocyanin is oxidized [18]. Pyocyanin is under the control of the Rhl and PQS systems and can accordingly be produced even in the absence of LasR after a delay [15]. As with the presence of \textit{lasR} mutants, high levels of sputum pyocyanin have been associated with advanced infection in cystic fibrosis patients [19]. Pyocyanin also serves as an antibiotic thanks to its redox activity, can act as a terminal electron...
LasR Cells Overproduce Pyocyanin

most previous laboratory studies of \textit{P. aeruginosa} quorum sensing have observed bacteria exponentially growing in shaking culture. Under such conditions, wild-type quorum-sensing behaviors begin during late exponential phase and continue into stationary phase, while LasR-independent behaviors do not begin to appear until the onset of stationary phase, between 8 and 24 h [15]. However, most bacterial cells in nature are not growing in optimal laboratory-like conditions. Instead, pathogens often form biofilms during infection [20]. The physiology of slowly growing bacteria resembles that of bacteria growing in biofilms [21], and \textit{P. aeruginosa} expresses the stationary-phase sigma factor RpoS both in clinical sputum samples [22] and in continuously fed biofilms in vitro [23]. Indeed, one reason for the treatment resistance of cells growing in biofilms is their relatively slow growth [24]. Therefore, I reasoned that slow-growing or stationary-phase cells maintained in longer-term culture might manifest phenotypes that reflect their behavior in a more physiologically relevant state. Here, I report that wild-type and \textit{lasR} cells exhibit clearly distinct yet complementary stationary-phase phenotypes. Moreover, wild-type/\textit{lasR} mixtures can collaborate to enact behaviors inaccessible to the individual strains.

Materials and Methods

Routine bacterial culture

\textit{Pseudomonas aeruginosa} and \textit{Escherichia coli} strains were routinely cultured on LB Lennox solid (1.5% agar) and liquid media at 37°C. Culture stocks were stored in 25% glycerol at -80°C, and fresh plates were grown for each experiment. The following antibiotics were used for selection/maintenance for \textit{P. aeruginosa}; the maintenance concentration was used for \textit{E. coli} culture: gentamycin (75/15 µg/ml) and tetracycline (75/25 µg/ml). Irgasan (25 µg/ml) was used as an \textit{E. coli}-specific selective agent. \textit{P. aeruginosa} strains are listed in Table 1. \textit{E. coli} strains are listed in Table S1 in File S1, plasmids are listed in Table S2 in File S1, and primer sequences are listed in Table S3 in File S1.

Specialized media

M63 medium contained 100 µM (13.6 g/L) KH₂PO₄, 15.14 mM (2 g/L) (NH₄)₂SO₄, and 0.36 µM (0.1 mg/L) FeSO₄. A 5X salts stock was adjusted to pH 7.0 with KOH before autoclaving. To make the final medium, the 5X stock was mixed with 0.2% casamino acids and 0.5% glycerol from 20% and 50% sterile stocks, respectively, and adjusted to 1X with sterile H₂O.

M9 medium was based on a salt solution of 12.8 g/L NaHPO₄·7H₂O, 3 g/L KH₂PO₄, 0.5 g/L NaCl, 1 g/L NH₄Cl. A 5X salts stock was prepared and autoclaved. To make the final medium, the 5X stock was mixed with 2 mM MgSO₄ and 0.1 mM CaCl₂ from sterile 1M stocks, the appropriate carbon sources (sodium caseinate was added from a 10% autoclaved stock), and was adjusted to 1X with sterile H₂O.

SCFM medium was made as described by Palmer \textit{et al.} [25] and was prepared and used freshly, as it displayed a short shelf life.

Specialized culture conditions

Static cultures of \textit{P. aeruginosa} were grown in 4-ml volumes in 12-well microtiter plates, in 2-ml volumes in 24-well plates, or in 200-µl volumes in 96-well plates. A 1% volume of stationary-phase LB starter culture (OD₆₀₀=2-3), adjusted to OD₆₀₀ = 1.0, was used for inoculation (hence, the starting OD₆₀₀ was equivalent to 0.01). Pure autoinducer molecules (3OC₁₂-HSL, C₄-HSL, and PQS; Sigma) were added from 100 mM stocks in DMSO, and Pure autoinducer molecules (3OC₁₂-HSL, C₄-HSL, and PQS; Sigma) were added from 100 mM stocks in DMSO, and equivalent volumes of DMSO were used for controls.

Reporter assays

Luminescent \textit{lux} reporter strains were grown in static 200-µL LB cultures in 96-well microtiter plates. The cultures were inoculated at an initial OD₆₀₀ of 0.01 (from starter cultures diluted to OD₆₀₀ = 1.0) and grown at 25°C and 40% relative humidity. At daily time points, the luminescence values of the plate were read with a BioTek Synergy 2 plate reader. Data were collected using BioTek Gen5 software. At the same time points, static cultures with isogenic backgrounds, bearing an empty reporter and prepared identically, were taken, vortexed to disperse cells

Figure 1. A lasR mutant overproduces pyocyanin under stationary-phase culture conditions. A. A simplified diagram showing some of the regulatory pathways linking the three quorum-sensing systems of \textit{P. aeruginosa} and exoproduct synthesis. Each system and its regulatory pathways is color coded. B. Plot of the number of live cells (colony-forming units) in static cultures of strains with wild-type (WT) \textit{PA14} (MTC172), \textit{PA14 lasR} (MTC774), and \textit{PA14 lasR rhlR} (MTC797) backgrounds in LB at 25°C. C. Pyocyanin quantification in static cultures of wild-type (WT) \textit{PA14} (MTC626) in LB at 25°C. The inset image shows the appearance of the cultures in a 12-well plate after 7 d. All plots show the mean ± standard deviation of 3 biological replicates.

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acceptor for \textit{P. aeruginosa}, and is a terminal signaling molecule in the quorum-sensing cascade [4]. It is therefore useful for monitoring quorum-sensing activity in \textit{P. aeruginosa}, especially given its bright blue color when oxidized.

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effective dispersion was verified by phase-contrast microscopy)
and serially diluted to obtain colony-forming unit (CFU) counts.
The resulting CFU counts were then used to normalize the
luminescence values of their respective reporter strains.

Pyocyanin extraction and quantification
Pyocyanin was extracted from the supernatants of liquid
cultures by adding an equal volume of chloroform (CHCl₃) and
vigorously vortexing. The lower, pyocyanin-containing organic
layer was then taken and vortexed with an equal volume of 0.2 M
HCl. The pink pyocyanin-containing aqueous layer was taken,
and its absorbance at 520 nm (A₅₂₀) was read in a BioTek Synergy
2 plate reader.

For some experiments, pyocyanin was quantified directly in
culture supernatants by reading the absorbance at 691 nm as
previously described [19]. Sterile medium was used as a blank.

Cheating experiments
For cheating experiments, PA14 cells or mixtures of PA14 or
PA14 Δphz cells with lasR cells were grown in liquid M9 medium
(6 mL) containing 1% casein at 25°C in a tube roller. Pyocyanin
was quantified as described above. The fraction of lasR cells within

| Strain   | Alias   | Relevant genotype or description | Source or reference |
|----------|---------|----------------------------------|---------------------|
| **P. aeruginosa** |         |                                  |                     |
| MTC1     | PA14    |                                  |                     |
| MTC63    | PA14 Δphz |                                  | [4]                 |
| MTC390   | PA14 ΔlasR |                                  | This study          |
| MTC498   | PA14 ΔrsiA |                                  | This study          |
| MTC500   | PA14 Δsial ΔlasI |                             | This study          |
| MTC537   | PA14 ΔppqA |                                  | This study          |
| MTC556   | PA14 Δrhl ΔppqA |                             | This study          |
| MTC625   | PA14 ΔlasR Δrhl |                             | This study          |
| MTC626   | PA14 ΔlasR Δrhl ΔI |                             | This study          |
| MTC628   | PA14 ΔlasR ΔppqA |                             | This study          |
| MTC637   | PA14 ΔlasR attB:CTX-1-aacC1 (Gen®) |    | This study          |
| MTC723   | PA14 attB:CTX-1-PPaRL-Lux |                       | This study          |
| MTC725   | PA14 attB:CTX-1-PPaRL-Lux |                       | This study          |
| MTC733   | PA14 attB:CTX-1-PpqsA-lux |                       | This study          |
| MTC735   | PA14 attB:CTX-1-PPaRL-Lux |                       | This study          |
| MTC737   | PA14 attB:CTX-1-PPaRL-Lux |                       | This study          |
| MTC745   | PA14 ΔlasR attB:CTX-1-PPaRL-Lux |                | This study          |
| MTC747   | PA14 ΔlasR attB:CTX-1-PPaRL-Lux |                | This study          |
| MTC755   | PA14 ΔlasR attB:CTX-1-PPaRL-Lux |                | This study          |
| MTC757   | PA14 ΔlasR attB:CTX-1-PPaRL-Lux |                | This study          |
| MTC759   | PA14 ΔlasR attB:CTX-1-PPaRL-Lux |                | This study          |
| MTC772   | PA14 attB:CTX-1-Lux |                       | This study          |
| MTC774   | PA14 ΔlasR attB:CTX-1-Lux |                       | This study          |
| MTC789   | PA14 ΔlasR Δrhl attB:CTX-1-PPaRL-Lux |             | This study          |
| MTC790   | PA14 ΔlasR Δrhl attB:CTX-1-PPaRL-Lux |             | This study          |
| MTC794   | PA14 ΔlasR Δrhl attB:CTX-1-PPaRL-Lux |             | This study          |
| MTC795   | PA14 ΔlasR Δrhl attB:CTX-1-PPaRL-Lux |             | This study          |
| MTC797   | PA14 ΔlasR Δrhl attB:CTX-1-PPaRL-Lux |             | This study          |
| MTC838   | PA14 ΔlasR ΔambB |                        | This study          |
| MTC842   | PA14 ΔlasR ΔphoB |                        | This study          |
| MTC949   | PA14 ΔlasR Δrhl ΔppqA |                        | This study          |
| 0007-2   | CF1     | lasR wild type (confirmed by sequencing) | [17]               |
| 0022-1   | CF2     | lasR79C-1 (W60STOP identified by sequencing) | [17]               |
| 0024-5   | CF3     | lasR wild-type (identified by sequencing) | [17]               |
| 0029-1   | CF4     | lasR577-578 (Q186P identified by sequencing) | [17]               |
| 0053-2   | CF5     | lasR520C-A (P174S identified by sequencing) | [17]               |
| 0063-6   | CF6     | lasR520C-520C (deletion and frameshift identified by sequencing) | [17]               |
a mixture was determined using a \textit{lasR} strain chromosomally marked with gentamycin resistance (MTC637). Cultures were serially diluted in 1X M9 salts and plated on LB or LB containing 5 µg/ml gentamycin to obtain CFU counts.

**Statistical analysis**

Comparisons between samples were analyzed using unpaired equal-variance two-tailed Student’s t-tests. The threshold for significance was set as $p \leq 0.01$.

**Results**

**Pyocyanin is overproduced by \textit{lasR} cells in extended stationary-phase culture**

To observe the behavior of stationary-phase cells over a time period of days rather than hours, as in traditional laboratory studies, I examined static liquid LB cultures of PA14 and a \textit{lasR} mutant derivative growing at 25°C. Under these conditions, cells grow to a high density that then very gradually falls over the course of several days (Fig. 1B) but do not exhibit the “death phase” that typically precedes long-term adaptation to stationary phase [26]. In shaking culture, wild-type cells produce noticeable pyocyanin beginning in late exponential phase [4], while \textit{lasR} cells begin to produce it by 24 h of culture [15]. After 3–4 days in static culture, I unexpectedly observed strong and continuing production of pyocyanin by stationary-phase \textit{lasR} cells that turned the cultures dark blue, while wild-type cells produced virtually no visible pyocyanin at any time during the experiment (Fig. 1C). This effect was strongest in LB at 25°C, but the same trend appeared in static cultures of minimal M63 medium and in a nutritional mimic of cystic fibrosis sputum [23] at both 25°C and 37°C (Fig. S1 in File S1). Therefore, the wild type and \textit{lasR} mutant display distinct stationary-phase phenotypes in that \textit{lasR} cells continually produce pyocyanin while wild-type cells barely produce any pyocyanin. The phenotype of the \textit{lasR} mutant was not due to additional mutations accumulated during the experiment, as cells from 6-day-old blue cultures displayed the same time course of pyocyanin production when inoculated into liquid LB, grown overnight at 37°C, and re-inoculated into static LB (Fig. S2 in File S1).

**Stationary-phase wild-type and \textit{lasR} cells express distinct quorum-regulated virulence genes**

Because stationary-phase wild-type and \textit{lasR} cells displayed distinct phenotypes with respect to pyocyanin production, I analyzed the expression of additional quorum-regulated genes with roles in virulence factor production. Two distinct expression patterns were apparent. The first, typified most strongly by \textit{lasB} but also seen for \textit{rhlA} (encoding the LasB elastase and a rhamnolipid biosynthesis protein, respectively), showed strong early expression in the wild-type but only weak expression in \textit{lasR} cells (Fig. 2). The second, seen most strongly for \textit{phzA1} but also for \textit{henA} (involved in phenazine and hydrogen cyanide synthesis, respectively), showed delayed but stronger expression by \textit{lasR} mutant cells but weaker expression by the wild type (Fig. 2). These results revealed that wild-type cells were successfully performing quorum sensing, as they very strongly expressed \textit{lasB} and also expressed \textit{rhlA}. However, \textit{phzA1} was notable for being largely turned off in the wild-type. The \textit{lasR} mutant displayed the opposite phenotype, most strongly expressing genes that were weakly expressed by the wild type. Among the sampled quorum-regulated virulence genes, the wild-type and \textit{lasR} strains thus showed distinct but complementary expression profiles, and the \textit{lasR} profile was characterized by strong \textit{phzA1} expression and pyocyanin production.

LasR-independent expression requires the Rhl and PQS quorum-sensing systems

Previously reported LasR-independent quorum sensing in shaking culture required the Rhl quorum-sensing system [15], in accord with its position in the quorum-sensing network (Fig. 1A). I thus tested whether the Rhl and PQS systems were also required for quorum expression in stationary-phase \textit{lasR} cells. Indeed, additional deletion of \textit{rhlR}, encoding the RhlR regulator, in a \textit{lasR} background abolished the expression of all tested genes (Fig. 2). Similarly, pyocyanin production did not occur in \textit{lasR rhl} or \textit{lasR pqsA} double mutants, which are unable to produce the Rhl autoinducer N-butyryl-l-homoserine lactone (C4-HSL) or 2-heptyl-4-quinolone (HHQ) and 2-heptyl-3-hydroxy-4-quinolone (PQS), respectively (Fig. 3A). Each of these double mutants could be complemented for pyocyanin production by exogenous addition of the appropriate autoinducer (C4-HSL or PQS), with stronger induction at 100 µM than at 10 µM (Fig. 3A). Consistent with these results, a triple \textit{lasR rhl} \textit{pqsA} mutant required the addition of both autoinducers to restore pyocyanin production (Fig. 3A). Moreover, exogenous addition of PQS alone or in combination with C4-HSL to the \textit{lasR} mutant accelerated pyocyanin production, while C4-HSL alone did not (Fig. 3B). This result is consistent with the idea that cellular RhlR levels are a limiting factor for LasR-independent pyocyanin production, as PQS signaling can stimulate \textit{rhlR} transcription [27] and addition of constitutively expressed plasmid-borne \textit{rhlR} greatly accelerated and increased pyocyanin production in a \textit{lasR} mutant in shaking culture [15].

A \textit{lasR pqsH} double mutant, which is unable to convert HHQ to PQS [28,29], was able to produce pyocyanin (Fig. 3C), suggesting that HHQ is itself a signaling molecule that can functionally substitute for PQS to induce pyocyanin production under stationary-phase conditions. This result contrasts with a previous report [29], but the difference may be due to the different strain background, culture media and conditions used in this work.

It has been suggested that LasR-independent quorum sensing and pyocyanin production may occur through the PhoB-mediated phosphate starvation pathway [27] or use the newly discovered signaling molecule IQS, whose synthesis requires the AmbB protein [30]. To test whether pyocyanin production by stationary-phase \textit{lasR} cells required either of these proteins in addition to Rhl and PQS quorum signaling, I constructed \textit{lasR phoB} and \textit{lasR ambB} double mutants and assayed them for pyocyanin production in static culture. Each of the double mutants produced pyocyanin indistinguishably from the \textit{lasR} mutant (Fig. S3 in File S1), showing that neither of these pathways is required for LasR-independent overproduction of pyocyanin in stationary-phase culture.

Repression by RsaL explains the different quorum profiles of wild-type and \textit{lasR} cells

The weak expression by wild-type cells of genes that were strongly expressed by the \textit{lasR} mutant suggested that they might be under negative regulation. Notably, \textit{phzA1} and \textit{henA}, which displayed the strongest LasR-independent expression and the weakest expression by the wild type, are direct targets of negative regulation by RsaL, a repressor whose primary role is to provide negative homeostatic feedback to Las quorum sensing [31]. Meanwhile, \textit{lasB} and \textit{rhlA}, which are not under RsaL repression [31], were strongly expressed in the wild type. Because expression of \textit{rsaL} is under LasR control [32], RsaL was an excellent candidate for a negative repressor that would be present in the wild type but absent in a \textit{lasR} mutant. Indeed, stationary-phase \textit{rsaL} expression in static culture was very strong in wild-type cells.
but quite weak in \textit{lasR} cells (Fig. 4A). Presumably, the RsaL protein produced during the initial peak of expression in wild-type cells continues to stably bind its target DNA sequences \cite{33}, such as \textit{phzA1}, in subsequent days, ensuring their continued repression. If RsaL were responsible for repressing genes such as \textit{phzA1} in otherwise quorum-active wild-type cells in stationary phase, inactivation of \textit{rsaL} in a wild-type background would relieve this repression. Consistent with this hypothesis, an \textit{rsaL} mutant in static culture displayed copious pyocyanin production that began significantly earlier than in a \textit{lasR} mutant (Fig. 4B), suggesting that RsaL normally blocks pyocyanin production by the wild type. Deletion of \textit{rsaL} also disrupts Las homeostasis, resulting in overabundance (25 \textmu M, compared to 2 \textmu M in the wild type) of the Las autoinducer \textit{N-3-oxo-dodecanoyl-L-homoserine lactone} (3OC12-HSL) \cite{31}. It was thus possible that high concentrations of 3OC12-HSL abetted the early production of pyocyanin. To correct for any such effect, I constructed an \textit{rsaL lasI} double mutant unable to produce 3OC12-HSL and exogenously added a low (100 nM) concentration of 3OC12-HSL at the time of inoculation. The double mutant displayed 3OC12-HSL-dependent early pyocyanin production that was even stronger than that of the \textit{rsaL} mutant (Fig. 4B), confirming that stationary-phase wild-type cells are capable of pyocyanin production but that it is repressed by the presence of the RsaL repressor. Therefore, expression of a specific set of quorum-regulated genes (including \textit{phzA1} and \textit{hcnA}) in \textit{lasR} cells is caused by LasI-independent Rhl and PQS quorum-sensing activity in combination with deactivation of RsaL-mediated repression.

\textit{lasR} cells contribute pyocyanin in mixed culture even under conditions that permit cheating

A \textit{lasR} mutant is a well-known example of a “cheater”. Typical cheating experiments use defined medium containing casein as the sole carbon source \cite{12,13,34}. Because casein utilization requires quorum-regulated extracellular proteases such LasB, whose production in early phases of growth is induced by the Las system \cite{35}, a \textit{lasR} mutant fails to grow on casein medium (Fig. S4 in File S1). When a wild-type strain is grown together with a \textit{lasR} mutant, the \textit{lasR} mutant benefits from the casein proteolysis performed by wild-type-derived LasB without the associated costs of producing quorum-regulated factors and thereby gains an advantage \cite{12,13}. In light of the distinct quorum-sensing profiles of stationary-phase wild-type and \textit{lasR} cells, I hypothesized that \textit{lasR} cells might be able to contribute quorum-regulated factors such as pyocyanin even while “cheating” with respect to nutrition. To test this hypothesis, I cultivated wild-type and \textit{lasR} cells alone and in a 1:4 mutant-to-wild-type mixture for several days in shaking liquid M9 medium with 1% casein, a typical cheating medium. As expected, the \textit{lasR} mutant alone did not grow in this medium (Fig. S4 in File S1), while the wild-type grew (Fig. S4 in File S1) and produced some pyocyanin (Fig. 5A), indicating quorum sensing. The mixture of the two strains, however, produced much more pyocyanin than the wild-type alone (Fig. 5A), suggesting that the \textit{lasR} mutant was contributing to pyocyanin production. To test this idea, I grew 1:4 \textit{lasR-to-phz} mixtures in which only the \textit{lasR} mutant could contribute pyocyanin. Such mixtures produced only slightly less pyocyanin than mixtures with the wild-type and substantially more pyocyanin than the wild-type alone (Fig. 5A), confirming that the \textit{lasR} mutant contributed the majority of pyocyanin in

*Figure 2. Wild-type and \textit{lasR} mutant cells display distinct virulence gene expression patterns in stationary-phase culture.* Gene expression was measured using chromosomally integrated \textit{lux} reporters in the listed strain backgrounds. Plots show the mean \pm standard deviation of 3 biological replicates each composed of 3 technical replicates. doi:10.1371/journal.pone.0088743.g002
mixtures. In such mixtures, the relative \textit{lasR} population increased from its initial 20% level to 30–40% after 7 days of culture (Fig. 5B), suggestive of a slight advantage gained by nutritional cheating. These results demonstrated that the \textit{lasR} mutant cooperated with respect to pyocyanin production even under conditions (casein medium) that forced it to cheat with respect to LasB. Notably, pyocyanin production by the \textit{lasR} cells was not detected until the third day of culture (Fig. 5A), explaining why this \textit{lasR} phenotype is not seen in cheating assays when cultures are diluted every 1–2 days [12,13].

Clinical \textit{lasR} isolates can overproduce pyocyanin

The overproduction of pyocyanin by stationary-phase \textit{lasR} cells in monoculture and in mixtures with wild-type cells raised the interesting possibility that \textit{lasR} cells may overproduce pyocyanin in clinical infections, thereby increasing virulence. A relationship between the presence of \textit{lasR} cells and high pyocyanin is at least suggested by separate studies associating high sputum pyocyanin [19] and \textit{lasR} cell presence [17] with \textit{P. aeruginosa} disease progression in cystic fibrosis patients. Assessing the relative contributions of \textit{lasR} and \textit{lasR} cells to pyocyanin or virulence factor production in actual chronic human infections is very difficult, due to the spatial and genetic complexity of human lung infections. However, as a simple test of principle, I subjected a small set of clinical cystic fibrosis isolates that were wild type or mutant for \textit{lasR} (Fig. 6A) to a static culture assay and looked for pyocyanin production. The \textit{lasR} strains exhibited minimal pyocyanin production, whereas \textit{lasR} mutant strains showed widely varied production ranging from minimal to very strong (Fig. 6B). This variability may reflect differences in the severity of the different \textit{lasR} mutant alleles (Fig. 6A) and other mutations in the genetic backgrounds of these strains that modulate pyocyanin production. Nonetheless, these results demonstrate that some clinical \textit{lasR} mutant strains have the ability to overproduce pyocyanin under stationary-phase conditions where \textit{lasR} isolates cannot.

**Discussion**

This work shows that the quorum response by \textit{lasR} mutants in slow-growth or stationary-phase conditions is distinct from the wild-type response and is characterized by strong expression of virulence factor genes that are repressed in wild-type cells by RsaL. For example, the pattern of low pyocyanin production by wild-type and high production by \textit{lasR} cells in static stationary-phase culture is a reversal of the pattern seen for cells growing exponentially in shaking culture, showing that “typical” lab conditions uncover only part of the full range of cell behaviors. Experiments conducted in shaking culture for 24 hours showed that \textit{lasR} cells could manifest a quorum response [15], but did not reveal the distinctions between the wild-type and \textit{lasR} stationary-phase phenotypes that develop after longer-term culture under slow-growth conditions.
Stationary-phase phenotypes are highly relevant for bacterial physiology in natural settings, including within infective biofilms [20,21,22]. The relative metabolic inactivity of some biofilm-embedded cells is one mechanism of resistance against killing by host defenses or by antibiotics [24]. Moreover, host environments like the cystic fibrosis lung contain hypoxic niches that slow bacterial growth [36,37]. It is therefore likely that many P. aeruginosa cells in long-term infections are enacting stationary-phase behaviors. In such conditions, the presence of lasR mutants within the P. aeruginosa population may permit the expression of important virulence factors such as pyocyanin that are repressed by RsaL in lasR+ cells, thus expanding the range of phenotypes available to the total population. In this way, niches containing lasR cells could make a key contribution to virulence.

If repression by RsaL prevents lasR+ cells from producing important virulence factors, why are mutations in rsaL not commonly isolated in clinical samples from chronic infections? One likely reason is because of the homeostatic function of RsaL in the normal quorum response. Cells lacking RsaL function display constitutive overproduction of quorum-regulated factors [38], perhaps making an rsaL cell population less competitive than wild-type cells under faster-growth conditions in the same way that wild-type cells can be cheated on by lasR cells. In contrast, a lasR mutant can be competitive under fast-growth conditions (as illustrated by its well-known cheating behavior) before overproducing a more narrowly defined set of quorum-regulated factors specifically during stationary phase. This fine tuning is made possible by a combination of three features of the quorum-sensing regulatory circuit: first, RsaL is under LasR control and thus is not produced in a lasR mutant [32]; second, RsaL has many other targets in addition to its homeostatic regulation of lasI [31]; and third, the Rhl and PQS systems, which are normally activated by LasR, can also self-activate in a lasR mutant [15,16].

The distinct contributions of lasR+ and lasR cells in a mixture allows them to collaborate to produce otherwise inaccessible phenotypes. This is seen most clearly in casein medium, where the lasR+ cells secrete LasB to break down casein and feed the lasR cells, and the lasR cells in turn produce high levels of pyocyanin. It is conceivable that such a division of labor, where lasR cells overproduce pyocyanin and other virulence factors, may have a role in host infection. In this scenario, slow-growing or stationary-phase lasR cells within an infecting population might continually produce pyocyanin under conditions where lasR cells do not. Overproduction of pyocyanin by some clinical lasR isolates under stationary-phase laboratory conditions suggests that they may do likewise in an infection setting, in accord with the findings that lasR strains [17] and high sputum pyocyanin [19] are both correlated with disease progression in cystic fibrosis patients. One corollary of this idea is that treatment strategies based on strong pharmacological inhibition of LasR (thus making cells functionally null for LasR) may in fact increase pyocyanin production by lasR+ cells in stationary phase.
LasR Cells Overproduce Pyocyanin

Figure 6. Clinical lasR isolates can overproduce pyocyanin. A. Locations of mutations in the LasR protein detected by sequencing. The mutations are in clinical isolates from Ref. 17. B. Appearance of static LB cultures of PA14 and lasR laboratory strains and of clinical isolates (CF1-6) (17) with and without lasR mutations. The cultures were grown for 12 days at 25°C to allow weaker phenotypes to develop.

Supporting Information

File S1 This file contains Tables S1–S3 and Figure S1–S4. Table S1, Escherichia coli strains used in this study. Table S2, Plasmids used in this study. Table S3, Primer sequences used in this study. Figure S1, Pyocyanin production in static culture. Figure S2, Pyocyanin production by lasR cells after overnight regrowth. Figure S3, LasR-independent pyocyanin production does not depend on AmbB or PhoB. Figure S4, Growth of wild-type PA14 or lasR cells in M9 medium with added casamino acids or casein.

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Author Contributions

Conceived and designed the experiments: MTC. Performed the experiments: MTC. Analyzed the data: MTC. Contributed reagents/materials/analysis tools: MTC. Wrote the paper: MTC.

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