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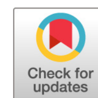
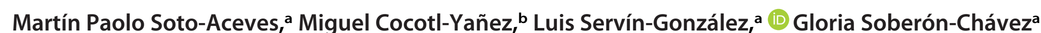
RESEARCH ARTICLE



The Rhl Quorum-Sensing System Is at the Top of the Regulatory Hierarchy under Phosphate-Limiting Conditions in *Pseudomonas aeruginosa* PAO1



Martín Paolo Soto-Aceves,[a] Miguel Cocotl-Yañez,[b] Luis Servín-González,[a] Gloria Soberón-Chávez[a]

[a]Departamento de Biología Molecular y Biotecnología, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Mexico City, México

[b]Departamento de Microbiología y Parasitología, Facultad de Medicina, Universidad Nacional Autónoma de México, Mexico City, México

ABSTRACT *Pseudomonas aeruginosa* is a major nosocomial pathogen that presents high-level resistance to antibiotics. Its ability to cause infections relies on the production of multiple virulence factors. Quorum sensing (QS) regulates the expression of many of these virulence factors through three QS systems: Las, Rhl, and PQS. The Las system positively regulates the other two systems, so it is at the top of a hierarchized regulation. Nevertheless, clinical and environmental strains that lack a functional Las system have been isolated, and, surprisingly, some of them still have the ability to produce virulence factors and infect animal models, so it has been suggested that the hierarchy is flexible under some conditions or with atypical strains. Here, we analyze the PAO1 type strain and its Δ*lasR*-derived mutant and report, for the first time, a growth condition (phosphate limitation) where LasR absence has no effect either on virulence factor production or on the gene expression profile, in contrast to a condition of phosphate repletion where the LasR hierarchy is maintained. This work provides evidence on how the QS hierarchy can change from being a strictly LasR-dependent to a LasR-independent RhlR-based hierarchy under phosphate limitation even in the PAO1 type strain.

IMPORTANCE *Pseudomonas aeruginosa* is an important pathogen, considered a priority for the development of new therapeutic strategies. An important approach to fight its infections relies on blocking quorum sensing. The Las system is the main regulator of the quorum-sensing response, so many research efforts aim to block this system to suppress the entire response. In this work, we show that LasR is dispensable in a phosphate-limited environment in the PAO1 type strain, which has been used to define the quorum-sensing response hierarchy, and that under this condition RhlR is at the top of the regulation hierarchy. These results are highly significant, since phosphate limitation represents a similar environment to the one that *P. aeruginosa* faces when establishing infections.

KEYWORDS LasR dispensability, *Pseudomonas aeruginosa*, RhlR-dependent regulation, antivirulence therapy, quorum-sensing hierarchy

Pseudomonas aeruginosa is a widespread bacterium (1, 2) that represents a deep concern in the medical field due to its intrinsic and acquired multidrug resistance (3) and the high incidence of its infections in immunocompromised individuals and cystic fibrosis (CF) patients (3). *P. aeruginosa* produces a variety of virulence factors, such as proteases (elastase and alkaline protease), pigments (pyocyanin and pyoverdin), and exotoxin A, among others. The production of many of its virulence factors is controlled by quorum-sensing (QS) systems at the transcriptional level. These systems consist of two principal components: a signal molecule, which is produced constitutively at low cell densities and secreted, and a regulatory protein, which is only active

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Address correspondence to Gloria Soberón-Chávez, gloria@biomedicas.unam.mx.

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when bound to the signal molecule and activates transcription of its target genes. They are population dependent, since the autoinducer concentration is proportional to the number of bacterial cells, so they activate phenotypes that are biologically relevant when produced at high cell concentration.

P. aeruginosa harbors three QS systems (4) that have been studied mainly in the PAO1 and PA14 type strains, showing a close conservation of this regulatory network in these and other well-studied *P. aeruginosa* strains (4). The Las system consists of the LasR regulatory protein and LasI, which synthesizes the signal molecule *N*-3-oxo-dodecanoyl-L-homoserine lactone (3O-C12-HSL). The Rhl system consists of the RhlR regulatory protein and the RhlI synthase of *N*-butanoyl-L-homoserine lactone (C4-HSL). The PQS system consists of the PqsR regulatory protein and the signal molecules, which are, in this case, 2-alkyl-4 (1h)-quinolones (4-hydroxy-2-heptylquinoline [HHQ] and 2-heptyl-3,4-dihydroxyquinoline [PQS]) that are synthesized by the proteins encoded by the *pqsABCDE* and *phnAB* operons and the *pqsH* gene (5). These three systems are arranged in a hierarchized way, where the Las system positively regulates the Rhl and PQS systems. Nevertheless, several reports where Las-defective strains are capable of producing virulence factors led to propose scenarios where the QS hierarchy is flexible (6–11). The high frequency of *lasR* mutants is particularly apparent among CF isolates (10), and one of these isolates has been shown to produce QS-regulated virulence factors using RhlR as the main transcriptional regulator (11). However, the altered QS hierarchy is mainly accepted to be representative of atypical strains, and, in the case of type strains, the absence of LasR has been associated with a delayed QS response (see Table S1 in the supplemental material), suggesting that the importance of LasR lies in the timing of the QS system activation (6, 7).

Phosphate limitation is a common stress condition that *P. aeruginosa* faces when establishing an infection, since the availability of phosphate in a healthy person is low (1.25 mM) and is even lower ($<$0.03 mM) in patients on chemotherapy or who have undergone a recent surgery (12–14). Furthermore, this stress condition is relevant, since it has been reported that virulence factor production is enhanced at low phosphate concentrations (13–15), especially for pyocyanin, which, besides being a toxin, is considered a terminal signaling factor of the QS cascade (16, 17). It has been proposed that phosphate limitation leads to the activation of a *P. aeruginosa* hypervirulent phenotype (18).

PhoB is the regulator protein that the PhoR kinase phosphorylates when phosphate is scarce. When PhoB is phosphorylated, it can regulate the transcription of its target genes by direct binding to the *pho* box (19). In *P. aeruginosa*, putative *pho* boxes have been located in some QS genes, such as *rhlR* and *lasR* (15), suggesting that phosphate availability influences QS.

As mentioned above, the production of the phenazine pyocyanin is enhanced under phosphate limitation. Pyocyanin production depends on the Las system, which positively regulates the Rhl system that then activates the transcription of both of the redundant operons *phzA1B1C1D1E1F1G1* (*phz1*) and *phzA2B2C2D2E2F2G2* (*phz2*) (20). The enzymes encoded by these operons are involved in the synthesis of the pyocyanin precursor phenazine-1-carboxylic acid (PCA) (21). Thus, the enhanced production of pyocyanin could be explained by PhoB-dependent *rhlR* expression.

In this work, we analyze the role of the Las and Rhl QS systems in virulence factor production and its control by the PhoB regulator under low and sufficient phosphate conditions using the PAO1 type strain as a model. We determined whether the QS hierarchy described previously for this type strain under nonstressed conditions applies to this nutrient-limited environment. Our results show that while under phosphate repletion LasR is indispensable to activate QS response, under phosphate limitation this regulator is completely dispensable, with RhlR at the top of the QS hierarchy. In addition, we show that under phosphate limitation, PhoB directly activates *rhlR* transcription and that the global transcription pattern of the PAO1-derived *lasR* mutant is indistinguishable from that of the PAO1 strain under phosphate-limited conditions. This

phenomenon of global LasR dispensability is supported by the fact that elastase LasB, a classical LasR target, becomes dependent only on the Rhl system under this condition.

RESULTS

Pyocyanin is overproduced under a low-phosphate condition. To characterize the effect of phosphate limitation on *P. aeruginosa* PAO1 pyocyanin production, low-phosphate (FDS) and sufficient-phosphate (FDS+) media containing 0.5 mM and 4.5 mM K_2HPO_4, respectively, were used to culture this bacterium. The growth in FDS was slightly lower, but this was not statistically significant (see Fig. S1 in the supplemental material).

We found that pyocyanin production was higher in FDS than FDS+ medium. This was evident from 16 h of growth (Fig. S2a), and at 24 h the level was eight times higher (Fig. S2b). This correlates with previous reports (14, 15) that proposed that this higher production can be explained by the regulation that PhoB exerts on *rhlR* transcription (15).

To determine whether rhamnolipids, a virulence-associated trait that is mainly dependent on RhlR/C4-HSL activity (22), were also overproduced in low-phosphate FDS medium, we detected these biosurfactants by thin-layer chromatography (TLC). We found that rhamnolipids were also overproduced in FDS (Fig. S2c), a result consistent with a previous report in which enhanced swarming, a rhamnolipid-related phenotype, was observed under phosphate limitation (23).

Taken together, these results suggest that under low-phosphate conditions, the production of RhlR-dependent phenotypes is increased.

PhoB directly regulates *rhlR* transcription. As mentioned above, the observed overexpression of the two phenotypes analyzed under the low-phosphate condition, pyocyanin and rhamnolipid production, can be explained by an increased expression of RhlR, as previously reported for the SCV 20265 strain (15). In that report, however, the phenomenon was observed at very late culture times of growth (38.5 and 45 h). Thus, we used an *rhlR′lux* transcriptional fusion to evaluate its expression in PAO1 cultured in FDS and FDS+ at earlier points in the stationary phase (18 to 24 h) (Fig. S3) and found, in accordance with this hypothesis, an increased expression in this period (Fig. S3), with 22 h being the time of highest increase of *rhlR* expression (Fig. S3), nearly doubling *rhlR* expression (Fig. 1a).

To test whether *rhlR* induction in low-phosphate medium was PhoB dependent, we first tested a *phoB* transcriptional fusion in FDS and FDS+ media to corroborate that the PhoR-PhoB system was more active under phosphate limitation. This fusion showed a four-times-higher activation on FDS than on FDS+ (Fig. 1a). We then evaluated the *rhlR* transcriptional fusion in a *phoB* mutant on FDS medium and found a reduced expression of about 40%, showing that *rhlR* transcription is partially PhoB dependent under low-phosphate conditions. Moreover, complementation of the *phoB* mutant with *phoB* in *trans* restored its expression (Fig. 1b). A putative *pho* box has been reported within the *rhlR* promoter region (14) (Fig. S4), so it has been proposed that *rhlR* regulation by PhoB is direct. To test this hypothesis, we performed an electrophoretic mobility shift assay (EMSA) using purified PhoB. This assay suggested a direct interaction, since the migration of a DNA fragment containing the *rhlR* promoter region is delayed in the presence of PhoB (Fig. 1c).

LasR expression is not affected by phosphate limitation. The existence of a putative *pho* box within the *lasR* promoter region has been reported (15) (Fig. S4), suggesting that PhoB could also regulate *lasR* in response to phosphate limitation. To test this, a *lasR′lacZ* fusion was evaluated in PAO1 grown in low-phosphate FDS and high-phosphate FDS+ media at 22 h. We found that its expression remained at the same level in both media (Fig. S5). This assay was also evaluated at the beginning of the stationary phase (16 h) (not shown), leading to the same conclusions. These results suggest that *lasR* is not subject to significant regulation by phosphate availability.

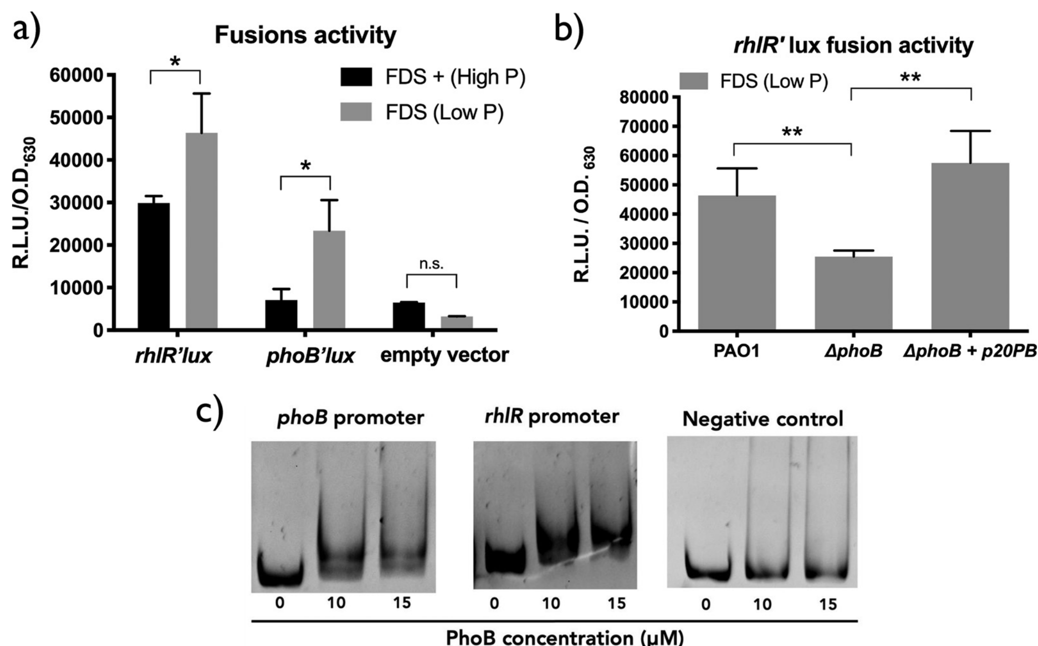

FIG 1 *rhlR* transcription is induced under low-phosphate conditions by PhoB. (a) *rhlR* and *phoB* transcriptional fusion activity in FDS+ and FDS media in PAO1 cultured for 22 h. R.L.U., relative light units. (b) *rhlR* transcriptional fusion activity in FDS+ and FDS media in PAO1, its *phoB* mutant (harboring empty vector pUCP20), and the complemented mutant (harboring p20PB). The results shown are the means from three biological replicates, and error bars denote the standard deviations. Asterisks indicate statistical significance by *t* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). n.s., nonsignificant. (c) PhoB EMSA with *rhlR* promoter fragment. *phoB* promoter fragment was used as a positive control, and a structural sequence (see Materials and Methods) was used as the negative control.

Pyocyanin production under a low-phosphate condition is LasR independent. The observation that *lasR* expression is not subject to phosphate regulation through PhoB (Fig. S5), in contrast to *rhlR* expression (Fig. 1a), led us to hypothesize that under low-phosphate conditions, the Rhl system is determinant for the QS system response, but the role of the Las and Pqs systems was not clear. To determine the relevance of the QS systems in pyocyanin overproduction under this condition, *lasR*, *rhlR*, and *pqsR* mutants were grown in FDS and FDS+ media.

In FDS+ medium, pyocyanin was detected only in the wild-type strain (Fig. 2). This result is in accordance with the reported QS regulation cascade in which the three systems have a role in pyocyanin production, since (i) the Las system activates the Rhl sys-

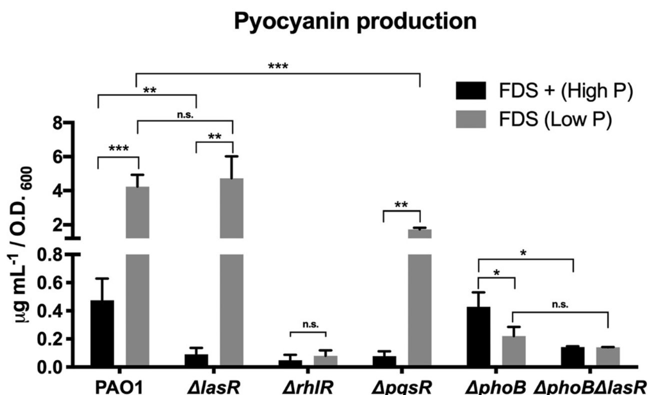

FIG 2 RhlR-dependent pyocyanin production under low-phosphate conditions is independent of LasR activity. Quantification of pyocyanin production by PAO1 and its isogenic *lasR*, *rhlR*, *pqsR*, *phoB*, and *phoB-lasR* mutants. The results shown are the means from three biological replicates, and error bars denote the standard deviations. Asterisks indicate statistical significance by *t* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). n.s., nonsignificant.

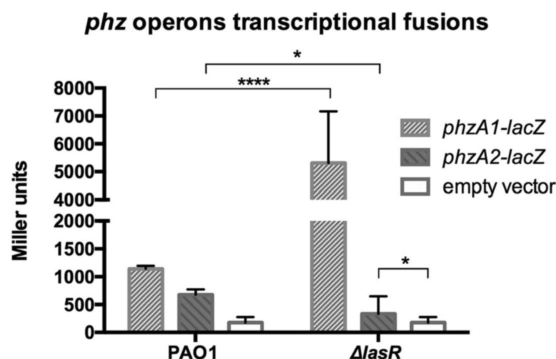

FIG 3 Increased pyocyanin production under low-phosphate conditions in the absence of LasR is only due to the expression of the *phz1* operon. Transcriptional fusion activity of *phzA1* and *phzA2* promoters of PAO1 and the *lasR* mutant grown in low-phosphate FDS medium. The results shown are the means from three biological replicates, and error bars denote the standard deviations. Asterisks indicate statistical significance by *t* test (*, $P < 0.05$; ****, $P < 0.0001$). n.s., nonsignificant.

tem (24, 25), (ii) the Rhl system is a positive regulator of the *phz* operons (20), and (iii) the Pqs system regulates pyocyanin production through PqsE, acting on RhlR activity (5).

Surprisingly, in low-phosphate FDS medium, pyocyanin was overproduced not only by the wild-type strain but also by the *lasR* mutant at practically the same levels (Fig. 2). This suggests that under low-phosphate conditions LasR is dispensable for pyocyanin production, while RhlR is still indispensable. The *pqsR* mutant was still able to produce about 50% of the pyocyanin produced by PAO1 (Fig. 2). This result confirms the importance of this system in pyocyanin production but suggests that it is not indispensable, since pyocyanin production is still possible in its absence.

Additionally, to assess the relevance of PhoB regulation, the *phoB* mutant was also evaluated. In FDS+ it produced levels similar to those of the PAO1 strain, as expected, since PhoB is inactive in this medium (Fig. S3). However, in FDS the *phoB* mutant was unable to overproduce pyocyanin (Fig. 2), evidencing the relevance of PhoB under this condition. In line with this, the *lasR-phoB* double mutant showed an affected production in FDS+, comparable to that of the *lasR* single mutant, since in this medium LasR is relevant but PhoB is inactive. On the other hand, in FDS, the production of the *lasR-phoB* mutant was similar to that of the *phoB* single mutant, since, under this condition, LasR is irrelevant and PhoB is active (Fig. 2).

Taken together, these results support the hypothesis that, under low-phosphate conditions, PhoB regulates RhlR, which becomes the main QS regulator for pyocyanin production, while LasR is dispensable.

The expression of the *phz1* operon is mainly responsible for LasR-independent pyocyanin production. To determine whether both *phz* operons contribute to LasR-independent pyocyanin production, transcriptional *lacZ* fusions to both promoter regions were evaluated in the *lasR* mutant grown in FDS and FDS+ media. In FDS+, no detectable activation of either operon was observed, correlating with the absence of pyocyanin production (Fig. 2). In FDS, the *phz1* operon promoter showed high transcription levels, while the *phz2* operon promoter showed only a slight activity (Fig. 3). These results suggest that the LasR-independent pyocyanin production relies on transcription of the *phz1* operon, which is directly regulated by RhlR (20), so its induction could be explained by RhlR overexpression in this medium.

LasR is globally dispensable under a low-phosphate condition. Since LasR turned out to be dispensable for pyocyanin production when the PAO1 strain was cultivated in low-phosphate FDS medium, we wondered whether this also applied to other QS-dependent phenotypes. To test this with another well-characterized RhlR-dependent phenotype (26), we visualized rhamnolipid production by TLC of the *lasR* mutant grown in FDS and FDS+ media. While in FDS+ medium rhamnolipids were not detected, in FDS they were produced at a lower but significant level in the *lasR*

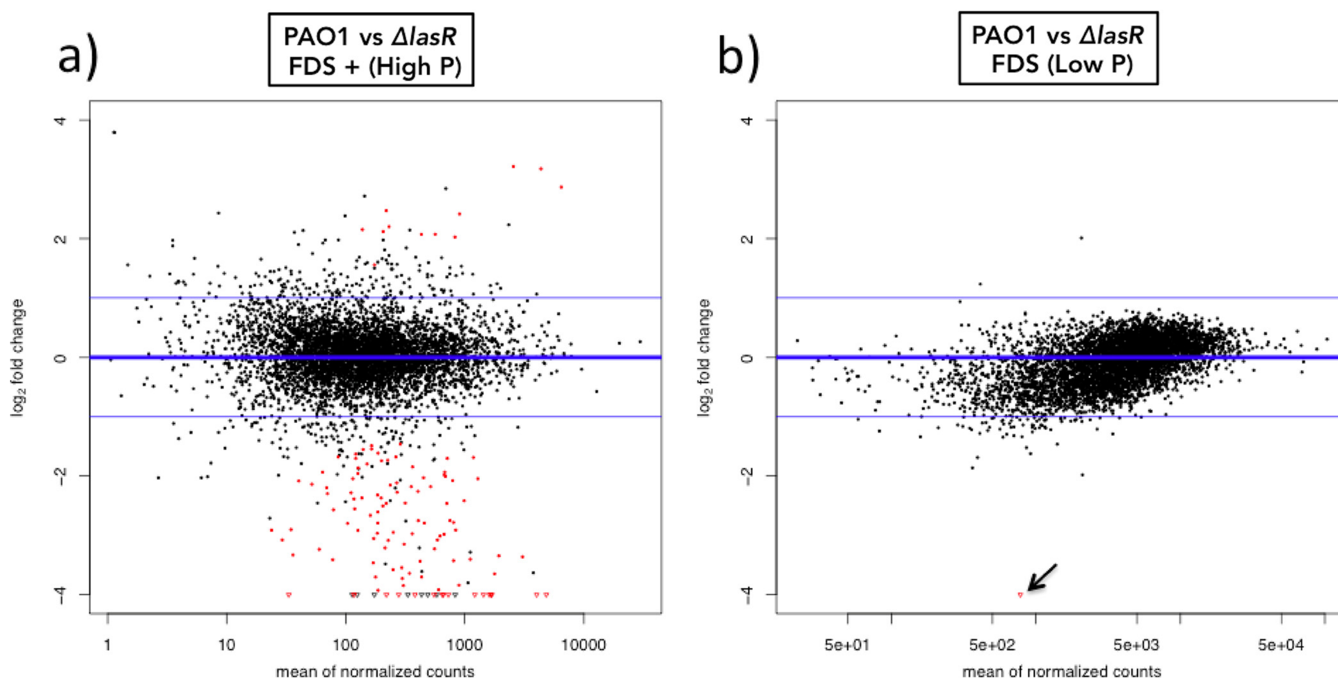

FIG 4 LasR transcriptional activity is not significant under low-phosphate conditions. MA plots based on DESeq2 differentially expressed genes. This plot represents each gene with a dot. The *x* axis is the average expression over the mean of normalized counts, and the *y* axis is the $\log_2$ fold change between conditions. Genes declared as differentially expressed are highlighted in red. (a) Comparison of gene expression between PAO1 and the *lasR* mutant grown in high-phosphate FDS+ medium. (b) Comparison of gene expression levels between PAO1 and *lasR* mutant grown in low-phosphate FDS medium. The point indicated with the arrow represents *lasR*, which naturally was the only statistically differentially expressed gene.

mutant compared to the level in the wild-type strain (Fig. S6). This led us to hypothesize that LasR dispensability under a low-phosphate condition was not restricted to pyocyanin production and could be a global effect.

To assess this question, a transcriptome sequencing (RNA-seq) analysis of the wild-type and *lasR* mutant strains grown in FDS and FDS+ media was performed. Comparison between the transcriptome of both strains in FDS+ medium showed 106 downregulated genes in the *lasR* mutant (Fig. 4a), which includes genes that are well-known targets of LasR positive regulation, such as *lasI* and *rhlR*.

We also found that the main contribution of LasR is as a transcriptional activator, since there are only 12 genes that are negatively regulated by LasR (Table 1 and Table S2); 4 of these 12 genes belong to the same efflux system (*mexAB-oprM* operon and *mexR*). The regulation of MexA by LasR has also been observed in a recent report (27). It has been suggested that 3O-C12-HSL is a substrate of this MexAB-OprM efflux pump (28). Thus, the biological significance of the LasR negative regulation of this pump could be in maintaining high concentrations of this signal molecule to favor Las system activity.

The comparison between PAO1 and its *lasR* mutant in FDS medium, surprisingly, showed no statistically significant differentially expressed genes (Fig. 4b). This conclusion is sustained by three statistical programs used to analyze the results (DEseq, edgeR, and limma-vomm) showing log fold change correlation values of >0.9956 and correlation *P* values of >0.9356 between each comparison method. These results show that LasR becomes dispensable under a low-phosphate condition not only for pyocyanin and rhamnolipid production but also at a global level. Thus, we can conclude that in the PAO1 type strain, which is representative of the canonical QS response, under low-phosphate conditions the classical hierarchy is not conserved, since expression of QS-dependent genes becomes LasR independent and solely dependent on RhlR.

Elastase production under a low-phosphate condition is LasR independent. To assess the global LasR dispensability in a well-characterized LasR-dependent

TABLE 1 Main differentially expressed genes in the *lasR* mutant compared to PAO1[a]

Locus and regulation status in *lasR* mutant	Gene	Log_2 fold change	*P* value
Downregulated			
PA1900	*phzB*	−6.4759458	4.60E−33
PA2515	*xylL*	−6.177013216	1.22E−28
PA2512	*Anta*	−5.631837667	2.65E−36
PA2300	*chiC*	−5.390440529	1.30E−28
PA2069	Probable carbamoyl transferase	−5.212351705	5.29E−30
PA1432	*lasI*	−5.019915647	1.92E−30
PA2514	*antC*	−4.904814066	4.58E−20
PA1899	*phzA*	−4.898585114	2.63E−20
PA2513	*antB*	−4.616625506	4.17E−19
PA4206	*mexH*	−4.492450527	3.20E−19
PA3479	*rhlA*	−4.384775865	8.20E−14
PA3724	*lasB*	−3.396848792	1.85E−14
PA3477	*rhlR*	−3.341738031	3.72E−15
PA2591	*vqsR*	−2.744781033	7.81E−07
PA3476	*rhlI*	−2.459061252	4.13E−06
PA1431	*rsaL*	−2.363228919	6.20E−07
Upregulated			
PA0090	*clpV1*	2.075320352	0.000953813
PA0424	*mexR*	2.077265258	8.33E−06
PA0078	*tssL1*	2.127012507	0.000530181
PA4918	*pcnA*	2.15701244	6.36E−06
PA0091	*vgrG1*	2.202895755	0.000525718
PA0070	*tagQ1*	2.417410024	0.000159362
PA1634	*kdpB*	2.473555242	0.000660378
PA0426	*mexB*	2.869241451	1.30E−12
PA0427	*oprM*	3.182374754	2.99E−15
PA0425	*mexA*	3.219524718	7.30E−15

[a]The 10 most upregulated and downregulated genes are shown. Shaded lines represent additional loci that are important, well-characterized LasR-dependent genes. The values shown correspond to DEseq2 analysis. No gene was statistically differentially expressed compared to levels in low-phosphate FDS medium.

phenotype, we measured the LasB elastase activity in both media. In FDS+, the activity was very low and the absence of either *lasR* or *rhlR* affected elastase activity (Fig. 5a). This was expected, since it has been reported that both regulators partially activate *lasB* expression in rich medium (9). Nevertheless, in FDS, this phenotype becomes LasR independent, and it depends exclusively on RhlR (Fig. 5b). Furthermore, the absence of *phoB* only affected this phenotype in FDS medium, as under this condition it is active and positively regulates *rhlR* expression. These results confirm the dispensability of

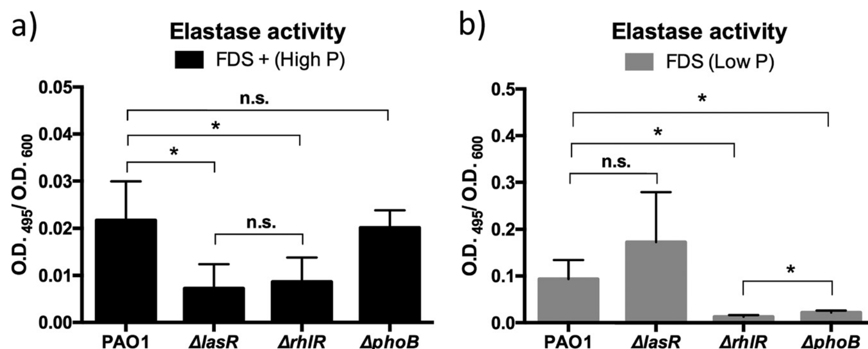

FIG 5 Elastase activity of PAO1 and its derived mutants. Quantification of elastase activity by PAO1 and its isogenic *lasR*, *rhlR*, and *phoB* mutants are shown. (a) FDS+ medium. (b) FDS medium. The results shown are the means from three biological replicates, and error bars denote the standard deviations. Asterisks indicate statistical significance by *t* test (*, $P < 0.05$). n.s., nonsignificant.

LasR under low-phosphate conditions due to the newly established hierarchy where RhlR is at the top of the QS response.

DISCUSSION

QS-dependent regulation of virulence factor production under phosphate limitation. As we have shown here, elastase LasB, an important LasR target, becomes LasR independent under a low-phosphate condition. In line with this, a recent report demonstrated that PhoB can bind the *lasI* promoter and activate its transcription (29). This supports the idea that LasR becomes dispensable even for the regulation of *lasI*, its main target gene. The LasR-independent regulation of *lasI* was previously reported, as it has been suggested that RhlR can induce *lasI* transcription in a heterologous *Escherichia coli* system (8). Nevertheless, under low-phosphate conditions, the biological relevance of the regulation that PhoB exerts on *lasI* might not have a great impact, since the concentration of 3O-C12-HSL is only slightly affected (29).

The synthesis of pyocyanin depends on the expression of enzymes encoded by the redundant operons *phz1* and *phz2*, which synthesize the pyocyanin precursor PCA, subsequently transformed by PhzM and PhzS to pyocyanin (21). The classical QS control of pyocyanin synthesis implies that LasR coupled with 3O-C12-HSL activates *rhlR* and *rhlI* transcription, and then RhlR/C4-HSL positively regulates both *phz* operons (30), although only the *phz1* operon has a predicted *las-rhl* box (31).

We have shown here that under phosphate-limited conditions, RhlR-dependent, LasR-independent pyocyanin production occurs, and this phenomenon depends mainly on the *phz1* operon, which shows high levels of transcription in the *lasR* mutant, while the *phz2* operon shows only a slight activation, even lower than the activity shown in the PAO1 wild-type strain. This difference could not be observed in the RNA-seq analysis due to the inability to distinguish between the transcripts coming from either operon due to the high nucleotide identity they share (>98%) (19).

RhlR directly activates transcription of the *phz1* operon, and there are reports that other transcriptional factors also act on the *phzA1* promoter. In this respect, it has been shown recently that PhoB can directly bind to the *phz1* operon (29). In addition, another element that participates in the regulation of the *phz* operons is RsaL, which has been shown to act as a negative regulator of the *phz1* operon (32) and a positive regulator of the *phz2* operon. Thus, the LasR-independent overexpression of pyocyanin under a low-phosphate condition can be caused not only by RhlR but also by PhoB and RsaL, as they can play a role in the regulation of *phz1*. However, our results suggest that the RhlR-dependent, LasR-independent induction under low-phosphate conditions of other QS-regulated phenotypes, such as rhamnolipid production, as well as the global dispensability of LasR under this stress condition, is mainly due to the LasR-independent *rhlR* expression.

Taken together, these results highlight the need for a new model for *P. aeruginosa* QS-dependent regulation of virulence factor production in phosphate limitation depending only on the Rhl system.

The flexibility of the QS hierarchy. *P. aeruginosa* is considered a major nosocomial pathogen; a better understanding of its virulence trait production is needed to design new strategies to control infections caused by this bacterium and especially those caused by the frequently isolated antimicrobial-resistant strains. The QS response is an important target for compounds that inhibit *P. aeruginosa* virulence, since it regulates the expression of many of the virulence factors produced by this opportunistic pathogen. The Las system was originally thought to be the main QS regulator and is still considered an important target for quorum-quenching (QQ)-based therapies (33, 34). Nevertheless, different works have provided evidence showing that under certain conditions in laboratory type strains (6–9) and isolates (11, 35–37), the Las system can be less relevant for QS activation. In Table S1 in the supplemental material, we have summarized some of the evidence reported in the literature of the flexible nature of the QS response of the PAO1 type strain, which is the strain used in this work, and PA14, which shows a very similar QS response (4). In these works, all performed under

conditions of phosphate repletion, LasR seems to be important only for the timing of the response, as the activation of QS-dependent phenotypes is affected by LasR at earlier times of growth and is unaffected at later times (8, 9). Nevertheless, in this work, we show that the LasR hierarchy relevance is maintained in the stationary phase in a high-phosphate medium (FDS+), since the absence of LasR has important effects on the phenotype and gene expression. On the other hand, in a low-phosphate medium (FDS), this LasR hierarchy seems to be irrelevant, since in the stationary phase its absence has no effect on the phenotype or on gene expression. These results expose a distinct pattern of how the LasR hierarchy acts and highlight the conclusion of different paths that can lead to LasR dispensability. It is important to note that our conclusions were reached by analyzing the PAO1 strain cultured in FDS+ at a time (18 to 22 h of growth) when the reported hierarchy of LasR as the master regulator is very clear, with cultivation at the same time points in FDS medium that show an RhlR-dependent hierarchy. It is possible that at later time points LasR dispensability could also be apparent even in the FDS+ medium, since at late stationary phase the QS response has been reported to be dependent on RhlR (8).

Occurrence and relevance of *lasR* mutants. An experimental evolution strategy has been used to select PAO1 *lasR* mutant derivatives that were still able to express RhlR-regulated virulence factors in casein broth (which is a high-phosphate condition), suggesting that the Las-independent production of virulence factors that we show to exist under low-phosphate conditions can be selected during the course of an infection (38). It is also possible that *lasR* mutants have increased antibiotic resistance due to the overexpression of the MexAB-OprM efflux pump and, thus, are positively selected.

It has been recognized that *lasR* mutants are frequently involved in *P. aeruginosa* infections, particularly in CF patients (10). Since LasR is considered the main transcriptional regulator and the first in the QS hierarchy, this phenomenon has opened the question of how it is possible that mutants lacking the main QS regulator can establish an infection. Social cheaters have been proposed as an explanation for this phenomenon (39). Nevertheless, there are several reports showing that *P. aeruginosa lasR* mutants can be virulent *per se* (11, 35, 36, 40), and recently it has been shown that Las-defective, RhlR-proficient *P. aeruginosa* isolates do not constitute a phylogenetic group but can originate from different clades (41).

In this context, the results presented here indicate that under some conditions, such as low phosphate availability, the LasR regulator becomes dispensable because RhlR is the head of the QS regulatory cascade. This can explain the success of *lasR* emergent strains but also demonstrates that LasR dispensability is not exclusive to the atypical strains, since this can occur in the well-studied PAO1 type strain. This is relevant for *P. aeruginosa* health impact, as this stress condition could represent an environment similar to the one that this bacterium faces when establishing an infection, since it has been reported that under certain clinical scenarios, such as patients that have undergone surgeries, the phosphate level is extremely low (14).

To our knowledge, this is the first report showing that under a specific environmental condition, LasR becomes completely dispensable for the expression of the *P. aeruginosa* QS response, which becomes only RhlR dependent; thus, it has a significant scientific value but is also relevant for the design of therapeutic strategies that are based on the inhibition of QS-dependent virulence factor production.

MATERIALS AND METHODS

Microbiological methods. The strains and plasmids used in this work are shown in Table S3 in the supplemental material. In all experiments we used strain MPAO1, provided by the University of Washington, referred to as PAO1. Precultures were grown in LB broth. Antibiotics were used at the following concentrations: for *E. coli*, 200 μg/ml carbenicillin (Cb), 15 μg/ml tetracycline (Tc), and 30 μg/ml streptomycin (Sm); for *P. aeruginosa*, 200 μg/ml Cb, 120 μg/ml Tc, and 200 μg/ml Sm. FDS medium [20 ml/liter glycerol, 10 g/liter DL-alanine, 50 μM iron(III) citrate, 0.1 M Na_2SO_4, 20 mM $MgCl_2$, 0.5 mM K_2HPO_4, pH 7.4] and FDS+ medium (same components, except 4.5 mM K_2HPO_4 was used) were used to analyze phenotypic traits as well as transcriptional and translational fusions and for the RNA extraction.

The FDS and FDS+ phosphate concentrations were set based on the levels used in previous reports (15, 19). Specifically, in FDS the concentration was even lower than that reported to maximize the differences, but the total density obtained in stationary phase, where QS is expressed, was not significantly affected (Fig. S1). Cultures were inoculated with washed cells at an optical density at 600 nm (OD_{600}) of 0.1. The flasks were incubated with continuous shaking (225 rpm) at 37°C. No antibiotics were added to FDS and FDS+ media.

Determination of virulence factor production. Phenotypic traits were analyzed at 24 h of growth. Pyocyanin concentration and elastase activity were measured as reported previously (9). The pyocyanin concentration and elastase activity are divided by the optical density (600 nm) of the culture to avoid the growth difference effect. Statistical significance was determined by Student's *t* test ($P < 0.05$), performed with Prism 6 software from GraphPad. Rhamnolipid thin-layer chromatography was performed as reported previously (9).

DNA manipulation and construction of mutants. Oligonucleotides used in this work are listed in Table S4. DNA was purified and manipulated by using standard techniques (42). All plasmids were routinely maintained in *E. coli* DH5α and were introduced by electroporation into *P. aeruginosa* as described previously (43). The *phoB* deletion was generated by the construction and insertion of the pXPBa plasmid into the PAO1 or Δ*lasR* strain to obtain the Δ*phoB* and Δ*phoB*-Δ*lasR* mutants, respectively. This plasmid insertion was followed by FLP-mediated cassette removal according to the protocol reported by Choi and Schweizer (44). pXPBa plasmid was constructed by cloning the *phoB* upstream and downstream flanking regions, with an apramycin resistance cassette in the middle, into the HindIII site of pEXGm18. The primers 6824 and 6825 were used to obtain the upstream *phoB* flanking region, while 6826 and 6827 were used to obtain the downstream *phoB* flanking region. Primers 9522 and 9523 were used to obtain the apramycin resistance cassette from pIJ773 (45). To complement the *phoB* mutant, the plasmid p20PB was constructed as follows. The structural sequence of *phoB* was obtained using oligonucleotides 3861 and 3862, and this fragment was cloned in pJET1.2/blunt to add restriction sites (XbaI and HindIII). Finally, the *phoB* coding sequence was cloned in pUCP20 in the restriction sites mentioned.

The *pqsR* mutant was generated with the same procedure but using the plasmid pEX-*pqsR*::Apra. This plasmid was constructed by cloning the *pqsR* upstream and downstream flanking regions, with an apramycin resistance cassette in the middle, into the HindIII site of pEXGm18. Oligonucleotides pqsRup and pqsR5Aa were used to obtain the upstream *pqsR* flanking region, while pqsR3Aa and pqsRdown were used to obtain the downstream *pqsR* flanking region.

Construction of transcriptional fusions. The *rhlR′-lux* (pMRRx) and *phoB′-lux* (pMPBx) plasmids were constructed by PCR amplification of each promoter region using the oligonucleotide pairs 1300-1301 and 2814-2815, respectively. The PCR products were cloned in the HindIII-PstI sites of mini-CTX-lux (46). *phzA1′-lacZ* (pLZA1z) and *phzA2′-lacZ* (pLZA2z) were constructed by PCR amplification of each promoter region using the oligonucleotide pairs 607-608 and 613-614, respectively. The PCR products were cloned in the XhoI-HindIII sites and XhoI-BamHI sites, respectively, in pLP170 (47).

Measurement of reporter fusion activity. β-Galactosidase activity of *phzA1′-lacZ*, *phzA2′-lacZ*, and *lasR′-lacZ* was measured using standard protocols (48). The activity of the *lux* transcriptional fusions was measured using cells cultured in flasks under the conditions stated in the text. Aliquots were put in 96-well black plates with clear bottoms, and luminescence measurements were made in the Synergy HT equipment (BioTek). Statistical significance was determined by Student's *t* test ($P < 0.05$) performed with Prism 6 software from GraphPad.

EMSA. The *rhlR* promoter fragment was obtained using primers 1300 and 1301. This fragment contains the four reported promoters and the putative *pho* box. The *phoB* promoter fragment used as a positive control was obtained using primers 2814 and 2815. The negative control was obtained using primers 4602 and 4603, which amplify a fragment within the *pqsD* gene. To express the PhoB protein, the plasmid pETPB was generated. pETPB was constructed by cloning the *phoB* structural gene into the BamHI and HindIII sites of pET21a (Novagene); the *phoB* structural gene was obtained by PCR amplification with oligonucleotides 8057 and 8058. PhoB-His was purified using the nickel-nitrilotriacetic acid spin kit (Qiagen) by following the provider's instructions. The DNA fragment and PhoB protein were incubated by following a protocol and with a buffer previously reported (18) using 80 ng of DNA and 0, 10, and 15 μM PhoB. The reaction samples were incubated at room temperature for 30 min. Electrophoresis was performed using 6% polyacrylamide native gels in 0.5× Tris-buffered saline buffer at 10 mA for 2 h at 4°C. The gels were stained using ethidium bromide and visualized with UV light.

RNA extraction, sequencing, and analysis. For RNA extraction, three independent cultures of strain PAO1 and its *lasR* mutant derivative were grown in FDS and FDS+ media for 22 h. Cells were harvested by centrifugation, and RNA isolation was carried out using the TRIzol (Invitrogen)-based protocol. Total nucleic acids were treated with RNase-free DNase I (Thermo Fisher) until no PCR product was detected after 30 cycles using primers 8598 and 2492. RNA integrity was analyzed by gel electrophoresis. rRNA was depleted by subtractive hybridization with the RiboMinus transcriptome isolation kit-bacteria (Thermo Fisher). mRNA libraries for each of the three independent cultures of each strain were constructed using the TrueSeq stranded mRNA library preparation kit (Illumina) and sequenced in Illumina NextSeq 500 equipment with up to 10^7 paired reads/sample. Differential expression was evaluated with packages DESeq2 1.22.1 (49), edgeR 3.24.1 (50), and limma-vomm 3.38.3 (51). Genes were considered differentially expressed if they showed a $\log_2$ fold change above 1.5 and a false discovery rate-controlled *P* value below 0.05 (52).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 2.2 MB.

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