Calcium-Regulated Protein CarP Responds to Multiple Host Signals and Mediates Regulation of *Pseudomonas aeruginosa* Virulence by Calcium

Michelle King,^a Aya Kubo,^a Leah Kafer,^a Reygan Braga,^a Daniel McLeod,^a Sharmily Khanam,^a Tyrrell Conway,^a
Marianna A. Patrauchan^a

^aDepartment of Microbiology and Molecular Genetics, Oklahoma State University, Stillwater, Oklahoma, USA

ABSTRACT *Pseudomonas aeruginosa* is an opportunistic pathogen causing life-threatening infections. Previously, we showed that elevated calcium (Ca^{2+}) levels increase the production of virulence factors in *P. aeruginosa*. In an effort to characterize the Ca^{2+} regulatory network, we identified a Ca^{2+} -regulated β -propeller protein, CarP, and showed that expression of the encoding gene is controlled by the Ca^{2+} -regulated two-component system CarSR. Here, by using a *Galleria mellonella* model, we showed that CarP plays a role in regulating *P. aeruginosa* virulence. By using transcriptome sequencing (RNA-Seq), reverse transcription (RT)-PCR, quantitative RT-PCR (RT-qPCR), and promoter fusions, we determined that *carP* is transcribed into at least two transcripts and regulated by several bacterial and host factors. The transcription of *carP* is elevated in response to Ca^{2+} in *P. aeruginosa* cystic fibrosis isolates and PAO1 laboratory strain. Elevated Fe^{2+} also induces *carP*. The simultaneous addition of Ca^{2+} and Fe^{2+} increased the *carP* promoter activity synergistically, which requires the presence of CarR. *In silico* analysis of the intergenic sequence upstream of *carP* predicted recognition sites of RhlR/LasR, OxyR, and LexA, suggesting regulation by quorum sensing (QS) and oxidative stress. In agreement, the *carP* promoter was activated in response to stationary-phase PAO1 supernatant and required the presence of elevated Ca^{2+} and CarR but remained silent in the triple mutant lacking *rhII*, *lasI*, and *pqsA* synthases. We also showed that *carP* transcription is regulated by oxidative stress and that CarP contributes to *P. aeruginosa* Ca^{2+} -dependent H_2O_2 tolerance. The multifactorial regulation of *carP* suggests that CarP plays an important role in *P. aeruginosa* adaptations to host environments.

IMPORTANCE *P. aeruginosa* is a human pathogen causing life-threatening infections. It is particularly notorious for its ability to adapt to diverse environments within the host. Understanding the signals and the signaling pathways enabling *P. aeruginosa* adaptation is imperative for developing effective therapies to treat infections caused by this organism. One host signal of particular importance is calcium. Previously, we identified a component of the *P. aeruginosa* calcium-signaling network, CarP, whose expression is induced by elevated levels of calcium. Here, we show that *carP* plays an important role in *P. aeruginosa* virulence and is upregulated in *P. aeruginosa* strains isolated from sputa of patients with cystic fibrosis. We also identified several bacterial and host factors that regulate the transcription of *carP*. Such multifactorial regulation highlights the interconnectedness between regulatory circuits and, together with the pleiotropic effect of CarP on virulence, suggests the importance of this protein in *P. aeruginosa* adaptations to the host.

KEYWORDS quorum sensing, transcriptional regulation, iron, oxidative stress, hydrogen peroxide, promoter activity, *Galleria mellonella*, iron regulation

Citation King M, Kubo A, Kafer L, Braga R, McLeod D, Khanam S, Conway T, Patrauchan MA. 2021. Calcium-regulated protein CarP responds to multiple host signals and mediates regulation of *Pseudomonas aeruginosa* virulence by calcium. Appl Environ Microbiol 87:e00061-21. <https://doi.org/10.1128/AEM.00061-21>.

Editor Harold L. Drake, University of Bayreuth

Copyright © 2021 American Society for Microbiology. All Rights Reserved.

Address correspondence to Marianna A. Patrauchan, m.patrauchan@okstate.edu.

Received 8 January 2021

Accepted 27 February 2021

Accepted manuscript posted online 5 March 2021

Published 27 April 2021

Pseudomonas aeruginosa is a ubiquitous Gram-negative opportunistic pathogen which is well known for infecting the lungs of patients with cystic fibrosis (CF) and burn wounds and for causing a variety of other infections, including keratitis, sepsis, and endocarditis (1–3). *P. aeruginosa* pathogenicity is defined by the diverse metabolic capabilities, extensive repertoire of virulence factors, biofilm formation, and resistance to host defenses of the pathogen (4, 5). The success of *P. aeruginosa* as a pathogen and the failure of antibiotic therapies to eliminate *P. aeruginosa* infections are thought to be due in part to its physiological plasticity, enabling the adaptability and resistance of this organism (reviewed in references 6 and 7). Current research efforts are focused on deciphering the intricate regulation of *P. aeruginosa* virulence and adaptive mechanisms in response to a multitude of host factors (8–11).

Several host factors are known to control the expression of virulence factors in *P. aeruginosa*. One of the most influential factors is iron, which is essential for both bacterial and host cells. Therefore, its availability is a continuous battle between host and invading bacteria. In order to compete for iron, bacteria produce multiple systems for sensing and sequestering the ion from the host environment. They include high-affinity siderophores, pyoverdine and pyochelin (12, 13). Reflecting the importance of iron, several virulence-related genes in *P. aeruginosa* are differentially regulated by iron availability, such as those encoding exotoxin A (14) and alkaline and PrpL proteases (15, 16). Iron also regulates the production of one of *P. aeruginosa* three quorum sensing (QS) systems, PQS, which has a secondary role as an iron scavenger (17). The availability of iron plays an important role in biofilm development, maturation, and dispersal (18–21). Other ions, whose homeostasis is tightly regulated by the host and has a major impact on *P. aeruginosa* virulence, include magnesium (22–24), zinc (25), and calcium. The role of calcium (Ca^{2+}) in regulating bacterial responses is outlined below. Besides ions, a number of small molecules produced by the host immune system have shown a regulatory impact on the expression of *P. aeruginosa* virulence factors. One of these molecules is H_2O_2 , generated by neutrophils and macrophages in response to infections as a key component of the pathogen-killing mechanism (26). Exposing *P. aeruginosa* to H_2O_2 leads to the development of an oxidative stress response that is orchestrated by the global regulator OxyR and includes production of pyocyanin, pyoverdine, exotoxins, and rhamnolipid (27–29).

Host Ca^{2+} plays both structural and signaling roles and is involved in regulating many essential processes, including gene transcription, cell apoptosis, and the immune response (reviewed in references 30 to 32). The intracellular levels of this ion are tightly regulated, and even minute disruptions in its cellular homeostasis may lead to or be caused by various human diseases. For example, in cystic fibrosis (CF), a dysfunctional cystic fibrosis transmembrane conductance regulator (CFTR) chloride pump causes an imbalance in ion homeostasis, affecting the levels of Ca^{2+} , iron, chlorine, sodium, and magnesium (33, 34). As a result, the levels of Ca^{2+} are increased in the lungs and nasal fluids of these individuals (35, 36). Such changes may generate cues that modulate the physiology and virulence of invading bacterial pathogens, including *P. aeruginosa* (37, 38). On the other hand, *P. aeruginosa* is able to disrupt Ca^{2+} homeostasis in a host by secreting QS and pyocyanin molecules, which leads to apoptosis (24, 39, 40). Overall, this knowledge reveals a significant role of Ca^{2+} in mediating host-pathogen interactions of *P. aeruginosa* and highlights the importance of characterizing the *P. aeruginosa* Ca^{2+} regulatory network, whose members may present novel targets for therapeutic treatments.

Previously, our group showed that Ca^{2+} positively regulates the production of multiple virulence factors in *P. aeruginosa*, including pyocyanin (41), secreted proteases (24, 42), pyoverdine, and rhamnolipid (M. Guragain, S. Khanam, M. King, A. Kubo, and M. Patrauchan, unpublished data). We have also shown that elevated Ca^{2+} enhances efflux pump-mediated tobramycin resistance, swarming motility, and biofilm formation in the pathogen (41–43). These virulence factors contribute to the ability of *P. aeruginosa* to colonize a host, evade the host immune system, and cause host cell damage

FIG 1 Role of *carP* in *P. aeruginosa* PAO1 virulence in *G. mellonella*. Survival of *G. mellonella* 24 h postinjection with 1 to 9 CFU of PAO1, $\Delta carP$, and $\Delta carP::carP$ strains grown at no (gray) or 10 mM (black) $CaCl_2$. Every point represents an individual experiment that included 10 worms for each condition and 5 control worms. Cells were grown in either BMM or LB. The data for the cultures grown in LB are indicated by larger symbols in circles. Statistical analyses (one-tailed non-paired *t* test with Welch's correction) was performed by using GraphPad Prism. The asterisk represents a *P* of <0.05.

(44–46). To characterize the molecular mechanisms of Ca^{2+} regulation in *P. aeruginosa*, we identified several components of the Ca^{2+} regulatory network (41, 47) and showed that intracellular Ca^{2+} homeostasis plays a key role in this regulation (Guragain et al., unpublished). We also identified a two-component system, CarSR, which is positively regulated by elevated Ca^{2+} and controls Ca^{2+} -dependent transcription of at least two genes, designated *carO* and *carP* (48). Interestingly, a homologous two-component system, BqsSR, in *P. aeruginosa* PA14 has been shown to regulate a *carP* homolog (PA14_04270) in response to elevated Fe^{2+} , but not Ca^{2+} (49, 50). This suggested that *carP* transcription can also be regulated by Fe^{2+} and thus be adjusted in the host environment during infection. *In silico* analyses predicted that *carP* encodes a β -propeller protein with a phytase-like domain. Phytases catalyze the hydrolysis of phytic acid (inositol hexakisphosphate) and release inorganic phosphate. We showed that *carP* is highly conserved in pseudomonads and that its disruption significantly reduced Ca^{2+} induction of pyocyanin production, tobramycin resistance, and swarming motility. Further, we showed that CarP is required for maintaining the basal level of intracellular Ca^{2+} , which may explain the role of this protein in *P. aeruginosa* tolerance to elevated extracellular Ca^{2+} (48).

Considering the predictions and the nature of CarP-dependent processes in *P. aeruginosa*, we hypothesized that the protein is involved in the interactions between the pathogen and its host. In this study, we tested the role of CarP in *P. aeruginosa* virulence by using a *Galleria mellonella* model. We also characterized the transcriptional regulation of *carP* in response to Ca^{2+} and other host factors by using quantitative reverse transcription-PCR (RT-qPCR) and monitoring luminescence produced by *carP* promoter fusion to a promoterless *luxCDABE* operon. Upon discovering the transcriptional response of *carP* to UV light and iron, we determined the role of the gene in *P. aeruginosa* resistance to H_2O_2 . Characterizing the physiological roles of the putative phytase and its transcriptional regulation in response to multiple bacterial and host factors provides a strong indication of the importance of this protein in *P. aeruginosa* virulence and interactions with the host.

RESULTS

CarP plays an important role during infection. To study the role of CarP in *P. aeruginosa* virulence *in vivo*, we used a *G. mellonella* infection model. *G. mellonella* larvae were injected with wild-type (WT) PAO1, $\Delta carP$, and $\Delta carP::carP$ strains grown with or without 10 mM $CaCl_2$. Death of the larvae was monitored for 24 h, which was selected to exclude the effect of cocooning that begins soon after. The data from at least four individual experiments, each using 10 worms per condition and 5 control worms, are shown in Fig. 1. *G. mellonella* injected with PAO1 grown in biofilm minimal medium (BMM) without added Ca^{2+} reached about 50% survival (Fig. 1, gray squares). A similar survival level was observed upon injection with the $\Delta carP$ mutant (gray triangles) or

FIG 2 Expression analysis of the *carP* mutant in PAO1 and CF clinical strains by RT-qPCR. The relative mRNA levels of the *carP* mutant were normalized to those of *rpoD* by using the $2^{-\Delta\Delta C_t}$ method ($n=3$) with standard errors of the mean. Averages and standard errors of results from three biological replicates are shown. Both two-factor analysis of variance (ANOVA) ($P < 0.05$ for all groups) and Student's *t* test were performed with *P* values of <0.1 (*) and <0.05 (**).

the $\Delta carP::carP$ complemented strain (gray circles) grown without added Ca^{2+} . In contrast, in the presence of 10 mM $CaCl_2$, survival of the wild type decreased to about 30% (black squares) and further dropped to below 20% in the larvae infected with the mutant (black triangles). Complementing the mutant recovered the WT phenotype (black circles). To test whether these results are medium dependent, the cultures were grown in rich Luria-Bertani (LB) medium instead of BMM. The medium switch did not alter the results (circled larger symbols). These data indicate that growth in the presence of Ca^{2+} slightly but consistently increases the ability of PAO1 to kill *G. melonella* larvae and that deletion of *carP* leads to an additional 1.6-fold increase in virulence of the bacterial cells grown at 10 mM $CaCl_2$. This suggests that *carP* plays a role in regulating *P. aeruginosa* virulence in a Ca^{2+} -dependent but medium-independent manner.

The comparison of *P. aeruginosa* transcriptional profiles during CF and soft tissue infections to those during *in vitro* culturing reported previously (51) showed that *carP* transcription increased at least 2-fold during infections. Further, according to the genomic data repository GEO (52), *carP* transcription was induced during growth in medium mimicking CF lung sputum (accession no. [GDS4250](#)), in *in vivo* growth in CF sputum ([GDS2869](#)), and in response to airway epithelia ([GDS2502](#)). To test *carP* transcription in CF clinical isolates and its response to elevated Ca^{2+} , we performed RT-qPCR in PAO1 (as a reference strain) and two CF sputum isolates (22014B_A1 and 31314A_B1) grown at different levels of Ca^{2+} (Fig. 2). First, in agreement with the reported study (51), although incubated *in vitro*, both CF isolates showed a slight but statistically significant increase in *carP* transcript abundance compared to that in strain PAO1. Second, with the addition of Ca^{2+} , *carP* transcription was increased in all three strains. The highest increase was detected in 31314A_A1 (8.9-fold), followed by PAO1 and 22014B_A1 (6.9- and 2.2-fold, respectively). The clinical isolates were collected from patients 31314A and 22014B, who were of different ages, 28 and 39 years old, respectively. It is possible that the differences in Ca^{2+} responses are due to the age, where the younger patient 31314A showed a significantly higher increase in *carP* transcription in response to elevated Ca^{2+} than the older patient, 22014B, thus indicating the potential importance of *carP* during the earlier stages of infection. However, a more expanded study is required to support this idea.

***In silico* analyses predicted multiple regulatory elements within the intergenic region upstream of *carP*.** Based on the observed role of *carP* in *P. aeruginosa* virulence and the reported transcriptional induction during infection and under infection-related conditions (51, 52), we hypothesized that the gene is differentially regulated in response to host factors commonly present during infections. To test this, we first performed an *in silico* analysis of the 326-bp intergenic region (IR) upstream of *carP* and predicted binding sites for RhlR/LasR (Virtual Footprint score, 9.98) and LexA (Virtual Footprint score, 9) and two putative canonical -35 and -10 promoter sequences (each) (Fig. 3A). The LexA binding region is located right upstream of the -35 element

A. Analysis of the regulatory region of *carP*

B. RT-PCR

C. RT-qPCR

FIG 3 Analyses of *carP* transcription and regulation. (A) Top, schematic representation of *in silico* predictions of the *carP* regulatory regions. The boxes represent the putative stem-loop (PSL), binding sites for OxyR, RhIR/LasR, and LexA, and *carP*. The -10 and -35 regions are represented by gray diamonds. Below the schematic representation, aligned with the coordinates, are the screen shots of the high-resolution Cappable RNA-Seq transcript reads on the plus strand obtained for cells grown with (5 mM) or without CaCl₂. The screen shots are shown to illustrate the identification of TSS. The abundances of *carP* transcripts were normalized and statistically analyzed and are presented in the text. The predicted transcriptional start sites (TSS1, -2, and -3) are indicated by arrowheads. At the bottom of this panel, the amplification regions for RT-PCR (T1, T2, and T3) and RT-qPCR (T1', T2', and T3') are indicated by black lines. The constructed promoter fusions C1, C2, and C3 for promoter activity assays are represented by arrows. Each number refers to the position relative to the first nucleotide of the *carP* open reading frame (ORF), defined as the +1 position. (B) Agarose gel electrophoresis of RT-PCR products amplified from the cDNA obtained from PAO1 grown in BMM with (10 mM) (+) or without (-) CaCl₂. T1, T2, and T3 indicate the PCR products designed as described for panel A. "Up" indicates the PCR

(Continued on next page)

near the 5' end of *carP*, indicating that it may serve as a transcriptional repressor, which, for example, was observed for the integrase gene *intl* (53). We also predicted the earlier reported (28) OxyR binding sequence, indicating its role as a transcriptional regulator of *carP*. These predictions suggest that *carP* transcription is controlled by quorum sensing (QS) via regulators RhlR/LasR and oxidative stress coordinated by OxyR. Expression of *carP* may also be a part of the SOS response, regulated by LexA. The corresponding regulatory signals commonly occur during infections in a host (29, 54–56).

RNA-Seq, RT-PCR, and RT-qPCR revealed *carP* multiple TSS. To characterize *carP* transcriptional start sites (TSS), we generated and sequenced a Cappable transcriptome sequencing (RNA-Seq) library for PAO1 grown in the presence (5 mM) or absence of Ca^{2+} . This approach enables mapping of TSS at single-nucleotide resolution (57). The RNA-Seq analyses revealed three putative TSS located at positions +7 (TSS1), –49 (TSS2), and –288 (TSS3) relative to the first nucleotide (+1) of the *carP* sequence (Fig. 3A), which indicates the potential for three different transcripts. To further investigate this, we performed reverse transcription-PCR (RT-PCR) using total RNA extracted from PAO1 cells grown without or with 10 mM Ca^{2+} , at which, according to our earlier microarray and RT-qPCR analyses, the transcription of *carP* increased by 4- to 13-fold (48). Three forward primers were designed to bind the transcripts at positions +22 (T1), –30 (T2), and –187 (T3) and were used in combination with the *carP* 3' reverse primer (Fig. 3A; Table 1). For a negative control, we used a forward primer binding to the region upstream of the TSS3 (–319) indicated as “up” (Fig. 3B) with the same 3' reverse primer. According to the RNA-Seq data, this region was not transcribed. All three expected PCR products with lengths of 0.94 kb (T1), 1.0 kb (T2), and 1.2 kb (T3) were detected when the cDNA (Fig. 3B), but not the RNA (see Fig. S1 in the supplemental material), was used as a template. These data support the transcription of the longest transcript with TSS3. However, the amplified shorter T1 and T2 fragments may be a mixture with the overlapping T2-T3 and T3 fragments, respectively. With the goal of distinguishing the transcripts based on their differential regulation by Ca^{2+} , we performed RT-qPCR and quantified the abundance of each transcript in the presence or absence of Ca^{2+} . The qPCR primer design is shown in Fig. 3A, and the results are summarized in Fig. 3C. While no difference was observed in T2' and T3' expression levels upon Ca^{2+} addition, the abundance of T1' increased by 2.2-fold in response to Ca^{2+} . Even though the primers for T1' and T2' may still amplify a mixture of overlapping transcripts, considering the observed differences in their abundances, we conclude that TSS1 and TSS3 transcripts were detected. Together with the RNA-Seq-based expression analyses, which showed that the abundance of the TSS1 and TSS2 transcripts increased in response to Ca^{2+} by 2- and 3-fold (false discovery rate [FDR] < 0.05, P < 0.05), respectively, these results suggest that *carP* is transcribed from at least two transcriptional starts, TSS1 and TSS3. The TSS3 transcript appears to not respond to Ca^{2+} , whereas the TSS1 transcript is induced by Ca^{2+} . Transcription from TSS2, although possibly regulated by Ca^{2+} , requires further studies. We also predicted a putative stem-loop (PSL) located at positions –289 to –215 (i.e., [–289, –215]). This 75-bp region was predicted to have a stable secondary structure fold, which may enable posttranscriptional regulation.

Considering the predicted binding sites for RhlR/LasR, OxyR, and LexA, we hypothesized that the 332-bp IR includes multiple regulatory elements adjusting *carP* transcrip-

FIG 3 Legend (Continued)

product corresponding to the [–319, +966] fragment. The 16S rRNA gene (16S) was used as an internal control, and genomic DNA (gDNA) was used as a positive control for each PCR amplification. To test for gDNA contamination, PCR was performed for each primer set in the RNA samples prior to cDNA synthesis (see Fig. S1 in the supplemental material). RT-PCR was repeated three times using independently isolated RNA. All primer sequences are provided in Table 1. (C) RT-qPCR using RNA obtained from PAO1 grown in BMM with (10 mM) or without CaCl_2 . T1', T2', and T3' represent the regions shown in panel A. The relative mRNA levels were normalized to those of *rpoD* using the $2^{-\Delta\Delta\text{Ct}}$ method, and the fold difference was calculated versus T3 at 0 mM Ca^{2+} . Median averages and standard errors from three biological replicates are shown. Both two-factor ANOVA (P < 0.05 for all groups) and Student's t test with a P of < 0.05 (*) were performed. ns, not significant (indicates Student's t test with a P of > 0.05).

TABLE 1 Strains, plasmids, and oligonucleotides used in this study

Strain, plasmid, or primer	Description or sequence	Reference or source
<i>P. aeruginosa</i> strains		
PAO1	Wild type strain, Alg [−]	111
<i>carP</i> ::Tn5 mutant	PA0327-B11::IS <i>phoA</i> /hah	71
Δ <i>carP</i> mutant	In PAO1 background	This study
Δ <i>lasI</i> Δ <i>rhII</i> Δ <i>pqsA</i> mutant	In PAO1 background	112
Δ <i>carR</i> :Gm ^r mutant	In PAO1 background	48
Δ <i>calC</i> mutant	In PAO1 background	Guragain et al., unpublished
Δ <i>carP</i> <i>attB</i> :: <i>carP</i> strain	<i>carP</i> complementation strain	This study
22014B_A1	CF sputum isolate (patient age, 39 yrs)	73
31314A_A1	CF sputum isolate (patient age, 28 yrs)	73
Plasmids		
pMS402	Promoterless <i>luxCDABE</i>	113
pMCP1	pMS402 with the −326 to +1 C1 region for promoter assay (Fig. 3A)	This study
pMCP2	pMS402 with the −326 to +36 C2 region for promoter assay (Fig. 3A)	This study
pMCP3	pMS402 with the −212 to +36 C3 region for promoter assay (Fig. 3A)	This study
Primers		
<i>carP</i> _up_F	TGCGCACCCGTGGAAATTAATTAAGGTACCGAATTCGGCAGGCCTTCCATGTGTTC	This study
<i>carP</i> _up_R	GACGCTCTGAGCATAGAGCCTGTACGCTG	This study
<i>carP</i> _down_F	GCTCTATGCT CAGGAGCGTCCCCTGGCC	This study
<i>carP</i> _down_R	TTATACGAGCCGGAAGCATAAATGTAAAGCAAGCTTAGCGTCTGTCTGGGCGACAC	This study
−326_Xho1 5'	F_TAAGCACTCGAGGCTGAATTCATGCA	This study
+1_BamH1 3'	R_TGCTTAGGATCCAGCATAGAGCCTGTA	This study
+36_BamH1 3'	R_AGAGAGGGATCCGGAAAACGGCTCGGGAG	This study
−212_Xho1 5'	F_GAGAGACTCGAGTGCGGCCAATAAGGGATC	This study
R1F	AGGCCATCGAGTACA	This study
R5F	CGTTTCCATACAGCGTACA	This study
R4F	TCTACGTGATCACCGACG	This study
R4R	GTTCTACGTCTTCAGCAAG	This study
<i>carPDS</i> -F	GCTCTATGCTCAGGAGCGTCCCCTGGCC	This study
<i>carPDS</i> -R	TTATACGAGCCGGAAGCATAAATGTAAAGCAAGCTTAGCGTCTGTCTGGGCGACAC	This study
PA0576_F1	GGGCGAAGAAGGAATGTGTC	114
PA0576_R1	CAGGTGGCGTAGGTGGAGAA	114
16S-533-F	GTGCCAGCAGCCGCGGTAA	115
16S-1100-R	AGGGTTGCGCTCGTTG	116
<i>carP</i> _44_F	GAGCTTTCACGCCTCGCCGT	This study
<i>carP</i> _192_R	CGAGCGCTCTTCCAGGCATC	This study
PA0327_BamHI_R	AGAGAGGGATCCTCAGGGCTCGCGC	73
Pser-up	CGAGTGGTTTAAGGCAACGGTCTTGA	100
Pser-down	AGTTCGGCCTGGTGGAGCAACTCG	100
New_ <i>carP</i> _R	TCAGGGCTGCGCCGGCTCGTC	73
F_IR_−187	CCAATCTGGTGGCGCTGGAA	This study
<i>carP</i> _up80_R	AGCTTAAAGTCAGCCAGCCG	This study
F_IR_−47	CGTTTCCATACAGCGTACA	This study
<i>carP</i> _53_R	GTGAAAGCTCGGGACATGGA	This study
F_ <i>carP</i> _+21	CCCGAGCCGTTTTCCATGTC	73
<i>carP</i> _123_R	GAAGCTCTGGCCGAGAAGG	This study

tion in response to different environmental stimuli. To study this, we generated three promoter fusion constructs, referred to as C1 [−326, +1], including the entire IR upstream of *carP*, C2 [−326, +36], and C3 [−212, +36] excluding the PSL (Fig. 3A). We cloned the corresponding fragments into pMS402 carrying a promoterless *luxCDABE* operon and monitored transcriptional activation by measuring the relative luminescence (RLU) normalized by the optical density at 600 nm (OD₆₀₀) to account for growth differences.

Ca²⁺ regulates *carP* transcription. The earlier reported microarray and RT-qPCR studies showed that the transcription of *carP* is increased at least 4-fold in PAO1 mid-log-phase cells grown in the presence of 10 mM Ca²⁺ compared to those grown without added Ca²⁺ (48). We aimed to determine whether *carP* transcription is growth

FIG 4 Impact of elevated extracellular Ca²⁺ on *carP* transcription. (A) Maximum signal detected for three promoter fusion constructs during growth at no, 5 mM, 10 mM, or 20 mM CaCl₂. (B) Activation of the two Ca²⁺-responsive promoter fusion constructs, C2 (left) and C3 (right), transformed into PAO1 (solid bars), the Δ*carR* mutant (bars with vertical lines), or the Δ*lasI* Δ*rhII* Δ*pqsA* mutant (bars with horizontal lines). The mutants were grown at no, 5 mM, 10 mM, or 20 mM CaCl₂. (C) Maximum signal detected for C2 (left) and C3 (right) transformed into PAO1 (solid bars) or the Δ*calC* mutant (checkered bars) during growth at no or 10 mM CaCl₂. The graphs represent the average of results for four biological replicates, and the error bars indicate standard deviation. Experiments were repeated twice to ensure data consistency. Statistical analysis was performed using GraphPad Prism with Student's *t* test at a *P* < 0.05 (*).

phase dependent and to identify the regulatory elements responding to Ca²⁺. For this, we monitored the activities of the promoter fusions during different phases of growth in the presence of different levels of Ca²⁺. First, PAO1 cells carrying C1, C2, or C3 constructs were grown for 24 h without Ca²⁺ or with the addition of 5 mM, 10 mM, or 20 mM Ca²⁺ (Fig. 4A). We observed no activity for C1. In contrast, the C2 and C3 constructs were moderately active at no Ca²⁺, and their activity increased 2.2- to 3.9-fold and 1.9- to 2.6-fold at 5 mM Ca²⁺ and 10 mM Ca²⁺, respectively. When 20 mM Ca²⁺ was added, the C2 activity continued to increase, whereas the activity of C3 decreased to that detected at 5 mM Ca²⁺. Both constructs showed the highest induction during the transition to stationary phase of growth (Fig. S2). The only exception was the C3 construct, which showed an earlier induction during log phase at 10 mM Ca²⁺. These data indicate that *carP* transcription is positively regulated by 5 to 20 mM Ca²⁺ in a growth phase-dependent manner. This induction requires the [+1, +36] fragment but not the [-326, -215] fragment carrying the PSL.

CarR is required for the Ca²⁺-dependent increase in *carP* promoter activity.

Previously we identified a Ca²⁺-induced two-component system, CarSR, which positively regulates *carP* in response to 10 mM Ca²⁺ (48). We aimed to determine whether the Ca²⁺ induction of the *carP* promoter described above requires CarR. For this, the Ca²⁺-responsive C2 and C3 constructs were transformed into the Δ*carR* background

and tested for promoter activity during growth at 5 and 10 mM Ca^{2+} (Fig. 4B). The lack of *carR* completely abolished Ca^{2+} induction of both C2 and C3. Interestingly, some activity was observed for both constructs during the transition to stationary phase (Fig. S2), alluding to the regulatory role of other factors in the absence of Ca^{2+} . These results suggest that CarR is required for Ca^{2+} induction of *carP* and that the [0, +36] fragment, shared by both Ca^{2+} -responding C2 and C3 constructs, contains the CarR binding region.

Challenging PAO1 with 10 mM Ca^{2+} activates *carP* transcription in a growth phase-dependent manner. To test the temporal response of *carP* promoters to addition of Ca^{2+} , we challenged PAO1 cells pregrown in the absence of Ca^{2+} and carrying Ca^{2+} -responsive C2 and C3 constructs with 5 or 10 mM Ca^{2+} during mid-log or stationary growth phases. The promoter activities were monitored for 2 h after the challenge. This timing was selected as the interval shorter than the PAO1 doubling time (119 min) under these growth conditions. Since growth at 5 mM Ca^{2+} induced C2 and C3 activities (Fig. 4A), we first challenged the cells with 5 mM Ca^{2+} . However, no significant response was detected (Fig. S3A). Furthermore, when cells were challenged with 10 mM Ca^{2+} , both C2 and C3 constructs showed increases in their activity during mid-log and stationary phases of growth (Fig. 5A; Fig. S3A). During mid-log-phase challenge, the activity increased for 60 min, followed by a rapid decrease. However, when cells were challenged during stationary phase, an increase of similar amplitude took only 30 min and was not followed by a decrease during the remaining 90 min of monitoring (Fig. 5A; Fig. S3A). To determine whether this response requires CarR, we challenged the ΔcarR mutant harboring each of the two promoter constructs with 10 mM Ca^{2+} (Fig. 5A). There was no activation for either construct, indicating that the temporal response to exogenously added Ca^{2+} is CarR dependent.

Changes in intracellular Ca^{2+} regulate the transcription of *carP*. Our previous studies showed that challenging PAO1 cells with Ca^{2+} generates a transient change in the intracellular levels of free Ca^{2+} ions ($\text{Ca}^{2+}_{\text{in}}$) (47). The generation of this transient change requires a balanced activity of two types of transporters: the Ca^{2+} channel CalC, which is responsible for Ca^{2+} influx (Guragain et al., unpublished), and several active transporters that are responsible for Ca^{2+} efflux (47). We have shown that the CalC-dependent $\text{Ca}^{2+}_{\text{in}}$ transients mediate Ca^{2+} regulation of a number of genes (Guragain et al., unpublished). To test the role of $\text{Ca}^{2+}_{\text{in}}$ transients in Ca^{2+} regulation of *carP* transcription, we transformed C2 and C3 constructs into the ΔcalC mutant and monitored their activity during growth at no or 10 mM Ca^{2+} (Fig. 4C; Fig. S4). The Ca^{2+} -dependent induction of C3 activity was abolished in the mutant, but no change was detected for the C2 construct. These results indicate that the regulatory impact of the CalC-dependent $\text{Ca}^{2+}_{\text{in}}$ requires the predicted PSL at the 5' end of the *carP* intergenic region.

QS regulates the transcription of *carP*. Considering the prediction of a LasR/RhlR binding site within the intergenic region of *carP* (Fig. 3A), we hypothesized that LasR and RhlR regulate *carP* transcription. Similar distances from the transcription start location of the LasR- and RhlR-recognized *lux* box were reported previously (58). These well-studied transcriptional regulators respond to 3-oxo- C_{12} -HSL and C_4 -HSL, correspondingly, and control the expression of many virulence factors in PAO1 (59–61). Supporting this hypothesis, the activities of C2 and C3 promoter constructs were elevated in the cells transitioning to stationary phase (Fig. S2) and the response to Ca^{2+} was higher in the cells in stationary phase (Fig. S3A), when cell density and therefore the level of secreted QS molecules are the highest (62, 63). To further test this hypothesis, we monitored the C2 and C3 activities in a mutant lacking the three QS synthases, the $\Delta\text{lasI} \Delta\text{rhlI} \Delta\text{pqxA}$ mutant in the isogenic PAO1 background. As anticipated, the activities of C2 and C3 promoter fusions were significantly impaired in the QS mutant under the tested conditions (Fig. 4B; Fig. S5). These data indicate that *carP* transcription is positively regulated by at least one of the three QS systems directly or indirectly via the QS-dependent regulators.

To support the observation that QS regulates the transcription of *carP*, we collected the supernatants of the stationary-phase strain PAO1 grown in BMM with or without

FIG 5 Impact of Fe²⁺ on *carP* transcription. Activation of two promoter fusion constructs, C2 (left) and C3 (right), transformed into PAO1 or the $\Delta carR$ mutant and challenged at mid-log phase with 10 mM CaCl₂ (gray diamonds) or 100 μ M FeSO₄ (white circles) added alone or in combination (black triangles). The arrows indicate the time of the challenge. The graphs represent the average results of four biological replicates, and the error bars indicate standard deviation. Experiments were repeated twice to ensure consistency. (B) Maximum signal detected for two promoter fusion constructs, C2 (left) and C3 (right), transformed into PAO1 during growth at no (black) or 10 mM (gray) CaCl₂ added to no-Fe BMM (diagonal), regular BMM containing 4 μ M FeSO₄ (solid), or BMM supplemented with 100 μ M FeSO₄ (squares). The graphs represent the average results of three biological replicates, and the error bars indicate standard deviation. Experiments were repeated twice to ensure data consistency. Statistical analysis was performed using GraphPad Prism with Student's *t* test at a *P* value of <0.005 (**) or <0.05 (*).

10 mM Ca²⁺ and, following filter sterilization, tested their effect on C2 and C3 activities in the $\Delta lasI \Delta rhII \Delta pqsA$ background. The mutant was grown with no or 10 mM Ca²⁺ (Fig. S6A). The C3 construct showed no response to the supernatant challenge (data not shown), and no activity was detected for the C2 construct carrying cells grown without Ca²⁺ (Fig. S6A). In contrast, the C2 activity in $\Delta lasI \Delta rhII \Delta pqsA$ cells grown at 10 mM Ca²⁺ showed a significant increase in response to the supernatants collected under both Ca²⁺ conditions (Fig. S6A). A similar response was observed when we tested the supernatants collected from the *carP*::Tn5 mutant grown with or without 10 mM Ca²⁺ (Fig. S6B). We also tested whether this effect requires CarR by challenging

the $\Delta carR$ mutant carrying the C2 construct, but no induction was observed (Fig. S6A). Overall, these data indicate that C2 activity is induced by a molecule secreted by PAO1 during stationary phase. The synthesis of this molecule is not affected by Ca^{2+} nor does it require CarP. The response in the C2 activity requires CarR and growth at elevated Ca^{2+} . Finally, we tested the effect of IQS, the more recently discovered QS system in *P. aeruginosa* (64). For this, we challenged the cells carrying the C2 construct and grown with or without 10 mM Ca^{2+} with IQS at final concentrations of 1 and 100 μM . No induction was detected (Fig. S6C), indicating that *carP* is not regulated by IQS.

Fe^{2+} and Ca^{2+} show a synergistic effect on *carP* transcription in a CarR-dependent manner. A homolog of *carSR* in *P. aeruginosa* PA14, *bqsSR*, has been shown to respond to elevated Fe^{2+} and increase the transcription of a *carP* homolog in this organism encoded by PA14_04270 (49, 50). Therefore, we hypothesized that CarSR-dependent transcription of *carP* in PAO1 is also controlled by Fe^{2+} . To test this, PAO1 strains carrying C1, C2, and C3 promoter fusions were grown in BMM without added Fe^{2+} (no-Fe BMM) or with the addition of 4 μM (regular BMM) or 100 μM Fe^{2+} . C1 showed almost no promoter activity under these conditions (data not shown). In the absence of Ca^{2+} , C2 and C3 showed no activities at no or 100 μM Fe^{2+} (Fig. 5B; Fig. S7). Both constructs were induced by 4 μM Fe^{2+} at least 10-fold. In the presence of 10 mM Ca^{2+} , we observed increased activities at no Fe^{2+} and further 2.5- to 7.4-fold induction at 4 μM Fe^{2+} for C2 and C3, respectively. The activity of both constructs at 100 μM Fe^{2+} was only slightly increased (Fig. 5B). Interestingly, most of the responses to Fe^{2+} occurred during the transition from exponential to stationary phase, whereas an earlier activation was observed in response to 4 μM Fe^{2+} , particularly for C3 carrying cells that showed activity during mid-log growth phase (Fig. S7). We also tested the constructs' activities in the $\Delta carR$ mutant, which showed no induction by Fe^{2+} (Fig. S7). The results suggest that the presence of Fe^{2+} and Ca^{2+} have a CarR-dependent synergistic effect on the activities of C2 and C3 promoter fusions. Elevating the level of Fe^{2+} from 4 to 100 μM , however, has an inhibiting effect.

To study the temporal response to Ca^{2+} and Fe^{2+} , cells carrying each of three constructs were grown in BMM and challenged with either 100 μM $FeSO_4$ alone or in combination with 10 mM $CaCl_2$ (Fig. 5A). Again, no change in C1 activity was detected (data not shown). However, a significant increase in the activities of C2 and C3 promoter fusions was observed within 1 h after Fe^{2+} addition (Fig. 5A). When Fe^{2+} and Ca^{2+} were added simultaneously, a striking two-phase increase in the activities of both C2 and C3 promoter fusions was registered. During the first 30 min, the promoter activity increased at a rate of about 2,000 RLU/OD₆₀₀/min. Then, after a 20-min plateau, the second phase of increase continued for the rest of the monitoring, with the activity increasing at a rate of about 5,000 RLU/OD₆₀₀/min (Fig. 5A). However, no response in C2 and C3 activities was detected in the $\Delta carR$ mutant, confirming the requirement of CarR for this induction (Fig. 5A).

To test the specificity of this induction, we also tested whether Zn^{2+} and Mg^{2+} , known to modulate *P. aeruginosa* virulence (24, 25), have any impact on *carP* promoter activity. For this, we challenged the mid-log-phase PAO1 cells carrying P2 or P3 promoter fusions with 100 μM $ZnCl_2$ or 0.5 mM $MgSO_4$ (the levels were selected based on the concentration in the human body or reported regulation by Zn^{2+} [65] and Mg^{2+} [66]). However, no response was observed (Fig. S3B), indicating that this regulation is Ca^{2+} and Fe^{2+} specific.

Oxidative stress regulates *carP* promoter activity. Based on the prediction of an OxyR binding site within the [−112, −97] region upstream of *carP*, we hypothesized its regulatory role in *carP* transcription. Considering that OxyR coordinates the development of the oxidative stress response in *P. aeruginosa* (28), we aimed to test whether *carP* responds to factors causing the oxidative stress response. Since H_2O_2 , the most common cause of oxidative stress, is not compatible with the *lux* system, we exposed PAO1 cells carrying C1, C2, or C3 constructs to UV light. UV treatment has been shown to induce oxidative stress in bacteria (67–69). The selected duration of the exposure (7 min) was optimized to ensure 100% cell survival. No UV effect on C1 activity and no UV effect on any of the constructs' activities under no- Ca^{2+} conditions were observed

FIG 6 Impact of oxidative stress on *carP* transcription. (A) Activation of C2 and C3 promoter fusion constructs transformed into PAO1 grown at 10 mM CaCl_2 to mid-log phase and exposed to 7 min of UV light (black line). The nonexposed control samples are shown in gray. The arrows indicate the time of the challenge. Each graph presents the average results for four biological replicates, and error bars indicate standard deviation. (B) H_2O_2 tolerance of PAO1 and the $\Delta carP$ mutant grown at 10 mM CaCl_2 until mid-log phase and exposed to 1 mM H_2O_2 for 30 min. After treatment, the cultures were serially diluted and plated for CFU counts before and after exposure, and the percent survival was calculated. Student's *t* test was performed for statistical analyses, with a *P* of <0.05 (*) or <0.005 (**). The experiment was repeated three times to ensure data consistency.

(data not shown). In contrast, when tested cells carrying C2 and C3 constructs were grown in the presence of 10 mM Ca^{2+} , C2 showed a slight increase, and C3 showed up to a 2- to 3-fold increase in response to the UV exposure (Fig. 6A). In addition to oxidative stress, UV treatment may trigger the SOS response, coordinated by LexA (56), whose binding site was also predicted upstream of *carP*. Therefore, we also tested the effect on the *carP* promoter fusions of mitomycin, a known inducer of the SOS response (70). However, no changes in C2 and C3 activities were observed upon treatment with 5 μg of the antibiotic (Fig. S8). This indicated that *carP* is induced by UV as a part of the oxidative stress response.

CarP contributes to PAO1 tolerance to H_2O_2 . Considering the important role OxyR plays in *P. aeruginosa* tolerance to H_2O_2 (28), and that C2 and C3 promoter fusions were activated in response to UV treatment, we aimed to determine the role of CarP in tolerance to H_2O_2 . For this, the wild-type (WT) and $\Delta carP$ cultures were grown at 10 mM Ca^{2+} to mid-log phase and exposed to 1 mM H_2O_2 for 30 min (Fig. 6B). The $\Delta carP$ mutant showed a statistically significant 2.3-fold decrease in cell survival in comparison to the WT (Fig. 6B). We conclude that CarP contributes to *P. aeruginosa* oxidative stress tolerance in the presence of elevated Ca^{2+} .

***carP*::Tn5 mutant.** The earlier reported experiments utilized a *carP*::Tn5 mutant obtained from the PAO1 two-allele library (71). For verification purposes, we sequenced the genome of the mutant at the Microbial Genome Sequencing Center. We would like to share that in comparison to the WT PAO1 strain, the mutant lacks a significant portion of its genome, including 105 genes. All the phenotypes observed in the transposon mutant that were reported earlier were verified by genetic complementation of *carP* (48). Here, we present the data based on the clean-deletion $\Delta carP$ mutant generated in this study.

DISCUSSION

Ca^{2+} has been shown to regulate bacterial virulence in a number of human pathogens, including *P. aeruginosa* (reviewed in reference 72). Uncovering novel components of the

Ca^{2+} regulatory network will likely reveal previously uncharacterized factors controlling and defining the ability of human pathogens to invade, persist, and cause diseases. Previously, we identified one such component, a putative β -propeller phytase, CarP, encoded within the core genome of *P. aeruginosa* (73), that plays an important role in maintaining $\text{Ca}^{2+}_{\text{in}}$ homeostasis and that is required for cell tolerance to elevated Ca^{2+} and Ca^{2+} regulation of pyocyanin production and swarming (48). Here, we show that in addition to the role of *carP* in *P. aeruginosa* virulence, its transcription is regulated by multiple host and bacterial regulatory factors (Fig. 7), which indicates its importance for *P. aeruginosa* adaptations to the host environment. Our data showed that CarP contributes to *P. aeruginosa* tolerance to H_2O_2 , commonly generated by neutrophils and macrophages during the respiratory burst to combat pathogens (74, 75). Although the function of CarP is not yet known, these findings underline the important role of CarP in *P. aeruginosa* virulence and adaptation to the human host, which is further supported by its increased expression in CF isolates compared to the laboratory reference strain.

During the course of infection, for example, in the lungs of CF patients, *P. aeruginosa* undergoes a transition from causing acute infection, when potent virulent factors damage the host tissues, to the mode of chronic infection (76). In the latter phase, *P. aeruginosa* can persist in the host for decades. Such a transition is controlled by complex regulatory rearrangements that switch off the production of aggressive virulence factors and turn on the factors contributing to biofilm formation and increased resistance (77). Elevated Ca^{2+} has been shown to contribute to the regulation of such a transition by enhancing the production of alginate and efflux pumps (41, 43) and by activating the Gac/Rsm pathway via interaction with the Gac-associated histidine kinase LadS (78). Using the acute infection model of *G. mellonella*, we here showed that deletion of *carP* enhanced *P. aeruginosa* virulence at 10 mM Ca^{2+} but had no impact at no/low Ca^{2+} . This suggests that the role of CarP in virulence is Ca^{2+} dependent. Considering these observations and that the levels of Ca^{2+} in the nasal and lung liquids increase during the progression of CF (79), CarP may be involved in the elevated Ca^{2+} -regulated switch to the chronic mode of *P. aeruginosa* interactions with the host.

β -Propeller proteins are found in all domains of life and have a wide range of functions. In bacteria, β -propeller proteins play important roles in nitrate reduction (80), uptake of zinc (81), sialic acid utilization (82), phytic acid catabolism (83), host-microbe interactions (84), and virulence (85). Based on the prediction of a phytase-like domain, CarP may potentially have an enzymatic function similar to a typical phytase, i.e., remove a phosphate(s) from phytic acid or similar substrates (86). This activity may result in manipulation of the host Ca^{2+} homeostasis through inositol-3-phosphate signaling (87). We predict that CarP is anchored to the inner membrane and faces the periplasm. This prediction is based on (i) the sequence of the transmembrane helix (TMH) and (ii) the high number of leucines toward the 5' end of the transmembrane helix, which is indicative of localization near the cytoplasm (88). Such localization sets the protein within the environment with a defined pool of accessible substrates. Future work will aim to validate the cellular localization and enzymatic function of the protein.

Transcriptional regulation of virulence factors in *P. aeruginosa* involves coordinated regulation by both bacterial and host factors. For example, production of pyoverdine is controlled by iron but also responds to QS and oxidative stress (20, 27, 89). This multifactorial regulation emphasizes that production of virulence factors is an adaptive process, tuning the physiology of the pathogen for survival under the pressure of the hostile host environment. Transcriptional regulation of *carP* in response to multiple host and bacterial factors provides another example of the ability of *P. aeruginosa* to integrate multiple environmental cues for a continuous enhancement of its fitness for survival in the host. Our data show that Ca^{2+} plays a major regulatory role in *carP* transcription (Fig. 4). The millimolar levels of Ca^{2+} ions in the extracellular fluids of the human body are tightly regulated and thus may feature the "inside host" environment and trigger adaptation programs in the invading pathogens. During diseases such as

cystic fibrosis (CF), Ca^{2+} levels in lung and nasal liquids and in saliva are elevated (90, 91), which may serve as a signal of a susceptible host. We show that *carP* regulation by Ca^{2+} involves two pathways. The first one is via the two-component regulatory system CarSR that is induced by elevated extracellular Ca^{2+} and regulates transcription of several genes, including *carP* in a Ca^{2+} -dependent manner (48). Since exclusion of the 5'-end 6 bp of the gene from the promoter fusion abolished Ca^{2+} induction, we concluded that this region is required for Ca^{2+} regulation and may contain the CarR binding region. The second pathway is via the changes in the intracellular Ca^{2+} , coordinated by Ca^{2+} channel CalC, required for the generation of the transient increase in the cytoplasmic Ca^{2+} (Guragain et al., unpublished). The transcriptional regulator responding to the $\text{Ca}^{2+}_{\text{in}}$ fluctuations is not yet known. The involvement of the two pathways in *carP* regulation provides novel evidence of their regulatory integration.

Individually added Ca^{2+} and Fe^{2+} significantly increase the transcription of *carP*; however, when added together, they showed a synergistically higher impact in a CarR-dependent manner. The Fe^{2+} regulation is in agreement with the work performed in PA14 on the homologous two-component system BqsSR (50). The genes *carP* and PA14_04270 share 98.4% nucleotide sequence identity, and their upstream 326-bp intergenic regions are 100% identical. Therefore, it is likely that PA14_04270 is regulated by Ca^{2+} as well, but either higher levels of the ion or stationary-phase cells need to be tested. Further, Kreamer et al. (49) identified two transcription start sites (TSS) for PA14_04270 (at positions -49 and $+26$). The -49 TSS is identical to the TSS2 identified here for *carP*. Although the Cappable RNA-Seq analysis did not identify the $+26$ TSS for *carP*, it detected two other TSS at positions $+7$ and -288 , which were supported by RT-PCR. Further sequence analyses detected ATG at position $+37$, which may serve as a translation initiation codon. In support of this possibility, we find a slightly atypical Shine-Dalgarno sequence (CGAGC) in the expected 8-bp upstream position (92) and a pyrimidine-rich region further upstream that may act as a ribosome recognition site (93). So, it is possible that the protein coding sequence begins at position $+37$, leaving the upstream untranslated regions of the three transcripts to promote posttranscriptional regulation and proper translation initiation. Further studies are required to confirm the predicted translation initiation and to characterize the roles and regulation of the multiple transcripts. Also in agreement with the observations for PA14_04270 (49, 50), we showed no impact of elevated Mg^{2+} and Zn^{2+} on the transcription of *carP*. Both homologs have a similar genomic environment, including genes predicted to encode polyamine utilization and transporters. Some polyamines, for example, spermidine, have been shown to increase resistance to antimicrobials and tolerance to oxidative stress in *P. aeruginosa* (94), which opens the possibility of a role of CarP in the biosynthesis of such compounds and may explain its contributions to H_2O_2 tolerance in PAO1.

The activation of *carP* promoter fusions by UV light raises the possibility that CarP is directly or indirectly involved in the *P. aeruginosa* oxidative stress response. This was supported by the prediction of an OxyR binding site and the observation that CarP contributes to PAO1 H_2O_2 tolerance. Although the exact mechanisms are not clear, both the UV activation of *carP* promoters and the CarP role in H_2O_2 tolerance required elevated Ca^{2+} . This adds another adaptation of *P. aeruginosa* to a host environment to the growing list of processes regulated by Ca^{2+} (72), which collectively indicate that Ca^{2+} triggers a multicomponent network enhancing the ability of the pathogen to persist in the host.

The increased transcription of *carP* during stationary phase of growth indicated the involvement of quorum sensing (QS) regulation. QS in *P. aeruginosa* plays a primary role in coordinating the increased cell density with the expression of several virulence factors. These factors include biofilm formation and production of toxins, pyocyanin, and pyoverdine (reviewed in reference 95). Supporting the hypothesis that QS controls *carP* expression, we observed abolished promoter activity in the ΔlasI ΔrhII ΔpqS triple synthase mutant (Fig. 4B). Further in agreement, the supernatant collected from

FIG 7 Current model summarizing the characterized and hypothetical transcriptional regulation of *carP* and its impact on Ca^{2+} -dependent phenotypes in *P. aeruginosa*. Three identified TSS are indicated by arrows. LasR/RhlR, OxyR, and CarR recognition sites, located upstream of *carP*, respond to QS, oxidative stress, and elevated exogenous $\text{Ca}^{2+}/\text{Fe}^{2+}$, respectively. The effect of $\text{Ca}^{2+}_{\text{in}}$ homeostasis mediated by CalC suggests the possibility of another transcriptional regulator responding to the changes in the $\text{Ca}^{2+}_{\text{in}}$ levels. This regulation leads to increased production of CarP, which contributes to the regulation of virulence, pyocyanin and pyoverdine production, tolerance to elevated Ca^{2+} and H_2O_2 , and swarming reported earlier (48) and presented here.

WT PAO1 activated the C2 fusion in the cells grown in the presence of 10 mM Ca^{2+} . Our data suggest that one of the three molecules 3-oxo- C_{12} -HSL, C_4 -HSL, or PQS, but not IQS, produced in a Ca^{2+} -independent manner directly or indirectly activates the *carP* promoter. This activation requires the [+1 to +6] region and CarR. It also requires the cells to be grown at elevated Ca^{2+} , which activates *carR* expression (48). Interestingly, since the C3 promoter fusion was not activated by the supernatants, the putative PSL lacking in this construct may play a negative role in this regulation. Further studies will aim to identify the specific QS molecule and to characterize its relationship with Ca^{2+} and CarR. Overall, the regulation of *carP* by QS, Ca^{2+} , and Fe^{2+} highlights the interconnectedness between the corresponding regulatory circuits.

Overall, the conditions shown to control *carP* transcription resemble those coexisting in the lungs of CF patients, where due to the dysfunctional CFTR, the ion homeostasis is drastically altered (96), the level of Ca^{2+} is elevated (35), and the activated migration of neutrophils causes elevated levels of reactive oxygen species (ROS) (97). In such an environment, *P. aeruginosa* persists for decades. Based on the observations presented here, we hypothesize that CarP is an important player in the physiological adjustments occurring in the pathogen during adaptations to the host environment, enabling its long-term survival. Although we showed that CarP plays a pleiotropic role in *P. aeruginosa* virulence and resistance and that *carP* expression is regulated by multiple bacterial and host factors, the function of CarP is not known (Fig. 7). Current studies are focused on identifying the role of CarP in the metabolic network of *P. aeruginosa* and, together with the ongoing protein work, are expected to elucidate the function of CarP and provide the molecular basis for its role in *P. aeruginosa* virulence.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Cultures were grown in biofilm minimal medium (BMM) (42), which contained (per liter) 9.0 mM sodium glutamate, 50 mM glycerol, 0.02 mM MgSO_4 , 0.15 mM NaH_2PO_4 , 0.34 mM K_2HPO_4 , 145 mM NaCl, 200 μl trace metals, and 1 ml vitamin solution. The trace metal solution consisted of (per liter of 0.83 M HCl) 5.0 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 5.0 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 5.0 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, and 2.0 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$. The vitamin solution contained (per liter) 0.5 g thiamine and 1 mg biotin. The pH of the medium was adjusted to 7.0. When required, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ was added to a final concentration of 5 mM, 10 mM, or 20 mM. The $\Delta\text{lasI} \Delta\text{rhlI} \Delta\text{pqS}$ mutant in the isogenic PAO1 background was a generous gift from Kangmin Duan (Manitoba University, Canada). The isogenic strain of wild-type *P. aeruginosa* PAO1 and sequence-verified transposon insertion mutants obtained from the University of Washington Two-Allele library (98) were used. The mutants contained *ISphoA/hah* or *ISlacZ/hah* insertions with a tetracycline

resistance cassette that disrupted the genes of interest. The mutations were also confirmed by two-step PCR: first, transposon-flanking primers were used to verify that the target gene is disrupted, and second, gene-specific primers were used to confirm the transposon insertion. The primer sequence is available at <https://www.gs.washington.edu>. For convenience, the mutants were designated PA::Tn5, where PA is the identifying number of the disrupted gene from *P. aeruginosa* PAO1 genome (<https://www.pseudomonas.com>). Primers were obtained from Integrated DNA Technologies, Inc. The antibiotic concentrations used were as follows: trimethoprim, 300 $\mu\text{g/ml}$; gentamicin, 30 $\mu\text{g/ml}$; tetracycline, 60 $\mu\text{g/ml}$. Whole-genome sequencing of the *carP*::Tn5 mutant was performed by the Microbial Genome Sequencing (MIGS) Center (NCBI accession number [PRJNA704188](https://www.ncbi.nlm.nih.gov/submit/PRJNA704188)). The MIGS also performed an *in silico* analysis of the sequence by using breseq, a computational pipeline for finding mutations relative to a reference sequence. The deleted regions of the chromosome were confirmed by PCR using primers flanking the deletion.

Generation of a *carP* deletion mutant and its genetic complement. The $\Delta carP$ deletion mutant was generated using Gibson cloning (99). In brief, 500-bp regions flanking *carP* were amplified using the primers carPupF, carPupR, carPdownF, and carPdownR (Table 1) and cloned into pEXG2 by using the HiFi DNA assembly kit (NEB). The generated plasmid, pcarPDM, was then electroporated into *Escherichia coli* SM10 for biparental mating with PAO1. The successful transformants were selected on *Pseudomonas* isolation agar (PIA) with 30 $\mu\text{g/ml}$ gentamicin. To facilitate homologous recombination, the cultures were grown in liquid LB for 6 h, followed by SacB-based counterselection using LB agar supplemented with 5% sucrose. The loss of the gene in the sucrose-sensitive and gentamicin-resistant transformants was verified by PCR using gene-specific primers carPupF and carPdownR (Table 1). The $\Delta carP$ mutant was complemented with the *carP* (PA0327) gene by following the protocol described previously (100). First, the region containing the entire sequence of *carP* (966 bp) and the 326-bp-upstream region was amplified using Q5 high-fidelity DNA polymerase (NEB) and the F_{IR} + 36bp_{carP}XhoI and PA0327_BamHI_R_Both primers, designed to include the XhoI and BamHI recognition sites (Table 1). The amplicon was inserted into XhoI- and BamHI-digested mini-CTX1 and confirmed by Sanger sequencing. The resulting plasmid, pASK302, was introduced into *E. coli* SM10 and then inserted into the $\Delta carP$ mutant strain by conjugation using SM10(pASK302) as a donor strain. The successful transconjugants were selected on PIA agar plates containing 60 $\mu\text{g/ml}$ of tetracycline. To remove the vector backbone, pFLP2 (101) encoding Flp recombinase was inserted into the transconjugants using electroporation. The successful transformants were selected on LB agar plates containing 300 $\mu\text{g/ml}$ of carbenicillin and counterselected on LB with 10% sucrose to cure from pFLP2. Carbenicillin- and tetracycline-sensitive clones were selected. To confirm the genotype, double PCR using the (i) F_{IR} + 36bp_{carP}XhoI and PA0327_BamHI_R_Both and (ii) Pser-up and Pser-down primers amplifying the upstream and downstream regions of the *attB* site on mini-CTX1 was performed.

H₂O₂ tolerance. A hydrogen peroxide tolerance assay was performed as described previously (41). Briefly, mid-log-phase bacterial cultures grown in BMM in the presence of 10 mM CaCl₂ were normalized to an OD₆₀₀ of 0.1, and 750 μl of the normalized culture was gently mixed with H₂O₂ to a final concentration of 1 mM and incubated at 37°C for 30 min. Then, 10- μl volumes of the cultures were serially diluted to determine the number of CFU before and after H₂O₂ treatment. The results are expressed as the mean survival percentages from three biological replicates. The experiment was repeated two times.

Virulence assay. To assess the impact of *carP* on *P. aeruginosa* virulence, the *Galleria mellonella* infection model was used as described previously (102) with modifications. This model has been established for studying bacterial pathogenesis (102, 103). Active *G. mellonella* larvae (Speedy Worms and American Cricket Ranch) of 2 to 3 cm in length, stored in the dark at 4°C without feeding for no more than 48 h, were chosen for injections. A new batch of larvae was received for each experiment. *P. aeruginosa* PAO1, $\Delta carP$, and $\Delta carP$::*carP* strains were grown in BMM or LB with 10 mM or no added CaCl₂ for 12 h (mid log). The concentration of Ca²⁺ in LB was measured by the QuantiChrom calcium assay kit by following the manufacturer's guidelines and was adjusted to 10 mM by adding the corresponding amount of CaCl₂. Cultures were diluted to contain approximately 1 to 9 CFU per injection. To verify the injected infection dose, the cultures prepared for injection were serially diluted and plated on Luria-Bertani (LB) agar plates for the CFU count. After gentle rinsing with 70% ethanol followed by 1 mg/ml rifampin, 10 larvae were injected with 5 μl of normalized cell suspension grown at 0 mM or 10 mM CaCl₂. In addition, five larvae were injected with 5 μl of phosphate-buffered saline (PBS) with or without 10 mM CaCl₂ for use as negative controls. The larvae were incubated at 37°C and tested for death every 2 h for 24 h. The time of death (TOD) was recorded for each larva, when no movement was observed in response to turning it over with a sterile toothpick. Killing curves were plotted and statistically analyzed by using GraphPad Prism 5 software. A total of 100 larvae were used for each experiment, which was repeated at least four times for validation of the results.

In silico analysis. The sequence of the 554-bp region upstream of *carP* was retrieved from <https://pseudomonas.com> (104). To predict regulatory elements, the sequence was analyzed by using bacterial promoter prediction algorithms BProm (105) and Virtual Footprint 3.0 (106). The transmembrane helix (TMH) was predicted with high accuracy using TMHMM v.1 (107).

RNA sequencing. RNA was isolated as previously described (108). Briefly, total RNA, in three replicates for each growth condition, was isolated from *P. aeruginosa* mid-log-phase cultures grown in BMM with no added Ca²⁺ or with 5 mM Ca²⁺ by using an RNeasy protect bacteria minikit (Qiagen) or ZR Fungal/Bacterial RNA MiniPrep (Zymo Research). The quality of the purified RNA was assessed using a Bioanalyzer 2100 (Agilent) and 1% agarose gel electrophoresis. RNA-Seq analysis was performed at Vertis Biotechnology AG, Germany, using differential RNA-Seq (dRNA-Seq) (57). The Cappable RNA library was generated by tagging the 5' triphosphate with 3'- desthiobiotin-TEG-guanosine 5'

triphosphate (DTBGTP) (NEB catalog no. N07615) by using the vaccinia capping enzyme (VCE) (NEB catalog no. M20805). The biotinylated RNA was captured on streptavidin beads, and the 5' triphosphate was converted to monophosphate. The RNA was then ligated onto an adaptor, and the library was amplified and sequenced on an Illumina NextSeq 500 system using a 75-bp read length. The NCBI accession number is [PRJNA703087](https://www.ncbi.nlm.nih.gov/submit/PRJNA703087). Sequence analyses of such a library enables identification of transcriptional starts at a single-base resolution (57). The transcript abundances were quantified and normalized. Differential expression of *carP* was considered with an FDR of <0.05 and a *P* of <0.05.

RT-PCR. To test the expression of three *carP* transcripts in PAO1, reverse transcription-PCR (RT-PCR) was performed as follows. Template cDNA was synthesized using RNA purified from PAO1 mid-log-phase cultures grown in BMM with or without 10 mM CaCl₂ as described previously (108). The quality of RNA was tested by agarose (1.2%) gel electrophoresis. The following three sets of primers were designed and used to amplify the portions of the expected transcripts (Fig. 3A; Table 1): F_ carP_+21/New_ carP_ R (T1), F_ IR_47/New_ carP_ R (T2), and F_ IR_187/New_ carP_ R (T3). Due to the overlapping nature of the transcripts, we expected that only the T3 primers would verify the presence of T3, whereas the T2 primers would amplify both T2 and T3 and the T1 primers would amplify all three transcripts. For a negative control, we designed F_ IR_320/New_ carP_ R primers to amplify the intergenic region upstream of *carP*, which according to the RNA-Seq data was not transcribed. For a positive control, we used 16S-533-F/16S-1100-R for the amplification of the 16S rRNA gene (Table 1). To ensure the absence of genomic DNA in the purified RNA, we tested the RNA samples by using all five sets of primers prior to cDNA synthesis. The specificity of the primers was first tested using DreamTaq green PCR master mix (Thermo Fisher Scientific) with PAO1 genomic DNA as a template. cDNA was synthesized using a Transcriptor first-strand cDNA synthesis kit (Roche, Indianapolis, IN). The PCRs were carried out using a C1000 Touch thermal cycler (Bio-Rad) and the following protocol: 98°C for 2 min and 30 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 40 s, followed by 72°C for 2 min. For the 16S rRNA gene, the protocol used was as follows: 95°C for 2 min and 30 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 1 min 30 s, followed by 72°C for 2 min for *carP*.

RT-qPCR was used to quantify the expression of three *carP* transcripts in PAO1 and to test the impact of elevated Ca²⁺ on *carP* transcription in PAO1 and two clinical isolates, 31314A and 22014B. The clinical isolates were isolated from the sputum of individuals with cystic fibrosis (CF) and received from E. I. Lutter's collection of clinical strains. The identity of isolates 31314A and 22014B was verified by sequencing their amplified 16S rRNA genes. Since clinical isolates do not consistently grow in BMM, the strains were grown in LB medium or LB supplemented with 10 mM CaCl₂ until mid-log phase (5 h; OD₆₀₀ of 0.30 ± 0.03). The level of Ca²⁺ was verified by using a QuantiChrom calcium assay kit (BioAssay Systems, Hayward, CA). Total RNA was isolated, and cDNA was synthesized as described above. The absence of genomic DNA in the RNA samples was confirmed by PCR amplification of *carP* and 16S rRNA genes prior to cDNA synthesis by using DreamTaq green PCR master mix. All the primers are listed in Table 1. They include 16S-533-F/16S-1100-R (16S rRNA), *carP* T1' (F_ carP_+21/carP_123_R), *carP* T2' (F_ IR_47/carP_53_R), *carP* T3' (F_ IR_187/carP_up80_R), *carP* (carP_44_F/carP_192_R), and *rpoD* (PA0576-F1/PA0576-R1). The primers' design for three *carP* transcripts is shown in Fig. 3A. Quantitative RT-PCR (RT-qPCR) was performed using SYBR green master mix (Roche, Indianapolis, IN) and a Light Cycler 480 Instrument II (Roche). The *rpoD* gene was used as an internal control. The relative fold changes in gene expression levels were calculated using the 2^{−ΔΔC_T} method (109).

Promoter activity reporter construction. To study the transcriptional regulation of *carP*, a promoterless *luxCDABE* reporter was used. pMS402 vector was generously shared by Kangmin Duan (Manitoba University, Canada). The 362-nt, 326-nt, and 248-nt regions upstream of *carP* were PCR amplified by using Phusion polymerase (a generous gift from Rolf Prade, Oklahoma State University) and primers −326_Xho1, +1_BamH1, +36_BamH1, and −212_Xho1 with flanking BamHI and XhoI restriction sites (Table 1) and cloned upstream of the *luxCDABE* operon. The resultant plasmids were designated pMCP1, pMCP2, and pMCP3 and transformed into the PAO1 wild type (WT) and the $\Delta carR$, $\Delta lasI$ $\Delta rhII$ $\Delta pqsA$, and $\Delta calC$ mutants (Table 1) by electroporation (110). Successful transformants were selected on LB agar plates containing 300 μ g/ml trimethoprim.

Promoter activity assay. Strains carrying promoter activity constructs were grown in 5 ml of BMM with shaking at 200 rpm for 12 h (mid-log phase). The OD₆₀₀ of the cultures was measured, and the cultures were diluted to an OD₆₀₀ of 0.3 using sterile BMM with the corresponding CaCl₂ level. Thus, normalized cultures were inoculated into a final volume of 200 μ l of BMM at a ratio of 1:100 in 96-well white clear-bottom plates (Grenier Bio-One) and incubated with shaking at 200 rpm at 37°C in an incubator. When needed, CaCl₂ to a final concentration of 5 mM, 10 mM, or 20 mM was added to BMM. OD₆₀₀ and luminescence were measured every 4 h for 24 h. Luminescence measurements were normalized by the OD₆₀₀ of the corresponding cultures, followed by subtraction of the empty-vector normalized luminescence values. To characterize a temporal effect of selected factors on *carP* promoter activity, the inoculated plates were incubated in a Synergy MX plate reader (Biotek) for 6 h (mid-log phase) or 10 h (stationary phase), with OD₆₀₀ and luminescence readings every 30 min. The plates were then taken out briefly, and 10 μ l of the solution of interest was added. After mixing for 1 s, the measurements of OD₆₀₀ and luminescence were resumed and taken every 10 min for 2 h. The following compounds were tested at their final concentrations: 5 mM CaCl₂, 10 mM CaCl₂, 100 μ M FeSO₄, 0.5 mM MgCl₂, 100 μ M ZnSO₄, 100 μ M IQS, and filtered cell supernatants. The supernatants were prepared by growing strain PAO1 or the *carP*::Tn5 mutant in 50 ml of BMM with or without 10 mM CaCl₂ until stationary phase. The cells were then pelleted by centrifugation at 29 × *g* for 10 min, and the supernatants were collected and filter sterilized using 0.2- μ m-pore-size filters (PALL Life Sciences). The supernatants were used immediately without storing. To test the effect of UV, cell cultures were grown in 96-well plates using a BioTek plate

reader using two rows: B (to be exposed to UV) and E (to serve as untreated controls). After reaching mid-log phase, the plate was placed into a NuAire class II biosafety cabinet, the lid was removed, and row E wells were covered with a sterile fitted lid of aluminum foil to prevent UV exposure. The uncovered samples (row B) were exposed to UV light for 7 min, and the plate was returned to the BioTek plate reader for the monitoring of the promoter activity that followed. To ensure that growth was not the defining factor in the luminescence changes, the measured relative luminescence was normalized by OD₆₀₀. Each experiment included three biological replicates and was repeated at least twice.

Data availability. The RNA-Seq data are available at NCBI under accession number [PRJNA703087](https://www.ncbi.nlm.nih.gov/submit/PRJNA703087). The genome sequence of the *carP::Tn5* mutant is available at NCBI under accession number [PRJNA704188](https://www.ncbi.nlm.nih.gov/submit/PRJNA704188).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

ACKNOWLEDGMENTS

We thank Joe Grissom for analysis of the RNA-Seq data. We thank members of the Matthew Cabeen lab for assistance in constructing the *carP* deletion mutant. We thank Rolf Prade and Biraj Kayastha, Oklahoma State University, for technical support and measuring calcium concentrations in medium preparations, respectively. We also thank Paul Williams at the University of Nottingham for sharing the purified IQS compound and E. I. Lutter from Oklahoma State University for sharing the CF clinical isolates.

The research was supported by NIH Centers of Biomedical Research Excellence (COBRE) grant no. P20GM103648.

REFERENCES

- Mahar P, Padiglione AA, Cleland H, Paul E, Hinrichs M, Wasiak J. 2010. *Pseudomonas aeruginosa* bacteraemia in burns patients: risk factors and outcomes. *Burns* 36:1228–1233. <https://doi.org/10.1016/j.burns.2010.05.009>.
- Stern GA. 1990. *Pseudomonas* keratitis and contact lens wear: the lens/eye is at fault. *Cornea* 9(Suppl 1):S36–S38. Discussion S39–S40. <https://doi.org/10.1097/00003226-199010001-00015>.
- Oliver A, Canton R, Campo P, Baquero F, Blazquez J. 2000. High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *Science* 288:1251–1254. <https://doi.org/10.1126/science.288.5469.1251>.
- Stewart PS, Costerton JW. 2001. Antibiotic resistance of bacteria in biofilms. *Lancet* 358:135–138. [https://doi.org/10.1016/S0140-6736\(01\)05321-1](https://doi.org/10.1016/S0140-6736(01)05321-1).
- Jesaitis AJ, Franklin MJ, Berglund D, Sasaki M, Lord CI, Bleazard JB, Duffy JE, Beyenal H, Lewandowski Z. 2003. Compromised host defense on *Pseudomonas aeruginosa* biofilms: characterization of neutrophil and biofilm interactions. *J Immunol* 171:4329–4339. <https://doi.org/10.4049/jimmunol.171.8.4329>.
- Fernández L, Breidenstein EB, Hancock RE. 2011. Creeping baselines and adaptive resistance to antibiotics. *Drug Resist Updat* 14:1–21. <https://doi.org/10.1016/j.drug.2011.01.001>.
- Breidenstein EB, de la Fuente-Núñez C, Hancock RE. 2011. *Pseudomonas aeruginosa*: all roads lead to resistance. *Trends Microbiol* 19:419–426. <https://doi.org/10.1016/j.tim.2011.04.005>.
- Everett J, Turner K, Cai Q, Gordon V, Whiteley M, Rumbaugh K. 2017. Arginine is a critical substrate for the pathogenesis of *Pseudomonas aeruginosa* in burn wound infections. *mBio* 8:e02160-16. <https://doi.org/10.1128/mBio.02160-16>.
- Park PW, Pier GB, Hinkes MT, Bernfield M. 2001. Exploitation of syndecan-1 shedding by *Pseudomonas aeruginosa* enhances virulence. *Nature* 411:98–102. <https://doi.org/10.1038/35075100>.
- Stempel N, Neidig A, Nusser M, Geffers R, Vieillard J, Lesouhaitier O, Brenner-Weiss G, Overhage J. 2013. Human host defense peptide LL-37 stimulates virulence factor production and adaptive resistance in *Pseudomonas aeruginosa*. *PLoS One* 8:e82240. <https://doi.org/10.1371/journal.pone.0082240>.
- Burrows LL. 2018. The therapeutic pipeline for *Pseudomonas aeruginosa* infections. *ACS Infect Dis* 4:1041–1047. <https://doi.org/10.1021/acscinfdis.8b00112>.
- Sriyosachati S, Cox CD. 1986. Siderophore-mediated iron acquisition from transferrin by *Pseudomonas aeruginosa*. *Infect Immun* 52:885–891. <https://doi.org/10.1128/IAI.52.3.885-891.1986>.
- Kang D, Revtovich AV, Chen Q, Shah KN, Cannon CL, Kirienko NV. 2019. Pyoverdine-dependent virulence of *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. *Front Microbiol* 10:2048. <https://doi.org/10.3389/fmicb.2019.02048>.
- Ochsner UA, Johnson Z, Lamont IL, Cunliffe HE, Vasil ML. 1996. Exotoxin A production in *Pseudomonas aeruginosa* requires the iron-regulated *pvdS* gene encoding an alternative sigma factor. *Mol Microbiol* 21:1019–1028. <https://doi.org/10.1046/j.1365-2958.1996.481425.x>.
- Shigematsu T, Fukushima J, Oyama M, Tsuda M, Kawamoto S, Okuda K. 2001. Iron-mediated regulation of alkaline proteinase production in *Pseudomonas aeruginosa*. *Microbiol Immunol* 45:579–590. <https://doi.org/10.1111/j.1348-0421.2001.tb01289.x>.
- Lamont IL, Beare PA, Ochsner U, Vasil AI, Vasil ML. 2002. Siderophore-mediated signaling regulates virulence factor production in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* 99:7072–7077. <https://doi.org/10.1073/pnas.092016999>.
- Minandri F, Imperi F, Frangipani E, Bonchi C, Visaggio D, Facchini M, Pasquali P, Bragonzi A, Visca P. 2016. Role of iron uptake systems in *Pseudomonas aeruginosa* virulence and airway infection. *Infect Immun* 84:2324–2335. <https://doi.org/10.1128/IAI.00098-16>.
- Yu S, Wei Q, Zhao T, Guo Y, Ma LZ. 2016. A survival strategy for *Pseudomonas aeruginosa* that uses exopolysaccharides to sequester and store iron to stimulate Psl-dependent biofilm formation. *Appl Environ Microbiol* 82:6403–6413. <https://doi.org/10.1128/AEM.01307-16>.
- Wiens JR, Vasil AI, Schurr MJ, Vasil ML. 2014. Iron-regulated expression of alginate production, mucoid phenotype, and biofilm formation by *Pseudomonas aeruginosa*. *mBio* 5:e01010-13. <https://doi.org/10.1128/mBio.01010-13>.
- Banin E, Vasil ML, Greenberg EP. 2005. Iron and *Pseudomonas aeruginosa* biofilm formation. *Proc Natl Acad Sci U S A* 102:11076–11081. <https://doi.org/10.1073/pnas.0504266102>.
- Dong Y, Zhang X-F, An S-W, Xu J-L, Zhang L-H. 2008. A novel two-component system BqsS-BqsR modulates quorum sensing-dependent biofilm decay in *Pseudomonas aeruginosa*. *Commun Integr Biol* 1:88–96. <https://doi.org/10.4161/cib.1.1.6717>.
- Song Y, Liu S. 2012. Magnesium for cardiovascular health: time for intervention. *Am J Clin Nutr* 95:269–270. <https://doi.org/10.3945/ajcn.111.031104>.

23. Mulcahy H, Lewenza S. 2011. Magnesium limitation is an environmental trigger of the *Pseudomonas aeruginosa* biofilm lifestyle. *PLoS One* 6: e23307. <https://doi.org/10.1371/journal.pone.0023307>.
24. Marquart ME, Dajcs JJ, Caballero AR, Thibodeaux BA, O'Callaghan RJ. 2005. Calcium and magnesium enhance the production of *Pseudomonas aeruginosa* protease IV, a corneal virulence factor. *Med Microbiol Immunol* 194:39–45. <https://doi.org/10.1007/s00430-003-0207-9>.
25. Gonzalez MR, Ducret V, Leoni S, Perron K. 2019. *Pseudomonas aeruginosa* zinc homeostasis: key issues for an opportunistic pathogen. *Biochim Biophys Acta Gene Regul Mech* 1862:722–733. <https://doi.org/10.1016/j.bbaggm.2018.01.018>.
26. Hyslop PA, Hinshaw DB, Scraufstatter IU, Cochrane CG, Kunz S, Vosbeck K. 1995. Hydrogen peroxide as a potent bacteriostatic antibiotic: implications for host defense. *Free Radic Biol Med* 19:31–37. [https://doi.org/10.1016/0891-5849\(95\)00005-1](https://doi.org/10.1016/0891-5849(95)00005-1).
27. Vinckx T, Mattheijs S, Cornelis P. 2008. Loss of the oxidative stress regulator OxyR in *Pseudomonas aeruginosa* PAO1 impairs growth under iron-limited conditions. *FEMS Microbiol Lett* 288:258–265. <https://doi.org/10.1111/j.1574-6968.2008.01360.x>.
28. Wei Q, Minh PN, Dotsch A, Hildebrand F, Panmanee W, Elfarash A, Schulz S, Plaisance S, Charlier D, Hassett D, Haussler S, Cornelis P. 2012. Global regulation of gene expression by OxyR in an important human opportunistic pathogen. *Nucleic Acids Res* 40:4320–4333. <https://doi.org/10.1093/nar/gks017>.
29. Melstrom KA, Jr, Kozlowski R, Hassett DJ, Suzuki H, Bates DM, Gamelli RL, Shankar R. 2007. Cytotoxicity of *Pseudomonas* secreted exotoxins requires OxyR expression. *J Surg Res* 143:50–57. <https://doi.org/10.1016/j.jss.2007.02.046>.
30. Clapham DE. 2007. Calcium signaling. *J Cell* 131:1047–1058. <https://doi.org/10.1016/j.cell.2007.11.028>.
31. Trebak M, Kinet J-P. 2019. Calcium signalling in T cells. *Nat Rev Immunol* 19:154–169. doi:10.1038/s41577-018-0110-7. <https://doi.org/10.1038/s41577-018-0110-7>.
32. Giorgi C, Marchi S, Pinton P. 2018. The machineries, regulation and cellular functions of mitochondrial calcium. *Nat Rev Mol Cell Biol* 19:713–730. <https://doi.org/10.1038/s41580-018-0052-8>.
33. Bhagirath AY, Li Y, Somayajula D, Dadashi M, Badr S, Duan K. 2016. Cystic fibrosis lung environment and *Pseudomonas aeruginosa* infection. *BMC Pulm Med* 16:174–174. <https://doi.org/10.1186/s12890-016-0339-5>.
34. Knowles MR, Robinson JM, Wood RE, Pue CA, Mentz WM, Wager GC, Gatzky JT, Boucher RC. 1997. Ion composition of airway surface liquid of patients with cystic fibrosis as compared with normal and disease-control subjects. *J Clin Invest* 100:2588–2595. <https://doi.org/10.1172/JCI119802>.
35. Roomans GM. 1986. Calcium and cystic fibrosis. *Scan Electron Microsc* 1986:165–178.
36. Blomfield J, Warton KL, Brown JM. 1973. Flow rate and inorganic components of submandibular saliva in cystic fibrosis. *Arch Dis Child* 48:267–274. <https://doi.org/10.1136/adc.48.4.267>.
37. Palmer KL, Mashburn LM, Singh PK, Whiteley M. 2005. Cystic fibrosis sputum supports growth and cues key aspects of *Pseudomonas aeruginosa* physiology. *J Bacteriol* 187:5267–5277. <https://doi.org/10.1128/JB.187.15.5267-5277.2005>.
38. Palmer KL, Aye LM, Whiteley M. 2007. Nutritional cues control *Pseudomonas aeruginosa* multicellular behavior in cystic fibrosis sputum. *J Bacteriol* 189:8079–8087. <https://doi.org/10.1128/JB.01138-07>.
39. Shiner E, Terentyev D, Bryan A, Sennoune S, Martinez-Zaguilan R, Li G, Gyorke S, Williams S, Rumbaugh K. 2006. *Pseudomonas aeruginosa* autoinducer modulates host cell responses through calcium signalling. *Cell Microbiol* 8:1601–1610. <https://doi.org/10.1111/j.1462-5822.2006.00734.x>.
40. Denning GM, Railsback MA, Rasmussen GT, Cox CD, Britigan BE. 1998. *Pseudomonas pyocyanine* alters calcium signaling in human airway epithelial cells. *Am J Physiol* 274:L893–L900. <https://doi.org/10.1152/ajplung.1998.274.6.L893>.
41. Sarkisova SA, Lotlikar SR, Guragain M, Kubat R, Cloud J, Franklin MJ, Patrauchan MA. 2014. A *Pseudomonas aeruginosa* EF-hand protein, EfpP (PA4107), modulates stress responses and virulence at high calcium concentration. *PLoS One* 9:e98985. <https://doi.org/10.1371/journal.pone.0098985>.
42. Sarkisova S, Patrauchan MA, Berglund D, Nivens DE, Franklin MJ. 2005. Calcium-induced virulence factors associated with the extracellular matrix of mucoid *Pseudomonas aeruginosa* biofilms. *J Bacteriol* 187:4327–4337. <https://doi.org/10.1128/JB.187.13.4327-4337.2005>.
43. Khanam S, Guragain M, Lenaburg DL, Kubat R, Patrauchan MA. 2017. Calcium induces tobramycin resistance in *Pseudomonas aeruginosa* by regulating RND efflux pumps. *Cell Calcium* 61:32–43. <https://doi.org/10.1016/j.ceca.2016.11.004>.
44. Basso P, Ragno M, Elsen S, Reboud E, Golovkine G, Bouillot S, Huber P, Lory S, Faudry E, Attrée I. 2017. *Pseudomonas aeruginosa* pore-forming exolysin and type IV pili cooperate to induce host cell lysis. *mBio* 8: e02250-16. <https://doi.org/10.1128/mBio.02250-16>.
45. Cigana C, Lorè NI, Riva C, De Fino I, Spagnuolo L, Sipione B, Rossi G, Nonis A, Cabrini G, Bragonzi A. 2016. Tracking the immunopathological response to *Pseudomonas aeruginosa* during respiratory infections. *Sci Rep* 6:21465–21465. <https://doi.org/10.1038/srep21465>.
46. Cigana C, Curcuro L, Leone MR, Ierano T, Lore NI, Bianconi I, Silipo A, Cozzolino F, Lanzetta R, Molinaro A, Bernardini ML, Bragonzi A. 2009. *Pseudomonas aeruginosa* exploits lipid A and mucopeptides modification as a strategy to lower innate immunity during cystic fibrosis lung infection. *PLoS One* 4:e8439. <https://doi.org/10.1371/journal.pone.0008439>.
47. Guragain M, Lenaburg DL, Moore FS, Reutlinger I, Patrauchan MA. 2013. Calcium homeostasis in *Pseudomonas aeruginosa* requires multiple transporters and modulates swarming motility. *Cell Calcium* 54:350–361. <https://doi.org/10.1016/j.ceca.2013.08.004>.
48. Guragain M, King MM, Williamson KS, Perez-Osorio AC, Akiyama T, Khanam S, Patrauchan MA, Franklin MJ. 2016. The *Pseudomonas aeruginosa* PAO1 two-component regulator CarSR regulates calcium homeostasis and calcium-induced virulence factor production through its regulatory targets CarO and CarP. *J Bacteriol* 198:951–963. <https://doi.org/10.1128/JB.00963-15>.
49. Kreamer NN, Costa F, Newman DK. 2015. The ferrous iron-responsive BqsRS two-component system activates genes that promote cationic stress tolerance. *mBio* 6:e02549-14. <https://doi.org/10.1128/mBio.02549-14>.
50. Kreamer NN, Wilks JC, Marlow JJ, Coleman ML, Newman DK. 2012. BqsR/BqsS constitute a two-component system that senses extracellular Fe(II) in *Pseudomonas aeruginosa*. *J Bacteriol* 194:1195–1204. <https://doi.org/10.1128/JB.05634-11>.
51. Cornforth DM, Dees JL, Ibberson CB, Huse HK, Mathiesen IH, Kirketerp-Møller K, Wolcott RD, Rumbaugh KP, Bjarnsholt T, Whiteley M. 2018. *Pseudomonas aeruginosa* transcriptome during human infection. *Proc Natl Acad Sci U S A* 115:E5125–E5134. <https://doi.org/10.1073/pnas.1717525115>.
52. Barrett T, Wilhite SE, Ledoux P, Evangelista C, Kim IF, Tomashevsky M, Marshall KA, Phillippy KH, Sherman PM, Holko M, Yefanov A, Lee H, Zhang N, Robertson CL, Serova N, Davis S, Soboleva A. 2013. NCBI GEO: archive for functional genomics data sets—update. *Nucleic Acids Res* 41: D991–D995. <https://doi.org/10.1093/nar/gks1193>.
53. Cambray G, Sanchez-Alberola N, Campoy S, Guerin E, Da Re S, Gonzalez-Zorn B, Ploy MC, Barbe J, Mazel D, Erill I. 2011. Prevalence of SOS-mediated control of integron integrase expression as an adaptive trait of chromosomal and mobile integrons. *Mob DNA* 2:6. <https://doi.org/10.1186/1759-8753-2-6>.
54. Williams P, Cámara M. 2009. Quorum sensing and environmental adaptation in *Pseudomonas aeruginosa*: a tale of regulatory networks and multifunctional signal molecules. *Curr Opin Microbiol* 12:182–191. <https://doi.org/10.1016/j.mib.2009.01.005>.
55. Liu Y-C, Chan K-G, Chang C-Y. 2015. Modulation of host biology by *Pseudomonas aeruginosa* quorum sensing signal molecules: messengers or traitors. *Front Microbiol* 6:1226. <https://doi.org/10.3389/fmicb.2015.01226>.
56. Cirz RT, O'Neill BM, Hammond JA, Head SR, Romesberg FE. 2006. Defining the *Pseudomonas aeruginosa* SOS response and its role in the global response to the antibiotic ciprofloxacin. *J Bacteriol* 188:7101–7110. <https://doi.org/10.1128/JB.00807-06>.
57. Conway T, Creecy JP, Maddox SM, Grissom JE, Conkle TL, Shadid TM, Teramoto J, San Miguel P, Shimada T, Ishihama A, Mori H, Wanner BL. 2014. Unprecedented high-resolution view of bacterial operon architecture revealed by RNA sequencing. *mBio* 5:e01442-14. <https://doi.org/10.1128/mBio.01442-14>.
58. Pessi G, Haas D. 2000. Transcriptional control of the hydrogen cyanide biosynthetic genes *hcnABC* by the anaerobic regulator ANR and the quorum-sensing regulators LasR and RhIR in *Pseudomonas aeruginosa*. *J Bacteriol* 182:6940–6949. <https://doi.org/10.1128/jb.182.24.6940-6949.2000>.
59. Kiratisin P, Tucker KD, Passador L. 2002. LasR, a transcriptional activator of *Pseudomonas aeruginosa* virulence genes, functions as a multimer. *J Bacteriol* 184:4912–4919. <https://doi.org/10.1128/jb.184.17.4912-4919.2002>.

60. Gambello MJ, Kaye S, Iglewski BH. 1993. LasR of *Pseudomonas aeruginosa* is a transcriptional activator of the alkaline protease gene (apr) and an enhancer of exotoxin A expression. *Infect Immun* 61:1180–1184. <https://doi.org/10.1128/IAI.61.4.1180-1184.1993>.
61. Mukherjee S, Moustafa D, Smith CD, Goldberg JB, Bassler BL. 2017. The RhlR quorum-sensing receptor controls *Pseudomonas aeruginosa* pathogenesis and biofilm development independently of its canonical homoserine lactone autoinducer. *PLoS Pathog* 13:e1006504. <https://doi.org/10.1371/journal.ppat.1006504>.
62. Pearson JP, Passador L, Iglewski BH, Greenberg EP. 1995. A second N-acylhomoserine lactone signal produced by *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* 92:1490–1494. <https://doi.org/10.1073/pnas.92.5.1490>.
63. McKnight SL, Iglewski BH, Pesci EC. 2000. The *Pseudomonas* quinolone signal regulates *rhl* quorum sensing in *Pseudomonas aeruginosa*. *J Bacteriol* 182:2702–2708. <https://doi.org/10.1128/jb.182.10.2702-2708.2000>.
64. Lee J, Wu J, Deng Y, Wang J, Wang C, Wang J, Chang C, Dong Y, Williams P, Zhang L-H. 2013. A cell-cell communication signal integrates quorum sensing and stress response. *Nat Chem Biol* 9:339–343. <https://doi.org/10.1038/nchembio.1225>.
65. Lhospice S, Gomez NO, Ouerdane L, Brutesco C, Ghssein G, Hajjar C, Liratni A, Wang S, Richaud P, Bleves S, Ball G, Borezée-Durant E, Lobinski R, Pignol D, Arnoux P, Voulhoux R. 2017. *Pseudomonas aeruginosa* zinc uptake in chelating environment is primarily mediated by the metallophore pseudopaline. *Sci Rep* 7:17132. <https://doi.org/10.1038/s41598-017-16765-9>.
66. Kilbourn JP. 1984. Composition of sputum from patients with cystic fibrosis. *Curr Microbiol* 11:19–22. <https://doi.org/10.1007/BF01567570>.
67. Pezzoni M, Tribelli PM, Pizarro RA, Lopez NI, Costa CS. 2016. Exposure to low UVA doses increases KatA and KatB catalase activities, and confers cross-protection against subsequent oxidative injuries in *Pseudomonas aeruginosa*. *Microbiology (Reading)* 162:855–864. <https://doi.org/10.1099/mic.0.000268>.
68. Hu ML, Tappel AL. 1992. Potentiation of oxidative damage to proteins by ultraviolet-A and protection by antioxidants. *Photochem Photobiol* 56:357–363. <https://doi.org/10.1111/j.1751-1097.1992.tb02171.x>.
69. Cabiscot CE, Tamarit SJ, Ros SJ. 2000. Oxidative stress in bacteria and protein damage by reactive oxygen species. *Int Microbiol* 3:3–8.
70. Hocquet D, Llanes C, Thouverez M, Kulasekara HD, Bertrand X, Plesiat P, Mazel D, Miller SI. 2012. Evidence for induction of integron-based antibiotic resistance by the SOS response in a clinical setting. *PLoS Pathog* 8:e1002778. <https://doi.org/10.1371/journal.ppat.1002778>.
71. Held K, Ramage E, Jacobs M, Gallagher L, Manoil C. 2012. Sequence-verified two-allele transposon mutant library for *Pseudomonas aeruginosa* PAO1. *J Bacteriol* 194:6387–6389. <https://doi.org/10.1128/JB.01479-12>.
72. King M, Kayastha B, Franklin M, Patrauchan MA. 2020. Calcium signaling regulates virulence in human pathogens. In Islam MS (ed), *Calcium signaling*. Springer, New York, NY.
73. Mares SE, King MM, Kubo A, Khanov AA, Lutter EI, Youssef N, Patrauchan MA. 2020. carP, encoding a Ca²⁺-regulated putative phosphatase, is evolutionarily conserved in *Pseudomonas aeruginosa* and has potential as a biomarker. *Microbiology (Reading)* 167:micr001004. <https://doi.org/10.1099/mic.0.001004>.
74. Linley E, Denyer SP, McDonnell G, Simons C, Maillard J-Y. 2012. Use of hydrogen peroxide as a biocide: new consideration of its mechanisms of biocidal action. *J Antimicrob Chemother* 67:1589–1596. <https://doi.org/10.1093/jac/dks129>.
75. Nauseef WM. 2007. How human neutrophils kill and degrade microbes: an integrated view. *Immunol Rev* 219:88–102. <https://doi.org/10.1111/j.1600-065X.2007.00550.x>.
76. Turner KH, Everett J, Trivedi U, Rumbaugh KP, Whiteley M. 2014. Requirements for *Pseudomonas aeruginosa* acute burn and chronic surgical wound infection. *PLoS Genet* 10:e1004518. <https://doi.org/10.1371/journal.pgen.1004518>.
77. Ciofu O, Tolker-Nielsen T. 2019. Tolerance and resistance of *Pseudomonas aeruginosa* biofilms to antimicrobial agents—how *P. aeruginosa* can escape antibiotics. *Front Microbiol* 10:913–913. <https://doi.org/10.3389/fmicb.2019.00913>.
78. Broder UN, Jaeger T, Jenal U. 2016. LadS is a calcium-responsive kinase that induces acute-to-chronic virulence switch in *Pseudomonas aeruginosa*. *Nat Microbiol* 2:16184. <https://doi.org/10.1038/nmicrobiol.2016.184>.
79. Lorin MI, Gaerlan PF, Mandel ID, Denning CR. 1976. Composition of nasal secretion in patients with cystic fibrosis. *J Lab Clin Med* 88:114–117.
80. Klünemann T, Preuß A, Adamczak J, Rosa LFM, Harnisch F, Layer G, Blankenfeldt W. 2019. Crystal structure of dihydro-heme d1 dehydrogenase NirN from *Pseudomonas aeruginosa* reveals amino acid residues essential for catalysis. *J Mol Biol* 431:3246–3260. <https://doi.org/10.1016/j.jmb.2019.05.046>.
81. Neupane DP, Fullam SH, Chacón KN, Yukl ET. 2019. Crystal structures of AztD provide mechanistic insights into direct zinc transfer between proteins. *Commun Biol* 2:308. <https://doi.org/10.1038/s42003-019-0542-z>.
82. Severi E, Muller A, Potts JR, Leech A, Williamson D, Wilson KS, Thomas GH. 2008. Sialic acid mutarotation is catalyzed by the *Escherichia coli* beta-propeller protein YjhT. *J Biol Chem* 283:4841–4849. <https://doi.org/10.1074/jbc.M70782200>.
83. Shin S, Ha N-C, Oh B-C, Oh T-K, Oh B-H. 2001. Enzyme mechanism and catalytic property of β propeller phytase. *Structure* 9:851–858. [https://doi.org/10.1016/s0969-2126\(01\)00637-2](https://doi.org/10.1016/s0969-2126(01)00637-2).
84. Lasica AM, Goulas T, Mizgalska D, Zhou X, de Diego I, Ksiazek M, Madej M, Guo Y, Guevara T, Nowak M, Potempa B, Goel A, Sztukowska M, Prabhakar AT, Bzowska M, Widziolek M, Thogersen IB, Enghild JJ, Simonian M, Kulczyk AW, Nguyen KA, Potempa J, Gomis-Ruth FX. 2016. Structural and functional probing of PorZ, an essential bacterial surface component of the type-IX secretion system of human oral-microbiomic *Porphyromonas gingivalis*. *Sci Rep* 6:37708. <https://doi.org/10.1038/srep37708>.
85. Siamer S, Gaubert S, Boureau T, Brisset M-N, Barny M-A. 2013. Mutational analysis of a predicted double β -propeller domain of the DspA/E effector of *Erwinia amylovora*. *FEMS Microbiol Lett* 342:54–61. <https://doi.org/10.1111/1574-6968.12108>.
86. Kumar V, Yadav AN, Verma P, Sangwan P, Saxena A, Kumar K, Singh B. 2017. β -Propeller phytases: diversity, catalytic attributes, current developments and potential biotechnological applications. *Int J Biol Macromol* 98:595–609. <https://doi.org/10.1016/j.ijbiomac.2017.01.134>.
87. Kania E, Roest G, Vervliet T, Parys JB, Bultynck G. 2017. IP(3) receptor-mediated calcium signaling and its role in autophagy in cancer. *Front Oncol* 7:140–140. <https://doi.org/10.3389/fonc.2017.00140>.
88. Baker JA, Wong W-C, Eisenhaber B, Warwicker J, Eisenhaber F. 2017. Charged residues next to transmembrane regions revisited: “positive-inside rule” is complemented by the “negative inside depletion/outside enrichment rule”. *BMC Biol* 15:66–66. <https://doi.org/10.1186/s12915-017-0404-4>.
89. Patriquin GM, Banin E, Gilmour C, Tuchman R, Greenberg EP, Poole K. 2008. Influence of quorum sensing and iron on twitching motility and biofilm formation in *Pseudomonas aeruginosa*. *J Bacteriol* 190:662–671. <https://doi.org/10.1128/JB.01473-07>.
90. Halmerbauer G, Arri S, Schierl M, Strauch E, Koller DY. 2000. The relationship of eosinophil granule proteins to ions in the sputum of patients with cystic fibrosis. *Clin Exp Allergy* 30:1771–1776. <https://doi.org/10.1046/j.1365-2222.2000.00988.x>.
91. Berchtold MW, Brinkmeier H, Müntener M. 2000. Calcium ion in skeletal muscle: its crucial role for muscle function, plasticity, and disease. *Physiol Rev* 80:1215–1265. <https://doi.org/10.1152/physrev.2000.80.3.1215>.
92. McCarthy JE, Brimacombe R. 1994. Prokaryotic translation: the interactive pathway leading to initiation. *Trends Genet* 10:402–407. [https://doi.org/10.1016/0168-9525\(94\)90057-4](https://doi.org/10.1016/0168-9525(94)90057-4).
93. Boni IV, Isaeva DM, Musychenko ML, Tzareva NV. 1991. Ribosome-messenger recognition: mRNA target sites for ribosomal protein S1. *Nucleic Acids Res* 19:155–162. <https://doi.org/10.1093/nar/19.1.155>.
94. Johnson L, Mulcahy H, Kanevets U, Shi Y, Lewenza S. 2012. Surface-localized spermidine protects the *Pseudomonas aeruginosa* outer membrane from antibiotic treatment and oxidative stress. *J Bacteriol* 194:813–826. <https://doi.org/10.1128/JB.05230-11>.
95. Balasubramanian D, Schnepel L, Kumari H, Mathee K. 2013. A dynamic and intricate regulatory network determines *Pseudomonas aeruginosa* virulence. *Nucleic Acids Res* 41:1–20. <https://doi.org/10.1093/nar/gks1039>.
96. Mall MA, Hartl D. 2014. CFTR: cystic fibrosis and beyond. *Eur Respir J* 44:1042–1054. <https://doi.org/10.1183/09031936.00228013>.
97. Conese M, Castellani S, D’Oria S, Di Gioia S, Montemurro P. 2017. Role of neutrophils in cystic fibrosis lung disease, p 119–141. In Khajaj MA (ed), *Role of neutrophils in disease pathogenesis*. InTech, Rijeka, Croatia.
98. Jacobs MA, Alwood A, Thaipisuttikul I, Spencer D, Haugen E, Ernst S, Will O, Kaul R, Raymond C, Levy R, Liu CR, Guenther D, Bovee D, Olson MV, Manoil C. 2003. Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* 100:14339–14344. <https://doi.org/10.1073/pnas.2036282100>.

99. Gibson DG, Young L, Chuang R-Y, Venter JC, Hutchison CA, Smith HO. 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Methods* 6:343–345. <https://doi.org/10.1038/nmeth.1318>.
100. Hoang TT, Kutchma AJ, Becher A, Schweizer HP. 2000. Integration-proficient plasmids for *Pseudomonas aeruginosa*: site-specific integration and use for engineering of reporter and expression strains. *Plasmid* 43:59–72. <https://doi.org/10.1006/plas.1999.1441>.
101. Hoang TT, Karkhoff-Schweizer RR, Kutchma AJ, Schweizer HP. 1998. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* 212:77–86. [https://doi.org/10.1016/s0378-1119\(98\)00130-9](https://doi.org/10.1016/s0378-1119(98)00130-9).
102. Ramarao N, Nielsen-Leroux C, Lereclus D. 2012. The insect *Galleria mellonella* as a powerful infection model to investigate bacterial pathogenesis. *J Vis Exp* 2012:e4392. <https://doi.org/10.3791/4392>.
103. Jander G, Rahme LG, Ausubel FM. 2000. Positive correlation between virulence of *Pseudomonas aeruginosa* mutants in mice and insects. *J Bacteriol* 182:3843–3845. <https://doi.org/10.1128/jb.182.13.3843-3845.2000>.
104. Winsor GL, Griffiths EJ, Lo R, Dhillon BK, Shay JA, Brinkman F. 2016. Enhanced annotations and features for comparing thousands of *Pseudomonas* genomes in the *Pseudomonas* genome database. *Nucleic Acids Res* 44:D646–D653. <https://doi.org/10.1093/nar/gkv1227>.
105. Solovyev V, Salamov A. 2011. Automatic annotation of microbial genomes and metagenomic sequences its applications in agriculture, p 61–78. *In* Li RW (ed), *Metagenomics and its applications in agriculture, biomedicine and environmental studies*. Nova Science Publishers, Hauppauge, NY.
106. Munch R, Hiller K, Grote A, Scheer M, Klein J, Schobert M, Jahn D. 2005. Virtual Footprint and PRODORIC: an integrative framework for regulon prediction in prokaryotes. *Bioinformatics* 21:4187–4189. <https://doi.org/10.1093/bioinformatics/bti635>.
107. Krogh A, Larsson B, von Heijne G, Sonnhammer EL. 2001. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J Mol Biol* 305:567–580. <https://doi.org/10.1006/jmbi.2000.4315>.
108. Lotlikar SR, Kayastha BB, Vullo D, Khanam SS, Braga RE, Murray AB, McKenna R, Supuran CT, Patrauchan MA. 2019. *Pseudomonas aeruginosa* β -carbonic anhydrase, psCA1, is required for calcium deposition and contributes to virulence. *Cell Calcium* 84:102080. <https://doi.org/10.1016/j.ceca.2019.102080>.
109. Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25:402–408. <https://doi.org/10.1006/meth.2001.1262>.
110. Smith AW, Iglewski BH. 1989. Transformation of *Pseudomonas aeruginosa* by electroporation. *Nucleic Acids Res* 17:10509–10509. <https://doi.org/10.1093/nar/17.24.10509>.
111. Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrenner P, Hickey MJ, Brinkman FS, Hufnagle WO, Kowalik DJ, Lagrou M, Garber RL, Goltry L, Tolentino E, Westbrook-Wadman S, Yuan Y, Brody LL, Coulter SN, Folger KR, Kas A, Larbig K, Lim R, Smith K, Spencer D, Wong GK, Wu Z, Paulsen IT, Reizer J, Saier MH, Hancock RE, Lory S, Olson MV. 2000. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* 406:959–964. <https://doi.org/10.1038/35023079>.
112. Otori CA, Dubern J-F, Chhabra SR, Cámara M, Hardie K, Williams P, Barrett DA. 2011. Simultaneous quantitative profiling of N-acyl-L-homoserine lactone and 2-alkyl-4(1H)-quinolone families of quorum-sensing signaling molecules using LC-MS/MS. *Anal Bioanal Chem* 399:839–850. <https://doi.org/10.1007/s00216-010-4341-0>.
113. Duan K, Dammel C, Stein J, Rabin H, Surette MG. 2003. Modulation of *Pseudomonas aeruginosa* gene expression by host microflora through interspecies communication. *Mol Microbiol* 50:1477–1491. <https://doi.org/10.1046/j.1365-2958.2003.03803.x>.
114. Savli H, Karadenizli A, Kolayli F, Gundes S, Ozbek U, Vahaboglu H. 2003. Expression stability of six housekeeping genes: a proposal for resistance gene quantification studies of *Pseudomonas aeruginosa* by real-time quantitative RT-PCR. *J Med Microbiol* 52:403–408. <https://doi.org/10.1099/jmm.0.05132-0>.
115. Relman DA, Schmidt TM, MacDermott RP, Falkow S. 1992. Identification of the uncultured *Bacillus* of Whipple's disease. *N Engl J Med* 327:293–301. <https://doi.org/10.1056/NEJM199207303270501>.
116. Turner S, Pryer KM, Miao VP, Palmer JD. 1999. Investigating deep phylogenetic relationships among cyanobacteria and plastids by small subunit rRNA sequence analysis. *J Eukaryot Microbiol* 46:327–338. <https://doi.org/10.1111/j.1550-7408.1999.tb04612.x>.