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MOLECULAR PATHOGENESIS

The Atypical Response Regulator AtvR Is a New Player in *Pseudomonas aeruginosa* Response to Hypoxia and Virulence

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ABSTRACT Two-component systems are widespread in bacteria, allowing adaptation to environmental changes. The classical pathway is composed of a histidine kinase that phosphorylates an aspartate residue in the cognate response regulator (RR). RRs lacking the phosphorylatable aspartate also occur, but their function and contribution during host-pathogen interactions are poorly characterized. AtvR (PA14_26570) is the only atypical response regulator with a DNA-binding domain in the opportunistic pathogen *Pseudomonas aeruginosa*. Macrophage infection with the *atvR* mutant strain resulted in higher levels of tumor necrosis factor alpha secretion as well as increased bacterial clearance compared to those for macrophages infected with the wild-type strain. In an acute pneumonia model, mice infected with the *atvR* mutant presented increased amounts of proinflammatory cytokines, increased neutrophil recruitment to the lungs, reductions in bacterial burdens, and higher survival rates in comparison with the findings for mice infected with the wild-type strain. Further, several genes involved in hypoxia/anoxia adaptation were upregulated upon *atvR* overexpression, as seen by high-throughput transcriptome sequencing (RNA-Seq) analysis. In addition, *atvR* was more expressed in hypoxia in the presence of nitrate and required for full expression of nitrate reductase genes, promoting bacterial growth under this condition. Thus, AtvR would be crucial for successful infection, aiding *P. aeruginosa* survival under conditions of low oxygen tension in the host. Taken together, our data demonstrate that the atypical response regulator AtvR is part of the repertoire of transcriptional regulators involved in the lifestyle switch from aerobic to anaerobic conditions. This finding increases the complexity of regulation of one of the central metabolic pathways that contributes to *Pseudomonas* ubiquity and versatility.

KEYWORDS atypical response regulator, hypoxia, macrophage, *Pseudomonas aeruginosa*, response regulator, virulence, anaerobic respiration, denitrification

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Two-component systems (TCSs) are signaling pathways that are widespread in bacteria and that sense and respond to environmental changes. These systems are also found in *Archaea* and *Eukarya*, but there is no evidence of their presence in animals (1), making these pathways good anti-infective drug targets.

The canonical TCS pathway has a histidine kinase that is autophosphorylated in response to an input signal and transfers its phosphoryl group to a cognate response regulator (RR). Once phosphorylated, the RR becomes active and initiates an appropriate cellular response to the initial stimulus, often by modulating gene expression (2–4).

Response regulators have a REC domain with a conserved secondary structure

[$(\beta\alpha)_5$] important for their activity. The REC domain classically possesses residues critical for RR activation, which includes an aspartate at the end of the third β-strand that can be phosphorylated by the histidine kinase (HK) (5). Despite their conservation, some RRs lack this aspartate residue or other conserved features and thus are considered atypical response regulators. A particular subgroup of atypical RRs, named aspartate-less receivers (ALR), was recently described, and proteins that belong to this subfamily have the replacement of an aspartate by any other amino acid (with the exception of tryptophan) in the phosphorylatable site (6).

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium able to cause disease in several hosts (7–9) and one of the major causes of hospital-acquired infections, such as acute pneumonia in ventilator-assisted patients, which is associated with high rates of mortality. This bacterium is also one of the major pathogens in the lungs of individuals with cystic fibrosis, leading to chronic pulmonary infection and impairment of quality of life (10–12).

Macrophages are at the first line of defense, recognizing the pathogen and orchestrating the host immune response against *P. aeruginosa* infection. The recognition of pathogen-associated molecular patterns (PAMPs) relies on their association with the cognate Toll-like receptors (TLR) in the immune cells, stimulating several signaling cascades, such as the proinflammatory NF-κB and mitogen-activated protein kinase pathways, leading to the production of cytokines, including tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), and IL-1 (13).

During acute lung injury, both airway edema and atelectasis result in low-ventilation and low-perfusion conditions, leading to a hypoxic environment within the affected areas of the lungs (14–16). The hostility posed by this environment induces several signaling pathways in *P. aeruginosa*, including the TCS NarXL. At the top of the anoxic response regulatory cascade is the transcriptional regulator Anr, whose activity is dependent on low oxygen levels. Anr is essential for *P. aeruginosa* growth in anoxia (17), inducing expression of the nitrate reductase gene (*nar*) clusters, important for anaerobic respiration via denitrification, as well as the transcriptional regulators *narXL* and *dnr*. The NarXL TCS responds to nitrate and nitrite, directly activates expression of the *nar* clusters, and indirectly regulates other denitrification genes via Dnr, a transcriptional factor that positively regulates the *nir* (nitrite reductase), *nor* (nitric oxide reductase), and *nos* (nitrous oxide reductase) gene clusters (18, 19). The timing of expression of the denitrification genes is consistent with the position of the role of their products in this electron transport chain. In the absence of oxygen and nitrate, *P. aeruginosa* can also use a fermentative state using the arginine deiminase pathway (*arc* gene clusters) or the pyruvate pathway (18). In hypoxia, the virulence factor pyocyanin also acts as a terminal electron receptor (18, 20).

P. aeruginosa has one of the largest sets of two-component systems known in bacteria, and these TCSs certainly contribute to its ability to thrive in a wide range of niches, including humans. *P. aeruginosa* PA14 presents at least 64 sensor kinases and 76 response regulators (2, 21–23). Using a bioinformatics approach, Maule and collaborators suggested that four *P. aeruginosa* RRs belong to the ALR subfamily (PA14_30650, PA14_26570, PA14_64050, PA14_03470) (6). About 4% of all analyzed REC domains in bacteria belong to this subfamily, highlighting the particular importance of the further characterization of these still unappreciated RRs.

No atypical RR in *P. aeruginosa* has been characterized to date, and in fact, these RRs are poorly characterized in other bacteria as well. Here we studied PA14_26570, a member of the ALR subfamily that possesses a DNA-binding domain. We show that the atypical RR PA14_26570, here named AtvR (atypical virulence-related response regulator), is important for *P. aeruginosa* virulence, activating the transcription of several genes involved in the hypoxia/anoxia response, as well as virulence-related genes. We also found that a strain lacking AtvR is less virulent in both *in vitro* and *in vivo* infection models. Taken together, our data demonstrate that AtvR should be underscored as an important contributor to the bacterium's arsenal against host defenses.

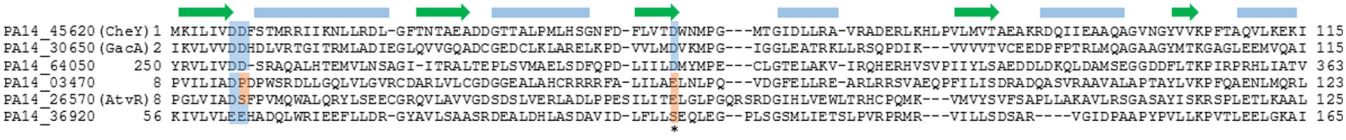

FIG 1 Multiple-sequence alignment of REC domains. Alignment of the REC domains from selected *P. aeruginosa* response regulators was performed with MUSCLE software. Green arrows above the sequences, β-sheets; blue rectangles, α-helices in the secondary structure; blue highlights, conserved residues in the catalytic active pocket in classical RRs; orange highlights, divergent residues in the atypical RRs PA14_03470, AtvR, and PA14_36920; asterisk, the phosphorylatable aspartate in classical RRs replaced by a glutamate in atypical RRs. The numbers correspond to the amino acid positions in the primary structure of each protein. The sequences were obtained from www.pseudomonas.com.

RESULTS

AtvR (PA14_26570) belongs to the ALR subfamily of response regulators. A multiple-sequence alignment using *P. aeruginosa* RR REC domains was performed. This domain's secondary structure is conserved across the RRs, as is the position of the aspartate that receives the phosphoryl group at the end of the third β-strand in classical RRs (Fig. 1, marked with an asterisk) (5). The CheY (PA14_45620) secondary structure was used as a representative of a typical REC domain in this analysis. In addition to the previously identified atypical response regulators in *P. aeruginosa*, we identified PA14_36920, which belongs to the aspartate-less receiver (ALR) subfamily (Fig. 1). In contrast to Maule et al. (6), who characterized the members of the ALR subfamily in several bacteria, a more refined analysis of only *P. aeruginosa* proteins was performed here. The proteins PA14_30650 and PA14_64050, classified by Maule et al. (6) to be atypical RRs, have an aspartate at the end of their third β-strand, characterizing them as typical RRs (Fig. 1). Moreover, PA14_30650 is the well-characterized GacA RR, phosphorylated by the HK GacS (24). PA14_64050 is annotated as GcbA, an RR containing a GGDEF domain in its C terminus (25).

AtvR sequence analysis showed two conserved domains, a REC domain and a helix-turn-helix (HTH) DNA-binding domain. This architectural structure is typical of that of the NarL family of response regulators (2). AtvR has one substitution in a conserved amino acid that is important for the stabilization of the phosphorylated aspartate (D15S) and in the phosphorylatable aspartate itself (D61E) (Fig. 1). Replacement of an aspartate by a glutamate usually leads to an always-on state by mimicking an active conformation state (26–28); therefore, the activity of AtvR is inferred to be independent of phosphorylation. Hence, AtvR activity should be constitutive and independent of a cognate histidine kinase, and its presence in the cell is probably enough for its transcription-inducing activity.

AtvR is important for virulence against macrophages *in vitro*. Macrophages play an important role against *P. aeruginosa* infections, leading to bacterial clearance and cytokine production. Pathogenic bacteria use several strategies to adapt to the host, among them being the inhibition of macrophage activation, which results in the reduction of both cytokine secretion and bacterial clearance (29). We asked whether AtvR would lead to bacterium adaptation to macrophages during infection, thus increasing *P. aeruginosa* virulence. To answer this question, we used an *in vitro* infection model with J774 macrophages incubated with the wild-type bacteria or the *atvR* deletion mutant (the Δ*atvR* mutant). First, we ascertained that the Δ*atvR* mutant grows as well as the wild type under conditions similar to those used in the macrophage infection assay (see Fig. S1 in the supplemental material).

Macrophages were infected and incubated for 1 h prior to gentamicin addition to kill extracellular bacteria. After 30 min (time zero), the cells were washed and fresh medium without antibiotics was added. The number of CFU of extracellular bacteria in the culture supernatants was assessed at 1 and 2 h (Fig. 2A). Macrophages were washed and lysed at 0, 1, and 2 h, and the released bacteria were enumerated (intracellular bacteria) (Fig. 2B). The counts of phagocytosed bacteria were similar in macrophages infected with the wild type and those infected with the Δ*atvR* mutant at all time points, suggesting no differences in the internalization process. However, we observed a

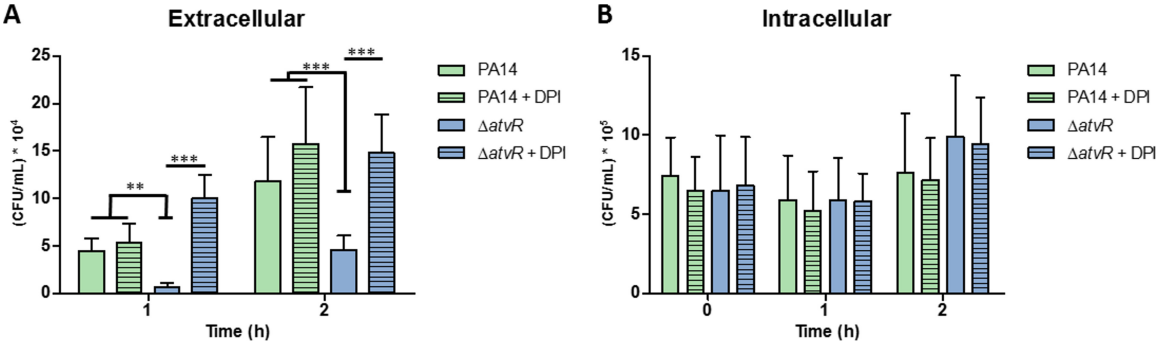

FIG 2 AtvR is important for resistance to macrophages and affects their activation. J774 macrophages were treated with DPI for 4 h. Control and DPI-treated macrophages were incubated with *P. aeruginosa* PA14 or the Δ*atvR* mutant at an MOI of 10. (A) The cells were washed with PBS, R-10 medium containing 200 μg/ml gentamicin was added to the wells, the plate was incubated for 30 min, the wells were washed, and fresh medium was added. At the indicated time points, the supernatants were collected and diluted, and the number of CFU of extracellular bacteria was determined. **, $P < 0.01$; ***, $P < 0.001$. (B) The macrophages were infected, treated as described in the legend to panel A, and lysed with Triton X-100. The released bacteria were diluted, and the number of CFU of intracellular bacteria was determined.

difference in the numbers of CFU of extracellular bacteria of the Δ*atvR* mutant and wild-type strain PA14 (Fig. 2A), indicating a role of AtvR in counteracting macrophage-mediated killing, leading to increased bacterial survival or reducing the amounts of harmful molecules produced by macrophages, such as reactive oxygen species. To uncover the oxidative state of macrophages responding to wild-type PA14 or the Δ*atvR* strain, they were incubated with diphenyleneiodonium (DPI), an NADPH oxidase inhibitor, prior to infection. In this setting, there were increased extracellular bacterial counts when macrophages were treated with DPI and infected with the Δ*atvR* mutant (compare the results for the Δ*atvR* mutant and the Δ*atvR* mutant from macrophages treated with DPI [Δ*atvR* + DPI] in Fig. 2A). The increase in the counts of extracellular PA14 bacteria from DPI-treated macrophages was less pronounced, indicating that the wild-type strain by itself is able to decrease the macrophage redox response, as we had already observed (29). The phagocytosis rate was the same when macrophages were treated or not treated with DPI, and the numbers of intracellular bacteria remained the same for all settings (Fig. 2B).

To determine if *P. aeruginosa* AtvR modulates macrophage activation, macrophages were infected with PA14, the Δ*atvR* mutant, the Δ*atvR*/pJN105 mutant (the Δ*atvR* mutant carrying an empty vector), or the Δ*atvR*/pAtvR mutant (the Δ*atvR* mutant carrying pAtvR, which is a pJN105 plasmid harboring *atvR* under the control of an arabinose-inducible promoter). After 3 h, supernatants were recovered and TNF-α levels were measured. Macrophages infected with the Δ*atvR* mutant secreted more TNF-α than macrophages infected with PA14 (Fig. 3A). This higher level of TNF-α production was due to a direct effect of the absence of AtvR, since the level of TNF-α secretion by macrophages infected with the complemented strain (the Δ*atvR*/pAtvR mutant) was

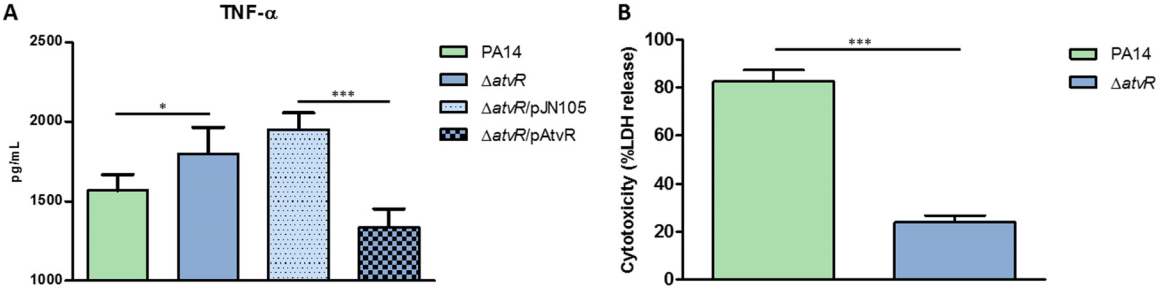

FIG 3 AtvR is important for virulence in a macrophage *in vitro* model. J774 macrophages were incubated with *P. aeruginosa* PA14 or the Δ*atvR*, Δ*atvR*/pJN105, or Δ*atvR*/pAtvR mutant at an MOI of 10. (A) After 3 h of infection, the supernatants were recovered and the level of TNF-α secretion was determined by ELISA. (B) For the cytotoxicity assay, after 2 h of infection, LDH release was determined as a measure of macrophage death. Data are the means ± SDs from three independent experiments performed in triplicate. *, $P < 0.05$; ***, $P < 0.001$.

restored to the wild-type level (Fig. 3A). Macrophage survival of bacterial infection was also increased when the macrophages were coincubated with the Δ*atvR* mutant over that when they were coincubated with PA14 (Fig. 3B), indicating that the AtvR function is needed for efficient killing of macrophages.

These *in vitro* infection results suggest that AtvR has a role in *P. aeruginosa* virulence, leading to higher bacterial counts and decreasing macrophage activation and survival. This hypothesis is strengthened by the results of the DPI assay, in which NADPH oxidase inhibition precludes the ability of the macrophages to kill Δ*atvR* bacteria (Fig. 2). We also found that AtvR positively regulates the expression of genes such as *katA* and *rahU* (Table 1), as discussed below, which are involved in modulating the oxidative status of macrophages (30, 31).

AtvR is essential for full *P. aeruginosa* virulence *in vivo*. Since AtvR was involved in virulence *in vitro*, the next step was to ascertain whether this atypical RR was also relevant in a mouse model of acute pneumonia. All mice infected intratracheally (i.t.) with PA14 were dead at 48 h postinfection (h.p.i.), while mice infected with the Δ*atvR* strain had higher survival rates, with 68% of the animals still being alive at 48 h.p.i. and 12% of the animals being alive after 7 days (Fig. 4A).

In this infection model, *P. aeruginosa* spreads quickly and reaches other organs, leading to animal death (29, 32). We assessed the bacterial burden in the primary infection site (lungs) as well as in secondary organs (liver and spleen) as an indication of sepsis. These organs were recovered at 24 h.p.i., and the bacterial load was evaluated. Animals infected with the Δ*atvR* mutant showed reduced bacterial burdens in all organs compared to those in wild-type strain-infected animals (Fig. 4B). This demonstrates that AtvR interferes with the host response against the bacterium, leading to reduced resolution of the bacterial infection by the host infected with wild-type strain PA14.

Because AtvR affects cytokine production *in vitro* (Fig. 3A), we asked whether AtvR also decreased immune system activation as well as neutrophil recruitment to the primary infection site. TNF-α and gamma interferon (IFN-γ) levels in the infected lungs were determined by enzyme-linked immunosorbent assay (ELISA) at 24 h.p.i., and the results showed that mice infected with the Δ*atvR* mutant released higher levels of both cytokines than PA14-infected or control mice (Fig. 4C and D). Higher levels of neutrophil (Ly6G/Ly6C$^+$, CD11b$^+$, F4/80$^-$) recruitment were also found in mice infected with the Δ*atvR* mutant than wild-type strain-infected or sham-infected mice (Fig. 4E). Myeloperoxidase (MPO) activity was observed in infected and control mice, but it was higher in animals infected with the Δ*atvR* mutant than in control or PA14-infected mice, confirming that the recruited neutrophils were activated and necessary for the full immune response (Fig. 4F). The numbers of macrophages in the lungs were similar for mice infected with either the wild-type or the mutant strain (data not shown), but the *in vitro* data suggest that they were activated more efficiently when mice were infected with the Δ*atvR* strain than when they were infected with PA14, recruiting more neutrophils to the infection site.

These results indicate the relevance of AtvR in promoting *P. aeruginosa* adaptation in the host, by disrupting immune activation and reducing proinflammatory cytokine production and neutrophil recruitment as well as their activation, thus promoting higher rates of host mortality.

AtvR affects expression of genes related to adaptation to low O_2 levels and virulence. To better understand the contribution of AtvR to *P. aeruginosa* adaptation to the host, which leads to higher virulence, we performed a high-throughput transcriptome sequencing (RNA-Seq) analysis to identify genes regulated by AtvR. The AtvR-overexpressing strain (PA14/pAtvR) and a control strain (PA14/pJN105) were grown under inducing conditions until the optical density at 600 nm (OD_{600}) was 1.0 at 37°C. A total of 287 genes were found to be differentially expressed; of these, 224 genes were upregulated (Table S3) and 63 genes were downregulated (Table S4) in PA14/pAtvR compared to their levels of regulation in PA14/pJN105. It is noteworthy that 44

TABLE 1 Genes differentially expressed in PA14/pAtvR and PA14/pJN105

Function and PA14 locus	PAO1 locus	Gene	Fold change in expression
Nitrate reductase			
PA14_13750	PA3877	*narK1*	3.62
PA14_13770	PA3876	*narK2*	5.44
PA14_13780	PA3875	*narG*	10.23
PA14_13800	PA3874	*narH*	10.67
PA14_13810	PA3873	*narJ*	9.09
PA14_13830	PA3872	*narI*	9.61
PA14_13840	PA3871		4.34
PA14_13850	PA3870	*moaA*	2.71
Nitrite reductase			
PA14_06650	PA0509	*nirN*	5.80
PA14_06660	PA0510	*nirE*	9.51
PA14_06670	PA0511	*nirJ*	7.20
PA14_06680	PA0512	*nirH*	5.86
PA14_06690	PA0513	*nirG*	5.05
PA14_06700	PA0514	*nirL*	4.49
PA14_06710	PA0515	*nirD*	3.80
PA14_06720	PA0516	*nirF*	3.23
PA14_06730	PA0517	*nirC*	3.93
PA14_06740	PA0518	*nirM*	4.58
PA14_06750	PA0519	*nirS*	3.23
PA14_06790	PA0521	*nirO*	2.41
PA14_06770	PA0520	*nirQ*	2.82
Nitric oxide reductase			
PA14_06810	PA0523	*norC*	6.12
PA14_06830	PA0524	*norB*	7.44
Nitrous oxide reductase			
PA14_20190	PA3393	*nosD*	4.02
PA14_20200	PA3392	*nosZ*	7.53
Arginine metabolism			
PA14_68330	PA5171	*arcA*	2.29
PA14_68340	PA5172	*arcB*	4.34
PA14_68350	PA5173	*arcC*	5.42
QS regulated			
PA14_36330	PA2193	*hcnA*	2.01
PA14_36320	PA2194	*hcnB*	3.19
PA14_36310	PA2195	*hcnC*	4.46
PA14_16250	PA3724	*lasB*	2.23
PA14_19100	PA3479	*rhlA*	2.60
Phenazine biosynthesis			
PA14_09400	PA4217	*phzS*	3.70
PA14_09410	PA4216	*phzG1*	7.16
PA14_09420	PA4215	*phzF1*	4.71
PA14_09440	PA4214	*phzE1*	5.88
PA14_09450	PA4213	*phzD1*	6.38
PA14_09460	PA4212	*phzC1*	9.14
PA14_09470	PA4211	*phzB1*	9.95
PA14_09480	PA4210	*phzA1*	6.11
PA14_09490	PA4209	*phzM*	2.69
PA14_39880	PA1905	*phzG2*	3.97
PA14_39890	PA1904	*phzF2*	4.71
PA14_39910	PA1903	*phzE2*	5.38
PA14_39925	PA1902	*phzD2*	6.52
PA14_39945	PA1901	*phzC2*	5.42
PA14_39960	PA1900	*phzB2*	6.57
PA14_39970	PA1899	*phzA2*	3.55

(Continued on next page)

TABLE 1 (Continued)

Function and PA14 locus	PAO1 locus	Gene	Fold change in expression
Redox related			
PA14_01490	PA0122	*rahU*	2.10
PA14_09150	PA4236	*katA*	2.33
Type VI secretion systems			
PA14_00820	PA0070	*tagQ1*	2.09
PA14_01030	PA0085	*hcp1*	2.11
PA14_44290	PA1656	*hsiA2*	2.06
PA14_43040	PA1657	*hsiB2*	3.27
PA14_43030	PA1658	*hsiC2*	3.00
PA14_43020	PA1659		2.46
PA14_43000	PA1660	*hsiG2*	2.58
PA14_42990	PA1661	*hsiH2*	2.48
PA14_42980	PA1662	*clpV2*	2.50
PA14_42970	PA1663	*sfa2*	2.35
PA14_42950	PA1665	*fha2*	3.68
PA14_42940	PA1666	*lip2.1*	2.76
PA14_42920	PA1667	*hsiJ2*	2.10
PA14_42910	PA1668	*dotU2*	2.74
PA14_42900	PA1669	*icmF2*	2.19
PA14_34020	PA2368	*hsiF3*	60.50

out of 224 upregulated genes (19.6%) belong to nine gene clusters involved in anaerobic respiration in the presence of nitrate (Table 1). These genes include genes encoding the complete denitrification pathway for nitrate reduction to molecular nitrogen, comprising the nitrate reductase (*nar* and *nap*), nitrite reductase (*nir*), nitric oxide reductase (*nor*), and nitrous oxide reductase (*nos*) gene clusters. The *arc* operon, which is required for fermentative growth of *P. aeruginosa* on arginine, and clusters related to pyocyanin biosynthesis (*phz*) were also upregulated (Table 1). These results suggest a role of AtvR in the regulation of anaerobic/hypoxic metabolism.

Because Anr also directly or indirectly regulates all denitrification clusters, we compared our data with those for the Anr regulon previously described (17). We found that only about 16% of the Anr regulon was also under the control of AtvR and 89% of the AtvR regulon was not under the control of Anr (Fig. S2). The AtvR regulon includes genes for type VI secretion systems, *lasB*, *rahU* (see below), and the operon that comprises the *aer2* aerotaxis transducer, which are not affected by the *anr* mutation (17, 33).

Some genes known to be relevant for virulence were also upregulated in the PA14/pAtvR strain, such as *hcnA*, *lasB*, *katA*, *rahU*, and genes for pyocyanin synthesis and type VI secretion systems (30, 31, 34–36). Catalase and RahU are implicated in modulating the production of reactive oxygen and nitrogen species, respectively, shutting down the macrophage response and therefore allowing an effective infection by *P. aeruginosa* (30, 31).

To validate the involvement of AtvR in the expression of denitrification and other genes, the transcription levels of *nar* (*narK1*, *narG*, and *narH*), *nir* (*nirC*, *nirS*, and *nirQ*), and phenazine biosynthetic genes (*phzS*, *phzM*), as well as cyanate biosynthesis (*hcnA*) and catalase (*katA*) genes, in the Δ*atvR*/pJN105 and Δ*atvR*/pAtvR strains were compared. Quantitative reverse transcription-PCR (RT-PCR) analysis of these genes confirmed their increased expression levels in the Δ*atvR* strain complemented for AtvR compared to their expression levels in the control, the Δ*atvR*/pJN105 mutant (Fig. 5). This finding supports the direct or indirect transcriptional regulation of genes involved in denitrification and virulence by AtvR and is consistent with the RNA-Seq data.

AtvR expression is induced in the presence of low O_2 levels and nitrate. Because AtvR regulates genes involved in the response to hypoxia and anoxia at the transcriptional level, we asked whether this atypical RR is involved in *P. aeruginosa* fitness in the presence of a low O_2 supply by regulating gene expression under this stressful

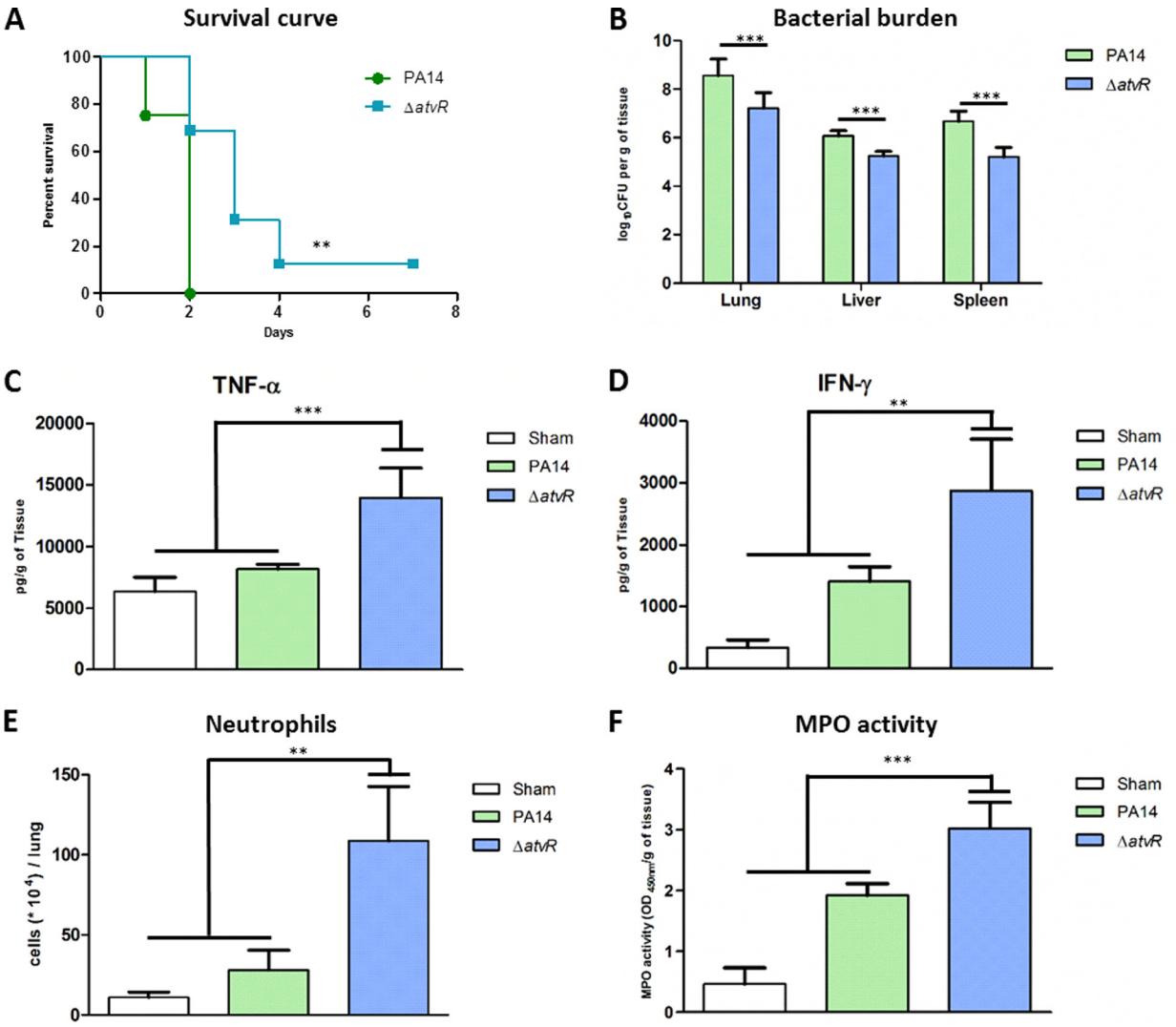

FIG 4 AtvR is important for *P. aeruginosa* virulence in a mouse model of acute pneumonia. BALB/c mice were infected i.t. with 2×10^6 bacteria of the wild-type strain PA14 or the Δ*atvR* mutant. (A) Mouse survival was followed during the course of the experiment. (B) At 24 h postinfection, animals were sacrificed, the organs were macerated, and the bacterial CFU were enumerated. (C, D) The levels of the cytokines TNF-α (C) and IFN-γ (D) in the lungs were determined by ELISA. (E) The animals were euthanized, the lungs were macerated, and the cell suspensions were labeled for neutrophils (Ly6G/Ly6C$^+$, F4/80$^-$) and analyzed by fluorescence-activated cell sorting. (F) The lung cells were lysed by sonication, and the suspension was centrifuged. The resulting supernatant was used for the MPO activity assay with TMB, and the OD at 450 nm was measured. (B to F) For all assays, samples were collected at 24 h postinfection. Data are the means ± SDs from three independent experiments. **, $P < 0.01$; ***, $P < 0.001$.

condition. The PA14, Δ*atvR*, Δ*atvR*/pJN105, Δ*atvR*/pAtvR, and Δ*anr* strains were grown under conditions of low O_2 tension with or without nitrate; alternatively, these strains were grown in normoxia with shaking. The Δ*anr* mutant was included as a hypoxia/anoxia-sensitive control (17, 37). After 24 h under low-O_2 conditions or 3 h of normoxia, the OD_{600} was measured and RNA was extracted. Under these conditions, no differences in the growth of cultures submitted to low O_2 levels without nitrate were observed (Fig. 6A). However, in the presence of nitrate and low levels of O_2, cultures of the Δ*atvR* or Δ*atvR*/pJN105 strain presented significant cell growth that was slightly lower than that of wild-type PA14 or the Δ*atvR*/pAtvR complemented strain, suggesting that AtvR may sense the presence of nitrate (Fig. 6A).

atvR expression was higher when PA14 was grown in the presence of low O_2 levels with nitrate than when it was grown in normoxia, indicating a role for AtvR under the former conditions (Fig. 6B). To strengthen this hypothesis, the expression of *narH*, one of the genes from the nitrate reductase cluster identified in the RNA-Seq analysis,

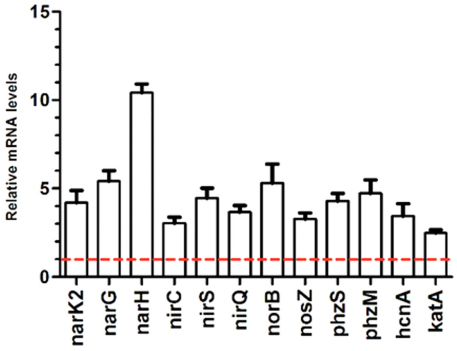

FIG 5 AtvR transcriptionally regulates denitrification and virulence-related genes. Quantitative RT-PCR was performed using cDNA made by reverse transcription of total RNA from Δ*atvR*/pJN105 or Δ*atvR*/pAtvR cells grown to an OD_{600} of 1 in LB medium at 37°C with 0.2% arabinose and 50 μg/ml gentamicin under agitation. Results are shown as values relative to the value for the Δ*atvR*/pJN105 control strain, which was considered 1 (red dashed line). Data are the means ± SDs from at least three independent experiments.

presented mRNA levels reduced 2-fold in the Δ*atvR* mutant compared to those in the wild type in the presence of nitrate in both hypoxia and normoxia (Fig. 6C). As expected, the complemented strain restored the level of *narH* expression to wild-type levels (Fig. 6D).

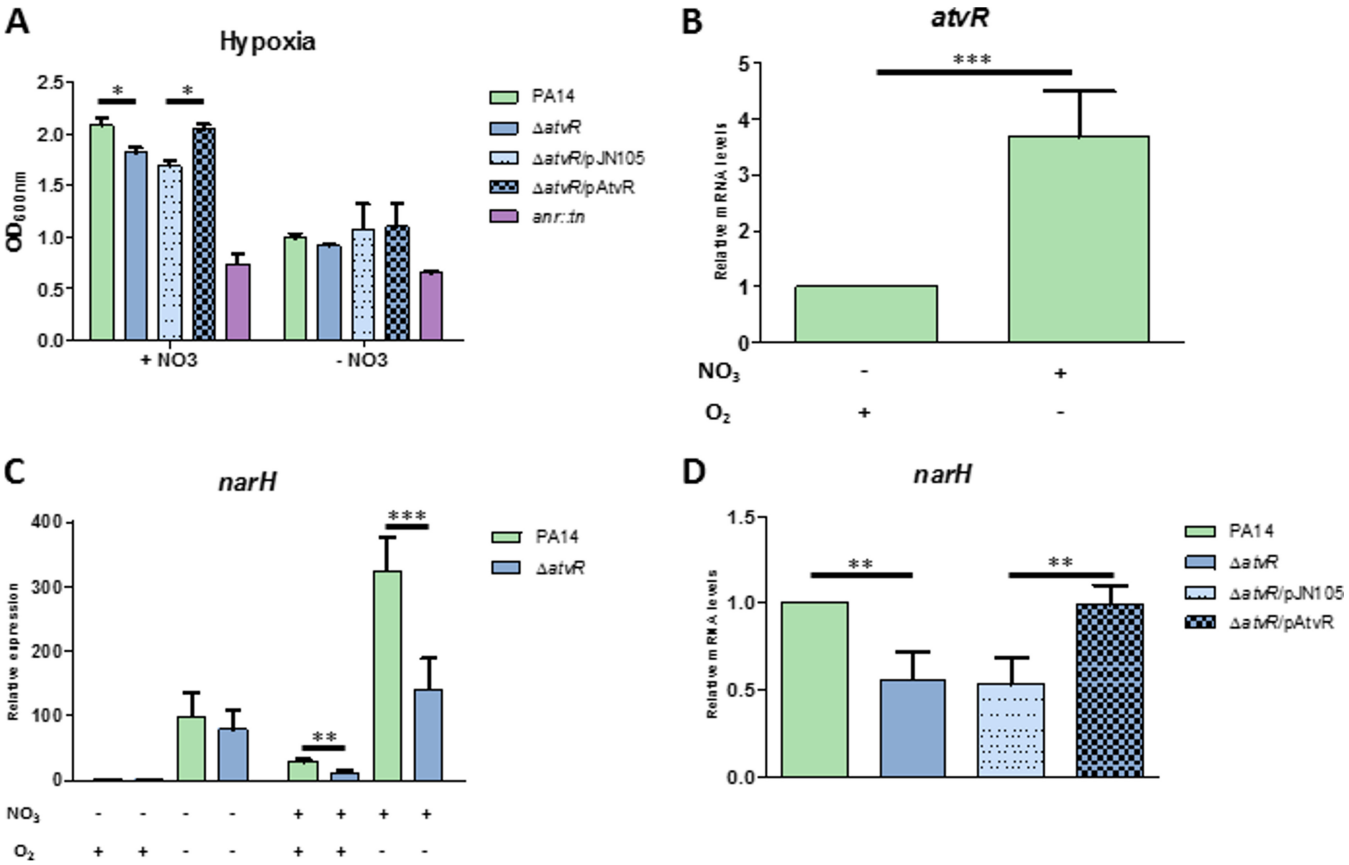

FIG 6 AtvR is expressed during low oxygen levels and regulates denitrification genes. (A) The wild-type PA14 strain, the Δ*atvR*, Δ*atvR*/pJN105, and Δ*atvR*/pAtvR strains, and the control Δ*anr* strain were grown under hypoxic conditions with or without 50 mM nitrate, and the OD_{600} was determined after 24 h. (B, C) Total RNA was extracted from the PA14 and Δ*atvR* strains in normoxia or hypoxia with or without 50 mM nitrate, as indicated at the bottom. (B) Level of *atvR* expression relative to that in the PA14 strain grown in normoxia without nitrate. (C) The level of expression of *narH* was determined under different conditions; the data were plotted relative to those for the PA14 strain grown in normoxia without nitrate. (D) The level of expression of *narH* during hypoxia with nitrate was analyzed and normalized to the levels in PA14 under the same conditions. Data are the means ± SDs from at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

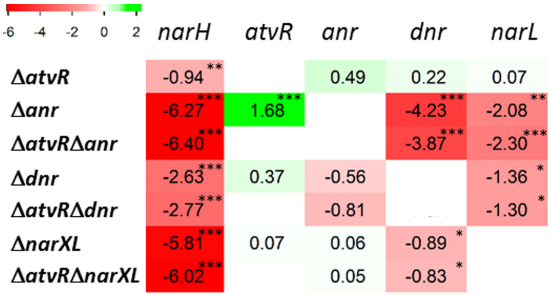

FIG 7 AtvR is not part of the known denitrification regulatory cascade, and it is repressed by Anr under low O_2 levels. The wild-type PA14/pJN105 strain and the Δ*atvR*/pJN105, Δ*anr*/pAtvR, Δ*dnr*/pJN105, Δ*narXL*/pJN105, Δ*atvR*Δ*anr*/pJN105, Δ*atvR*Δ*dnr*/pJN105, and Δ*atvR*Δ*narXL*/pJN105 mutants were grown in LB medium with 50 mM nitrate under aerobic conditions until the OD_{600} was 2. At this time point, cell cultures were transferred to a candle jar for 4 h, RNA was extracted, and qRT-PCR was performed for the genes indicated at the top. Relative expression levels for each gene were normalized to those in PA14/pJN105 and are depicted as $\log_2$ values. The upregulated genes are marked in green and the downregulated in red, with shades corresponding to the degree of regulation. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

AtvR acts in parallel to the Anr-NarXL-Dnr denitrification regulatory cascade. To investigate whether the AtvR regulatory role acts in concert with the denitrification regulatory pathway, strains with a deletions of *anr*, *dnr*, or *narXL* were constructed both in the wild type and in the Δ*atvR* mutant backgrounds, and the AtvR-overexpressing plasmid pAtvR or the control plasmid pJN105 was introduced into the mutants. Because the *anr* mutant barely grew under our low-O_2 conditions, all strains were incubated in LB medium with 50 mM KNO_3 under normoxic conditions until the OD_{600} was 2. At this point, cultures were transferred to a candle jar and total RNA was extracted after 4 h. In this setting, no growth differences were observed for any strain in normoxia (Fig. S1) and hypoxia (data not shown).

AtvR has no effect on the levels of *anr*, *dnr*, and *narL* mRNA, while the other transcriptional factors, Anr, Dnr, and NarXL, are part of an intricate regulatory network in which Anr is the master, inducing *dnr* and *narL* expression (Fig. 7 and S3) (18). As expected, we observed that Dnr and NarXL do not control *anr* expression but they regulate each other, as a lack of *dnr* resulted in lower levels of expression of *narL* and a lack of *narXL* led to reduced *dnr* mRNA levels. Dnr and NarXL had no influence on *atvR* mRNA levels, but, surprisingly, Anr had a negative role (Fig. 7 and S3).

To further dissect the role of AtvR in regulating the expression of denitrification genes, we evaluated the effect of combining the *atvR* deletion with the *anr*, *dnr*, and *narXL* deletions on *narH* mRNA levels. As expected, we observed a reduction of ~70-fold and a reduction of ~60-fold in *narH* mRNA levels in the Δ*anr* and Δ*narXL* strains, respectively, compared to those in the wild-type strain. A 6-fold reduction was observed in the Δ*dnr* strain, which is also in agreement with previously described data (19). As we observed before (Fig. 6A), a lack of AtvR resulted in a 2-fold reduction in the level of *narH* compared to that in the wild type (Fig. 7). It is important to note that *narH* expression was not restored by AtvR overexpression in the single, double, or triple mutants, but it was in the Δ*atvR* strain (Fig. S3).

These data, added to the fact that the growth of the Δ*anr* mutant in the presence of low O_2 levels was not complemented by overexpression of AtvR (not shown), suggest that AtvR does not have an additive function with the functions of Anr, Dnr, or NarXL on *narH* expression and that AtvR may control denitrification gene expression in an alternative manner.

DISCUSSION

Two-component systems are among the most studied signaling pathways in bacteria. Atypical RRs, especially those with a replacement of the phosphorylatable aspartate by glutamate, probably lack phosphorylation control and, therefore, the histidine kinase-mediated signal transduction pathway. Atypical response regulators with DNA-

binding domains are associated with several functions, such as regulating *Helicobacter pylori* cell growth, controlling *Streptomyces* development, and inducing a pathway that results in *Synechococcus* sp. bleaching under conditions of nutrient deprivation. To our knowledge, the only atypical RR previously associated with virulence was *Chlamydia trachomatis* ChxR, but this was inferred by the regulation of putative virulence genes, and no evidence of a direct link of this atypical RR to infection has been shown (38–46).

The importance of an atypical RR to virulence, impairment of the host immune response, and regulation of expression of virulence-related genes is shown here for the first time. Among those genes that encode well-established virulence factors are the genes of the phenazine cluster (*phz*), which is required for the synthesis of pyocyanin, a redox-active molecule; *hcn*, responsible for cyanide production; the catalase gene; and genes for the type VI secretion system (30, 35, 47, 48). Quorum sensing (QS)-regulated genes, such as *lasB* and *rhlB*, are also under the influence of AtvR and may account for the reduced virulence of the Δ*atvR* mutant as well, maybe due to an uncoordinated response of the QS cascade, as supported by the upregulation of the *phz* cluster as well. Curiously, QS-regulated genes are upregulated both in our AtvR-overexpressing strain and in an *anr* mutant (33), indicating opposite roles in this case and agreeing with the repression of *atvR* by Anr. Catalase, encoded by the *katA* gene, is an enzyme that reduces the oxygen peroxide that originates from the macrophage oxidative burst (30), and *rahU* codes for an extracellular enzyme that was shown to inhibit macrophage intracellular nitric oxide synthase (iNOS), being also part of the bacterial arsenal against host defenses (31).

Several genes involved in denitrification were induced in the AtvR-overexpressing strain. During acute and chronic infections, *P. aeruginosa* may find microaerobic conditions, and the presence of a complex regulatory system in response to low oxygen tension is not surprising. At least three main systems were shown to regulate anaerobic respiration in *P. aeruginosa*, namely, the NarXL, Anr, and Dnr systems (49, 50). NarXL and Anr directly regulate the nitrate reductase gene cluster and indirectly regulate other anaerobic respiration genes via the Dnr transcription factor. The importance of those systems in *P. aeruginosa* adaptability is reinforced by the reduced virulence of the *anr*, *nar*, and *nir* mutants, as *anr*, *nar*, and *nir* allow *P. aeruginosa* to survive under anoxic/hypoxic conditions when nitrate is present (49, 51, 52). Interestingly, the type III secretion system is not expressed in PA14 *nirS* mutants, but it is recovered by adding exogenous NO generators, suggesting that NO acts as a signaling molecule for cytotoxicity (53). During bacterial infections, the overall nitrate pool is increased (54); hence, it could be used as an electron acceptor to support bacterial alternative respiration. In fact, anaerobic respiration was already shown to be important in other pathogens, such as *Salmonella enterica* serovar Typhimurium, in which the presence of periplasmic nitrate reductase is essential for virulence during colitis (55).

Therefore, AtvR contributes to *P. aeruginosa* virulence because it enables this bacterium to spread during infection, colonizing primary and secondary organs, leading to a severe acute infection which results in host death. AtvR is required for *P. aeruginosa* resistance against macrophage killing, as the deletion of AtvR resulted in higher levels of TNF-α production and higher levels of bacterial clearance *in vitro*. The Δ*atvR* strain was defective in the mouse model of acute pneumonia, being cleared more efficiently and allowing neutrophils to be recruited to the site of infection. These data suggest that AtvR plays an important role in *P. aeruginosa* adaptation against host defense by modulating the innate immune response.

The roles of transcriptional regulators in fine-tuning adjustments in gene expression, leading to just-in-time gene expression, are very important for a highly adaptable bacterium to respond to a plethora of conditions that it encounters during the colonization of different niches. Survival in the absence of oxygen or in the presence of very low oxygen concentrations is crucial for *P. aeruginosa* to thrive during an infection, which is reflected by the numerous regulators involved in the metabolic switch to anaerobic respiration. To those already characterized activators, comprising Anr, NarL, and Dnr, we add the atypical RR AtvR (Fig. 8). We show here that AtvR does not fit into

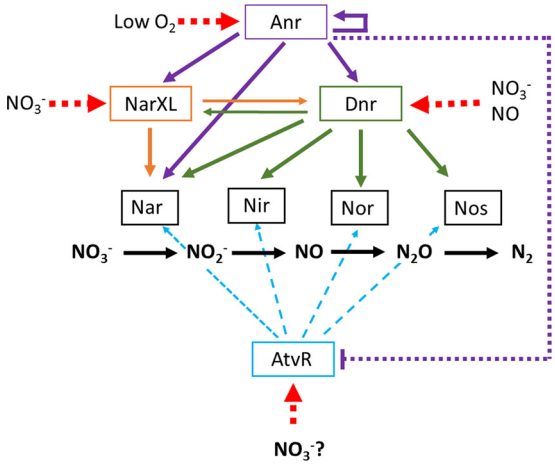

FIG 8 AtvR and the complex regulation of denitrification genes in *P. aeruginosa*. The structural genes for each electron transfer complex, nitrate (Nar), nitrite (Nir), nitric oxide (Nor), and nitrous oxide (Nos) reductases (gray boxes), are regulated by the Anr transcription activator, the TCS NarXL, the Dnr activator, and the newly discovered AtvR. Black arrows, electron transfer pathway; colored and solid arrows, positive transcriptional regulation; red dashed arrows, factors that affect the activity of the regulators are shown, in which the arrows point to the targets.

the already known denitrification regulatory cascade that has Anr as the master activator and Dnr and NarXL as downstream regulators. *atvR* mRNA levels are not under the regulation of Dnr and NarXL but are decreased by Anr (Fig. 7; see also Fig. S3 in the supplemental material). Although part of the AtvR regulon is superposed onto those regulators, it may respond to slightly different conditions by activating other virulence-relevant genes (Table 1). The AtvR function is not able to suppress the lack of Anr (Fig. 7 and S3), even though it can positively regulate the entire denitrification pathway.

The challenge now is to understand how the expression and activity of AtvR are regulated, because the lack of an aspartate rules out regulation by phosphorylation. Would it be simply by modulating its transcription in the presence of nitrate, or are there more subtle mechanisms that regulate the stability of the message and/or the activity of the protein itself? How do all those regulators interact to warrant that the denitrification process is induced when needed? Those open questions need to be addressed to better understand one of the central metabolic pathways that contribute to *Pseudomonas* ubiquity and versatility.

MATERIALS AND METHODS

Bacterial strains, plasmids, oligonucleotides, and culture conditions. The strains and plasmids used in this study are listed in Table S1 in the supplemental material. *P. aeruginosa* strains were grown at 37°C in LB broth supplemented with 250 μg/ml of kanamycin, 20 μg/ml nalidixic acid, or 50 μg/ml of gentamicin with or without 0.2% arabinose, when required. *Escherichia coli* strains were grown in LB medium supplemented with 100 μg/ml of ampicillin, 50 μg/ml of kanamycin, or 10 μg/ml of gentamicin, when required.

To construct the unmarked in-frame deletion of *atvR* (PA14_26570) or *anr* (PA14_44490), primers flanking the upstream and downstream regions of *atvR* or *anr* were designed (Table S2). *atvR* amplicons were cloned into pNPTS138 at the HindIII and EcoRI sites to generate pNPTS138Δ*atvR*. The *anr*, *dnr*, and *narXL* amplicons were cloned into pEX18Ap using sequence- and ligation-independent cloning (56) to generate pEX18Δ*anr*, pEX18Δd*nr*, and pEX18Δ*narXL*. The resulting constructs were used to introduce the *atvR*, *anr*, *dnr*, or *narXL* deletion into the wild-type strain PA14 genome or in the strain with the Δ*atvR* background by homologous recombination (57). Mutant clones were confirmed by PCR.

The *atvR*-overexpressing plasmid was constructed by amplifying the *atvR* coding region, cloning it into the pGEM-T vector, and digesting the resulting plasmid at the EcoRI and SpeI sites. This fragment was gel purified and cloned into pJN105 to generate pAtvR. The pAtvR and pJN105 plasmids were introduced into the wild-type strain and the Δ*atvR*, Δ*dnr*, Δ*anr*, and Δ*narXL* mutants, generating the PA14/pJN105, PA14/pAtvR, Δ*atvR*/pJN105, Δ*atvR*/pAtvR, Δ*dnr*/pJN105, Δ*dnr*/pAtvR, Δ*anr*/pJN105, Δ*anr*/pAtvR, Δ*narXL*/pJN105, and Δ*narXL*/pAtvR strains.

Growth curves. For growth curves, cultures were incubated overnight in LB or R-10 medium (RPMI 1640 supplemented with 2 mM glutamine, 10% fetal bovine serum [FBS], and 40 μg/ml gentamicin) and adjusted to an OD_{600} of 0.1 in LB or R-10 medium with or without gentamicin, and 1-ml samples were

transferred to 24-well plates. The growth was monitored by determination of the OD_{600} at 15-min intervals with a SpectraMax Paradigm multimode microplate reader at 37°C with low orbital agitation. Alternatively, bacterial strains were grown to an OD_{600} of 2.0 in LB medium with or without gentamicin and arabinose. The bacteria were then diluted to 5×10^6 bacteria/ml in R-10 medium with (for the Δ*atvR*/pJN105 and Δ*atvR*/pAtvR strains) or without (for the PA14 and Δ*atvR* strains) 50 μg/ml of gentamicin and 0.2% arabinose. Then, 200 μl of the bacterial suspensions was added to a 96-well plate and the plate was incubated for 4 h 30 min at 37°C in 5% CO_2, the same conditions used for the *in vitro* virulence assays. At the time points indicated above, each culture was serially diluted and plated and the numbers of CFU were determined.

RNA sequencing analysis. PA14/pJN105 or PA14/pAtvR cultures were grown in LB medium with 0.2% arabinose and 50 μg/ml gentamicin at 37°C to an OD_{600} of 1.0. Two biological replicates were analyzed for each strain. Cells were harvested after addition of RNAprotect (Qiagen), total RNA was isolated using an RNeasy minikit (Qiagen), and RNA quality was assessed using a Bioanalyzer instrument. mRNA was enriched using a Ribo-Zero rRNA removal kit (for Gram-negative bacteria; Illumina), and rRNA depletion was confirmed by use of the Bioanalyzer instrument. Paired-end libraries were constructed using a TruSeq RNA library preparation kit (v2). The mean fragment size was determined by use of the Bioanalyzer instrument, and each library concentration was determined using a Kapa library quantification kit (Kapa Biosystems) according to the manufacturer's protocol. All samples were sequenced in an Illumina MiSeq system in the paired-end mode.

Gene expression quantification. Sequence reads were separated according to their barcode, and barcode sequences were removed. For each sample, read quality was assessed using a FastQC sequencer, and the 3′ end was trimmed using a Fastx tool kit. After this step, the sequences were mapped to the *P. aeruginosa* PA14 reference strain genome and counted using the EDGE-pro program (58). The R package EDGE-R (59) was used for differential expression analysis. Genes were classified as differentially expressed if they presented a $\log_2$-fold change greater than 1 or less than -1 and if the Benjamini-Hochberg method-corrected *P* value (*P*-adj) was less than 0.05.

Hypoxia. Experiments were performed under hypoxic conditions as described before with some modifications (60). Briefly, bacterial strains were grown aerobically in LB medium and diluted in 1 ml LB medium with or without 50 mM nitrate in a 1.5-ml microtube. The bacterial cultures were maintained for 24 h in a candle jar. After this incubation, the OD_{600} was determined and total RNA was extracted as described below.

qRT-PCR. PA14/pJN105 or PA14/pAtvR was grown under the same conditions described above for the RNA-Seq experiments. For hypoxic conditions, samples were incubated in a candle jar and collected after 3 h (normoxia) or 24 h after inoculation (hypoxia). Alternatively, the strains with mutated *anr*, *dnr*, *narXL*, and/or *atvR* were grown to an OD_{600} of 2 in LB medium with 50 mM KNO_3. Samples were incubated in a candle jar and collected after 4 h. All RNA samples were extracted with the TRIzol reagent (Invitrogen), treated with DNase I (Thermo Scientific), and used for cDNA synthesis with RevertAid reverse transcriptase (Thermo Scientific) and random hexamer primers (Thermo Scientific). The cDNA was used as the template for quantitative RT-PCR (qRT-PCR) using Maxima SYBR green–carboxy-X-rhodamine quantitative PCR master mix (Thermo Scientific) and a StepOne Plus real-time PCR system (Applied Biosystems). The primers used are listed in Table S2. Expression was normalized against that of *nadB*, and the relative expression from at least three biological replicates was calculated. Expression is given as the fold change with the standard deviation (SD) (61).

Cell culture. The macrophage cell line J774 was maintained in R-10 medium at 37°C in 5% CO_2. Cells were counted using a Neubauer chamber, and dead cells were excluded by trypan blue exclusion assay. Macrophages were seeded at 1×10^5 cells per well (96-well plates) or 5×10^5 cells per well (24-well plates) in R-10 medium without antibiotics or in R-10 medium with 50 μg/ml gentamicin and 0.2% arabinose. Macrophages were primed overnight with 10 ng/ml IFN-γ at 37°C in 5% CO_2.

***In vitro* infection experiments.** All strains were grown in LB broth to an OD_{600} of 2.0. Bacteria were diluted in R-10 medium without antibiotics. For the phagocytosis and evasion assays, macrophages that had previously been seeded in 96-well plates with R-10 medium were pretreated or not pretreated with the NAPDH oxidase inhibitor diphenyleneiodonium (DPI) for 4 h. Bacteria were added at a multiplicity of infection (MOI) of 10, and at 1 h postinfection, the supernatant was removed and fresh R-10 medium containing 200 μg/ml of gentamicin was added to the cell cultures to kill the remaining extracellular bacteria. After 30 min of incubation with gentamicin, cells were washed once with phosphate-buffered saline (PBS) and fresh medium was added. This point was defined as time zero. After the times indicated above, the supernatant was collected and serially diluted, the bacteria were plated, and the numbers of CFU, corresponding to the extracellular bacteria, were determined. To evaluate the amount of intracellular bacteria, macrophages were washed once and lysed with PBS with 0.1% Triton X-100; the lysates were serially diluted and plated, and the numbers of CFU were determined.

To quantify TNF-α, macrophages were seeded in 24-well plates and infected at an MOI of 10. At 3 h postinfection, the supernatants were removed, centrifuged, and stored at -20°C. Cytokine quantification was performed by ELISA (R&D Systems) by following the manufacturer's instructions. For the cytotoxicity assay, macrophages were seeded in a 96-well plate and infected as described above. Cytotoxicity was measured for samples taken at the times indicated above by quantifying the release of lactate dehydrogenase (LDH) using a CytoTox 96 nonradioactive cytotoxicity assay according to the manufacturer's instructions.

Ethics statement. All animal experiments were performed in agreement with the Ethical Principles in Animal Research adopted by the Brazilian Conselho Nacional de Controle da Experimen-

tação Animal (CONCEA) and in accordance with the recommendations in the *Guide for Care and Use of Laboratory Animals* of the National Research Council (62). The animal protocol was approved by the Internal Animal Care and Use Committee of the Instituto de Química, Universidade de São Paulo (approval no. 17/2015).

Animals. Female BALB/c mice (8 to 12 weeks old) were obtained from the in-house animal facility of the Biotério de Produção e Experimentação da Faculdade de Ciências Farmacêuticas e do Instituto de Química da Universidade de São Paulo. Mice were kept on a 12-h light/12-h dark cycle with free access to food and water and were maintained under specific-pathogen-free conditions. All mice were euthanized in a CO_2 chamber, and every effort was made to minimize suffering.

Animal inoculation with bacteria. The PA14 and Δ*atvR* strains were used for intratracheal (i.t.) inoculation as described before (29) with a few modifications. Bacteria were grown as described above, harvested by centrifugation at 12,000 × *g* for 2 min, washed twice in sterile PBS, and suspended in PBS at a concentration of 2×10^6 bacteria/60 μl. The number of CFU per milliliter was validated by plating serial dilutions of the suspensions. Each mouse received 60 μl of a bacterial suspension. A ketamine-xylazine mixture was injected intraperitoneally to anesthetize the mice before surgery. A midventral incision was made, and the trachea was exposed. The bacterial suspension was inoculated i.t. Controls were inoculated i.t. with 60 μl sterile PBS.

***In vivo* CFU determination.** At 24 h after infection, the lungs, spleen, and liver were harvested for determination of the numbers of CFU and cytokine measurements. The tissues were homogenized in 1 ml PBS for the lung and spleen and in 2 ml PBS for the liver. The supernatants were collected, and the numbers of CFU were determined by serial dilution and plating on LB plates. For cytokine measurements, the lungs were homogenized, and the supernatants were centrifuged at 12,000 × *g* for 10 min at 4°C. The cytokines TNF-α, IFN-γ, and IL-10 were quantified by ELISA (R&D Systems) by following the manufacturer's instructions.

Flow cytometry. At 24 h after infection, the lungs were harvested, minced, and digested with collagenase for 30 min at 37°C. Red blood cells (RBC) were lysed by adding NH_4Cl lysis buffer. Cells were resuspended in PBS with 3% FBS and stained with different combinations of conjugated antibodies, including F4/80-phycoerythrin (PE)-Cy5 (BM8), CD11c-fluorescein isothiocyanate (HL3), CD11b-PE (M1/70), and Ly6G/Ly6C-allophycocyanin (RB6-8C5), followed by incubation for 20 min on ice. Finally, the cells were washed and resuspended for flow cytometry analysis. FlowJo software (Tree Star) was used to analyze the data.

Myeloperoxidase activity assay. The myeloperoxidase activity assay was performed as previously described with a few modifications (63, 64). Animals were infected as described above, and the lungs were harvested, mechanically lysed in the presence of 50 mM sodium phosphate, pH 5.4, 5 mM EDTA, and 0.5% cetyltrimethylammonium bromide, sonicated, and centrifuged. The supernatant (50 μl) was mixed with an equal volume of 3 mM 3,3′,5,5′-tetramethylbenzidine dihydrochloride (TMB) for 2 min. The reaction was stopped by the addition of 25 μl of 2 M H_2SO_4. The optical density (OD) at 450 nm was measured.

Survival. After i.t. infection with wild-type PA14 or the Δ*atvR* mutant ($n = 16$ per group), animals were observed for survival. All deaths reported were from moribund/euthanized mice. Mice with labored or rapid breathing, decreased motility, ruffled or abnormal-looking fur, or other obvious signs of distress were considered moribund, as described before (65).

Statistical analyses. Prism (v5) software (GraphPad Inc.) was used for all statistical analyses, except for statistical analysis of the data from RNA-Seq analysis. Kaplan-Meier survival curves were plotted, and significance was calculated using the log rank test. Data were compared using a *t* test and one-way or two-way analysis of variance (ANOVA) followed by Bonferroni's multiple-comparison test.

Data availability. The raw RNA-Seq data were deposited at BioProject (https://www.ncbi.nlm.nih.gov/bioproject) under accession number PRJNA375803.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00207-17.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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G.H.K., R.L.B., and S.R.D.A. conceived and designed the experiments. G.H.K., L.C.D.B., J.R.F.D.A., and T.D.O.P. performed the virulence experiments. G.H.K., G.G.N., and A.L.B. performed the RNA-Seq experiment and analysis. R.L.B. and S.R.D.A. wrote the paper. G.H.K., G.G.N., T.D.O.P., R.L.B., and S.R.D.A. contributed reagents, materials, and analysis tools.

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