RpoN Modulates Carbapenem Tolerance in *Pseudomonas aeruginosa* through *Pseudomonas* Quinolone Signal and PqsE

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The ability of *Pseudomonas aeruginosa* to rapidly modulate its response to antibiotic stress and persist in the presence of antibiotics is closely associated with the process of cell-to-cell signaling. The alternative sigma factor RpoN (σ^{54}) is involved in the regulation of quorum sensing (QS) and plays an important role in the survival of stationary-phase cells in the presence of carbapenems. Here, we demonstrate that a $\Delta rpoN$ mutant grown in nutrient-rich medium has increased expression of *pqsA*, *pqsH*, and *pqsR* throughout growth, resulting in the increased production of the *Pseudomonas* quinolone signal (PQS). The link between *pqsA* and its role in carbapenem tolerance was studied using a $\Delta rpoN \Delta pqsA$ mutant, in which the carbapenem-tolerant phenotype of the $\Delta rpoN$ mutant was abolished. In addition, we demonstrate that another mechanism leading to carbapenem tolerance in the $\Delta rpoN$ mutant is mediated through *pqsE*. Exogenously supplied PQS abolished the biapenem-sensitive phenotype of the $\Delta rpoN \Delta pqsA$ mutant, and overexpression of *pqsE* failed to alter the susceptibility of the $\Delta rpoN \Delta pqsA$ mutant to biapenem. The mutations in the $\Delta rpoN \Delta rhIR$ mutant and the $\Delta rpoN \Delta pqsH$ mutant led to susceptibility to biapenem. Comparison of the changes in the expression of the genes involved in QS in wild-type PAO1 with their expression in the $\Delta rpoN$ mutant and the $\Delta rpoN$ mutant-derived strains demonstrated the regulatory effect of RpoN on the transcript levels of *rhIR*, *vqsR*, and *rpoS*. The findings of this study demonstrate that RpoN negatively regulates the expression of PQS in nutrient-rich medium and provide evidence that RpoN interacts with *pqsA*, *pqsE*, *pqsH*, and *rhIR* in response to antibiotic stress.

Pseudomonas aeruginosa, an opportunistic Gram-negative bacterium, is the leading cause of infections in immunocompromised patients and those suffering from cystic fibrosis. To cause disease, *P. aeruginosa* expresses and secretes virulence factors that promote colonization and survival within its host. *P. aeruginosa* infections are particularly difficult to treat due to their high levels of resistance to antimicrobials. In contrast to antibiotic resistance, which is attributed to a wide repertoire of mechanisms, *P. aeruginosa* displays another phenotype known as antibiotic tolerance, characterized by the presence of a small population of antibiotic-tolerant cells that persist in the presence of antibiotics (1). The presence of antibiotic-tolerant cells results in relapse of the bacterial infection after cessation of antibiotic treatment; therefore, determining the precise mechanism for antibiotic tolerance is a prerequisite for adequately fighting persistent infections caused by *P. aeruginosa*.

P. aeruginosa is capable of overcoming the effects of virtually any commercially available antibiotic, including β -lactam antibiotics, such as carbapenems. Despite this, carbapenems are still commonly used to treat infections caused by *P. aeruginosa*. The main advantage of carbapenems is their resistance to the β -lactamase AmpC and their ability to bind to multiple different penicillin binding proteins (PBPs) and inhibit cell wall synthesis (2, 3). It has been demonstrated, however, that the presence of carbapenems induces cell rounding, a morphotype that promotes β -lactam tolerance (4).

However, the molecular mechanism underlying antibiotic tolerance is largely unknown. Environmental signals, including prolonged nutrient deprivation (5), oxidative stress (6), DNA-damaging agents (7), and activation of the stringent response (5, 8), promote antibiotic tolerance. *P. aeruginosa* closely regulates its response to environmental conditions through a cell-to-cell communication system or quorum sensing (QS). In *P. aeruginosa*,

three interconnected QS systems control the global gene regulation implicated in the production of virulence factors and biofilm formation (9, 10). Two QS systems, the LasR/I and RhIR/I systems, modulate gene transcription in response to increasing concentrations of the *N*-acyl-L-homoserine lactones synthesized by LasI [*N*-(3-oxododecanoyl) homoserine lactone] and RhII (*N*-butanoyl homoserine lactone) (11–13). In addition to the *las* and *rhl* regulatory systems, *P. aeruginosa* has a third QS system, known as the *Pseudomonas* quinolone signal (PQS), which acts as a link between the *las* and *rhl* systems and signals via 4-hydroxy-2-alkylquinolines (HAQs) (14, 15).

The quinolone signaling network is encoded by the *pqsABCDE* operon, which is positively regulated by the transcriptional regulator PqsR (also known as MvfR) (15). The first four genes in the operon are required for the synthesis of 2-heptyl-4-quinolone (HHQ), which is oxidized to PQS through the action of PqsH (15). In addition to HHQ and PQS, the synthesis of the other main

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HAQ, 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO), depends on PqsABCD; however, its synthesis is not dependent on the presence of HHQ (15). Transcription of *pqsABCDE* leads to the expression of the fifth gene in the operon, PqsE. The precise function of PqsE is not understood; however, structural analysis indicated that PqsE is related to the metallo- β -lactamase superfamily and is not required for HAQ biosynthesis but independently regulates genes that are under PQS control (16–18). PqsE is required for swarming motility and biofilm formation and can activate virulence in the absence of a functional *pqs* system (19). PqsE is closely tied with the elements of the *rhl* signaling system (18, 20). PQS positively regulates the *rhl* system, and *rhl* can negatively impact the production of PQS (21–23). PQS controls the production of the virulence factors pyocyanin (17) and elastase and influences biofilm development (21). Furthermore, PQS plays a role in antibiotic stress through its dual pro- and antioxidant functions, serving as a bacterial stress regulator (24).

Major factors contributing to antibiotic tolerance are transcriptional regulators, such as sigma factors, which initiate transcription by associating with the core RNA polymerase enzyme and direct promoter-specific transcription, thus affecting the expression of a wide range of genes (25). The RpoS sigma factor (σ^S) is a master regulator of the stress response and is closely linked with the regulation of QS, production of the virulence factors pyocyanin and exotoxin A (26), and biofilm formation (27). Furthermore, RpoS activity serves as a defense mechanism in the presence of carbapenems and fluoroquinolones (28, 29). Another alternative sigma factor, RpoN (σ^{54}), controls the expression of genes involved in nitrogen metabolism and motility (30–33). Recent studies showed the complex control of expression of the type VI secretion system (T6SS), an important factor of the bacterial pathogenesis, by RpoN (34). RpoN has been linked with the regulation of QS by repressing *lasR-lasI* and *rhlR-rhlI* (35). Depending on nutrient availability, RpoN can also influence biofilm formation in *P. aeruginosa* (36). The goal of this study was to expand on previous work from our laboratories demonstrating the role of RpoN in antibiotic tolerance to carbapenems in *P. aeruginosa* (37).

To investigate the mechanism by which RpoN contributes to carbapenem tolerance, we initially constructed an *rpoN* in-frame deletion mutant and demonstrated that the $\Delta rpoN$ mutant has increased expression of *pqsA*, *pqsH*, and *pqsR* and higher levels of PQS. This observation prompted us to investigate the link between increased PQS levels and the carbapenem-tolerant phenotype of the $\Delta rpoN$ mutant. We constructed an in-frame deletion of *pqsA* in the $\Delta rpoN$ mutant background and confirmed that *pqsA* positively affects carbapenem tolerance in the $\Delta rpoN$ mutant. In addition, we also show that the *pqsE*, *pqsH*, and *rhlR* genes have a positive effect on the survival of the $\Delta rpoN$ mutant in the presence of carbapenems, suggesting that PQS and PQS-associated virulence factors mediate tolerance to carbapenems. Deletion of *vqsR* in the $\Delta rpoN$ mutant background did not affect the bacterial response to carbapenems. Using the results of transcriptional analysis of the wild-type PAO1, $\Delta rpoN$ mutant, and $\Delta rpoN$ mutant-derived strains, we provide further insights into the regulatory network involved in carbapenem tolerance.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains, plasmids, and primers used and generated in this study are shown in Table 1. Bac-

teria were routinely cultured at 37°C in Luria-Bertani (LB) medium or on L-agar plates supplemented with 1 mM L-glutamine, and when necessary, with 10% sucrose. Vogel-Bonner minimal medium (VBMM) (38) was used in mating experiments. Antibiotics for plasmid selection and propagation were added as required: gentamicin at 20 μ g/ml for *Escherichia coli* or 100 μ g/ml for *P. aeruginosa*.

Reagents. Biapenem was purchased from Meiji Seika Pharma Co., Ltd. (Yokohama, Japan), and doripenem was purchased from Shionogi & Co., Ltd. (Osaka, Japan). Both were used at a concentration of 32 μ g/ml. Synthetic PQS was obtained from Toyama Chemical Co., Ltd. (Tokyo, Japan), was solubilized in methanol, and was used at a concentration of 50 μ M for killing assays.

Antibiotic susceptibility testing. The MIC of each agent was determined using the broth microdilution method as previously described (39), with the following modifications: bacterial suspensions were incubated in LB medium at a density of 1×10^6 CFU/ml. MICs were determined after 24 h of incubation at 37°C. The MIC was defined as the lowest concentration of antimicrobial agent that completely inhibited the growth of the organism, as detected by the unaided eye.

Time-kill assays. For time-kill studies, approximately 10^8 CFU/ml of stationary-phase cells grown in 10 ml of LB medium in 50-ml conical tubes at 37°C were centrifuged at $3,000 \times g$ for 15 min. Subsequently, sedimented cells were resuspended in fresh 10-ml aliquots of LB medium and allowed to grow with antimicrobial agents in a shaker (200 rpm) at 37°C for 24 h. Samples were collected at several time points, 10-fold serial dilutions were prepared with 0.85% NaCl, and 100- μ l samples were plated onto L-agar plates in duplicate. Microbial killing was assessed at defined time points by counting the colonies and calculating the percent survival relative to the number of untreated cells at time zero. Plates with 30 to 300 colonies were used for CFU counts. Data were collected from at least three independent experiments.

Generation of mutant strains. Unmarked deletions of *rpoN*, *vqsR*, *pqsE*, *pqsA*, *rhlR*, and *pqsH* were constructed in *P. aeruginosa* PAO1 using the pEX18Gm suicide vector (40), which uses the *sacB*-based counterselection method (41). A strain with the single deletion of *rpoN* was first constructed, and that mutant was then used to generate the $\Delta rpoN \Delta vqsR$, $\Delta rpoN \Delta pqsA$, and $\Delta rpoN \Delta pqsE$ double mutants. To delete *rpoN*, the following procedure was used: a PCR fragment containing 545 nucleotides upstream of the *rpoN* start site and 150 nucleotides within the open reading frame was digested with EcoRI-BamHI and then inserted into pEX18Gm that had been digested with the same enzymes and linked to a BamHI-HindIII-digested PCR fragment containing 39 nucleotides of the *rpoN* gene and 516 nucleotides downstream of the *rpoN* stop codon. The deletion includes the helix-turn-helix motif and the *rpoN* box. The pEX18Gm- $\Delta rpoN$ construct was sequenced to confirm that PCR did not introduce a mutation before the construct was mobilized from *E. coli* S17-1 into *P. aeruginosa*. The transconjugants carrying the integrated plasmid on the chromosome were selected on L-agar plates containing 10% (wt/vol) sucrose, and sucrose-resistant colonies were screened using colony PCR to identify mutants. To generate double deletions, the aforementioned procedure was used. The *vqsR* and *pqsE* upstream and downstream fragments were generated using the primer pairs listed in Table 1. The two PCR products for each gene were digested with SacI-BamHI (upstream) and BamHI-SphI (downstream) for *vqsR* and with EcoRI-BamHI (upstream) and BamHI-HindIII (downstream) for *pqsE*. Inserts were then cloned into pEX18Gm to generate pEX18Gm- $\Delta vqsR$ and pEX18Gm- $\Delta pqsE$, respectively. The constructs were transferred from *E. coli* S17-1 to the *P. aeruginosa* $\Delta rpoN$ mutant. To delete *pqsA*, flanking upstream and downstream regions of approximately 650 bp were amplified from the chromosome of PAO1. The resulting DNA fragments contained a 15-bp overlap and were joined by overlap extension PCR. This final PCR product was cloned into pEX18Gm using the SacI and HindIII restriction sites to generate pEX18Gm- $\Delta pqsA$. In-frame deletion constructs of *rhlR* and *pqsH* were performed using an In-Fusion HD cloning kit (Clontech Laboratories, Inc.). The upstream fragment of each gene

TABLE 1 Bacterial strains, plasmids, and primers used in the study

Bacterial strain, plasmid or primer	Relevant characteristics ^a	Reference or source
Bacterial strains		
<i>E. coli</i> S17-1	<i>thi pro hsdR recA</i> Tra ⁺	These laboratories
<i>P. aeruginosa</i>		
PAO1	Wild type	
PAO1 $\Delta rpoN$	PAO1 with an in-frame deletion of <i>rpoN</i>	This study
PAO1 $\Delta rpoN \Delta vqsR$	PAO1 with an in-frame deletion of <i>rpoN</i> and <i>vqsR</i>	This study
PAO1 $\Delta rpoN \Delta pqsA$	PAO1 with an in-frame deletion of <i>rpoN</i> and <i>pqsA</i>	This study
PAO1 $\Delta rpoN \Delta pqsE$	PAO1 with an in-frame deletion of <i>rpoN</i> and <i>pqsE</i>	This study
PAO1 $\Delta rpoN \Delta pqsH$	PAO1 with an in-frame deletion of <i>rpoN</i> and <i>pqsH</i>	This study
PAO1 $\Delta rpoN \Delta rhlR$	PAO1 with an in-frame deletion of <i>rpoN</i> and <i>rhlR</i>	This study
Plasmids		
pEX18Gm	Broad-host-range gene replacement vector; <i>sacB</i> Gm ^r	41
pJN105	Arabinose-inducible gene expression vector; pBRR-1 MCS; <i>araC</i> -P _{BAD} Gm ^r	42
pEX18Gm- $\Delta rpoN$	<i>rpoN</i> deletion suicide vector	This study
pEX18Gm- $\Delta vqsR$	<i>vqsR</i> deletion suicide vector	This study
pEX18Gm- $\Delta pqsA$	<i>pqsA</i> deletion suicide vector	This study
pEX18Gm- $\Delta pqsE$	<i>pqsE</i> deletion suicide vector	This study
pEX18Gm- $\Delta pqsH$	<i>pqsH</i> deletion suicide vector	This study
pEX18Gm- $\Delta rhlR$	<i>rhlR</i> deletion suicide vector	This study
Primers		
Primers for construction of mutant strains		
rpoN-upF	ATAGAATTCCGATCTCGGTCGGCGACATC	
rpoN-upR	ATAGGATCCCCTGGAGGTCCAGGGTGGATAG	
rpoN-downF	ATAGGATCCGGCATAGCCCCTTCGAGCGAG	
rpoN-downR	ATAAAGCTTCTCCGGCAGCTCCCTGGCTA	
vqsR-upF	ATAGAGCTCGCCGGTGACCAACACGGTGG	
vqsR-upR	ATAGGATCCGACGCACGCTAGTGCGCCGC	
vqsR-downF	ATAGGATCCTGCGATATCCACACAACACTACTCCTC	
vqsR-downR	ATAGCATGCGGCATAGCGATTGCCGGGAT	
pqsA-upF	CTAATGGAGCTCAGGGAAGCCTGCAAAATGGCAG	
pqsA-upR	GTGATAAAGGGTGTGCTGCGCTGGGAGAGAATGTA	
pqsA-downF	GACACCCCTTATCACGACAACCTTCCGGAGG	
pqsA-downR	CTAATGAAGCTTGCAATCCAGGTTGGGCCGGCA	
pqsE-upF	ATAGAATTCCGCCGGCGAGAGTCTCGAA	
pqsE-upR	ATAGGATCCAAGCCTCAACATGGCCGGT	
pqsE-downF	ATAGGATCCCCTGGACTGAGACGGGACAT	
pqsE-downR	ATAAAGCTTAGGCTGGACAGGCCATGC	
pqsH-upF	CGACTCTAGAGGATCGTAAGGGGTTGACAGGAG	
pqsH-upR	CACCGGCGGCTACTGGGTCATCCGTTGCTCCTT	
pqsH-downF	CAGTAGCCGCGGGTGCCA	
pqsH-downR	CCATGATTACGAATTGGACGACATCCGGCTTCA	
rhlR-upF	CGACTCTAGAGGATCCGCCTACGCGCCACTGGG	
rhlR-upR	GATGAGACCCAGCGCCCTCATTCAGTAAGCCC	
rhlR-downF	GCGCTGGGTCTCATCTGAAG	
rhlR-downR	CCATGATTACGAATTAGCGCGAAAGCTCCCAG	
pqsESD-F	TTGGGCTAGCGAATTAGGAAACAGCTATGTTGAGGCTTTCGG	
pqsES-R	TGGCGGCCGCTCTAGTCAGTCCAGAGGCAGCGCCTGGC	
Primers for qRT-PCR		
vqsR-F	GCTGTCGATCGCCACTATCA	
vqsR-R	TCGGAGTGGGACTTCACGTT	
pqsA-F	CATCTCGCCGAACAGATTCC	
pqsA-R	CCAACTTGCCGTTGTCGTT	
pqsR-F	GCAGCTGATCGCGACAT	
pqsR-F	CAGCAGCACCCGGAGATT	
pqsH-F	GCGCGATCGAGTTCATC	
pqsH-R	CAGGGCGATTCCCACTGA	
rhlR-F	TTCGCGCTCCTGGAAAAG	
rhlR-R	CGCCATAGGCGTAGTAATCGA	
rpoS-F	CACTTCCTTCTCTCCAAACAACA	
rpoS-R	AGCTGCGTTGCGTCCAA	
omlA-F	CGAACTATCAACCAGCTGGTG	
omlA-R	GCTGTGCTCTGCAGGTTGTG	

^a Gm^r, resistance to gentamicin; MCS, multiple-cloning site. In the oligonucleotide sequences, introduced restriction sites are underlined, and the sequences introduced for overlap extension PCR are in italics.

contained a 15-bp overlap complementary to a 15-bp region of the downstream fragment. The amplified fragments contained a 5' end homology to the vector pEX18Gm and were fused together using in-fusion cloning in order to produce the deletion constructs pEX18Gm- Δ *rhlR* and pEX18Gm- Δ *pqsH*. The Δ *rpoN* Δ *rhlR* mutant and Δ *rpoN* Δ *pqsH* mutant were generated by allelic exchange of the *rhlR* and *pqsH* genes, respectively, in the Δ *rpoN* mutant background with pEX18Gm- Δ *rhlR* and pEX18Gm- Δ *pqsH*, respectively, using sucrose counterselection.

Construction of inducible overexpression of *pqsE*. To construct an expression plasmid for PqsE, the *pqsE* gene was amplified from *P. aeruginosa* PAO1 chromosomal DNA using the primers pqsESD-F and pqsESD-R. Using the In-Fusion cloning kit, this PCR product was cloned under the control of an arabinose-inducible promoter in the pJN105 plasmid (42) that had been digested with EcoRI-XbaI, resulting in plasmid pJN105-*pqsE*, which was further verified by sequence analysis. The pJN105 and pJN105-*pqsE* plasmids were introduced into wild-type *P. aeruginosa* PAO1 and the Δ *rpoN* Δ *pqsA* mutant via conjugation. For *pqsE* overexpression experiments, 0.2% arabinose was added to the stationary-phase culture 30 min prior to biapenem treatment.

PQS extraction and quantification. To analyze PQS production, wild-type PAO1 and the Δ *rpoN* mutant were used to inoculate 10-ml cultures. Following 16 h of incubation at 37°C, the cultures were washed and used to inoculate 10-ml subcultures to a final optical density at 595 nm (OD₅₉₅) of 0.01. Cultures were grown for 6 h, 10 h, and 24 h and extracted with acidified ethyl acetate. The phases were separated by centrifugation, and the organic phase was collected and dried. Extracts were resuspended in 20 μ l of methanol. Aliquots (10 μ l) of extracts were loaded onto silica gel thin-layer chromatography (TLC) plates (60F₂₅₄; Merck) that had previously been soaked in 5% KH₂PO₄ for 30 min and activated at 100°C for 1 h. Two microliters of the 10 mM PQS standard was used as a control. Samples were resolved using a solvent mixture consisting of dichloromethane-methanol (95:5). Fluorescent spots were visualized under long-wave UV light and photographed.

RNA isolation and qRT-PCR analysis. Strains were grown overnight in 10 ml of LB medium at 37°C for 16 h, after which time the cells were washed and used to inoculate 10-ml subcultures in LB medium to a final OD₅₉₅ of 0.01. The cultures were incubated at 37°C and were sampled at 6 h, 10 h, and 24 h. Total RNA was isolated from cells using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. To remove traces of contaminating DNA, total RNA was treated with Turbo DNase (Ambion) as suggested by the manufacturer. cDNA was generated from 550 ng of DNase-treated RNA using a Transcriptor first-strand cDNA synthesis kit (Roche Applied Science). Quantitative real-time PCRs (qRT-PCRs) were carried out in a LightCycler apparatus (Roche Molecular Biochemicals) using a LightCycler FastStart DNA Master^{Plus} SYBR green I kit (Roche Applied Science) according to the specifications of the supplier. Data were analyzed using LightCycler software (version 3.5). To correct for the differences in the amount of starting material, *omlA* was used as a reference gene. The software calculated the concentrations of the transcripts studied using a standard curve. The oligonucleotide primers used to detect the expression of each gene of interest are listed in Table 1. At least three technical replicates were performed for each cDNA sample analyzed.

Pyocyanin quantification. The *P. aeruginosa* PAO1, Δ *rpoN* mutant, and Δ *rpoN* mutant-derived strains were grown for 16 h in LB medium; pyocyanin was extracted from 5 ml of bacterial supernatant with chloroform and then reextracted with 0.2 N HCl to yield a pink solution. The absorbance at 520 nm was measured, and the amount of pyocyanin was calculated as previously described (43).

Growth assay. To assess the growth of the wild-type PAO1, Δ *rpoN* mutant, and Δ *rpoN* mutant-derived strains, cultures grown overnight were washed and used to inoculate 10-ml subcultures in LB medium to an OD₅₉₅ of ~0.01. The absorbance at 595 nm was monitored every 2 h for 12 h, with an additional measurement being taken at 24 h.

RESULTS

RpoN represses the expression of *pqsA*, *pqsR*, *pqsH*, and *lasR*.

We have previously shown that RpoN downregulates the expression of the QS transcriptional regulator VqsR and that of sigma factor PvdS (37). VqsR has been implicated in the regulation of another transcriptional regulator, QscR (44), as well as in the regulation of siderophore synthesis and genes in the PQS operon (45, 46). The connection between PQS and iron is well established; PQS is known to chelate iron and induce the expression of genes involved in the regulation of *pvdS* and the biosynthesis of pyoverdine and is essential for pyocyanin production (19, 21, 47, 48).

The implication of the involvement of RpoN in the regulation of the *las* and *rhl* QS systems prompted us to expand on the potential role of RpoN in regulating PQS. To this end, we isolated RNA from wild-type strain PAO1 and the Δ *rpoN* mutant that had been grown in LB medium for 6 h (OD₅₉₅, ~0.6), 10 h (OD₅₉₅, ~0.9), and 24 h (OD₅₉₅, ~1.1) and compared the transcript levels of *pqsA*, the first gene in the *pqsABCDE* operon, in each strain. The most pronounced difference was observed at the 6-h and 10-h time points, at which time the Δ *rpoN* mutant expressed 6 and 7 times more *pqsA*, respectively, than the wild type. By 24 h, the levels of *pqsA* transcripts in the *rpoN* mutant were only 1.3 times higher than those in the wild type (Fig. 1A).

The expression of the *pqsABCDE* operon requires the LysR-type transcriptional activator PqsR, which binds to the *pqsA* promoter. PqsR binding is increased in the presence of PQS (23, 49); therefore, we also measured *pqsR* expression. As we suspected, the Δ *rpoN* mutant had higher levels of *pqsR* than the wild type at all time points (Fig. 1B).

Having established the role of *rpoN* in the regulation of *pqsA* and *pqsR*, we subsequently sought to confirm that the increased levels of *pqsA* and *pqsR* correlate with an increase in the expression of *pqsH*, which is responsible for the conversion of HHQ into PQS and is directly controlled by LasR (15). We found that *pqsH* expression was upregulated in the Δ *rpoN* mutant relative to its expression in the wild type (Fig. 1C).

The close regulatory link between LasR and PQS, with LasR being a dominant regulator of *pqsR* and *pqsH* expression (23, 50), prompted us to investigate the transcriptional levels of *lasR* in wild-type PAO1 and in the Δ *rpoN* mutant. The level of *lasR* expression in the Δ *rpoN* mutant was consistently higher than that in wild-type PAO1 at all tested time points (Fig. 1D). Based on the findings presented above, we conclude that in nutrient-rich medium, RpoN promotes the downregulation of the *pqsA*, *pqsR*, and *pqsH* genes through the regulatory mechanism governed by LasR.

RpoN negatively affects PQS production. To correlate the transcriptional data with the production of PQS in the wild-type and Δ *rpoN* mutant, the strains were cultured and sampled as described above for the transcriptional analysis, and the levels of PQS, which included intracellular, extracellular, and membrane-associated PQS, were examined using TLC. Figure 2 demonstrates that PQS was detected in cell extracts prepared from the wild-type strain in early-stationary-phase LB medium cultures at 6 h of growth (OD₅₉₅, ~0.6); however, less PQS was detected in the Δ *rpoN* mutant at the same time point, as determined by UV inspection. In contrast, PQS levels were higher in the Δ *rpoN* mutant than in wild-type PAO1 at 10 h postinoculation, and this difference was even more pronounced at the 24-h time point, where the Δ *rpoN* mutant demonstrated significantly higher levels of PQS.

FIG 1 Expression of *pqsA* (A), *pqsR* (B), *pqsH* (C), and *lasR* (D) in wild-type PAO1 and the $\Delta rpoN$ mutant throughout growth. Overnight cultures of PAO1 and the $\Delta rpoN$ mutant were diluted to an OD₅₉₅ of 0.01 in LB medium supplemented with 1 mM glutamine, grown at 37°C, and harvested at an OD₅₉₅ of ~0.6 (6 h), 0.9 (10 h), and 1.1 (24 h) for total RNA isolation. The levels of the *pqsA*, *pqsR*, *pqsH*, and *lasR* transcripts were measured by qRT-PCR and normalized to the level of *omlA* expression. All results are the averages from at least three independent experiments, and the error bars represent SDs.

Somewhat surprisingly, although transcriptional analysis demonstrated increased expression of *pqsA*, *pqsR*, and *pqsH* at the 6-h time point, this did not correlate with the PQS levels observed. Although we cannot offer a conclusive explanation for this discrepancy, we hypothesize that the amount of substrate for PQS synthesis might be limited in the $\Delta rpoN$ mutant at this point during growth. Furthermore, it is possible that at this time point, the regulation of *pqsA* might be under the control of some intermediary regulatory network(s) other than PqsR-PQS. The increased levels of *pqsA* could be a consequence of the effect that RhlR has on *pqsA* expression. The *pqsA* promoter region has a distinct transcriptional site controlled by RhlR that exerts posttranscriptional regulation of *pqsA* to fine-tune the production of PQS (51). However, we do not exclude the possibility that a low level of expression of *pqsH* in the early growth phase may be responsible for undetectable levels of PQS in the $\Delta rpoN$ mutant, despite increased *pqsA* expression. In addition, the PQS levels observed at the 24-h time point were significantly higher than those found in the wild

type, even though the $\Delta rpoN$ mutant demonstrated a modest 1.3-fold upregulation of *pqsA* expression relative to the wild type.

We favor the proposed negative autoregulatory model for PQS regulation in which, once maximal physiological levels of PQS are reached, PQS leads to downregulation of the *pqsA* operon (20). These results additionally confirm that RpoN negatively affects the synthesis of PQS through the downregulation of *pqsA*, *pqsR*, and *pqsH* expression.

***pqsA*, *pqsE*, and *pqsH* confer tolerance to carbapenems in the $\Delta rpoN$ mutant.** Our previous observation demonstrated that stationary-phase cells of the $\Delta rpoN$ mutant display a significant tolerant phenotype in the presence of carbapenems (37). One of the characteristics of antibiotic-tolerant cells is that their mechanism usually does not reflect MIC values, which remain unchanged. The MIC values of biapenem and doripenem for the mutant strains remained comparable to those observed for the wild-type strain PAO1 and were 0.5 $\mu\text{g/ml}$ for both antibiotics for the wild-type PAO1 strain and the PAO1 $\Delta rpoN$, $\Delta rpoN \Delta vqsR$, $\Delta rpoN \Delta pqsA$, $\Delta rpoN \Delta pqsE$, and $\Delta rpoN \Delta pqsH$ mutant strains.

The goal of this study was to find the target(s) through which RpoN modulates the effect of carbapenems. The $\Delta rpoN$ mutant displayed a higher degree of tolerance to 32 $\mu\text{l/ml}$ biapenem (Fig. 3A) and doripenem (Fig. 3B) than the wild-type strain.

The increased expression of *vqsR* in the $\Delta rpoN$ mutant prompted us to look at the role of and requirement for VqsR in surviving antibiotic stress. The $\Delta rpoN \Delta vqsR$ mutant demonstrated a trend of susceptibility to carbapenems similar to that of the $\Delta rpoN$ mutant, suggesting that VqsR is not directly involved in the antibiotic tolerance observed in the $\Delta rpoN$ mutant (Fig. 3C and D).

To integrate the information gained in our transcriptional studies indicating that the $\Delta rpoN$ mutant has increased expression of *pqsA* and, consequently, increased production of PQS, we sought to determine whether *pqsA* is a target for the interaction with carbapenems in the $\Delta rpoN$ mutant. To assess the role of *pqsA* in the susceptibility to carbapenems in the $\Delta rpoN$ mutant, we

FIG 2 PQS production in wild-type PAO1 and the $\Delta rpoN$ mutant as measured by TLC. Overnight cultures of PAO1 and the $\Delta rpoN$ mutant were diluted to an OD₅₉₅ of 0.01 in LB medium supplemented with 1 mM glutamine, grown at 37°C, harvested at an OD₅₉₅ of ~0.6 (6 h), 0.9 (10 h), and 1.1 (24 h), and then assayed for PQS production. Lane 1, 2 μl (10 mM) synthetic PQS; lane 2, PAO1 (6 h of growth); lane 3, $\Delta rpoN$ mutant (6 h of growth); lane 4, PAO1 (10 h of growth); lane 5, $\Delta rpoN$ mutant (10 h of growth); lane 6, PAO1 (24 h of growth); lane 7, $\Delta rpoN$ mutant (24 h of growth). A representative photograph of TLC-separated extracts from PAO1 and the $\Delta rpoN$ mutant is shown.

FIG 3 Killing assays of wild-type PAO1, the $\Delta rpoN$ mutant, and the $\Delta rpoN$ mutant-derived strains in the presence of biapenem (A, C, E, G) and doripenem (B, D, F, H) at 32 $\mu\text{g/ml}$. Under the assumption that the survival at time zero was 100%, the numbers of CFU were converted to percentages. The experiment was performed in triplicate. Error bars, SDs from three experiments.

performed the killing assay with the $\Delta rpoN \Delta pqsA$ mutant in the presence of biapenem (Fig. 3E) and doripenem (Fig. 3F). The $\Delta rpoN \Delta pqsA$ mutant exhibited a steep decrease in viability, measured as a decrease in the number of CFU, indicating the complete loss of the tolerant phenotype observed in the $\Delta rpoN$ mutant.

The observations suggesting a direct role of *pqsA* in tolerance to carbapenems in the $\Delta rpoN$ mutant prompted us to further address the significance of *pqsE* in the interaction with carbapenems. PqsE, which is coregulated together with the *pqsABCD* genes, is not required for HAQ synthesis and instead controls the production of PQS-regulated factors, such as pyocyanin (20). The loss of *pqsE* in the $\Delta rpoN$ mutant background resulted in a decline in viability in the presence of carbapenems (Fig. 3G and H).

To confirm that the loss of *pqsE* does not affect PQS production in the $\Delta rpoN$ mutant background and to gain additional information regarding the effect of *vqsR* on PQS production, we examined the production of PQS in the $\Delta rpoN \Delta pqsE$ and $\Delta rpoN \Delta vqsR$ mutants using TLC. The results demonstrated that neither the mutation in *pqsE* nor the mutation in *vqsR* affected the levels of PQS in the $\Delta rpoN$ mutant (Fig. 4). From these data, we concluded that the mechanism underlying the tolerance of stationary-phase $\Delta rpoN$ mutant cells is linked with the upregulation of the *pqsA* gene and the consequent PQS production.

We also demonstrated that *pqsE* does not require PQS synthesis to promote survival in the presence of carbapenems in the $\Delta rpoN$ mutant, suggesting that PqsE might affect the response to carbapenems through the regulation of PQS-controlled virulence factors, such as pyocyanin. Therefore, we sought to define the

correlation between pyocyanin production and carbapenem tolerance in the wild-type and mutant strains. Whereas mutations in *pqsA* and *pqsE* in the *rpoN* mutant background profoundly affected pyocyanin production, the $\Delta rpoN$ and the $\Delta rpoN \Delta vqsR$ mutants demonstrated levels of pyocyanin equal to those ob-

FIG 4 PQS production in the $\Delta rpoN \Delta pqsE$ and $\Delta rpoN \Delta vqsR$ mutants as measured by TLC. Overnight cultures of the $\Delta rpoN \Delta pqsE$ and $\Delta rpoN \Delta vqsR$ mutants were diluted to an OD_{595} of 0.01 in LB medium supplemented with 1 mM glutamine, grown at 37°C, harvested at an OD_{595} of ~ 0.6 (6 h), 0.9 (10 h), and 1.1 (24 h), and then assayed for PQS production. Lane 1, 2 μl (10 mM) synthetic PQS; lane 2, $\Delta rpoN \Delta vqsR$ mutant (6 h of growth); lane 3, $\Delta rpoN \Delta pqsE$ mutant (6 h of growth); lane 4, $\Delta rpoN \Delta vqsR$ mutant (10 h of growth); lane 5, $\Delta rpoN \Delta pqsE$ mutant (10 h of growth); lane 6, $\Delta rpoN \Delta vqsR$ mutant (24 h of growth); lane 7, $\Delta rpoN \Delta pqsE$ mutant (24 h of growth). A representative photograph of TLC-separated extracts from the $\Delta rpoN \Delta vqsR$ and $\Delta rpoN \Delta pqsE$ mutants is shown.

FIG 5 Biapenem (32 µg/ml) killing assay of wild-type PAO1, the $\Delta rpoN$ mutant, and the $\Delta rpoN\Delta pqsH$ mutant. Under the assumption that the survival at time zero was 100%, the numbers of CFU were converted to percentages. The experiment was performed in triplicate. Error bars, SDs from three experiments.

served in wild-type PAO1 (see Fig. S1 in the supplemental material).

Finally, we addressed the question of whether HHQ, a direct precursor of PQS that produces PQS through the action of PqsH, which is positively regulated by *lasR* (15), might play a role in the response to carbapenems. The sensitive phenotype of the $\Delta rpoN\Delta pqsA$ mutant might be due to the loss of PQS or any other HAQ because, upon deletion of *pqsA*, the production of PQS was abolished, as was the production of many other HAQs, such as HQNO and HHQ. To address the role of HHQ production on survival upon carbapenem exposure, we constructed an $\Delta rpoN\Delta pqsH$ mutant and performed a time-kill assay using biapenem at a concentration of 32 µl/ml. Assessment of the susceptibility of the stationary-phase cells of the $\Delta rpoN\Delta pqsH$ mutant to biapenem compared to the survival rate of the $\Delta rpoN$ mutant displayed a clear difference; the *rpoN* and *pqsH* deletions abolished the biapenem-tolerant phenotype of the $\Delta rpoN$ mutant (Fig. 5). The growth of the wild-type and mutant strains was shown to be comparable (see Fig. S2 in the supplemental material). These results indicate that the loss of PQS synthesis through the mutation in the *pqsH* contributes to carbapenem tolerance.

RpoN requires the contribution of both PQS and PqsE to overcome the effect of carbapenems. To determine if the sensitive phenotype of the $\Delta rpoN\Delta pqsA$ mutant is a consequence of a loss of PQS or PqsE, since the mutation in *pqsA* negatively affects the expression of *pqsE* (19), we initially addressed this question by performing a time-kill assay using wild-type PAO1 and the $\Delta rpoN\Delta pqsA$ mutant with 32 µl/ml biapenem in the presence of 50 µM PQS, which is a biologically relevant concentration. The killing curve demonstrated that the addition of PQS completely restored the susceptibility of the $\Delta rpoN\Delta pqsA$ mutant, increasing the survival by 100-fold. In wild-type PAO1, susceptibility to biapenem

FIG 6 Biapenem (32 µg/ml) killing assay of wild-type PAO1 and the $\Delta rpoN\Delta pqsA$ mutant in the presence of PQS (50 µM) (A) and *pqsE* overexpression using an arabinose-inducible pJN105-*pqsE* construct (B). (A) Time-kill assay for the $\Delta pqsA$ mutant in the presence of biapenem (32 µg/ml). Arabinose (0.2%) was added 30 min before the addition of biapenem. The viability of wild-type PAO1, the $\Delta rpoN\Delta pqsA$ mutant, and the $\Delta pqsA$ mutant is expressed as percent survival.

also decreased in the presence of exogenously supplied PQS (Fig. 6A). The biapenem killing curve observed for the $\Delta pqsA$ mutant was similar to that observed for the wild type (Fig. 6A).

Next, we evaluated the contribution of PqsE overexpression to the survival of the $\Delta rpoN\Delta pqsA$ mutant in the presence of biapenem. We introduced pJN105-*pqsE*, expressing PqsE under the control of the arabinose promoter, in wild-type PAO1 and the $\Delta rpoN\Delta pqsA$ mutant. The biapenem time-kill assay profiles of wild-type PAO1 and the $\Delta rpoN\Delta pqsA$ mutant, which were compared in the presence and absence of a vector carrying *pqsE* induced by 0.2% arabinose in the medium, demonstrated that the inducible overexpression of *pqsE* completely abrogated the susceptibility to biapenem in wild-type PAO1 and produced a minor effect in the $\Delta rpoN\Delta pqsA$ mutant (Fig. 6B). Because exogenously provided PQS positively affects the expression of the entire *pqs* operon, including *pqsE*, the results clearly demonstrate that RpoN, through the contribution of both PQS and the PqsE-mediated network, defines the response to carbapenems.

rhIR modulates the response to carbapenems in the $\Delta rpoN$ mutant. The evidence that *las* signaling is placed at the top of the QS hierarchical system exerting transcriptional control over both *rhIR* and *rhII* and that the production of PQS depends on *lasR*, which positively regulates *pqsR* and directly controls the expression of *pqsH* (13, 15, 50), prompted us to extend our studies investigating the importance of *lasR* in mediating carbapenem tolerance in the $\Delta rpoN$ mutant. To address this question, we tried to construct a $\Delta rpoN\Delta lasR$ mutant but were unable to create this mutant after multiple attempts.

Throughout growth, the $\Delta rpoN$ mutant demonstrated significant increases in the levels of the transcriptional regulator *rhIR*. To further establish the importance of *rhIR* in carbapenem tolerance, we examined the effect of an *rhIR* mutation in the $\Delta rpoN$ mutant on biapenem survival. Treatment with biapenem at 32 µl/ml led to an increase in the sensitivity of the $\Delta rpoN\Delta rhIR$ mutant after 12 h of growth (Fig. 7). Assessing the PQS levels in the $\Delta rpoN\Delta rhIR$ mutant, we observed that the $\Delta rpoN\Delta rhIR$ mutant expressed wild-type levels of PQS (Fig. 8). All together, these results confirm

FIG 7 Biapenem (32 μg/ml) killing assay of wild-type PAO1, the $\Delta rpoN$ mutant, and the $\Delta rpoN\Delta rhIR$ mutant. Under the assumption that the survival at time zero was 100%, the numbers of CFU were converted to percentages. The experiment was performed in triplicate. Error bars, SDs from three experiments.

that RhIR is an important participant in mediating tolerance to carbapenems.

Transcriptional expression of the *rhIR*, *vqsR*, and *rpoS* genes in the $\Delta rpoN$ mutant and $\Delta rpoN$ mutant-derived strains. Given that *pqsA* and *pqsE* mediate the response to carbapenems through interactions with RpoN, we further asked whether some of the major regulators of *P. aeruginosa* QS (RhIR, VqsR, and RpoS) previously shown to be negatively regulated by RpoN might also aid in modulating the response to carbapenems. To do this, we used qRT-PCR to compare the transcriptional expression of *rhIR*, *vqsR*, and *rpoS* in the wild-type PAO1, $\Delta rpoN$ mutant, and $\Delta rpoN$ mutant-derived strains. The levels of the *rhIR*, *vqsR*, and *rpoS* transcripts (Fig. 9A to C, respectively) were consistently higher in the $\Delta rpoN$ mutant than in the wild type at all tested time points, with the most pronounced difference being observed at the 24-h time point. These results confirm previously obtained data that indicate that RpoN predominantly exerts a negative regulation of *rhIR*, *vqsR*, and *rpoS* throughout growth (28, 35, 37).

Next, we investigated the contribution of the quorum-sensing transcriptional regulator VqsR on the expression of *rhIR* and *rpoS* in the $\Delta rpoN$ mutant background. In the $\Delta rpoN\Delta vqsR$ mutant, *rhIR* levels were 2-fold lower than those in the $\Delta rpoN$ mutant at the 24-h time point (Fig. 9A). This confirms that VqsR plays a role in RhIR regulation and suggests that RpoN exerts part of its negative effect on RhIR by downregulating VqsR. Given the evidence that RpoN negatively affects the expression of sigma factor RpoS in stationary phase (28) and that VqsR is involved in the positive regulation of RpoS (45), we investigated the expression of *rpoS* in the $\Delta rpoN\Delta vqsR$ mutant. At the 24-h time point, *rpoS* levels in the $\Delta rpoN\Delta vqsR$ mutant were 3-fold lower than those in the $\Delta rpoN$ mutant (Fig. 9C). These results suggest that some of the interaction between RpoN and RpoS may be mediated through VqsR; however, the exact role of VqsR in the response to carbapenems remains to be elucidated, particularly in light of the fact that carbapenem susceptibility remained unchanged in the $\Delta rpoN\Delta vqsR$ mutant, despite the downregulation of both RhIR and RpoS.

To gain more insights into how the RpoN network connects with *pqsA*, we also examined the transcript levels of *rhIR*, *vqsR*, and

FIG 8 PQS production in wild-type PAO1, the $\Delta rpoN$ mutant, and the $\Delta rpoN\Delta rhIR$ mutant as measured by TLC. Overnight cultures of PAO1, the $\Delta rpoN$ mutant, and the $\Delta rpoN\Delta rhIR$ mutant were diluted to an OD_{595} of 0.01 in LB medium supplemented with 1 mM glutamine, grown at 37°C, harvested at an OD_{595} of ~1.1 (24 h), and then assayed for PQS production. Lane 1, 2 μl (10 mM) synthetic PQS; lane 2, PAO1 (24 h of growth); lane 3, $\Delta rpoN$ mutant (24 h of growth); lane 4, $\Delta rpoN\Delta rhIR$ mutant (24 h of growth); lane 5, 2 μl (10 mM) synthetic PQS. A representative photograph of TLC-separated extracts from PAO1, the $\Delta rpoN$ mutant, and the $\Delta rpoN\Delta rhIR$ mutant is shown.

rpoS in the $\Delta rpoN\Delta pqsA$ mutant and compared them to the transcript levels observed in the wild type and the $\Delta rpoN$ mutant. The effect of the *pqsA* deletion in the $\Delta rpoN$ mutant on *rhIR* expression was obvious at all time points, as *rhIR* levels decreased by 3.6- and 3-fold at the 10-h and 24-h time points, respectively, relative to those in the $\Delta rpoN$ mutant (Fig. 9A). The most pronounced difference in *vqsR* expression between the $\Delta rpoN\Delta pqsA$ and $\Delta rpoN$ mutants was observed at the 24-h time point, with a 7-fold difference in expression being detected (Fig. 9B). At the 10-h time point, *rpoS* transcript levels were 3.1-fold lower in the $\Delta rpoN\Delta pqsA$ mutant than in the $\Delta rpoN$ mutant (Fig. 9C). These results demonstrate that elimination of the synthesis of PQS and the activity of RhIR, VqsR, and RpoS modulates the response to carbapenems.

Using the same approach, we analyzed the effects of deleting *pqsE* in a $\Delta rpoN$ mutant background on the expression of *rhIR*, *vqsR*, and *rpoS*. All three genes, which are involved in QS regulation, followed a similar profile of downregulation in the $\Delta rpoN\Delta pqsE$ mutant and the $\Delta rpoN$ mutant. At the 24-h time point, *rhIR* transcript levels were 5-fold lower (Fig. 9A) and *vqsR* levels were 2.6-fold lower (Fig. 9B) in the $\Delta rpoN\Delta pqsE$ mutant than in the $\Delta rpoN$ mutant, and for *rpoS* levels, the same pattern seen with *vqsR* levels was observed, in which *rpoS* levels decreased by 2-fold in the $\Delta rpoN\Delta pqsE$ mutant compared with those in the $\Delta rpoN$ mutant (Fig. 9C).

All together, these results demonstrate that RpoN acts on QS regulators, including RhIR, VqsR, and RpoS, and that these elements of QS may define the mechanism by which RpoN mediates the response of *P. aeruginosa* to carbapenems.

DISCUSSION

To escape the bactericidal effect of antibiotics, *P. aeruginosa* employs its complex regulatory network to coordinate and promote adaptation to antibiotic stress. Of the several genes in *P. aeruginosa* known to modulate the response to antimicrobials (5, 8, 24, 28, 29, 37), an important role is played by sigma factor RpoN. This sigma factor is involved in the regulation of nitrogen assimilation, motility, QS, biofilm formation, secretion systems, and many others (30–36).

One important mechanism through which RpoN modulates

FIG 9 Expression levels of *rhlR* (A), *vqsR* (B), and *rpoS* (C) in wild-type PAO1, the $\Delta rpoN$ mutant, and the $\Delta rpoN$ mutant-derived strains. Cultures of PAO1, the $\Delta rpoN$ mutant, and the $\Delta rpoN$ mutant-derived strains grown overnight were diluted to an OD_{595} of 0.01, grown at 37°C in LB medium, and harvested at an OD_{595} of ~0.6 (6 h), 0.9 (10 h), and 1.1 (24 h) for total RNA preparation. Transcript levels were measured by quantitative RT-PCR and normalized to the level of *omlA* expression. All results are averages from at least three independent experiments, and the error bars represent SDs.

the bactericidal effect of antimicrobials stems from its role in the regulation of QS, a target for antimicrobial therapy (52). In this study, we demonstrated that RpoN, in addition to its ascribed role in the regulation of the *las* and *rhl* QS systems, interacts with the PQS QS system, negatively regulates the expression of *pqsA*, *pqsR*, and *pqsH*, and, consequently, negatively affects the production of PQS. The regulatory network intercepted between RpoN and the elements of the QS system is presented in Fig. 10.

Our initial studies on RpoN demonstrated that RpoN has a marked negative effect on the QS transcriptional regulator VqsR (37). Available evidence suggests that VqsR regulates QS systems

(*las*, *rhl*, and *pqs*) by directly downregulating the expression of *qscR* (44), and the *las* system seems to be the dominant regulator of *vqsR*, *pqsR*, and *pqsH* (15, 53). We postulate that the observed activation of *pqsR* and *vqsR* in the $\Delta rpoN$ mutant is due to the upregulation of *lasR*, which in turn enhances *pqsA* expression. Furthermore, PqsR-PQS autoinduces PQS synthesis and further activates *rhlR* and *rhlI* expression.

In addition to increased expression of *vqsR*, the $\Delta rpoN$ mutant demonstrated significantly increased levels of the *rpoS* transcript throughout growth, suggesting that the absence of σ^{54} facilitates an increase in σ^S RNA polymerase binding, thus triggering the

FIG 10 Diagram of the proposed model of RpoN regulation in *P. aeruginosa*. The detailed network of regulation is described in the Discussion. Solid lines with arrows and dashed lines with blunt ends, genes that are positively and negatively affected, respectively. IM, inner membrane; OM, outer membrane; PS, periplasmic space.

survival mode in the presence of an antibiotic. The fact that the RpoS-dependent pathway is involved in the response to carbapenems suggested that the carbapenem-tolerant phenotype of the $\Delta rpoN$ mutant in the stationary phase may be due to increased levels of RpoS (29). VqsR positively affects the expression of the *pqsABCDE* operon and of *pqsH* (46), and it has been demonstrated that PQS enhances RpoS expression (21); therefore, we presume that VqsR might modulate the expression of RpoS through PQS, reinforcing mutual control between PQS, VqsR, and RpoS.

We have previously reported that RpoN has an impact on iron-regulated genes irrespective of iron availability in the growth medium, and consequently, RpoN negatively affects the expression of the alternative sigma factor PvdS (37). Other findings also point to the fact that iron, QS, and RpoN are closely connected (54). To integrate the RpoN network into the regulation of PvdS, we postulate that both the control of the expression of iron-regulated genes, such as *pvdS* and *pchA* to *pchG*, by VqsR (44, 45, 55) and the iron-chelating properties of PQS (47, 48) could account for the increased expression of *pvdS*.

Using a time-kill assay, we were further able to demonstrate that *pqsA*, *pqsE*, *pqsH*, and *rhlR* play a role in mediating the increased survival of the $\Delta rpoN$ mutant in the presence of carbapenems because their inactivation in the $\Delta rpoN$ mutant altered the carbapenem-tolerant phenotype of the $\Delta rpoN$ mutant. The evidence that RpoN regulates several transcriptional regulators involved in the regulation of QS, including VqsR, RpoS, and PqsR, in addition to LasRI and RhlRI (35), suggests that the complex network between the elements of the QS systems contributes to the increased survival of the $\Delta rpoN$ mutant in the presence of carbapenems. This observation is consistent with a previous finding that the number of *P. aeruginosa* persister cells increases in response to QS and virulence factors (6). Additionally, it has been reported that the loss of *rpoN* through the deregulation of glucose metabolism increases gluconate production, which correlates with reduced susceptibility to several antibiotics (56).

A strategy for overcoming the effects of carbapenems requires RpoN to mediate this response through the regulators, which are likely to be membrane associated because carbapenems gain entrance into the cell through the outer membrane proteins (OMPs), and once they are located in the periplasmic space, they trigger lethality through interactions with transmembrane penicillin binding proteins (PBPs), leading to cell wall disintegration (57). One of the targets might be the transcriptional regulator AmpR, a potential inner membrane-associated protein (58). This hypothesis is supported by the observations that in the presence of β -lactams, the $\Delta rpoN$ mutant stimulated a significant increase in the levels of the *ampR* gene and the gene encoding β -lactamase, *ampC* (58), suggesting that this increase in *ampR* and *ampC* levels might aid in explaining the carbapenem-tolerant phenotype of the $\Delta rpoN$ mutant. Additionally, the $\Delta ampR$ mutant exerted a negative effect on the expression of the major QS regulators LasR, RhlR, PqsR, and PQS and the pyochelin and pyoverdine genes (59).

Another potential strategy to overcome the effect of carbapenems is through the role of PQS and its association with the outer membrane. Because PQS has membrane-altering activities linked with the formation of outer membrane vesicles (OMVs) (60, 61) and more than 62.5% of the PQS produced is deposited in OMVs (62), we assume that the increased production of PQS and its

association with the cell membrane would lower the effective concentration of carbapenems taken up by the cells.

An additional explanation for the role of PQS in modulating the response to carbapenems might come from the observation that OprF, a major outer membrane protein, is important in virulence factor production and in the formation of OMVs through modulation of PQS levels (63). Therefore, any perturbation in this network might affect the susceptibility to carbapenems.

Growing evidence suggests that one of the mechanisms by which cells react to antibiotic stress is through increased vesiculation (64). Adding to the complexity of PQS regulation, PQS has both antioxidant and pro-oxidant activities (5). The pro-oxidant activity of PQS increases the sensitivity of *P. aeruginosa* to peroxide and various antibiotics (5, 24), possibly resulting in cell lysis and DNA release. Our results with exogenously supplemented PQS demonstrate that PQS protected the cells from the effect of carbapenems. We presume that the difference in the survival potential between the cells exposed to PQS and those not exposed could be explained, in part, by the action of the alternative sigma factor RpoS, which could counterbalance the pro-oxidant effect of PQS by eliciting a stress response.

Furthermore, it is important to note that treatment with β -lactams leads to activation of the SOS response, which halts bacterial cell division and allows survival (65). Efflux pumps have been implicated as an important component of oxidative stress, providing protection against hydroxyl radicals by expelling damaged proteins outside the cells and promoting tolerance (66). To support this hypothesis, PQS is associated with the MexGHI-OpmD efflux pump, which controls antibiotic susceptibility through the production of PQS and *N*-acyl-L-homoserine lactone QS signal molecules and is also regulated by VqsR (44, 67). A recent study demonstrates that MexG is capable of binding PQS (68). The studies by Wu et al. demonstrated that the pro-oxidant paraquat promoted antibiotic tolerance, underscoring the importance of oxidative stress in antibiotic tolerance (69).

The fact that the induction of *pqsE* in the $\Delta rpoN \Delta pqsA$ mutant produced merely a basal decrease in carbapenem susceptibility suggests that at least two factors, PQS and PqsE, cooperate to alleviate the effect of carbapenems in the $\Delta rpoN$ mutant. In addition to *pqsA*, the information that we gathered from the time-kill assay for the $\Delta rpoN \Delta pqsH$ mutant reveals the direct involvement of PQS in the response to carbapenems in the $\Delta rpoN$ mutant. The fact that the $\Delta pqsH$ mutant is defective in vesicle formation (15, 61) additionally accentuates the importance of PQS in the interaction with the cell wall-targeting antibiotics. Furthermore, *pqsH* is directly controlled by LasR and not by PqsR (15). It is therefore conceivable that the LasR regulatory control over *pqsH* is important in tolerance to carbapenems. In the same line of experiments, we demonstrated that *pqsE* plays a role in tolerance to carbapenems in the $\Delta rpoN$ mutant and that its role in tolerance is not dependent on PQS levels because they remained as high in the $\Delta rpoN \Delta pqsE$ mutant as in the $\Delta rpoN$ mutant. These observations led us to conclude that the loss of tolerance to carbapenems might also be accounted for by the loss of PQS-mediated virulence factors because PqsE influences the production of the secondary metabolites, such as pyocyanin, through interactions with RhlR (18, 20). Given that *rhlR* is controlled by RpoN and the regulation of RhlR and PqsE is interdependent, we addressed the possibility of whether suppressing the production of virulence factors via a mutation in *rhlR* in the $\Delta rpoN$ mutant would correspond to the sus-

ceptibility observed in the $\Delta rpoN \Delta pqsE$ mutant. The loss of *rhlR* in the $\Delta rpoN$ mutant background affected survival in the presence of carbapenems and led to the decreased production of PQS, indicating that PQS production is important for biapenem tolerance mediated through RhlR.

In this study, we demonstrate that RpoN shuts down major metabolic systems, such as the QS systems, to communicate environmental cues between networks and to reduce energy costs. In this way, RpoN promotes the activation of only those cellular functions required for cells to enter into a less metabolically active state and persist. In addition, it was demonstrated that during growth under nutrient-restricted conditions the $\Delta rpoN$ mutant downregulates the expression of *pqs* signaling (70). Low metabolic activity and stressed environmental conditions are connected with the increase in the levels of the alarmone ppGpp, a regulator of the stringent response, which is involved in the regulation of QS systems, the nitrogen stress response, and antibiotic tolerance (5, 8, 71, 72) and is essential for sustaining the survival of the cells. Therefore, we postulate that another level of RpoN regulation is mediated through ppGpp. Finally, the observations presented herein contribute to a better understanding regarding the way in which *P. aeruginosa* integrates multiple regulatory signals to alleviate the effect of carbapenems. A complete understanding of the regulatory pathways that interfere with RpoN during antibiotic stress remains to be elucidated.

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