rpoN Gene of *Pseudomonas aeruginosa* Alters its Susceptibility to Quinolones and Carbapenems

**Running title:** *rpoN* gene and tolerance in *Pseudomonas aeruginosa*

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The alternative sigma factor $\sigma^{54}$ has been implicated in diverse functions within the cells. In this study, we have constructed an rpoN mutant in Pseudomonas aeruginosa and investigated its importance as a target for antimicrobial agents, such as quinolones and carbapenems. The stationary phase cells of the rpoN mutant displayed an approximately 15 times higher survival rate to quinolones and carbapenems than the wild-type cells. The stationary phase led in P. aeruginosa rpoN mutant to substantial production of pyoverdine. Pyoverdine synthesis correlated with decreased susceptibility to antimicrobial agents. Quantitative real-time PCR (qRT-PCR) revealed that the stationary phase cells of the rpoN mutant grown without antimicrobial agent had approximately 4-140 and 2-14-fold higher transcripts of pvdS and vqsR genes, respectively, than the wild type strain. In the presence of antimicrobial agent, pvdS and vqsR transcripts were 400 and 5-fold, respectively, elevated in comparison to the wild-type. Flow cytometry assays using green fluorescent protein (GFP) reporter demonstrated increased expression of the vqsR gene in the rpoN mutant throughout the growth. A pvdS mutant of P. aeruginosa, deficient in pyoverdine production, was shown to be susceptible to biapenem.
These findings suggest that \textit{rpoN} is involved in the tolerance to antimicrobial agents in \textit{P. aeruginosa} and its tolerant effect is partly dependent on increased pyoverdine production and \textit{vqsR} gene expression.

\section*{INTRODUCTION}

\textit{P. aeruginosa} is an opportunistic pathogen that infects immunocompromised hosts causing infections that are especially difficult to eradicate. \textit{P. aeruginosa} has evolved a mechanism to partly escape from the effect of antimicrobial agents without necessarily expressing a resistance mechanism. This mechanism has been introduced in the literature as antimicrobial tolerance. Antimicrobial tolerance can be defined as the intrinsic ability of bacteria to survive the killing effects of antimicrobial agents (23). The molecular basis of the tolerance is virtually unexplored. Upon certain environmental conditions, such as alteration in the nutritional supply, entry into the stationary phase, high cell density, temperature, pH or osmolarity, planktonic cells can turn on stress-response genes and switch to a more tolerant phenotype (12). Stress-response genes are regulated by different linked signals, such as quorum sensing, ppGpp and poly P kinase. We have recently
reported that increased basal levels of ppGpp under nongrowing conditions might be a signal leading to tolerance to quinolones in *P. aeruginosa* (25). Transcriptional regulators such as sigma factors are key elements in the bacterial adaptive responses needed for pathogenesis. For example, it has been shown that RpoS, a central regulator of stress response, also plays a role in tolerance to quinolones and carbapenems in *P. aeruginosa* (11). RpoN is another important sigma factor, which also appears to regulate virulence in *P. aeruginosa*. Work on RpoN has revealed that this sigma factor is important not only for the expression of flagella and pili (24, 26), but also has been recognized to govern a number of distinct functions, all of which seem to be important for the adaptation and survival under unfavorable environmental conditions (1). Moreover, the role of RpoN in the regulation of virulence factors and its global negative control on the quorum sensing system in *P. aeruginosa* has been demonstrated (2, 22). Studies performed in *Escherichia coli* showed the relationship between a mutation in the *rpoN* gene and resistance to novobiocin, the coumarin antibiotic that inhibits DNA supercoiling by blocking the B subunit of DNA gyrase (4). The role of RpoN in the development and maintenance of tolerance to antimicrobial agents has not yet been defined. Taking into account that different metabolic
activities within the cells were co-related with RpoN, we further wondered if RpoN could
play a role as a novel target for antimicrobial agents in \textit{P. aeruginosa}.

The data presented here demonstrate that \textit{rpoN} mutant during the stationary phase
of growth encounters an iron limited condition characterized by the secretion of the
siderophore, pyoverdine. We suggest that the tolerant effect to antimicrobials in the \textit{rpoN}
mutant may be connected with increased pyoverdine synthesis, and \textit{vqsR} gene expression.

\textbf{MATERIALS AND METHODS}

\textbf{Bacterial strains, plasmids and growth conditions.} The bacterial strains and plasmids
that were used and constructed in this study are described in Table 1. The bacteria were
grown at 37°C in Luria-Bertani broth (Difco Laboratories), unless otherwise indicated.

Antibiotics were added as required at the following concentrations for work with \textit{E. coli},
with the concentrations used for \textit{P. aeruginosa} shown in brackets: ampicillin, 50 µg/ml;
kanamycin, 25 µg/ml; carbenicillin (400 µg/ml), gentamicin, 15 µg/ml (200 µg/ml),
tetacycline, 10 µg/ml (100 µg/ml), and sucrose 5 % for \textit{P. aeruginosa}. 1 mM
L-glutamine was included in the media for the overnight growth of PAO1 and rpoN mutant. When indicated, the media were also supplemented with FeCl₃ at a concentration of 100 µM.

**DNA analysis and manipulation.** Restriction enzymes and DNA polymerases were purchased from New England Biolabs (Beverly, MA), Toyobo (Osaka, Japan), TaKaRa Shuzo (Kyoto, Japan) and were used under the conditions recommended by the manufacturers. Plasmid DNA was isolated using a Qiafilter plasmid Maxi kit (Qiagen, Maryland, USA) or a Plasmid Miniprep Kit according to the protocol provided by the manufacturer (Bio Rad, Calif.). Chromosomal DNA was purified from *P. aeruginosa PAO1* using a Bacterial DNA Kit (Omega Bio-Tek Inc., Doraville, GA). Treatment of DNA with enzymes, subcloning of DNA and transformation of plasmids into *E. coli* and *P. aeruginosa* was carried out using standard methods (16). Where required, DNA fragments were isolated from agarose gels using a Qiagen gel extraction kit (QIAGEN, Valencia, Calif.). Standard methods were used for the preparation of competent cells and for plasmid electroporation into *E. coli* (16). *P. aeruginosa* electrocompetent cells were prepared as described elsewhere (20).
Construction of the *rpoN* mutant. To construct an *rpoN* knockout mutant, PCR amplification was used to obtain a 1.5-kb fragment of *rpoN* using primers:

5'-ACCCGTAGTAGTGGATGGTGC-3' and 5'-CAACGTCACACCAGTCGCTTG-3'.

The amplified fragment was cloned into pGEM-T vector creating pRN. Next, the *BssHII* fragment was deleted from the *rpoN* and replaced with a 1.3 kb Tc' gene using *BssHII* linkers to form pRN1. The constructed plasmid was transformed in PAO1 by electroporation, and the presence of the *rpoN::Tc* allele at the proper location in the *P. aeruginosa* chromosome was confirmed using PCR with primers that hybridize outside and inside the *rpoN* gene, and by DNA sequencing using BigDye Terminator Cycle Sequencing Ready reaction Kit and ABI PRISM 3100 (Applied Biosystems, Inc.).

Complementation of the *rpoN* mutant. For the complementation experiments, a 2.5-kb *EcoRI-HindIII* fragment encompassing the *rpoN* gene was amplified and digested with *EcoRI* and *HindIII*. The generated fragment was subsequently ligated into an *EcoRI-HindIII* digested broad host range vector, pMMB67EH, to yield pDVR. In this construct, the *rpoN* gene conserved its own promoter and Shine-Dalgarno sequence and was inserted in the opposite orientation with respect to the *tac* promoter.
Construction of the \textit{pvdS} mutant. For construction of the insertion within \textit{pvdS}, primers pvdS1s; (5’-TCTCCCTCCATCATTCGCAG-3’) and pvdS1a; (5’-AGGACAACGCTGGGAAAGGAG-3’) were used to PCR amplify a 0.9 kb fragment encompassing the \textit{pvdS} gene. The amplified fragment was inserted into a pGEM-T vector to yield pPVD. A gentamicin cassette was amplified from pAC\textit{Ω}Gm (17) as a \textit{StuI} restriction fragment and cloned into the unique \textit{StuI} site of the \textit{pvdS} fragment creating pPVD1. Plasmid pPVD2 was constructed by insertion of the MOB cassette from pMOB3 (18) as a \textit{NotI} fragment into \textit{NotI} digested pPVD2. Biparental mating between \textit{E. coli} S17-1 (19) (pPVD2) and \textit{P. aeruginosa} PAO1 was used to replace the wild-type gene with the mutant \textit{pvdS::Gm}$. Double crossovers were selected on plates containing 5% sucrose and gentamicin (200 µg/ml). The resultant \textit{pvdS} mutant was confirmed by PCR.

\textbf{Construction of \textit{vqsR-gfp} transcriptional fusion.} For construction of the \textit{vqsR-gfp} gene fusion, a 240 bp \textit{vqsR} promoter region was PCR-amplified from the genomic DNA of \textit{P. aeruginosa} PAO1 using primers vqsR1s; 5’-CGGAATTCGTCGAATAACGCCAGTGCACAA-3’ and vqsR1a; 5’-CGGGATCCCGCTTGAGCAACTTTCCCA-3’ (underlined nucleotides
represent engineered restriction enzyme sites EcoRI and BamHI, respectively). Amplified fragment was digested with EcoRI and BamHI and then ligated into EcoRI-BamHI digested GFP reporter vector p67GFP (K. Murakami, unpublished data).

**Antimicrobial agents.** Ofloxacin (Sigma, St. Louis, Mo.), ciprofloxacin (Bayer Pharma, Germany), biapenem and imipenem (Meiji Seika Kaisha, LTD, Tokyo, Japan) were used in the study.

**Susceptibility testing.** The MIC and the MBC (minimal bactericidal concentration) of each agent were determined using the broth microdilution method, as previously described (10), with the following modification: the bacterial suspensions at a density of $1 \times 10^6$ cells/ml were incubated in LB broth. MICs were determined after 24 h of incubation at 37°C. The MIC was defined as the lowest concentration of the antimicrobial agent that completely inhibited the growth of the organism, as detected by the unaided eye. MBCs were measured by removing 10 µl from all wells containing no visible growth and plating the samples on LB agar plates for further incubation at 37°C for 24 h.

**Time-kill study.** For the time-kill studies, stationary phase cells grown for 12-16 h and logarithmic phase cells were used. Cells were harvested by centrifugation and resuspended...
in fresh LB broth before the incubation with antimicrobial agents. Aliquots (0.1 ml) were taken after 0-12 hours of incubation, and plated in duplicate on agar plates after serial dilutions to enumerate the surviving bacteria after 24-48 h of incubation at 37°C. Cell viability at each time point was expressed as the percentage of viable cells (CFU/ml) at time zero.

RNA isolation, RT-PCR and quantitative real-time PCR (qRT-PCR) analysis.

Overnight LB broth-grown cultures were washed once and resuspended in fresh LB medium before the start of the experiment. Cells were sampled at time 0 and at 1, 3 and 5 h after the resuspension in the fresh medium with or without 8 µg/ml of ofloxacin, and their RNA was immediately stabilized with RNAprotect Bacteria Reagent (Qiagen) and stored at -80°C. Total RNA was isolated with the RNeasy spin column (including an on-column DNase digestion step) according to the manufacturer (Qiagen), treated with RQ1 Dnase I (Promega) for 1 h at 37°C and repurified through an RNeasy column. Approximately 650 ng of RNA was converted to cDNA. cDNA was synthesized using the SuperScriptTM First-Strand Synthesis System (Invitrogen, Groningen, Netherlands). The oligonucleotide probes for RT-PCR and qRT-PCR were synthesized by Hokkaido System Science Co.,
RT-PCR and qRT-PCR were performed using primers; pvdS2s; 5’-AGATGTGGTCCAGGATGCGT-3’ and pvdS2a; 5’-GTGTTGCAGGGTCGCGTAGT-3’, vqsR3s; 5’-TTGCGGATATCGTCTCCGAA-3’, vqsR3a; 5’-TTTTCATCAGCGCGATGACC-3’, rpsLs; 5’-CGAACTATCAACCAGCTGGTG-3’, and rpsLa; 5’-GCTGTGCTCTTGAGTTTG-3’. As a control for RNA contamination by DNA, the PCR reaction was performed on the same samples without first strand synthesis. A Roche LightCycler (Roche Molecular Biochemicals) real-time PCR machine (software version 3.5) was used for the quantification of cDNA. For quantitative analysis of the pvdS, vqsR or rpsL transcripts by qRT-PCR, PCRs were performed using a LightCycler® FastStart DNA MasterPLUS SYBR Green I kit (Roche Applied Science, Mannheim, Germany) according to the specifications of the supplier.

qRT-PCRs were performed in 10-µl mixtures containing 2 µl of Master Mix, 1 µl of cDNA, and a 0.4 µM of each of the forward and reverse primers. For quantitation, pvdS and vqsR amplicons were first cloned into the pGEM-T and pCR2.1-TOPO cloning vector, respectively, and then purified recombinant plasmid DNAs containing the amplicon of
interest were tenfold serially diluted and used to generate external standard curves according to the manufacturer’s instructions. For construction of rpsL external standard curve, genomic PAO1 DNA was used. PCRs were performed in triplicate for each gene and sample. The 230-bp PAO1 rpsL and pvdS and a 210-bp fragment of the PAO1 vqsR gene were amplified using the following cycles: 95°C for 10 minutes, and 35 cycles of 95°C for 10 s, 52-60°C for 10 s, 72°C for 5 s and 72°C for 30 s. To correct for differences in the amount of starting material, the ribosomal rpsL gene was chosen as a reference gene. Results were read with the ‘second derivative maximum’ algorithm of the software provided. The LightCycler software generated a standard curve by plotting ‘crossing cycle number’ versus logarithms of the given concentrations for each control. The software calculated the concentrations of the studied genes with the aid of the standard curve.

Flow cytometric analysis. P. aeruginosa cultures were maintained in LB broth supplemented with carbenicillin (400 µg/ml). To study growth-phase-dependent vqsR expression by flow cytometry, shuttle vector, p67GFP, containing GFP reporter under the control of P. aeruginosa vqsR promoter region was electroporated into P. aeruginosa PAO1 and DVR, and the transformants were designated PGV and RGV, respectively.
Overnight cultures of PGV and RGV were diluted to an OD$_{595}$ of 0.01 in LB broth supplemented with carbenicillin 400 µg/ml and incubated at 37°C with shaking. For each assay the optical density at 595 nm was determined at hourly intervals and the experiment was continued for 24 h. In another experiment, overnight cultures of PGV and RGV were washed, resuspended in fresh LB broth and then supplemented with ofloxacin at a concentration of 8 µg/ml. Samples (0.5 ml) were taken after 0-5 hours of incubation and assayed for flow cytometry. Prior to the measurement, bacterial cells were washed once in phosphate-buffered saline (PBS), resuspended in 0.5 ml of PBS and then serially diluted. A Coulter Epics XL™ flow cytometer (Beckman Coulter, Inc.) was used to measure the intensity of fluorescence of vqsR-$gfp$ producing bacteria. Fluorescence and scatter data were collected for 20 000 events and mean fluorescence intensity was calculated. The relative fluorescent units represent the fluorescence values corrected for background (PAO1 without $gfp$).

RESULTS
Susceptibility testing. To address whether \textit{rpoN} serves as a possible target for antimicrobial agents, insertional inactivation of the \textit{rpoN} gene was performed in \textit{P. aeruginosa} PAO1 and was designated DVR. The MICs and the MBCs of antimicrobial agents for the study strains are summarized in Table 2. No significant difference was observed in the MIC values between the wild-type strain and the \textit{rpoN} mutant. The MBC values for quinolones in the \textit{rpoN} mutant were slightly higher in comparison to the wild-type. In contrast, the MBC values for carbapenems in the \textit{rpoN} mutant were 8-16 times higher than those seen in the wild-type strain. The MIC and the MBC values of the \textit{pvdS} mutant and the wild-type were found to be almost the same, except for MBC of imipenem which was 4 times higher than in the wild-type cells. The MBC is not a suitable or reliable measure of tolerance because it is defined as the endpoint survival of more than 99.9% cells, therefore the assessment of both the rate and the extent of killing was performed using time-kill assays.

Impact of \textit{rpoN} inactivation on tolerance to quinolones and carbapenems. An examination of growth kinetics in LB medium supplemented with 1 mM glutamine showed that the growth rate of the mutant did not differ from the wild-type rate indicating
that the rpoN gene mutation does not have any effect on growth rate under the conditions tested (Fig. 1C). For overnight growth, the wild-type and rpoN mutant cells were supplemented with 1 mM glutamine. The addition of glutamine had no effect on the susceptibility to antimicrobial agents following resuspension, therefore glutamine was omitted from the resuspension. The time-kill studies in the presence of ofloxacin 8 µg/ml and ciprofloxacin 2 µg/ml are shown in Fig. 1A and 1B, respectively. The survival rate of the rpoN mutant in the presence of quinolones was 15 times higher than that of the wild-type. To confirm that this phenotype was due to loss of rpoN and not a secondary mutation, we constructed a complementation plasmid encoding a wild-type copy of rpoN under the control of its native promoter contained on pDVR1, a pMMB67-based shuttle vector. The survival of the DVR1 complemented strain harboring pDVR1 (Fig. 1A and 1B) was as low as that of the wild-type. The killing curves for biapenem 32 µg/ml and imipenem 16 µg/ml are shown in Fig. 2A and 2B, respectively. The stationary phase cells of the rpoN mutant displayed reduced susceptibility to carbapenems, being approximately 15 times less sensitive to biapenem and imipenem addition after 12 h than the wild-type strain. The complemented strain DVR1 restored parental phenotype in the presence of
carbapenems (Fig. 2A and 2B). It was apparent that the effect of killing was due to antibiotics, not to natural dying (data not shown). For growth of the log-phase culture, overnight cultures were diluted 1,000-fold in fresh medium and bacteria were cultured for ~3 h to an OD$_{600}$=0.25. Assessing the sensitivity of logarithmic phase cells of rpoN mutant strain grown in LB to the addition of biapenem by comparing the survival rate with wild-type strain also showed a clear difference. The logarithmic phase cells were treated with biapenem 32 µg/ml and the killing curve is presented in Fig. 3. We observed that exposing logarithmic phase cells of the rpoN mutant to biapenem, without glutamine supplementation, produced tolerant effect to biapenem addition. In contrast, supplementing rpoN mutant with glutamine, tolerant effect was abolished. In the wild-type strain, glutamine addition had no effect on susceptibility to biapenem during the logarithmic phase. From these data we initially concluded that the mechanism underlying the tolerance in the logarithmic phase cells of the rpoN mutant differs from the one in the stationary phase cells. The stationary phase cells of rpoN mutant trigger pyoverdine production. During the course of this study, we have observed that upon transition in the stationary
phase and following resuspension in the fresh medium, \( rpoN \) mutant significantly synthesized pigment. The observations raised the question as to whether the pigment produced by \( rpoN \) mutant could be a siderophore, pyoverdine, and would in a way represent iron limitation. In order to approach this problem, we allowed for two possibilities; a) we postulated that parental susceptibility in the \( rpoN \) mutant could be restored in the presence of excess iron throughout the growth and therefore proving that iron limitation in \( rpoN \) mutant led to increased tolerance to antimicrobials b) using a \( pvdS \) mutant, deficient in pyoverdine synthesis, we asked whether \( pvdS \) gene disruption affects the susceptibility to antimicrobials. To prove the hypothesis mentioned above, we grew \( rpoN \) mutant and the wild-type strain overnight in the presence of 100 µM \( \text{FeCl}_3 \) and 1mM glutamine. Before the start of the experiment, the cells were washed once and resuspended in fresh medium supplemented with quinolones and 100 µM \( \text{FeCl}_3 \) (Fig. 4A and 4B). The addition of \( \text{FeCl}_3 \) suppressed pyoverdine production and restored parental phenotype in the presence of quinolones. The susceptibility of the \( rpoN \) mutant in the presence of carbapenems and \( \text{FeCl}_3 \) is demonstrated in Fig. 2A and 2B. Addition of \( \text{FeCl}_3 \) during biapenem killing-assay eliminated tolerant effect in \( rpoN \) mutant, and in case of imipenem,
re-growth occurred after 6 hours. To further investigate the importance of pyoverdine production in survival of the *rpoN* mutant to antimicrobial agents, we constructed a *pvdS* mutant by insertional inactivation and determined its susceptibility to antimicrobials by killing curve assays. A *pvdS* mutant exhibited no change in the antibiotic susceptibility relative to the wild-type in the presence of ofloxacin (Fig. 1A). However, the survival of the *pvdS* mutant was affected by the presence of biapenem 32 µg/ml (Fig. 2A). These data initially suggested that *pvdS* gene is probably implicated in the tolerance to biapenems but not to quinolones. To gain more insight into the pigment produced, we measured the absorbance of the filtered supernatant of the wild-type strain and *rpoN* mutant after overnight growth. The absorption spectrum is shown in Fig. 5. We observed a peak at 403 nm, which is characteristic of pyoverdine (3). To determine whether siderophore production was related to the growth medium, we grew the *rpoN* mutant in NBY (2) and PTSB (13) media commonly used for the growth of *P. aeruginosa*. We observed that in both media, the *rpoN* mutant showed the same growth kinetics as when grown in LB medium; however, we did not observe siderophore production and spectrometric observations did not reveal increase in the absorbance between $A_{350} - A_{450}$ nm (data not
This observation implies that increased pyoverdine production seen in the \textit{rpoN} mutant might be only LB medium-restricted or dependent. Taken together, we propose that the \textit{rpoN} mutant of \textit{P. aeruginosa} PAO1 secreted pyoverdine under conditions that repressed production of pyoverdine in the wild-type strain.

\textbf{Transcriptional expression of \textit{pvdS} and \textit{vqsR} genes.} Previous studies in \textit{Vibrio harveyi} (8) have demonstrated that \textit{rpoN} together with \textit{luxO} is implicated in the siderophore production. PvdS is an alternative sigma factor controlling the expression of the genes required for pyoverdine synthesis, an iron-chelating compound secreted by \textit{P. aeruginosa} (7). Since a link between \textit{rpoN} and siderophore production has not been demonstrated in \textit{P. aeruginosa}, our unexpected finding of increased production of the pyoverdine in the stationary phase cells of the \textit{rpoN} mutant led us therefore to begin our studies by assessing the transcript levels of \textit{pvdS} following resuspension of the stationary phase cells of the \textit{rpoN} mutant and the wild-type in the fresh medium without or with antibiotic addition.

RT-PCR was performed on total RNA (~650 ng). qRT-PCR was used to confirm the results obtained using RT-PCR. The \textit{rpsL} gene was used as an internal control to ensure that the same amount of total RNA from the wild-type and the mutant strain was used. The
starting quantity of cDNA from the wild-type and the mutant was normalized using \( rpsL \) gene. The results showed that without quinolone addition, at 1-h time point, 140-fold increase of \( pvdS \) transcripts in the \( rpoN \) mutant was observed in comparison to the wild-type. At 3-h and 5-h time point, \( pvdS \) transcripts remained approximately 10- and 5-fold, respectively, higher than in the wild type strain (Fig. 6A). To examine the contribution of antibiotic addition on \( pvdS \) expression pattern, we performed qRT-PCR on the cDNA obtained from the wild-type and \( rpoN \) mutant after addition of 8 µg/ml ofloxacin. The results demonstrated a substantial increase of \( pvdS \) transcripts in the \( rpoN \) mutant; at the 3-h time point, the \( rpoN \) mutant showed 30-fold increase in \( pvdS \) transcripts when compared to the wild-type. Moreover, at 5-h time point, the 400-fold increase of the \( pvdS/rpsL \) ratio was observed in the \( rpoN \) mutant (Fig. 6C). These results together indicate that antibiotic addition actually increased \( pvdS \) transcripts in the \( rpoN \) mutant. This observation prompted us to investigate if some other genes might be upregulated by this significant increase in \( pvdS \) transcripts in the \( rpoN \) mutant. Since it was previously reported (5, 6) that \( VqsR \) controls the expression of the genes required for siderophore biosynthesis, we tested in both the wild-type and the \( rpoN \) mutant the levels of transcripts of the \( vqsR \)
gene. When we assayed vqsR transcripts in the rpoN mutant without ofloxacin addition, interestingly, at 1-h time point, we observed 15-fold higher expression of vqsR in the rpoN mutant, which clearly correlated with increased transcripts of pvdS at 1-h time point. At 3-h time point, vqsR transcripts decreased reaching wild-type levels and further increased about 2-fold in the rpoN mutant at 5-h time point (Fig. 6B). We further analyzed the vqsR transcripts after addition of ofloxacin, and results clearly demonstrated that the transcription of the vqsR gene in the rpoN mutant was increased ~ 2.5-5-fold in comparison to the wild-type strain (Fig. 6D).

Analysis of vqsR-gfp expression using flow cytometry. For these studies, we transformed both wild-type (PAO1) and rpoN mutant (DVR) with P_{vqsR-gfp} reporter contained on a MMB67-based shuttle vector (p67GFP) and measured GFP expression over time by flow cytometry. As shown in Fig. 7A, in the rpoN mutant, the expression of vqsR-gfp increased about 10-fold at 24-h time point. Despite the lower expression, an increase of vqsR-gfp expression at the stationary phase was also observed in the wild-type strain. When we measured the expression of vqsR-gfp in the stationary phase cultures in the presence of 8 µg/ml of ofloxacin, the rpoN mutant, when compared to parental strain...
PAO1, exhibited a notable increase in vqsR-gfp expression (Fig. 7B). At time 0, the rpoN mutant displayed approximately 45-fold increase in the expression of vqsR-gfp in comparison to wild-type strain. Five hours after the addition of ofloxacin, the expression of vqsR-gfp in the rpoN mutant increased about 8-fold versus that in the wild-type strain. Presented results suggest that in P. aeruginosa a regulatory link between rpoN, pvdS and vqsR exists.

**DISCUSSION**

In this study, we have shown that insertional inactivation of the rpoN gene of P. aeruginosa affects the activity of quinolones and carbapenems, leading to the decreased susceptibility to these antimicrobial agents. The results presented suggest that rpoN mutant depending on growth phase and nutritional balance in its environment, allows different trigger for survival during the stress conditions such as antibiotic addition. Studies done in P. putida reveal that RpoN, while not playing a key role in survival to various nutritional and environmental stresses, the rpoN mutant maintained a significantly higher viability than the wild-type strain when exposed to stress conditions, such as oxidative damage and
hyperosmotic environment (1). In support of the involvement of RpoN in the antibiotic
stress response in *P. aeruginosa*, we offer the following arguments.

The transition to stationary phase and prolonged stationary phase led to increased
pyoverdine production in the *rpoN* mutant. Using qRT-PCR, we followed the
accumulation pattern of the pyoverdine by quantifying *pvdS* transcripts in the absence or
presence of antimicrobial agent. During the growth in the antibiotic-free medium, *pvdS*
transcripts in the *rpoN* mutant were 5-140 higher than in the wild-type. The addition of
ofloxacin significantly increased pyoverdine synthesis in the *rpoN* mutant. At first glance,
our findings were surprising in light of the role of RpoN in pyoverdine synthesis during the
stationary phase. In *P. aeruginosa*, pyoverdine synthesis is regulated by PvdS, an
alternative sigma factor. The role of pyoverdine extends beyond its role in chelating and
transporting iron in the cells during iron limitation, but also serves as a signaling molecule
representing a mechanism by which *P. aeruginosa* cells may respond to increased cell
density (7). Moreover, pyoverdine expression positively regulates the expression of genes
for synthesis of exotoxin A, PrpL protease and pyoverdine itself (7). Several pieces of
evidence are consistent with the notion that increased cell density might control
pyoverdine synthesis (7, 21). The involvement of RpoN in the regulation of quorum
sensing (2, 22) and our observations of pyoverdine production solely in the stationary
phase, led us to propose that high cell density and altered nutrient supply may induce
certain metabolic imbalance in the rpoN mutant followed by upregulation of the stationary
phase survival genes. On account of its role as a quorum-sensing regulator and its impact
on iron homeostasis in P. aeruginosa (5), it was possible that VqsR actually regulated pvdS
expression and played a role in the antibiotic-stress response in the rpoN mutant. While
real-time PCR and flow cytometry studies presented herein clearly demonstrated VqsR
upregulation, we do not yet know if the siderophore trigger seen in the rpoN mutant is
regulated by VqsR, quorum-sensing or by some other unknown pathway. It is unlikely that
increased pyoverdine production presented in this study is due to an upregulation of VqsR
alone, because the levels of vqsR transcripts seem to be unaltered in the rpoN mutant to
FeCl₃ addition (data not shown), suggesting that additional levels of regulation are
probably implicated. In addition, transcription pattern of the pvdS during the growth in the
antibiotic-free medium parallels the one seen for vqsR, and provides for possible pvdS
upregulation by VqsR. On the contrary, antibiotic addition demonstrated that pvdS
upregulation is probably mediated by some other antibiotic stress-regulated gene and is not only \(\text{VqsR}\)-dependent. At the level of regulation, \(\text{rpoN vqsR}\) double mutant would probably shed further light on the requirement and role of \(\text{VqsR}\) in pyoverdine synthesis and survival during the antibiotic stress. The upregulation of quorum sensing-related genes in the \(\text{rpoN}\) mutant such as \(\text{vqsR}\) suggests that \(\text{rpoN}\) gene downregulates quorum sensing gene expression in the wild-type strain, either directly or indirectly.

Another interest in this work was to assess the importance of pyoverdine in the antimicrobial stress-response of the \(\text{rpoN}\) mutant by constructing a pyoverdine-deficient mutant of \(\text{P. aeruginosa}\) PAO1. Killing-curve assays suggested that \(\text{pvdS}\) gene is involved in the tolerance of stationary phase cells to biapenem. At this point, it is worth considering that \(\text{mexAB-oprM}\) operon has been involved in the uptake of pyoverdine and also in the transport of certain \(\beta\)-lactam antibiotics (14), suggesting that possible selectivity for uptake between pyoverdine and carbapenems might exist, thus explaining partially tolerant phenotype of \(\text{rpoN}\) mutant with imipenem and \(\text{FeCl}_3\). While the wild-type susceptibility of the \(\text{pvdS}\) mutant to ofloxacin, complicates the explanation of increased survival of the \(\text{rpoN}\) mutant by overproduced pyoverdine, it also suggests that pyoverdine probably requires
another regulator for direct interaction with antimicrobials, such as quinolones. Nutrient limitation (absence of glutamine) from the medium allowed log-phase cells to overcome the action of biapenem. We hypothesized that the physiological adaptation of the logarithmic-phase cells of rpoN mutant to environmental conditions could be attributed to ppGpp. In support of our observations, Powell et al. (15) proposed that ppGpp controls glutamine metabolism in the cells lacking adequate nitrogen metabolism. Recent studies by Merrell et al. (9) demonstrated in Helicobacter pylori a strong level of coregulation of genes involved in nitrogen metabolism and iron starvation. The loss of σ^{54} activity leads to diminished glutamine metabolism, therefore we presume that this selectivity in nitrogen usage may affect in some instance the uptake of the iron in the cell or some other pathways for iron uptake may not be available. The data presented here suggest that the link between pyoverdine production and vqsR gene expression is a participant, but not the only one in rendering rpoN mutant tolerant to antimicrobials. While the mechanism behind observed pyoverdine and its role in the antibiotic-stress response is obviously complex, it seems that iron alters the outcome of the P. aeruginosa response to antimicrobial agents. Further studies will be aimed at answering the questions that lie in the background of the tolerance
to antimicrobial agents seen in the rpoN mutant.

ACKNOWLEDGMENTS

We thank Herbert P. Schweizer for providing plasmids pACΩGm and pMOB3, and M. Tsuda for providing plasmid pMMB67EH. This work was supported by a grant-in-aid for scientific research (no. 17591914) to T. O. from Japan Society for Promotion of Science.

REFERENCES

1. Cases, I., and V. de Lorenzo. 2001. The limits to genomic predictions: role of sigma

35:217-221.


GeneChip expression analysis of the VqsR regulon of *Pseudomonas aeruginosa* TB.


**Table 1.** Strains and plasmids used and constructed in this study

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant feature</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO1</td>
<td>prototroph</td>
<td></td>
</tr>
<tr>
<td>DVR</td>
<td><em>rpoN::Tc</em></td>
<td>this study</td>
</tr>
<tr>
<td>DVR1</td>
<td><em>rpoN::Tc</em> carrying pDVR</td>
<td>this study</td>
</tr>
<tr>
<td>PGV</td>
<td>PAO1 carrying pGV</td>
<td>this study</td>
</tr>
<tr>
<td>RGV</td>
<td><em>rpoN::Tc</em> carrying pGV</td>
<td>this study</td>
</tr>
<tr>
<td>PVD</td>
<td><em>pvdS::Gm</em></td>
<td>this study</td>
</tr>
<tr>
<td>527</td>
<td><em>E. coli</em></td>
<td></td>
</tr>
<tr>
<td>528</td>
<td>XL1-Blue</td>
<td>General cloning host</td>
</tr>
<tr>
<td>529</td>
<td>S17-1</td>
<td><em>thi endA recA hsdR</em> with</td>
</tr>
<tr>
<td>530</td>
<td></td>
<td>RP4-2Tc::Mu-Km::Tn7 integrated in the chromosome</td>
</tr>
<tr>
<td>531</td>
<td>SSC110</td>
<td><em>rpsL</em> (Str&lt;sup&gt;f&lt;/sup&gt;) <em>thr leu endA thi-l lacY galK galT</em></td>
</tr>
<tr>
<td>532</td>
<td></td>
<td><em>ara tonA tsx dam dcm supE44D</em> (lac-proAB)</td>
</tr>
<tr>
<td>533</td>
<td></td>
<td>[F&lt;sup&gt;+&lt;/sup&gt; traD36 proAB lacI&lt;sup&gt;rew&lt;/sup&gt; ZDM15]</td>
</tr>
<tr>
<td>534</td>
<td>Plasmids</td>
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<td>535</td>
<td>pGEM-T</td>
<td>TA cloning vector, high copy number, Ap&lt;sup&gt;r&lt;/sup&gt;</td>
</tr>
<tr>
<td>536</td>
<td>pCR2.1-TOPO</td>
<td>TA cloning vector, high copy number, Ap&lt;sup&gt;r&lt;/sup&gt;, Km&lt;sup&gt;r&lt;/sup&gt;</td>
</tr>
<tr>
<td>537</td>
<td>pACΩGm</td>
<td>pACYC184 derivative carrying Ω fragment, Gm&lt;sup&gt;r&lt;/sup&gt;</td>
</tr>
<tr>
<td>538</td>
<td>pMOB3</td>
<td>Km&lt;sup&gt;r&lt;/sup&gt; Cm&lt;sup&gt;r&lt;/sup&gt; 5% sucrose sensitive</td>
</tr>
<tr>
<td>539</td>
<td>pVqsR</td>
<td>pCR2.1-vqsR</td>
</tr>
<tr>
<td>540</td>
<td>pRN</td>
<td><em>rpoN</em> in pGEM-T</td>
</tr>
<tr>
<td>541</td>
<td>pRN1</td>
<td><em>rpoN::Tc&lt;sup&gt;r&lt;/sup&gt;</em> in pGEM-T</td>
</tr>
<tr>
<td>542</td>
<td>pPVD</td>
<td><em>pvdS</em> in pGEM-T</td>
</tr>
</tbody>
</table>
pPVD1: pvdS::Gm\(^r\) in pGEM-T this study

pPVD2: pvdS::Gm\(^r\) and mob in pGEM-T this study

pMMB67EH: IncQ broad-host-range cloning vector, Ap\(^r\)/Cb\(^r\) Tsuda M.

pDVR: pMMB67EH containing a functional rpoN gene in opposite orientation this study

to the tac promoter, Ap\(^r\)/Cb\(^r\)

p67GFP: broad host range vector carrying GFP, Ap\(^r\)/Cb\(^r\) this laboratory

pGV: p67GFP carrying the vqsR promoter region this study

Abbreviations: Ap\(^r\), Gm\(^r\), Cb\(^r\), Km\(^r\), and Tc\(^r\), stand for resistance to ampicillin, gentamicin, carbenicillin, kanamycin and tetracycline, respectively.
Table 2. Susceptibility of the wild-type, *rpoN* mutant, and *pvdS* mutant to antimicrobial agents

<table>
<thead>
<tr>
<th>Antimicrobial agents</th>
<th>Wild-type (PAO1)</th>
<th><em>rpoN</em> mutant</th>
<th><em>pvdS</em> mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC (µg/ml)</td>
<td>MIC (µg/ml)</td>
<td>MIC (µg/ml)</td>
</tr>
<tr>
<td>Biapenem</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Imipenem</td>
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<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.125</td>
<td>0.25</td>
<td>0.125</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>2</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>2</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>1</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1</td>
<td>0.25</td>
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<tr>
<td></td>
<td>1</td>
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<td>0.25</td>
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<td>0.5</td>
<td>1</td>
<td>0.25</td>
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<tr>
<td></td>
<td>0.5</td>
<td>1</td>
<td>0.25</td>
</tr>
</tbody>
</table>
**FIGURE LEGENDS:**

**FIG. 1.** Time-dependent killing study for 8 µg/ml of ofloxacin (A) and 2 µg/ml of ciprofloxacin (B) for stationary grown cells of PAO1, DVR and PVD. Assuming that the survival at 0 time was 100%, CFU was changed to a percentage. PAO1; wild-type (circles on solid line), DVR; rpoN mutant (squares on dotted line), PVD; pvdS mutant (diamonds on dotted line). Complementation studies were performed by introducing the rpoN gene on a pMMB67EH into DVR (triangles on solid line). (C) Growth curves showing culture absorbance at 595 nm plotted against time; Wild-type; PAO1 (circles on solid line) and rpoN mutant; DVR (squares on dotted line). The experiment was performed in triplicate, and the error bars represent the standard deviations. Where error bars are not shown, the standard deviation was within the size of the symbol.

**FIG. 2.** Time-dependent killing study in the presence of biapenem at a concentration of 32 µg/ml (A) and imipenem 16 µg/ml (B) for stationary phase cells of PAO1, DVR and PVD. Complementation studies were performed by introducing the rpoN gene on a pMMB67EH into DVR (triangles on solid line). Survival of DVR supplemented with FeCl₃ in the presence of carbapenems (squares on solid line). Assuming that the survival at 0 time was
100%, CFU was changed to a percentage. PAO1; wild-type (circles on solid line), DVR; rpoN mutant (squares on dotted line), PVD; (diamonds on dotted line). Error bars represent the standard deviations of three determinations.

**FIG. 3.** Time-dependent killing study in the presence of biapenem 32 µg/ml for logarithmic phase cells of PAO1 and DVR (with or without addition of 1 mM glutamine). Assuming that the survival at 0 time was 100%, CFU was changed to a percentage. PAO1; wild-type (circles on solid line), DVR; rpoN mutant (no glutamine addition) (squares on dotted line), DVR; rpoN mutant (glutamine added) (triangles on solid line). Error bars represent the standard deviations of three determinations.

**FIG. 4.** Kinetics of killing by ofloxacin and ciprofloxacin in the presence of FeCl₃. CFUs were determined at different time points after incubation with 100 µM FeCl₃ in LB medium supplemented with ofloxacin 8 µg/ml (A) and ciprofloxacin 2 µg/ml (B). PAO1; wild-type (circles on solid line), DVR; rpoN mutant (squares on dotted line). Error bars represent the standard deviations of three determinations.
FIG. 5. Spectrum of culture supernatants of the wild-type (PAO1) and rpoN mutant (DVR) grown till the stationary phase in LB medium supplemented with 1mM glutamine. PAO1; wild-type (solid line), DVR; rpoN mutant (dotted line).

FIG. 6. Transcriptional expression of pvdS and vqsR genes. Stationary phase cells of wild-type (PAO1) and rpoN mutant (DVR) were grown in the presence or absence of ofloxacin at a concentration of 8 µg/ml and total RNA was isolated at time 0 and at 1, 3, and 5 h. pvdS (A) and vqsR (B) transcripts in the absence of ofloxacin, and pvdS (C) and vqsR (D) transcripts in the presence of ofloxacin for the wild-type and rpoN mutant strain. Error bars represent the standard deviations of three determinations.

FIG. 7. Expression of the vqsR-gfp transcriptional fusion. Samples from wild-type (PAO1) and rpoN mutant (DVR) were transformed with the indicated reporter construct and analyzed by flow cytometry. vqsR-gfp expression was analyzed throughout the growth (A) and in the stationary phase in the presence of 8 µg/ml of ofloxacin (B). Mean fluorescence intensities for vqsR-gfp were plotted against time on the representative graphs. Each graph represents the average of three experiments.
FIG. 1.
FIG. 2.
FIG. 4.
FIG. 5.
FIG. 6.
FIG. 7.

A

Mean fluorescence (arbitrary units)

0 2 4 6 8 10 12 24

Time (h)

B

Mean fluorescence (arbitrary units)

0 1 2 3 4 5

Time (h)

wild-type

DVR