四国歯誌　18(1)：133～147，2005：Thesis

学位論文

# Mechanism of Quinolone Tolerance in *Pseudomonas aeruginosa*

Darija Viducic

Keywords：*Pseudomonas aeruginosa,* tolerance, quinolone

Abstract：The capacity of bacteria to survive, but not grow during antibiotic treatment may be defined as antibiotic tolerance. In bacteria, significant physiological changes occur depending on various environmental conditions; low metabolic activity, slow growth, iron limitation and different stress conditions may be considered as factors playing a role in the tolerance to antimicrobial agents in *Pseudomonas aeruginosa*. One of the factors underlying these conditions is an alarmone, ppGpp that accumulates in the cells during transient starvation and nutritional deficiency. In order to investigate the possible role of ppGpp in the antibiotic tolerance in *P. aeruginosa*, the knockout mutants in the genes involved in the stringent response such as *relA*, *spoT* and *dksA* were constructed and investigated for their antibiotic susceptibility to quinolones. The susceptibility of these mutants to quinolone was determined using minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) testing and colony forming units per ml (CFU/ml). MIC/MBC ratios for ofloxacin in the *dksA* and the *spoT* knockout mutant were 1/4 and 0.5/2, respectively, in the comparison with the wild type strain, 1/1. The survival rate of the *dksA* and *spoT* knockout mutant in the presence of 8 μg/ml of ofloxacin showed to be approximately 100 times higher than the same for the wild type strain. In the presence of 1 μg/ml of ciprofloxacin, the survival rate for *dksA* and *spoT* mutant was 10-50 times higer than seen in the wild type strain. In comparison with the wild type, the *relA* and the *relA spoT* mutant displayed increased sensitivity to quinolones. The intracellular levels of ppGpp determined by high performance liquid chromatography (HPLC) demonstrated that *spoT* and *dksA* mutants possess higher basal levels of ppGpp. In conclusion, the data indicate that significantly elevated levels of ppGpp might be responsible for rendering these mutants tolerant to quinolones. The data presented in this study not only furnish some additional insights into the pleiotropic effect of ppGpp but also expand the importance of ppGpp as an antimicrobial target in *P. aeruginosa*.

Moreover, given the facts that RpoN, an alternative sigma factor activates the expression of wide variety of environmentally regulated genes and is required for virulence in a variety of pathogens, we hypothesized that it might additionally be a target for interaction with antimicrobial agents. Two knockout mutants in the *rpoN* gene were constructed and investigated for their response to quinolone treatment. Using different construction approach, we obtained *rpoN*::$Tc^r$ which displayed tolerance to quinolone and responded to quinolone addition by increased production of siderophore; in addition, *rpoN*::$Gm^r$ showed increased susceptibility to quinolone treatment. Results obtained imply that RpoN exerts its response to antibiotics through different pathways of regulation, and one of them might be due to the production of siderophore.

Department of Microbiology, Graduate School of Dentistry, The University of Tokushima

## INTRODUCTION

When threatened by environmental stress, bacteria display rapid molecular response which allows the adaptation to certain stimulus in their environment. In bacteria, nutritional limitation usually triggers accumulation of ppGpp, an effector molecule of stringent response. ppGpp modulates the response to starvation by slowing down the transcriptional rate and protein synthesis[1] and DNA replication[2]. The metabolism of ppGpp is mediated by two regulatory proteins, RelA and SpoT. RelA is ribosome-associated ppGpp synthetase that produces ppGpp during amino acid starvation. SpoT is bifunctional enzyme that is capable of synthesizing ppGpp during carbon starvation, and is also responsible for ppGpp hydrolysis[3]. Another gene under the control of stringent response is *dksA*. Recent observations have suggested that overexpressed *dksA* is involved in the inhibition of the gene expression belonging to the quorum sensing system[4]; moreover, *dksA* plays a role in *rpoS* expression through ppGpp[5]. Cellular levels of ppGpp change throughout the growth, tending to increase in the stationary phase when the nutritional supply for the cells is decreasing. Therefore ppGpp in bacteria serves as a protection factor during nutrient exhaustion. The connection between intracellular levels of ppGpp and different physiological processes such as quorum sensing in *P. aeruginosa*[6], biofilm formation in *Escherichia coli* and *Listeria monocytogenes* has already been described[7, 8]. Increased levels of ppGpp lead to enhanced tolerance to multiple stresses in *Lactococcus lactis*[9]. Furthermore, recent results by Korch et al.[10] suggest that ppGpp plays a crutial role in high persistence shown in *hipA7* mutant. Among many different stresses that bacteria encounter during the growth, another source of stress represents antibiotic addition. In order to escape the lethal effects of antibiotics, the bacteria respond by using different survival strategies such as inducing antibiotic-modifying enzyme(s), lowering the antibiotic sensitivity of the targets, lowering the penetration rate of antibiotics by loss of the antibiotic-permeable porin and pumping out antibiotics by active extrusion systems[11]. Apart from the antibiotic resistance, where the bacteria become insensitive to the antibiotic addition and continue to multiply during antibiotic treatment, antibiotic tolerance may be defined as a state where bacteria neither grow nor lyse in the presence of antibiotic[12]. Once the source of the stress has been removed from the environment, the bacteria readjust their transcription profile to less strict conditions in the environment. The mechanism by which bacteria partly overcome the effect of antibiotics is poorly understood, however, there are several lines of evidence that the supression of cell death and high persistence is probably governed by ppGpp[10]. It was proposed that persisters represent dormant, non-dividing cells which serve as a survival mechanism upon exposure to stress conditions by allowing the entrance into antibiotic-insensitive state or by enhancing the switching rate from normal to persister cells[10]. We postulate that potential harmful effect of antibiotic would be decreased by increasing ppGpp basal levels, because the protective role of ppGpp would lead to transition to the state which allows the survival while the cells are exposed to antibiotic. Taken all together, we propose a model by which tolerance to quinolone treatment is governed by increased basal ppGpp levels and that tolerant effect is a result of the effects that ppGpp exerts at the posttranscriptional or posttranslational levels. However, at this point, we suggest that the mechanism of antimicrobial tolerance may be multifactorial.

RpoN (sigma factor 54) is one of the subunits of RNA polymerase that target specific promoters sharing consensus sequences located at the position -24/-12 upstream from the transcriptional start site. The strong interaction of sigma factor 54 with its promoter keeps the sigma 54-holoenzyme promoter complex in a closed conformation which may then require activation by another protein to induce local DNA melting and initiate transcription[13]. The roles of *rpoN* vary among various microbial species. According to the data reported so far, the *rpoN* gene has been involved in different activities in cells.The *rpoN* is an indispensable factor for the expression of the nitrogen regulated genes and nitrogen fixation[14]. Besides nitrogen regulation, recent studies have suggested that the physiological functions of *rpoN* go far beyond the nitrogen regulation system; *rpoN* is involved in dicarboxylic acid transport[15], pillin and flagellin synthesis[16], toluene and xylene metabolism[17], and hydrogen metabolism[18]. Recently, 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 reported[19]. Studies performed in *E. coli* demonstrated 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[20]. Taking into account that different metabolic activities within cells were correlated with RpoN, we further wondered if *rpoN* could play a role as a novel target for antimicrobial agents in *P. aeruginosa*. We describe the construction of the *rpoN* mutants of *P. aeruginosa* and investigate the link between these mutant strains and the effect they display to antimicrobial agents, in particular, quinolones.

## MATERIAL AND METHODS

### Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are described in the Table 1. The bacteria were grown at 37℃ in Luria-Bertani (LB) broth, unless otherwise indicated. Antimicrobial agents were added as required at the following

Table 1. Bacterial strains and plasmids constructed in this study

| Strain or plasmid | Relevant feature[a] | Source or reference |
|---|---|---|
| *P. aeruginosa* | | |
| PAO1 | wild type, prototroph | |
| SM21 | $Sm^r$ strain derived from PAO1 | this laboratory |
| DVR1 | SM21 *relA*::$Gm^r$ | this study |
| DV14 | SM21 *relA*::$Gm^r$, *spoT*::$Tc^r$ | this study |
| MFM | SM21 *spoT*::$Gm^r$ | this study |
| DV31.7 | SM21 *dksA*::$Tc^r$ | this study |
| DRN | *rpoN*::$Gm^r$ | this study |
| DVR | *rpoN*::$Tc^r$ | this study |
| *E. coli* | | |
| XL1-Blue | *recA*1 *endA*1 *gyr*98 *thi*-1 *hsdR*17 *supE*44 *relA*1F`*proAB lacI*$^q$ ZDM15 Tn10 ($Tet^r$) | Stratagene |
| S17-1 | *thi endA recA hsdR* chromosome::RP4-2Tc::Mu-Km::Tn7 | M.Tsuda |
| Plasmid | | |
| pACYC184 | $Cm^r$ $Tc^r$ | Nippon Gene |
| pACΩGm | pACYC184 derivate carrying Ω fragment | Schweizer et al. |
| pGEM-T& | general purpose | |
| pGEM-T easy | cloning vector, *ori* (ColE1); $Amp^r$ | Promega Inc. |
| pDV1 | *relA* in pGEM-T | this study |
| pDVG2 | *relA*::$Gm^r$ pGEM-T | this study |
| pDV6 | *spoT* in pGEM-T | this study |
| pDV9 | *spoT*::$Gm^r$ in pGEM-T | this study |
| pDV7 | *spoT*::$Tc^r$ in pGEM-T | this study |
| pDV3 | *dksA* in pGEM-T | this study |
| pDV31.7 | *dksA*::$Tc^r$ in pGEM-T | this study |
| pRPON | *rpoN* in pGEM-T | this study |
| pRPON1 | *rpoN*::$Tc^r$ in pGEM-T | this study |
| pRPON2 | *rpoN* in pGEM-T easy | this study |
| pRPON3 | *rpoN*::$Gm^r$ in pGEM-T easy | this study |
| pRPON4 | *rpoN*::$Gm^r$ in pACYC184 | this study |
| pRPON5 | *rpoN*::$Gm^r$ in pACYC184 (*Hin*cII deletion within *rpoN*) | this study |

[a]Abbreviations: $Amp^r$, $Cm^r$, $Gm^r$, $Tp^r$, $Tc^r$, and $Sm^r$, stand for resistance to ampicillin, chloramphenicol, gentamicin, trimethoprim, tetracycline, and streptomycin, respectively

concentration for work with *E. coli*, with the concentrations used for *P. aeruginosa* shown in brackets: trimethoprim 25 μg/ml; streptomycin (1000 μg/ml); ampicillin, 50 μg/ml; gentamicin, 20 μg/ml (100 μg/ml), teteracycline, 10 μg/ml (100 μg/ml), chloramphenicol 10 μg/ml (50 μg/ml). *rpoN* mutant strains were supplemented with 1mM L-glutamine when grown in LB medium. For the induction of pyoverdine production, King B broth was used[21]. Media were also supplemented with $FeCl_3$ (100 μg/ml), or ethylenediamine (*o*-hydroxy) phenylacetic acid (EDDA) (400 μg/ml), as required. When indicated, LB medium was supplemented with serine hydroxamate (SHX) at the concentration of 200 μM for the induction of stringent response.

**DNA analysis and manipulation**

Restriction enzymes and DNA polymerases were purchased from New England Biolabs, Inc. (Beverly, MA, USA), Toyobo (Osaka, Japan), TaKaRa Shuzo (Kyoto, Japan) and were used under the conditions recommended by the manufacturers. Oligonucleotides were purchased from Hokkaido System Science Co., LTD (Sapporo, Japan). Plasmid DNA was isolated using Plasmid Miniprep Kit according to the protocol provided by the manufacturer (Bio Rad, CA, USA). Chromosomal DNA was isolated from *P. aeruginosa* SM21

and PAO1 using Bacterial DNA Kit (Omega Bio-Tek Inc., Doraville, GA, USA). Treatment of DNA with enzymes, subcloning of DNA, transformation of plasmids into *E. coli* and *P. aeruginosa* was carried out using standard methods[22]. Where required, DNA fragments were isolated from agarose gels using Qiagen gel extraction kit (Qiagen, Valencia, CA, USA). Standard methods were used for the preparation of competent cells and for plasmid electroporation into *E. coli*[22]. *P. aeruginosa* electrocompetent cells were prepared as described elsewhere[23].

**Construction of *P. aeruginosa spoT* mutant**

To generate a *spoT* mutant, a 2,050-bp fragment was amplified by polymerase chain reaction (PCR) using genomic SM21 DNA as a template and a pair of primers: 5'-CTGGG AAAACGACAAGCCGAC-3' and 5'-GCATTTTTCCGGG TTGTGGCG-3', that hybridize to the sequences upstream and downstream of the *spoT* gene, respectively. Amplified fragment was purified and inserted into a pGEM-T vector (Promega Corporation, Madison, WI, USA). An 817 bp deletion between the *Bss*HII sites within the *spoT* gene was created. The gentamycin-resistance gene from the Ω fragment of pACΩGm[24] was inserted into the *Bss*HII sites of *spoT* and constructed plasmid was transferred into *P. aeruginosa* by electroporation. Clones that showed homologous recombination were selected as gentamycin resistant and were further checked for the correct insertion of the *spoT*::Gm$^r$ in the chromosomal DNA of *P. aeruginosa* SM21.

**Construction of a *P. aeruginosa dksA* mutant**

A mutant strain, derived from SM21, which carries a tetracycline gene (Tc$^r$) replacing a major part of the *dksA* gene was constructed as follows; a 0.64 kb fragment containing the *dksA* gene was amplified by PCR using SM21 genomic DNA as the template and a pair of the primers 5'-CGCCAA GGTTATCGTTCTGGTA-3' and 5'-TACATAGGAAGAGGT CATGGCG-3'. The fragment was cloned into a pGEM-T vector. The resulting plasmid pDV3 was digested with *Bss*HII which recognizes two sites within the PCR amplified fragment of the *dksA* gene. A 0.27 bp internal fragment from the *dksA* coding sequence was excised. The linearized plasmid with *Bss*HII digestion was ligated to the 1.3 kb Tc$^r$ amplified from pACYC184 (Nippon Gene, Japan) using a pair of primers; 5' -TTGGCGCGCCAAGAAGTCAGCCCCATACGATA-3' and antisense 5'-TTGGCGCGCCAAGAGTGGTGATGAATCCG TTAGCGA-3'. The constructed pDV31.7 was electroporated in the *P. aeruginosa* SM21 and tetracycline-resistant colonies were selected and checked for the loss of carbenicillin resistance indicating a double cross-over to yield the *dksA* mutant strain.

**Construction of *relA* and double *relA spoT* mutant**

A *relA* knockout mutant was obtained following the same construction protocol as mentioned above for the *dksA* and the *spoT* mutant strains. The mutant was selected as having Gm$^r$ insertion within the *relA* gene, and therefore conferring the resistance to gentamicin. For the construction of *relA spoT* double knockout mutant the same strategy was applied as mentioned above. Tc$^r$ as resistance marker with flanking *Bss*HII linkers was used for insertion in the *Bss*HII sites of *spoT* in order to generate pGEM- Δ*spoT*::Tc$^r$. The constructed plasmid pDV7 was electroporated into the *relA*::Gm$^r$ mutant and double knockout mutant was selected as confirming resistance to gentamicin and tetracycline. In all mutants, the replacement of the wild type gene with the corresponding gene with a resistance marker insertion was confirmed by PCR and DNA sequencing.

**Construction of *rpoN* knockout mutant**

To construct an *rpoN* knockout mutant, 2 strategies were applied. First, PCR amplification was used to obtain a 1.5-kb fragment of *rpoN* using the pair of primers: 5'-ACCCGTA GTAGTGGATGGTGC-3' and 5'-CAACGTCACACCAGT CGCTTG-3'. The amplified fragment was cloned into pGEM-T vector creating pRPON. Next, *Bss*HII fragment was deleted from the *rpoN* and replaced with 1.3 kb Tc$^r$ gene with *Bss*HII linkers to form pRPON1. In the second strategy, the amplified *rpoN* gene was cloned into pGEM-T easy vector to yield pRPON2. Fragment between two *Bss*HII sites within the *rpoN* was excised and replaced with Gm$^r$ cassette from pACΩGm plasmid. The resulting construction containing Gm$^r$ cassette insertion is called pRPON3. The *Eco*RI fragment containing the *rpoN*::Gm$^r$ construct from pRPON3 was subsequently cloned into pACYC184 resulting in pRPON4. Further, *Hin*cII deletion was performed within the *rpoN* to yield pRPON5. This construction was used for introduction of the *rpoN*::Gm$^r$ allele onto the chromosome of *P. aeruginosa* PAO1. The presence of the *rpoN*::Gm$^r$ 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.

**Antimicrobial agents**

Ofloxacin obtained from Sigma (St. Louis, Mo.) and ciprofloxacin from Bayer Pharma (Germany) was used in the study.

**Susceptibility testing**

The MIC and the MBC of each agent were determined by the microbroth dilution method[25] with the following modification: the bacterial suspensions of *P. aeruginosa*

SM21, *P. aeruginosa* PAO1 and mutant strains were used at a density of $1 \times 10^6$ CFU/ml. MICs were determined after 18 to 22h of incubation at 37℃. 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. Minimal bactericidal concentrations (MBCs) were measured by removing 10μl from all wells containing no visible growth and plating the samples on LB agar plates for further incubation at 37℃ for 24 h.

For the time-kill studies, stationary phase cells grown for 12-14 h were used in the study. The culture was centrifuged at 3000xg for 15 min and resuspended in the fresh LB broth before the incubation with antimicrobial agent; ofloxacin 8 μg/ml, ciprofloxacin 1 or 2μg/ml. Aliquots (0.1ml) were taken after 0-5 hours of incubation, and plated in duplicate on agar plates after serial dilutions to enumerate the surviving bacteria after 24-48h of incubation at 37℃ . Cell viability at each time point was expressed as the percentage of viable cells (CFU/ml) at time zero. Each time-kill experiment was performed on three independent occasions.

**Purification of nucleotide and assay of nucleotide pools by HPLC**

The nucleotide, ppGpp was purifeid from the bacterial cells using method described by Payne et al.[26] with slight modifications. ppGpp was isolated from 10 ml samples of cultures grown overnight in LB broth. Bacterial cultures were washed and resuspended in fresh LB broth. At indicated time before and after the addition of antibiotic, cells were collected by rapid (<1 min) vacuum filtration using Millipore AP15 glass fiber prefilters ( Millipore Corp., MA, USA) and extracted with cold 1 M formic acid for 30 min. Samples including formic acid were collected, centrifuged at 4℃ to remove all cell debris, and filtered through 0.45 μm filter. Thereafter, the supernatant was lyophilized. The residue after lyophilization was dissolved in 100 μl of deionized distilled water and refiltered using syringe driven filter unit 0.45 μm (Millex® - HV, Millipore). Nucleotides in 20 μl of cell extracts were separated by HPLC and quantitated by measuring absorbance at 254 nm as described[27]. The intracellular concentration of nucleotides was assayed by HPLC (L-7000 Hitachi, Tokyo, Japan) on a Partisil 10 SAX column (4.6 x250mm, Whatman, International Ltd., England). The nucleotides were eluted at a flow rate of 1ml/min using the gradient made of low (7 mM $KH_2PO_4$, adjusted to pH 4.0 with $H_3PO_4$) and high (0.5 M $KH_2PO_4$+0.5 M $Na_2SO_4$, adjusted to pH 5.4 with KOH) ionic strength buffers. The percentage of buffer with high ionic strength was increased for 20 min from 0 to 100%, and remained like that for another 25 minutes. The area under the peak corresponding to ppGpp was determined and converted to picomoles per cell dry weight (CDW).

**Analysis of outer membrane proteins**

Outer membranes were prepared using the sarcosyl solubilization method as described by Filip et al.[28]. The bacterial pellet was obtained after centrifugation of 20 ml of culture of *P. aeruginosa* PAO1 and *rpoN* mutants grown in LB broth, LB broth plus 8 μg/ml ofloxacin or King's B broth, following disruption via sonication and harvesting of the outer membrane protein's fraction by centrifugation[28].

**SDS-PAGE**

Pellets containing the outer membrane proteins were resuspended in 150μl of sterile distilled water and the protein concentration was determined using the Bio-Rad protein assay with bovine serum albumin as a standard. The proteins were solubilized in sodium dodecyl sulfate (SDS) sample buffer and boiled for 5 min, separated by discontinuous SDS polyacrylamide gel electrophoresis (PAGE) using 5% stacking gel and 12% separating gel following staining with Coomassie brilliant blue G-250.

**Measurement of siderophore production**

Siderophore production was measured using the liquid chromeazurol S assay as described by Schwyn and Neilands[29].

**RNA isolation and RT-PCR analysis of mRNA levels**

Overnight LB broth-grown cultures were washed once and resuspended in fresh medium before the start of the experiment. Cells were sampled at time 1, 3, 5 h after the addition of antibiotic, and their RNA was immediately stabilized with RNAprotect Bacteria Reagent (Qiagen) and stored at -80℃. 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 30 min at 37℃ and repurified through an RNeasy column. cDNA was synthesized using the SuperScript™ First-Strand Synthesis System (Invitrogen, Groningen, The Netherlands). RT-PCR for detection of *pvdS* mRNA was done using a pair of primers; pvdS1s; 5'-ATGTCGGAACAACTGTCTACCC-3' and pvdS1a; 5'-TCCCTTGGCGATGTCCTTCTGT-3'. As a control for RNA contamination by DNA, the PCR reaction was done on the same samples, without first strand synthesis.

## RESULTS

**Role of *dksA* and *spoT* in tolerance to quinolone**

To address whether the *relA*, *spoT* and *dksA* genes are required for tolerance to quinolone, and serve as possible targets for antimicrobial agents, we constructed a set of

deletion mutants in the above mentioned genes in *P. aeruginosa* SM21.

MIC and MBC values for wild type strain and mutant strains are shown in the Table 2. Whereas the MIC values for *dksA* and *spoT* mutants were almost the same as the MIC for the wild type, the MBC values showed to be at least 4 times higher than those for the wild type strain. MICs are commonly used to assess the *in vitro* activities of antimicrobial agents. However, they provide little information on the pattern of bacterial activities. As the time-kill studies with extensive sampling allow assessment of both the rate and extent of bacterial killing and regrowth[30], we performed time-kill studies of the stationary phase cultures at drug concentrations equal to 8 to 16 times the MIC of that drug for a bacterial strain. The optimal bactericidal concentrations of quinolones are close to eight times their MIC[31]. The time-kill studies in the presence of ofloxacin for the *dksA* mutant are shown in Fig. 1A. The survival rate of the mutant at 5 h after addition of ofloxacin was approximately 100 times higher than that seen for the wild type strain. The *spoT* mutant showed significantly lower growth rate than seen in the wild type strain (data not shown). When the *spoT* mutant strain was incubated for 5 h with ofloxacin, tolerant effect to antibiotic was 100 times higher than that seen in the wild type strain (Fig. 2A). When the cells of *dksA* and *spoT* mutant strain were exposed to ciprofloxacin at the concentration of 1 μg/ml which corresponds to 8 times MIC of that drug, the tolerant effect was however slightly decreased, showing the *dksA* and *spoT* mutant approximately 50 to 10 times more tolerant than the wild type strain (Fig. 1B and 2B). As no differences were observed in the MIC values for quinolones in the constructed mutants and the wild type, it suggests that higher tolerant effect seen in these knockouts is not due to expression of a resistance mechanism. Further, we wondered if the tolerant effect was cell density dependent. We exposed the log phase cells to antimicrobial agents at the concentration mentioned above. Tolerant effect was however slightly decreased but still present when compared to the wild type strain (data not shown). Then, we investigated the influence of the growth medium on the tolerant effect to antimicrobial agents; the mutant cells were grown in the morpholinepropanesulfonic acid (MOPS) minimal medium supplemented with 0.5% casamino acids for the *dksA* mutant strain and the tolerant effect showed to be almost the same as when the cells were grown in the LB medium (data not shown). Since the *spoT* mutant has been already characterized by increased basal levels of ppGpp, mostly on account of lost ppGpp hydrolase activities, and chloramphenicol is known as an inhibitor of protein synthesis, as well as inhibitor of ppGpp synthesis, we wondered if chloramphenicol addition would affect the degradation of basal levels of ppGpp and consequently influence the response towards quinolone treatment. To assess the effect of chloramphenicol on ppGpp, the *spoT* mutant and wild type cells were exposed to quinolone in the presence of the chloramphenicol at sub-MIC concentration of 50 μg/ml (Fig. 3). The results showed that reduced the amount of ppGpp by the presence of sub-MIC concentration of chloramphenicol produced increased tolerant effect to quinolone. It suggests that the amount of ppGpp present in the cells at the time of exposure to antibiotic does not necessarily play the main role, and that some other factors may be crutial in rendering these mutants less susceptible to quinolone. The addition of choramphenicol reduced the amount of ppGpp as verified by HPLC but the reduction of tolerance was not seen. Next, we tried to verify whether the protective role of ppGpp would still be present by increasing the ppGpp levels in the cells. To examine this possibility, wild type and mutant cells were treated with ciprofloxacin at concentration of 1 or 2 μg/ml and 200 μM SHX. The treatment with SHX began 5 min prior to ciprofloxacin addition (Fig. 4). Overproduction of ppGpp by SHX addition in wild type and mutant cells led to protective role to ciprofloxacin treatment. However, by increasing the concentration of antibiotic to 2 μg/ml, the protective role of overproduced ppGpp was not seen or was not significantly changed. The result suggested that the tolerant effect per se is a mechanism distinct from the amount of ppGpp levels in the cell at the moment of the exposure to

Table 2. Susceptibility of wild type and mutant strains to antimicrobial agents

| Strain | ofloxacin | | ciprofloxacin | |
|---|---|---|---|---|
| | MIC | MBC | MIC | MBC |
| | μg/ml | | μg/ml | |
| SM21 (wild type) | 1 | 1 | 0.125 | 0.25 |
| DVR1 (*relA*::Gm$^r$) | 0.5 | 1 | 0.25 | 0.25 |
| MFM (*spoT*::Tc$^r$) | 0.5 | 2 | 0.125 | 0.25 |
| DV12 (*relA*::Gm$^r$ *spoT*::Tc$^r$) | 1 | 1 | 0.125 | 0.125 |
| DV31.7 (*dksA*::Tc$^r$) | 1 | 4 | 0.125 | 1 |

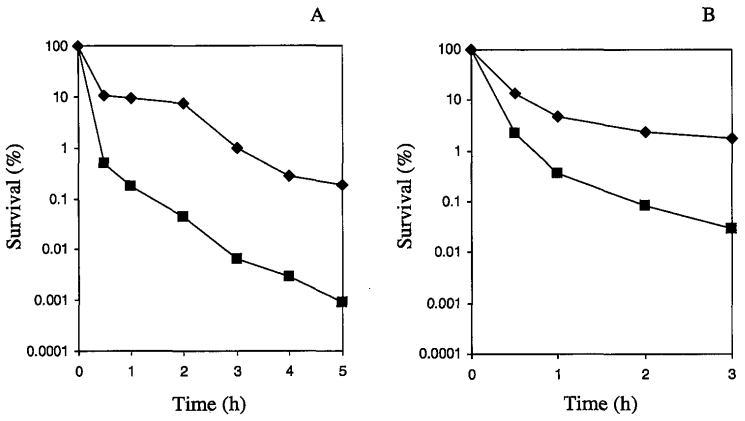


Fig. 1 Survival of the wild type (squares) and *dksA* mutant (diamonds) in the presence of ofloxacin 8 μg/ml (A) and ciprofloxacin 1 μg/ml (B).

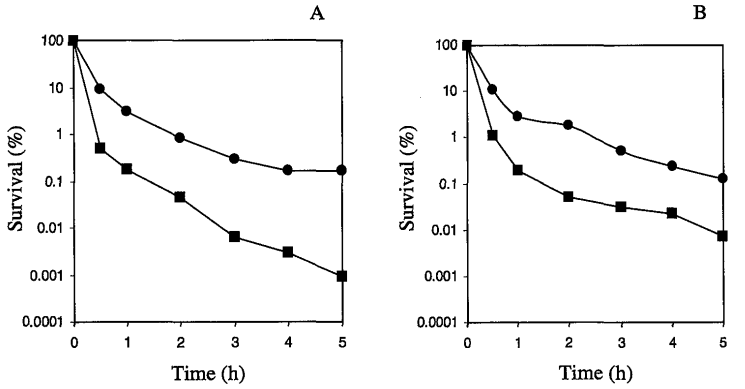


Fig. 2 Time-kill studies for wild type (squares) and *spoT* mutant (diamonds) in the presence of ofloxacin 8 μg/ml (A) and ciprofloxacin 1 μg/ml (B).

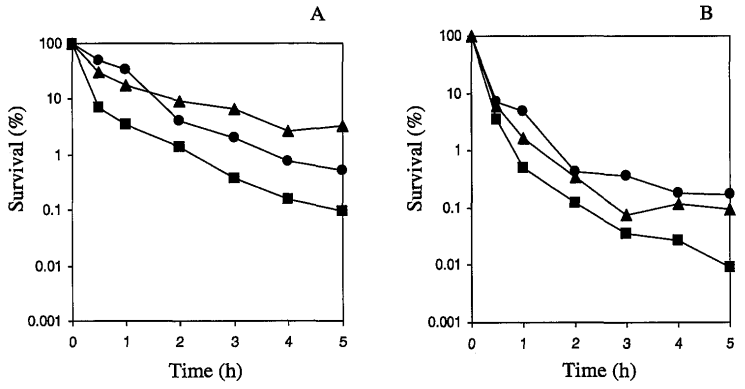


Fig. 4 Time-kill studies in the presence of serine hydroxamate (SHX) at the concentration of 200 μM and ciprofloxacin 1 μg/ml (A) and 2 μg/ml (B), for wild type (squares), *dksA* mutant (circles) and *spoT* mutant (triangles). Induction of ppGpp was achieved by adding SHX to stationary phase cells 5 min prior ciprofloxacin treatment.

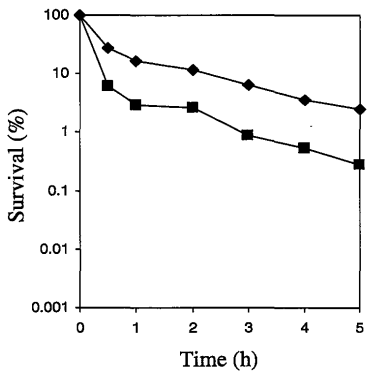


Fig. 3 Time-kill study for wild type (squares) and *spoT* mutant (diamonds) in the presence of ciprofloxacin 1 μg/ml and chloramphenicol 50 μg/ml. Chloramphenicol treatment began 5 min prior to ciprofloxacin addition.

antimicrobial agent. We further determined the response of the *relA* and *relA spoT* mutant strains to quinolone treatment. The *relA* and the double mutant *relA spoT* showed almost the same survival rate as the wild type, or being even more sensitive to antibiotic treatment than the wild type strain as seen for the *relA* mutant (Fig. 5A, 5B and 5C).

**Quantification of ppGpp levels by HPLC imply for increased basal levels of ppGpp in *dksA* and *spoT* mutant**

For the measurement of the ppGpp by HPLC, ppGpp was isolated from the stationary phase cells to reflect the same conditions as those used for time-kill studies. First, we measured the basal levels of ppGpp in both mutant strains and a wild type strain. As the *spoT* mutant strain shows low degrading activities of ppGpp, basal ppGpp levels found in

the *spoT* mutant were approximately 3 times higher than that those seen in the *dksA* mutant (Fig. 6). The addition of ciprofloxacin caused slight decrease in the ppGpp levels in the *spoT* mutant from 220 pmol to 100 pmol/mg CDW, however, in the *dksA* mutant, the ppGpp levels remained almost unchanged to ciprofloxacin addition (Fig. 7). Moreover, we measured the levels of ppGpp after exposure to SHX in the presence of ciprofloxacin. Shortly after addition of SHX (Fig. 8), ppGpp levels in the *spoT* mutant increased significantly in comparison with the *dksA* mutant that demonstrated almost constant levels of ppGpp. Furthermore, we were interested to see how chloramphenicol addition would reflect the levels of ppGpp in the *spoT* mutant. In treatment with chloramphenicol 5 min prior ciprofloxacin addition, chloramphenicol reduced the ppGpp levels approximately 2 times when compared to ppGpp levels found in the presence of ciprofloxacin (Fig. 9). However, under the conditions of this study we were not able to detect ppGpp levels in the wild type strain or in the *relA* mutant or double *relA spoT* mutant strain. One explanation would be that very low basal levels present in these mutant cells and wild type (if present at all) are due to very fast degradation in LB broth, in which the synthesis rate is very low and the degradation rate of ppGpp is very fast.

**Role of *rpoN* and siderophore production in tolerance to quinolone**

MIC data for study strains are summarized in the Table 3. No significant difference was observed in the MIC values for wild type strain and the *rpoN* mutant strains, however, MBC values for the *rpoN*::Tc$^r$ were slightly increased in comparison with the *rpoN*::Gm$^r$ mutant and the wild type. In order to gain more insights on the antibiotic's bactericidal activity in mutants constructed in this study, the time-kill studies were performed. The antimicrobial agents, ofloxacin and ciprofloxacin were used at a concentration of 8 μg/ml and 2 μg/ml, respectively, and the time-kill studies are shown in Fig. 10A and 10B. The *rpoN*::Tc$^r$ showed to be 10 times more tolerant to antibiotic treatment when compared to the wild type strain; on the other hand, the *rpoN*::Gm$^r$ mutant displayed almost the same susceptibility to killing to ofloxacin as wild type strain or being even more susceptible to ciprofloxacin treatment than wild type strain. Interestingly, 1 h after the addition of antibiotics, the *rpoN*::Tc$^r$ knockout showed increased accumulation of a green fluorescent pigment which correlated with the increase in the tolerant effect to quinolone. The results raised the question if the pigment produced by *rpoN*::Tc$^r$ mutant could be a siderophore, pyoverdine, characteristic of fluorescent *Pseudomonas*. In order to check if the transcription from the *pvdS* gene correlates with the accumulation of pigment, RT-PCR was performed on total

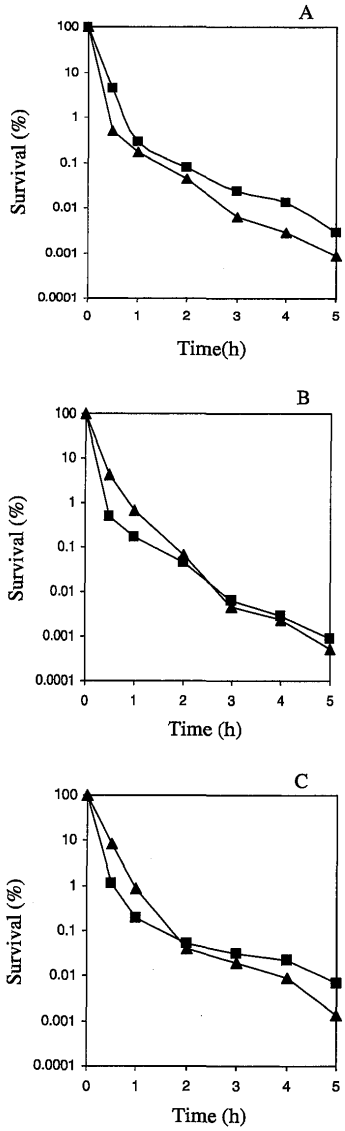


Fig. 5 Time-kill study for wild type (squares) and *relA* mutant (triangles) (A) in the presence of ofloxacin 8 μg/ml. Time-kill studies for wild type (squares) and *relA spoT* mutant (triangles) in the presence of ofloxacin 8 μg/ml (B) and ciprofloxacin 1 μg/ml (C).

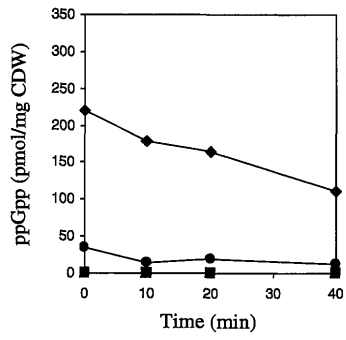


Fig. 6 Basal levels of ppGpp found in wild type strain (squares), *dksA* mutant (circles), and *spoT* mutant (diamonds).

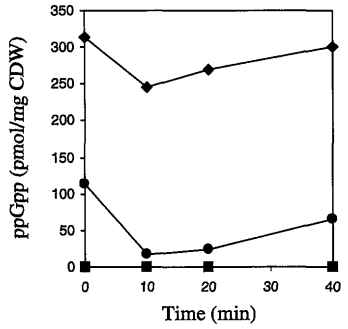


Fig. 7 Changes in the ppGpp content after the addition of ciprofloxacin 1 μg/ml for stationary phase cells; wild type (squares), *dksA* mutant (circles), *spoT* mutant (diamonds).

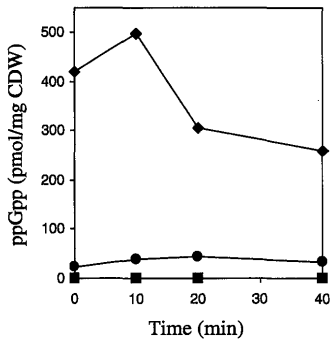


Fig. 8 Changes in the ppGpp content after the addition of ciprofloxacin 1 μg/ml and SHX 200 μM for stationary phase cells; wild type (squares), *dksA* mutant (circles), *spoT* mutant (diamonds).

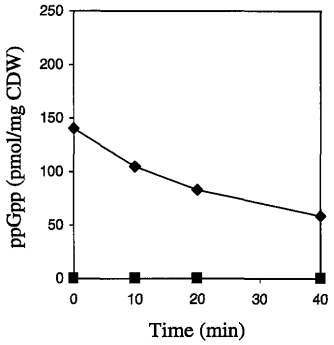


Fig. 9 Changes in the ppGpp content after the addition of ciprofloxacin 1 μg/ml and chloramphenicol 50 μg/ml for wild type (squares), *spoT* mutant (diamonds).

Table 3. Susceptibility of wild type and *rpoN* mutant strains to antimicrobial agents

| Strain | ofloxacin | | ciprofloxacin | |
|---|---|---|---|---|
| | MIC | MBC | MIC | MBC |
| | μg/ml | | μg/ml | |
| PAO1 (wild type) | 1 | 1 | 0.125 | 0.5 |
| DRN (*rpoN*::Gm$^r$) | 0.5 | 1 | 0.25 | 0.5 |
| DVR (*rpoN*::Tc$^r$) | 1 | 2 | 0.25 | 2 |

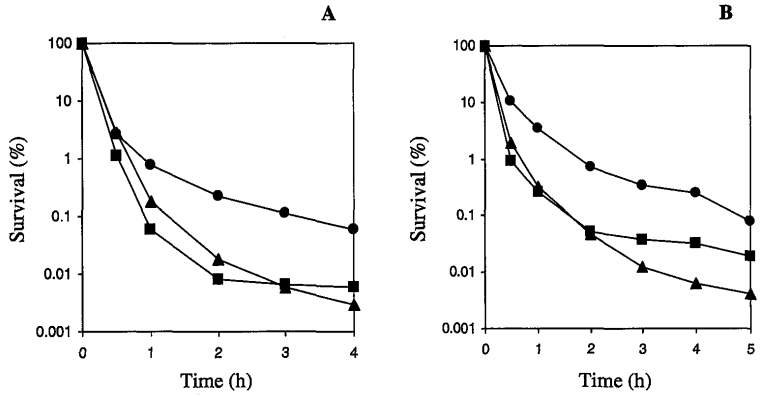


Fig. 10 Time-kill studies for wild type strain (squares), *rpoN*::Tc$^r$ (circles), *rpoN*::Gm$^r$ (triangles) in the presence of ofloxacin 8 μg/ml (A) and ciprofloxacin 2 μg/ml (B).

RNA isolated during the time-kill study in the presence of ofloxacin at working concentration of 8 μg/ml. As shown in Fig. 11 and 12, RT-PCR results showed that the addition of antibiotic increased the transcription of the *pvdS* gene significantly in the *rpoN*::Tc$^r$ mutant, and less prominent increase was seen in the *rpoN*::Gm$^r$ knockout mutant. To further characterize the produced pigment as possibly being a siderophore and representing iron limitation in the cell, overnight culture of the wild type and the *rpoN*::Tc$^r$ mutant were resuspended in the fresh medium supplemented with 100 μg/ml $FeCl_3$ in order to complement the iron deficiency and were exposed to antibiotic treatment (Fig. 13A and 13B). Addition of $FeCl_3$ had an inverse tolerant effect for the *rpoN*::Tc$^r$ showing that the iron limited conditions were playing a part in the tolerance to ofloxacin. Positive tolerant effect was lost after allowing repleted iron conditions. However, the treatment with ciprofloxacin and $FeCl_3$ did not affect the tolerance in *rpoN*::Tc$^r$, implying that even iron

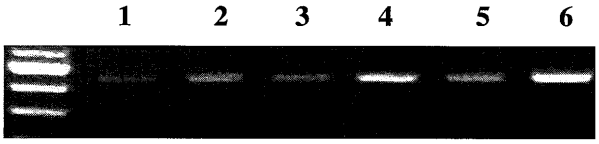


Fig. 11 The effect of ofloxacin addition on the expression of *pvdS* gene
Stationary phase cells were exposed to ofloxacin and total RNA was isolated at 1, 3, 5 hours after the addition of antibiotic. RT-PCR was performed using primers; *pvdS*1s and *pvdS*1a. PAO1 (lane 1, 3, 5) and *rpoN*::Tc$^r$ (2, 4, 6).

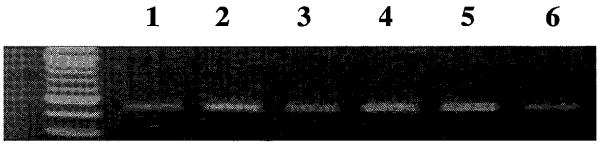


Fig. 12 The effect of ofloxacin addition on the expression of *pvdS* gene for wild type and *rpoN*::Gm$^r$ mutant strain. The same procedure was performed as in Fig. 11. PAO1 (lane 1, 3, 5) and *rpoN*::Gm$^r$ (lane 2, 4, 6).

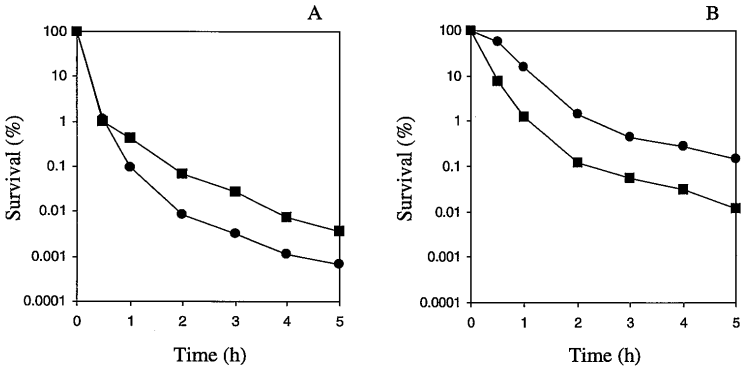


Fig. 13 Time-kill studies for wild type strain (squares), *rpoN*::Tc$^r$ (circles), in the presence of ofloxacin 8 μg/ml (A) and ciprofloxacin 2 μg/ml (B), respectively. $FeCl_3$ at the concentration of 100 μg/ml was added in the medium.

repleted conditions in the cells were not sufficient to repress the production of the siderophore. Next, in order to check how complete iron depletion would affect the response to antibiotic treatment, the time-kill study was performed in the presence of EDDA which serves as an iron chelator. When the *rpoN*::Tc$^r$ mutant was exposed to antibiotic and EDDA at a working concentration of 400 μg/ml, after 5 h of incubation the production of the pigment increased significantly, however, the same was not seen for the wild type strain. In the wild type strain, killing in the presence of EDDA and ofloxacin showed to be almost the same as seen in the control group in which EDDA was omitted (Fig. 14A and 14B). The measurement of siderophore production in the *rpoN* mutant strains did not reveal significant difference with the wild type strain (Table 4). An examination of growth kinetics in LB medium supplemented with 1 mM L-glutamine showed that growth rates of both mutant strains did not differ significantly from the wild type rates (data not shown). Furthermore, outer membrane proteins demonstrated the difference in the expression profile in the wild type and mutant strains (Fig. 15).

## DISCUSSION

In the present study we show two different factors that play a role in the tolerance to antimicrobial agents in *P. aeruginosa*. Entrance in the antibiotic insensitive state by rapid transition to a dormant state upon application of stress is a way for the cells to survive while exposed to the stress. In the present study we propose the mechanism by which the tolerant effect to quinolone treatment might be mediated by the effector molecule, ppGpp. Besides amino acid starvation, it is also known that carbon starvation, nutritional deprivation such as the entrance in the stationary phase, lead to inhibition of stable RNA synthesis accompanied by accumulation of ppGpp[1]. The entry into stationary phase represents a nutritional deficiency and growth arrest and shuts off as soon as the cells become able to resume growth. In this way, increased basal levels of ppGpp would provide the pathways for rapid and reversible modulation of transcription by inducing the genes involved in the cell survival[32]. Some recent reports have linked the stringent effector molecule, ppGpp with premature activation of quorum-sensing-regulated virulence factors[33]. Since ppGpp was recognized as a pleiotropic effector molecule involved in different pathways such as changing the transcription pattern of regulatory genes, rapid adjustment to the stress, we began constructing a set of mutants in the genes involved in the stringent response, such as the *relA* and *spoT* gene. In *E. coli*, the *relA* encodes the (p)ppGpp synthetase and SpoT is a bifunctional enzyme with both (p)ppGpp synthetase and (p)ppGpp hydrolase activities. SpoT-dependent accumulation

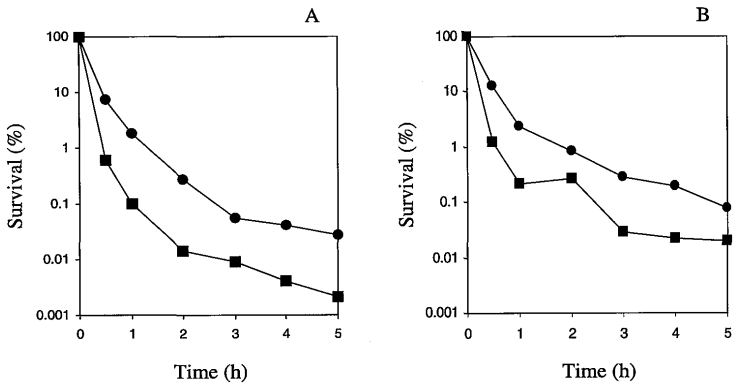


Fig. 14 Time-kill studies for wild type strain (squares), *rpoN*::Tc$^r$ (circles), in the presence of ofloxacin 8 μg/ml (A) and ciprofloxacin 2 μg/ml (B), respectively. EDDA at the concentration of 400 μg/ml was added in the medium.

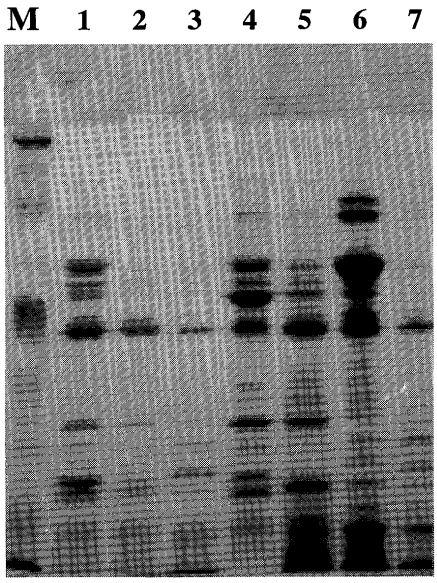


Table 4. Siderophore production using Chrome azurol S liquid assay.

| Strain | Siderophore units (%) |
|---|---|
| PAO1 wild type | 86.04 ± 2.19 |
| DRN *rpoN*::Tc$^r$ | 82.15 ± 1.10 |
| DVR *rpoN*::Gm$^r$ | 73.48 ± 1.85 |

Fig. 15 Analysis of outer membrane proteins from *P. aeruginosa* PAO1 and *rpoN* mutant strains. Outer membranes were prepared from wild type and mutant strains of *P. aeruginosa* PAO1 grown under different culture conditions and analysed by SDS-PAGE. The positions of molecular weight standard proteins are shown. PAO1, DVR, DRN grown in LB broth (lane 1, 2, 3, respectively), PAO1 grown in LB broth + 8 μg/ml of ofloxacin (lane 4), DVR grown in LB broth + 8 μg/ml of ofloxacin (lane 5), PAO1 grown in King B (lane 6), DVR grown in King B (lane 7).

of guanosine tetraphospahte and loss of degrading activities in the *spoT* mutant led us to investigate the role that increased basal levels of ppGpp may play towards antibiotic tolerance to quinolone. In rich medium such as LB with large free amino acid pools, SpoT synthetase activity will be low and SpoT hydrolase activity will be high. The basal level of ppGpp during exponential growth depends therefore, in part, on how the opposing activities of the *spoT* product are balanced[1]. Elevation of ppGpp by SpoT activity can account for *rpoS* induction even in a strain with *relA* deletion[34]. These results suggest that increased levels of *rpoS* as a consequence of the *spoT* dependent accumulation of ppGpp could possibly be another factor playing a role in the tolerance to stress such as antibiotic addition. We have recently reported that *rpoS* plays a role in the tolerance to stationary phase cells[35]. Moreover, our attention was drawn to the pleiotropic regulator DksA because mutants defective in the *dksA* show phenotypes similar to those of ppGpp° mutants. In *E. coli*; the *dksA* has been recently suggested to be required for *rpoS* induction by the nutrient stress signal ppGpp[5]. The deletion of *dksA* blocked *rpoS* induction by ppGpp, whereas overproduction of *dksA* induced *rpoS* independently of ppGpp[30]. The mechanism of persistence has been introduced in the literature through the first discovery of *hipA* mutant[36] that showed increased tolerance to antibiotic treatment. Important issue about the mechanism of persistence is that the persisters are not resistant to antibiotics and do not exhibit an increased MIC which usually reflects the expression of specific resistance mechanisms. Interestingly, we found that the *dksA* and *spoT* mutant showed almost the same MIC as the wild type strain, however, we found increased MBCs in these mutant cells, which may be a characteristic of tolerant cells. The transition from active growing state into the nongrowing state leads to production of persistence at an increasing frequency as the cell density in the bacterial population increases. It was possibly due to the entrance into the stationary phase and activation of quorum sensing system that may be governed by ppGpp. Knockout mutants, *dksA* and *spoT*, have intact *relA* gene, capable of synthesizing ppGpp, and it was shown that inactivation of *relA* diminishes the *hipA7* mutant to confer

high persistence phenotype[10]. Furthermore, increased survival rate after quinolone treatment in this study may be explained by the fact that increased basal levels of ppGpp at some point affect DNA replication; increased intracellular concentration of ppGpp seems to lead to decrease in the DnaA protein concentration and inhibits the initiation of DNA replication[2]. In the conclusion, the mechanism by which these knockout mutants decrease the process of lysis following addition of antibiotic might be explained through the increased basal levels of ppGpp which will in turn act by rapid turnover in the transcription gene profile allowing the entrance in the dormant protective state characterized as antibiotic insensitive state. Moreover, ppGpp may retard the contact of the antibiotic with the target such as DNA gyrase. However, further studies will help to elucidate the role of the ppGpp in antibiotic tolerance in *P. aeruginosa*.

The second part of the study deals with the response that the *rpoN* mutants display towards antibiotic treatment. The mechanism and ability of antibiotics to reach certain targets within the cells may largely depend on the physiological state of the cell, implying that environmental conditions, nutrient limitation and cell status may produce different response to the stress such as antibiotic treatment. Intercellular communication systems allow bacteria to control the conditions in the environment and accordingly to coordinate the expression of several genes particularly those involved in the production of extracellular products and virulence factors. Since the *rpoN* gene of *P. aeruginosa* has been linked with the regulation of virulence-related factors such as alginate synthesis[37] and negative control of quorum sensing[38], it prompted us to try to establish possible link between the *rpoN*, quorum sensing, iron limitation and antimicrobial tolerance. The *rpoN* is controlling the quorum sensing system by down regulating the expression of GacA at low and high cell densities and on the other side having positive effect on the expression of Vfr especially during late growth phase. Increased transcription of *lasR/lasI* and *rhlR/rhlI* in the *rpoN* mutant as observed by Heurlier et al.[19] brought to attention that such increase of the components of quorum sensing may play part in reduced susceptibility to antimicrobial agents in the *rpoN*::Tc$^r$ mutant. Hassett et al.[39] have reported that the quorum sensing controls the expression of the catalase and superoxide dismutase genes and mediates the susceptibility to hydrogen peroxide. Iron deficiency seems to lead to inhibition of oxygen transfer and enhanced formation of virulence factors as reported by Kim et al.[40]. Stintzi et al.[41] showed that cell density while not an essential signal for siderophore production may play a key role in modulating pyoverdine biosynthesis and that *lasI* mutants show decreased levels of pyoverdine. According to the data obtained in this part of the study, iron concentration in the medium might be the factor influencing the susceptibility to antimicrobial agents. Incubation of the *rpoN*::Tc$^r$ mutant with quinolone increased the production of green pigment, suggesting for siderophore. It should be pointed out that the culture conditions used in this study for growing the *rpoN* mutants were iron rich and that observed production of siderophore was a result of antibiotic addition to the culture of the *rpoN*::Tc$^r$ mutant and was not provocated by using low iron medium which is usually used for assessing the production of pyoverdine. Time-kill studies were performed in LB broth which is considered to be a rich medium and the concentration of free iron is far sufficient for the cells. As increased production of siderophore was observed after the addition of antibiotic to the culture of the *rpoN*::Tc$^r$ mutant, we investigated the transcriptional levels of the gene responsible for the pyoverdine synthesis, *pvdS*, by performing RT-PCR. Transcription of the *pvdS* gene was significantly increased in the *rpoN*::Tc$^r$ mutant when compared with the *rpoN*::Gm$^r$ mutant or the wild type strain. Although the mechanism by which the *rpoN*::Tc$^r$ mutant displays a tolerant effect to quinolone treatment remains poorly understood, we attempted to understand the background of the deletions in the *rpoN* gene and antibiotic tolerance through the following explanations; we believe that fluorescent pigment secreted by *rpoN*::Tc$^r$ mutant influenced the tolerant effect to antimicrobial agents, ofloxacin and ciprofloxacin. However, at this point, we suggest that the tolerant effect was not only limited to quinolone group of antibiotics, because other antimicrobial agents displayed almost the same pattern of the tolerant effect in both knockout mutants (data not shown). It is well known that quinolones have two essential bacterial enzymes as their targets, DNA gyrase (topoisomerase II) and DNA topoisomerase IV. Binding of quinolone to the gyrase blocks DNA replication and transcription accounting for the bacteriostatic activity of the drugs. Short incubation time and higher concentration of the drug are needed for the lethal action leading to cell death[42]. However, the mechanism of action of ofloxacin and ciprofloxacin at this point seems to be different. Ciprofloxacin possibly possesses an additional mode of killing cells and it seems that iron concentration in the cell, representing iron deficiency or sufficiency, is not playing important role in developing tolerance to ciprofloxacin. Brooun et al.[43] demonstrated that planktonic and biofilm cells were equally responding to ofloxacin treatment, however the same effect was not shown for the treatment with ciprofloxacin. Under the conditions of this study, we used ofloxacin concentration which corresponds to 8 times MIC for the *rpoN*::Tc$^r$ mutant and 16 times for the *rpoN*::Gm$^r$ mutant and wild type strain, respectively. This discrepancy in ofloxacin concentrations could possibly encounter for the

slight difference we observed during the time-kill studies for the *rpoN*::Tc$^r$ mutant and the *rpoN*::Gm$^r$ mutant, but would not completely explain the differences found between these two mutants. In order to further characterize the production of the pigment in the *rpoN*::Tc$^r$ mutant, we measured the absorption spectra of the culture supernatant after the exposure to antibiotic treatment (data not shown). We found that the culture supernatants showed absorption spectra with residual peaks at 400nm, which were only slightly shifted by acidification. This data led us to conclude that the fluorescence emitted by the mutant strain after exposure to antibiotic was not probably due to the native pyoverdine molecule, but rather a secreted compound that might be precursor of pyoverdine or its degraded form. PvdS has been recognized as an extracytoplasmic sigma factor, required for expression of pyoverdine synthesis genes in *P. aeruginosa*[44]. According to the data obtained, we speculate that in the *rpoN*::Tc$^r$ mutant, *pvdS* upregulates one of the genes whose product would be involved in the later step of pyoverdine synthesis, e.g. formation of functional domain important not only for the emission of fluorescence but also for chelating of the ferric ion. Next, we wondered if such precursor or degraded form might be influenced by the presence of antibiotics and/or could possibly tend to interact with the antibiotic by forming a "siderophore-antibiotic conjugates" that use specific iron uptake pathway for the entry in the cells. Moreover, we asked if the conditions in the environment and cells-state provoke some kind of competition between iron and antibiotic for binding to siderophore. Up to date, several studies demonstrated that siderophore-antibiotic conjugates can act against pathogenic bacteria which produce and use siderophore to acquire iron.[45, 46]. It is not unreasonable to assume that such a complex of siderophore-antibiotic might lead to decreased susceptibility of the cells to antibiotic. Data obtained from the outer membrane protein profiles revealed that there are slight differences in the expression of OMPs in both mutant strains when compared to the wild type strain, in the presence of antibiotic or when grown in King B medium. However, we cannot explain that these differences might be responsible for tolerant effect towards antibiotics in these mutants, but it is likely that surface structures can be altered till some extend by growth in the presence of antibiotics[47]. If this is to be the case, the inhibition of expression of surface proteins required for iron acquisition may encounter for tolerant effect to antibiotics. One of the most intriguing results encountered in this study was the fact that mutant strains showed completely different response to quinolone treatment. Such a different response to quinolone treatment is possibly only quinolone-specific and only DNA synthesis specific. The studies involving the antibiotics that do not target the DNA synthesis such as carbapenems both mutants show almost the same response to antibiotic and same MIC/MBC values (data not shown). Further, different antibiotic susceptibility in these mutant strains to quinolone might be influenced by different construction protocol used in generating the mutants in this study. Recent report suggests that sigma factor 54 possesses several domains that may function independently one of the other[48]. The fact that *rpoN* has been involved in the regulation of hydrogen uptake and that iron deficiency leads to inhibition of oxygen transfer and enhanced formation of virulence factors may be another aspect to consider the complex mechanism of regulation connected with *rpoN*. An understanding of antibiotic tolerance mediated by ppGpp, deletions within the *rpoN* gene, and iron deficiency may provide the key link with antimicrobial agents.

## ACKNOWLEDGMENTS

I would like to express my thanks to Prof. Yoichiro Miyake for his support and enouragement during my PhD thesis. Moreover, I am very grateful to Prof. Tsuneko Ono (Major in Laboratory Science, Tokushima University School of Health Science) for insightful discussions, excellent instruction and help with HPLC. In particular, I am greatly indebted to Prof. Takafumi Noma, Department of Molecular Biology and Prof. Katsuhiko Yoshimoto, Department of Medical Pharmacology for reading this manuscript and their invaluable suggestions.

## REFERENCES

1) Cashel M, and Rudd KE (1987) in *Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology (Ingraham J, Low KB, Magasanik B, Neidhardt FC, Schaecter N, and Umbarger HE, eds) Vol.2, pp.1410-1438, American Society for Microbiology, Washington, D.C.

2) Szalewska-Palasz A, Wegrzyn A, Herman A, and Wegrzyn G. The mechanism of the stringent control of lambda plasmid DNA replication. EMBO J 13, 5779-5785 (1994)

3) Xiao H, Kalman M, Ikehara K, Zemel S, Glaser G, and Cashel M. Residual guanosine 3',5'-bispyrophosphate synthetic activity of *relA* null mutants can be eliminated by *spoT* null mutations. J Biol Chem 266, 5980-5990 (1991)

4) Branny P, Pearson JP, Pesci EC, Kohler T, Iglewski BH, and Van Delden C. Inhibition of quorum sensing by a *Pseudomonas aeruginosa dksA* homologue. J Bacteriol 183, 1531-1539 (2001)

5) Brown L, Gentry D, Elliott T, and Cashel M. DksA affects ppGpp induction of RpoS at a translational level. J Bacteriol 184, 4455-4465 (2002)

6) Jude F, Kohler T, Branny P, Perron K, Mayer MP, Comte R, and van Delden C. Posttranscriptional control of quorum-sensing-dependent virulence genes by DksA in *Pseudomonas aeruginosa*. J Bacteriol 185, 3558-3566 (2003)
7) Balzer GJ, and McLean RJ. The stringent response genes *relA* and *spoT* are important for *Escherichia coli* biofilms under slow-growth conditions. Can J Microbiol 48, 675-680 (2002)
8) Taylor CM, Beresford M, Epton HA, Sigee DC, Shama G, Andrew PW, and Roberts IS. *Listeria monocytogenes relA* and *hpt* mutants are impaired in surface-attached growth and virulence. J Bacteriol 184, 621-628 (2002)
9) Rallu F, Gruss A, Ehrlich SD, and Maguin E. Acid- and multistress-resistant mutants of *Lactococcus lactis*: identification of intracellular stress signals. Mol Microbiol 35, 517-528 (2000)
10) Korch SB, Henderson TA, and Hill TM. Characterization of the *hipA7* allele of *Escherichia coli* and evidence that high persistence is governed by (p)ppGpp synthesis. Mol Microbiol 50, 1199-1213 (2003)
11) Nakajima A, Hoshikawa M, and Nakae T. Antibiotic stress induces a large amount of outer membrane protein in *Pseudomonas aeruginosa*. FEMS Microbiol Lett 165, 261-265 (1998)
12) Robertson GT, Zhao J, Desai BV, Coleman WH, Nicas TI, Gilmour R, Grinius L, Morrison DA, and Winkler ME. Vancomycin tolerance induced by erythromycin but not by loss of *vncRS*, *vex3*, or *pep27* function in *Streptococcus pneumoniae*. J Bacteriol 184, 6987-7000 (2002)
13) Popham D, Szeto D, Keener J, and Kustu S. Function of a bacterial activator protein that binds to transcriptional enhancers. Science 243, 629-635 (1989)
14) Gussin GN, Ronson CW, and Ausubel FM. Regulation of nitrogen fixation genes.
Annu Rev Genet 20, 567-591 (1986)
15) Ronson CW, Astwood PM, Nixon BT, and Ausubel FM. Deduced products of C4-dicarboxylate transport regulatory genes of *Rhizobium leguminosarum* are homologous to nitrogen regulatory gene products. Nucleic Acids Res 15, 7921-7934 (1987)
16) Totten PA, Lara JC, and Lory S. The *rpoN* gene product of *Pseudomonas aeruginosa* is required for expression of diverse genes, including the flagellin gene. J Bacteriol 172, 389-396 (1990)
17) Merrick MJ. In a class of its own--the RNA polymerase sigma factor sigma 54 (sigma N). Mol Microbiol 10, 903-909 (1993)
18) Hendrickson EL, Plotnikova J, Mahajan-Miklos S, Rahme LG, and Ausubel FM. Differential roles of the *Pseudomonas aeruginosa* PA14 *rpoN* gene in pathogenicity in plants, nematodes, insects, and mice. J Bacteriol 183, 7126-7134 (2001)
19) Heurlier K, Denervaud V, Pessi G, Reimmann C, and Haas D. Negative control of quorum sensing by RpoN (sigma54) in *Pseudomonas aeruginosa* PAO1. J Bacteriol 185, 2227-2235 (2003)
20) Jovanovic M, Lilic M, Janjusevic R, Jovanovic G, and Savic DJ. tRNA synthetase mutants of *Escherichia coli* K-12 are resistant to the gyrase inhibitor novobiocin. J Bacteriol 181, 2979-2983 (1999)
21) King EO, Ward MK, and Raney DE. Two simple media for the demonstration of pyocyanin and fluorescin. J Lab Clin Med 44, 301-307 (1954)
22) Sambrook J, Fritsch EF, and Maniatis T. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. 1989.
23) Smith AW, and Iglewski BH. Transformation of *Pseudomonas aeruginosa* by electroporation. Nucleic Acids Res 17, 10509 (1989)
24) Schweizer HP. Small broad-host-range gentamycin resistance gene cassette for site-specific insertion and deletion mutagenesis. BioTechniques 15, 831-834 (1993)
25) Miyake Y, Fujiwara S, Usui T, and Suginaka H. Simple method for measuring the antibiotic concentration required to kill adherent bacteria. Chemotherapy 38, 286-290 (1992)
26) Payne SH, and Ames BN. A procedure for rapid extraction and high-pressure liquid chromatographic separation of the nucleotides and other small molecules from bacterial cells. Anal Biochem 123, 151-161 (1982)
27) Takahashi K, Kasai K, and Ochi K. Identification of the bacterial alarmone guanosine 5'-diphosphate 3'-diphosphate (ppGpp) in plants. Proc Natl Acad Sci U S A 101, 4320-4324 (2004)
28) Filip C, Fletcher G, Wulff JL, and Earhart CF. Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium-lauryl sarcosinate. J Bacteriol 115, 717-22 (1973)
29) Schwyn B, and Neilands JB. Universal chemical assay for the detection and determination of siderophores. Anal Biochem 160, 47-56 (1987)
30) White R, Friedrich L, Burgess D, Warkentin D, and Bosso J. Comparative in vitro pharmacodynamics of imipenem and meropenem against *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 40, 904-908 (1996)
31) Fung-Tomc JC, Gradelski E, Valera L, Kolek B, and Bonner DP. Comparative killing rates of quinolones and

cell wall-active agents. Antimicrob Agents Chemother 44, 1377-1380 (2000)

32) Chang DE, Smalley DJ, and Conway T. Gene expression profiling of *Escherichia coli* growth transitions: an expanded stringent response model. Mol Microbiol 45, 289-306 (2001)

33) van Delden C, Comte R, and Bally AM. Stringent response activates quorum sensing and modulates cell density-dependent gene expression in *Pseudomonas aeruginosa*. J Bacteriol 183, 5376-5384 (2001)

34) Hirsch M, and Elliott T. Role of ppGpp in *rpoS* stationary-phase regulation in *Escherichia coli*. J Bacteriol 184, 5077-5087 (2002)

35) Murakami K, Ono T, Viducic D, Kayama S, Mori M, Hirota K, Nemoto K, and Miyake Y. Role for *rpoS* gene of *Pseudomonas aeruginosa* in antibiotic tolerance. FEMS Microbiol Lett (Article in press)

36) Moyed HS, and Broderick SH. *hipA*, a newly recognized gene of *Escherichia coli* K-12 that affects frequency of persistence after inhibition of murein synthesis. J Bacteriol 155, 768-775 (1983)

37) Garrett ES, Perlegas D, and Wozniak DJ. Negative control of flagellum synthesis in *Pseudomonas aeruginosa* is modulated by the alternative sigma factor AlgT (AlgU). J Bacteriol 181, 7401-7404 (1999)

38) Thompson LS, Webb JS, Rice SA, and Kjelleberg S. The alternative sigma factor RpoN regulates the quorum sensing gene *rhlI* in *Pseudomonas aeruginosa*. FEMS Microbiol Lett 220, 187-195 (2003)

39) Hassett DJ, Sokol PA, Howell ML, Ma JF, Schweizer HT, Ochsner U, and Vasil ML. Ferric uptake regulator (Fur) mutants of *Pseudomonas aeruginosa* demonstrate defective siderophore-mediated iron uptake, altered aerobic growth, and decreased superoxide dismutase and catalase activities. J Bacteriol 14, 3996-4003 (1996)

40) Kim EJ, Sabra W, and Zeng AP. Iron deficiency leads to inhibition of oxygen transfer and enhanced formation of virulence factors in cultures of *Pseudomonas aeruginosa* PAO1. Microbiology 149, 2627-34 (2003)

41) Stintzi A, Evans K, Meyer JM, and Poole K. Quorum-sensing and siderophore biosynthesis in *Pseudomonas aeruginosa*: *lasR/lasI* mutants exhibit reduced pyoverdine biosynthesis. FEMS Microbiol Lett 166, 341-345 (1998)

42) Chen CR, Malik M, Snyder M, and Drlica K. DNA gyrase and topoisomerase IV on the bacterial chromosome: quinolone-induced DNA cleavage. J Mol Biol 258, 627-37 (1996)

43) Brooun A, Liu S, and Lewis K. A dose-response study of antibiotic resistance in *Pseudomonas aeruginosa* biofilms. Antimicrob Agents Chemother 44, 640-646 (2002)

44) Tsuda M, Miyazaki H, and Nakazawa T. Genetic and physical mapping of genes involved in pyoverdin production in *Pseudomonas aeruginosa* PAO. J Bacteriol 177, 423-431 (1995)

45) Diarra MS, Lavoie MC, Jacques M, Darwish I, Dolence EK, Dolence JA, Ghosh A, Ghosh M, Miller MJ, and Malouin F. Species selectivity of new siderophore-drug conjugates that use specific iron uptake for entry into bacteria. Antimicrob Agents Chemother 40, 2610-2617 (1996)

46) Hennard C, Truong QC, Desnottes JF, Paris JM, Moreau NJ, and Abdallah MA. Synthesis and activities of pyoverdin-quinolone adducts: a prospective approach to a specific therapy against *Pseudomonas aeruginosa*. J Med Chem 44, 2139-2151 (2001)

47) LeVatte MA, and Sokol PA. Effects of sub-inhibitory concentrations of antibiotics on surface expression of ferripyochelin-binding protein in *Pseudomonas aeruginosa*. J Antimicrob Chemother 24, 881-895 (1989)

48) Kelly MT, and Hoover TR. Mutant forms of *Salmonella typhimurium* sigma54 defective in transcription initiation but not promoter binding activity. J Bacteriol 181, 3351-3357 (1999)