Effect of rpoS Mutation on the Stress Response and Expression of Virulence Factors in Pseudomonas aeruginosa

SANG-JIN SUH,1,2* LAURA SILO-SUH,2 DONALD E. WOODS,3 DANIEL J. HASSELT,4 SUSAN E. H. WEST,5 AND DENNIS E. OHMAN2

Department of Microbiology and Immunology, University of Tennessee, and Veterans Affairs Medical Center, Memphis, Tennessee 38104; Department of Microbiology and Immunology, Medical College of Virginia campus of Virginia Commonwealth University, and McGuire Veterans Affairs Medical Center, Richmond, Virginia 23298-0678; Department of Microbiology and Infectious Diseases, University of Calgary, Calgary, Alberta, Canada T2N 4N1; Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati College of Medicine, Cincinnati, Ohio 45257; and Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin—Madison, Madison, Wisconsin 53706

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The sigma factor RpoS (σS) has been described as a general stress response regulator that controls the expression of genes which confer increased resistance to various stresses in some gram-negative bacteria. To elucidate the role of RpoS in Pseudomonas aeruginosa physiology and pathogenesis, we constructed rpoS mutants in several strains of P. aeruginosa, including PA01. The PA01 rpoS mutant was subjected to various environmental stresses, and we compared the resistance phenotype of the mutant to that of the parent. The PA01 rpoS mutant was slightly more sensitive to carbon starvation than the wild-type strain, but this phenotype was obvious only when the cells were grown in a medium supplemented with glucose as the sole carbon source. In addition, the PA01 rpoS mutant was hypersensitive to heat shock at 50°C, increased osmolarity, and prolonged exposure to high concentrations of H2O2. In accordance with the hypersensitivity to H2O2, catalase production was 60% lower in the rpoS mutant than in the parent strain. We also assessed the role of RpoS in the production of several exoproducts known to be important for virulence of P. aeruginosa. The rpoS mutant produced 50% less exotoxin A, but it produced only slightly smaller amounts of elastase and LasA protease than the parent strain. The levels of phospholipase C and casein-degrading proteases were unaffected by a mutation in rpoS in PA01. The rpoS mutation resulted in the increased production of the phenazine antibiotic pyocyan and the siderophore pyoverdine. This increased pyocyanin production may be responsible for the enhanced virulence of the PA01 rpoS mutant that was observed in a rat chronic-lung-infection model. In addition, the rpoS mutant displayed an altered twitching-motility phenotype, suggesting that the colonization factors, type IV fimbriae, were affected. Finally, in an alginate-overproducing cystic fibrosis (CF) isolate, FRD1, the rpoS101::aacC1 mutation almost completely abolished the production of alginate when the bacterium was grown in a liquid medium. On a solid medium, the FRD1 rpoS mutant produced approximately 70% less alginate than the wild-type strain. Thus, our data indicate that although some of the functions of RpoS in P. aeruginosa physiology are similar to RpoS functions in other gram-negative bacteria, it also has some functions unique to this bacterium.

Microorganisms continuously sense and respond to environmental stimuli, such as starvation, desiccation, osmotic stress, oxidative stress, and changes in temperature. In many instances, an appropriate response to an environmental stimulus requires changes in the levels of specific gene products. One way to accomplish this is by modulating gene expression through the replacement of the main sigma factor of RNA polymerase with an alternative sigma factor that recognizes a specific promoter of the stimulus response gene (19, 39). The sigma factor RpoS (σS) was originally identified in Escherichia coli and Salmonella typhimurium as an alternative sigma factor that activates the expression of numerous genes required to maintain cell viability during the stationary phase of growth when cells are experiencing nutrient starvation (for reviews, see references 30 and 38). Activation of these genes makes the bacterium more resistant to environmental stresses, such as prolonged starvation, osmotic stress, and oxidative stress. RpoS also plays an important role in exponentially growing cells that are exposed to increased osmolarity (22). In E. coli and S. typhimurium, RpoS induces the expression of more than 30 genes in response to various stresses (33, 42). RpoS also activates the genes necessary for virulence in several bacteria, including E. coli, S. typhimurium, and Shigella flexneri (3, 31, 48, 55). Among pseudomonads, the role of RpoS as a general stress response regulator has been described for Pseudomonas putida (43, 51) and Pseudomonas fluorescens (52). In P. fluorescens, RpoS also modulates the production of several antibiotics and affects the biological control activity of the bacterium. Pseudomonas aeruginosa is a gram-negative bacterium that is found in various environmental niches, including soil, water, plants, hospital environments, and human infections. As an opportunistic pathogen, this bacterium primarily infects patients who are immunocompromised (11). During infection, P. aeruginosa must survive major environmental changes, including changes in temperature, pH, ionic strength, and the presence of reactive oxygen intermediates and antibodies. With the exception of several genes that have been identified as being involved in the oxidative stress response, such as katA, katB,
sodA, sodB, and alginate genes (6, 8, 21, 40, 41, 62), little is known about the stress responses of _P. aeruginosa_. However, some information regarding the stress responses of this bacterium can be obtained from studies on the regulation of its various exoproducts. _P. aeruginosa_ secretes a battery of extracellular products that are important for virulence: exotoxin A, elastase, LasA protease, phospholipase C, alkaline protease, rhamnolipid, pyocyanin, and alginate. Many of these toxic exoproducts are involved in procuring nutrients and/or protecting the bacterium from the host immune system (14). Furthermore, some of these exoproducts are synthesized in response to various stresses that are limiting, respectively. Thus, the synthesis of some of these exoproducts may be regulated by various stresses that are not well understood. In this study, we present evidence that RpoS mediates the general stress response and modulates synthesis of several exoproducts, including some secondary metabolites.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and media.** Bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were grown in L broth (LB) or LB supplemented with appropriate antibiotics at 37°C with aeration, unless otherwise indicated. For minimal salts medium, no-carbon–E minimal medium (NCE) (12) supplemented with either glucose (10 mM) or succinate (20 mM) as a carbon source was used. For exotoxin A assays, iron-depleted Trypticase soy broth dialysate was used as previously described (47). For phospholipase C assays and pyocyanin extractions, 1% peptone broth supplemented with 1% glycerol and dialysate was used as previously described (47). For phospholipase C assays and pyocyanin extractions, 1% peptone broth supplemented with 1% glycerol and 1% NaCl was used as the growth medium. For pyoverdin assays, _P. aeruginosa_ was grown in King B medium (28). Following conjugations, pseudomonas isolation agar was used to counterselect against _E. coli_. Media were solidified with 1.5% Bacto Agar (Difco). The following antibiotic concentrations (per mL) were used: ampicillin, 100 μg for _E. coli_; carbenicillin, 300 μg for _P. aeruginosa_; gentamicin, 20 μg for _E. coli_ and 500 μg for _P. aeruginosa_; kanamycin, 50 μg for _E. coli_. Antibiotics were purchased from Sigma (St. Louis, Mo.).

**DNA manipulations, transformations, and conjugations.** _E. coli_ DH10B or HB101 was routinely used as a host strain for cloning. Plasmids were purified with QIAprep spin miniprep columns (Qiagen), according to the manufacturer's instructions. DNA was introduced into _E. coli_ or _P. aeruginosa_ by electroporation or by conjugation. A standard electroporation procedure was used for _E. coli_ with the _E. coli_ gene pulser (Bio-Rad, Hercules, Calif.). For _P. aeruginosa_, electrocompetent cells were prepared as follows. Cells were grown to mid-log phase (optical density at 600 nm [OD_600]) of 0.5 to 0.7) in 100 mL of LB and harvested by centrifugation. Following two washes with 10 mL of 0.3 M sucrose, the cells were resuspended in 1 mL of 0.3 M sucrose, and 40 μL of this cell suspension was used for transformation. The conditions used to electroporate _P. aeruginosa_ were 200 V, 200 μF, and 1.6 kV. For conjugations, triparental matings were performed with the helper plasmid pRK2013 as previously described (17), with the following modifications. Briefly, 50 to 100 μL of overnight cultures of donor, helper, and recipient strains were mixed in 1 mL of saline, centrifuged, washed with 1 mL of saline, and resuspended in 50 μL of saline. The cell suspension was spotted on an LB plate and incubated overnight at 30°C. Following incubation, the cells were scraped with a sterile cotton swab, resuspended in 2 mL of saline, and plated on selective plates. Introduction of plasmid DNA into _P. aeruginosa_ FRD1 was done exclusively by conjugation.

**Construction of _P. aeruginosa_ rpoS mutants.** To generate _P. aeruginosa_ rpoS mutants, the suicide plasmid pSS1, which carries the _rpoS1::aacCI_ null allele, was constructed. Briefly, pDB18R, which carries _P. aeruginosa_ rpoS on a 1.8-kb KpnI-HindIII fragment, was digested with SphiI to cut once within _rpoS_. Following T4 DNA polymerase digestion to blunt the protruding ends, the linearized plasmid was digested with HinflII to delete approximately 420 nucleotides within the _rpoS_ gene. The _aacCI_ gene, which encodes gentamicin resistance (Gm^r^) and which was obtained from pUCGMI as a SmalI fragment, was inserted to generate the _rpoS1::aacCI_ allele. pSS1 was introduced into _P. aeruginosa_ PA01 and PA103 by electroporation, and the potential _rpoS_ mutants were isolated as having Gm^r^.
pSS1

P. aeruginosa chromosome

FIG. 1. Construction of P. aeruginosa rpoS101::aacCI (Gm²) mutants. To generate a null allele of the P. aeruginosa rpoS gene, approximately 420 bp of the rpoS coding sequence on a 1.8-kb fragment was replaced with the aacCI gene, which encodes Gm², to form pSS1. The wild-type rpoS was replaced with the rpoS101::aacCI allele carried on the suicide plasmid pSS1, as described in Materials and Methods, to generate P. aeruginosa rpoS mutants.

Alginate assays. To quantitate the amount of alginic acid produced when it was grown in liquid medium, cells were grown in LB for 20 to 24 h at 37°C with aeration. Alginate from the culture supernatant was separated from the cells by centrifugation (17,000 × g for 10 min) and dialyzed at 4°C against deionized water for 24 h with three changes of water. Dialysis tubing with a molecular weight cutoff of 10,000 (Spectrum, Houston, Tex.) was used. To quantitate the alginic acid produced on solid medium, overnight cultures of FRD1 or SS138 were serially diluted in saline (0.85% NaCl), and 0.1 ml of diluted cultures was plated on LB plates. The plates were incubated at 37°C for 25 to 30 h; alginic acid was collected by scraping the cells and alginic acid, following centrifugation to separate the alginic from the cells. The alginic thus collected was subjected to 24 h of dialysis at 4°C against three changes of deionized water. The amount of alginic acid present in the samples was quantitated by the carbazole method. Rat chronic-lung-infection assay. The rat chronic-lung-infection assay was performed as previously described (60). Briefly, agar beads embedded with approximately 3 × 10⁸ cells of either the wild-type bacteria or the rpoS mutant were placed in the left lungs of rats. The lungs of three animals from each group were isolated at days 7 and 14 for bacterial quantification. The lungs of two additional animals from each group were isolated at the same times for histopathological examination as previously described (56). Briefly, sagittal slices of the entire left lobes of the fixed lungs were dehydrated in graded alcohols, embedded in paraffin, and cut into 6-μm-thick sections. Mounted sections were stained with hematoxylin and eosin. Infiltration of the lung by inflammatory cells and exudate was measured microscopically with a Zeiss integrating eyepiece. The number of points overlaying the surface area of the infiltrate was divided by the total number of points counted over the entire surface area of the left lobe to measure the percent infiltration. This procedure was repeated with left lobe slices from each animal’s lung.

RESULTS

Construction of P. aeruginosa rpoS mutants. P. aeruginosa rpoS mutants were isolated in order to better understand the potential role of RpoS (σ²) in P. aeruginosa physiology and pathogenesis. Figure 1 shows the schematic diagram for constructing the suicide plasmid, pSS1, which carries the null P. aeruginosa rpoS101::aacCI allele, and the subsequent generation of P. aeruginosa rpoS mutants with pSS1. We generated a
null allele of rpoS by deleting the 420-bp HincII-SphI fragment of the rpoS coding sequence and replacing it with an 820-bp SmalI fragment that confers the Gm’ encoded by aacCI. We introduced pSS1 into P. aeruginosa and isolated colonies that had undergone the allelic exchange reaction replacing the wild-type rpoS with the rpoS101::aacCI allele. The inheritance of the rpoS101::aacCI allele in P. aeruginosa was confirmed by Southern blot hybridization analysis and/or PCR analysis (data not shown). The rpoS mutants of PAO1, PA103, and FRD1 were designated SS24, SS6, and SS138, respectively (Table 1).

Response of rpoS mutants to environmental stresses. To determine whether RpoS confers cross-protection for P. aeruginosa against various stresses, the resistance of the parent (PAO1) and rpoS mutant (SS24) against prolonged starvation, heat shock, increased osmolarity, and \( \text{H}_2\text{O}_2 \) was determined. To study its response to prolonged carbon starvation, we grew P. aeruginosa on three different growth media to determine whether growth on a specific carbon source affected the survival of P. aeruginosa. The media used were minimal medium with glucose, minimal medium with succinate, and LB. For P. aeruginosa, glucose is a less preferred carbon source than succinate. There was no difference in growth rate between the parent and rpoS mutant in each of three growth media (data not shown). When bacteria were grown with glucose as the sole carbon source, we observed increased sensitivity by the rpoS mutant to prolonged starvation (Fig. 2A). However, the difference in survival between the parent and the rpoS mutant was not as pronounced as that observed in other bacteria. For example, an E. coli rpoS mutant is almost 50-fold more sensitive to starvation than the parent strain after 9 days of incubation (33). In P. aeruginosa, we consistently observed only a four- to eightfold difference between the parent and rpoS mutant after 12 to 14 days of incubation. In contrast, when bacteria were grown with succinate as a sole carbon source (Fig. 2B) or in LB (Fig. 2C), we observed little difference in survival between the rpoS mutant and the parent. The most striking difference observed among the three growth conditions was the relative resistance of P. aeruginosa to prolonged starvation when the cells were grown with glucose as the carbon source. When cells were grown on glucose as the sole carbon source, approximately 12% of the parent cells and 4% of the rpoS mutant cells were viable after 15 days of starvation. In contrast, when cells were grown on succinate, approximately 1% of the parent cells and 0.8% of the rpoS mutant cells were viable after 13 days of incubation. When cells were grown in LB, no viable cells were recovered after 10 to 16 days of continued incubation (data not shown). Thus, P. aeruginosa is sensitive to prolonged starvation, and RpoS had little effect in protecting the bacterium from prolonged starvation. In addition, the growth of P. aeruginosa on glucose appeared to decrease the sensitivity of the bacterium to prolonged starvation. Although only three substrates were tested, it appeared that survival of P. aeruginosa was enhanced when a poorer substrate was used.

When stationary-phase cultures of the parent (PAO1) and the rpoS mutant (SS24) were exposed to a sudden shift in temperature from 24° to 50°C, the rpoS mutant was more sensitive to heat shock at 50°C. After 8 min of incubation at 50°C, there was a 50-fold difference in survival between the
parent and the rpoS mutant (Fig. 2D). When the cells were subjected to an increase in osmotic pressure caused by the addition of a high concentration of salt, the rpoS mutant was more sensitive to a 2 M concentration of sodium chloride (Fig. 2E). After 6 h of exposure, there was approximately a 37-fold difference in relative viability between the parent and the rpoS mutant. Thus, RpoS was essential for resistance against osmotic stress caused by exposure to salt. Against nonionic osmotic stress, the wild type was only slightly more resistant (an approximately threefold difference) after 6 h of exposure) to 2 M sucrose than the rpoS mutant (data not shown). This indicated that the role of RpoS in protecting P. aeruginosa from nonionic osmotic stress may be small.

We also assayed whether RpoS mediated protection against H$_2$O$_2$ in P. aeruginosa (Table 2). In exponentially growing cells, there was no difference in sensitivity to H$_2$O$_2$ between the parent and the rpoS mutant. However, when stationary-phase cells were tested, the zone of inhibition caused by H$_2$O$_2$ was approximately 20% larger for the rpoS parent versus 5,809 $\pm$ 0.6% for the rpoS mutant. This suggested that RpoS modulates the level of pyocyanin and pyoverdine, the primary siderophore produced by P. aeruginosa, in culture supernatant

Effect of rpoS mutation on production of exprodcts. P. aeruginosa produces and secretes various toxins, proteases, and secondary metabolites that contribute to the pathogenesis of the bacterium. We investigated whether RpoS could influence the production and secretion of exotoxin A, elastase, LasA protease, casein-degrading proteases, phospholipase C, pyocyanin, and pyoverdine. The data are shown in Table 3.

Exotoxin A is an ADP-ribosyltransferase that inhibits eu-karyotic protein synthesis (36). Exotoxin A activity in the culture supernatants was decreased by 50% in the PAO1 rpoS mutant (SS24). In the exotoxin A-overproducing PA103 and its respective rpoS mutant (SS6), the RpoS$^\Delta$ defect resulted in a 70% reduction in exotoxin A activity (18,502 $\pm$ 1.276 for the parent versus 5,809 $\pm$ 407 for the rpoS mutant). We also determined the effect of the rpoS mutation on the extracellular accumulation of proteases and phospholipase C in the PAO1 background. Culture supernatants of the PAO1 rpoS mutant consistently had 20% less elastase activity and 10% less LasA protease activity. The decreased accumulation of these extracellular proteins in the culture supernatant of rpoS mutant was fully restored to the level of the parental strain when RpoS was provided in trans from a plasmid (data not shown). A measure of casein degradation detects many proteases produced by the bacterium (i.e., elastase and alkaline protease). The difference in the production of casein-degrading proteases between the parent strain and the rpoS mutant was negligible (data not shown). Similarly, little difference in the production and secretion of total phospholipase C (Table 3) or hemolytic phospholipase C (data not shown) between the parent and the rpoS mutant was observed. Based on these data, we concluded that RpoS was required for the maximum production of exotoxin A but that it was not required for the maximum production of casein-degrading proteases or phospholipase C.

Pyocyanin and pyoverdine are secondary metabolites produced by P. aeruginosa. Pyocyanin is a blue compound that plays an important role in P. aeruginosa pathogenesis (59). Pyocyanin kills bacteria and inhibits lymphocyte proliferation and ciliary function. The color of P. aeruginosa rpoS mutant culture supernatants was an intense blue relative to parent supernatants from the rpoS mutants were producing and secreting more pyocyanin. Measurements showed that culture supernatants from the rpoS mutant contained twice as much pyocyanin as the parent strain PAO1. Conversely, the overproduction of RpoS from a high-copy-number plasmid in PAO1 resulted in a decrease in pyocyanin levels in culture supernatants (data not shown). Thus, RpoS was required for the maximum production of pyocyanin.

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Effect of rpoS mutation on alginate production. Alginate is an exopolysaccharide that is overproduced by most of the P. aeruginosa strains isolated from CF patients suffering from chronic lung infections (18). The overproduction of alginate increases the persistence of the bacterium in the CF lung. To determine whether RpoS is involved in the production of alginate, we examined SS138, the rpoS101::lacI mutant of FRD1 (an alginate-overproducing CF isolate). As shown in Fig. 3, when the cells were grown in a liquid medium, alginate production was almost completely abolished in the FRD1 rpoS mutant. In contrast, when grown on plates (seeded with 10$^2$ CFU/plate), the rpoS mutant produced approximately 35% of the alginate produced by the wild-type strain. Interestingly, when agar plates were seeded with a high cell density (i.e., $\geq 10^5$ CFU), the rpoS mutant accumulated as much alginate as...
the wild-type FRD1 (data not shown). Because FRD1 and the rpoS mutant grew at similar rates (data not shown), the difference in alginate production could not be attributed to a growth defect. Thus, RpoS, in combination with cell density, was required for maximum alginate synthesis in *P. aeruginosa*. Providing RpoS in trans on a multicopy plasmid restored the alginate production of the rpoS mutant to that of the parental strain FRD1 (data not shown).

**Effect of rpoS mutation on the rat chronic-lung-infection model.** We assessed the role of RpoS in the virulence of *P. aeruginosa* using the rat chronic-lung-infection model. In this model, the bacteria are embedded in agar beads which protect them from the host immune response (60). The results showed that the rpoS mutant survived as well as the wild-type bacteria when the mutant was embedded in agar beads and placed in rat lungs. Interestingly, the rpoS mutant appeared to cause greater damage to lung tissues than the wild-type strain (Table 4). The increased damage inflicted on the lungs of rats by the rpoS mutant may be due to the overproduction of pyocyanin.

Although the rat chronic-lung-infection model is a good model for assessing the roles of extracellular products in infection, it does not provide information about colonization functions and other initial stages of infection. The colonization of *P. aeruginosa* is mediated by type IV fimbriae (13), which also mediate the twitching motility. We examined the twitching motility of the rpoS mutant to determine whether RpoS may be involved in *P. aeruginosa* colonization. As shown in Fig. 4, the zone of motility of the rpoS mutant (SS24) was approximately 60 to 70% of that of the parent (PAO1). In addition, the shape of the zone observed for the rpoS mutant was typically elliptical, rather than the circular shape that was observed for the parent strain. Finally, the rpoS mutant demonstrated altered swarming motility on the top of the agar. These data suggest that RpoS may be involved in the colonization of *P. aeruginosa* as mediated by the type IV fimbriae.

**DISCUSSION**

The ability of *P. aeruginosa* to resist, adapt, and survive in a wide variety of environments makes it a ubiquitous bacterium in nature and likely contributes to its opportunistic pathogenic behavior. As an initial effort to elucidate and understand the stress responses of this bacterium, we isolated and characterized *P. aeruginosa* rpoS mutants to determine the effect of an RpoS+ defect on the stress responses and the synthesis of virulence factors in this bacterium.

Our data demonstrate both similarities and differences between the role of RpoS in *P. aeruginosa* and its role in other bacteria. Some of the similarities include the RpoS-mediated resistance of *P. aeruginosa* to heat-shock, H$_2$O$_2$, and osmotic stress. These data indicate that RpoS is a general stress response regulator in *P. aeruginosa*. However, because the physiology and metabolism of *P. aeruginosa* are different from those of *E. coli*, we do not know whether RpoS-mediated resistance to various stresses in *P. aeruginosa* is similar or even proceeds via analogous genes. For example, the *E. coli* rpoS mutant is hypersensitive to 15 mM H$_2$O$_2$ (33), while the *P. aeruginosa* rpoS mutant is resistant to 100 mM H$_2$O$_2$ (data not shown), despite reduced catalase production in the *P. aeruginosa* rpoS mutant. In *E. coli*, RpoS regulates the expression of two catalase biosynthetic genes, katE and katG, in order to detoxify

**TABLE 4. Effect of rpoS mutation on chronic lung infection caused by *P. aeruginosa***

<table>
<thead>
<tr>
<th>Parent or mutant</th>
<th>Initial inoculum (CFU/ml)</th>
<th>Quantitative bacteriology (CFU/ml) on day $^a$:</th>
<th>Quantitative pathology (% damage) on day $^c$:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>14</td>
</tr>
<tr>
<td>RpoS+ (PAO1)</td>
<td>$3.5 \times 10^4$</td>
<td>$(4.4 \pm 4.3) \times 10^5$</td>
<td>$(1.3 \pm 1.2) \times 10^5$</td>
</tr>
<tr>
<td>RpoS- (SS24)</td>
<td>$2.8 \times 10^4$</td>
<td>$(2.3 \pm 2.2) \times 10^5$</td>
<td>$1.2 \times 10^6 \pm 9.2 \times 10^5$</td>
</tr>
</tbody>
</table>

$^a$ Values are means ± standard deviations.
$^b$ Infiltration index.
$^c$ Significantly different from RpoS+ value ($P < 0.05$) by Student’s *t* test.
Alginate production may be cell density dependent. Our data show a higher cell density can be reached. Thus, RpoS-mediated O-acetylation of alginate under low-cell-density conditions when the expression of rpoS itself is induced by high cell density? Perhaps the basal level of rpoS expressed in FRD1 at a low cell density may be sufficient for the induction of alginate production and/or secretion. Alternatively, due to the close proximity of the cells growing on a solid surface, a few cells may be sufficient to induce the quorum-sensing system.

In conclusion, the mutation in rpoS affects a variety of functions in P. aeruginosa. These include the general stress response, the accumulation of virulence factors, and twitching motility. Our data suggest that one of the functions of RpoS in P. aeruginosa is to fine-tune the cell metabolism, which includes modulating the production of several secondary metabolites, in order to maintain the optimal conditions for cells in stationary phase. The two-dimensional gel analysis on PAO1 and SS24 indicated that the rpoS mutation results in an alteration in the production and accumulation of at least 37 proteins (data not shown). Studies to identify the genes regulated by RpoS in P. aeruginosa are in progress.

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