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Elevated Mutation Frequency in Surviving Populations of Carbon-Starved *rpoS*-Deficient *Pseudomonas putida* Is Caused by Reduced Expression of Superoxide Dismutase and Catalase▽

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RpoS is a bacterial sigma factor of RNA polymerase which is involved in the expression of a large number of genes to facilitate survival under starvation conditions and other stresses. The results of our study demonstrate that the frequency of emergence of base substitution mutants is significantly increased in long-term-starved populations of *rpoS*-deficient *Pseudomonas putida* cells. The increasing effect of the lack of RpoS on the mutation frequency became apparent in both a plasmid-based test system measuring Phe$^+$ reversion and a chromosomal *rpoB* system detecting rifampin-resistant mutants. The elevated mutation frequency coincided with the death of about 95% of the cells in a population of *rpoS*-deficient *P. putida*. Artificial overexpression of superoxide dismutase or catalase in the *rpoS*-deficient strain restored the survival of cells and resulted in a decline in the mutation frequency. This indicated that, compared to wild-type bacteria, *rpoS*-deficient cells are less protected against damage caused by reactive oxygen species. 7,8-Dihydro-8-oxoguanine (GO) is known to be one of the most stable and frequent base modifications caused by oxygen radical attack on DNA. However, the spectrum of base substitution mutations characterized in *rpoS*-deficient *P. putida* was different from that in bacteria lacking the GO repair system: it was broader and more similar to that identified in the wild-type strain. Interestingly, the formation of large deletions was also accompanied by a lack of RpoS. Thus, the accumulation of DNA damage other than GO elevates the frequency of mutation in these bacteria. It is known that oxidative damage of proteins and membrane components, but not that of DNA, is a major reason for the death of cells. Since the increased mutation frequency was associated with a decline in the viability of bacteria, we suppose that the elevation of the mutation frequency in the surviving population of carbon-starved *rpoS*-deficient *P. putida* may be caused both by oxidative damage of DNA and enzymes involved in DNA replication and repair fidelity.

Accumulation of reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, and hydroxyl radicals leads to nucleic acid, protein, and cell membrane damage. ROS have been implicated in cancer, aging, and various diseases in humans but also in the death of microorganisms (53). Many microorganisms are continuously faced with ROS derived from different sources. For example, during infection, pathogenic bacteria are exposed to the exogenous oxidative stress that phagocytes use as a host defense mechanism (25, 47). Additionally, ROS are constantly generated as by-products of aerobic metabolism. To counteract oxidative stress, both prokaryotic and eukaryotic cells maintain inducible defense systems to detoxify oxidants and repair damage (14, 32). Gram-negative bacteria commonly synthesize both cytoplasmic and periplasmic isozymes of superoxide dismutases (SOD) to eliminate superoxide anions (40). Hydrogen peroxide is scavenged in most organisms by peroxidases and catalases (13, 32). Bacteria use distinct sensing mechanisms for the detection of discrete forms of oxidative stress. For instance, the SoxRS system is a regulator of superoxide stress whereas the regulators OxyR and PerR respond to hydrogen peroxide stress (32).

RpoS is a sigma subunit of RNA polymerase that is involved in the induction of a large number of genes when the environment becomes unable to sustain the growth of bacteria. The *rpoS* gene (also named *katF*) was initially characterized as a locus which affects the synthesis of catalase (38). Later it was shown that RpoS is a central regulator of the general stress response in *Escherichia coli*, where it controls the expression of at least 100 genes and is involved in cross-protection against osmotic, acidic, and oxidative stress (27, 78). RpoS-deficient cells of *E. coli* are less protected against oxidative or osmotic stress and are less viable in the stationary phase (35, 42).

Several lines of evidence indicate that RpoS positively regulates many mutagenic processes in growth-limiting environments of bacteria (reviewed in references 20 and 22). RpoS enhances spontaneous mutations by upregulating specialized DNA polymerase Pol IV in *E. coli* (36). Pol IV is responsible for 50 to 80% of the Lac$^+$ revertants that occur because of 1-bp deletions in runs of iterated bases in the F′ episome in *E. coli* strain FC40 (19, 43). The emergence of Lac$^+$ revertants is reduced about 10-fold in the absence of RpoS (36, 39). Additionally, RpoS controls a switch that changes normally error-free double-strand break repair into an error-prone process under stress (60). RpoS is also required for base substitution mutations in aging *E. coli* colonies (8). Some evidence indi-

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cates that downregulation of mismatch repair proteins MutS and MutH by RpoS (17, 75) increases mutation rates in starving *E. coli* (8, 21, 24, 79). Phase variation in *Pseudomonas* sp. strain PCL1171 is also dependent on RpoS (76).

We have studied the mechanisms of mutagenic processes in bacteria by using *Pseudomonas putida* as a model organism. The genus *Pseudomonas* represents one of the largest groups of bacteria including both pathogenic and nonpathogenic species. Bacteria of this genus are known for their abilities to colonize multiple habitats and to adapt rapidly to new environments. *P. putida* is a fast-growing bacterium found in most temperate soil and water habitats where oxygen is present. It is also able to colonize surfaces of living organisms. We have found that RpoS can act as a positive regulator in the transposition of Tn*3* family transposon Tn*4652* in *P. putida* (31). Additionally, the occurrence of 2- to 3-bp deletions, characteristic of long-term starvation, was not detected in an RpoS$^-$ background (66). RpoS also promotes genome instability in other *Pseudomonas* species (76).

In the present study, we focused on the role of RpoS in the occurrence of point mutations in starving *P. putida*. The results presented herein demonstrate a negative role for RpoS in the emergence of base substitutions in this organism. We found that the formation of base substitutions, estimated with both plasmid-based and chromosomal test systems, was significantly elevated in *rpoS*-deficient *P. putida* after 5 to 7 days of carbon starvation. The increased mutation frequency in a starving population coincided with the rapid death of the majority of the cells. Suppression of the effects of the lack of RpoS by overexpression of SOD or catalase indicated that damage caused by ROS reduces survival and elevates the mutation frequency in a starving *rpoS*-deficient strain.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are described in Table 1. Complete medium was Luria-Bertani medium (46), and minimal medium was M9 (1). Solid medium contained 1.5% Difco agar. Casamino Acids and glucose were added to the minimal medium at final concentrations of 0.4 and 0.2%, respectively. Phenol minimal plates contained 2.5 mM phenol as the sole carbon and energy source. Antibiotics were added at the following concentrations: ampicillin at 100 μg/ml, kanamycin at 50 μg/ml, tetracycline at 50 μg/ml, chloramphenicol at 1,500 to 3,000 μg/ml, streptomycin at 500 μg/ml, rifampin at 100 μg/ml, and carbenicillin at 1,500 to 3,000 μg/ml. Potassium tellurite was added at a final concentration of 25 μg/ml. *E. coli* was incubated at 37°C, and *P. putida* was incubated at 30°C. *E. coli* and *P. putida* were electrotransformed as described by Sharma and Schimke (68). *E. coli* strains DH5α (Invitrogen) and CC118 λpir (28) were used for DNA cloning procedures, and HB101 (10) was used as a host for helper plasmid pRK2013 (18), which was necessary for the mobilization of nonconjugative plasmids. CC118 λpir was also used as a host for the R6K replicon-based helper plasmid providing the Tn*7* transposase proteins (6).

Measurement of intracellular amounts of superoxide and SOD activities and detection of catalase expression in bacteria. In order to measure the amount of superoxide radicals (SO) in carbon-starved *P. putida* cells, wild-type (WT) strain PaW85 and its *rpoS*-deficient derivative were grown overnight in M9 minimal medium supplemented with glucose and Casamino Acids. Approximately 1×10^9 cells were spread onto minimal plates and incubated under carbon starvation conditions. Cells from three agar plates were resuspended in 1.5 ml of M9 buffer at the beginning of starvation (on day 2) and after prolonged starvation (on day 8). Cell suspensions were divided into two equal amounts (each part was 750 μl). In the first half of the suspension, the cells were disrupted by sonication and the cell lysate (lysate I) cleared by centrifugation at 16,000 × *g* for 25 min at 4°C was used for measurement of the total amount of SO. The second half of the cell suspension was centrifuged at 12,000 × *g* for 3 min, and the supernatant (supernatant I) was collected for the measurement of SO. Cells from the bottom of an Eppendorf tube were resuspended in 750 μl of M9 buffer and harvested by centrifugation to obtain supernatant II for the measurement of SO. Finally, after the removal of supernatant II, the cells were resuspended in 750 μl of M9 buffer, disrupted by sonication, and cleared by centrifugation at 16,000 × *g* for 25 min at 4°C, yielding lysate II for the measurement of SO. Hydroethidine was added to cell lysates I and II or collected supernatants I and II at a final concentration of 63.5 μM. The reaction mixtures were incubated for 30 min at 30°C in the dark. The amount of superoxide in the reaction mixtures was expressed as hydroethidine dehydrogenation to ethidium bromide (EB) by superoxide anion. The quantity of EB generated was determined by the intensity of EB fluorescence measured with a computerized fluorometer Tecan Genios-Plus at 485-nm excitation and 595-nm emission wavelengths. The amount of SO was calculated either per plate or as the relative amount of SO per 1×10^6 CFU in a 1-ml reaction mixture. All measurements were repeated at least four times in triplicate.

To measure SOD activity in stationary-phase *P. putida* cells, cells starved for 8 days were resuspended and sonicated in phosphate buffer (100 mM Na_2HPO_4/KH_2PO_4 [pH 7]). SOD activity was measured in cell lysates by using the SOD Assay Kit-WST (Fluka) according to the protocol provided by the manufacturer. Still, the reaction was performed at 30°C, which is the optimal growth temperature of *P. putida*. SOD activity was expressed as the percent inhibition of tetrazolium salt reduction to formazan dye (absorbance at 440 nm) by superoxide anion. The percent formazan formation per 1.0 μg of total protein was measured.

The expression of catalase was monitored by determining the formation of O_2 bubbles in a cell suspension after the addition of an H_2O_2 solution. A 100-μl volume of overnight-grown bacterial cultures was diluted into 2 ml of M9 buffer, and 20 μl of 30% H_2O_2 was added to detect the catalase reaction. The reaction tubes were photographed after 1 min of incubation at room temperature.

Construction of *P. putida* strains overexpressing SOD or catalase. In order to overexpress SOD in *P. putida* (strain PaWRpoSSodAB), we cloned the *sodA* and *sodB* genes of *P. putida* under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible P_{tac} promoter and the LacI repressor and introduced the *lacI*^{q}-P_{tac}-*sodAB* expression cassette into the bacterial chromosome by using mini-Tn*5*-based plasmid pJMT6 (65). The genomic DNA of *P. putida* PaW85 was amplified with primers sodA1 and sodA2 to clone the *sodA* gene and with primers sodB1 and sodB2 to clone the *sodB* gene. The primers used for amplification are shown in Table 2, and the constructed plasmids are described in Table 1. First, the PCR product of the *sodB* gene was cloned into the EcoRV site of pBluescript KS(+) to obtain pKSsodB. Subsequently, the PCR product of the *sodA* gene was inserted into the SmaI site of pKSsodB, yielding plasmid pKSsodAB. To obtain the *lacI*^{q}-P_{tac}-*sodAB* expression cassette, the XbaI- and SalI-generated DNA fragment carrying the *sodAB* genes was cloned into the pBRlacItac vector, resulting in plasmid pBRlacItacsodAB. The expression cassette was then subcloned within a BamHI-cleaved DNA fragment into pUC18Not (resulting in plasmid pUC18NotlacItacsodAB) to gain NotI sites for the cloning of this cluster into pJMT6. The resulting plasmid, pUTtellacItacsodAB, which does not replicate in hosts other than *E. coli* strain CC118 λpir, was conjugatively transferred into the *P. putida* WT strain and its *rpoS*-deficient derivative by using helper plasmid pRK2013. Transconjugants carrying random insertions of mini-Tn*5* containing the *lacI*^{q}-P_{tac}-*sodAB* expression cassette in the *P. putida* chromosome were isolated.

To overexpress the catalase gene *katA* in *P. putida* (strain PaWRpoSKatA), the P_{tac}-*katA* transcriptional fusion was constructed and introduced into the *att*Tn*7* site of the *P. putida* chromosome by using a mini-Tn*7*-based integration system (34). To insert the gene for the LacI repressor into the *P. putida* chromosome, the Eco47III- and SmaI-generated DNA fragment containing the *lacI*^{q} gene from plasmid pREP4 was inserted into pUC18Not to obtain pUC18NotlacI. The latter construct was cleaved with NotI to subclone this gene into mini-Tn*5* delivery plasmid pUTmini-Tn*5* Cm, yielding pUTCmlacI. Finally, the *lacI*^{q} gene was introduced into the *P. putida* chromosome as a result of random insertions of mini-Tn*5* as described above. The *katA* gene-containing DNA region of plasmid pAM10.6 (57) was amplified with primers katA1 and katA2 (Table 2). The PCR product was cut with XbaI and NotI and inserted into XbaI- and NotI-cleaved pBluescript (KS) derivative pKStac containing the P_{tac} promoter, resulting in plasmid pKStackatA. Plasmid pKStac was obtained by cloning the P_{tac} promoter from plasmid pACtac (71) within the BamHI- and HindIII-cleaved DNA fragment into pBluescript KS(+) opened with the same restriction endonucleases. The P_{tac}-*katA* transcriptional fusion was cut from pKStackatA with restriction endonucleases ApaI and NotI and inserted into Tn*7*-based mini-Tn-carrying vector pBK-miniTn7-ΩSm1 (34) opened with the same enzymes, yielding plasmid pAKNtackatA. A strain overexpressing both SodAB and KatA was constructed by using strain PaWRpoSKatA as a recipient.

Transconjugants were tested for resistance to carbenicillin. Only clones that

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or construction	Source or reference
Strains		
E. coli		
DH5α	*supE44* Δ*lacU169* (ϕ80d*lacZ*ΔM15) *recA1 endA1 hsdR17 thi-1 gyrA96 relA1*	Invitrogen
HB101	*subE44 subF58 hsdS3*(r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	10
CC118 λpir	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA1* λ*pir* phage lysogen	28
P. putida		
PaW85	WT	7
PaWRpoS	PaW85, *rpoS*::*km* (the same as PKS54)	55
PaWRpoSLacI	PaWRpoS, *lacI*^{q} gene in chromosome	This study
PaWRpoSSodAB	PaWRpoS, *lacI*^{q}-P_{tac}-*sodAB* expression cassette in chromosome	This study
PaWRpoSKatA	PaWRpoS, P_{tac}-*katA* transcriptional fusion and *lacI*^{q} gene in chromosome	This study
PaWRpoSSodABKatA	PaWRpoS, *lacI*^{q}-P_{tac}-*sodAB* and P*tac-katA* expression cassettes in chromosome	This study
Plasmids		
pBluescript KS(+)	Cloning vector (Ap^r)	Stratagene
pUC18Not	Cloning vector (Ap^r)	28
pUTmini-Tn*5* Cm	Delivery plasmid for mini-Tn*5* Cm (Ap^r Cm^r)	65
pJMT6	Delivery plasmid for mini-Tn*5* Tel (Ap^r Tel^r)	65
pRK2013	Helper plasmid for conjugal transfer of mini-Tn-carrying plasmids (Km^r)	18
pBK-miniTn*7*-ΩSm1	pUC19-based delivery plasmid for mini-Tn*7*-ΩSm1 (Sm^r Ap^r)	34
pUX-BF13	R6K replicon-based helper plasmid providing Tn*7* transposase proteins (Ap^r Mob$^+$)	6
pAM10.6	Biodegradative plasmid carrying catalase gene *katA*	57
pBRlacItac	pBR322 carrying P_{tac} promoter and *lacI*^{q} gene for LacI repressor	55
pKSsodB	pBluescript KS (+) containing PCR-amplified *sodB* gene region inserted into EcoRV-opened vector plasmid	This study
pKSsodAB	PCR-amplified *sodA* gene in SmaI site of pKSsodB	This study
pBRlacItacsodAB	pBRlacItac with *sodAB* genes cloned from pKSsodAB within XbaI- and SalI-generated DNA fragment	This study
pUC18NotlacItacsodAB	pUC18Not containing *lacI*^{q}-P_{tac}-*sodAB* expression cassette cloned within BamHI-generated DNA fragment from pBRlacItacsodAB	This study
pUTtellacItacsodAB	mini-Tn*5* delivery plasmid pJMT6 containing *lacI*^{q}-P_{tac}-*sodAB* expression cassette cloned from pUC18NotlacItacsodAB in NotI site	This study
pACtac	pACYC184 containing P_{tac} promoter	71
pKStac	pBluescript KS(+) containing P_{tac} promoter cloned from plasmid pACtac within BamHI- and HindIII-generated DNA fragment	This study
pKStackatA	PCR-amplified *katA* gene from plasmid pAM10.6 inserted into XbaI- and NotI-cleaved pKStac	This study
pAKNtackatA	pBK-miniTn*7*-ΩSm1 containing P_{tac}-*katA* transcriptional fusion inserted into ApaI- and NotI-cleaved vector plasmid	This study
pREP4	LacI repressor-expressing plasmid	Qiagen
pUC18NotlacI	pUC18Not containing *lacI*^{q} gene from pREP4 cloned within Eco47III- and SmaI-generated fragment into SmaI site	This study
pUTCmlacI	*lacI*^{q} gene from pUC18NotlacI inserted within NotI fragment into mini-Tn*5* delivery plasmid pUTmini-Tn*5* Cm	This study
pKTpheA56+A	Test system for detection of Phe$^+$ revertants occurring due to 1-bp deletions	74
pKTpheA22TAG	Test system for detection of Phe$^+$ revertants occurring due to base substitutions	74

were sensitive to carbenicillin represent true transposition event and not integration of the whole plasmid. The desired strains were confirmed by PCR analysis. To study the phenotypic effects of the constructed strains, we examined several clones carrying the same transcriptional fusions which gave the reproducible effects. *P. putida* carrying only the *lacI*^{q} gene insertion into the chromosome was used as a reference when we compared the effects of overexpression of SOD or catalase on the survival and mutagenesis of *rpoS*-deficient bacteria.

Isolation and analysis of Phe$^+$ revertants and Rif^r mutants. The assay system we used to record the frequencies of different types of point mutations in *P. putida* was based on activation of the phenol monooxygenase gene *pheA*, enabling bacteria to utilize phenol as a growth substrate and to form colonies on selective plates. The reporter gene *pheA* was altered in RSF1010-derived tester plasmids either by +1 frameshift mutation or by introducing a TAG translational stop codon into the *pheA* gene to monitor the occurrence of 1-bp deletions or base substitutions, respectively (74). Conditions for the isolation of phenol-degrading Phe$^+$ revertants were the same as in our previous studies (67, 74). The viability of bacteria was determined on the same plates that were used for the isolation of Phe$^+$ revertants. An approximately 350-bp DNA region covering the area of the *pheA* gene containing potential reversion mutations was analyzed by DNA sequencing in the Phe$^+$ revertants as described previously (74). The frequency of spontaneous Rif^r mutations in independent growing cultures of the *P. putida* WT strain and its *rpoS*-deficient derivative was calculated per 1×10^9 cells by using the Lea-Coulson method of the median (37, 63). The same cultures were plated onto phenol minimal plates, and after 10 days of starvation, the cells were collected by resuspension from the agar surface and plated onto selective Luria-Bertani medium plates containing 100 μg/ml rifampin. The number of Rif^r colonies was determined after 24 h of incubation, and the median value of the frequency of mutations was determined in cells that survived the long-term starvation conditions. Data were analyzed by a program for statistical analyses (Statgraphics Centurion XV; Statpoint Inc.). Differences between average accumulation rates of Phe$^+$ mutants were analyzed with the Student *t* test. A chi-square test of independence was used to compare DNA sequencing results.

TABLE 2. Oligonucleotides used in this study

Purpose	Designation	Sequence and position[a]
Cloning of *P. putida sodA* gene	sodA1	5′-CGCTGCCAAGCCGGATGT-3′, complementary to positions −32 to −15 upstream of ATG start codon of *sodA*
	sodA2	5′-TTACTTCAGGGCTTCAAGGTA-3′, complementary to positions 610 to 627 downstream of ATG start codon of *sodA*
Cloning of *P. putida sodB* gene	sodB1	5′-CGGCCTTGCGCAAACCGC-3′, complementary to positions −28 to −45 upstream of ATG start codon of *sodB*
	sodB2	5′-TTAGGCCTTGAAGGTCTTGCC-3′, complementary to positions 547 to 591 downstream of ATG start codon of *sodB*
Cloning of *katA* gene from pAM10.6	katA1	5′-AATTTCTAGAAACGGGAGTTAATAGTATGAG-3′, complementary to positions −16 to + 5 relative to ATG start codon of *katA*
	katA2	5′-TTAAGCGGCCGCTTAAAGAAAACTTGGTAAACCT-3′, complementary to positions 1499 to 1520 downstream of ATG start codon of *katA*

[a] Restriction enzyme sites are underlined (XbaI site for katA1 and NotI site for katA2).

RESULTS

RpoS is involved in the avoidance of base substitution mutations in stationary-phase *P. putida* populations. In order to study the involvement of RpoS in the occurrence of point mutations in long-term-starved populations of *P. putida*, we used two tester plasmids, pKTpheA56+A for the detection of restoration of the reading frame of the phenol monooxygenase gene *pheA* by −1 frameshift and pKTpheA22TAG to estimate various base substitutions eliminating the TAG stop codon from the *pheA* gene (74). Phenol minimal plates were inspected for the emergence of phenol-utilizing (Phe^+) revertants each day, and the frequency of accumulation of Phe^+ revertants per CFU determined from the same phenol minimal plates was calculated (Fig. 1A). The frequency of accumulation of 1-bp deletion mutants did not differ between the WT strain and its *rpoS*-deficient derivative (Fig. 1B). At the same time, as shown in Fig. 2A, the emergence of base substitution mutants was approximately 1.5-fold higher in the *rpoS*-deficient *P. putida* strain than in the WT strain during the initial period of starvation (days 4 to 6). After the first week of starvation, the frequency of accumulation of the Phe^+ revertants increased in the *rpoS*-deficient *P. putida* strain and thereafter remained approximately up to seven times higher than that measured in the WT strain.

To examine how general the elevated rate of the generation of base substitutions in starving cells of the *rpoS*-deficient *P. putida* strain is, we compared the mutation frequencies in starving WT and *rpoS*-deficient *P. putida* by using a chromosomal *rpoB* gene encoding the β subunit of the RNA polymerase as a reporter. The results presented in Fig. 2B show that the median frequencies of Rif^r mutants in growing cultures of the WT strain and its *rpoS*-deficient strain were similar at 3.7×10^{-9} and 3.0×10^{-9}, respectively. After 10 days of starvation, the frequency of Rif^r mutants in the *rpoS*-deficient *P. putida* strain increased more than 50-fold. Thus, the use of both plasmid-based and chromosomal test systems revealed that the

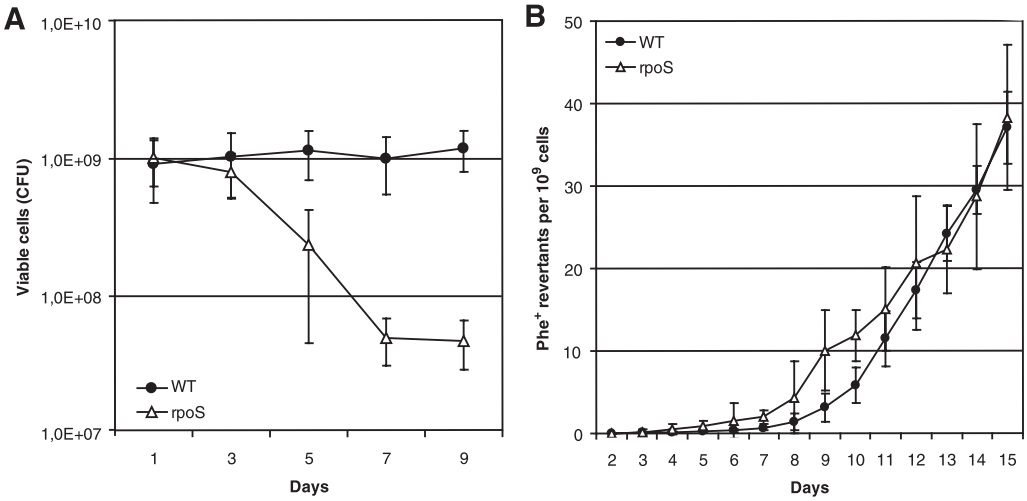

FIG. 1. Effects of RpoS on *P. putida* survival under carbon starvation conditions and frequency of accumulation of 1-bp deletions. (A) Viability of *P. putida* WT strain PaW85 and its *rpoS*-deficient derivative on phenol minimal plates. (B) Accumulation of Phe^+ revertants on phenol minimal plates in *P. putida* WT strain PaW85 and its *rpoS*-deficient derivative carrying plasmid pKTpheA56+A. About 5×10^8 *P. putida* cells from independent cultures grown overnight in liquid M9 medium were plated onto phenol minimal plates. Data for at least five parallel experiments are presented. In all cases, means ± standard deviations (error bars) for 10 plates calculated per 1×10^9 cells are shown.

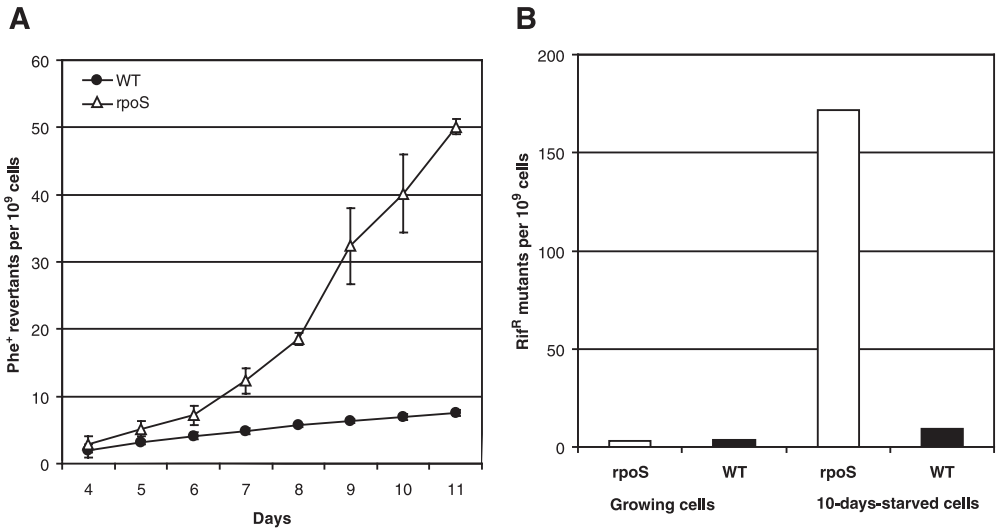

FIG. 2. Effect of RpoS on the frequency of base substitution mutations in *P. putida* under starvation conditions. (A) Accumulation of Phe^+ revertants on phenol minimal plates in *P. putida* WT strain PaW85 and its *rpoS*-deficient derivative (rpoS) carrying plasmid pKTpheA22TAG. About 5×10^8 *P. putida* cells from independent cultures grown overnight in liquid M9 medium were plated onto phenol minimal plates. Data for at least five parallel experiments are presented. Means ± standard deviations (error bars) for 10 plates calculated per 1×10^9 cells are shown. (B) Frequency of Rif^r mutants in growing cells and cells of *P. putida* WT strain PaW85 and its *rpoS*-deficient derivative starved for 10 days. The frequency of spontaneous Rif^r mutations in 50 independent growing cultures of the *P. putida* WT strain and its *rpoS*-deficient derivative was calculated per 1×10^9 cells by using the Lea-Coulson method of the median (37, 63). The same cultures were plated onto phenol minimal plates, and after 10 days of starvation, the frequency of Rif^r mutations was determined in the cells that survived the long-term starvation conditions. Median values for 10 plates calculated per 1×10^9 cells are shown. In total, 100 and 200 plates were examined for Rif^r mutants in populations of the WT and its *rpoS*-deficient derivative, respectively.

absence of RpoS caused an elevated frequency of base substitutions in long-term-starved *P. putida*.

Elevated mutation frequency in starving *rpoS*-deficient *P. putida* is suppressed by artificial overexpression of SOD or catalase. ROS such as superoxide, hydrogen peroxide, and hydroxyl radicals are constantly generated as by-products of aerobic metabolism. ROS can damage proteins, lipids, and nucleic acids and may lead to the death of cells. It is shown that expression of SOD is required for the survival of *E. coli* during the stationary phase (2). Importantly, the time period when the frequency of accumulation of base substitution mutants increased in *rpoS*-deficient *P. putida* coincided with the rapid death of about 95% of the cells in the population (Fig. 1A and 2A). Hence, we hypothesized that starving *rpoS*-deficient *P. putida* could be less protected against damage caused by ROS than the WT strain.

The results obtained by Park et al. (56) indicated that the response to superoxide stress in *P. putida* may be quite different from the SoxR mechanism in *E. coli*. However, the control mechanism has remained unknown. We compared SOD expression in stationary-phase cells of *P. putida* WT and *rpoS*-deficient strains. The results presented in Fig. 3 show an approximately fivefold decline in the level of SOD activity in the *rpoS*-deficient strain compared to that observed in the WT. These data suggest that SOD expression is under the positive control of RpoS in *P. putida*. Thus, it is possible that the reduced level of expression of SOD in starving *rpoS*-deficient *P. putida* cells is one of the mechanisms responsible for the accumulation of ROS and therefore causes a drop in viability and the elevation of mutations.

In order to examine the possibility that limitation of SOD in *rpoS*-deficient bacteria can elevate the accumulation of base substitutions under the conditions of long-term-starvation of bacteria, we studied the effect of overexpression of this enzyme on the mutation frequency in starving bacteria. It is known that SOD is functional in *P. putida* stationary-phase cells as a SodAB heterodimer (26). Therefore, we constructed a chro-

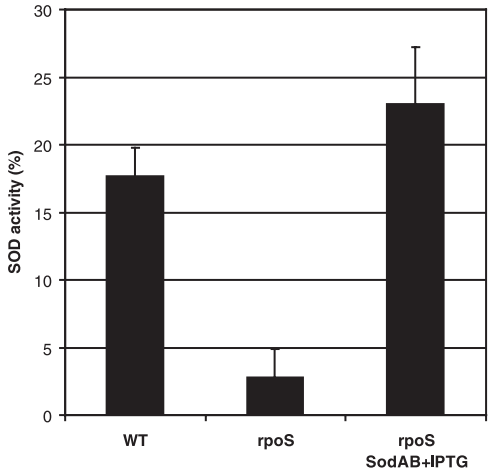

FIG. 3. Effects of RpoS on the expression of SOD activity in carbon-starved *P. putida*. SOD activity was measured in cells of the WT strain, the *rpoS*-deficient strain (rpoS), and the *rpoS*-deficient strain overexpressing SodAB (rpoS SodAB plus IPTG) that were starved for 8 days. SOD activity is expressed as the percent inhibition of tetrazolium salt reduction to formazan dye (absorbance at 440 nm) by superoxide anion.

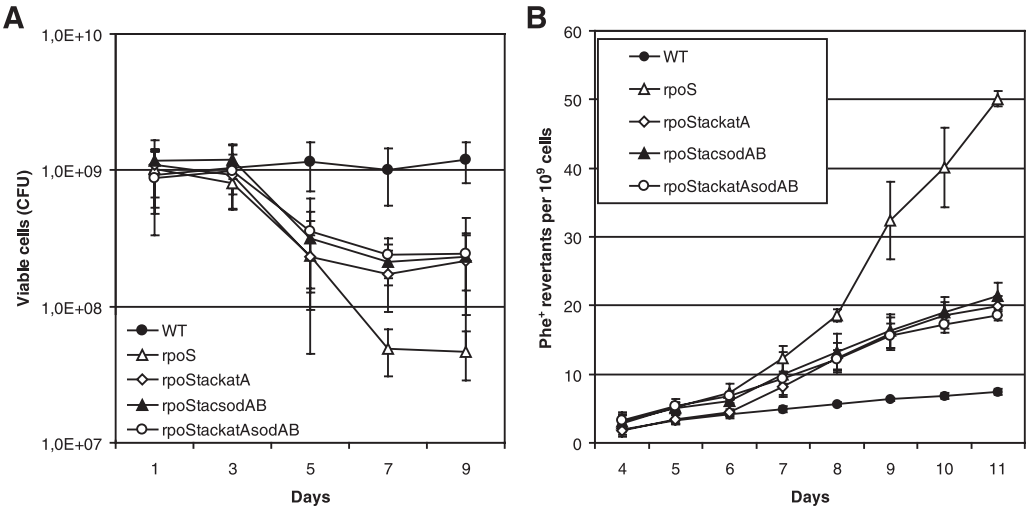

FIG. 4. Effects of overexpression of SOD and catalase on the survival of *rpoS*-deficient *P. putida* cells under carbon starvation conditions and on the frequency of accumulation of base substitution mutations. (A) Viability of the *P. putida rpoS*-deficient strain overexpressing catalase (rpoStackatA), SodAB (rpoStacsodAB), or both catalase and SodAB (rpoStackatAsodAB) on phenol minimal plates. The viability of the WT strain and its *rpoS*-deficient derivative without SOD or catalase overexpression is shown as well. (B) Accumulation of Phe^+ revertants on phenol minimal plates in starving populations of the same strains shown in panel A. All strains carried the tester plasmid pKTpheA22TAG. Data for at least five parallel experiments are presented. In all cases, means ± standard deviations (error bars) for 10 plates calculated per 1×10^9 cells are shown.

mosomal *sodA* (PP0946) and *sodB* (PP0915) overexpression cassette where these genes are expressed as a single transcriptional unit under the control of the IPTG-inducible P_{tac} promoter. The *rpoS*-deficient *P. putida* strain carrying the P_{tac}-*sodAsodB* expression cassette exhibited a level of SOD activity comparable to that measured in the WT strain (Fig. 3). Notably, the overexpression of SOD increased the survival of the *rpoS*-deficient *P. putida* strain about 10-fold during long-term-starvation (Fig. 4A). Also, the frequency of accumulation of Phe^+ revertants was approximately 2.5-fold lower in this strain than in the *rpoS*-deficient strain lacking the SOD overexpression cassette (Fig. 4B). At the same time, SOD overexpression did not affect the mutation frequency in WT cells (data not shown). Thus, our results indicate that RpoS is important in the regulation of the *sodA* and *sodB* genes, protecting cells against oxidative damage, and that the increased amount of superoxide promotes mutagenesis in starving populations of *P. putida*. However, the limitation of SOD in the *rpoS*-deficient strain was not the only factor responsible for cell death and elevation of the mutation frequency.

The expression of two catalases, KatA and KatB, is under the control of the OxyR regulator in *P. putida* (29). It is also known that RpoS positively regulates the expression of KatB (29, 48). Additionally, Ramos-González and Molin (62) have shown that the *rpoS*-deficient *P. putida* strain has an increased sensitivity to hydrogen peroxide in the stationary phase. We observed significant differences between the native levels of expression of catalase in starving WT and *rpoS*-deficient *P. putida* cells (Fig. 5). This indicated that limitation of catalase in starving *rpoS*-deficient *P. putida* cells may also affect the viability and mutagenic processes of bacteria. Therefore, we studied the effect of artificial overexpression of catalase on the survival of and the frequency of base substitutions in starving *rpoS*-deficient *P. putida* cells. For that purpose, we constructed the *P. putida* strain overexpressing *katA*-encoded catalase derived from *P. fluorescens* plasmid pAM10.6 (57). The gene was cloned under the control of the IPTG-inducible P_{tac} promoter and introduced into the *P. putida* chromosome. The overexpression of KatA was verified by the production of oxygen bubbles in a cell suspension after the addition of hydrogen peroxide. Remarkably, O_2 production was significantly enhanced under the conditions of overexpression of *P. fluorescens* catalase KatA in a culture of *rpoS*-deficient bacteria grown overnight (Fig. 5), thereby confirming the finding of the previously published studies (57) that *P. fluorescens* KatA is a catalase which is functional in *P. putida*. We also confirmed that long-term-starved cells of the *rpoS*-deficient strain containing the *P. fluorescens katA* expression construct exhibit higher catalase activity than the same strain without this construct (data not shown). Similar to the effect of the overexpression of SOD, the IPTG-induced expression of this catalase increased the survival of starving cells lacking RpoS and reduced the frequency of accumulation of base substitution mu-

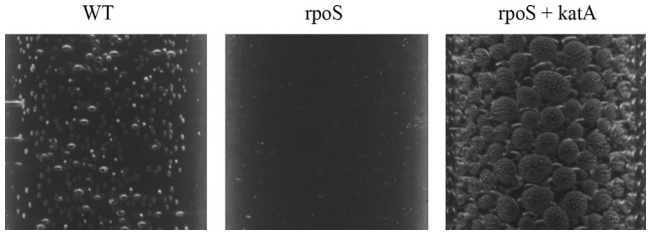

FIG. 5. Detection of catalase expression in the *P. putida* in WT strain, its *rpoS*-deficient derivative (rpoS), and the *rpoS*-deficient strain overexpressing the catalase gene *katA*. Amounts of oxygen bubble formation were compared in cell suspensions of overnight cultures of these strains 1 min after the addition of a hydrogen peroxide solution.

TABLE 3. Amounts of superoxide in lysates and supernatants of cells of the *P. putida* WT strain and its *rpoS*-deficient derivative

Strain (day of sampling)	Amt of superoxide/ plate	Amt of superoxide/ 10^6 CFU[c]
WT strain		
Lysate I[a] (2)	35,956 ± 5,740	32.0 ± 10.1
Supernatant I (2)	11,509 ± 1,501	11.2 ± 2.1
Supernatant II (2)	561 ± 251	0.8 ± 0.07
Lysate II[b] (2)	28,938 ± 4,772	33.0 ± 22.8
Lysate I[a] (8)	32,677 ± 4,649	42.9 ± 9.8
Supernatant I (8)	14,910 ± 2,722	21.0 ± 6.2
Supernatant II (8)	1,829 ± 452	3.7 ± 1.0
Lysate II[b] (8)	20,101 ± 1,888	39.7 ± 13.8
RpoS-deficient strain		
Lysate I[a] (2)	82,677 ± 11,407	111 ± 13.3
Supernatant I (2)	27,033 ± 2,780	43.8 ± 8.3
Supernatant II (2)	3,165 ± 1,324	7.4 ± 0.4
Lysate II[b] (2)	36,667 ± 4,598	62.9 ± 12.1
Lysate I[a] (8)	73,189 ± 11,972	8,832 ± 1,599
Supernatant I (8)	32,812 ± 6,370	4,302 ± 803
Supernatant II (8)	11,177 ± 5,446	1,861 ± 994
Lysate II[b] (8)	28,103 ± 4,324	4,481 ± 1,062

[a] Cells from agar plates sonicated within their resuspension buffer (see preparation of lysate I in Materials and Methods).

[b] Cells sonicated after washing with M9 (see preparation of lysate II in Materials and Methods).

[c] Relative amount of SO per 1×10^6 CFU in 1 ml reaction mixture; values for supernatant I and supernatant II are given per 1×10^6 CFU in 1 ml of cell suspension before and after washing of cells, respectively.

tations (Fig. 4). Taken together, our results indicated that the limitation of SOD and catalase in starving *rpoS*-deficient *P. putida* is one of the most important factors resulting in the death of the majority of the cells and elevation of the mutation frequency in the surviving population under long-term carbon starvation conditions. Nevertheless, simultaneous overexpression of SOD and KatA catalase in the *rpoS*-deficient strain did not further improve cell survival and the frequency of mutations, thereby indicating that the defense mechanisms provided by SOD and catalase overexpression cannot fully prevent the death of cells under the conditions studied (Fig. 4A and B).

The total amount of SO per plate is increased in *rpoS*-deficient bacteria. As our results clearly demonstrated that overexpression of SOD or catalase in the *P. putida rpoS*-deficient strain increased the viability of cells and reduced the mutation frequency under conditions of long-term starvation, we decided to measure the cellular amount of SO in the WT strain and its *rpoS*-deficient derivative at the beginning of starvation (on day 2) and after prolonged starvation (on day 8). Cells from phenol minimal plates were resuspended in equal amounts of M9 buffer, and SO was measured in cleared lysates prepared from cell suspensions obtained directly from the plates (lysate I) and those prepared from cells washed with M9 buffer to get rid of lysed cells (lysate II). The results presented in Table 3 demonstrate that the amount of SO calculated per 10^6 CFU in the reaction mixtures was significantly increased in the case of *rpoS*-deficient bacteria. We detected a two- to threefold greater SO amount in this strain than in the WT strain when the cells of *rpoS*-deficient bacteria were starved for 2 days on minimal plates and a more-than-100-fold increase when the *rpoS*-deficient strain was starved for 8 days.

As already mentioned above, the number of CFU was reduced drastically during the prolonged starvation of *rpoS*-deficient bacteria (Fig. 1A). Therefore, it is possible that other cells (dead ones with cell wall integrity and those staying in a viable but nonculturable state) were sources of superoxide as well. Indeed, the total amounts of this oxygen radical measured per plate differed only twofold between the WT strain and its *rpoS*-deficient derivative. Interestingly, a large amount of SO was already detectable in the supernatant of cells that were centrifuged without sonication (supernatant I). Supernatants derived from the additional washing of the cells (supernatant II) also contained SO. The presence of large amounts of SO in supernatants I and II indicated that many cells derived from the surface of agar plates lysed during the initial resuspension and subsequent washing, thereby releasing SO into the environment. Additionally, there is a possibility that at least a part of the SO molecules measured in supernatants I and II diffused from surviving cells. This idea is supported by the results of measurement of SO in the WT strain grown overnight in liquid M9 medium plus glucose. When cells from the liquid culture were collected by centrifugation and washed with M9 buffer, about 20% of the total amount of SO measured in cells plus their washing solution was detectable in the washing solution alone (data not shown). Taken together, the results of our experiments indicate that the total amount of SO per plate is increased if bacteria lack RpoS whereas a large amount of SO is derived from cells which are either dead or not able to form colonies. It is difficult to say how much the intracellular amount of SO is increased in the surviving cells under the conditions studied. However, since the overexpression of SOD significantly increased the viability of *rpoS*-deficient cells during long-term carbon starvation (Fig. 4A), an inability to detoxify ROS seems to be a major reason for the death of the cells.

The spectrum of mutations characterized in starving RpoS-deficient bacteria is different from that identified in bacteria lacking the GO repair system. Based on the results demonstrating that the decreased survival of *rpoS*-deficient *P. putida* under carbon starvation conditions is caused by limitation of enzymes responsible for detoxification of ROS, we supposed that the elevation of the mutation frequency under such conditions is caused by the incomplete removal of DNA lesions resulting from ROS attack. It is known that oxidative damage of DNA is an important source of mutation (9) and that the guanine oxidation product 7,8-dihydro-8-oxoguanine (GO) can give rise to mutations in starving *E. coli* (11, 12). Also, our previous results demonstrated that the absence of GO repair enzymes in starving *P. putida* leads to an increase in the mutation frequency and the characteristic spectrum of base substitution mutations (67). Therefore, in order to examine the possibility of whether the GO repair system could became saturated in *rpoS*-deficient *P. putida* under starvation conditions, we analyzed the *pheA* gene sequence of Phe^+ colonies that were picked up from selective plates on days 7 to 10, when the mutation frequency was significantly elevated. We expected to see a pattern of mutations in the *rpoS*-deficient background similar to that observed in strains lacking the GO repair enzymes. Surprisingly, the analysis of the nucleotide sequence of the Phe^+ revertants (Table 4) revealed that the spectrum of mutations identified in the *rpoS*-deficient strain was distinct from that identified by us previously in strains lacking either GO repair enzyme MutY or MutT (67). The

TABLE 4. Reversion of nonsense mutation (TAG) in Phe$^+$ mutants accumulating in the *P. putida* WT strain and its RpoS-deficient derivative

Change in TAG DNA sequence	No. of occurrences[a] (% of total)			
	WT	*mutT*	*mutY*	*rpoS*[b]
T → C	164 (77)	14 (8.3)	24 (12)	75 (40)[c]
T → G	19 (9)	34 (20)	5 (2)	25 (13)
T → A	1 (0.5)	0	0	4 (2)
G → T	12 (5.6)	1 (0.6)	176 (85)	18 (10)
A → C	0	118 (70)	0	1 (1)
A → G	13 (6)	2 (1.1)	3 (1)	48 (26)[c]
A → T	4 (1.9)	0	0	4 (2)
Deletions	0	0	0	11 (6)[c]

[a] The spectra of mutations in the WT and GO repair-deficient strains are from reference 67.

[b] Phe$^+$ mutant colonies used for identification of stationary-phase mutations were picked up on days 7 to 15. We did not notice remarkable changes in the spectra of mutations in revertants derived from either the early or the late starvation period in the WT and GO repair-deficient derivative strains (67).

[c] Statistically significantly different ($P < 0.05$) from WT strain value.

proportions of G-to-T and A-to-C transversions, which were strictly increased in *mutY*- and *mutT*-deficient bacteria, respectively, were not significantly increased in the *rpoS* mutant strain (Table 4). These data indicate that the higher frequency of mutations in long-term-starved *rpoS*-deficient *P. putida* was not caused by saturation of the GO repair system due to accumulation of the guanine oxidation product GO.

At the same time, the spectrum of mutations identified in the *rpoS* mutant background differed from that characterized in the WT strain. For example, the proportion of A-to-G transitions increased from 6% in the WT strain to 26% in the *rpoS*-deficient strain, whereas the proportion of T-to-C transitions decreased from 77% in the WT strain to 40% in the strain lacking RpoS. Interestingly, the absence of RpoS in starving *P. putida* stimulated the appearance of large in-frame deletions from the *pheA* sequence encompassing the introduced stop codon. Importantly, no deletions have been recorded with this test system in any of the other genetic backgrounds studied by us so far. In the case of *rpoS*-deficient bacteria, we identified one 69-nucleotide (nt)-long deletion, two identical 63-nt-long deletions, six identical 45-nt-long deletions, and one 30-nt-long deletion. One CTG deletion was found as well. Thus, as the absence of RpoS in starved *P. putida* affected the spectrum of mutations, certain types of DNA damage distinct from GO accumulate in starving RpoS-deficient *P. putida*.

DISCUSSION

The present study demonstrates that the absence of RpoS results in a significant elevation of base substitution mutations in starving *P. putida*. Additionally, the formation of some larger deletions was also accompanied by a lack of RpoS function in starving bacteria. The rise in the mutation frequency coincided with the death of the majority of *rpoS*-deficient cells after 5 to 7 days of starvation on minimal agar plates. Our results indicate that the decreased survival of carbon-starved *rpoS*-deficient *P. putida* on agar plates, i.e., in a structured environment, is caused by ROS. (i) SOD and catalase activities were reduced in starving *P. putida* lacking RpoS (Fig. 3 and 5). (ii) Overexpression of SOD or catalase KatA increased the survival of the *rpoS*-deficient strain by 10-fold and lowered the mutation frequency (Fig. 4A and B). Additionally, measurement of the amounts of SO indicated that *rpoS*-deficient cells have a reduced ability to detoxify ROS compared to that of WT cells (Table 3). The increase in the amount of SO in the case of the *rpoS*-deficient strain already became evident at the beginning of starvation (on day 2). Surprisingly, the total amounts of SO per plate measured in the *rpoS*-deficient strain on day 2 exceeded those measured on day 8. One possible explanation for this is that the number of CFU in populations of the *rpoS*-deficient strain starved for 8 days was reduced about 50-fold compared to that at the beginning of starvation and therefore the samples prepared on day 2 contained more SO-producing cells. At the same time, dead cells and those unable to form colonies could be a source of SO in our assays as well. Indeed, supernatants of the cells resuspended from agar plates (supernatant I in Table 3) contained a large amount of SO, whereas the SO values were the highest in the supernatants of the *rpoS*-deficient cells sampled on day 8. Unfortunately, direct measurement of SO in a fraction of viable (and colony-forming) cells in starved populations of *rpoS*-deficient bacteria was complicated due to the uncertainty of cell sorting by flow cytometry. The majority of *rpoS*-deficient cells derived from agar plates already stained with propidium iodide (PI) at the beginning of starvation, and the proportion of PI-stained cells even increased if the cells were washed before PI staining (data not shown). Although PI is frequently used to distinguish dead cells from viable ones because PI penetrates only bacteria with damaged membranes, bacteria having compromised membranes are not always dead (see, e.g., the instructions for LIVE/DEAD *Bac*Light Bacterial Viability Kits; Molecular Probes, Invitrogen). Some of our unpublished results indicate that the cell membrane of *rpoS*-deficient *P. putida* is more permeable than that of the WT strain when the bacteria are incubated on agar plates. Additionally, many cells in long-term-starved populations of bacteria could be in a viable but nonculturable state, which complicates the separation of surviving and potentially mutating cells from the rest of the cells on selective plates even more.

Although the overexpression of SOD or catalase in the *P. putida rpoS*-deficient strain increased the survival of bacteria under carbon starvation conditions and reduced the mutation frequency in the surviving cells, the frequency of accumulation of Phe$^+$ revertants was still about twofold higher than that estimated in the WT populations. Thus, it is possible that, in addition to the accumulation of superoxide and hydrogen peroxide, some other hazardous components accumulate in starving *rpoS*-deficient *P. putida* as well. One may imagine that the death of *rpoS*-deficient cells could be explained by changes in pH during the prolonged incubation of bacteria on selective plates. Since the growth medium of the bacteria was buffered with M9 solution, we did not expect to see pH changes. Indeed, direct measurement of pH on minimal agar plates after prolonged incubation of bacteria did not reveal any changes (data not shown).

The increase in the frequency of base substitutions in the cells that survived was observed in the cases of both the plasmid-based test system measuring Phe$^+$ reversion and the chromosomal Rif^r-based test system. This indicates that long-

term-starved *rpoS*-deficient *P. putida* cells, or at least a subpopulation of these cells, have a genome-wide elevated mutability. GO is known to be one of the most stable and frequent base modifications resulting from oxygen radical attack on DNA (9). Bacteria have evolved a GO repair system to avoid mutations occurring due to GO (45). Analysis of the mutation spectrum of the Phe^+ revertants in the *P. putida rpoS*-deficient strain did not reveal any similarity to the spectra identified by us previously in *P. putida* derivatives lacking GO repair enzyme MutT or MutY (67). While the lack of particular DNA repair functions specifically elevated certain types of mutations, A to C or G to T in strains lacking MutT or MutY, respectively (67), the mutation spectrum characterized in the *rpoS*-deficient strain was broader and more similar to that of the WT. These data imply that the GO repair system is functional in the *rpoS*-deficient strain.

The proportion of A-to-G transitions was increased in the absence of RpoS. Another significant dissimilarity from the WT strain was the occurrence of large deletions from the *pheA* sequence. Thus, it is possible that the occurrence of certain types of DNA damage not recognized by the GO repair system is responsible for these mutations in the *rpoS* mutant background. It is known that GO is highly susceptible to further oxidation products which are highly mutagenic (51). 2-Hydroxyadenine also induces mutations (33). Indeed, the results of our recent study imply that adenine oxidation products might also be an important source of mutation in starving bacteria (67). Additionally, aging cells are particularly subject to alkylation damage (73) and loss of enzymes that remove alkyl groups from damaged bases increases the mutation frequency in nongrowing bacteria (reviewed in reference 20). Our preliminary results indicate that long-term-starved *rpoS*-deficient bacteria accumulate DNA damage which is repaired by the nucleotide excision repair pathway (data not shown). The substrate repertoire of nucleotide excision repair extends from bulky lesions to other damage, such as certain oxidative base lesions, alkyl lesions, abasic (AP) sites, etc. (49). These results also support the idea that the accumulation of certain types of DNA damage produced by ROS and/or by methylation agents may facilitate the generation of mutations in *rpoS*-deficient *P. putida*. Furthermore, damage of DNA bases, if not repaired, and generation of AP sites due to limitation of AP endonuclease may cause accumulation of DNA strand breaks, thereby inducing RecA and facilitating recombination processes in starving cells. DNA synthesis occurring during recombinational repair may be error prone due to the involvement of DNA damage-induced specialized DNA polymerases. One may also hypothesize that the appearance of large deletions, especially in the *rpoS*-deficient background of *P. putida*, might be caused by ROS-stimulated recombination. Additionally, nonhomologous end joining (NHEJ), an essential pathway responsible for the repair of double-strand breaks and composed of Ku and a multifunctional DNA ligase (LigD), has recently been identified in many prokaryotes, including *Pseudomonas* species (58, 69). The DNA repair provided by the bacterial NHEJ system has been shown to be inaccurate, resulting in single nucleotide additions or deletions with various lengths at the break site (23, 41, 72). Recent studies also suggest that NHEJ acts under conditions where DNA replication is reduced or absent, such as in spores or in the stationary phase (59, 72, 77). Hence, it is possible that the deletions identified by us in this study were due to NHEJ taking place in starving cells of *rpoS*-deficient *P. putida*.

On the other hand, we suppose that the elevation of the mutation frequency in starved *rpoS*-defective *P. putida* cannot be caused by DNA damage only. This assumption is based on the findings that the rise in mutation frequency in the starving population of the *rpoS*-deficient strain coincided with the death of the majority of the cells and that the overexpression of SOD or catalase had stronger effects on the survival of the cells than on their mutation frequency. It is argued that the increased mutation frequency in stationary-phase cells does not limit the cellular life span and DNA does not seem to be the primary target of oxidative damage in the cells (53). Instead, the oxidative damage of proteins and membrane lipids is the major reason for aging and mortality (53). Bacterial stasis results in an increase in the oxidation and differential oxidation of target proteins (15). Oxidative modifications may inhibit or alter the activity of proteins, whereas carbonylated proteins have increased susceptibility to proteolysis (54). Studies by Dukan and Nyström (15) have demonstrated that protein carbonylation is increased in *E. coli rpoS* mutant cells starved for 1 to 2 days and that stasis-induced oxidation targets specific proteins. Importantly, the results presented by Dukan and Nyström (15) revealed that protein carbonylation can be mitigated by overproducing SOD. Also, some earlier studies have revealed that the lack of SOD or catalase activity in *E. coli* elevates the mutation frequency at doses of various oxidants that were nonmutagenic for WT cells (61, 64). Similar to the results of our studies, the contribution of GO to mutagenesis seemed to be negligible in the studies by Ruiz-Laguna et al. (64).

Oxidative damage of proteins is connected to errors in the translational machinery: damage to components of protein synthesis increases mistranslation, and vice versa, mistranslated proteins are more susceptible to oxidative damage (16). Mistranslation can be promoted by various factors in aging cells. For example, amino acid starvation can lead to a 10- to 100-fold increase in mistranslation (70). As first proposed in 1991 by Ninio (52) and later confirmed by others (3, 4, 5, 30), mistranslation of DNA repair and replication proteins can create a transient mutator phenotype. Hence, taking these results together, we suppose that, in addition to oxidative DNA lesions, mistranslation and oxidative damage of proteins may also affect DNA replication fidelity and thereby increase the mutation frequency in *rpoS*-deficient bacteria under the conditions studied. So far, direct evidence supporting this hypothesis is lacking. However, oxidative modification of replication proteins has recently been reported in eukaryotic systems (44, 50). Additionally, hypermutagenesis in *E. coli mutA* cells mistranslating aspartate as glycine due to a mutation in the glycine tRNA anticodon was mediated by modifications of DNA polymerase III due to elevated mistranslation (3).

The results of the present study (Fig. 1B) did not reveal any differences in the frequency of accumulation Pol IV-dependent 1-bp deletion mutants in starving *P. putida* WT and *rpoS*-deficient cells. In the light of the idea that genome-wide elevation of base substitutions occurs in long-term-starved *rpoS*-deficient *P. putida* as a result of an increased amount of ROS attacking both DNA and proteins, it is difficult to explain why

the frequency of 1-bp deletions was not affected. DNA polymerase Pol IV has been shown to be upregulated by RpoS in starving *E. coli* cells (36). On the contrary, we found that the transcription of the Pol IV *dinB* gene in *P. putida* was not dependent on RpoS (74). However, we cannot exclude the possibility that RpoS indirectly controls the stability of Pol IV and/or the activity and access of Pol IV to the DNA replication machinery. If this is true, then two opposite effects, the reduced amount of Pol IV-provided error-prone DNA synthesis and increased mutagenesis due to ROS-induced damage may balance each other in starving *rpoS*-deficient cells. Further studies are needed to investigate this possibility.

To sum up, the results presented in this study indicate that the stationary-phase sigma factor RpoS is important for the survival of *P. putida* in the stationary phase. RpoS-deficient cells with limiting amounts of SOD and catalase activities (due to their positive control by RpoS) are more exposed to ROS on a solid surface under conditions of carbon starvation, and this may be lethal to bacteria because of the oxidative damage to proteins and the cell membrane. So far, the role of oxidative damage to proteins in DNA integrity has been underestimated. It certainly needs more thorough investigation. We suggest that the surviving population of carbon-starved *rpoS*-deficient *P. putida* has an elevated mutation frequency as a result of the accumulation of both DNA damage and a decline in DNA replication and repair fidelity due to oxidative damage of enzymes and/or errors occurring during the translation of proteins.

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