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SpoT-Triggered Stringent Response Controls *usp* Gene Expression in *Pseudomonas aeruginosa*▽†

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The universal stress proteins (Usps) UspK (PA3309) and UspN (PA4352) of *Pseudomonas aeruginosa* are essential for surviving specific anaerobic energy stress conditions such as pyruvate fermentation and anaerobic stationary phase. Expression of the respective genes is under the control of the oxygen-sensing regulator Anr. In this study we investigated the regulation of *uspN* and three additional *P. aeruginosa usp* genes: *uspL* (PA1789), *uspM* (PA4328), and *uspO* (PA5027). Anr induces expression of these genes in response to anaerobic conditions. Using promoter-*lacZ* fusions, we showed that P_{uspL}-*lacZ*, P_{uspM}-*lacZ*, and P_{uspO}-*lacZ* were also induced in stationary phase as described for P_{uspN}-*lacZ*. However, stationary phase gene expression was abolished in the *P. aeruginosa* triple mutant Δ*anr* Δ*relA* Δ*spoT*. The *relA* and *spoT* genes encode the regulatory components of the stringent response. We determined pppGpp and ppGpp levels using a thin-layer chromatography approach and detected the accumulation of ppGpp in the wild type and the Δ*relA* mutant in stationary phase, indicating a SpoT-derived control of ppGpp accumulation. Additional investigation of stationary phase in LB medium revealed that alkaline pH values are involved in the regulatory process of ppGpp accumulation.

Bacteria are well prepared to adapt to a variety of growth-inhibiting stress conditions (reviewed in reference 48). One major factor controlling bacterial growth is the availability of oxygen. *Pseudomonas aeruginosa* prefers aerobic respiration and is capable of using nitrate or nitrite as a terminal electron acceptor under anaerobic growth conditions (10). Additionally, respiration-independent pathways like arginine fermentation (53) and pyruvate fermentation (15) support survival of *P. aeruginosa* in the absence of any terminal electron acceptors. Anaerobic growth and survival are essential for chronic *P. aeruginosa* infections in immunocompromised individuals, including those affected by the genetic disorder cystic fibrosis (1, 37, 46, 57, 61).

In previous studies we investigated the regulation of two anaerobically induced *P. aeruginosa* genes, *uspK* (PA3309) and *uspN* (PA4352), and demonstrated their significance for a successful adaptation to anaerobic energy stress (6, 44). Both genes encode proteins containing domains with a universal stress protein (Usp) signature (Pfam accession number PF0582). Members of the Usp family have been shown to confer survival to a variety of stress conditions in *Escherichia coli* (20, 26, 35) and have been additionally linked to motility, adhesion, and iron metabolism (34). In contrast, the *P. aeruginosa* UspK and UspN proteins increase survival under specific anaerobic stress conditions. UspK supports long-term survival during pyruvate fermentation (44), and UspN increases survival in anaerobic stationary phase (6). In addition to UspK and UspN, three additional Usp-encoding genes, *uspL* (PA1789), *uspM* (PA4328), and *uspO* (PA5027), are expressed in response to oxygen limitation (1; also N. Boes and M. Schobert, unpublished data). In contrast to the UspK protein, which contains a single Usp domain, UspN and the three additional Usp-type stress proteins, UspL, UspM, and UspO, contain two Usp domains in tandem. They share similar molecular masses and an overall moderate identity from 23 to 31% to UspN (see Fig. S1 in the supplemental material). But no information about gene regulation or function of these three proteins is available.

We demonstrated that anaerobic induction of *uspK* and *uspN* gene expression is dependent on the Anr regulator (6, 44). Anr is a homolog of the *E. coli* oxygen-sensing Fnr regulator and mediates the adaptation process from aerobic to anaerobic conditions (17, 43). In addition to Anr-dependent induction of *uspK* and *uspN*, we observed that gene expression of *uspK* and *uspN* is induced in stationary phase by a yet unknown regulator. In *E. coli*, the stringent response was shown to induce *usp* genes in stationary phase (20).

The stringent response is one of the global regulatory networks in bacteria, providing a rapid adaptation to a variety of growth-inhibiting stress conditions (8). The regulatory components of the stringent response are the guanosine nucleotides pppGpp and ppGpp. The accumulation of pppGpp and ppGpp in the bacterial cell alters the transcriptional profile (13, 16, 51) and promotes growth arrest by repressing transcription of genes involved in protein biosynthesis. In *E. coli* the stringent response has recently been shown to induce a large-scale restructuring of metabolic gene expression including genes involved in central metabolism (51). Two distinct enzymes, RelA and SpoT, control the accumulation of ppGpp in *E. coli*. In response to amino acid deprivation, pppGpp synthesis of RelA is activated (8). The second regulatory mechanism of the strin-

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gent response controls the balance between the synthetase and hydrolase activity of ppGpp by SpoT (8, 59). In contrast to RelA, SpoT-controlled ppGpp accumulation is triggered by a variety of stimuli, e.g., inhibition of fatty acid metabolism (19, 45), carbon deprivation (59), or membrane-perturbating agents (50).

In *P. aeruginosa* research on the stringent response focused primarily on RelA. It has been shown that overexpression of *relA* increases *rpoS* transcription and additionally activates quorum sensing (52). Moreover, a Δ*relA* mutant displayed decreased levels of the quorum-sensing-dependent elastase LasB and showed a reduced virulence in a *Drosophila melanogaster* infection model (14, 52).

In this study we investigated the transcriptional regulation of three new *usp* genes, PA1789 (*uspL*), PA4328 (*uspM*), and PA5027 (*uspO*). All three genes encode proteins with tandem Usp domains, and transcriptional regulation of all three is similar to that of *uspN*. They are induced in response to oxygen limitation in an Anr-dependent manner and in stationary phase. We determined that stationary phase gene expression is caused by the stringent response and can be abolished only in a Δ*relA* Δ*spoT* double mutant but not in a Δ*relA* single mutant. Further characterization of the Δ*relA* Δ*spoT* double mutant identified a SpoT-mediated response triggered by alkaline pH. To our knowledge this is the first research in *P. aeruginosa* indicating an alkaline pH-mediated SpoT-controlled stringent response.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are shown in Table 1. For standard molecular biology protocols, *E. coli* and *P. aeruginosa* strains were grown in Luria Bertani (LB) medium as described previously (44).

Construction and testing of promoter-*lacZ* reporter gene fusions. Chromosomal promoter-*lacZ* reporter gene fusions were constructed using the mini-CTX-*lacZ* vector (4). To analyze the *uspL* (PA1789) promoter, a 462-bp PCR product upstream of the translational start of *uspL* (PA1789) was generated using primer oKS20 (5′-GGAATTCTCTTCAGGATCGCCAGCAC-3′) and primer oKS21 (5′-CGCGGATCCACGAGGATGCTGCGAATG-3′). Furthermore, a 448-bp PCR product upstream of the translational start of *uspM* (PA4328) was generated using primer oKS22 (5′-GGAATTCACAGCAGAAC CTCGATCTC-3′) and primer oKS23 (5′-CGCGGATCCACCACCAGCAGGT TATG-3′), and a 453-bp PCR product upstream of the translational start of *uspO* (PA5027) was generated using primer oKS24 (5′-GGAATTCACCACATCGG CAGCATGTA-3′) and primer oKS25 (5′-CGCGGATCCATCATGGCGGAC TCCGT-3′). Primers oKS20, oKS22 and oKS24 contained an EcoRI restriction site at the 5′ end (underlined), and oKS21, oKS23, and oKS25 contained a restriction site for BamHI also at the 5′ end (underlined). The EcoRI- and BamHI-digested PCR products were cloned between the EcoRI and BamHI sites of mini-CTX-*lacZ* to generate pNB001 (P_{uspL}-*lacZ*), pNB002 (P_{uspM}-*lacZ*), and pNB004 (P_{uspO}-*lacZ*).

To monitor the expression of the stringent response-controlled promoter of P_{uspN}, we used $P_{uspN\Delta Anr}$, which was constructed in previous studies (6) and which contains a nonfunctional Anr box. Transfer of promoter-*lacZ* fusions harboring plasmids in *P. aeruginosa* was carried out by a diparental mating using *E. coli* S17 λ*pir* as the donor. The CTX integrase of the parental mini-CTX-*lacZ* vector promoted integration of the plasmid into the *attB* site of the *P. aeruginosa* genome. Constructed plasmids were transferred into *P. aeruginosa* PAO1 wild type and the *anr* mutant strain PAO6261 to generate the *P. aeruginosa* strains NB005 (PAO1 with P_{uspL}-*lacZ*), NB006 (PAO1 with P_{uspM}-*lacZ*), NB008 (PAO1 with P_{uspO}-*lacZ*), NB021 (PAO1 Δ*anr* with P_{uspL}-*lacZ*), NB022 (PAO1 Δ*anr* with P_{uspM}-*lacZ*), and NB024 (PAO1 Δ*anr* with P_{uspO}-*lacZ*) (Table 1). pNB19 was introduced in PA14 wild type, PAO1 Δ*relA* Δ*spoT*, and in diverse transposon mutants to generate the strains NB104 (PA14 wild type), NB171 (Δ*relA* Δ*spoT*), NB202 (ΔPA0149), NB203 (ΔPA0472), NB205 (ΔPA1912), NB208 (ΔPA2050), NB210 (ΔPA2896), NB216 (ΔPA0762), NB217 (ΔPA1455), NB218 (ΔPA2426), NB219 (ΔPA4462), and NB220 (ΔPA3622). In these strains parts of the mini-CTX-*lacZ* vector containing the tetracycline resistance cassette were deleted using a FLP recombinase encoded on the pFLP2 plasmid (22). β-Galactosidase assays were performed as outlined before in detail (15, 42), and activity is reported in Miller units (29).

To analyze the activation of the *usp* promoters under anaerobic conditions, cells were incubated aerobically in LB medium and were transferred to anaerobic flasks at an optical density at 578 nm (OD_{578}) of 0.7. Activities were determined before and 15, 30, 60, and 180 min after the cultures were shifted to anaerobic conditions.

β-Galactosidase activities of aerobically grown cultures were monitored from exponential phase to stationary phase in LB medium.

Construction of *P. aeruginosa* Δ*relA* and Δ*relA* Δ*spoT* mutant strains. Unmarked gene deletion mutants were obtained using well-established strategies based on *sacB* counter-selection and FLP recombinase excision (22). The suicide vector pKS18 used to replace *relA* has been described previously (44). To achieve a deletion of *spoT*, the suicide vector pNB54 was constructed. The BamHI-digested gentamicin resistance cassette of pPS858 was cloned between two PCR fragments of *spoT* in the multiple cloning site of pEX18Ap. The two PCR fragments contained DNA homologous to the upstream and downstream areas of *spoT*. A 636-bp fragment containing the upstream promoter region of the *spoT* was amplified using primer oNB33 (5′-CGAGCTCAGGACAGCGACGAGGT GAT), containing a SacI restriction site at the 5′ end (restriction sites are underlined), and oNB34 (5′-CGCGGATCCTTCACCCCCTGCCCGTA-3′), containing a BamHI site. The primers oNB35 (5′-CGCGGATCCACGGCA ACAT CGAGAAG-3′), containing a BamHI site, and oNB36 (5′-CCAAGCT TCCGGCTTA CTCGAGGACG-3′), containing a HindIII site, amplified 622 bp of the corresponding downstream region of *spoT*. In order to create Δ*relA* and Δ*relA* Δ*spoT* mutant strains, we used pKS18 to replace *relA* in NB021, NB022, NB023, NB024, NB071, and PAO1 wild type to create NB134, NB146, NB135, NB136, NB160, and NB159, respectively. We used the suicide vector pKS18 to replace *relA* with a gentamicin cassette by *sacB*-based counter-selection. FLP recombinase encoded on the pFLP2 plasmid removed the Flp recognition target (FRT)-flanked gentamicin cassette. To introduce Δ*spoT* in the created Δ*relA* mutant strains, we used the suicide vector pNB54 to replace *spoT* with a gentamicin cassette by *sacB*-based counter-selection to create the strains NB147, NB152, NB148, NB149, NB171, and NB170. FLP recombinase encoded on the pFLP2 plasmid removed the FRT-flanked gentamicin cassette once more.

Culture conditions for pppGpp/ppGpp assays. In order to monitor the accumulation of ppGpp during the transition from exponential to stationary phase, cultures were inoculated to an initial OD_{578} of 0.05 and were incubated aerobically at 37°C. For each ppGpp assay, aliquots of cultures were labeled with $^{32}P_i$ (0.1 mCi/ml) for 1 h prior to harvest.

To investigate the accumulation of both ppGpp and pppGpp during stationary phase in LB medium, cultures of *P. aeruginosa* (wild-type PAO1, Δ*relA*, and Δ*relA* Δ*spoT*) were grown for 8 h aerobically to stationary phase (OD_{578} of 5.0 to 7.0). Aliquots of 60 μl were labeled with $^{32}P_i$ (0.2 mCi/ml) for 1 h prior to harvest. In order to determine ppGpp and pppGpp levels in stationary phase with defined pH values, cultures were buffered with 0.1 M morpholinepropanesulfonic acid (MOPS) at either pH 7.0 or 8.5 prior to labeling with $^{32}P_i$ (0.2 mCi/ml) for 30 min.

ppGpp and pppGpp accumulation in response to alkaline pH values or serine hydroxamate (SHX) was carried out in exponential growth phase under anaerobic conditions. Cultures of *P. aeruginosa* were inoculated from overnight cultures to an initial OD_{578} of 0.05. Aliquots of 60 μl were incubated at 37°C in LB medium with 50 mM nitrate. To achieve anaerobic conditions, cultures were overlaid with mineral oil. Cells were incubated for 4 h to reach exponential phase with an OD_{578} of 0.5 and were labeled with $^{32}P_i$ (2 mCi/ml) for 15 min prior to treatment with either SHX (3 mM) or NaOH (20 mM, pH 9) and were incubated for an additional 15 min before harvest.

In control experiments we performed ppGpp assays with longer equilibration periods. We equilibrated stationary-phase cultures for 4 h and exponential-phase cultures for 2 h prior to NaOH or SHX treatment and obtained the same results as with short incubation periods. However, longer equilibration times resulted in higher background activity caused by the incorporation of radioactive phosphate in cell components such as membrane, RNA, DNA, etc. (data not shown).

Detection of ppGpp and pppGpp using a TLC approach. To obtain the same cell quantities of each growth phase for each ppGpp assay, appropriate volumes were harvested by centrifugation for 1 min at 12,000 × *g* (100 μl of a culture with an OD_{578} of 1.0, 20 μl of a culture with an OD_{578} of 5.0). Pellets were resus-

TABLE 1. Strains and plasmids used in this study

Bacterial strain or plasmid	Genotype or phenotype	Reference or source
Strains		
P. aeruginosa		
PAO1	Wild type	12
PA14	Wild type	27
PAO6261	PAO1 Δ*anr*	60
NB021	PAO6261 *attB*::(P_{uspL}-*lacZ*)	This study
NB022	PAO6261 *attB*::(P_{uspM}-*lacZ*)	This study
NB023	PAO6261 *attB*::(P_{uspN}-*lacZ*)	6
NB024	PAO6261 *attB*::(P_{uspO}-*lacZ*)	This study
NB134	PAO6261 *attB*::(P_{uspL}-*lacZ*) Δ*relA*	This study
NB135	PAO6261 *attB*::(P_{uspN}-*lacZ*) Δ*relA*	This study
NB136	PAO6261 *attB*::(P_{uspO}-*lacZ*) Δ*relA*	This study
NB146	PAO6261 *attB*::(P_{uspM}-*lacZ*) Δ*relA*	This study
NB147	PAO6261 *attB*::(P_{uspL}-*lacZ*) Δ*relA* Δ*spoT*	This study
NB148	PAO6261 *attB*::(P_{uspN}-*lacZ*) Δ*relA* Δ*spoT*	This study
NB149	PAO6261 *attB*::(P_{uspO}-*lacZ*) Δ*relA* Δ*spoT*	This study
NB152	PAO6261 *attB*::(P_{uspM}-*lacZ*) Δ*relA* Δ*spoT*	This study
NB159	PAO1 Δ*relA*	This study
NB170	PAO1 Δ*relA* Δ*spoT*	This study
PA0149::*MAR2xT7*[a]	ΔPA0149 (mutant ID 26541)[b]	27
PA0472::*MAR2xT7*	ΔPA0472 (mutant ID 25794)	27
PA1912::*MAR2xT7*	ΔPA1912 (mutant ID 5297)	27
PA2050::*MAR2xT7*	ΔPA2050 (mutant ID 35909)	27
PA2896::*MAR2xT7*	ΔPA2896 (mutant ID 23265)	27
PA0762::*MAR2xT7*	ΔPA0762 (mutant ID 40799)	27
PA1455::*MAR2xT7*	ΔPA1455 (mutant ID 28485)	27
PA2426::*MAR2xT7*	ΔPA2426 (mutant ID 34241)	27
PA4462::*MAR2xT7*	ΔPA4462 (mutant ID 44482)	27
PA3622::*MAR2xT7*	ΔPA3622 (mutant ID 32095)	27
NB202	PA0149::*MAR2xT7 attB*::($P_{uspN\Delta Anr}$-*lacZ*)	This study
NB203	PA0472::*MAR2xT7 attB*::($P_{uspN\Delta Anr}$-*lacZ*)	This study
NB205	PA1912::*MAR2xT7 attB*::($P_{uspN\Delta Anr}$-*lacZ*)	This study
NB208	PA2050::*MAR2xT7 attB*::($P_{uspN\Delta Anr}$-*lacZ*)	This study
NB210	PA2896::*MAR2xT7 attB*::($P_{uspN\Delta Anr}$-*lacZ*)	This study
NB216	PA0762::*MAR2xT7 attB*::($P_{uspN\Delta Anr}$-*lacZ*)	This study
NB217	PA1455::*MAR2xT7 attB*::($P_{uspn\Delta Anr}$-*lacZ*)	This study
NB218	PA2426::*MAR2xT7 attB*::($P_{uspn\Delta Anr}$-*lacZ*)	This study
NB219	PA4462::*MAR2xT7 attB*::($P_{uspn\Delta Anr}$-*lacZ*)	This study
NB220	PA3622::*MAR2xT7 attB*::($P_{uspn\Delta Anr}$-*lacZ*)	This study
NB104	PA14 *attB*::($P_{uspn\Delta Anr}$-*lacZ*)	This study
NB071	PAO1 *attB*::($P_{uspn\Delta ANR}$-*lacZ*)	6
NB160	PAO1 Δ*relA attB*::($P_{uspn\Delta Anr}$-*lacZ*)	This study
NB171	PAO1 Δ*relA* Δ*spoT*, *attB*::($P_{uspn\Delta Anr}$-*lacZ*)	This study
KS11	PAO1 *attB*::(mini-CTX-*lacZ*)	44
E. coli		
DH10B	F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX74 deoR recA1 endA1 araD139* Δ(*ara leu*)*7697 galU galK* λ^- *rpsL nupG*	GibcoBRL (Invitrogen)
S17 λ*pir*	*pro thi hsdR*$^+$ Tp^r Sm^r chromosome::RP4-2 Tc::Mu-Km::Tn*7* λ*pir*	11
ST18	S17 λ*pir* Δ*hemA*	Sabrina Thoma (unpublished)
SM10	*thi-1 thr leu tonA lacY supE recA*::RP4-2-Tc::Mu (Km^r)	11
Plasmids		
pEX18Ap	Ap^r *oriT*$^+$ *sacB*$^+$; gene replacement vector with MCS from pUC18	22
mini-CTX-*lacZ*	Tc^r; promoterless *lacZ* gene	4
pPS858	Ap^r Gm^r; source of gentamicin cassette	22
pFLP2	Ap^r; source of FLP recombinase	22
pNB001	Tc^r; mini-CTX-*lacZ* containing a 462-bp fragment of the putative promoter region of the PA1789 gene between EcoRI and BamHI	This study
pNB002	Tc^r; mini-CTX-*lacZ* containing a 448-bp fragment of the putative promoter region of the PA4352 gene between EcoRI and BamHI	This study
pNB003	Tc^r; mini-CTX-*lacZ* containing a 465-bp fragment of the putative promoter region of the PA4352 gene between EcoRI and BamHI	6
pNB004	Tc^r; mini-CTX-*lacZ* containing a 453-bp fragment of the putative promoter region of the PA4352 gene between EcoRI and BamHI	This study
pNB54	Ap^r Gm^r; pEX18Ap with a 636-bp promoter *spot*; Gm^r-*gfp* fragment from pPS858 and 622 bp downstream of the coding region of *spoT* between SacI and HindIII	This study
pKS18	Ap^r Gm^r; pEX18Ap with 796-bp promoter of *relA* (PA0934); Gm^r-*gfp* fragment from pPS858, and 824 bp of the 3′ coding region of *relA* between EcoRI and HindIII	44
pNB19	Tc^r; mini-CTX-*lacZ* containing a 465-bp fragment of the putative promoter region, with a mutation in the Anr-box (TTGATGTGCATCAA → TTGATGTGCATACG) of the PA4352 gene between EcoRI and BamHI	6

[a] *MAR2xT7* is a transposon based on *mariner* (27).
[b] Identification (ID) numbers of the mutants are from the *P. aeruginosa* PA14 Insertion Mutant Library (27).

pended in 10 μl of 1 M formic acid and were frozen at −20°C. To extract pppGpp and ppGpp, acidic mixtures were frozen at −20°C and thawed three times; cell debris was removed by centrifugation at 14,000 rpm for 2 min, and 5 μl of supernatant was spotted onto a polyethylenimine cellulose plate (PEI cellulose-F; Merck). Separation of pppGpp and ppGpp by thin-layer chromatography (TLC) was performed with 1.5 M KH_2PO_4 (pH 3.4) as running solvent. Dried plates were exposed to a K-Screen (Kodak) and were analyzed with a phosphorimager (FX-Scanner; Bio-Rad).

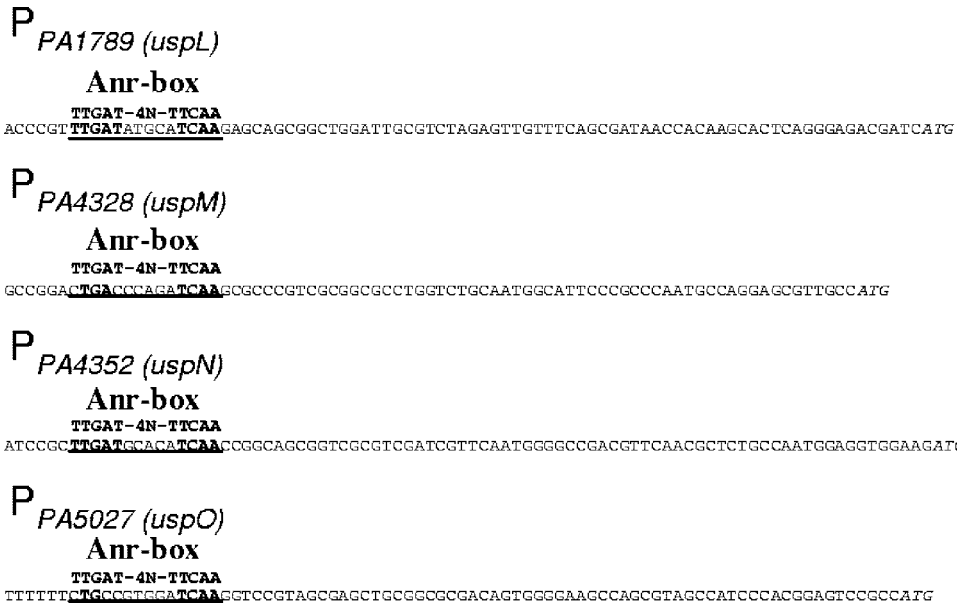

FIG. 1. Promoter regions of *uspL*, *uspM*, *uspN*, and *uspO*. Promoter regions of *uspL*, *uspM*, *uspN*, and *uspO* upstream of the translational start codon are shown (italic). The putative Anr box determined by the Virtual Footprint tool (32) is underlined, and conserved bases are shown in bold. The published consensus sequence (47) of the Anr homologue Fnr is given on top of each putative Anr box.

RESULTS

Anaerobic induction of P_{uspL}-*lacZ*, P_{uspM}-*lacZ*, and P_{uspO}-*lacZ* reporter gene expression is dependent on the oxygen-sensing regulator Anr. Previously we demonstrated Anr-dependent gene expression of *uspK* (PA3309) and *uspN* (PA4352) in response to anaerobiosis (6, 44). Investigations of the *P. aeruginosa* transcriptome under anaerobic conditions identified three additional *usp* genes, *uspL* (PA1789), *uspM* (PA4328), and *uspO* (PA5027), to be expressed in response to anaerobiosis (1; also Boes and Schobert, unpublished). Promoter analyses of all putative *usp* genes in *P. aeruginosa* using the Virtual Footprint tool of the PRODORIC database (32) indicated the presence of conserved Anr binding sites in the promoter regions of *uspL*, *uspM*, and *uspO* (Fig. 1). In order to investigate Anr-dependent gene expression of *uspL*, *uspM*, and *uspO*, we used transcriptional promoter-*lacZ* fusions.

We introduced P_{uspL}-*lacZ*, P_{uspM}-*lacZ*, and P_{uspO}-*lacZ* separately in the *P. aeruginosa* wild type and a Δ*anr* mutant strain to monitor β-galactosidase activities under aerobic and anaerobic conditions. Since a Δ*anr* mutant strain fails to grow anaerobically, we performed a shift experiment. *P. aeruginosa* wild-type and Δ*anr* mutant strains were grown in LB medium aerobically to the mid-log phase (OD_{578} of 0.7). Immediate transfer of the cultures to hermetically sealed serum bottles generated anaerobic conditions within 3 min (15). During aerobic exponential growth, *P. aeruginosa* had doubling times of about 30 min. Transfer to anaerobic conditions resulted in a growth arrest of both strains, wild type and the Δ*anr* mutant, since no nitrate was added to the growth medium. Aliquots for β-galactosidase activity determination were taken prior to the shift to anaerobic incubation and after 15, 30, 60, and 180 min of anaerobic incubation (Fig. 2). β-Galactosidase activities of all strains harboring promoter-*lacZ* constructs increased during anaerobic conditions in the wild type, while no increase was detected in Δ*anr* mutant strains, demonstrating Anr-dependent gene expression of P_{uspL}-*lacZ*, P_{uspM}-*lacZ*, and P_{uspO}-*lacZ* in vivo (Fig. 2) and confirming our previous results for P_{uspN}-*lacZ* (6).

Induction of P_{uspL}-*lacZ*, P_{uspM}-*lacZ*, P_{uspN}-*lacZ*, and P_{uspO}-*lacZ* reporter gene expression in aerobic stationary phase is mediated by the stringent response. In our previous studies, we monitored increased promoter activities using primer extension and P_{uspN}-*lacZ* reporter gene fusions during aerobic and anaerobic stationary phase. However, we excluded the involvement of RpoS, RelA, RhlR, or LasR in stationary-phase induction of P_{uspN}-*lacZ* (6).

We have already demonstrated that Anr contributes in part to aerobic stationary-phase induction of P_{uspN} (6) since respiration of *P. aeruginosa* at high cell densities in stationary phase results in an oxygen-limited environment (9). To exclude the impact of Anr, we determined β-galactosidase activities of P_{uspL}-*lacZ*, P_{uspM}-*lacZ*, P_{uspN}-*lacZ*, and P_{uspO}-*lacZ* in Δ*anr* mutant during aerobic stationary phase. All promoter-*lacZ* reporter gene fusions were induced in a Δ*anr* mutant strain during stationary phase, confirming Anr-independent induction (Fig. 3A). To investigate the involvement of the stringent response in promoter activation, we deleted both genes encoding the enzymes regulating ppGpp concentration, RelA and SpoT, in a Δ*anr* strain. First, we introduced a *relA* deletion to create Δ*anr* Δ*relA* double mutants harboring the respective four promoter-*lacZ* fusions. Deletion of *relA* results in a strong decrease in ppGpp levels in *E. coli* (33). While P_{uspL}-*lacZ* and P_{uspM}-*lacZ* showed almost no decrease in β-galactosidase activities as a consequence of a *relA* deletion in stationary phase, after 13 h we observed a decrease in the induction patterns of P_{uspN}-*lacZ* and P_{uspO}-*lacZ* in the Δ*anr* Δ*relA* double mutant compared to the Δ*anr* strain (Fig. 3A). To abolish residual ppGpp accumulation in Δ*relA* strains of *E. coli*, deletion of *spoT* is required (59). Consequently, we deleted *spoT* in *P. aeruginosa* to

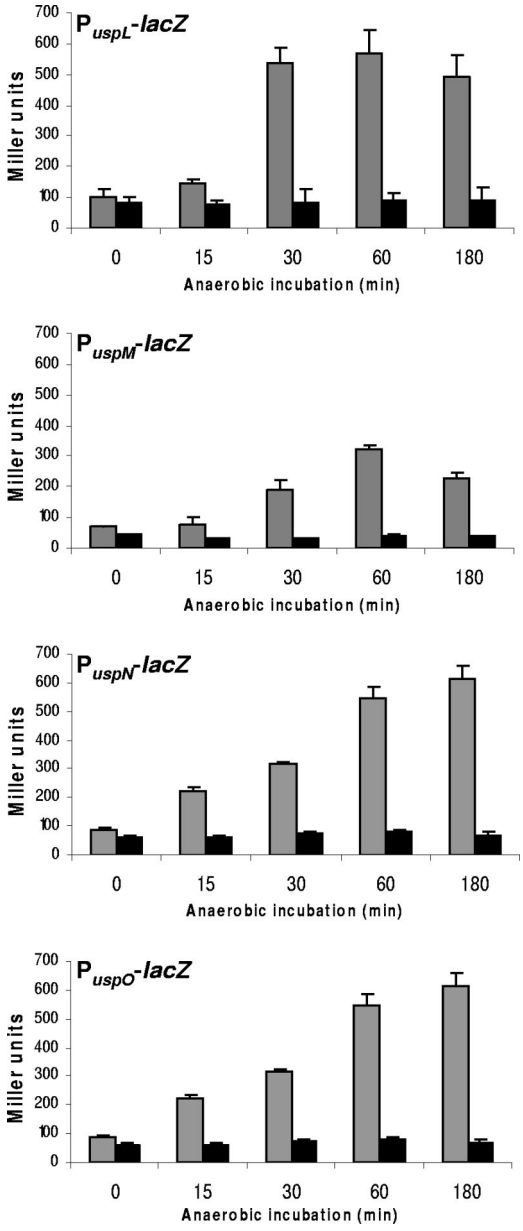

FIG. 2. β-Galactosidase activities in *P. aeruginosa* wild type (gray bars) and the Δ*anr* mutant (black bars) containing the respective P$_{uspL}$-*lacZ*, P$_{uspM}$-*lacZ*, P$_{uspN}$-*lacZ*, and P$_{uspO}$-*lacZ* fusions during anaerobiosis. Strains were grown aerobically at 37°C in LB medium to an OD_{578} of 0.7 (time point 0) and transferred to anaerobic conditions. Since the medium contained no alternative electron acceptor, the shift to anaerobic conditions resulted in a growth arrest for 4 h. Aliquots after 15, 30, 60, and 180 min were taken for β-galactosidase assays. All experiments were repeated three times; standard deviations are indicated.

create Δ*anr* Δ*relA* Δ*spoT* triple mutant strains and determined β-galactosidase activities of P$_{uspL}$-*lacZ*, P$_{uspM}$-*lacZ*, P$_{uspN}$-*lacZ*, and P$_{uspO}$-*lacZ* harboring strains in stationary phase. Activation of all tested *usp* promoters ceased in the absence of Anr, RelA, and SpoT. These results clearly demonstrated a stringent response-controlled induction of P$_{uspL}$-*lacZ*, P$_{uspM}$-*lacZ*, P$_{uspN}$-*lacZ*, and P$_{uspO}$-*lacZ* in stationary phase.

Accumulation of ppGpp in *P. aeruginosa* during stationary phase requires *relA* and *spoT*. The activation of all tested stringent response-controlled *usp* promoters in *relA*-deficient strains was abolished by the introduction of a *spoT* deletion, indicating a residual ppGpp accumulation in Δ*relA* single mutants in stationary phase. To confirm these results, we determined ppGpp levels simultaneously in the β-galactosidase samples during the transition from exponential to stationary phase, using a TLC approach.

For each ppGpp assay, samples were labeled with $^{32}P_i$ (0.1 mCi/ml) for 1 h, and identical cell numbers were harvested. Cell lysates were applied to a PEI cellulose-F TLC plate. ppGpp was separated using 1.5 M K_2HPO_4 as a running solvent. Results of ppGpp accumulation in correlation with growth phase, time of cultivation, and pH of cultures are shown in Fig. 3B. Accumulation of ppGpp was detected after 6 h of incubation dependent on the presence of SpoT, when the pH of cultures reached a value of 8.3. At this time point similar amounts of ppGpp were detected either in the absence or presence of RelA, indicating that ppGpp accumulation is strictly initiated by SpoT. Introducing a *spoT* deletion in a Δ*relA* mutant abolished any ppGpp accumulation during stationary phase, as expected. A longer time course of ppGpp accumulation revealed clearly lower ppGpp levels in the absence of RelA. The accumulation pattern of ppGpp during 24 h of cultivation is characterized by a peak after 8 h of cultivation in the presence of RelA and SpoT and a peak after 7 h of cultivation in the absence of RelA.

***P. aeruginosa* accumulates ppGpp but not pppGpp during stationary phase.** In order to analyze both ppGpp and pppGpp levels during stationary phase, we applied samples in parallel on a TLC plate. Wild-type, Δ*relA*, and Δ*relA* Δ*spoT* strains were labeled for 1 h prior to harvest with $^{32}P_i$ from stationary phase after 8 h of cultivation (compare growth curves in Fig. 3B). As shown in Fig. 4A, we detected accumulation of ppGpp in stationary phase in the wild type and in a Δ*relA* single mutant, as already shown in Fig. 3B. Interestingly, no corresponding accumulation of the precursor pppGpp was detected in the wild-type or Δ*relA* strain.

Alkaline pH triggers the stringent response during stationary phase in *P. aeruginosa*. To elucidate conditions that might be involved in controlling an accumulation of ppGpp in stationary phase, we assumed one of these conditions to be alkaline pH. Increased pH in stationary phase of cultures grown in LB medium has been described previously (21). To confirm our assumption that alkaline pH triggers ppGpp accumulation during stationary phase, we buffered stationary phase cultures of the wild type with 0.1 M MOPS buffer at either pH 7.0 or 8.5 prior to labeling with $^{32}P_i$ for 30 min (Fig. 4B). Neutralizing the growth medium to pH 7 resulted in a strongly decreased accumulation of ppGpp compared to alkaline pH values of 8.5, indicating a pH-regulated accumulation of ppGpp. We observed no accumulation of the precursor pppGpp in stationary phase, indicating a typical SpoT-controlled accumulation of ppGpp (see Discussion).

Accumulation of ppGpp/pppGpp in response to alkaline pH or SHX treatment. To examine the effect of alkaline pH values on the stringent response, we treated cells during exponential growth with 20 mM NaOH to induce a pH shift from 7.5 to 9. During exponential growth, cells without SHX or NaOH treat-

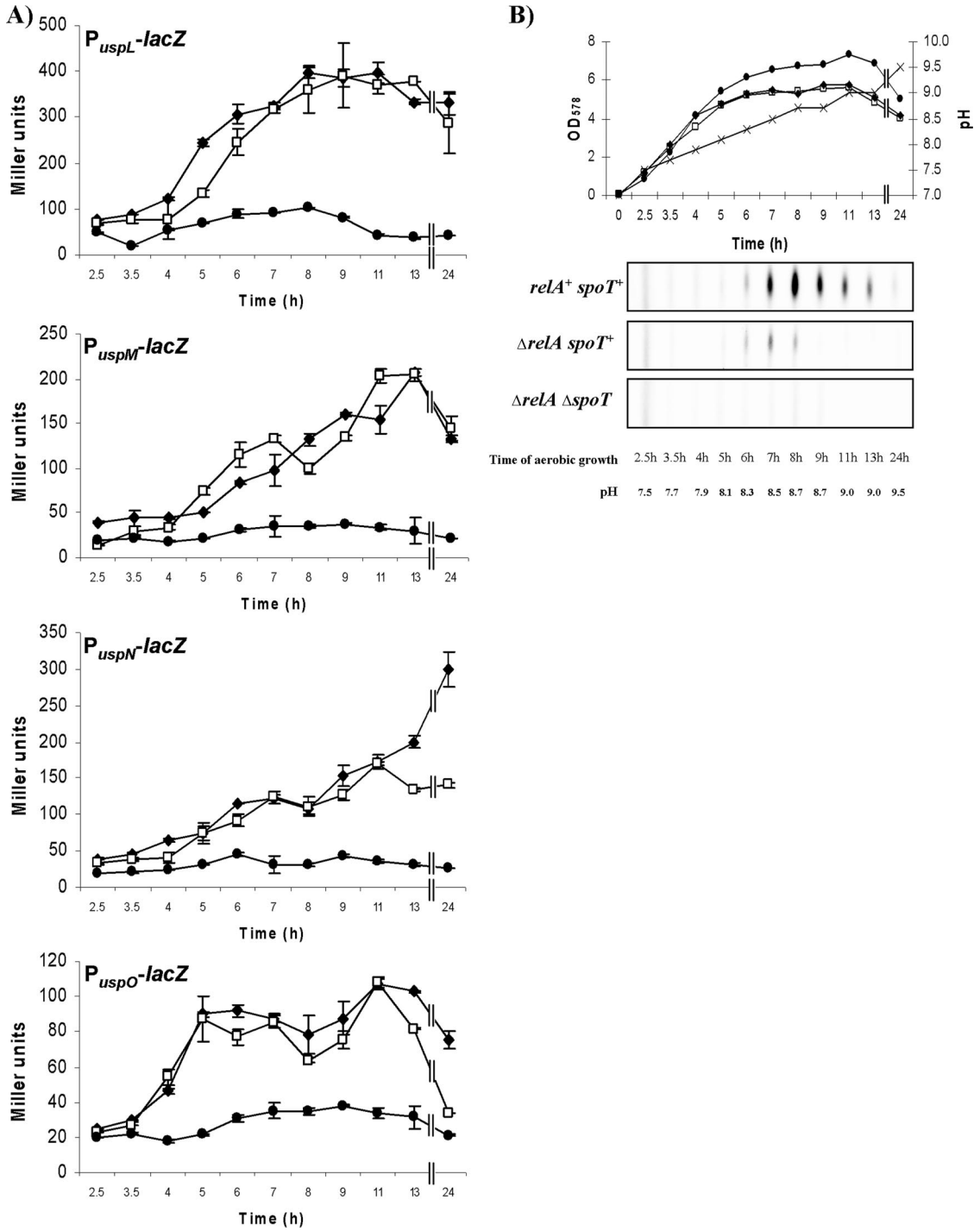

FIG. 3. β-Galactosidase activities of P_{usp}-*lacZ* (A) and accumulation of ppGpp (B) in stationary phase in various *P. aeruginosa* mutant strains. (A) β-Galactosidase activities of P_{uspL}-*lacZ*, P_{uspM}-*lacZ*, P_{uspN}-*lacZ*, and P_{uspO}-*lacZ* were determined in the following *P. aeruginosa* mutant strains: Δ*anr* mutant (filled diamonds), Δ*anr* Δ*relA* double mutant (open squares), and Δ*anr* Δ*relA* Δ*spoT* triple mutant (filled circles). Cells were grown in LB medium aerobically at 37°C. β-Galactosidase activities were determined every hour from exponential to stationary phase (see Materials and Methods). All experiments were repeated three times; standard deviations are indicated. (B) Growth curves, pH of cultures, and ppGpp accumulation shown as an example of *P. aeruginosa* strains harboring P_{uspL}-*lacZ*, described in panel A, during the transition from exponential to stationary phase. P_{uspL}-*lacZ* harboring strains NB021 (Δ*anr*) (filled diamonds), NB134 (Δ*anr* Δ*relA*) (open squares), and NB146 (Δ*anr* Δ*relA* Δ*spoT*) (filled circles) were inoculated to an initial OD_{578} of 0.05 in LB medium and were grown at 37°C aerobically to the stationary phase. pH (crosses), OD_{578}, and ppGpp accumulation were monitored from exponential phase to stationary phase. Aliquots of cultures for ppGpp determination were taken and labeled for 1 h with $^{32}P_i$. OD_{578} and pH values of cultures were determined prior to harvesting the cells for a ppGpp assay. Nucleotides were separated on a PEI cellulose-F TLC plate using 1.5 M K_2HPO_4, pH 3.6, as a solvent. Dried plates were exposed to a K-Screen (Kodak) and analyzed by a phosphorimager (FX-Scanner; Bio-Rad).

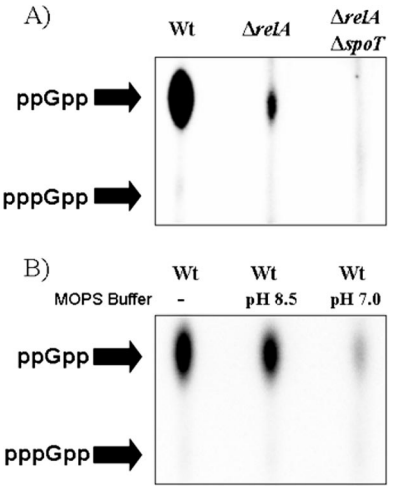

FIG. 4. Accumulation of ppGpp in *P. aeruginosa* during stationary phase confirmed by TLC. Cells were grown to stationary phase (OD_{578} of 5.0 to 7.0) and were labeled with $^{32}P_i$ for 1 h. Nucleotides were separated on a PEI cellulose-F TLC plate using 1.5 M K_2HPO_4, pH 3.6, as a solvent. Dried plates were exposed to a K-Screen (Kodak) and analyzed by a phosphorimager (FX-Scanner; Bio-Rad). (A) ppGpp accumulation in *P. aeruginosa* wild type, Δ*relA*, and Δ*relA* Δ*spoT* during stationary phase in LB medium. (B) ppGpp accumulation in *P. aeruginosa* wild type grown in unbuffered LB medium or LB medium buffered at pH 8.5 or pH 7.0. Wt, wild type.

ment showed only low to undetectable levels of ppGpp. A pH shift from 7.5 to 9 was followed by accumulation of ppGpp in the wild type (Fig. 5), supporting our assumption that high pH values do induce the stringent response in *P. aeruginosa*. Again, no corresponding accumulation of the precursor pppGpp could be detected, indicating a SpoT-controlled accumulation of ppGpp (see discussion). To demonstrate the difference between RelA- and SpoT-controlled accumulation of ppGpp, we induced the stringent response using SHX, commonly used to simulate amino acid limitation and to initiate a RelA-controlled accumulation of ppGpp. In this case we observed accumulation of both the precursor pppGpp and ppGpp (Fig. 5).

Involvement of alternative sigma factors in ppGpp-mediated gene expression of the $P_{uspN\Delta Anr}$-*lacZ* reporter gene. Positive effects of ppGpp on gene expression can be mediated by the housekeeping sigma factor σ^{70} (38) or by the increased

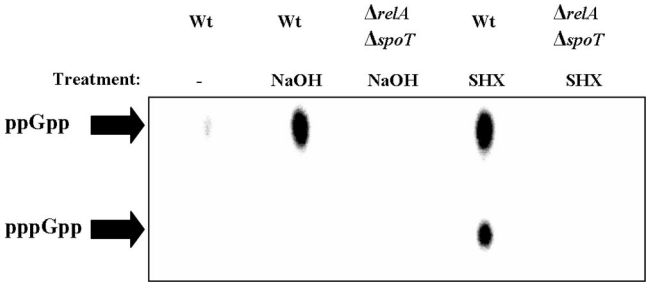

FIG. 5. Accumulation of ppGpp and pppGpp induced by either NaOH or SHX during exponential growth phase. Cells were grown anaerobically to exponential growth phase (OD_{578} of 0.5) and were labeled with $^{32}P_i$ for 15 min prior to treatment with NaOH (20 mM; pH 9) or SHX (3 mM) as indicated. Nucleotides were separated as described in Materials and Methods. Wt, wild type.

TABLE 2. β-Galactosidase activities of $P_{uspN\Delta Anr}$-*lacZ* in diverse mutants deficient in given putative sigma factors or regulatory components

Genotype of parent strain[a]	Description or function of disrupted gene[b]	β-Galactosidase activity (Miller units) with $P_{uspN\Delta Anr}$-*lacZ* in stationary phase[c]
Wild type		280.0 (± 20.0)
ΔPA0762 (Δ*algU*)	Sigma factor AlgU	500.6 (± 35.0)
ΔPA1455 (Δ*fliA*)	Sigma factor FliA	472.9 (± 8.0)
ΔPA2426 (Δ*pvdS*)	Sigma factor PvdS	437.3 (± 20.0)
ΔPA4462 (Δ*rpoN*)	Sigma 54 factor/sigma factor RpoN	329.5 (± 27.9)
ΔPA3622 (Δ*rpoS*)	Sigma factor RpoS	425.7 (± 6.6)
ΔPA0472 (Δ*fiuI*)	Sigma factor FiuI	376.6 (± 25.8)
ΔPA0149	Probable sigma 70 factor, ECF subfamily	441.5 (± 5.9)
ΔPA1912	Probable sigma 70 factor, ECF subfamily	372.3 (± 11.3)
ΔPA2050	Probable sigma 70 factor, ECF subfamily	434.3 (± 29.9)
ΔPA2896	Probable sigma 70 factor, ECF subfamily	399.1 (± 10.7)
ΔPA0934/ΔPA5338 (Δ*relA* Δ*spoT*)	GTP pyrophosphokinase/ guanosine-3′,5′-bis (diphosphate) 3′-pyrophosphohydrolases	60.3 (± 2.5)

[a] $P_{uspN\Delta Anr}$-lacZ was integrated in transposon mutant strains from the *P. aeruginosa* PA14 Insertion Mutant Library (27) in the wild-type and in the constructed Δ*relA* Δ*spoT* strain (this study).
[b] ECF, extracytoplasmic function.
[c] Background β-galactosidase activity of the donor vector mini-CTX-*lacZ* was determined to be 70.2 (± 14.5) Miller units in the wild type.

competitiveness of alternative sigma factors during the stringent response (23). In order to identify alternative sigma factors mediating ppGpp-dependent gene expression of P_{uspN} in stationary phase, we tested 10 transposon mutants of putative sigma factors (Table 2) using strains from the *P. aeruginosa* PA14 Insertion Mutant Library (27). To monitor exclusively the stringent response-activated promoter of *uspN* in strains from the PA14 Insertion Mutant Library, it was again necessary to exclude gene expression induced by Anr of P_{uspN}-*lacZ*. Previously, we showed that the mutation of the Anr binding site in P_{uspN} did not influence stationary-phase gene expression (6). Hence, we used a mutated promoter of *uspN*, designated $P_{uspN\Delta Anr}$, with a nonfunctional Anr box (6). We introduced $P_{uspN\Delta Anr}$-*lacZ* in a wild-type strain, in a Δ*relA* Δ*spoT* double mutant, and in various transposon mutant strains listed in Table 2. We observed a loss of activation of $P_{uspN\Delta Anr}$-*lacZ* in stationary phase in the Δ*relA* Δ*spoT* double mutant, confirming a stringent response dependence of $P_{uspN\Delta Anr}$-*lacZ* (Table 2). All tested transposon mutants showed induction patterns of $P_{uspN\Delta Anr}$-*lacZ* in stationary phase similar to the pattern of the wild type, indicating that none of the tested sigma factors was acting with ppGpp in stationary phase to coregulate induction of P_{uspN}.

DISCUSSION

Expression of *usp* genes in *P. aeruginosa*. Expression of genes encoding the universal stress proteins UspK, UspL,

UspM, UspN, and UspO in response to oxygen limitation has been observed using transcriptome analysis for *P. aeruginosa* under microaerobic conditions and in biofilm-grown cells (1, 28, 54). Extensive phenotypic screenings of *P. aeruginosa uspL*, *uspM*, and *uspO* mutants in our laboratory revealed no stress-related phenotype similar to *usp* mutants in *E. coli* or anaerobic phenotypes as described for *uspK* or *uspN* mutants (6, 44) in *P. aeruginosa* (data not shown). Nevertheless, we found identical transcriptional control of the *uspL*, *uspM*, and *uspO* promoters compared to the *uspN* promoter. Using promoter-*lacZ* fusions, we demonstrated Anr-dependent gene expression of *uspL*, *uspM*, and *uspO* under anaerobic conditions, similar to *uspN* (Fig. 2), confirming bioinformatics predictions of Anr binding sites in the corresponding promoter regions (Fig. 1). Usp-type stress proteins were also reported to be produced under oxygen-limiting conditions in *Mycobacterium* (36), indicating that Usps might be important for adaptation to anaerobic environments in other pathogenic bacterial species.

We observed increased activities of the tested *usp* promoter-*lacZ* fusions P_{uspL}-*lacZ*, P_{uspM}-*lacZ*, and P_{uspO}-*lacZ* in stationary phase that were similar to the results for P_{uspN}-*lacZ*. We showed that the expression of these genes is dependent on the presence of RelA and SpoT, the two regulatory components of the stringent response. The positive control by pppGpp and ppGpp has also been reported for *uspA*, *uspC*, *uspD*, and *uspE* in *E. coli* (20, 25) and *uspA3* in *Corynebacterium glutamicum* (7). In *E. coli* regulation of *usp* gene expression by the stringent response seems plausible since the corresponding *usp* mutant phenotypes indicate that Usps contribute to survival in response to a variety of different stress conditions (5, 20, 34, 35). A recent publication indicates that *E. coli uspA* is additionally regulated by fructose-6-phosphate, which points to additional levels of regulation via metabolic intermediates (39). Whether metabolic intermediates also regulate *usp* gene expression in *P. aeruginosa* is an interesting question. Although a broad range of phenotypes has been described for *usp* deletion strains of *E. coli* and *P. aeruginosa*, the exact biological function of Usps remains unknown. It is also interesting that phenotypic characterization of *P. aeruginosa uspN* and *uspK* mutants indicates that these proteins are essential for surviving specific stress conditions. In contrast, *E. coli* Usp-type proteins contribute to survival in response to more universal stress conditions. This specific role of the *P. aeruginosa* Usps might also explain why even an extensive screening did not result in identification of a phenotype for mutants defective in *uspL*, *uspM*, or *uspO* genes although they are transcriptionally regulated in a manner similar to *uspN*.

SpoT-controlled stringent response. Depending on growth conditions and medium, a variety of regulators and sigma factors contribute to stationary-phase gene expression in *P. aeruginosa*. We showed that the oxygen-sensing Anr regulator contributes to stationary-phase gene expression in a culture incubated under aerobic conditions (6). This Anr-dependent gene expression is due to oxygen-limiting conditions caused by respiration of *P. aeruginosa* at high cell densities (9).

Published research on the stringent response in *P. aeruginosa* has focused on RelA and amino acid limitation-induced stringent response (14, 52). RelA is well studied in *E. coli*, and was described as a ribosome-associated protein that catalyzes the synthesis of pppGpp. This synthetase activity is greatly enhanced by unloaded tRNAs that enter the ribosome (56), leading to a stringent response evoked by amino acid limitation (Fig. 6A and C). However, accumulation of pppGpp and ppGpp is additionally controlled by SpoT, which is present in a number of gram-negative bacteria (30).

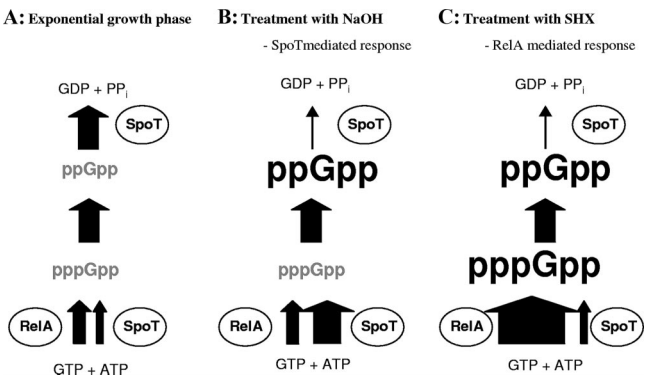

FIG. 6. Regulation of the stringent response in *P. aeruginosa* wild type during exponential growth (A) and in response to either NaOH (B) or SHX (C) treatment. Accumulation of nucleotides is indicated by bold letters, undetectable nucleotide levels are represented by gray letters, and synthesis or degradation rates are indicated by the size of arrows. Conversion of pppGpp to ppGpp is mediated by a polyphosphate kinase (Ppx). (A) ppGpp and pppGpp metabolism during exponential phase is characterized by an equal rate of pppGpp synthesis and ppGpp degradation. (B) Treatment with NaOH induces a SpoT-regulated stringent response. The accumulation of ppGpp is achieved by a decrease in the ppGpp degradation process, resulting in lower ppGpp degradation rates than synthesis rates. (C) Treatment with SHX induces mainly a RelA-mediated stringent response, resulting in increased synthesis rates of pppGpp. In this case both, pppGpp and ppGpp accumulate in the cell. However, a reduction in ppGpp degradation by SpoT was also described under these conditions in *E. coli* (41).

A SpoT-controlled stringent response appears to be more complex since SpoT was shown to respond to a broad range of stress factors and to be associated with enzymes of seemingly unrelated function. On one hand, it was shown to be linked by CgtA to ribosomes (58), and, on the other hand, it was shown to interact with the acyl carrier protein, a protein involved in fatty acid biosynthesis (3).

Several lines of evidence indicate that in our experimental setup the stringent response is triggered by SpoT and, to a minor extent, by RelA. Only a Δ*anr* Δ*relA* Δ*spoT* mutant but not a Δ*anr* Δ*relA* mutant completely lost the ability to induce *lacZ* reporter gene fusions of the *uspL*, *uspM*, *uspN*, and *uspO* promoters in aerobic stationary phase. This result is in accordance with our TLC-based ppGpp determination. We detected ppGpp accumulation in *P. aeruginosa* during stationary phase in a Δ*relA* single mutant (Fig. 3B and 4A). After 6 h of aerobic incubation, we observed similar ppGpp concentrations in the wild type and the Δ*relA* single mutant, demonstrating a ppGpp accumulation strictly accomplished by SpoT (Fig. 3B). From these results we assume that the balance between the synthetase and hydrolase activity of SpoT is shifted toward pppGpp synthesis at this time point, initiating the accumulation of ppGpp completely independent of RelA (Fig. 7, compare B and E). Further incubation in stationary phase results in a decrease in ppGpp in the wild type and a reduction to almost undetectable levels in a Δ*relA* single mutant (Fig. 7C and F).

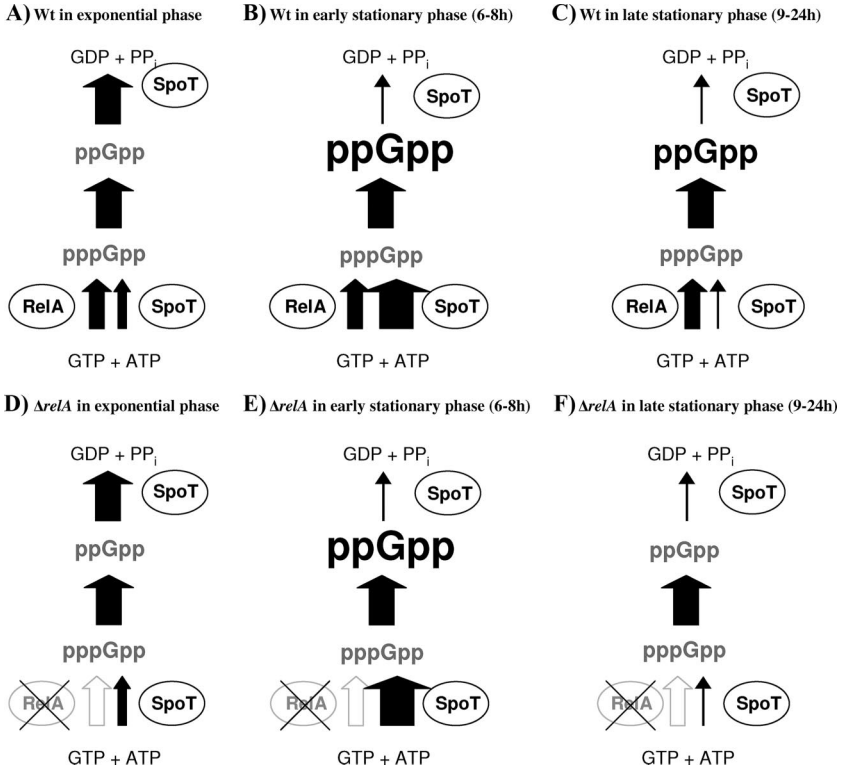

FIG. 7. Scheme of ppGpp accumulation in *P. aeruginosa* wild type (A to C) and the Δ*relA* mutant (D to F) during growth from exponential to stationary phase. Accumulation of nucleotides is indicated by bold letters, undetectable nucleotide levels are represented in gray letters, and synthesis or degradation rates are indicated by the size of arrows. During exponential growth a balance between synthesis and degradation of ppGpp is regulated by SpoT (A and D). During early stationary phase (6 to 8 h of cultivation) (compare Fig. 3 B), SpoT increases its synthesis activity and decreases the degradation rates for ppGpp (indicated by arrow size), resulting in the accumulation of ppGpp (bold letters) (B and E). In late stationary phase, pppGpp synthesis by SpoT ceases, resulting in the accumulation of ppGpp, which is dependent on a complex interplay of the pppGpp synthesis rates of RelA and diminished degradation activity of SpoT in the wild type (C and F). Loss of SpoT-dependent pppGpp synthesis results in levels of ppGpp that are lower in late stationary phase than in early stationary phase, indicated by letter size (compare with Fig. 3B).

This observation correlates with the functionally unstable character of SpoT synthetase activity shown in *E. coli* (33). Murray and Bremer reported that SpoT synthetase activity is dependent on continuous protein biosynthesis since synthetase activity of SpoT in *E. coli* has an average functional lifetime of about 40 s or less after translation of *spoT* mRNA, whereas the ppGpp degradation activity and its control are independent of the SpoT protein's lifetime. Taking into account that translation is generally downregulated during stationary phase, it is plausible that ppGpp accumulation decreases over the course of time during stationary phase because of the loss of SpoT-dependent synthetase activity (compare Fig. 7B and C). Prolonged cultivation during stationary phase results in higher ppGpp levels in the wild type than in the Δ*relA* mutant. This observation is clearly dependent on the basic production of pppGpp by RelA, which is consequently absent in the Δ*relA* mutant.

The lowered ppGpp levels in the Δ*relA* mutant still allowed almost wild-type induction of the promoter-*lacZ* reporter gene fusions in stationary phase between 5 and 11 h of incubation (Fig. 3A). Interestingly, we observed a decrease in induction of P_{uspO}-*lacZ* and P_{uspN}-*lacZ* in the Δ*relA* mutant after 13 h of incubation.

An additional indication for a SpoT-triggered stringent response during stationary phase is the increase in ppGpp but not pppGpp levels. A RelA-triggered stringent response usually results in ppGpp and pppGpp accumulation (Fig. 6C). This is caused by a massive increase in pppGpp synthesis activity by RelA itself and additionally by a reduction in ppGpp degradation by SpoT (Fig. 6C) (41). In a SpoT-triggered stringent response, SpoT itself is presumed to control ppGpp levels mainly on the level of ppGpp degradation since synthesis activity is unstable in the absence of protein biosynthesis (18, 33). This, in turn, leads to the accumulation of ppGpp and not its precursor pppGpp. We observed the accumulation of ppGpp alone in stationary phase (Fig. 4A), while a RelA-controlled stringent response induced by SHX results clearly in an accumulation of both ppGpp and pppGpp (Fig. 5 and 6C).

Alkaline pH elicits a SpoT-controlled stringent response. We observed an increase in pH up to 8.3 at the end of the exponential growth phase and usually measured pH values of 8.5 in early stationary phase, as previously described by others (21). The following two experiments indicated that alkaline pH is the trigger for SpoT-controlled ppGpp accumulation in stationary phase. First, ppGpp levels in LB medium buffered at pH 7.0 were drastically reduced compared to levels in LB medium buffered at pH 8.5 or unbuffered LB medium (pH 8.7) (Fig. 4B). Second, we also demonstrated clearly that a shift to

alkaline pH induces an accumulation of ppGpp during exponential growth (Fig. 5). Induction of ppGpp accumulation during exponential growth in response to alkaline pH is not limited to LB medium but could also be initiated in a defined minimal medium containing succinate as the sole carbon source (see Fig. S2 in the supplemental material). The accumulation of ppGpp but not the precursor pppGpp in response to alkaline pH again indicated a SpoT-controlled accumulation of ppGpp (Fig. 5 and 6B).

Induction of the stringent response as a consequence of a pH shift was also reported for *Helicobacter pylori* (31, 55). Despite the fact Wells et al. (55) used acidic pH stress to induce the stringent response in *H. pylori*, they observed the same accumulation pattern for only ppGpp and not the precursor pppGpp, as we showed with alkaline stress in *P. aeruginosa*. The mechanism triggering the accumulation of ppGpp in response to pH stress might be linked to the pH-dependent perturbation of the proton gradient since treatment with the protonophor carbonyl cyanide *m*-chlorophenylhydrazone has also been shown to induce a SpoT-regulated stringent response by a reduction of the rate of ppGpp degradation in *E. coli* (50). Nevertheless, the capability of SpoT to detect and respond to a variety of apparently unrelated stress factors remains a highly complex and greatly unsolved network, which now can be additionally linked to environmental alkaline pH.

Stringent response-mediated induction of $P_{uspN\Delta Anr}$ in stationary phase is independent of 10 tested alternative sigma factors. Gene expression by ppGpp occurs either directly by an interplay of DksA, a transcriptional cofactor of stringent response, and the housekeeping sigma factor σ^{70} (38) or indirectly by an increase in competitiveness of other alternative sigma factors for the core of RNA polymerase (2). Additionally, expression of genes transcribed by alternative sigma factors like RpoS and RpoN has been shown to be dependent on the presence of ppGpp (24, 49). In order to identify sigma factors involved in ppGpp-mediated induction of $P_{uspN\Delta Anr}$ in stationary phase, we screened for reduced β-galactosidase activities in diverse transposon mutants deficient in known alternative sigma factors (*algU*, *fliA*, *pvdS*, *fiuI*, *rpoN*, and *rpoS*) or putative sigma factors (PA0149, PA1912, PA2050, and PA2896) (for a review, see reference 40). All tested mutant strains showed similar or even higher expression levels of $P_{uspN\Delta Anr}$-*lacZ* than the wild type, which excluded the tested sigma factors as essential cofactors in ppGpp-mediated induction of $P_{uspN\Delta Anr}$-*lacZ*. That the expression values of $P_{uspN\Delta Anr}$ in the tested mutant strains (329 to 500 Miller units) (Table 2) were higher than the wild type (280 Miller units) supports the assumption that a different sigma factor is controlling P_{uspN} in concert with ppGpp. From these results we assume that ppGpp induction of P_{uspN} might be directly mediated by σ^{70} and DksA. However, we cannot exclude the possibility that other unknown regulatory components or sigma factors coregulate ppGpp-mediated induction of $P_{uspN\Delta Anr}$ during stationary phase. This question is currently under investigation in our laboratory.

ACKNOWLEDGMENTS

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