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RpoS Is Necessary for Both the Positive and Negative Regulation of Starvation Survival Genes during Phosphate, Carbon, and Nitrogen Starvation in *Salmonella typhimurium*

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The starvation stress response of *Salmonella typhimurium* encompasses the genetic and physiologic changes that occur when this bacterium is starved for an essential nutrient such as phosphate (P), carbon (C), or nitrogen (N). The responses to the limitation of each of these nutrients involve both unique and overlapping sets of proteins important for starvation survival and virulence. The role of the alternative σ factor RpoS in the regulation of the starvation survival loci, *stiA*, *stiB*, and *stiC*, has been characterized. RpoS (σ^S) was found to be required for the P, C, and N starvation induction of *stiA* and *stiC*. In contrast, RpoS was found to be required for the negative regulation of *stiB* during P and C starvation-induced stationary phase but not during logarithmic phase. This role was independent of the *relA* gene (previously found to be needed for *stiB* induction). The role of RpoS alone and in combination with one or more *sti* mutations in the starvation survival of the organism was also investigated. The results clearly demonstrate that RpoS is an integral component of the complex interconnected regulatory systems involved in *S. typhimurium*'s response to nutrient deprivation. However, differential responses of various *sti* genes indicate that additional signals and regulatory proteins are also involved.

The starvation stress response (SSR) of *Salmonella typhimurium*, and other enteric bacteria, encompasses the genetic and physiologic changes that occur upon starvation for an essential nutrient, e.g., phosphate (P), carbon (C), or nitrogen (N). The SSR can be subdivided into the P starvation, C starvation, and N starvation stress responses, each of which involves unique and overlapping genes and proteins (34–37, 42–44, 46; reviewed in references 24, 25, 41, and 45). Many of the components of the SSR are also important in the bacterial response to (and survival during) other environmental stresses such as acid pH, oxidative stress, osmotic stress, heat shock, and anaerobiosis (2, 9, 10, 16, 18, 33, 37, 43, 45). The importance of the SSR becomes evident when we consider that many of the conditions that salmonellae encounter, as they move through various microenvironments within their hosts and natural aquatic and terrestrial microcosms, are frequently limiting for bacterial growth in terms of the availability of essential nutrients (14, 19, 48). Evidence from several laboratories indicates that *S. typhimurium* encounters microenvironments within the host (e.g., within host macrophages) that induce both unique and overlapping sets of stress-inducible proteins. Several of these proteins are regulated by P, C, and/or N starvation (1, 3, 7, 8, 29, 49). Consequently, the ability of *Salmonella* spp. to respond to and survive periods of nutrient deprivation can have a profound influence on the epidemiology and pathogenesis of disease caused by salmonellae.

To address the question of how *S. typhimurium* responds to and survives starvation conditions, we have identified a number of P starvation-inducible (*psi*), C starvation-inducible (*csi*), and multiple-nutrient starvation-inducible (*sti*) loci by Mu d-directed *lac* fusion techniques (11, 38, 42, 44, 46). Three of the *sti* loci identified by this method, *stiA*, *stiB*, and *stiC*, are essential for bacterial survival during simultaneous P, C, and N starvation (PCN starvation). Isogenic strains carrying single mutations in any one of these three loci exhibit 50- to 75-fold-reduced levels of survival compared with wild type strains. Moreover, double *sti* mutants exhibit 500- to 2,000-fold-lower levels of survival, indicating a kind of synergistic relationship among these three loci (44). The expression of these loci exhibits a complex interconnected network of regulation involving positive control by the *relA* locus (i.e., guanosine tetraphosphate [ppGpp]) (4) and negative control by the *crp* gene (i.e., cyclic AMP [cAMP] receptor protein [22]) (44). Interestingly, the regulation of the *stiB* locus (unlike that of *stiA* and *stiC*) was unaffected by a *cya* mutation, suggesting that cAMP receptor protein acts alone or with a different signal molecule in the repression of *stiB* (44).

Recently, the alternative sigma factor RpoS (also called KatF or σ^S [30, 47]) has been implicated in the regulation of genes and proteins during C starvation and stationary phase following logarithmic growth in rich medium (8, 20a, 24, 26, 27, 49). We report here the role of RpoS in the regulation of the starvation survival genes *stiA*, *stiB*, and *stiC* during P, C, and/or N starvation. We also characterize the relationship between the *rpoS* gene and the *stiA*, *stiB*, and *stiC* loci in the long-term starvation survival of *S. typhimurium*.

MATERIALS AND METHODS

Bacterial strains and phage used. The bacterial strains used in this study are derivatives of *S. typhimurium* SL1344 or JF235 and are listed in Table 1. Transductions were performed with

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TABLE 1. List of bacterial strains used in this study

Strain	Genotype (pertinent phenotype[a])	Source or reference
SL1344	*hisG46*	B. B. Finlay (15)
SF1005	14028s *katF* (*rpoS*)::Amp^r	8
SMS438	SL1344 *rpoS*::Amp^r	This study
ST66	SL1344 *stiA1*::MudJ (*lac* Kan^r)	This study
SMS465	ST66 *rpoS*::Amp^r	This study
ST67	SL1344 *stiB2*::MudJ (*lac* Kan^r)	This study
SMS466	ST67 *rpoS*::Amp^r	This study
SMS482	SMS465 *relA21*::Tn*10* (Tet^r)	This study
SMS523	ST67 *relA21*::Tn*10* (Tet^r)	This study
ST68	SL1344 *stiC4*::MudJ (*lac* Kan^r)	This study
SMS467	ST68 *rpoS*::Amp^r	This study
JF235	Δ*nadA100*	J. W. Foster
SMS470	JF235 *rpoS*::Amp^r	This study
JF807	JF235 Δ*stiA18*	44, 46
SMS471	JF807 *rpoS*::Amp^r	This study
JF1142	JF235 Δ*stiB13*	44, 46
SMS472	JF1142 *rpoS*::Amp^r	This study
JF1145	JF235 Δ*stiC15*	44, 46
SMS473	JF1145 *rpoS*::Amp^r	This study
SMS392	JF235 Δ*stiA18 stiB2*::MudJ (*lac* Kan^r)	44
SMS474	SMS392 *rpoS*::Amp^r	This study
SMS393	JF235 Δ*stiA18 stiC4*::MudJ (*lac* Kan^r)	44
SMS475	SMS393 *rpoS*::Amp^r	This study
SMS399	JF235 Δ*stiB13 stiC4*::MudJ (*lac* Kan^r)	44
SMS476	SMS399 *rpoS*::Amp^r	This study

[a] Amp^r, ampicillin resistance; Kan^r, kanamycin resistance; Tet^r, tetracycline resistance.

the high-transducing derivative of *S. typhimurium* bacteriophage P22, P22 HT 105/1 *int* (HT phage) (5). In all cases, the strains used in this study were determined to be nonlysogens for P22 phage by growth on green indicator agar plates (6) and by sensitivity to the H5 derivative of P22 phage (23).

Culture media and antibiotics used. The minimal media used in this study were modifications of a minimal MOPS (morpholinepropanesulfonic acid)-buffered salts (MS) medium (32) and were described in detail previously (44, 46). Nicotinic acid and histidine were added to minimal media, as needed, at a final concentration of 0.1 mM. Rich media used included Luria-Bertani (LB) broth and agar (6).

Antibiotics were used, as needed, at the following final concentrations: 30 μg of ampicillin ml^{-1}, 10 (minimal medium) or 20 (rich medium) μg of tetracycline ml^{-1}, or 100 μg of kanamycin ml^{-1}.

Confirmation of regulatory mutant phenotypes. Modified SMG medium (39) was used to confirm the *relA* mutant phenotype. The *rpoS* mutant phenotype was confirmed qualitatively by mixing a colony of the desired strain from an LB agar plate with 50 μl of 10% H_2O_2 and observing for the evolution of oxygen (bubbling) (31).

Starvation induction assays and kinetics of expression. Starvation induction and induction kinetics were measured as previously described (44). Briefly, overnight cultures grown in nonlimiting minimal medium (MS hiPCN [see below]) were washed and resuspended in MS buffer (no P, C, or N sources) and then were used to inoculate (i) nonlimiting PCN (MS hiPCN [25 mM KH_2PO_4/K_2HPO_4, pH 7.4–0.4% glucose–10 mM NH_4Cl]), (ii) limiting P (MS loP [0.113 mM KH_2PO_4/K_2HPO_4]), (iii) limiting C (MS loC [0.025% glucose]), and (iv) limiting N (MS loN [1 mM NH_4Cl]) media to an A_{600} of ca. 0.03 (time zero). Cultures were incubated at 37°C with aeration. At specified time intervals, growth was monitored by A_{600}, and 0.1- or 0.5-ml aliquots were removed and assayed for β-galactosidase activity by the method of Miller (28).

Starvation survival assays. Long-term starvation survival was assayed as previously described (44). Briefly, overnight cultures were washed, resuspended, and used to inoculate 3 ml of MS loPCN medium (1 mM KH_2PO_4/K_2HPO_4, pH 7.4–0.25% glucose–10 mM NH_4Cl) to ca. 4×10^7 to 5×10^7 CFU ml^{-1}. This culture was then grown with aeration at 37°C to ca. 4×10^8 CFU ml^{-1}. At this point, 1 ml of this culture was diluted in 9 ml of MS buffer. At specified time intervals, aliquots of the culture were removed, immediately serially diluted in MS buffer, and plated onto LB agar plates plus antibiotic, as needed, to determine viable plate counts. Time zero was the point at which the culture entered starvation-invoked stationary phase (cell density of ca. 3×10^8 CFU ml^{-1}). Survival was calculated as the percentage of the maximum cultural viability achieved.

RESULTS

RpoS is required for *stiA* and *stiC* induction during phosphate, carbon, and nitrogen starvation. The alternative σ factor RpoS, or σ^S, is essential for the development of starvation-induced general resistance to a variety of stresses in both *Escherichia coli* and *S. typhimurium* (8, 18, 20a, 24). Therefore, since the *stiA* and *stiC* loci are required for starvation survival and both are induced during starvation for phosphate, carbon, and nitrogen (44, 46), we wanted to determine if RpoS plays a role in the regulation of these two starvation survival genes. To accomplish this, an *rpoS*::Amp^r insertion mutation (8) was transduced into each of the *sti*::MudJ (*lac* Kan^r) (*sti-lac*) transcriptional fusion-containing strains. Prior to transduction, complementation of the *rpoS* mutation with a cloned wild-type *rpoS* gene on a ColE1 plasmid vector was performed. The cloned *rpoS* gene restored full *spvB* expression as measured from the *spvB-lac* translational fusion on plasmid pFF14 (7), and the gene also restored resistance to low pH and hydrogen peroxide exposure to wild-type levels (data not shown).

Figure 1 illustrates the results from subsequent starvation induction assays with the wild-type parent and the *rpoS*::Amp^r derivatives of the *stiA-lac* and *stiC-lac* fusion strains. As can be seen in Fig. 1, the *rpoS* mutation eliminated the normal induction of these loci during C and N starvation. Interestingly, the *rpoS* mutation also eliminated the normal P starvation induction of these two loci. This supports recent findings demonstrating that RpoS levels increase during phosphate limitation and provides direct evidence that RpoS is involved in phosphate starvation-regulated gene expression. Moreover, the *rpoS* mutation is the first mutation shown to affect the P starvation induction of these *sti* loci. We previously reported that the P starvation induction of these genes is *phoP* independent (13, 44). The *phoB* and *phoR* homologs of *S. typhimurium* have not been identified, so a role for these genes has not been determined. Curiously, expression of the *stiC-lac* fusion was still slightly induced during P and N starvation (but not C starvation) in an *rpoS* background. This suggests that regulatory mechanisms, in addition to RpoS, are involved in *stiC* (but not *stiA*) expression during P or N starvation. Nonetheless, it is clear from these results that RpoS is involved in the positive regulation of both *stiA* and *stiC* during C, N, and P starvation.

RpoS functions in the negative regulation of *stiB*. The *stiB* locus is also required for the starvation survival of *S. typhimurium* and is induced during the transition from log phase to P or C starvation-induced (but not N starvation-induced) stationary phase (44). Thus, we wanted to determine if RpoS also plays a role in the regulation of this starvation survival

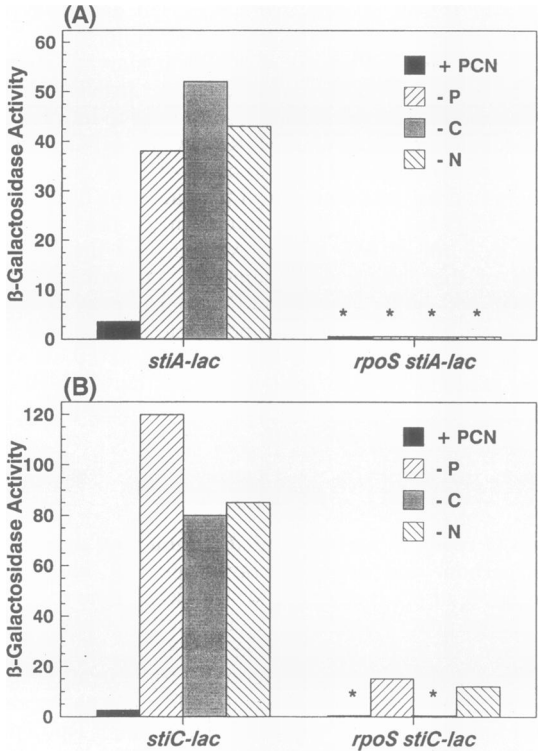

FIG. 1. Role of the alternative σ factor RpoS, or σ^S, in the regulation of the starvation survival genes *stiA* and *stiC*. $rpoS^+$ and *rpoS* derivatives of strains carrying either *stiA-lac* (ST66 and SMS465) (A) or a *stiC-lac* (ST68 and SMS467) (B) fusion were grown and treated as described in Materials and Methods in nonlimiting (MS hiPCN [+PCN]), limiting P (MS loP [−P]), limiting C (MS loC [−C]), or limiting N (MS loN [−N]) medium. β-Galactosidase activity was measured at several predetermined intervals throughout log phase and starvation-induced stationary phase in the various starvation media. The activities shown represent the maximal level of induction achieved under each starvation condition. Maximal levels of induction for both *stiA* and *stiC* typically occur at about 4 to 6 h into starvation. β-Galactosidase was assayed at the same intervals for both the wild-type (e.g., $rpoS^+$ $relA^+$ parent) and mutant fusion-containing strains. β-Galactosidase activity is expressed in Miller units (28). The asterisks (*) indicate that β-galactosidase activity was undetectable in our assay procedure. The data presented are from a representative experiment from at least three separate trials.

locus. As illustrated in Fig. 2A, starvation induction assays performed on wild-type and *rpoS* derivatives of the *stiB-lac* fusion strain yielded an unexpected and extremely interesting finding. The *rpoS* mutation resulted in a two- to threefold-higher level of induction of *stiB* during P starvation and a five- to sixfold-higher level of induction during C starvation, suggesting that RpoS is involved in the negative regulation of the *stiB* locus.

As shown in Fig. 2B, the *rpoS* mutation does not alter the timing of *stiB* induction; it alters only the rate and level to which it is induced. Thus, RpoS appears to be involved in limiting the expression of *stiB* during P and C starvation-induced stationary phase but has no effect on basal expression during logarithmic growth in minimal media.

An *rpoS* mutation suppresses the effect of a *relA* mutation on *stiB* induction. Induction of the *stiB* locus is dependent on the *relA* gene during C starvation but not P starvation (44). However, the *rpoS* mutation was found to suppress the effect of a *relA* mutation on the C starvation-induced expression of *stiB*, because the *rpoS* mutation restored the C starvation induction of *stiB* in the presence of a *relA* null mutation (Fig. 2A). The fact that RpoS is an alternative σ factor (47) suggests that *rpoS* controls the expression of a negative regulator of *stiB*, rather than *stiB* itself. ppGpp would then be necessary to overcome the action of this putative *stiB* repressor in this model.

Effect of an *rpoS* mutation alone or in combination with *stiA*, *stiB*, and/or *stiC* mutations on survival during prolonged PCN starvation. We previously reported (44) that *stiA*, *stiB*, and *stiC* mutations, separately (50- to 75-fold) or in combination (500- to 2,000-fold), reduced survival during prolonged PCN starvation. Other investigators have reported that *rpoS* mutants are more sensitive to the effects of prolonged carbon starvation than wild-type parent strains (8, 20a, 27). Since the *stiA*, *stiB*, and *stiC* loci are regulated by RpoS, we wanted to examine the combined effects of *rpoS* and *sti* mutations on starvation survival. Results presented in Fig. 3A show that strains possessing the *rpoS* mutation alone lost viability more quickly and exhibited a 75- to 100-fold reduction in survival, after 15 to 20 days of starvation, compared with the parent strain. This supports the findings of previous studies with *E. coli* and *S. typhimurium* (8, 20a, 27), which were performed for shorter periods, and further confirms the effectiveness of our starvation protocol. However, the extent to which viability was compromised was comparable to that of $rpoS^+$ strains possessing single mutations in either the *stiA*, *stiB*, or *stiC* locus (compare Fig. 3A and C) (44).

Moreover, we found that combining an *rpoS* mutation with individual *sti* mutations produced only a slight additional effect on starvation survival compared with $rpoS^+$ *sti* mutants (compare Fig. 3A and C). Because *rpoS* mutants would presumably be defective in all *rpoS*-dependent systems, one might expect that inactivating *rpoS*-dependent genes in an *rpoS* mutant would not significantly decrease survival any further. However, this does not appear to be the case. This point is demonstrated by the finding that combining an *rpoS* mutation with pairs of *sti* mutations caused diminished starvation survival even beyond that of *rpoS* or *rpoS sti* mutants (compare Fig. 3A and B). This in itself was not that surprising, since we previously reported that *sti* double mutants are much more sensitive to long-term starvation than *sti* single mutants (45) (compare Fig. 3C and D). The extent to which *rpoS stiA stiB*, *rpoS stiA stiC*, and *rpoS stiB stiC* triple mutants lost viability was comparable to that of $rpoS^+$ *sti* double mutants (compare Fig. 3B and D). Taken as a whole, these results suggest that even basal levels of expression (levels occurring in the absence of RpoS) of the *rpoS*-dependent survival genes can contribute to starvation survival and/or, perhaps, that a separate cryptic or unknown mechanism that can partially compensate for the loss of RpoS might be involved. However, if the expression of two (or more) of these *sti* survival genes is completely abolished, then the effect on starvation survival becomes even more pronounced.

There is one other possibility as to why *rpoS* mutants survive starvation better than double *sti* mutants. This alternative explanation stems from the fact that our survival assays are performed under conditions of simultaneous P, C, and N starvation, whereas the role of RpoS in regulation was assessed under conditions of starvation for individual nutrients. Thus, the possibility that the *sti* loci are regulated differently during PCN starvation and are less RpoS dependent under these conditions existed. However, this was ruled out for two reasons: (i) *sti-lac* expression was measured under conditions which were the same as those used for our survival assays, and (ii) analogous starvation survival assays were performed under conditions in which mutants were starved for only the C

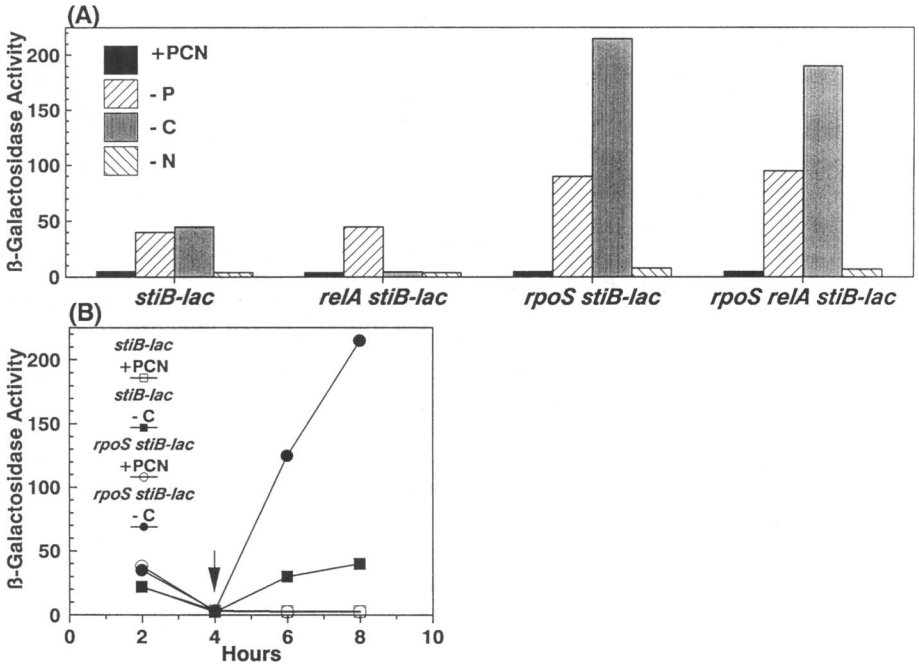

FIG. 2. Role of the alternative σ factor RpoS, or σ^S, in the regulation of the starvation survival gene *stiB*. (A) *rpoS*$^+$ and *rpoS* derivatives of a *stiB-lac* fusion strain (ST67 and SMS466) were grown and treated as described in Materials and Methods in nonlimiting (MS hiPCN [+PCN]), limiting P (MS loP [−P]), limiting C (MS loC [−C]), or limiting N (MS loN [−N]) medium. β-Galactosidase activity was measured at several predetermined intervals throughout log phase and starvation-induced stationary phase in the various starvation media. The activities shown represent the maximal level of induction achieved under each starvation condition. Maximal levels of induction for *stiB* typically occur at about 4 to 6 h into starvation. β-Galactosidase was assayed at the same intervals for both the wild-type (e.g., *rpoS*$^+$ *relA*$^+$ parent) and mutant fusion-containing strains. (B) *rpoS*$^+$ and *rpoS* derivatives of a *stiB-lac* fusion strain were grown and treated as described in Materials and Methods in MS hiPCN (+PCN) or MS loC (−C) medium. The arrow indicates the point at which the limiting C culture stopped growing because of exhaustion of glucose. β-Galactosidase activity is expressed in Miller units (28). The data presented are from a representative experiment from at least three separate trials.

source. The results suggest that starving for P, C, and N sources simultaneously is not significantly different from starving for only the carbon source in terms of (i) the regulation of *stiA*, *stiB*, or *stiC* or (ii) the effects of mutations in these loci on starvation survival (data not shown).

Results similar to those presented in Fig. 3C and D were previously reported (44) and are included here for the purpose of comparison. The growth rates of *rpoS* mutants with and without one or more *sti* mutations were not significantly different from each other during logarithmic growth (data not shown).

DISCUSSION

The data presented indicate that the alternative σ factor RpoS has a central role in both the positive and the negative regulation of starvation-inducible gene expression in *S. typhimurium*. RpoS, or σ^S, typically functions as a positive regulator redirecting the selectivity of core RNA polymerase (E) complexes for specific promoters (47), e.g., starvation-inducible promoters and stationary-phase-inducible promoters. This appears to be the case for *stiA* and *stiC*.

RpoS has been implicated in the regulation of C and N starvation-inducible gene expression in *E. coli* (20a, 27) and C starvation-inducible gene expression in *Salmonella* spp. (8, 38, 49). However, this is the first report to directly demonstrate the involvement of RpoS in P starvation-inducible gene expression. A role for RpoS in P starvation-inducible gene expression is supported by the recent finding that RpoS itself is induced during P starvation (12). Although RpoS is required for the P, C, and N starvation induction of *stiA* and *stiC*, separate additional signals appear to be involved in the response to individual starvation conditions as well as other conditions that induce RpoS-dependent genes. For example, we have identified *rpoS*-dependent genes induced during P and C starvation (but not N starvation) as well as *rpoS*-dependent genes induced only during C starvation (38). Moreover, *stiA* and *stiC* are not induced during the transition from logarithmic phase to stationary phase in rich medium (LB) or nonlimiting minimal medium (MS hiPCN) (44, 46), while many other *rpoS*-regulated genes are induced under these conditions (21, 31, 40). Therefore, it seems likely that $E\sigma^S$ holoenzyme complexes interact with signals or regulatory components unique to a specific condition(s), e.g., P or C starvation or transition to stationary phase following logarithmic growth in LB medium. Furthermore, there are genes induced during these conditions that do not require RpoS for their induction (38, 47). Thus, the signals generated or regulatory proteins functioning under these conditions may also interact with other RNA polymerase holoenzymes, e.g., $E\sigma^{70}$ holoenzymes (47). One thing is clear from the complexity of SSR gene regulation: one must exercise caution when trying to characterize so-called stationary-phase gene expression because many environmental and growth conditions can result in stationary phase without necessarily causing the induction of certain genes.

One possible signal molecule is ppGpp (synthesized by the *relA* gene product [4]), which we previously found to be required for the induction of *stiA* and *stiC* during C and N

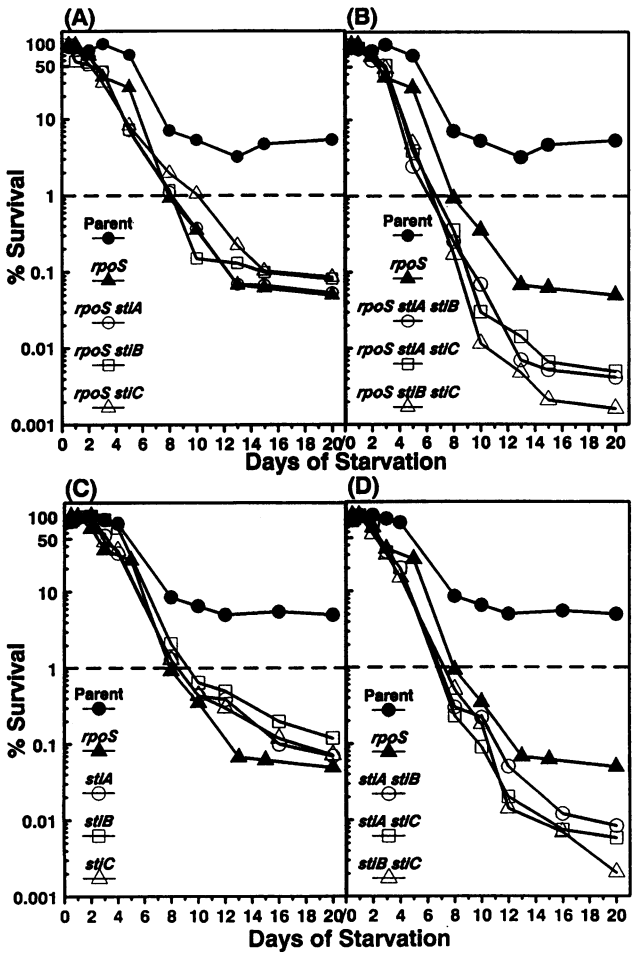

FIG. 3. Starvation survival of strains carrying an *rpoS* mutation in combination with one or two *sti* mutations. Desired strains were grown and starved by dilution in MS buffer as described in Materials and Methods. Maximum survival was measured between 12 and 48 h following entry into starvation-induced stationary phase (day 0) for each culture. Subsequent viability determinations of each culture were expressed as percentages of the maximum viability for that culture (% survival). The same parent strain (JF235) and *rpoS* mutant strain (SMS470) were used in panels A through D. (A) SMS471 (*rpoS stiA*), SMS472 (*rpoS stiB*), SMS473 (*rpoS stiC*). (B) SMS474 (*rpoS stiA stiB*), SMS475 (*rpoS stiA stiC*), SMS476 (*rpoS stiB stiC*). (C) JF807 (*stiA*), JF1142 (*stiB*), JF1145 (*stiC*). (D) SMS392 (*stiA stiB*), SMS393 (*stiA stiC*), SMS399 (*stiB stiC*). The data presented are from a representative experiment from at least three separate trials.

starvation (but not P starvation) (44). Thus, a model whereby ppGpp interacts directly with $E\sigma^S$ holoenzyme complexes to further alter or perhaps strengthen their interaction with certain promoters, e.g., *sti* promoters, can be postulated. However, a recent report by Gentry et al. (12) demonstrating that ppGpp positively regulates RpoS synthesis during C, P, or amino acid starvation in *E. coli* can also explain why *relA* null mutants are defective in *stiA* and *stiC* induction during C and N starvation, assuming that a similar phenomenon occurs in *S. typhimurium*. The findings that (i) ppGpp levels apparently do increase during P starvation, (ii) RpoS is induced during P starvation in a ppGpp-dependent manner, and (iii) the P starvation induction of *stiA* and *stiC* is independent of *relA* but is still *rpoS* dependent suggest an interesting scenario. ppGpp has been shown to be synthesized by both the *relA* and *spoT* gene products (4). With this in mind, a model for the *spoT*-dependent, *relA*-independent accumulation of ppGpp during P starvation can be postulated. Thus, the ppGpp-dependent induction of RpoS, and consequently RpoS-dependent genes, would be *spoT* dependent during P starvation and *relA* dependent during C starvation. The *spoT*-dependent, *relA*-independent accumulation of ppGpp has been demonstrated to occur in response to fatty acid deprivation in *E. coli* (40a). In that study the researchers postulated a link between fatty acid starvation and carbon starvation in terms of the signal transduction mechanisms responsible for ppGpp accumulation. However, this link is based on reports that ppGpp accumulation during C starvation is *relA* independent but *spoT* dependent (4). Our studies seem to contradict the *relA* independence of ppGpp accumulation during C starvation since the C starvation induction of *stiA*, *stiB*, and *stiC* is *relA* dependent (44). Therefore, the respective roles of RelA and SpoT in the accumulation of ppGpp under various starvation and stress conditions requires further study.

Another interesting and unexpected finding reported here is that *stiB* is overexpressed during P and C starvation (but not during log phase) in an *rpoS* genetic background. This suggests, given its function as a σ factor, that RpoS may act indirectly by positively regulating another gene which negatively regulates *stiB* expression during P and C starvation. This putative repressor would not function during log phase; the repression of *stiB* during log phase is mediated by cAMP receptor protein (22) in a cAMP-independent manner (44). The function of this putative *stiB* repressor appears to be to limit the level of induction of *stiB* under inducing conditions. The reason for the need to limit *stiB* expression is under investigation.

As with *stiA* and *stiC*, the induction of *stiB* is dependent on the *relA* gene product during C starvation (but not P starvation) (44). However, we report here that an *rpoS* null mutation can suppress the effect of a *relA21*::Tn*10* insertion mutation (39) on *stiB* expression. A possible explanation is that, as proposed above, RpoS may be required for the expression of a putative *stiB* repressor that limits the expression of *stiB* during C starvation. ppGpp would then be needed to relieve the effect of this putative repressor on *stiB* expression during C starvation. Thus, in an *rpoS* or *rpoS relA* mutant the *stiB* repressor would not be expressed, and in turn the *stiB* locus would not be repressed during C starvation. Therefore, ppGpp (or the *relA* gene product) would not be required to relieve the repression of *stiB* under these conditions. In a *relA rpoS*$^+$ background, the putative *stiB* repressor would be synthesized, repressing *stiB* expression, but since ppGpp synthesis is defective, the repression could not be relieved and *stiB* would not be induced. According to this model, however, if the same *stiB* repressor functions during C and P starvation, then either (i) a signal transduction system other than ppGpp must be involved during P starvation or (ii), as stated above, ppGpp synthesis during P starvation involves a *relA*-independent mechanism, e.g., the *spoT* gene product.

The *stiA*, *stiB*, *stiC*, and *rpoS* loci are all needed for long-term starvation survival of *S. typhimurium* (8, 44). On the basis of the fact that *stiA*, *stiB*, and *stiC* are all regulated by RpoS, one might predict that *rpoS* mutants exhibit increased sensitivity to the effects of C starvation because *stiA* and *stiC* are not induced and/or because *stiB* is overexpressed in an *rpoS* mutant. Because RpoS regulates genes, in addition to these three *sti* loci, that are likely to be important for starvation survival, one might expect *rpoS* mutants to be even more susceptible to starvation effects than *sti* single mutants. However, *rpoS* singly or in combination with *stiA*, *stiB*, or *stiC* only slightly increased

sensitivity to the effects of starvation relative to *stiA*, *stiB*, or *stiC* mutations in an *rpoS*$^+$ background (compare Fig. 3A and C). This implies that *stiA* and *stiC*, at least, are key starvation survival genes in the *rpoS*-dependent system because a deficiency of either locus compromises starvation survival to about the same extent as an *rpoS* mutation. Interestingly, if *sti* mutations are combined in an *rpoS*$^+$ or *rpoS* background (compare Fig. 3B and D), the effect on starvation survival is even more dramatic than that with an *rpoS sti*$^+$ mutant. This suggests that even basal levels of *rpoS*-dependent gene products synthesized in the absence of a functional RpoS protein can partially protect cells during starvation. Also, the fact that *rpoS* double *sti* mutants are only slightly more sensitive to the effects of prolonged starvation than isogenic *rpoS*$^+$ strains suggests that the loss of two *rpoS*-dependent *sti* survival genes produces a near maximal effect on starvation survival. In such a case, *rpoS*-independent mechanisms may account for residual levels of survival. In addition, genetic (8, 17) and biochemical (47) analyses have demonstrated that at least some RpoS-regulated genes are not absolutely dependent upon RpoS for their expression. Rather, RpoS serves to amplify the expression of specific genes possessing additional independent regulatory systems during starvation conditions. Thus, this may also account for phenotypic differences between strains harboring mutations in *rpoS* or in specific *rpoS*-regulated genes.

What is clear from our findings (11, 38, 42–46, 49) and the work of other laboratories (1–3, 8, 12, 20–21, 24–27, 30, 31, 34–37, 40–41, 47) is that the regulation of the SSR and of stationary-phase gene expression is carried out by a very complex network involving both unique and overlapping signals and regulatory proteins. The signal molecules identified, thus far, include cAMP and ppGpp. The regulators identified to date include the cAMP receptor protein and the alternative σ factor RpoS. Recently, reports on *E. coli* have also implicated Lrp and integration host factor in the RpoS-dependent stationary-phase induction of *osmY* (20). This suggests that these proteins may also play a role in the regulation of stationary-phase gene expression in general. Interestingly, these regulatory components can have both positive and negative effects on starvation-regulated gene expression.

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