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Synthesis of the Stationary-Phase Sigma Factor σ^S Is Positively Regulated by ppGpp

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Strains of *Escherichia coli* which lack detectable guanosine 3′,5′-bispyrophosphate (ppGpp) display a pleiotropic phenotype that in some respects resembles that of *rpoS* (*katF*) mutants. This led us to examine whether ppGpp is a positive regulator of σ^s synthesis. σ^s is a stationary-phase-specific σ factor that is encoded by the *rpoS* gene. We found that a ppGpp-deficient strain is defective in σ^s synthesis as cells enter stationary phase in a rich medium, as judged by immunoblots. Under more-defined conditions we found that the stimulation of σ^s synthesis following glucose, phosphate, or amino acid starvation of wild-type strains is greatly reduced in a strain lacking ppGpp. The failure of ppGpp-deficient strains to synthesize σ^s in response to these starvation regimens could indicate a general defect in gene expression rather than a specific dependence of *rpoS* expression on ppGpp. We therefore tested the effect of artificially elevated ppGpp levels on σ^s synthesis either with mutations that impair ppGpp decay or by gratuitously inducing ppGpp synthesis with a P_{tac}::*relA* fusion. In both instances, we observed enhanced σ^s synthesis. Apparently, ppGpp can activate σ^s synthesis under conditions of nutrient sufficiency as well as during entry into stationary phase. This finding suggests that changes in ppGpp levels function both as a signal of imminent stationary phase and as a signal of perturbations in steady-state growth.

The product of the *rpoS* gene of *Escherichia coli* is a positive regulator of many genes selectively expressed as cells enter stationary phase (9). The *rpoS* gene is also known as *katF* because of its regulatory effects on *katE*, which encodes a stationary-phase-specific catalase (catalase II). The product of *rpoS* was first proposed to alter transcription specificity of RNA polymerase because the deduced KatF protein sequence is similar to that of RNA polymerase sigma factors (22). Also, promoters specifically induced upon entry into stationary phase were viewed as not matching the consensus sequence for σ^{70} promoters, suggesting that an alternative sigma factor may be involved in stationary-phase gene expression (1). These features led Lange and Hengge-Aronis (15) to rename the *katF* gene *rpoS* and designate its product σ^s. Stationary-phase gene expression is not solely regulated by σ^s. Some stationary-phase-specific genes are not dependent on *rpoS* (3, 38), and the predicted σ-like function of σ^s on in vitro transcription has been verified for some, but not other, stationary-phase-specific promoters (25, 34). Furthermore, differences in consensus sequences between σ^{70} and σ^s-dependent promoters are less clear (25, 34). Although the regulatory functions governing stationary-phase-specific gene expression are complex, the ability of *rpoS* mutants to block expression of many stationary-phase induced genes indicates that σ^s is a central regulatory element in this process (9).

Identification of the components that activate *rpoS* gene expression is puzzling because entry into stationary phase is achieved by limitation for nutritional sources of either nitrogen, carbon, or phosphate. There is no known regulatory element common to all of these starvation events in *E. coli* although about 15 proteins, detected by two-dimensional gels, do accumulate in common with all three modes of starvation (19). One solution, as proposed by Hengge-Aronis (9), is that there is a distinction between total nutrient exhaustion in stationary phase and the less-than-complete limitation that accompanies known nutrient-specific responses. However, slow growth alone is sufficient to induce *rpoS* expression. In vivo evidence with *rpoS* transcriptional and translational fusions to *lacZ* suggests that *rpoS* expression begins to increase during slow growth, well before growth stops. Mulvey et al. (23) as well as Schellhorn and Stones (30) show that nutrients need not be completely exhausted by demonstrating that the activity of analogous fusions can be stimulated by adding spent culture medium or weak acids to exponentially growing cells.

It is plausible that there are multiple signals capable of activating *rpoS*, perhaps each matched to a specific starvation regimen. A study of *Salmonella typhimurium* starvation-inducible fusions showed them to be regulated to varying degrees, often with overlap, negatively by *cya* or *crp* and positively by *relA* (32). In *E. coli*, more than 30 proteins are estimated from two-dimensional gels to show *rpoS*-dependent expression (19). These observations reveal interesting features of genes presumably regulated by *rpoS* but provide only a questionable link to signals regulating *rpoS* itself.

Participation of guanosine 3′,5′-bispyrophosphate (ppGpp) as an activator of *rpoS* expression is also plausible because intracellular levels of this nucleotide increase in response to amino acid or energy source limitation (4) as well as to limitation for inorganic nitrogen source (11). Phosphate limitation is, however, reported as having at most only mild effects on ppGpp levels (16, 18, 26).

Accumulation of ppGpp depends upon activities of the products of two genes, *relA* and *spoT* (4). During amino acid starvation, the ppGpp synthetic activity of the RelA protein is activated by uncharged tRNA. The accumulation of ppGpp in response to energy source starvation occurs by blocking degradation of ppGpp, catalyzed by a ppGpp 3′-pyrophosphohydrolase encoded by the *spoT* gene. Apparently, the RelA

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TABLE 1. Strains used in this study

Strain	Genotype	Source or reference
MG1655	λ^- F^-; prototroph	B. Bachman
CF1693	MG1655; *ΔrelA251 ΔspoT207*	37
CF4941	MG1655; *galK2 zib563*::Tn*10 ΔrelA251*	This work
CF4977	CF4941; *spoT202*	This work
CF4943	CF4941; *spoT203*	This work
CF4969	CF4941; *spoT204*	This work

mechanism specifically responds to amino acid starvation and the SpoT mechanism specifically responds to carbon source limitation.

When both *relA* and *spoT* are deleted in the same strain, ppGpp is not detectable under any starvation condition so far tested (7, 10, 37). The ppGpp-deficient (ppGpp^0) strains display a pleiotropic phenotype which may reflect a loss of ppGpp-dependent functions. Since some of these features are reminiscent of *rpoS* mutants and because ppGpp levels are known to be responsive to the availability of amino acids and energy sources, we tested the possibility that ppGpp activates σ^S synthesis.

MATERIALS AND METHODS

Strains, plasmids, media, growth conditions, and enzyme assays. The strains used in this report are listed in Table 1. MOPS (morpholinepropanesulfonic acid) medium was made as described by Neidhardt et al. (24) with the modifications of Bochner and Ames (2). Unless otherwise noted, glucose was added to 0.2% and the final phosphate concentration was 5 mM. Amino acids were added to the concentrations suggested by Davis et al. (6), except that serine was added to a final concentration of 200 μg/ml. Thiamine was added to a final concentration of 1 μg/ml. Luria-Bertani (LB) broth was prepared as described previously (21). Cultures were grown at 32°C (for acidic phosphatase assays) or 37°C. Strains were constructed by P1 transduction (21).

Cultures were started as follows. A few colonies were scraped off plates inoculated the previous night and resuspended in 5 ml of appropriate medium. The resuspended cells were then diluted to an A_{600} of 0.01 in the appropriate medium and grown to an A_{600} of 0.5, when they were diluted again to an A_{600} of 0.01. Alternatively, the cultures were initially diluted to an A_{600} of 0.005 and grown without further dilution. If care is not taken to dilute the cultures appropriately, basal levels of σ^S are inconsistent. Cultures are grown from resuspended, freshly grown colonies as opposed to overnight liquid cultures because the ppGpp^0 strain tends to accumulate suppressor mutations when grown for long periods.

Acidic phosphatase levels were measured as described by Dassa et al. (5) except that 10-min rates of *p*-nitrophenol phosphate hydrolysis at 37°C were measured in a microtiter dish and scanned for A_{415}. Units of activity are expressed as nanomoles of product hydrolyzed per minute normalized to cell culture A_{600} values. Colonies were stained with iodine vapors as described by Lange and Hengge-Aronis (15).

Starvation protocols. Starvation for specific nutrients was achieved by either of two ways. Starvation by depletion of glucose or phosphate was reached by growing cultures in MOPS medium with 0.02% glucose or 0.5 mM KH_2PO_4 with no added amino acids. Depletion of each nutrient was determined by cessation of growth of the culture. Abrupt starvation was achieved by growing cultures in MOPS medium with all amino acids, glucose, and phosphate to an A_{600} between 0.3 and 0.4, at which time 20 ml of culture was then filtered, washed twice with 5 ml of prewarmed MOPS medium lacking the specific nutrient being depleted, and resuspended in 5 ml of prewarmed MOPS medium lacking the specific nutrient. Equal portions of the washed, resuspended cells were then added to 20 ml of prewarmed MOPS medium lacking the tested nutrient and to a control culture containing all nutrients. The cultures were then grown for 20 (for glucose starvation) or 30 min, when a 10-ml sample was taken to assay for σ^S. All cultures were grown at 37°C. To confirm that the cultures were starved for the nutrient being tested, after 30 min the missing nutrient was added and the cultures were monitored every 10 min for an additional 40 min. We concluded that the proper nutrient was depleted if the growth rate of the cell increased to that of the control culture following the addition of the missing nutrient. In all cases, this was found to be true.

SDS-polyacrylamide gel electrophoresis. Whole-cell extracts were prepared by resuspending pelleted whole cells in sodium dodecyl sulfate (SDS) sample buffer (2% SDS, 0.75 M β-mercaptoethanol, 250 mM Tris-HCl, pH 6.8) such that the concentration was equivalent to 0.01 A_{600} units/μl and heated at 100°C for 2.0 min. In cases in which total protein was determined, the cells were split and one half were resuspended in sample buffer and the other half were resuspended in H_2O. The protein concentration of the samples in H_2O was determined by the bicinchoninic acid assay (Pierce). Alternatively, cells were lysed with SDS sample buffer and total protein was precipitated by the addition of 4 volumes of acetone followed by 30 min in dry ice. The total precipitated protein was then pelleted, washed twice with acetone, and resuspended in 2% SDS. Protein content was then determined by the bicinchoninic acid assay. Peptides were separated on 9% polyacrylamide–SDS gels.

Immunological methods. σ^S levels were measured via Western blot (immunoblot) analysis with the monoclonal antibody 1RS1, raised against antigen purified from an overproducing strain (25). Transfer of proteins to polyvinylidene difluoride membranes from SDS-polyacrylamide gels was performed as described by Towbin et al. (35). The final SDS concentration in the transfer buffer was 0.05%. Proteins were transferred at 250 mA for 1 h at room temperature. Filters were blocked with 5% nonfat dry milk (BLOTTO) in phosphate-buffered saline (PBS) for 30 or more min prior to the addition of antibody. Ascites fluid containing anti-σ^S antibody was diluted 1/1,000 in 0.5% BLOTTO and incubated for at least 2 h. The filters were washed with excess PBS three times for at least 20 min each time. Goat anti-mouse horseradish peroxidase-conjugated second antibody was then incubated with the filters at a dilution of 1/1,000 for at least 1 h. The filters were washed as before and developed with the ECL reagent system from Amersham according to instructions. When necessary, filters were stripped of antibody by incubation in 2% SDS–10 mM dithiothreitol for 1 h at 65°C, after which the blots were blocked for 2 h to overnight and probed with antibody to the α subunit of RNA polymerase as described for probing with anti-σ^S.

Quantitation of Western blots. The relative intensities of the bands shown in Fig. 6 were quantitated by image analysis. Images were captured with a video camera attached to a Macintosh IICi computer and analyzed with the NIH Image (version 4.8) program. Because the last point shown in Fig. 6 was not linear on the exposure used to scan the other points, a lighter exposure was scanned and the intensity of the last point was determined relative to the point preceding it. This relative intensity was used to calculate the intensity relative to that of the other datum points and plotted as shown in Fig. 6B.

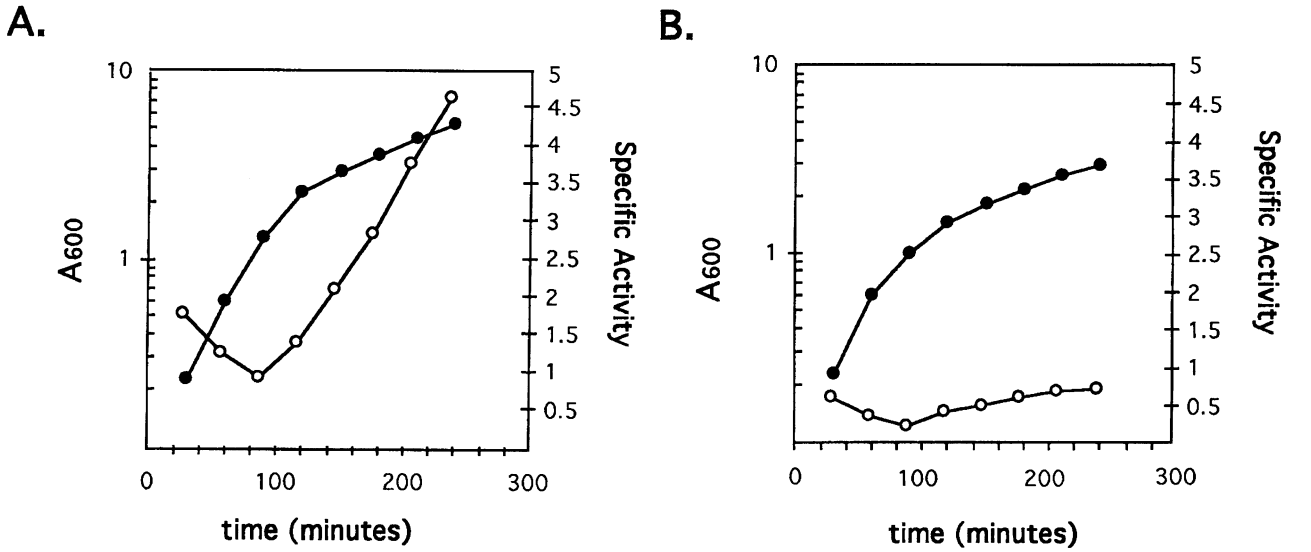

FIG. 1. Effect of ppGpp deficiency on acidic phosphatase activity. Cultures grown in LB medium were monitored for growth (filled circles; A_{600}) and acidic phosphatase specific activity (open circles; nanomoles of *p*-nitrophenolphosphate hydrolyzed per minute at A_{600}) and plotted versus time after initial dilution. (A) Wild-type strain MG1655; (B) ppGpp° strain CF1693.

RESULTS

ppGpp° mutant strains share phenotypic traits with *rpoS* mutants. The phenotype of ppGpp° strains includes an inability to grow on minimal media lacking almost a full complement of amino acids, inviability in stationary phase, susceptibility to killing during heat shock despite unimpaired expression of heat shock genes, cold shock adaptation, salt-sensitive growth, elongated cell morphology during growth, and abnormal morphology in stationary phase (3a, 13, 36, 37). Some features are analogous to the *rpoS* mutant phenotype, though they are less severely affected in ppGpp° strains. Features that are similar but more mildly affected in ppGpp° strains include viability effects, morphological alterations, and salt sensitivity (9). *rpoS* mutants, however, do not display amino acid requirements.

We explored the phenotypic similarities between ppGpp deficiency and *rpoS* deficiency by quantitatively measuring acidic phosphatase activity, encoded by the *appA* gene. The *rpoS*-dependent expression of the *appA* gene is well documented since *appR* regulatory mutants were later identified as *rpoS* alleles (5, 15). Figure 1 shows acidic phosphatase-specific activities of a wild-type prototrophic strain and its otherwise isogenic ppGpp° derivative during the transition from exponential growth on LB medium to slower growth and early entry into stationary phase. The ppGpp° strain shows a mild defect in the ability to induce acidic phosphatase activity during this transition relative to the parental strain. The activity differences shown in the figure are transient; overnight cultures show activities that are nearly equivalent, differing only two- to fourfold (data not shown).

Lange and Hengge-Aronis (15) reported that glycogen accumulation, measured by staining colonies with iodine vapors, is *rpoS* dependent. This simple test allowed them to isolate an *rpoS* insertion mutant. Also, Romeo and Preiss (27) have reported the involvement of ppGpp in glycogen accumulation during exponential growth. While the wild-type parent readily stains brown, colonies of the ppGpp° strain remain as resistant to iodine staining as an *rpoS*-deleted derivative (data not shown). The *rpoS* gene has been reported to be frequently inactivated by an amber mutation in *E. coli* K-12 strains (14) and generally susceptible to mutagenic activity after prolonged exposure to stationary phase (20). We infer from iodine staining that our isolates of MG1655 possess a functional *rpoS* gene.

Two phenotypic features of *rpoS* mutants examined in 24-h LB medium cultures were unaffected in the ppGpp° strain—sensitivity to hydrogen peroxide and to 2.5 M NaCl (data not shown).

σ^s synthesis upon entry into stationary phase after growth in rich medium is abolished in a ppGpp° strain. Most previous assays of *rpoS* expression during entry into stationary phase used transcriptional or translational *lacZ* fusions. Studies using such fusions suggest that both transcriptional and posttranscriptional components are involved in the regulation of *rpoS* expression and that the posttranscriptional component plays the major role (17, 18), although this conclusion was not reached by others (30). Here we have used immunoblots with an anti-σ^s monoclonal antibody to measure regulation of σ^s synthesis in both wild-type (strain MG1655) and ppGpp° (strain CF1693) cells during the transition between exponential growth in LB medium and stationary phase. As shown in Fig. 2, the wild-type parent does not accumulate appreciable σ^s until growth slows, as expected from fusion activity evidence. The ppGpp° strain displays a different pattern of σ^s synthesis, failing to accumulate σ^s during the period of changing growth rate and showing sluggish σ^s synthesis much later than the wild-type culture (Fig. 2). Overnight cultures of the ppGpp° strain have σ^s levels approaching those of the wild-type strain (data not shown) akin to the expression of *appA* in the same strain.

As a control for gel loading and to provide an internal standard, the blot was also probed with a monoclonal antibody recognizing the α subunit of RNA polymerase. We found that the cellular content of the α subunit remains relatively constant under most conditions, with a notable exception being following amino acid starvation, mentioned below. As shown in Fig. 2, the levels of α subunit per total protein did not vary during entry into stationary phase.

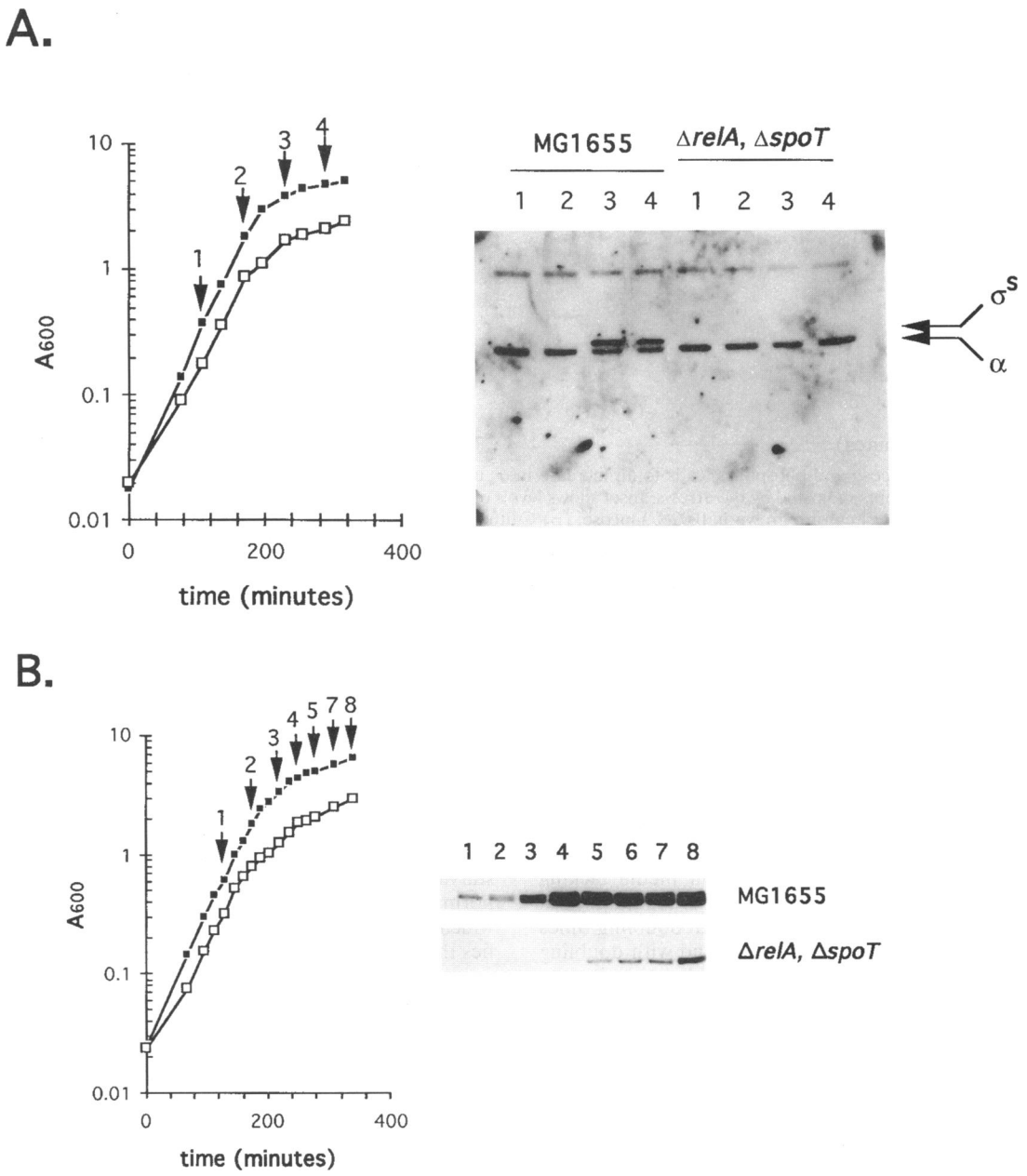

FIG. 2. Pattern of σ^S accumulation in a wild-type and a ppGpp^0 strain during growth in LB medium. (A) Cultures of MG1655 (wild type) and CF1693 (Δ*relA* Δ*spoT*) were grown in LB medium and monitored at A_{600}. At the points indicated by the arrows, samples were taken, concentrated by centrifugation, and prepared for Western blot analysis as described in Materials and Methods. Closed squares, strain MG1655; open squares, strain CF1693. In this experiment, 10 μg of total protein was loaded per lane and the blots were probed with anti-σ^S and anti-α subunit of RNA polymerase antibody simultaneously. The band running above α and σ^S is reactive with the anti-α antibody (unpublished data) and is the size of an α dimer. (B) Same as panel A except that the blots were probed with anti-σ^S antibody only and that 20 μg of total protein was loaded to show slight accumulation of σ^S in the Δ*relA* Δ*spoT* strain.

Glucose or phosphate starvation elevates σ^S levels in wild-type cells. It is difficult to know which specific nutrient is limiting for growth under the conditions shown in Fig. 2. It is likely that stationary phase in LB medium results not only from nutrient limitation but also from the buildup of secondary metabolites, both of which contribute to the stimulation of σ^S synthesis (23, 30). We therefore measured σ^S abundance under defined starvation conditions with wild-type strains to confirm that σ^S synthesis increases in response to limiting glucose or phosphate in minimal medium. As shown in Fig. 3, depletion of either glucose or phosphate clearly leads to a large increase in σ^S accumulation.

σ^S synthesis in response to glucose, phosphate, or amino acid starvation is defective in a ppGpp^0 strain. We attempted to induce carbon source limitation in the ppGpp^0 strain as described for the wild-type strain and obtained only slowing of growth and not an abrupt cessation of growth (data not shown). This complication arises because growth of the

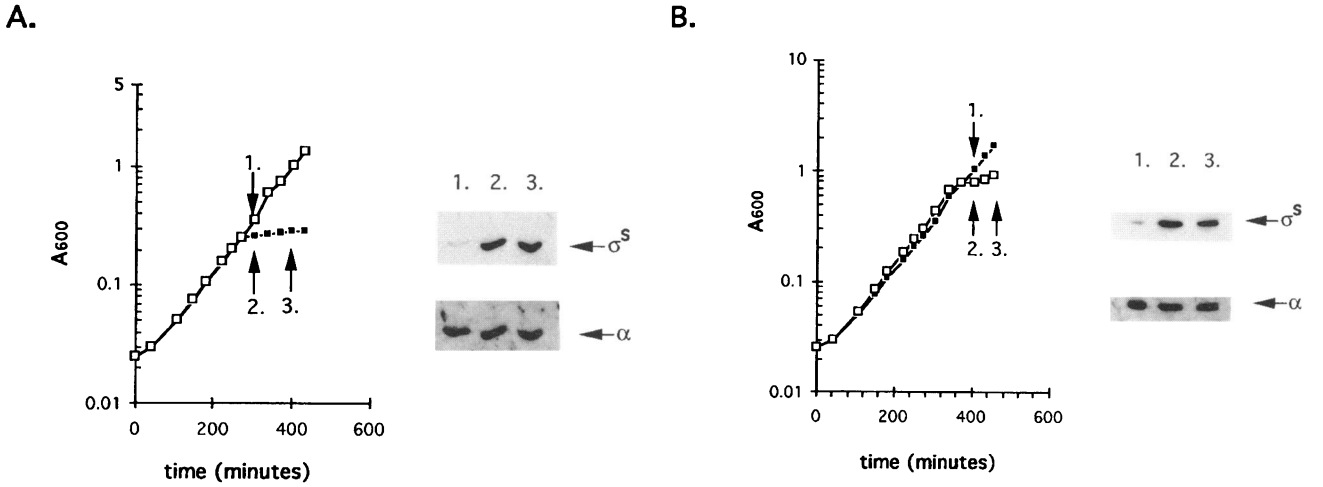

FIG. 3. Starvation for glucose and phosphate leads to an increase in σ^s in strain MG1655. (A) Cultures were grown in limiting glucose, and samples were taken at the points indicated by the arrows. Inset shows levels of σ^s and α subunit at each of the points. Open squares, culture grown in 0.2% glucose; closed squares, culture grown in 0.02% glucose. (B) Cultures were grown in limiting phosphate, and samples were taken at the times indicated by the arrows. Closed squares, culture grown in 5 mM KH_2PO_4; open squares, culture grown in 0.5 mM KH_2PO_4. Inset shows the levels of σ^s and of the α subunit of RNA polymerase at each of the points. The equivalent of 0.15 A_{600} units was loaded per lane. After being probed with anti-σ^s antibody, the membranes were stripped and reprobed with anti-α subunit of RNA polymerase antibody as a control for loading.

ppGpp° strain requires supplementing glucose minimal medium with a full complement of amino acids (37). Presumably, continued growth after exhaustion of glucose in the medium containing all amino acids is due to the use of some amino acids as alternative carbon sources.

We achieved glucose-limited growth in the presence of all amino acids by a filtration protocol (Materials and Methods) in which cells growing on glucose and amino acids were filtered, washed free of glucose, and resuspended in media lacking glucose but containing all amino acids. In this medium, both the wild type and the ppGpp° strain grew with doubling times of about 30 min when glucose was present and with doubling times of about 60 min following removal of glucose during the brief period of measurement. With this filtration downshift method, growth is again not completely stopped, but is limited by the absence of glucose since prefiltration growth rates are restored by resupplementation with glucose (data not shown). Figure 4A shows that the increase in σ^s levels shown by the wild-type strain under these conditions is similar to behavior shown in Fig. 3. With the ppGpp° strain, a small increase in σ^s concentration is seen to accompany starvation, but the level of induction is much lower than that in the wild-type strain.

We compared σ^s synthesis in wild-type and ppGpp° strains during phosphate starvation also by a filtration protocol. As shown in Fig. 4B, the wild-type strain exhibits a large increase in σ^s levels while the ppGpp° strain, again, exhibits only a small increase during phosphate limitation. With a phosphate exhaustion protocol as opposed to the filtration protocol, the pattern of σ^s synthesis for the ppGpp° strain is the same as that shown in Fig. 4B.

Phosphate starvation has different effects on growth of the two strains. The turbidity of the wild-type strain slowly increased for at least 1 h after filtration and resuspension in a phosphate-free medium, with an apparent doubling time of 120 min. The increase in turbidity shown by the ppGpp° strain stops abruptly and then decreases slightly over the next 30 min. The source of this difference is unknown, but it could reflect differences in phosphate scavenging or storage abilities.

To determine whether amino acid starvation induces σ^s synthesis in a ppGpp-dependent manner, we again used filtration to remove amino acids from an otherwise nutritionally complete medium, which results in a transient amino acid deprivation in the prototrophic parental strain. Figure 4C shows that σ^s levels increase in the wild-type, but not the ppGpp°, strain. The levels of σ^s actually appear to decrease in the amino acid-free culture of the ppGpp° strain. In addition, the levels of α subunit also decreased following amino acid starvation. We believe that the decrease in the α subunit levels normally occurs during amino acid starvation in a manner which is apparently ppGpp independent. This behavior qualifies the use of the α subunit as an internal sample standard for amino acid starvation conditions.

Exponentially growing *spoT* mutant strains with abnormally high ppGpp basal levels have high σ^s levels. The above experiments implicate a requirement for ppGpp for activation of σ^s synthesis. We examined the effects of artificially manipulating higher-than-normal ppGpp levels on σ^s synthesis to directly test this possibility.

A series of *spoT* mutants have been isolated as aminotriazole-resistant (AT^r) suppressors of AT^s *relA* mutants. These mutations cause increased basal levels of ppGpp in exponentially growing cells. Alleles *spoT202* to *204* confer incremental ppGpp basal level elevations of about 6-, 8-, and 12-fold, respectively (29). These mutations map in the amino-terminal portion of the *spoT* structural gene and are thought to selectively impair ppGppase activity of the SpoT protein (28). If ppGpp can act as a positive regulator of σ^s synthesis independently of starvation, then increasing basal levels of ppGpp should increase basal levels of σ^s in cells even during steady-state growth. As shown in Fig. 5, the *spoT* mutants, relative to a wild-type control, do have increased levels of σ^s during logarithmic growth. Apparently the levels of σ^s do not show a graded *spoT* allele-specific response, suggesting a low ppGpp concentration threshold for σ^s synthesis, similar to AT resistance (29).

Induction of ppGpp without starvation induces σ^s synthesis. An alternative method for increasing ppGpp in unstarved cells involves abrupt induction to levels approximating those

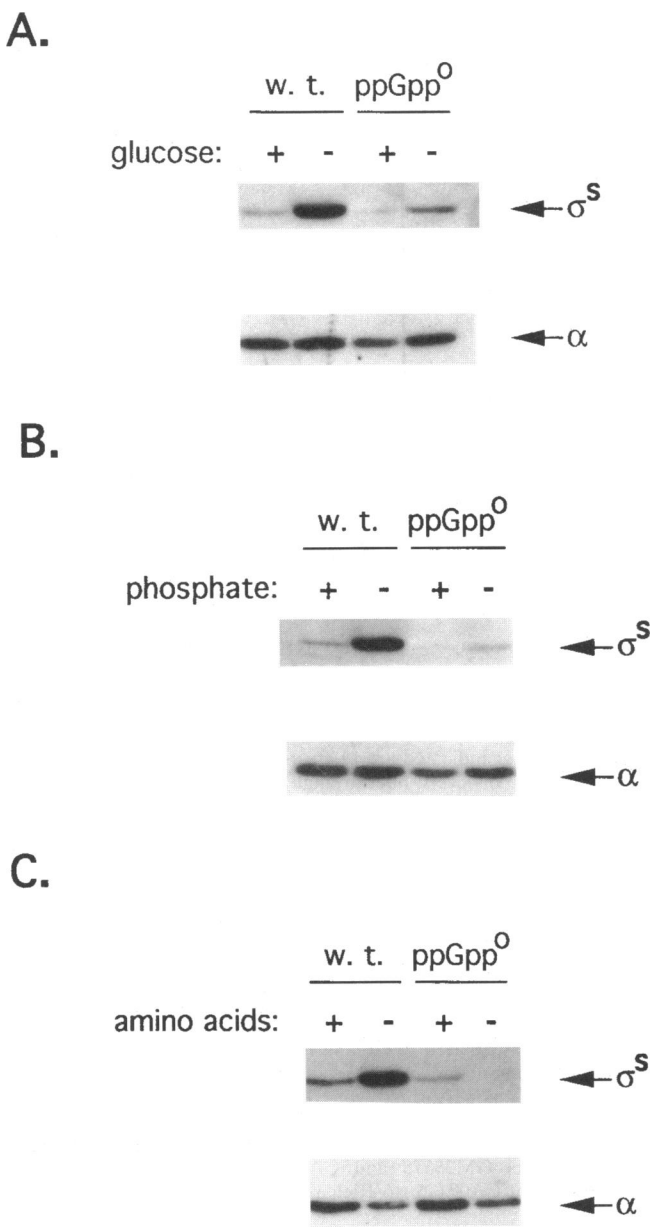

FIG. 4. Glucose, phosphate, and amino acid starvation in the ppGpp° strain leads to impaired induction of σ^s levels. Cultures were grown and starved by the filtration protocol outlined in Materials and Methods and analyzed for σ^s. (A) Glucose starvation; (B) phosphate starvation; (C) amino acid starvation. The equivalent of 0.2 A_{600} units was loaded per lane. After being probed with anti-σ^s antibody, the membranes were stripped and reprobed with anti-α subunit of RNA polymerase antibody as a control for loading. w.t., wild type.

achieved during amino acid or carbon source starvation. This method uses a plasmid bearing a truncated *relA* gene under the control of an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible promoter which produces a catalytically active fragment of the RelA protein (31). The induction of the pALS13 plasmid with IPTG leads to the overproduction of a constitutive ppGpp synthetic activity that is independent of ribosomes (31, 33). Uninduced levels of ppGpp remain low in strains carrying this plasmid probably because the truncated protein is unstable, unlike the full-length RelA protein (31). We measured σ^s levels in response to induction of pALS13 in rapidly growing, wild-type cells. As shown in Fig. 6, the levels of σ^s increase rapidly when ppGpp synthesis is induced. Quantitation of the immunoblot indicates that σ^s levels increase about 60-fold, with no significant change in the levels of the α subunit.

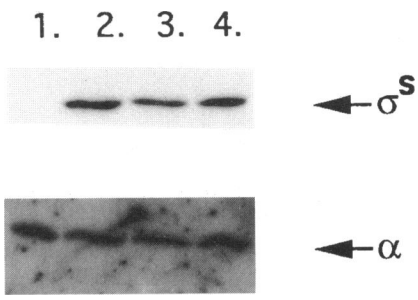

FIG. 5. *spoT* mutants that have high basal levels of ppGpp also have high basal levels of σ^s. Strains CF4941 (wild type), CF4977 (*spoT202*), CF4943 (*spoT203*), and CF4969 (*spoT204*) were grown in LB medium to an A_{600} of about 0.5 (0.53, 0.5, 0.53, and 0.54, respectively) and assayed for σ^s by Western analysis. The equivalent of 0.15 A_{600} units was loaded per lane. After being probed with anti-σ^s antibody, the membranes were stripped and reprobed with anti-α subunit of RNA polymerase antibody. Lane 1, *spoT*$^+$; lane 2, *spoT202*; lane 3, *spoT203*; lane 4, *spoT204*.

DISCUSSION

We interpret these experiments to indicate that ppGpp has a major regulatory role in the activation of σ^s synthesis occurring early in the transition from exponential growth to stationary phase. This possibility was suggested by shared phenotypic features of ppGpp° strains and *rpoS* mutants. The defects in the ppGpp° strain are more severe at the time of entry into stationary phase than later in stationary phase. We have noticed with immunoblots that σ^s synthesis eventually does increase in the ppGpp° strain at times beyond the transition period. A major role for the ppGpp-dependent activation of σ^s synthesis may, therefore, relate to the timing of changes occasioned by these transitions.

A ppGpp° strain shows defective σ^s synthesis when growth is limited in LB medium as well as when growth is limited for lack of glucose, phosphate, or amino acids (Fig. 2 to 4). Since artificially elevated levels of ppGpp stimulate σ^s synthesis in nonstarved wild-type strains (Fig. 5 and 6), the simplest explanation for defective σ^s synthesis in starved ppGpp° strain is that ppGpp is directly involved in regulating σ^s levels. The mechanism by which σ^s levels increase in wild-type strains as they enter stationary phase is uncertain but is thought to contain transcriptional and posttranscriptional components (17, 18). The activity of *rpoS-lacZ* operon and protein fusions in wild-type cells is induced about three- and about sixfold, respectively (8). Although differences in protein and mRNA stability arising from the different constructions provide possible alternative explanations for this behavior, the simplest conclusion is that both transcriptional and posttranscriptional components are involved. We are currently determining which component ppGpp affects.

The effect of gratuitous IPTG induction of ppGpp is an increase in σ^s synthesis that is very rapid (Fig. 6) and, from previous estimates of ppGpp accumulation (31, 33), is probably virtually coincident with ppGpp accumulation. Since σ^s-dependent promoters now appear to have sequences similar to those

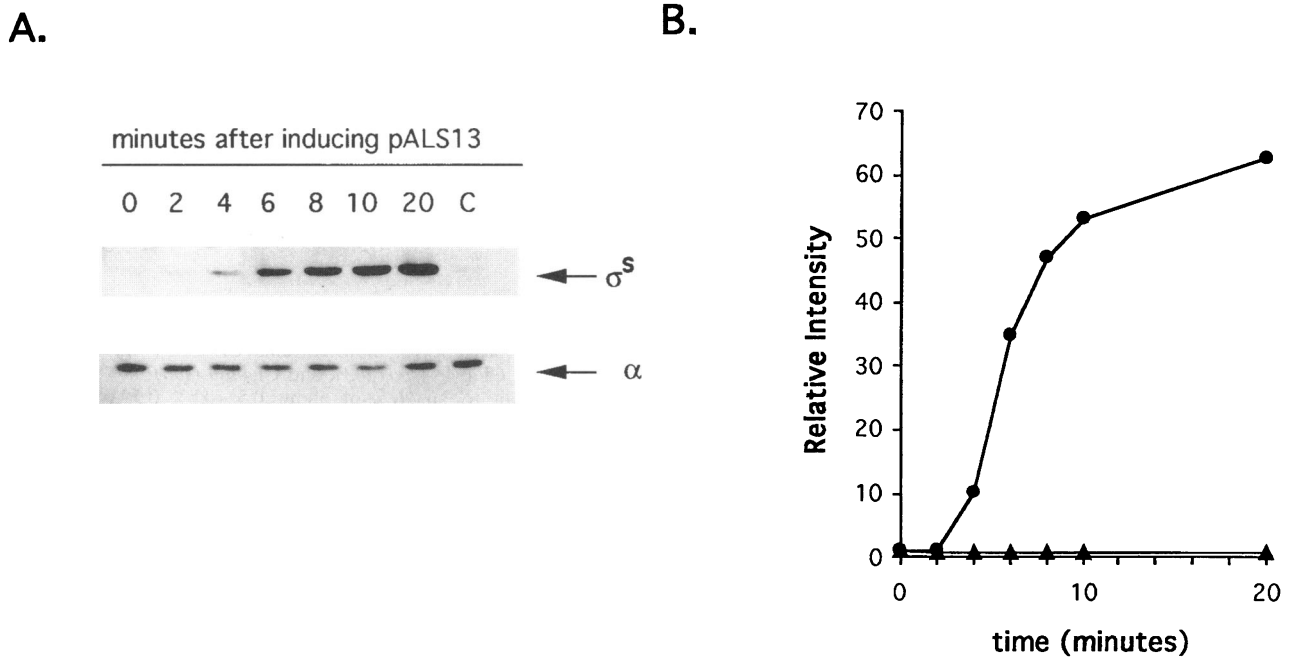

FIG. 6. Induction of ppGpp synthesis leads to an induction of σ^S accumulation. Strain MG1655 (pALS13) was grown to an A_{600} of 0.3, when the culture was divided and half was treated with 1.0 mM IPTG. Samples were taken at the indicated time points and assayed for σ^S and the α subunit of RNA polymerase by Western blots. (A) Western blot. Lane C contains an equivalent amount of protein from the control culture 20 min after the addition of IPTG. (B) Quantitation of blot in panel A by image analysis. Values indicated are relative to the uninduced sample taken at time 0.0. The equivalent of 0.15 A_{600} units was loaded per lane. Filled circles, relative σ^S levels; filled triangles, relative α levels.

of σ^{70} promoters (9, 25) and since some σ^{70} promoters are apparently also recognized by σ^S (34), it is possible that some of the transcriptional effects ascribed to ppGpp are primarily due instead to σ^S. This underscores the need to examine transcriptional effects accompanying elevation of ppGpp in *rpoS* deletion strains. An involvement of σ^S in these processes might explain why documentation of ppGpp effects on in vitro transcription has been contradictory and elusive (4, 12).

From the viewpoint of ppGpp regulation, it is not surprising that starvation for amino acids or glucose results in ppGpp-dependent effects, since starvation for these nutrients is well documented as leading to increases in ppGpp levels. We are unaware of published evidence of ppGpp accumulation during limiting growth in LB medium. Given that ppGpp levels increase following depletion of nutrients, it would be surprising if ppGpp levels remained unchanged as cells enter stationary phase in LB medium. In addition, phosphate starvation has been reported to result in no increase in ppGpp (8, 26). The σ^S accumulation reported here has led us to reexamine ppGpp accumulation following phosphate starvation. We find that ppGpp levels do significantly increase following phosphate starvation (10a), confirming a previous but unappreciated finding (16). We do not know whether this feature of phosphate starvation can be generalized to other strains and growth conditions.

The finding that σ^S synthesis is activated in response to gratuitous induction of ppGpp also raises the need to reevaluate the physiological role of the stringent response. Previously, the stringent response to amino acid starvation could be thought of as one extreme of a spectrum of steady-state conditions without considerations of stationary-phase development (4, 29). If anything, amino acid starvation was thought to block the transition into stationary phase. The results shown here suggest that ppGpp elevation activates σ^S synthesis and thereby may provide a point of entry for the changes in gene expression accompanying stationary-phase development.

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