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RelA Is a Component of the Nutritional Stress Activation Pathway of the *Bacillus subtilis* Transcription Factor σ^B

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The general stress regulon of *Bacillus subtilis* is induced by the activation of the σ^B transcription factor. Activation of σ^B occurs when one of two phosphatases (RsbU and RsbP), each responding to a unique type of stress, actuates a positive regulator of σ^B by dephosphorylation. Nutritional stress triggers the RsbP phosphatase. The mechanism by which RsbP becomes active is unknown; however, its activation coincides with culture conditions that are likely to reduce the cell's levels of high-energy nucleotides. We now present evidence that RelA, a (p)ppGpp synthetase and the key enzyme of the stringent response, plays a role in nutritional stress activation of σ^B. An insertion mutation that disrupts *relA* blocks the activation of σ^B in response to PO_4 or glucose limitation and inhibits the drop in ATP/GTP levels that normally accompanies σ^B induction under these conditions. In contrast, the activation of σ^B by physical stress (e.g., ethanol treatment) is not affected by the loss of RelA. RelA's role in σ^B activation appears to be distinct from its participation in the stringent response. Amino acid analogs which induce the stringent response and RelA-dependent (p)ppGpp synthesis do not trigger σ^B activity. In addition, neither a missense mutation in *relA* (*relA240GE*) nor a null mutation in *rplK* (*rplK54*), either of which is sufficient to inhibit the stringent response and RelA-dependent (p)ppGpp synthesis, fails to block σ^B activation by PO_4 or glucose limitation.

The general stress regulon of *Bacillus subtilis* is a collection of approximately 127 genes (15, 26, 27, 37) whose expression is elevated when the bacterium is exposed to physical (e.g., heat or osmotic shock, ethanol treatment) or nutritional (e.g., glucose or PO_4 limitation, azide treatment) stress (7, 8, 15, 19, 37, 39, 40). The regulon is controlled by the activity state of the σ^B transcription factor. As illustrated in Fig. 1, σ^B is inactive in unstressed *B. subtilis* due to an inhibitor protein (RsbW) that binds and holds σ^B unavailable to RNA polymerase (6, 11). σ^B is released from RsbW when a second regulatory protein (RsbV) binds to RsbW in lieu of σ^B (11). RsbV is normally inactive due to a RsbW-catalyzed phosphorylation (3, 11, 39). When *B. subtilis* is exposed to stress, one of two stress-specific phosphatases (RsbP and RsbU) is activated to dephosphorylate phosphorylated RsbV, thereby allowing RsbV to displace σ^B from the RsbW-σ^B complex (36, 44).

RsbP is the nutritional stress phosphatase. In vivo activation of RsbP requires RsbQ, a predicted hydrolase or acyltransferase, whose gene is cotranscribed with *rsbP* (9, 36). The metabolic inducer of the RsbP/Q system is unknown. Activation of σ^B by this system is commonly initiated by allowing *B. subtilis* cultures to enter stationary phase in rich (i.e., Luria-Bertani) medium, but it can also be brought on by any of a number of treatments which are likely to reduce the cell's energy charge level (e.g., glucose or phosphate limitation, azide treatment) (40). This common feature of the inducing conditions suggested that changes in ATP levels might be the signal that activates RsbP/Q.

RsbU is the phosphatase that responds to physical stress (19, 40, 44). Exposure to environmental insult empowers a positive regulator of this pathway, RsbT, to inactivate its inhibitor (RsbS) by phosphorylation and induce the RsbU phosphatase to reactivate phosphorylated RsbV (44). The RsbS-RsbT interactions are thought to be modulated by RsbR and a family of related proteins; however, the actual roles of the RsbR-like proteins in this process remain undefined (1, 2, 12). As is the case with the nutritional stress pathway, the signal that physical stress generates and the mechanism by which it is conveyed to RsbT are unknown. There is evidence, however, that a ribosome-mediated process may be involved in sensing or signaling physical stress. This notion is based on the failure of physical but not nutritional stress to activate σ^B in *B. subtilis* strains that are either deficient in a ribosome-associated GTP-binding protein (Obg) or missing ribosome protein L11 (30, 31, 36).

Strains lacking ribosome protein L11 are not only blocked in the activation of σ^B by physical stress, but also have an impaired growth rate and are unable to activate the stringent response (33, 43, 46). The stringent response is a process in which amino acid deficiencies can trigger a ribosome-associated protein (RelA) to initiate the synthesis of (p)ppGpp and modulate transcription (10). The loss of RelA has been found by others to be accompanied by lowered induction of some *B. subtilis* stress response proteins (41). This observation, when taken with the requirement for protein L11 in both the activation of σ^B by physical stress and the activation of RelA by amino acid starvation, prompted us to examine the potential role of RelA in the activation of σ^B. We find that RelA does play a role in the activation of σ^B. However, unlike L11, RelA is not involved in physical stress induction of σ^B, but is instead an element of σ^B activation in response to nutritional stress.

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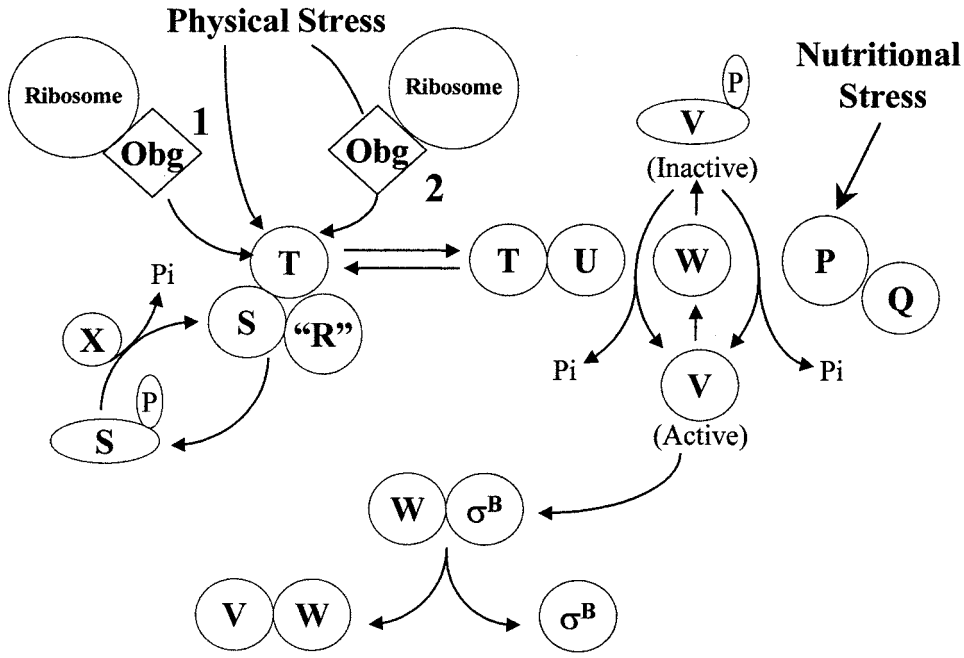

FIG. 1. Model of σ^B activation. σ^B is held inactive in unstressed *B. subtilis* as a complex with an anti-σ^B protein, RsbW (W). σ^B is freed from RsbW when a release factor, RsbV (V), binds to RsbW. In unstressed *B. subtilis*, RsbV is inactive due to an RsbW-catalyzed phosphorylation (V-P). Physical stress activates an phosphorylated RsbV phosphatase, RsbU (U), which reactivates RsbV. RsbT (T) is the RsbU activator. RsbT is normally bound to a negative regulator, RsbS (S), which inhibits its activity. RsbR and a family of related proteins (R) also bind to RsbS and RsbT and are believed to facilitate their interactions. Upon exposure to stress, RsbT phosphorylates and inactivates RsbS and then activates the RsbU phosphatase. A ribosome-mediated process and Obg, a ribosome associated GTPase that can bind to RsbT, are required for stress to trigger the activation of RsbT. It is unknown whether ribosome- and Obg-dependent processes serve as separate facilitators for stress to activate RsbT (1) or comprise a linear pathway through which stress directly communicates to RsbT (2). RsbS-P is dephosphorylated and reactivated by a phosphatase, RsbX (X), that is encoded by one of the genes downstream of the *sigB* operon's σ^B-dependent promoter. RsbX levels become elevated when σ^B is active, which may facilitate a return of RsbT to an inactive complex with RsbS. Energy depletion activates a separate pathway in which a novel phosphorylated RsbV phosphatase (P) and its associated activator (Q) are triggered, by unknown means, to reactivate RsbV. The model is based on references 1, 4, 6, 8, 11, 18, 19, 30, 39, 40, 42, 44, and 46.

MATERIALS AND METHODS

Bacterial strains. All strains and plasmids used in this study are listed in Table 1. The BSA, BSJ, and BSZ strains are derivatives of PY22. BSA46 carries a specialized SPβ prophage, encoding a translational fusion of the σ^B-dependent gene *ctc* to the *Escherichia coli lacZ* gene (SPβ *ctc*::*lacZ*). This fusion allows β-galactosidase activity to be monitored as a measure of σ^B activity (4). BSZ7 is BSA46 transformed to RelA$^-$ by means of a plasmid (pUKRE1) that is incapable of autonomous replication in *B. subtilis* but carries an internal fragment of the *B. subtilis relA* gene.

pUKRE1 was constructed by ligating a PCR-amplified portion of the *relA* sequence (nucleotides 3 to 542 of the 2,202-nucleotide *relA* gene [http://www.pasteur.fr/Bio/SubtiList]) from PY22 into pUK19 (17). Transformation of this plasmid into *B. subtilis*, followed by selection for a plasmid-encoded antibiotic resistance, yields transformants in which the plasmid integrated into the *B. subtilis* chromosome disrupting *relA*. *relA* is the promoter-proximal gene of a bicistronic operon (i.e., *relA yrvI*). Therefore, BSZ7 is not only RelA$^-$ but also has the downstream open reading frame (*yrvI*) separated from the *relA* promoter by the vector sequence. BSZ28 is BSA46 transformed with a plasmid (pUS-RCT) that carries the carboxy-terminal sequence (nucleotides 1987 to 2202) of *relA* in PUS-19 (5). BSZ28 is RelA$^+$ but, like BSZ7, has a plasmid sequence separating *yrvI* from its putative promoter.

To create a copy of *relA* for complementation, *relA* and its promoter were amplified from PY22 with a pair of oligonucleotides that hybridize 146 bases upstream of the *relA* open reading frame and at the coding sequence for the *relA* carboxy terminus. The resulting 2.35-kbp fragment was cloned into an *amyE* integrating vector (pLD30 *amyE*::*spec*) and subsequently transformed into PY22, selecting for spectinomycin resistance and Amy$^-$. The resulting strain (BSZ66) was then sequentially transformed with chromosome DNA from BSZ6 (*relA*::pUK-RE1) with selection for kanamycin resistance (*relA3-542*), and from BSA46 (SPβ *ctc*::*lacZ*) with selection for chloramphenicol resistance to yield BSZ68 (*relA*::pUK-RE1[*relA3-542*] *amyE*::*relA* SPβ *ctc*::*lacZ*). Test transformations of chromosome DNA from BSZ68 into wild-type *B. subtilis* verified the presence of the *relA3-542* allele (small-colony Rel$^-$ phenotype linked to kana-

TABLE 1. Strains and plasmids

Strain or plasmid	Relevant genotype	Construction, reference, or source
Bacillus strains		
PY22	*trpC2*	Laboratory strain
IS58	*trpC2 lys*	33
IS56	*trpC2 lys relA240GE*	33
BSJ18	*trpC2 lys* SPβ *ctc*::*lacZ*	SPβ *ctc*::*lacZ*→IS58
BSJ19	*trpC2 lys relA240EG* SPβ *ctc*::*lacZ*	SPβ *ctc*::*lacZ*→IS56
BSA46	*trpC2* SPβ *ctc*::*lacZ*	5
BSZ7	*trpC2* SPβ *ctc*::*lacZ relA*::*pUK-RE1*	pUK-RE1→BSA46
BSZ5	*trpC2* SPβ *ctc*::*lacZ relA*::*pUS-RE1*	pUS-RE1→BSA46
BSZ28	*trpC2* SPβ *ctc*::*lacZ relA*::*pUS-RCT*	pUS-RCT→BSA46
BSZ66	*trpC2 amyE*::*spc*::*relA*	pSp-RelA→PY22
BSZ67	*trpC2 relA*::*pUK-RE1 amyE*::*spc*::*relA*	BSZ7→BSZ66
BSZ68	*trpC2 relA*::*pUK-RE1 amyE*::*spc*::*relA* SPβ *ctc*::*lacZ*	BSA46→BSZ67
BSZ9	*trpC2* SPβ *ctc*::*lacZ rplK57*	46
Plasmids		
pUS-19	Ap^r Spc^r	5
pUK-19	Ap^r Km^r	17
pUS-RCT	Ap^r Spc^r *relA* 1987–2202	This study
pUK-RE1	Ap^r Km^r *relA*3–542	This study
pUS-RE1	Ap^r Spc^r *relA*3-542	This study
pSp-RelA	Ap^r *amyE*::*Spc*::*relA*	This study

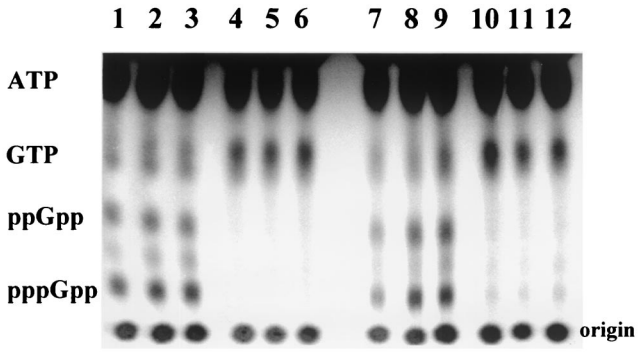

FIG. 2. Induction of (p)ppGpp synthesis by amino acid limitation. Congenic *B. subtilis* strains BSA46 (RelA$^+$) [lanes 1 to 3] and BSZ7 (*relA3-542*) [lanes 4 to 6] and BSJ18 (RelA$^+$) [lanes 7 to 9] and BSJ19 (*relA240GE*) [lanes 10-12] were grown to optical density 0.3 in synthetic medium with Casamino Acids and $^{32}[P]H_3PO_4$ (100 μCi/ml), pelleted and resuspended in the same medium without Casamino Acid but with norvaline (1 mg/ml). Samples were taken at 20 min (lanes 1, 4, 7, and 10), 40 min (lanes 2, 5, 8, and 11), and 60 min (lanes 3, 6, 9, and 12) after resuspension and analyzed by thin-layer chromatography as described in Materials and Methods. The positions of ATP and GTP were determined with nucleotide markers. The (p)ppGpp positions were inferred from their mobilities relative to GTP (13).

mycin resistance, AmyE$^-$ linked to spectinomycin resistance, and no linkage between kanamycin resistance and spectinomycin resistance).

Culture conditions. Strains to be stressed by ethanol were grown in LB (29). Glucose and phosphate limitation was investigated in a synthetic medium (35) in which the glucose concentration was lowered from 0.2% to 0.05% or the PO_4 concentration was lowered from 0.6 mM to 0.15 mM.

Analysis of (p)ppGpp GTP and ATP levels. Cells were grown in the synthetic medium (35) used for the glucose/PO_4 limitation studies and incubated for at least two generations in the presence of 100 μCi/ml $[^{32}P]H_3PO_4$ before the first sample was taken. Cultures were allowed to grow without inhibitor or treated at an optical density of approximately 0.3 with amino acid analogs (norvaline [1.5 mg/ml] or norvaline [1.0 mg/ml] and serine hydroxamate [1 mg/ml]) to trigger amino acid starvation and induce the stringent response. The medium for the norvaline treatment contained tryptophan but lacked Casamino Acids. The 100-μl samples were pelleted, resuspended in 100 μl of 3 M formic acid, subjected to two cycles of freezing and thawing, and centrifuged in an Eppendorf microfuge for 2 min to pellet debris. Supernatant samples (1 to 5 μl) extracted from equivalent numbers of cells (based on optical density), were spotted onto polyethylenemine-cellulose plates (Selecto Scientific) for separation by thin-layer chromatography in 1.5 M KH_2PO_4 (pH 3.4) (13, 41). Labeled nucleotides were visualized following chromatography by autoradiography and quantified by densitometry with an Alpha Imager 2000 (Alpha Innotech Corp., San Leandro, Calif.) and its associated software. Unlabeled ATP and GTP was spotted on the plates as markers and visualized after chromatography by UV light induced fluorescence. The identities of the labeled pppGpp and ppGpp were inferred by their positions in the chromatograph relative to the origin and GTP (13).

RNA analysis. Cells were grown in synthetic medium with Casamino Acids to an optical density of 0.5, pelleted, and resuspended in a similar medium either lacking PO_4 or without Casamino Acids but containing norvaline (0.05%). Samples were harvested at 2, 40, and 70 min after resuspension and processed for RNA extraction with the Q-BioGene Fast RNA Pro Blue kit (Q-BioGene) following the manufacturer's instructions.

RNA samples were subjected to reverse transcription-PCR with avian myeloblastosis virus reverse transcriptase/*Taq* DNA polymerase and oligonucleotide primer pairs that would hybridize to nucleotides 44 to 61 and 285 to 304 of the P_A transcript region and nucleotides 33 to 52 and 171 to 210 of the P_B transcript region. Amplified DNAs were analyzed by polyacrylamide gel electrophoresis (6% acrylamide) and ethidium bromide staining. To verify that the PCR products were the result of RNA amplification and not contaminating DNAs, the amplifications were repeated either without reverse transcriptase or by attempting to PCR amplify the reaction mixtures, reverse transcribed with the oligonucleotide for one of the transcripts (e.g., P_A), with the oligonucleotides specific for the second transcript (e.g., P_B). In neither case was a DNA product detected.

General methods. *B. subtilis* transformation was carried out as described by Yasbin (45). β-Galactosidase assays were performed on chloroform-permeabilized cells, as described by Kenny and Moran (20).

RESULTS

RelA is a component of the σ^B nutritional stress pathway. During a characterization of RelA in *B. subtilis*, it was noted that a RelA$^-$ strain had reduced levels of a number of σ^B-dependent proteins (41). Given RelA's association with ribosomes and our previous evidence for possible ribosome involvement in σ^B's activation (31, 46), we sought to determine whether RelA could influence either of the σ^B activation pathways. For this purpose, a null mutation was created in *relA* (*relA3-542*) by targeting an integrating plasmid into the interior of *relA*. RelA$^-$ transformants (BSZ5, BSZ7), selected on the basis of the plasmid-encoded antibiotic resistance, had a growth rate that was approximately one third that of their congenic parent (BSA46) (data not shown) and failed to accumulate (p)ppGpp following amino acid starvation (Fig. 2, lanes 4 to 6).

RelA$^+$ and RelA$^-$ cultures were subjected to conditions that activate either the nutritional stress (PO_4 or glucose limitation) or physical stress (4% ethanol) pathways for σ^B induction (40). As seen in Fig. 3, RelA$^+$ *B. subtilis* but not the RelA$^-$ variant activated σ^B-dependent transcription when growth slowed in response to PO_4 (Fig. 3A) or glucose (Fig. 3B) limitation. In contrast to the failure of nutritional stress to activate σ^B in RelA$^-$ *B. subtilis*, ethanol treatment resulted in rapid induction of σ^B activity in both wild-type and mutant *B.*

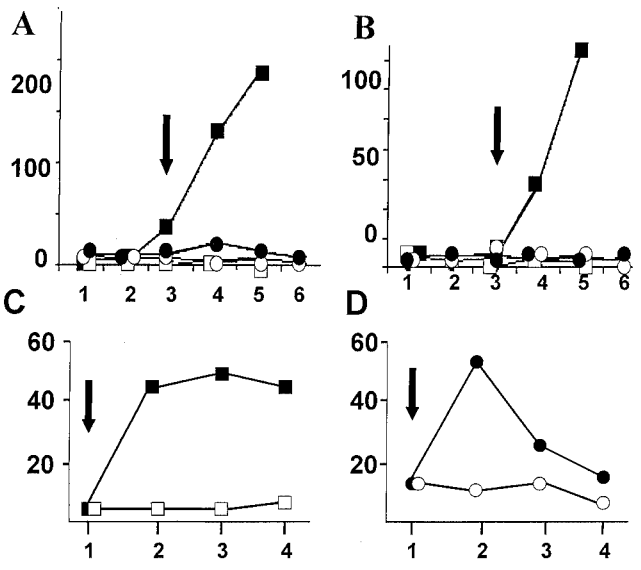

FIG. 3. Induction of σ^B by phosphate limitation or ethanol treatment in Rel$^{+/-}$ *B. subtilis.* BSA46 (RelA$^+$ □, ■) or BSZ5 (RelA$^-$ ○, ●) were grown in a synthetic medium containing 0.15 mM (■, ●) or 0.6 mM (□, ○) PO_4 (panel A) or 0.05% (■, ●) or 0.2% (□, ○) glucose (panel B). Samples were taken at 30-min intervals and assayed for σ^B-dependent β-galactosidase activity. The arrows show the times at which the cultures left exponential growth. In panels C and D, BSA46 (C) and BSZ5 (D) were grown continuously in LB (≤) or exposed to 4% ethanol (■, ●) at the point indicated by the arrows. Vertical axes, β-galactosidase (Miller units). Horizontal axes, time (30-min intervals).

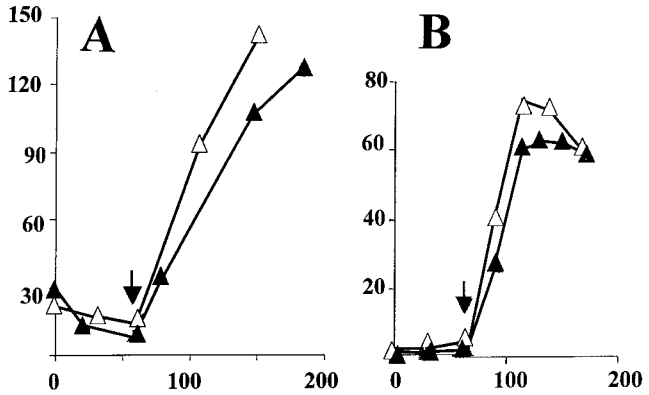

FIG. 4. σ^B activation by PO_4 limitation in *relA*::pUS-RCT and *relA3-542 amyE*::*spc*::*relA* strains. BSA46 (RelA$^+$ [Δ], panels A and B), BSZ28 (RelA$^+$ YrvI$^-$ [▲], panel A), and BSZ68 (*relA3-542 amyE*::*relA* [▲], panel B) were grown in low-phosphate synthetic medium, with samples taken at the indicated times for β-galactosidase analyses. The arrows depict the times at which the cultures left exponential growth. Vertical axes, β-galactosidase (Miller units). Horizontal axes, time (minutes).

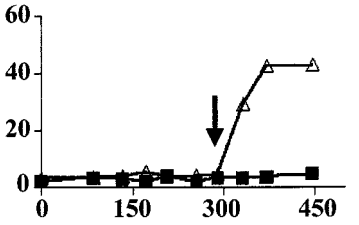

FIG. 5. Induction of σ^B by PO_4 limitation in RplK$^-$ *B. subtilis*. *B. subtilis* strain BSZ9 (*rplK57*) was grown in low (Δ) or high (■) PO_4 synthetic medium. Samples were taken at the indicated times (minutes) and assayed for σ^B-dependent β-galactosidase activity (Miller units). The arrow indicates the time as which the cultures left exponential growth.

subtilis (Fig. 3C and D). These results, as well as the β-galactosidase measurements that follow, depict representative data from assays that were repeated three or more times.

relA is the promoter-proximal gene in a bicistronic operon (41; http://www.pasteur.fr/Bio/SubtiList). The function of the second gene of the operon, *yrvI*, is unproven, but based on homologies, it appears to be a D-tyrosyl-tRNA deacylase. Although it seemed unlikely that a block in *yrvI* expression could account for the failure of BSZ7 to activate σ^B, we tested this possibility with a second integrating plasmid, whose cloned *relA* element included the 3′ end of the *relA* gene. *B. subtilis* strains with this plasmid integrated into *relA* would express an intact *relA*, but carry the transcriptional disruption of *yrvI* present in the RelA$^-$ strain. RelA$^+$ *B. subtilis*, with the *relA* operon interrupted by this plasmid, grew normally and responded like the wild-type strain to either phosphate (Fig. 4A) or glucose limitation (data not shown). To further verify that the loss of RelA was responsible for the block in σ^B induction, a *relA3-542* strain (BSZ68) was constructed which contained a cloned copy of the *B. subtilis relA* gene and its promoter at the *amyE* locus. The second copy of *relA* corrected both the slow-growth phenotype and the σ^B activation defect (Fig. 4B) of the *relA3-542* allele. We conclude that RelA is dispensable for σ^B induction by physical stress, but it is necessary for the activation of σ^B's nutritional stress pathway when triggered by either PO_4 or glucose limitation.

The stringent response doesn't activate σ^B. A requirement for RelA in activating the nutritional stress pathway of σ^B was unexpected. We and others had previously found no induction of σ^B following amino acid limitation, the classical activator of RelA (23, 40). Two experiments were undertaken to verify this finding. The first involved examining the ability of a *B. subtilis* strain, lacking ribosome protein L11, to respond to PO_4 limitation and activate σ^B. The gene encoding L11 (*rplK*) was originally designated *relC* based on the inability of strains with mutations at this locus to initiate the stringent response (32, 33, 43). As seen in Fig. 5, the *rplK57* strain (BSZ9), unlike the RelA$^-$ strain, is still able to respond to PO_4 limitation and activate σ^B. This result demonstrates that even though the nutritional stress pathway has a RelA-dependent component, this component does not require a ribosome protein that is necessary for RelA activation by amino acid limitation.

To directly examine the possible connection between the traditional stringent response and σ^B activation, we compared the effects of norvaline, an inducer of the stringent response, to that of PO_4 limitation on the activity of a σ^B-dependent promoter. The *sigB* operon has two promoters, a σ^A-dependent promoter providing basal expression of the operon, and an internal σ^B-dependent promoter that upregulates the expression of the downstream half of the operon (18, 42). With reverse transcription-PCR techniques to follow the activity of these promoters, we observed (Fig. 6) that the activity of σ^B-dependent promoter (P_B) was stimulated by PO_4 limitation, but diminished following induction of the stringent response. The activity of the σ^A promoter (P_A) was reduced by both limitation for PO_4 and norvaline treatment. Thus, the abundance of the σ^B-dependent transcript increases relative to the σ^A-dependent transcript following PO_4 limitation, but not norvaline treatment. The observation that a mutation (*rplK56*),

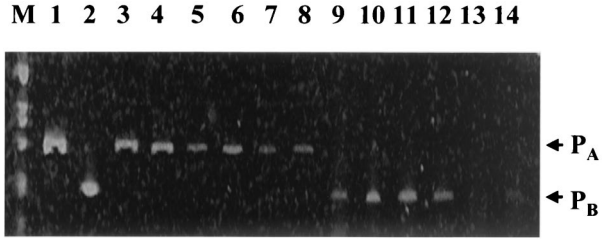

FIG. 6. Effect of PO_4 or amino acid limitation on the activities of the sigB operon promoters. BSA46 (wild-type *B. subtilis*) was grown in a complete synthetic medium to an optical density of 0.5, pelleted, and resuspended in either a medium without PO_4 (lanes 3 to 5, 9 to 11) or lacking Casamino Acids but containing norvaline (0.05%) (lanes 6 to 8, 12 to 14). Samples were taken at 2 (lanes 3, 6, 9, and 12), 20 min (lanes 4, 7, 10, and 13), and 70 min (lanes 5, 8, 11, and 14) after resuspension and analyzed by reverse transcription-PCR with oligonucleotides that would amplify nucleotides +44 to +304 of the σ^A-dependent transcript (P_A) and nucleotides +32 to +210 of the σ^B-dependent transcript (P_B). After amplification, the DNAs were fractionated by polyacrylamide gel electrophoresis (6% acrylamide) and stained with ethidium bromide. Lanes 1 and 2 illustrate the PCR products amplified from *B. subtilis* chromosomal DNA with the P_A- and P_B-specific oligonucleotides, respectively. M, 1-kbp DNA ladder (Invitrogen).

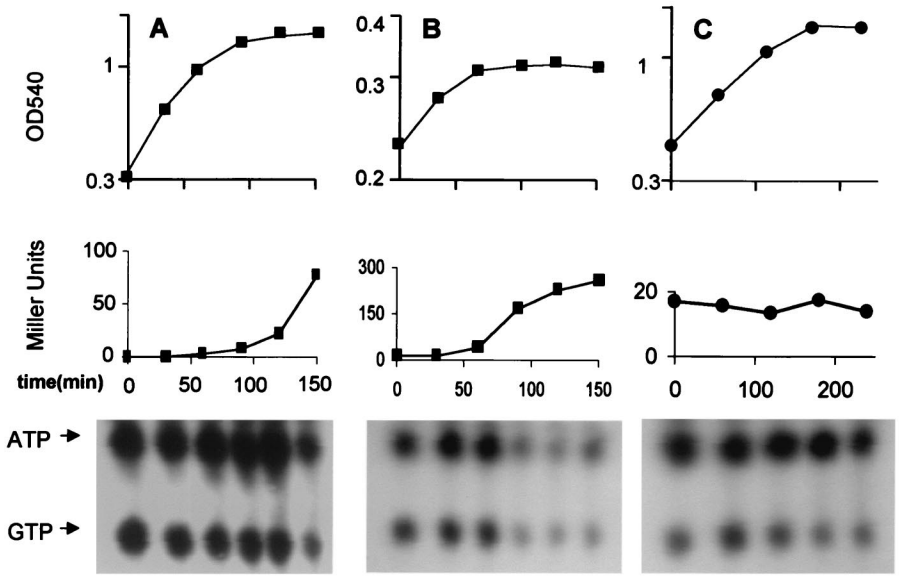

FIG. 7. Changes in ATP/GTP levels by RelA$^{+/-}$ *B. subtilis* in low-PO_4 medium. Parallel cultures of wild-type *B. subtilis* (BSA46) or a congenic RelA$^-$ strain (BSZ7) were grown in synthetic medium with [^{32}P]H_3PO_4 (100 μCi/ml), for labeling of nucleotides, or without [^{32}P]H_3PO_4, for growth and σ^B-dependent β-galactosidase measurements. (A) BSA46 grown in synthetic medium with high glucose and phosphate. (B) BSA46 grown in low-phosphate medium, (C) BSZ7 grown in low-phosphate medium. Samples were taken and analyzed for growth (optical density) (top panels), β-galactosidase activity (Miller units) (middle panels), and ATP/GTP (thin-layer chromatography) (bottom panels). The positions of ATP and GTP in the thin-layer chromatography system employed were determined with markers as in Fig. 2.

which blocks the RelA-dependent stringent response, fails to prevent σ^B induction by PO_4 limitation, and our confirmation of the finding that amino acid limitation does not activate σ^B, argue that the RelA-dependent contribution to σ^B activation is distinct from its role in the stringent response.

Nucleotide changes during activation of σ^B. RelA's role as a component of the nutritional stress pathway for σ^B activation is likely to be tied to a unique aspect of its activity during glucose and PO_4 starvation and not the accumulation of (p)ppGpp. Previous models of the induction of σ^B by nutritional stress envisioned a drop in high energy nucleotides as a possible signal for σ^B activation. We therefore asked whether this might be the case in the RelA directed activation and if nucleotide levels are differentially altered during PO_4 limitation in RelA$^{+/-}$ strains.

To examine this, RelA$^+$ and RelA$^-$ strains of *B. subtilis* were grown to stationary phase in PO_4-limited medium. Parallel cultures with or without $^{32}PO_4$ were sampled for changes in ATP/GTP abundance by thin layer chromatography and σ^B activity by σ^B-dependent β-galactosidase activity, respectively. As illustrated in Fig. 7, coincident with a cessation of growth in PO_4-limited medium, the RelA$^+$ strain displayed an increase in σ^B activity and a drop in ATP/GTP (Fig. 7B). Neither the drop in ATP/GTP nor the activation of σ^B was evident when the Rel$^+$ strain entered stationary phase in a medium with adequate glucose and phosphate (Fig. 7A). Continued incubation of this culture did however lead to a decrease in nucleotide levels and a rise in σ^B activity after 1.5 h in stationary phase. As expected, the RelA$^-$ strain failed to induce σ^B activity upon entry into stationary phase in the PO_4-deficient medium. In addition, it did not display the dramatic decline in ATP/GTP levels that occurred in the RelA$^+$ strain (Fig. 7C).

To estimate the degree to which ATP and GTP declined in these cultures, we quantitated several representative chromatograms by densitometry. In PO_4-limited medium, the levels of ATP and GTP, present in the stationary phase RelA$^+$ extract dropped to around 20% of that seen in the exponentially growing culture, (Table 2). In contrast, the ATP/GTP levels of either the RelA$^+$ culture in high PO_4 medium, or the RelA$^-$ culture in either low or high-PO_4 medium, persisted at levels that were approximately 80% to 90% of that seen in exponentially growing cultures. Activation of the stringent response by the addition amino acid analogs triggered a predictable drop in GTP levels, due to ppGpp synthesis, but no decline in ATP (Table 2). These results reveal a coincidence between the RelA$^+$ phenotype, nutritional conditions that trigger σ^B activation, and a drop in ATP/GTP levels.

RelA's activation of σ^B does not correlate with (p)ppGp accumulation. PO_4 and glucose limitation can induce both σ^B activity and the synthesis of (p)ppGpp. *E. coli* has two enzymes, RelA and SpoT, that are capable of synthesizing (p)ppGpp (10). RelA responds to amino acid starvation, while SpoT is triggered by glucose/PO_4 limitation. In *B. subtilis* (p)ppGpp synthesis appears to be solely dependent on a single RelA/SpoT homolog (41). Given that the synthesis of (p)ppGpp has been reported to cause a significant drop in GDP/GTP levels in *B. subtilis* (16, 25), it seemed plausible that the RelA dependent drop in ATP/GTP levels seen following PO_4 limitation might involve such a reaction.

With thin-layer chromatography, we asked whether *B. subtilis*, subjected to PO_4 limitation, with its accompanying decrease in ATP/GTP levels, would display a concomitant accumulation of (p)ppGpp. If an increase in (p)ppGpp paralleled the decrease in ATP/GTP, it would suggest a mechanism by

TABLE 2. Changes in ATP and GTP

Strain	Culture conditions[a]	Relative nucleotide levels[b] (%)	
		ATP	GTP
BSA46 (RelA$^+$)	Stationary phase 0.6 mM PO_4^{2-}	104.9 ± 2.9	79.25 ± 5.25
	Stationary phase 0.15 mM PO_4^{2-}	19.6 ± 9.3	14.8 ± 3.2
	Norvaline (1 mg/ml) + serine hydroxamate (1 mg/ml)	114.5 ± 5.5	31.75 ± 4.75
BSZ-7 (RelA$^-$)	Stationary phase 0.6 mM PO_4^{2-}	82.8 ± 16	75 ± 7
	Stationary phase 0.15 mM PO_4^{2-}	87.3 ± 9	88.5 ± 12

[a] *B. subtilis* grown in [$^{32}PO_4$] was harvested 1 h after growth slowed in either high or low PO_4 medium or 40 min after the addition of the indicated amino acid analogs.
[b] Samples were extracted with 3 M formic acid and analyzed as in Fig. 3. Autoradiograms of the separated nucleotides were quantitated by densitometry (Alpha Imager 2000) and compared to the amount of that nucleotide extracted from a similar concentration of cells (based on the O D_{540}) harvested during exponential growth. The data (average of two separate experiments) are expressed as a percentage of the labeled nucleotide in a mid-log-phase culture growing in the same medium.

which RelA might directly reduce the cell's ATP/GTP levels. Extracts of $^{32}PO_4$-labeled *B. subtilis* entering stationary phase in either a low PO_4 or a high PO_4 medium, or exposed to an amino acid analogs to activate the stringent response, were separated by thin-layer chromatography and examined for ATP, GTP and (p)ppGpp by autoradiography. The PO_4-limited culture (Fig. 8 lanes 1 to 4) displayed the anticipated decrease in ATP/GTP, but failed to accumulate (p)ppGpp at levels comparable to the decline in ATP/GTP or the amount detected in the culture exposed to an amino acid analog (Fig. 8 lanes 9 to 11). Extended (5x) exposure of the chromatogram detected small amounts of ppGpp in both the low and high PO_4 cultures (not shown). The failure of (p)ppGpp to accumulate at levels equivalent to the levels of ATP/GTP decline argues that either the ATP/GTP decline isn't a direct result of (p)ppGpp synthesis or that the (p)ppGpp, formed under these conditions, is being rapidly turned over.

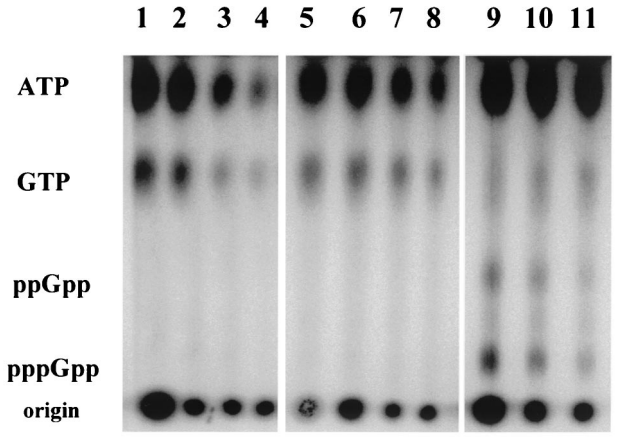

FIG. 8. Changes in ATP, GTP, and (p)ppGpp by B. subtilis following entry into stationary phase in low or high PO_4 medium or amino acid limitation. Wild-type *B. subtilis* (BSA46) grown in synthetic medium with [^{32}P]H_3PO_4 (100 μCi/ml) for labeling nucleotides and either limiting (lanes 1 to 4) or nonlimiting PO_4 (lanes 5 to 11). At an optical density of 0.4, norvaline (1 mg/ml) and serine hydroxamate (1 mg/ml) were added to a portion of the culture with nonlimiting PO_4 (illustrated in lane 5) to mimic amino acid starvation. Samples were taken at 20-min intervals for both the untreated (lanes 6 to 8) and amino acid-limited (lanes 9 to 11) cultures. The low-PO_4 and high-PO_4 cultures ceased exponential growth at the times represented by lanes 3 and 7, respectively. Growth of the amino acid-limited cultures slowed by the first sample taken after the addition of the amino acid analogs (lane 9). Samples were analyzed by thin-layer chromatography as in Fig. 2.

BSJ19 (IS*56*) is a relaxed *B. subtilis* strain. It carries a *relA* mutation in which a glycine residue, conserved in both RelA and SpoT, is replaced by glutamate (41). *B. subtilis* carrying this *relA* allele (*relA240GE*) has approximately 2% of the (p)ppGpp synthetase activity of wild-type strains (41). BSJ19's inability to synthesize (p)ppGpp while still encoding a RelA protein allows us to ask whether the RelA-dependent drop in ATP/GTP and the inducibility of σ^B is linked to RelA's (p)ppGpp synthetase activity. In a preliminary experiment to verify the strain's Rel$^-$ phenotype, we subjected BSJ19 and its congenic parent (BSJ18) to amino acid limitation and examined their accumulation of (p)ppGpp by thin-layer chromatography. As illustrated in Fig. 2, the RelA$^+$ strain (lanes 7 to 9) readily accumulated (p)ppGpp, while these nucleotides were barely detected in the *relA240GE* strain (lanes 10 to 12). We also noted that, unlike our *relA* null strain, whose growth rate was only one third of that of its RelA$^+$ parent, the growth rates of BSJ18 and BSJ19 were indistinguishable in rich (LB) medium.

The BSJ18/19 strains were cultured in PO_4-limited medium with samples taken to assess both σ^B induction and possible ATP/GTP changes. As illustrated in Fig. 9, the strain with the *relA240GE* allele (Fig. 9B) activated σ^B to a level that was similar (70%) to that of its wild-type counterpart (Fig. 9A) and displayed a reduction in ATP/GTP that was virtually indistinguishable from the RelA$^+$ strain as the cultures' growth slowed. This result suggests that RelA's role in activating the nutritional stress pathway of σ^B, is not only distinct for its known role in triggering the stringent response, but may involve an activity other than (p)ppGpp synthesis.

DISCUSSION

Induction of σ^B by the nutritional stress occurs via the activation of the RsbQ/P phosphatase (9, 36), a process that is triggered by culture conditions consistent with reducing the cells' overall energy level (40). It is plausible, although unproven, that the drop in nucleoside triphosphate that occurs concomitant with σ^B activation could itself be the signal that communicates nutritional stress to RsbP/Q. The current work reaffirms the coincidence of σ^B activation with a drop in nucleoside triphosphates, but adds a possible role for RelA in this process.

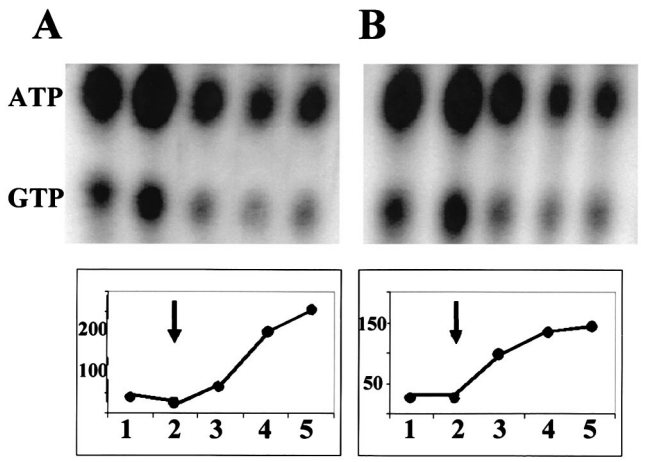

FIG. 9. σ^B induction and ATP/GTP levels in *relA240GE B. subtilis*. BSJ18 (RelA$^+$) (A) and its congenic *relA240GE* strain (BSJ19) (B) were grown in a synthetic medium with limiting PO_4. [^{32}P]H_3PO_4 (100 μCi/ml) was present in parallel cultures for labeling of nucleotides. Samples were taken at 20-min intervals (represented by the numbers on the *x* axis) and analyzed as in Fig. 7 for σ^B-dependent β-galactosidase activity (Miller units) and ATP/GTP. The arrows indicate the onset of stationary phase for each culture.

In an earlier study characterizing *relA* in *B. subtilis*, σ^B-dependent gene products were among a group of proteins whose levels appeared to increase following amino acid depletion in a RelA$^+$ but not a RelA$^-$ strain of *B. subtilis* (41). Although we and others were not been able to induce σ^B by depletion of amino acids in past (23, 40) or current work (Fig. 6), we now find that RelA influences the RsbP-dependent pathway of σ^B activation in response to glucose or PO_4 limitation. In the original RelA study (41), the authors raised the possibility that the observed induction of σ^B-dependent proteins might have been due to oxygen limitation rather than amino acid deprivation. Given that oxygen limitation is an inducing condition for σ^B's nutritional stress pathway (36), this circumstance could reconcile our findings. RelA is clearly involved in the activation of σ^B by nutritional stress, but, the mechanism by which RelA contributes to this process is not clear.

RelA's best characterized activity is that of a (p)ppGpp synthetase; however it is unlikely that (p)ppGpp itself is the signal that triggers σ^B induction. Amino acid limitation, an effective inducer of (p)ppGpp synthesis fails to activate σ^B. A notable difference between the RelA$^{+/-}$ strains is the decline in ATP/GTP levels in the RelA$^+$ strain and the persistence of these nucleotides at relatively stable levels in the RelA$^-$ strain, when growth slowed in PO_4-limiting medium. In light of the observation that inhibitors of GTP or ATP synthesis (i.e., decoynine, azide) can induce σ^B in otherwise rich medium (24, 40), this result raises the possibility that a RelA-dependent effect on nucleotide levels, rather than (p)ppGpp synthesis itself, could be the connection between glucose or phosphate limitation and the activation of σ^B. In support of the idea that RelA's role in σ^B activation involves facilitating a decline in energy charge, we have observed (S. Zhang, unpublished data) that azide treatment can activate σ^B in a RelA$^-$ strain.

Assuming that RelA can activate σ^B by directing a decline in ATP/GTP, can this decline be attributed to its activity as a (p)ppGpp synthetase? The synthesis of (p)ppGpp is known to result in a significant drop in GTP/GDP levels (10, 16, 22, 35). The GTP drop has two causes: the use of GTP as a substrate for (p)ppGpp synthesis, and a (p)ppGpp-dependent inhibition of IMP dehydrogenase, an enzyme in the guanine nucleotide biosynthetic pathway (22). This phenomenon is known to affect another stress-activated response of *B. subtilis*, entry into sporulation. GTP/GDP levels are the intracellular indicators of nutrient availability that trigger endospore formation (21, 22, 28, 34). An order of magnitude drop in GTP, but not ATP, occurs when *B. subtilis* cultures are induced to sporulate, with RelA's (p)ppGpp synthetase contributing to this GTP decline (21, 22).

It could be speculated that under the culture conditions which we use to activate σ^B, the synthesis of (p)ppGpp in glucose/PO_4 starved cells drops GTP/ATP to a sufficiently low level so as to activate σ^B's nutritional stress pathway, while adequate carbon and PO_4 reserves could prevent a sufficient drop in nucleotide pools during (p)ppGpp synthesis induced by amino acid limitation. This might allow σ^B activation under one (p)ppGpp inducing condition but not the other.

Two of our findings are not consistent with this model. First, the levels of (p)ppGpp that are formed in response to PO_4 limitation are significantly less than the marked drop in ATP/GTP levels that occurs in the culture under this condition (Fig. 8). If the drop in ATP/GTP is a consequence of the channeling of these nucleotides into (p)ppGpp, ppGpp turnover under this condition will need to be invoked to explain its low abundance. Second, a *relA* variant (*relA240GE*), whose product has only 2% of the (p)ppGpp synthetase activity of the wild-type RelA (Fig. 2), displays near normal levels of σ^B activity and ATP/GTP decline following PO_4 limitation (Fig. 9). A more likely model might envision a yet to be defined RelA function that affects nucleotide levels, but is not directly tied to RelA's activity as a (p)ppGpp synthetase. The existence of such an unspecified property is suggested by the slow growth phenotype of our "null" *relA* mutant. If RelA's function is limited to (p)ppGpp synthesis and decay, it is not obvious why *relA* null mutants, created by us and others (41), display impaired growth, while the missense mutant (*relA240GE*), whose lesion affects (p)ppGpp synthetase activity, does not.

In light of our previous finding of the likely involvement of a putative ribosome associated GTP binding protein (Obg) in activating the physical stress pathway of σ^B induction (30, 31, 46), it is intriguing that RelA, another ribosome associating protein, might be involved in activating σ^B's nutrient-dependent pathway. This coincidence raises the possibility that the ribosome associated processes could be common elements in both pathways of σ^B activation, with ribosome-associated proteins sensing unique stress-triggered changes in ribosome status and communicating this to the stress-activators of σ^B.

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