JOURNAL OF BACTERIOLOGY, July 1995, p. 3771–3780
0021-9193/95/$04.00+0

Vol. 177, No. 13

Separate Mechanisms Activate σ^B of *Bacillus subtilis* in Response to Environmental and Metabolic Stresses

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Received 18 January 1995/Accepted 28 April 1995

σ^B is a secondary sigma factor that controls the general stress response of *Bacillus subtilis*. σ^B-dependent transcription is induced by the activation of σ^B itself, a process that involves release of σ^B from an inhibitory complex with its primary regulator, RsbW. σ^B becomes available to RNA polymerase when RsbW forms a complex with an additional regulatory protein (RsbV) and, because of this, fails to bind σ^B. Using Western blot (immunoblot) analyses, reporter gene fusion assays, and measurements of nucleotide pool sizes, we provide evidence for two independent processes that promote the binding of RsbW to RsbV. The first occurs during carbon limitation or entry into stationary phase. Activation of σ^B under these circumstances correlates with a drop in the intracellular levels of ATP and may be a direct consequence of ATP levels on RsbW's binding preference. The second activation process relies on the product of a third regulatory gene, *rsbU*. RsbU is dispensable for σ^B activation during carbon limitation or stationary phase but is needed for activation of σ^B in response to any of a number of different environmental insults (ethanol treatment, salt or acid shock, etc.). RsbU, or a process dependent on it, alters RsbW binding without regard for intracellular levels of ATP. In at least some instances, the effects of multiple inducing stimuli are additive. The data are consistent with RsbW being a regulator at which distinct signals from separate effectors can be integrated to modulate σ^B activity.

σ^B is a secondary sigma factor of *Bacillus subtilis* (7, 14, 15). It is activated to direct transcription of a subset of genes when *B. subtilis* stops exponential growth in sporulation-suppressing media or is subjected to any of a number of environmental stresses (e.g., heat, salt, ethanol, and peroxide) (3, 5, 8–10, 16, 17, 34). Regulation of σ^B is complex, with at least four proteins known to function in its control (3, 4, 10, 16, 33, 36). Three of these proteins (RsbV, RsbW, and RsbX) are the products of genes that are cotranscribed with the σ^B structural gene (*sigB*) in an operon whose promoter is recognized by the σ^B-containing form of RNA polymerase (E-σ^B) (3, 10, 18). The fourth protein (RsbU) is the product of a gene which lies immediately upstream of the *sigB* operon (33, 36). The *rsbU* transcription unit includes three additional genes of undefined function (36). There is evidence that the *rsbU* transcript can extend into the *sigB* operon; however, the significance of this is unknown (33, 35, 36).

Of the four proteins recognized as controlling σ^B activity, RsbW is the primary σ^B regulator, with RsbW activity modulated by the other three proteins (3, 10, 12, 33). RsbW is a σ^B binding protein (6). Its association with σ^B prevents the sigma factor's complexing with RNA polymerase (6, 12, 33). A second regulator, RsbV, is essential for σ^B release from RsbW inhibition (3, 10). RsbW can form a mutually exclusive complex with either RsbV or σ^B (6, 12). In the absence of RsbV, σ^B remains bound to RsbW, thereby blocking σ^B-dependent transcription (6, 12). The basis of RsbW's binding decision is unknown, but it may be influenced by the ratio of ATP to ADP present within the bacterium. In vitro studies of another *B. subtilis* sigma factor (σ^F) controlled by proteins homologous to RsbW and RsbV demonstrated that the RsbW homolog (SpoIIAB) preferentially binds to σ^F when the ATP/ADP ratio is high and to SpoIIAA, the RsbV counterpart, when the ratio is low (1). On the bases of the similarity between the σ^F and σ^B regulators and the observation that at least some conditions that induce σ^B are also likely to lower the intracellular concentrations of ATP, Alper et al. proposed that RsbW, like SpoIIAB, may be sensitive to ATP levels and preferentially bind to σ^B when the ATP/ADP ratio is high and to RsbV when it is low (1).

Besides there being a possible role for ATP in directly influencing RsbW's binding preference, ATP is also a phosphate donor in a RsbW-catalyzed reaction that phosphorylates RsbV (12). Both RsbV and SpoIIAA are phosphorylated by their corresponding anti-σ factors (1, 12, 24). The phosphorylated form of RsbV is not found associated with RsbW (12). It is probable that phosphorylation of RsbV directs some aspect of σ^B regulation; however, it is unclear whether RsbV phosphorylation represents a device to specifically inactivate RsbV or is the result of a reaction that RsbW undergoes in order to exchange ATP for ADP. The remaining two σ^B regulators, RsbU and RsbX, appear to influence the binding preference of RsbW in response to a subset of activation signals. The findings of our previous genetic and biochemical experiments argued that RsbU, or a process dependent on RsbU, can promote RsbW binding to RsbV, thereby allowing E-σ^B formation (33). RsbU-dependent activation of σ^B was most evident in strains which lacked the fourth σ^B regulator, RsbX (33). Thus, RsbX behaves as a negative regulator of RsbU-dependent σ^B activation.

Although RsbU could be shown to elevate σ^B activity to high levels, the conditions under which it normally does so were not entirely clear. We saw no significant contribution of RsbU to σ^B activation when *B. subtilis* entered the stationary phase of growth in Luria broth (LB). However, we did note that RsbU could contribute to σ^B activation during other forms of stress (33). An effect of RsbU on σ^B activity was also reported by Wise and Price (36). These authors documented less σ^B activ-

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TABLE 1. *B. subtilis* strains and plasmids used for this study

Strain or plasmid	Relevant genotype or feature(s) of plasmid	Construction or reference
Strains		
168Δ*atp*2	Δ*atpFHAGDC*::*kan*	28
PY22	*trpC2*	P. Youngman, University of Georgia
BSA46	SPβ *ctc*::*lacZ*	3
BSA132	*rsbV312 rsbX*::pWH25[a] SPβ *ctc*::*lacZ*	33
BSA140	*rsbU*Δ*Nde*I *rsbX*::pWH25 SPβ *ctc*::*lacZ*	33
BSA142	*rsbU*::pAL127[b] SPβ *ctc*::*lacZ*	33
BSA158	*rsbU*Δ*Nde*I *rsbX*::pWH25	BSA140⇒PY22[c]
BSA159	*rsbV312 rsbX*::pWH25	BSA132⇒PY22
BSA179	*rsbX*::pWH25	pWH25⇒PY22
BSA180	SPβ *ctc*::*sigE41*	SPβ *ctc*::*sigE41* × PY22[d]
BSA181	SPβ *gsiB*::*sigE41*	SPβ *gsiB*::*sigE41* × PY22
BSA182	SPβ *sigB*::*sigE41*	SPβ *sigB*::*sigE41* × PY22
BSA191	*rsbU*Δ*Nde*I *rsbX*::pWH25 SPβ *ctc*::*sigE41*	SPβ *ctc*::*sigE41* × BSA158
BSA192	*rsbU*Δ*Nde*I *rsbX*::pWH25 SPβ *gsiB*::*sigE41*	SPβ *gsiB*::*sigE41* × BSA158
BSA193	*rsbU*Δ*Nde*I *rsbX*::pWH25 SPβ *sigB*::*sigE41*	SPβ *sigB*::*sigE41* × BSA158
BSA267	SPβ *ctc*::*sigE41*	pGB3C⇒ZB307
BSA268	SPβ *gsiB*::*sigE41*	pGB3G⇒ZB307
BSA269	SPβ *sigB*::*sigE41*	pGB3S⇒ZB307
BSA270	SPβ *ctc*::*lacZ* Δ*atpFHAGDC*::*kan*	168Δ*atp*2⇒BSA46
BSA271	*rsbU*Δ*Nde*I *rsbX*::pWH25 SPβ *ctc*::*lacZ* Δ*atpFHAGDC*::*kan*	168Δ*atp*2⇒BSA140
BSA272	*sigB*::Δ*Hin*dIII-*Eco*RV::*cat*	ML6⇒PY22
ML6	*sigB*::Δ*Hin*dIII-*Eco*RV::*cat*	16
ZB307	SPβc2Δ2::pSK10Δ6	38
Plasmids		
pGB3	*bla spc sigE41*	This study
pGB3C	*bla spc ctc*::*sigE41*	This study
pGB3G	*bla spc gsiB*::*sigE41*	This study
pGB3S	*bla spc sigB*::*sigE41*	This study
pKSB52	*bla sigB*	This study
pKSC8	*bla ctc*	22
pKSG1	*bla gsiB*	22
pBluescriptKS	*bla*	27
pKSSP	*bla* P_B[e]	This study
pV312	*bla* P_B *rsbV312*	3
pUS19	*bla spc*	4

[a] The integrative plasmid pWH25 contains a 2-kb *Eco*RI-*Sph*I fragment including the 3′ end of *rsbX* and 1.9 kb downstream of *rsbX*. Strains transformed with this plasmid are $RsbX^+$.

[b] The integrative plasmid pAL127 contains a 207-bp *Pst*I-*Eco*RI fragment that includes the 3′ end of *rsbU* and the *sigB* promoter. The presence of pAL127 leaves *rsbU* intact and prevents potential readthrough from *rsbU* into the *sigB* operon.

[c] An arrow indicates the construction of the strain by transformation.

[d] A cross indicates the construction of the corresponding strain by transduction with a SPβ-based specialized transducing phage.

[e] The P_B plasmids contain the σ^B-dependent promoter of the *sigB* operon.

ity in $RsbU^-$ *B. subtilis* than that seen in $RsbU^+$ strains when both were grown in LB medium supplemented with glucose and glutamine (LBGG) (36). In this paper, we examine the role of RsbU in σ^B activation in detail and present evidence for at least two distinct mechanisms of σ^B activation: an RsbU-independent mechanism, which is likely to be effected by a drop in the intracellular ATP level and is responsible for σ^B activation upon entry into stationary phase, and an RsbU-dependent mechanism which activates σ^B in response to environmental stress (salt shock, ethanol exposure, heat shock, etc.). This latter mechanism appears independent of ATP levels.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *B. subtilis* strains and the plasmids used in this study are listed in Table 1. All BSA strains are derivatives of PY22, which was originally obtained from P. Youngman (University of Georgia). We transferred the *rsbU*Δ*Nde*I, *rsbV312*, *sigB*::Δ*Hin*dIII-*Eco*RV::*cat*, and *rsbX*::pWH25 alleles into PY22 by transformation with chromosomal DNA from BSA140, BSA132, and ML6, respectively (Table 1). The presence of the alleles was verified by PCR with the use of the primers described previously (33).

The *ctc*, *gsiB*, and *sigB* promoters were fused to the *sigE41* allele so that σ^B-dependent promoter activity could be monitored by Western blot (immunoblot) with a previously described anti-σ^E antibody (32). *sigE41* encodes a truncated, inactive form of the *B. subtilis* sporulation-specific sigma factor σ^E (32). The plasmid pGB3 was created by cloning a 1.1-kb *Pst*I fragment that carries the *sigE41* allele into pUS19 (4). Plasmid pGB3C is a *ctc*::*sigE41* transcriptional fusion containing the *ctc* promoter and its N terminus on a 627-bp *Eco*RI-*Hin*cII fragment from pKSC8 cloned into the *Eco*RI-*Sma*I sites of pGB3. Plasmid pGB3G is a *gsiB*::*sigE41* transcriptional fusion. It was formed from pGB3 by ligating a 617-bp *Sac*I-*Pvu*II fragment of pKSG1 with pGB3 digested with *Sac*I and *Sma*I. pGB3G encodes an intact GsiB protein without the transcriptional terminator. A *sigB*::*sigE41* transcriptional fusion was constructed by first cloning a 207-bp *Pst*I-*Eco*RI fragment of pV312 (3) that contains the σ^B-dependent promoter of *sigB* and the first two codons of *rsbV* into pBluescriptKS to obtain the needed restriction endonuclease sites. The resulting plasmid, pKSSP, was digested with *Sac*I and *Hin*cII, and a 276-bp fragment carrying the *sigB* promoter was cloned into *Sac*I-*Sma*I-digested pGB3 to yield pGB3S. pGB3 and its derivatives cannot replicate in *B. subtilis* but can confer resistance to spectinomycin after integration into the chromosome by single-site homologous recombination. The plasmids pGB3C, pGB3G, and pGB3S were transformed into *B. subtilis* ZB307. ZB307 carries a temperature-inducible SPβ prophage with sequences homologous to the pUS19 vector (38). Phage lysates were prepared from these transformants and screened for the ability to transduce the *spc* marker. Three such transformants (BSA267, BSA268, and BSA269) were identified as sources for specialized transducing-phage lysates (with pGB3C, pGB3G, and pGB3S,

respectively). With these lysates, *ctc*::*sigE41*, *gsiB*::*sigE41*, and *sigB*::*sigE41* were transduced into PY22 and BSA158, generating strains BSA180 to -182 and BSA191 to -193, respectively (Table 1). BSA270 and BSA271 were constructed by transforming BSA46 and BSA140 with chromosomal DNA from 168Δ*atp*2 (28). *Escherichia coli* TG2 (27) was routinely grown in LB (27) and used as host for DNA manipulation.

Growth conditions. In all experiments, the *B. subtilis* strains were cultivated with vigorous agitation (250 rpm) at 37°C. For the investigation of the effect of NaCl, heat shock, acid, or ethanol, the bacteria were grown in LB. LB buffered with potassium phosphate (8) was used for the experiments involving the addition of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; an uncoupler of oxidative phosphorylation). This medium was reported to trigger a stronger CCCP induction of *ctc-lacZ* than LB alone (2). Substitution of LB buffered with potassium phosphate for LB did not significantly change the induction of *ctc-lacZ* by other stresses or entry into stationary phase. The following environmental stresses were investigated: heat shock, by transfer of bacteria to a 48°C environment; salt stress, by addition of NaCl to a final concentration of 2.5% (wt/vol); ethanol stress, by addition of ethanol to a final concentration of 4% (vol/vol); acid shock, by addition of 1 M HCl to shift the pH of the medium from 6.5 to 5.25; and CCCP, which was added to a final concentration of 2 μM. With the exception of heat shock, all stresses shifted the generation time from approximately 22 to 35 min. *B. subtilis* strains grew faster after the shift to 48°C than at 37°C. The availability of oxygen was limited by reducing the shaking frequency of the culture to 50 rpm. This reduction slowed the growth rate of the culture to a generation time of 280 min. The effects of limiting the availability of specific nutrients were tested by using a synthetic medium described previously (31). Glucose limitation and phosphate limitation were accomplished by growing the bacteria in medium with 0.05% glucose and 0.18 mM KH_2PO_4, respectively. A limitation for tryptophan was triggered by growing the bacteria in medium with 62 nM L-tryptophan. Starvation for nitrogen was accomplished by using a synthetic medium without glutamate and reducing the amount of $(NH_4)_2SO_4$ to 1 mM. Growth limitation for the intended factor was verified by the addition of the limited nutrient and observation of the resumption of growth.

Quantification of the ATP-ADP pool. The levels of ATP and ADP were measured as described previously (22). The procedure involved harvesting of cells by filtration through a cellulose nitrate filter, immediate addition of ice-cold 10% (wt/vol) trichloroacetic acid, and extraction of nucleotides for 30 min at 4°C. The extracts were treated four times with water-saturated diethyl ether and mixed with 1 volume of twofold concentrated high-pressure liquid chromatography (HPLC) buffer. HPLC buffer consists of 125 mM potassium phosphate, 10 mM tetrabutylammonium hydrogen phosphate, and 6% (vol/vol) methanol (pH 6.5; adjusted with potassium hydroxide). After centrifugation (5 min, 12,000 × *g*, 4°C), 50 μl of the extract was injected into the HPLC system. Nucleotides were separated on a reversed-phase column (SUPELCOSIL LC18T; C_{18}, 4.6-mm internal diameter, 3-μm pore size) with HPLC buffer as a mobile phase. Analysis was performed isocratically at a flow rate of 1.5 ml/min.

SDS-PAGE and Western blot analysis. Bacteria were disrupted by passage through a French press. The protein concentration was determined with a Bio-Rad protein assay according to the manufacturer's instructions, and identical amounts of protein were applied to each lane. For the detection of σ^B or σ^E, the extracts were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 10% acrylamide. All additional steps of the Western blot analysis were performed as described previously (4). The monoclonal antibodies against RsbV, RsbW, σ^B, and σ^E have been described elsewhere (4, 5, 32).

Assay of β-galactosidase activity. Cells were harvested by centrifugation and frozen at −20°C. β-Galactosidase assays were done by the method of Kenney and Moran (19), with chloroform and SDS to permeabilize the bacteria. The activity was expressed in Miller units $[10^3 \times A_{420}/(\text{volume} \times \text{time} \times A_{540})]$ (23). An alternative assay was performed to more precisely measure the low uninduced levels of Ctc-LacZ in the studies with Δ*atp* mutations. This process involved resuspending cell pellets in Z buffer (23) and disrupting bacteria by passage through a French pressure cell. After removal of the cell debris by centrifugation, dilutions of the supernatant were assayed for β-galactosidase activity (23). Protein concentrations were determined by a Bio-Rad protein assay as recommended by the manufacturer. β-Galactosidase activity was calculated by the following formula: $\Delta A_{420} \times 1{,}000 \times \text{min}^{-1} \times \text{mg of protein}^{-1}$.

RNA isolation and slot blot analysis. Total RNA of *B. subtilis* was isolated as described previously (34). Decreasing amounts of total RNA were transferred onto a positively charged nylon membrane by slot blotting and hybridized with digoxygenin-labeled RNA that had been synthesized in vitro from the linearized plasmid pKSB52. pKSB52 was constructed by inserting a 610-bp *Hin*dIII-*Cla*I fragment coding for σ^B into pBluescriptII KS. After linearization with *Sac*I, digoxygenin-labeled RNA was synthesized from the noncoding strand with T3 RNA-polymerase and used as a probe for the hybridization. The chemiluminograms were quantified with a Personal Densitometer from Molecular Dynamics.

General methods. DNA manipulations and the transformation of *E. coli* were performed according to standard protocols (27). Transformation of competent *B. subtilis* cells was carried out by the method of Yasbin et al. (37) with transformants selected on LB agar plates containing 10 μg of kanamycin per ml or 200 μg of spectinomycin per ml.

RESULTS

RsbU-dependent induction of *ctc*::*lacZ*. Expression of a *sigB* operon containing a null *rsbX* allele yields a high level of σ^B activity in $RsbU^+$ but not $RsbU^-$ strains of *B. subtilis* (33). This result suggests the existence of an RsbU-dependent σ^B activation mechanism that is regulated by RsbX; however, the circumstances that induce RsbU-dependent activation of σ^B are unknown. In earlier studies, we saw no obvious effect of RsbU on σ^B activation by the entry into the stationary phase of growth (3, 4, 33). We and others noted that a number of environmental stresses, besides entry into stationary phase, lead to σ^B activation (5, 8, 34). We therefore tested whether the RsbU pathway participated in σ^B activation following exposure to any of these stresses.

Exponentially growing *B. subtilis* that carries a translational fusion of the σ^B-dependent *ctc* gene to *E. coli lacZ* was exposed to various stimuli and analyzed for σ^B activity by assaying *ctc*-dependent β-galactosidase levels. Salt shock and ethanol treatment had been previously shown to be potent inducers of σ^B activity (8, 34). This effect is reproduced in our present study (Fig. 1A and B), in which moderate salt or ethanol stress triggered a significant induction of *ctc*::*lacZ* in wild-type *B. subtilis* (BSA46). The induction was transient, with maximum β-galactosidase levels measured at 20 min after application of the stress. When similar experiments were conducted with a *B. subtilis* strain (BSA140) with a null mutation in *rsbU* (*rsbU*Δ*Nde*I), there was no induction of the *ctc* promoter following salt or ethanol stress (Fig. 1A and B).

The response of *ctc*::*lacZ* expression to acid shock was also tested. In preliminary experiments, using the addition of hydrochloric acid to change the culture pH, we determined that the maximum *ctc* induction occurred when the pH of the LB was shifted from 6.5 to 5.25 (data not shown). The effect of this pH shift on *ctc*::*lacZ* levels is illustrated in Fig. 1C. Unlike the previous two conditions, the loss of RsbU only partially blocked σ^B activation following acid shock. The strain carrying the *rsbU* deletion accumulated approximately half as much β-galactosidase as that measured in the $RsbU^+$ strain. These data imply that salt stress and ethanol stress activate σ^B by an RsbU-dependent process, whereas activation of σ^B by acid shock has both RsbU-dependent and RsbU-independent components. σ^B activation by these stresses is independent of potential readthrough from the upstream *rsbU* operon. Insertion of an integrating plasmid (33) between the *rsbU* and *sigB* operons had virtually no effect on the stress inductions (data not shown).

We previously showed that RsbU-dependent activation of σ^B requires an intact *rsbV* allele (33). This requirement is also true for the activation of σ^B in response to the stresses described above. *ctc*::*lacZ* expression does not increase significantly following salt stress, ethanol treatment, or acid shock if the *B. subtilis* strain under investigation carries a null mutation in *rsbV* (*rsbV312*) (data not shown). Thus, all three stresses activate σ^B via RsbV regardless of their degree of dependence on RsbU.

RsbU-independent induction of *ctc*::*lacZ*. The first condition recognized to induce σ^B-dependent genes is the cessation of exponential growth in a sporulation-suppressing medium (e.g., LB) (17). Unlike the σ^B activation that occurs following the stresses described above, *ctc* induction upon entry into stationary phase does not appear to have an RsbU-dependent component. There is no appreciable difference in the stationary-phase levels of β-galactosidase from *ctc*::*lacZ* in wild-type *B. subtilis* and a strain deficient in RsbU (Fig. 1D).

The signal that triggers stationary-phase induction of σ^B

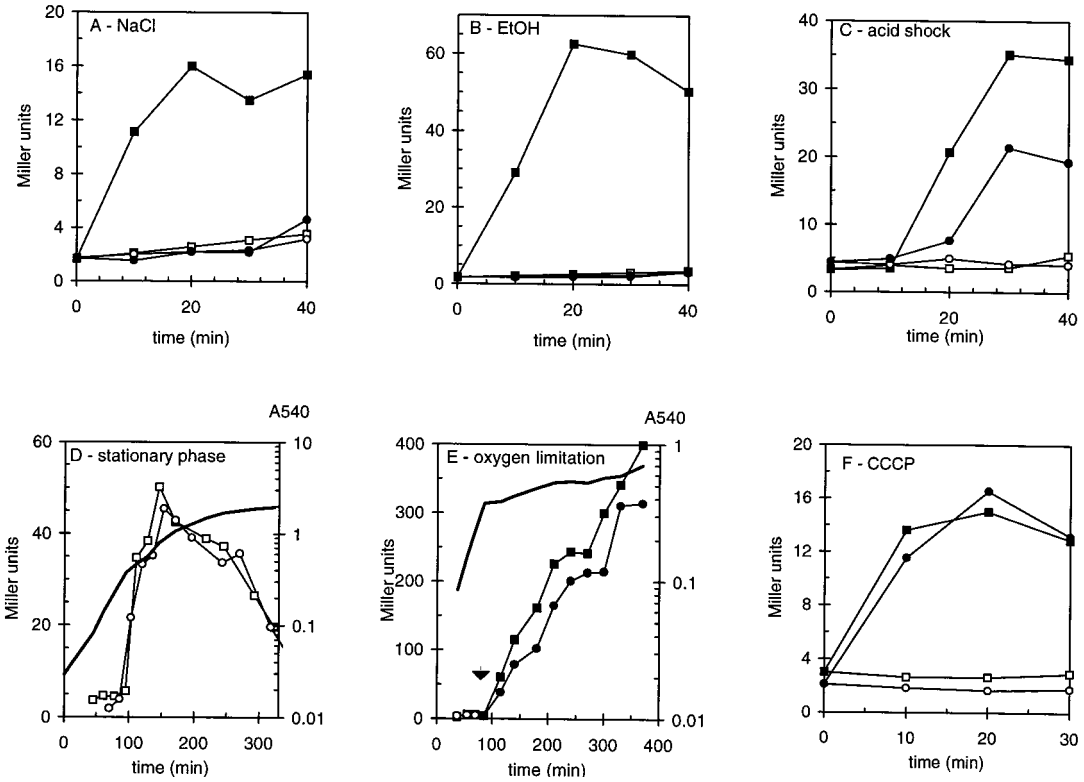

FIG. 1. Stress-induced *ctc*::*lacZ* expression. *B. subtilis* strains carrying SPβ *ctc*::*lacZ* were grown in LB with potassium phosphate (F) (8) or LB (A to E). During exponential growth, cultures were divided and one half of each was exposed to 2.5% (wt/vol) NaCl (A), 4% (vol/vol) ethanol (EtOH) (B), acid shock by pH shift from 6.5 to 5.25 (C), reduced aeration by reduction of the shaking frequency from 250 to 50 rpm (E), or 2 μM CCCP (F). Panel D displays activity of *ctc*::*lacZ* upon entry into stationary phase. Bacteria were treated at time zero (A to C and F) or at the time indicated by the arrow (E). β-Galactosidase activity levels were determined as described in Materials and Methods. Open symbols represent control samples, whereas the filled symbols depict stressed cultures. □ and ■, BSA46 (wild type); ○ and ●, BSA140 (*rsbU*Δ*Nde*I). The solid thick lines in panels D and E represent the growth rate of BSA46. BSA140 growth was similar.

activity is obscure, but it may be related to changes in intracellular ATP levels. Both Alper et al. (1) and Diederich et al. (11) reported that low ATP levels can cause the release of *B. subtilis* σ^F from an inhibitory complex with its anti-σ partner. One of these groups also showed that σ^B activity is likely to be influenced by ATP levels (1). Other studies demonstrated a correlation between induction of the σ^B-dependent *gsiB* promoter and a drop in the intracellular ATP level under some growth conditions (e.g., carbon or phosphate limitation) (22). Given that a drop in ATP is likely to occur when cells enter the stationary phase of growth, it seemed that RsbU-independent activation of σ^B at stationary phase might be a result of a direct effect of reduced ATP levels on the binding preference of RsbW. To test this notion, we examined the effects of other conditions that might influence ATP abundance on the activity of σ^B in $RsbU^+$ and $RsbU^-$ strains. A failure to provide adequate aeration should curtail *B. subtilis*' ability to form ATP. We therefore restricted aeration by reducing the frequency at which flask cultures of *B. subtilis* were shaken. This change not only significantly decreased the cultures' rate of growth but also caused a very pronounced RsbU-independent induction of *ctc* (Fig. 1E). Presumably, this induction was due to a limitation of oxygen and a resulting drop in intracellular ATP.

Among the experiments that led Alper et al. (1) to propose a direct nucleotide involvement in σ factor release was the use of CCCP, an uncoupler of oxidative phosphorylation, to lower ATP levels and induce *ctc* expression. We repeated this experiment to investigate whether this inducing condition is RsbU dependent. We found that treatment of exponentially growing cultures of *B. subtilis* with CCCP, at a concentration (2 μM) which reduced its growth rate by approximately 40%, resulted in a modest but reproducible induction of *ctc*::*lacZ* expression (Fig. 1F). This induction occurred at similar levels regardless of whether the strains were $RsbU^+$ or $RsbU^-$ (Fig. 1F). Thus, CCCP treatment represents an additional means of inducing RsbU-independent σ^B activation.

Entry into stationary phase, O_2 limitation, and CCCP treatment are likely to lower intracellular ATP levels in addition to inducing *ctc*. This coincidence suggests that the RsbU-independent activation of σ^B may be a consequence of a drop in ATP levels. In keeping with the notion that this effect would occur as a result of a shift in the binding of RsbW from σ^B to RsbV, we find that σ^B activation under these conditions occurs only in cells with a functional *rsbV* gene (data not shown). As was true for the RsbU-dependent inductions, the three circumstances which induced RsbU-independent σ^B activation did so even when potential readthrough from the upstream *rsbU* operon was blocked (data not shown).

RsbU-dependent and -independent inductions of σ^B activity are additive. In previous studies, we determined that only a very small proportion of the σ^B present within a cell becomes activated following exposure to inducing conditions (33). This fact implies that there is a substantial reserve of inactive σ^B which may be activated in response to multiple inducing stimuli. A simple test of this idea would involve exposing *B. subtilis* to both RsbU-dependent and RsbU-independent stresses si-

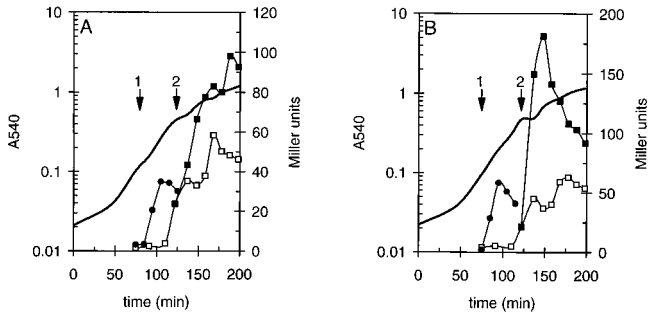

FIG. 2. Additive effects of RsbU-dependent and RsbU-independent inductions of *ctc*::*lacZ*. *B. subtilis* BSA46 (SPβ *ctc*::*lacZ*) was grown in LB and exposed to acid shock (A) or ethanol stress (B) during exponential growth (arrow 1) or at the onset of stationary phase (arrow 2). The solid thick lines represent the growth rates of the untreated culture. β-Galactosidase levels were determined as for the experiment represented in Fig. 1. ●, β-galactosidase activity after exposure to stress during exponential growth; □, stationary-phase induction of β-galactosidase; ■, β-galactosidase activity after exposure to stress at the onset of stationary phase.

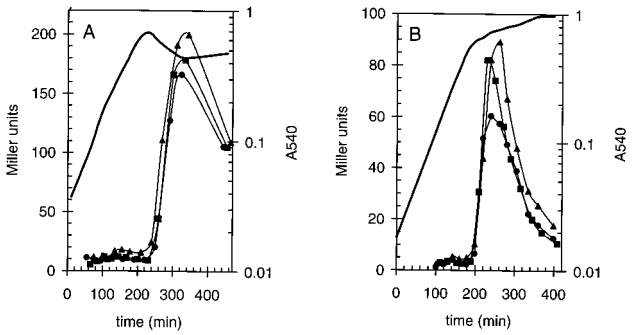

FIG. 3. RsbU-independent induction of *ctc* by starvation for glucose or phosphate. *ctc*::*lacZ* expression was monitored by determining the β-galactosidase activity as described for Fig. 1. Bacteria were grown in a synthetic medium (31) with growth-limiting amounts of either glucose (A) or phosphate (B). The solid thick line displays the bacterial growth rate (BSA46). BSA140 and BSA142 growth rates were similar. ■, BSA46 (wild type); ●, BSA140 (*rsbU*ΔNdeI); ▲, BSA142 (*rsbU*::pAL127).

multaneously, to investigate whether the resulting levels of σ^B-dependent transcription are additive.

We therefore subjected *B. subtilis* to either acid shock or ethanol treatment while the cultures were either actively growing or entering stationary phase. The results of this experiment are illustrated in Fig. 2. Either a drop in pH (Fig. 2A) or ethanol addition (Fig. 2B) at the time when the culture entered stationary phase dramatically elevated the level of *ctc*::*lacZ* expression above that seen with either the application of the stress or entry into stationary phase alone. The enhancement of *ctc* induction seen by the combination of ethanol or acid stress and entry into stationary phase did not occur in RsbU$^-$ bacteria (data not shown). Boylan et al. (8) also noted a similar additive effect of salt stress on the stationary-phase induction of *ctc* expression. We interpret these data as evidence that σ^B activation has a reserve of induction potential which allows heightened σ^B activity in response to multiple stimuli.

Changes in the level of adenosine nucleotides correlate with the activation of σ^B by nutrient limitation but not stress. Entry into stationary phase, exposure to CCCP, and limitation of oxygen (Fig. 1D to F) all induced σ^B via an RsbU-independent mechanism. To investigate the possibility that this process was due to changes in cellular adenosine nucleotide levels, we monitored the intracellular concentrations of ATP and ADP which accompany RsbU-dependent or RsbU-independent activation of σ^B. The expectation was that ATP levels should fall in response to RsbU-independent inducing stimuli but not necessarily fall under conditions that activate the RsbU-dependent pathway. Measurements of nucleotide pools were performed in a defined, synthetic medium, whereby the influence of specific limitations on the activation of σ^B could be studied. In a preliminary experiment, we tested the effect of limiting different nutrients on RsbU-independent *ctc* induction. As can be seen from the data in Fig. 3, either phosphate or glucose deprivation triggers an increase in *ctc-lacZ* expression regardless of whether the strain is the wild type (BSA46) or RsbU$^-$ (BSA140) or contains a plasmid integrated between *rsbU* and the *sigB* operon to disrupt potential readthrough transcription from the upstream *rsbU* operon (BSA142). In keeping with the properties of RsbU-independent σ^B activation, a null mutation in *rsbV* prevented the induction (data not shown). The σ^B activation was specific for carbon or phosphate limitation. Other growth-limiting conditions such as withholding of a required amino acid or starvation for nitrogen did not induce the expression of *ctc* (data not shown).

We chose glucose limitation in minimal medium as an example of an RsbU-independent stimulus. Ethanol treatment was chosen as an example of an RsbU-dependent stimulus. Ethanol treatment in a glucose minimal medium results in an RsbU-dependent induction of the *ctc-lacZ* fusion that is similar to the induction seen with LB (data not shown). The changes in the ATP and ADP pools following glucose limitation and ethanol treatment were determined. Limitation of glucose resulted in a 60 to 80% drop in the level of ATP relative to the level seen in exponentially growing cells (Fig. 4B). In contrast, the level of ATP increased 40% after the treatment with 4% ethanol. ADP levels were not significantly changed by either treatment. The differences in ATP levels following exposure to each of the inducing conditions support the argument that a drop in ATP is a potential signal for RsbU-independent σ^B activation (e.g., stationary-phase induction due to carbon limitation), but such a drop is unlikely to be the inducer of RsbU-dependent σ^B activation (e.g., ethanol stress). If the ATP were the sole regulator of σ^B activity, the increase in ATP following ethanol treatment should have failed to induce or even inhibit σ^B activation. Apparently, RsbU-dependent σ^B activation is independent of the ATP levels and can override the putative ATP regulation of σ^B induction. σ^B activation following acid shock has both RsbU-

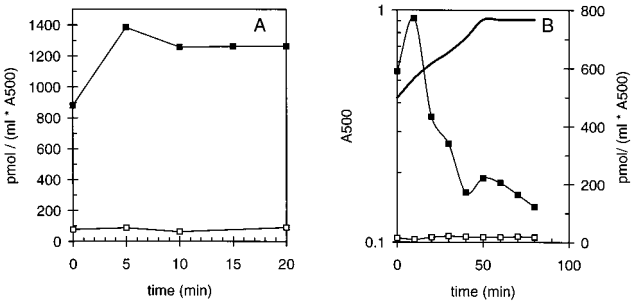

FIG. 4. Measurement of the ATP-ADP pools. *B. subtilis* BSA46 (wild type) was grown in a synthetic medium and subjected to ethanol stress (4% final concentration) added at time zero (A) or growth-limiting amounts of glucose (B). The solid thick line in panel B shows the bacterial growth rate. The sizes of the nucleotide pools were determined as described in Materials and Methods. ■, ATP; □, ADP.

dependent and -independent components (Fig. 1C). We therefore examined acid-shocked *B. subtilis* to determine whether the RsbU-independent σ^B activation that occurs under this condition could be a reflection of a drop in intracellular ATP and found a 20 to 25% drop in ATP levels that was apparent within 10 min of the application of stress (data not shown). This drop in the ATP level is substantially smaller than the 60 to 80% drop that occurs during carbon starvation. RsbU-independent σ^B activation during acid shock is only about 10% of that seen during glucose limitation. Thus, this modest drop in ATP level may be sufficient to account for the relatively small amount of RsbU-independent σ^B activity seen in these circumstances.

F_0F_1 ATP synthase is involved in the formation of ATP via oxidative phosphorylation. Santana et al. (28) created a *B. subtilis* strain lacking the F_0F_1 ATP synthase genes (168Δ*atp*2). The Δ*atp*2 deletion strain has lower ATP levels than that found in its parental strain and, as a consequence, is compromised for growth (28). This strain provides an additional test for the role of ATP in σ^B activation. If a low level of ATP is a σ^B inducing condition, strains deficient in F_0F_1 ATP synthase, which are hence relatively ATP poor, should exhibit elevated σ^B activity. To examine this question, we moved the Δ*atp*2 mutation into our wild-type laboratory strain (BSA46) and measured the effect of the mutation on *ctc*::*lacZ* expression during growth in glucose minimal medium. Santana et al. reported that ATP levels in the Δ*atp*2 strain are half the levels seen in a wild-type strain during growth in minimal medium (28). We found that the uninduced level of *ctc* expression was three times higher in the Δ*atp*2 strain than that seen in its wild-type isogenic parent (228 versus 75 U, respectively). A similar increase was also observed when the Δ*atp*2 mutation was placed in an $RsbU^-$ strain; however, we have no data on ATP levels in this double-mutant strain and so the significance of this result is uncertain. There is thus a correlation between a reduction in ATP levels and RsbU-independent elevation of σ^B-dependent transcription.

***ctc*, *sigB*, and *gsiB* display coordinated induction patterns.** Up to this point, we have measured the activity of σ^B by using a *ctc-lacZ* translational fusion as the reporter system. It is formally possible that the effects which we have observed and are attributing to σ^B activity are specific for *ctc* and not σ^B-dependent transcription in general. As a test of the general aspects of the response, we constructed transcriptional fusions of the σ^B-dependent promoters of *gsiB* (22, 34) *sigB* (18), and *ctc* (17) to a *B. subtilis sigE* allele (*sigE41*) whose product is an inactive σ^E with a characteristic mobility during SDS-PAGE (32). We monitored changes in promoter activity of strains carrying such fusions by Western blot, using antibody previously prepared against σ^E (32). In preliminary experiments, we determined that the appearance of σ^{E41} from these promoters is absolutely dependent on σ^B activity (data not shown). The activity of the *sigB* promoter was also monitored by using anti-σ^B antibody to directly probe σ^B levels (4). Figure 5 illustrates the Western blot analyses. Although the results are only semiquantitative, it is apparent that the levels of σ^B and the activities of the *sigB*, *ctc*, and *gsiB* promoters show parallel increases following treatment of wild-type bacteria with ethanol, NaCl, CCCP, or HCl (Fig. 5A, lanes 2, 3, 5, and 7, respectively). Similar increases also occur when the cells are subjected to O_2 limitation (Fig. 5B, lane 3) or enter stationary phase (Fig. 5A, lane 4). When activities of the promoters of $RsbU^+$ and $RsbU^-$ strains were compared, ethanol induction of all three promoters was found to require RsbU, while O_2 limitation remained as an RsbU-independent inducer of σ^B-dependent transcription (Fig. 5B). These results argue that

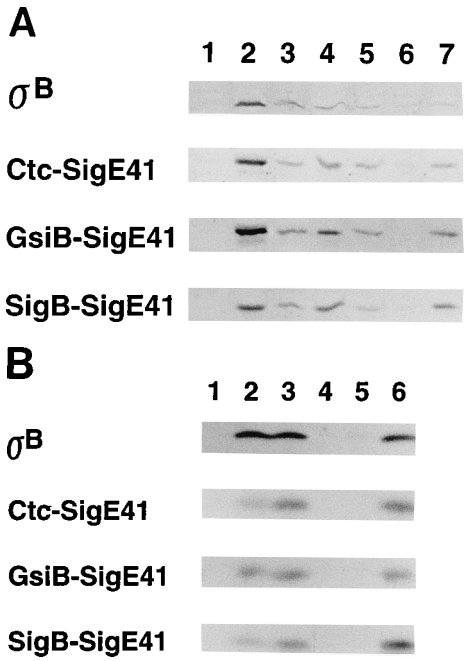

FIG. 5. Induced expression of *ctc*, *gsiB*, and *sigB*. Expression of σ^B-dependent promoters was monitored by using *ctc*::*sigE41*, *gsiB*::*sigE41*, and *sigB*::*sigE41* transcriptional fusions. The level of SigE41 and the level of σ^B from the *sigB* operon were estimated by Western blot analysis. (A) Bacteria grown in LB with potassium phosphate (lanes 1 to 5) or LB (lanes 6 and 7). During exponential growth, the culture was divided and exposed to 4% ethanol (lane 2), 2.5% NaCl (lane 3), 2 μM CCCP (lane 5), and acid shock (pH shift from 6.5 to 5.25 by the addition of HCl) (lane 7). The bacteria were harvested 30 min after the exposure to the stimuli. Lanes 1 and 6 represent controls of exponentially growing bacteria. The sample for lane 4 was harvested after the cells entered the stationary phase. Strains BSA180 (*ctc*::*sigE41*), BSA181 (*gsiB*::*sigE41*), and BSA182 (*sigB-sigE41*) were used. (B) Activation of σ^B in wild-type bacteria (lanes 1 to 3) and in strains with a deletion in *rsbU* (lanes 4 to 6) by treatment with 4% ethanol (lanes 2 and 5) or oxygen limitation (lanes 3 and 6) during exponential growth in LB. Protein extracts from untreated growing bacteria were applied to lanes 1 and 4. The harvested cells were processed as described in Materials and Methods, with 50 μg of protein loaded into each lane for analysis.

expressions of the three σ^B-dependent promoters are similarly regulated and that the findings based on *ctc* are likely to apply to σ^B-dependent transcription in general.

High-level induction of σ^B activity in LBGG requires RsbU and is mediated by changes in the pH. A curiosity associated with the regulation of σ^B-dependent promoters has been that their expression is induced to a significantly higher level when cells enter stationary phase in LB supplemented with 5% glucose and 0.2% glutamine (LBGG) than when they enter stationary phase in LB alone (17) (Fig. 6B). *B. subtilis* excretes excess carbon into the culture medium in the form of organic acids (e.g., acetate-pyruvate) during growth in high-concentration glucose media (30). When glucose becomes limiting, these excreted acids are reassimilated and metabolized through the Krebs TCA pathway. Glucose represses the TCA cycle, and at least one σ^B-dependent promoter has been shown to be hyperactive in *B. subtilis* strains with mutations in TCA cycle genes (17). These facts suggest a possible explanation for heightened σ^B induction in glucose-containing media. Acid shock activation of σ^B due to organic acid excretion may be superimposed on the normal stationary-phase induction of σ^B. Such a circumstance would be similar to the enhanced *ctc* expression that was observed when we artificially reduced the culture pH at the onset of stationary phase (Fig. 2A). To see

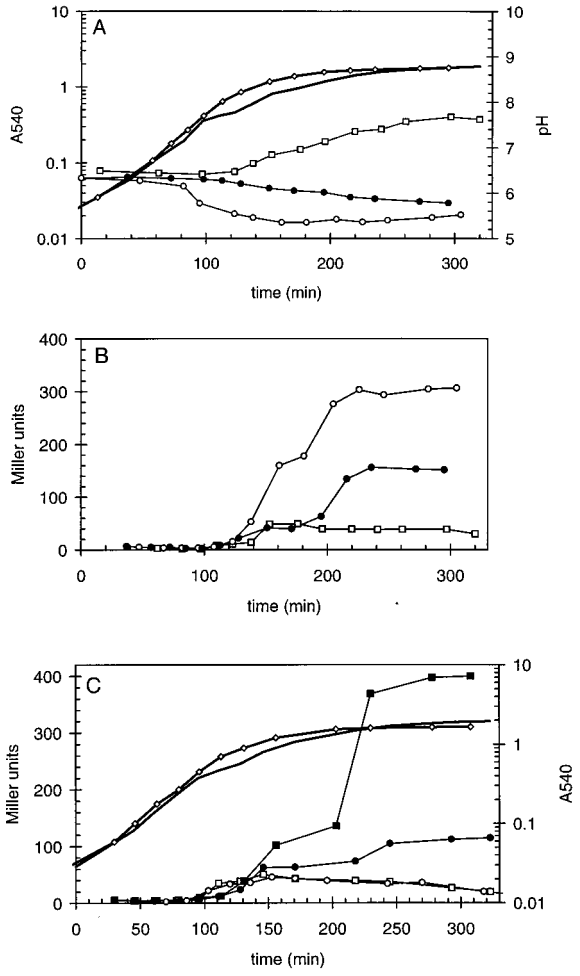

FIG. 6. Medium-dependent expression of *ctc*::*lacZ* and pH changes during entry of *B. subtilis* into stationary phase. The growth rates of BSA46 in LB (thick line) and LBGG (◇) are displayed. Growth of BSA140 was similar. (A) pH changes of the media during growth of BSA46 (wild type) in LB (□), LBGG (○), or LBGG buffered with 100 mM MOPS (pH 6.5) (●). (B) *ctc*-dependent β-galactosidase activity of BSA46 grown in LB (□), LBGG (○), or LBGG buffered with 100 mM MOPS (●). (C) β-Galactosidase activity of BSA46 (□ and ■) and BSA140 (*rsbU*Δ*Nde*I; ○ and ●) during growth in LB (open symbols) or LBGG (filled symbols).

whether the anticipated acid excretion was in fact influencing the pH of the culture, we measured the pH of the culture medium when wild-type bacteria were grown in either LB or LBGG. The pH of the LB culture remained essentially constant during exponential growth and increased to about 7.5 when growth ceased (Fig. 6A). In contrast, the pH of the LBGG medium decreased from 6.5 to 5.5, with the largest drop occurring at the end of exponential growth (Fig. 6A). Thus, the strong induction of *ctc-lacZ* observed with LBGG may be partly due to acidification of the medium. To test this idea, we attempted to block the pH change by buffering the medium with MOPS (morpholinepropanesulfonic acid). Because of the amount of acid excreted and our reluctance to alter the osmolarity of the medium by adding a high concentration of buffer, this tactic did not completely prevent the acidification of the medium but did reduce it (Fig. 6A). When the stationary-phase induction of *ctc*::*lacZ* in buffered LBGG was measured, it was found to be approximately half that in the unbuffered LBGG (Fig. 6B). Attempts to buffer the culture medium with Tris produced similar results (data not shown). The addition of MOPS to unsupplemented LB blocked the rise in pH found in this medium but did not alter the pattern of *ctc* expression from that observed in the MOPS-free LB medium (data not shown). The ability of MOPS to partially block both the acidification of the culture medium and the glucose-glutamine enhancement of the stationary-phase *ctc* induction suggests that acid shock may be a contributor to σ^B activation under this condition.

Acid shock induction of *ctc* is at least partially dependent on *rsbU*. If acid shock contributed to the strong induction of *ctc* observed with LBGG, a part of this induction should depend on RsbU. Fig. 6C displays the expression of *ctc*::*lacZ* in $RsbU^+$ and $RsbU^-$ strains that were grown and entered stationary phase in either LB or LBGG. The stationary-phase induction of the β-galactosidase in LBGG but not LB was reduced fourfold by the loss of RsbU. Wise and Price observed a similar effect for the loss of RsbU on *ctc* expression in LBGG (36). We interpret our results as evidence that the entry into the stationary phase of growth can induce σ^B activation by multiple means, depending on the medium in which cultivation takes place and the stresses that develop in that medium. Entry into stationary phase in LB appears to involve a single σ^B induction component that is relatively independent of RsbU (possibly ATP), while stationary-phase induction in LBGG has a significant RsbU-dependent element (possibly acid shock) added to the RsbU-independent component.

Heat shock activation of σ^B involves an *rsbU rsbV*-dependent component. Activation of σ^B-dependent transcription by heat shock is unique in that it appears to have an RsbV-independent component (5, 8). Given that RsbU is involved in σ^B activation by other forms of stress, we wished to learn the degree to which it might also participate in heat shock activation of σ^B. Induction of σ^B-dependent transcription by heat shock is not readily quantified. The most commonly used reporter gene product, *E. coli* β-galactosidase, is labile in *B. subtilis* at elevated temperatures (5). In addition, our *B. subtilis* strains carrying SPβ *ctc*::*sigE41* as a reporter system, unlike their PY22 parent, were found to be temperature sensitive for growth. Given these circumstances, we resorted to monitoring the accumulation of σ^B itself as a measure of *sigB* operon expression and σ^B activity. σ^B increase following heat shock in $RsbV^-$ *B. subtilis* is, in fact, the principal evidence for σ^B's thermal activation having an RsbV-independent component (5, 8). Extracts from control and heat-shocked bacteria were examined for changes in σ^B abundance by Western blot analysis. Although these data are only semiquantitative, they show that both the $RsbV^-$ and $RsbU^-$ strains accumulate less σ^B following heat shock than does a wild-type strain (Fig. 7A). This result argues for a role for both RsbV and RsbU in normal heat shock induction of σ^B.

To quantitate the relative differences in *sigB* expression following heat shock in these strains, RNA was extracted prior to and after a shift from 37 to 48°C and analyzed by slot blot hybridization with a *sigB*-specific probe. The results, quantitated by densitometry, are illustrated in Fig. 7B. *sigB*-specific RNA was found to increase 25-fold following application of heat shock in wild-type *B. subtilis* and 4- to 5-fold in the $RsbU^-$ and $RsbV^-$ strains. Although we had previously interpreted the RsbV-independent increase of *sigB* operon products following heat shock to be a result of σ^B activation, recent data now call this notion into question. RNA specific for the σ^B-dependent *gsiB* gene increases almost 500-fold in wild-type *B. subtilis* following heat shock but only 6-fold in an $RsbV^-$ strain (21). This disparity in the expression of a σ^B-dependent gene following heat shock in $RsbV^+$ or $RsbV^-$ strains suggests that

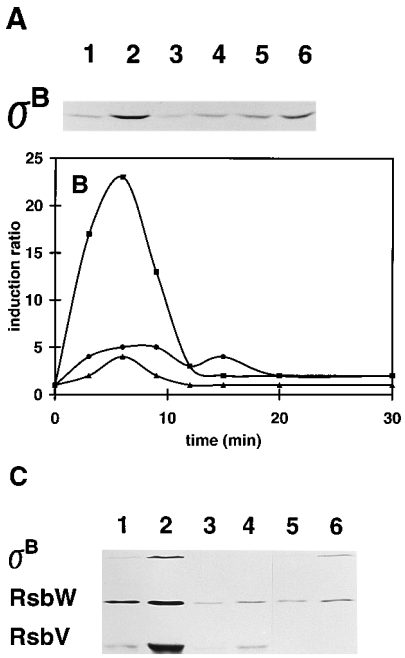

FIG. 7. Influence of mutations in *rsbV* and *RsbU* on the heat shock induction of σ^B. Bacteria were grown in LB. During exponential growth at 37°C, the culture was divided and one half was shifted to 48°C. (A) Influence of heat shock on the level of σ^B in PY22 (wild type; lanes 1 and 2), BSA158 (*rsbU*Δ*Nde*I; lanes 5 and 6), and BSA159 (*rsbV312*; lanes 3 and 4). Bacteria were harvested during exponential growth (lanes 1, 3, and 5) or 30 min after the transfer to 48°C (lanes 2, 4, and 6). Crude extracts were prepared, and 100-μg samples of protein were analyzed by Western blot. The blot was probed with a monoclonal antibody raised against σ^B. (B) Analysis of the level of *sigB* mRNA before and after the transfer to 48°C conditions. Bacteria were subjected to heat shock, and the level of *sigB* mRNA was determined as described in Materials and Methods. The amount of *sigB* mRNA before heat shock was set as one, and induction relative to this level is displayed. ■, BSA179 (wild type); ●, BSA158 (*rsbU*Δ*Nde*I); ▲, BSA159 (*rsbV312*). (C) Levels of RsbW, σ^B, and RsbV in PY22 (wild type; lanes 1 and 2), BSA272 (*sigB*::Δ*Hin*dIII-*Eco*RV::*cat*; lanes 3 and 4), and BSA159 (*rsbV312*; lanes 5 and 6) before (lanes 1, 3, and 5) and 30 min after the transfer to 48°C conditions (lanes 2, 4, and 6). A sample (100 μg) of protein extract was loaded into each lane, and the nitrocellulose filter was probed with antibodies raised against RsbW, σ^B, and RsbV.

the increased σ^B found in the $RsbV^-$ strain may not be due to σ^B activation in this genetic background. We therefore reexamined the accumulation of *sigB* operon products by the use of antibodies against several of these proteins (RsbV, RsbW, and σ^B) and included a strain deficient in σ^B in the analysis. Figure 7C shows that RsbW, an operon product common to both the $SigB^-$ and $RsbV^-$ strains, rose to a similar levels following heat shock in these two strains. This result argues that a significant portion of the RsbV-independent increase in σ^B protein levels following heat shock is likely to be unrelated to σ^B activation and that heat shock activation of σ^B, like the σ^B activation which occurs following other environmental insults, is primarily an RsbU-RsbV-dependent process.

DISCUSSION

There is mounting evidence that E-σ^B transcribes a subset of genes which are activated as part of the general stress response in *B. subtilis* (5, 8, 34). Although the explicit role of the σ^B regulon in the stress response has yet to be determined, the diverse environmental insults that activate σ^B suggest that σ^B-dependent transcription will be found to enhance *B. subtilis*' ability to respond to stress. In support of such an idea, we have recently observed that the absence of σ^B blocks the ability of some, but not all, *B. subtilis* strains to resume growth after exposure to salt shock (35). The basis of this phenomenon is under investigation.

σ^B-dependent transcription occurs when σ^B is released from RsbW-mediated inhibition. σ^B is one of a growing family of σ factors, including σ^F and σ^G in *B. subtilis* and σ^F in *Salmonella typhimurium*, that are negatively regulated by anti-σ factor proteins (6, 13, 20, 25, 26, 29). These proteins bind to particular σ factors and, by doing so, block σ availability to RNA polymerase. For the two best-studied of these σ factors (σ^B and σ^F of *B. subtilis*), release of σ from its corresponding anti-σ protein involves the binding of the anti-σ to an alternative protein in lieu of σ. For σ^B, the anti-σ^B is RsbW and its counteracting protein is RsbV (6). There is good evidence, from both in vitro and in vivo studies, that RsbW forms a mutually exclusive complex with either σ^B or RsbV and that a shift from the RsbW-σ^B to the RsbW-RsbV complex occurs concomitant with the appearance of E-σ^B and the transcription of σ^B-dependent genes (6, 12, 33).

The process causing RsbW to bind to either RsbV or σ^B is poorly understood, but the intracellular ratio of ATP to ADP has been proposed as an influence on the RsbW binding decision (1). The potential importance of the ATP/ADP ratio was revealed by in vitro studies wherein the binding preference of the RsbW homolog (SpoIIAB) could be shifted from its target σ factor (σ^F) to the RsbV homolog (SpoIIAA) by altering the ATP/ADP ratio present in the reaction mixture (1, 11). It was determined that a high ATP/ADP ratio would favor binding of the anti-σ factor to its σ factor target while a low ATP/ADP ratio would result in the formation of an anti-σ factor–antagonist complex and σ release. Although these experiments were performed with σ^F regulatory proteins, the similarities between the σ^F and σ^B regulators as well as the likelihood that at least some σ^B-activating circumstances were also conditions where ATP levels would be low prompted the investigators to propose that regulation by ATP-ADP may be common to both systems and that σ^B is also activated by a drop in the ATP/ADP ratio (1). In the current paper, we present evidence that a low level of ATP is a promising signal for σ^B activation under some but not all σ^B inducing conditions. There appear to be at least two pathways that signal σ^B activation. These pathways can be distinguished by their dependence on RsbU. The first responds to environmental insults (ethanol treatment, salt stress, acid stress, heat shock, etc.), is dependent on the *rsbU* gene product, and does not appear to be influenced by ATP levels. The second is independent of RsbU and appears to activate σ^B in response to conditions (reduced aeration, entry into stationary phase, treatment with CCCP, and the presence of the Δ*atp*2 mutation) that are likely to cause a reduction in the intracellular levels of ATP.

Our data indicate that some inducing conditions uniquely activate one or another particular pathway while other inducing conditions are likely to turn on multiple routes of σ^B activation, resulting in an enhanced response. Examples of single-pathway inductions include activation of σ^B by carbon limitation, an RsbU-independent condition which is accompanied by a 60 to 80% drop in the level of intracellular ATP, and ethanol treatment, which does not involve a drop in the ATP level but instead depends on RsbU. In contrast, multipathway induction by both RsbU-dependent and RsbU-independent pathways occurs as a consequence of entry into stationary phase in LBGG. The RsbU-dependent activation of σ^B in this circumstance is at least partially due to pH stress. The RsbU-independent activation presumably involves a stationary-

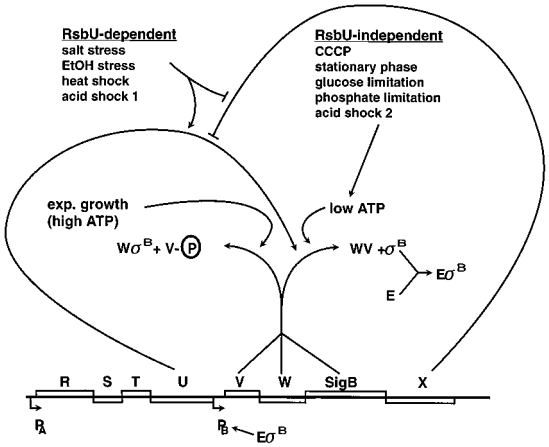

FIG. 8. Pathways of σ^B activation. RsbW is the primary regulator of σ^B. It forms mutually exclusive complexes with σ^B and RsbV. If the RsbW-σ^B complex forms, σ^B is unavailable to RNA polymerase, while formation of the RsbV-RsbW complex leaves σ^B free to form an RNA polymerase holoenzyme (E-σ^B). Once formed, E-σ^B initiates the transcription of several operons, including *sigB* itself (represented along the bottom line). Two major pathways, distinguishable by their dependence on a regulatory protein (RsbU) encoded by an upstream operon are proposed as facilitators of the formation of RsbW-RsbV and the release of σ^B. Pathway 1 is RsbU dependent and promotes RsbW-RsbV complex formation in response to environmental stresses such as salt, ethanol (EtOH), and heat or acid shock. The RsbU-dependent pathway is negatively regulated by RsbX, an additional protein factor. It is unknown whether the stress induction indicated for pathway 1 involves a release of RsbU from the negative regulation of RsbX or an independent activation of RsbU. Activated RsbU, or a process under its control, then stimulates the association of RsbW with RsbV. Pathway 2 is RsbU independent and is activated by conditions that are likely to reduce the intracellular levels of ATP. Low ATP concentrations may directly provoke the binding of RsbW to RsbV while preventing the phosphorylation of RsbV that inhibits its activity. exp., exponential. V-Ⓟ, phosphorylated RsbV.

phase- and/or pH-dependent drop in the intracellular ATP level.

The most poorly understood of the σ^B inducing conditions is heat shock. We and others had previously noted that σ^B accumulation following heat shock persists in the absence of RsbV (5, 8). We interpreted this persistence as evidence for an RsbV-independent component for σ^B activation; however, our recent data argue that the major RsbV-independent component of enhanced σ^B levels does not involve σ^B activation but occurs by other means. In the present study, we still observe RsbV-independent σ^B accumulation but note a significant RsbV-dependent component. Maximum levels of σ^B following heat shock require both RsbU and RsbV. Given that ATP levels are known to rise following heat shock (22), the RsbV component of σ^B activation following this stress is probably restricted to the RsbU-dependent pathway.

Our data can be condensed into the model illustrated in Fig. 8. The figure depicts RsbW as the primary inhibitor of σ^B which is antagonized by the binding of RsbV. The binding of RsbW to RsbV is favored by low intracellular ATP levels which may be caused by the conditions indicated in the figure. The finding that ATP levels correlate with the RsbU-independent activation of σ^B provides support for the model, whereby RsbW serves as a sensor of the cell's energy charge and directly activates or inactivates σ^B accordingly. We had previously shown that RsbW can phosphorylate RsbV and that only unphosphorylated RsbV is found bound to RsbW (12). This distinction suggests a simple mechanism by which RsbW could serve as a sensor of ATP levels. At high ATP levels, RsbV is efficiently phosphorylated by RsbW, inactivating RsbV and favoring the formation of RsbW-σ^B complexes. At low ATP levels, the phosphorylation of RsbV may not occur efficiently. RsbV remains unphosphorylated and bound to RsbW, thereby freeing σ^B to form an RNA polymerase holoenzyme. Consistent with this view are the findings of recent in vitro experiments of Alper et al. that show that interactions between RsbV and RsbW are strongly influenced by the concentration of ATP and ADP (2). Alternatively, and irrespective of ATP levels, the RsbV-dependent activation of σ^B can be facilitated by RsbU in response to a number of environmental stresses. The mechanism of this reaction is unknown; however, it is likely to involve the phosphorylation state of RsbV. We had noted in earlier studies that RsbU-dependent activation of σ^B, like the RsbU-independent reaction, is accompanied by the binding of unphosphorylated RsbV to RsbW (33). Perhaps the RsbU pathway restricts the ability of RsbW to phosphorylate RsbV, regardless of the ATP levels, or alternatively, releases a phosphatase activity that keeps a portion of the RsbV population active to bind to RsbW.

Although more pieces to the puzzle of σ^B regulation are being discovered, many are still to be found. Among the more intriguing questions now are what the ATP-independent signal sent to the RsbU-dependent pathway by environmental stress is and how RsbU communicates this signal to RsbW.

ACKNOWLEDGMENTS

We thank R. Losick for stimulating discussions and communication of results prior to publication and P. Glaser for providing the Δ*atp*2 mutant.

This work was supported by National Institutes of Health grant GM48220 to W.G.H. and grants from Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie to M.H. U.V. is the recipient of a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft.

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