JOURNAL OF BACTERIOLOGY, Aug. 1999, p. 4653–4660 Vol. 181, No. 15
0021-9193/99/$04.00+0

Obg, an Essential GTP Binding Protein of *Bacillus subtilis*, Is Necessary for Stress Activation of Transcription Factor σ^B

JANELLE M. SCOTT AND W. G. HALDENWANG*

Department of Microbiology, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284-7758

Received 12 April 1999/Accepted 24 May 1999

σ^B, the general stress response σ factor of *Bacillus subtilis*, is activated when intracellular ATP levels fall or the bacterium experiences environmental stress. Stress activates σ^B by means of a collection of regulatory kinases and phosphatases (the Rsb proteins), which catalyze the release of σ^B from an anti-σ factor inhibitor. By using the yeast dihybrid selection system to identify *B. subtilis* proteins that could interact with Rsb proteins and act as mediators of stress signaling, we isolated the GTP binding protein, Obg, as an interactor with several of these regulators (RsbT, RsbW, and RsbX). *B. subtilis* depleted of Obg no longer activated σ^B in response to environmental stress, but it retained the ability to activate σ^B by the ATP responsive pathway. Stress pathway components activated σ^B in the absence of Obg if the pathway's most upstream effector (RsbT) was synthesized in excess to the inhibitor (RsbS) from which it is normally released after stress. Thus, the Rsb proteins can function in the absence of Obg but fail to be triggered by stress. The data demonstrate that Obg, or a process under its control, is necessary to induce the stress-dependent activation of σ^B and suggest that Obg may directly communicate with one or more σ^B regulators.

σ^B is a transcription factor that directs RNA polymerase (RNAP) to promoters of the *Bacillus subtilis* general stress regulon (14). Induction of the regulon occurs when σ^B is activated by either a decline in ATP levels or the onset of any of a number of environmental stresses (e.g., heat shock, acid salt, and ethanol) (5, 7, 14, 18, 34, 38). σ^B is present in the prestressed cell, but it is held inactive in a complex with an anti-σ^B protein, RsbW (6, 9). σ^B is released from RsbW when a second protein (RsbV) binds to RsbW (9). The genes for RsbV, RsbW, σ^B, and five additional σ^B regulatory proteins (RsbR, -S, -T, -U, and -X) are cotranscribed as an eight-gene operon from a promoter (P_A) that is recognized by the *B. subtilis* housekeeping σ factor, σ^A (40). An internal σ^B-dependent promoter (P_B) enhances expression of the downstream four genes during periods of σ^B activity (i.e., P_A *rsbR rsbS rsbT rsbU* P_B *rsbV rsbW sigB rsbX*) (4, 5, 7). A likely mechanism for σ^B control by the Rsb proteins is illustrated in Fig. 1. In unstressed cells, RsbV, the effector of σ^B release, is inactive due to RsbW-catalyzed phosphorylation (9, 37). Under conditions of low ATP (e.g., entry into the stationary phase of growth) this phosphorylation reaction is thought to be inefficient, and σ^B remains active (2, 37, 38). In addition to this ATP-responsive activation, diverse environmental stresses initiate a sequence of Rsb-dependent processes which reactivate RsbV (18, 37, 38, 40, 41). In stressed *B. subtilis*, RsbT, normally inactive and complexed to RsbS, phosphorylates RsbS and becomes free to activate the RsbV-P phosphatase, RsbU (12, 41). RsbU then dephosphorylates RsbV-P, allowing RsbV to displace σ^B from RsbW. Negative regulation is reestablished when RsbX, a RsbS-P phosphatase, dephosphorylates RsbS-P (41). This enables RsbS to again bind and inactivate RsbT. Although much has been learned about how the Rsb proteins function to alternatively silence or activate σ^B, the mechanism by which stress communicates with the Rsb proteins is unknown. Neither the signal that is generated by environmental stress nor the regulator that is its target has been identified. In *E. coli*, protein denaturation and chaperone activation play key roles in communicating environmental stress to stress response transcription factors (8, 13, 43). Although similar processes appear to control chaperone expression in *B. subtilis* (23, 24, 27), we and others have not found an obvious correlation between chaperone activity and σ^B activity in *B. subtilis* (23, 29). In addition, the known Rsb proteins appear to be inadequate to respond to environmental stress and activate σ^B when they are expressed in *Escherichia coli* (29). Taken together, these results argue that the signal communicating environmental stress to the Rsb proteins is likely to be novel and bacillus specific. Assuming that an unidentified *B. subtilis* protein communicates stress signals to the Rsb phosphatase/kinase cascade, we attempted to identify candidate proteins, based on their ability to physically interact with key Rsb proteins in the yeast Gal4 dihybrid system. By this approach, several *B. subtilis* genes were identified as Rsb interactors. Although many of the interactions appear to be fortuitous, a number of biologically relevant associations were found. These included several known interacting Rsb proteins, as well as Obg, an essential GTP-binding protein (21, 31, 32) which proved to be needed for stress activation of σ^B. The pattern of σ^B induction in various mutant *B. subtilis* strains indicated that Obg, or a process under its control, plays a role in activating RsbT, the most upstream effector of σ^B's stress-induced pathway.

MATERIALS AND METHODS

Bacterial strains, plasmids, and cultivation. All of the strains and plasmids used here are listed in Table 1. All BSJ and BSA strains are derivatives of PY22. Bacteria were grown in Luria-Bertani medium (LB [22]) at 37°C. The cells were exposed to ethanol or sodium azide during exponential growth at final concentrations of 4% or 2 mM, respectively. P_{xyl} and P_{spac} were induced by xylose (0.5%) or IPTG (isopropyl-β-D-thiogalactopyranoside; 1 or 0.1 mM), respectively. *E. coli* TG-2 was used as the host for cloning (26).

Library construction and yeast transformation. Libraries of *B. subtilis* DNA::GAL4 activator domain (AD) fusions were constructed from PY22 chromosomal DNA that had been partially digested with either *Sau*IIIA or *Tsp*509I and ligated into either the *Bam*HI sites in pACT-2, pGAD-GL, and pGAD-10 (Clontech Laboratories, Inc., Palo Alto, Calif.) or the *Eco*RI sites of pACT-2 and

* Corresponding author. Mailing address: Department of Microbiology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Dr., San Antonio, TX 78284-7758. Phone: (210) 567-3957. Fax: (210) 567-6612. E-mail: Haldenwang@UTHSCSA.edu.

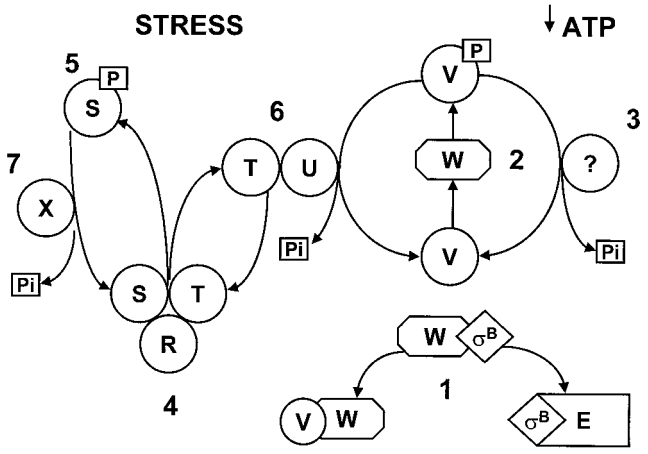

FIG. 1. Model of σ^B regulation. (Step 1) The anti-σ^B protein RsbW (W) can form a mutually exclusive complex with either σ^B or RsbV (V) (6, 9). When bound to RsbW, σ^B is unavailable to RNA polymerase (E) (6). RsbV binding to RsbW allows the release of σ^B and the formation of a σ^B RNAP polymerase holoenzyme (E-σ^B) (6). (Step 2) With ATP as a phosphate donor, RsbW can phosphorylate RsbV (2, 9). RsbV-P (V-P) is inactive as a σ^B release factor (2, 9). During growth, relatively high ATP levels favor the phosphorylation and inactivation of RsbV, leaving σ^B bound to RsbW (2, 38). If ATP levels fall, as when *B. subtilis* enters stationary phase, the phosphorylation of RsbV may be inefficient, leading to the persistence of active RsbV, the formation of RsbV-RsbW complexes, and the release of σ^B (2, 38). (Step 3) The magnitude of the low-ATP activation of σ^B is enhanced by dephosphorylation of a portion of the RsbV-P by an unknown mechanism (37). (Step 6) Environmental stress (e.g., heat shock, osmotic shock, and ethanol treatment) activates an RsbV-P phosphatase, RsbU (U), which creates active RsbV, regardless of ATP levels (37). (Step 4) RsbT (T), the activator of RsbU, is normally inactive due to an association with a negative regulator RsbS (S) (41). RsbR (R), an additional regulatory protein, binds to RsbS and RsbT (31) and is believed to play a structural role and to facilitate RsbT-RsbS interactions (1, 12). (Step 5) Upon exposure to stress, RsbT phosphorylates and inactivates RsbS and activates the RsbU phosphatase (12, 31). (Step 7) RsbS-P is dephosphorylated and reactivated by a phosphatase, RsbX (X) (31), which is encoded by one of the genes downstream of the *sigB* operon's σ^B-dependent promoter (17). RsbX levels increase with increasing σ^B activity (10). This may serve to limit the activation process and return RsbT to an inactive complex with RsbS.

pGAD-GL, respectively. These clonings created potential translational fusions between the *Sau*IIIA fragments and the Gal4 AD in all three reading frames and between the *Tsp*509I fragments and the Gal4-AD in two reading frames. The libraries were transformed, according to established protocols (Clontech Laboratories) into yeast strain Y190 containing resident plasmids that encoded binding domain (BD) fusions to RsbU, RsbT, and RsbX (pUV31, pUV76, or pUV145) (36). Plasmids that encoded interacting proteins were selected by GAL4-dependent histidine prototrophy and screened for GAL4-dependent β-galactosidase activity (36). The *B. subtilis* DNA fragments of interest were amplified by PCR from positive colonies by using primers specific for the sequences immediately outside of the multiple cloning sites of the vectors. These DNAs were verified as the coding elements for proteins that interact with the *rsb* fusions by recloning into the AD vector and transformation into yeast cells carrying the appropriate *rsb* fusion. The DNA sequences that encoded interacting peptides were determined and their predicted protein sequences compared to the *B. subtilis* genome database (30a) to identify the genes from which they were derived.

Construction of plasmids for integration into target genes. pUSX19 was created by cloning a 1.7-kb *Bam*HI-*Sph*I DNA fragment containing the xylose promoter and repressor from pX-2 (20) into similar sites of pUS19 (4). The 5′ ends (500 to 700 bp) of the genes that encoded protein fragments, which interacted with the Rsb proteins in the yeast two-hybrid system, were PCR amplified and cloned into the *Bam*HI and *Sac*I sites of pUSX19 by using sites that had been introduced during the amplification. This cloning placed the 5′ ends of the genes downstream of P_{xyl} in pUSX19. When these plasmids were transformed into PY22, a Campbell-like integration of the plasmid into the *B. subtilis* chromosome disrupted the resident copy of the gene and positioned the sole intact copy of the target gene downstream of P_{xyl}. These strains were then grown with or without xylose to determine whether the isolated genes were essential for growth. A *ctc*::*lacZ* reporter system was introduced into these strains by transformation with BSA46 (3) chromosomal DNA and selection for a *ctc*::*lacZ*-linked antibiotic resistance (*erm* and *cat*). To assess the possible need for each Rsb interacting protein in σ^B stress activation, strains containing the P_{xyl} fusions were grown in LB with or without xylose and exposed to stress (4% ethanol) during the exponential phase of growth (optical density at 540 nm of 0.2). The σ^B activity was monitored by reporter gene (*ctc*::*lacZ*) expression. To determine the effects of enhanced levels of the interacting proteins on stress activation of σ^B, the cultures were grown in the presence of 2% xylose, which induces P_{xyl} several 100-fold (20), and stressed as described above.

Construction of plasmids and strains for Obg and RsbT manipulations. pJM2, a P_{spac}::*obg* fusion plasmid, was constructed by cloning the *obg* gene, including its ribosomal binding site, which had been amplified by PCR into pBluescript (26), by using *Bam*HI sites that had been placed at each end during the amplification. The orientation of the fragment in the plasmid was determined by restriction endonuclease digestion analysis. The *obg* segment was excised with *Hin*dIII and *Xba*I and cloned into the multiple cloning site of pDG148. pJM4 was constructed by PCR amplification of a 250-bp DNA fragment containing 150 bp of the 5′ end of *obg* and 100 bp of upstream DNA. *Bam*HI and *Bgl*II sites, introduced into the ends of the fragment during amplification, were used to clone the fragment into the *Bam*HI site of pUSX19, creating a P_{xyl}::obg_{150} fusion. pHV501T contains the *Hin*dIII/*Bam*HI piece of *rsbT* from pDT11 (30). The fragment was cloned into pHV501 downstream of P_{spac}. The resulting plasmid was cut with *Cla*I and *Bam*HI to remove *lacZ*, made blunt with the Klenow fragment of DNA polymerase, and religated. Transformation of PY22 with pHV501T places P_{spac} between *rsbS* and *rsbT* within the *sigB* operon (BSA400), due to a Campbell-like integration of the plasmid into *rsbT*.

General methods. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Western blotting, and β-galactosidase activity assays were performed as previously described (10, 19). DNA manipulations were performed according to standard protocols (26). Transformation of naturally competent *B. subtilis* cells was carried out as described by Yasbin et al. (42). Yeast β-galactosidase assays were performed as done previously (36). Predicted protein translations of the DNA fragments were determined by using DNAMAN software (Lynnon Biosoft Co.). Sequencing of DNA fragments identified in the yeast two-hybrid system screen was performed by the University of Texas Health Science Center at San Antonio Center for Advanced DNA Technologies.

RESULTS

Identification of Rsb interactors. We considered RsbT, RsbU, and RsbX as the most likely of the Rsb proteins to be targeted by stress-generated signals. To identify possible protein mediators of such signals, we screened *B. subtilis* chromosomal DNA for genes whose products could be shown to physically interact with RsbT, -U, or -X in the yeast dihybrid system. As described in Materials and Methods, yeast strains carrying Gal4-activatable *his* and *lacZ* genes, as well as plasmids encoding *rsbT*, *rsbU*, or *rsbX* fused to the Gal4 DNA BD, were transformed with plasmid libraries of *B. subtilis* chromosomal DNA. The plasmid libraries contain potential translational fusions between various *B. subtilis* genes and the Gal4 AD. Transformants in which the cloned *B. subtilis* DNA encoded a Gal4-AD protein which could associate with an Rsb/Gal4-BD and create a functional gene activator protein were selected as histidine prototrophs and screened for a Lac^+ phenotype. After we verified that the His^+ Lac^+ activities of the positive clones were due to an interaction between the AD fusion proteins and the Rsb-BD proteins (Materials and Methods), the AD fusion plasmids were recovered from the yeast cells, transformed into *E. coli*, and screened by restriction endonuclease-Southern blot analyses to eliminate duplicate clones. A total of 31 unique *B. subtilis* DNA inserts were isolated from approximately 400 His^+ Lac^+ transformants. The insert DNA was sequenced by using DNA primers complementary to the vectors' translational fusion junctions. The identity of the *B. subtilis* element that had become fused to the Gal4-AD was determined by searching the *B. subtilis* genome sequence database (30a) for the sequences contained at the Gal4-AD fusion junctions. The results of this exercise are listed in Table 2. Eleven predicted intergenic regions were among the *B. subtilis* chromosome segments that displayed Gal4 activity when cloned into the AD vector. Presumably, these represent fortuitously generated AD fusion proteins that could pair with particular Rsb::Gal4-BD chimeras. In previous studies, where interactions between the various *rsb* genes were

TABLE 1. *B. subtilis* strains and plasmids used

Strain or plasmid	Relevant genotype	Reference, source, or construction
Bascillus strains		
SJV6	*P_{spac}::obg*	A. Grossman
PY22	*trpC2*	P. Youngman
AG514	*spo0A::cat*	A. Grossman
BSA46	*trpC2 Spβ ctc::lacZ*	3
XS15	*trpC2 rsbX::spc rsbU194VA Spβ ctc::lacZ*	30
BSA400	*trpC2 rsbT*::pHV501T *(P_{spac}::rsbT)*	pHV501T→PY22
BSA419	*trpC2 P_{spac}::rsbT Spβ ctc::lacZ*	BSA46 →BSA400
BSJ-5	*trpC2 P_{spac}::obg*	SJV6→PY22
BSJ-6	*trpC2 P_{spac}::obg Spβ ctc::lacZ*	BSA46→BSJ-5
BSJ-9	*trpC2 P_{spac}::obg* (pJM2) *Spβ ctc::lacZ*	pJM2→BSA46
BSJ-10	*trpC2 P_{spac}::obg rsbX::spc rsbU194VA Spβ ctc::lacZ*	XS15→BSJ-6
BSJ-11	*trpC2 P_{xyl}::obg*	pJM4→PY22
BSJ-12	*trpC2 P_{xyl}::obg Spβ ctc::lacZ*	BSA46→BSJ-11
BSJ-13	*trpC2 P_{xyl}::obg P_{spac}::rsbT Spβ ctc::lacZ*	BSJ-11→BSA419
BSJ-16	*trpC2 spo0A::cat*	AG514→PY22
BSJ-17	*trpC2 spo0A::cat Spβ ctc::lacZ*	BSA46→BSJ-16
Plasmids		
pUS19	Ap^r $Spec^r$	4
pUSX19	Ap^r $Spec^r$ *P_{xyl} xylR*	This study
pACT-2	Ap^r vector with AD of GAL4	Clontech
pGAD-10	Ap^r vector with AD of GAL4	Clontech
pGAD-GL	Ap^r vector with AD of GAL4	Clontech
pX-2	Ap^r *P_{xyl} xylR*	20
pUV31	Ap^r protein fusion of RsbX with BD of GAL4	36
pUV76	Ap^r protein fusion of RsbT with BD of GAL4	36
pUV145	Ap^r protein fusion of RsbU with BD of GAL4	36
pJM2	Ap^r Kan^r *P_{spac}::obg*	This study
pJM4	Ap^r $Spec^r$ *P_{xyl}::obg_{150} xylR*	This study
pDG148	Ap^r Kan^r *P_{spac} lacI*	P. Stragier
pHV501	Ap^r Erm^r *P_{spac} lacI lacZ*	S. D. Ehrlich
pHV501T	Ap^r Erm^r *P_{spac}::rsbT lacI*	This study
pDT11	Ap^r Kan^r *P_{spac}::rsbT lacI*	30
pBluescript	Ap^r	26

examined in the yeast dihybrid system, no RsbX interactions were detected with any of the other Rsb genes, but interactions were found between RsbT and RsbS/-R/-U and between RsbU and RsbV/-T (36). Our current screening of the *B. subtilis* genome detected two of these four associations: the interactions of RsbT with both RsbR and RsbS (Table 2).

The remaining 18 cloned segments defined portions of 16 different predicted or known genes (Table 2). Two of these genes (*yvgO* and *yjlD*) were detected twice in overlapping fragments. Four of the genes were RsbT interactors, eight were RsbU interactors, and four were found in pairings with RsbX. As described in Materials and Methods, we attempted to distinguish biologically relevant associations from fortuitous biochemical interactions by reducing or elevating the expression of the identified genes in *B. subtilis* and then asking whether either of these manipulations would affect σ^B activity during growth or stress (data not shown). Only *obg*, a gene isolated in the RsbX pairing, was found to have a significant effect on σ^B's activity in this analysis. It remains possible that one or more of the other *B. subtilis* genes could have a less obvious role in Rsb function; however, our inability to readily demonstrate this prompted us to focus on *obg*.

Obg is necessary for stress activation of σ^B. Obg is an essential *B. subtilis* protein (21, 31, 32). In order to study its possible role in σ^B activation, we used a *B. subtilis* strain (BSJ-6) in which *obg*'s expression was under the control of an IPTG-inducible promoter (32). Withholding IPTG from this strain leads to a depletion of Obg and a concomitant cessation of growth (32). Cultures of BSJ-6 were resuspended in LB with or without IPTG and monitored until growth had slowed in the culture lacking IPTG. Portions of the cultures were then stressed by treatment with ethanol. The σ^B activity in both cultures was estimated by using a reporter gene fused to a σ^B-dependent promoter (i.e., *ctc::lacZ*). *B. subtilis* with Obg (i.e., the IPTG-containing culture) immediately activated σ^B after ethanol treatment; however, the strain in which the Obg levels had been depleted failed to activate σ^B in response to ethanol stress (Fig. 2). Similar results were obtained with heat shock as the stress and Western blot analyses to judge the inducibility of the Rsb genes that are downstream of the *sigB* operon's σ^B-dependent promoter (i.e., *rsbV* and *-W*, *sigB*, and *rsbX*) (data not shown).

We had previously characterized two mechanisms by which σ^B is activated (38). One relies on the above-described stress induction pathway. A second type of σ^B activation occurs under culture conditions where ATP levels are low (e.g., glucose limitation, Mn^{2+} treatment, and entry into stationary phase) (38). Presumably, under low ATP conditions RsbV remains active due to ineffective phosphorylation by RsbW (Fig. 1) (2, 38). To determine whether Obg is needed by the ATP responsive pathway, we treated BSJ-6 (*P_{spac}::obg*), which was either growing in LB with IPTG or had ceased growth due to Obg depletion in LB lacking IPTG, with sodium azide, a compound which blocks oxidative phosphorylation and lowers ATP levels in *B. subtilis* (16). Azide addition resulted in enhanced σ^B activity regardless of whether or not the culture was depleted

TABLE 2. Interacting proteins and intergenic regions identified in the yeast two-hybrid system[a]

Bait protein	Interacting protein	Predicted function	Protein size (no. of amino acids)	Amino acid segment in BD fusion
RsbT	ProB	γ-Glutamyl kinase	354	321–354
	RsbR	Regulator of σ^B	274	116–256
	RsbS	Regulator of σ^B	121	8–117
	YfmR	Similar to ABC transporter	629	572–629
	YhcH	Similar to ABC transporter	305	113–203
	YorL	Similar to DNA polymerase III, α subunit	1,305	1,211–1,240
RsbU	PpsD	Peptide synthetase	3,603	1,261–1,351
	RpoC	RNA polymerase, B subunit	1,200	671–811
	SpsB	Spore coat polysaccharide biosynthesis	472	53–100
	SpsJ	Spore coat polysaccharide biosynthesis	315	7–47
	YjlD	Similar to NADH dehydrogenase	392	1–23, 1–46
	YlaG	Similar to GTP binding elongation factor	612	267–372
	YoaR	Unknown, no similarity to known proteins	303	222–303
	YorL	Similar to DNA polymerase III, α subunit	1,305	1,211–1,240
RsbX	Obg	GTP binding protein	428	285–328
	YrkN	Unknown, no similarity to known proteins	185	32–185
	YtgA	Similar to ABC transporter	307	140–296
	YvgO	Unknown, no similarity to known proteins	161	29–100, 55–100

[a] The table lists each of the Rsb proteins fused to the Gal4::BD (bait protein), the identity of the *B. subtilis* genes that were isolated against them as interacting Gal4::AD fusions (interacting protein), and the predicted function of the genes. The predicted size of the gene products (protein size) and the particular amino acid segment of the proteins that were present as part of the fusion proteins are also listed. The nucleotide designations of the *B. subtilis* intergenic regions which yield "fortuitous" fusion proteins are noted as follows: for RsbT, 1886017–1885806; for RsbU, 3334901–3335145, 4094152–4094628, 521710–521628, 1298509–1298682, 3990625–3990466, 135661–135552, 3648551–3648811, 262440–262510, 2564095–2564127, and 2359371–2359314.

of Obg (Fig. 3). Although σ^B activity increased in both cultures, the level of reporter gene activity in the Obg-depleted culture (Fig. 3B) was approximately one-half of that seen in the culture in which Obg was not limiting (Fig. 3A).

Continued incubation of Obg-induced and -depleted cultures led to the expected activation of σ^B as the Obg^+ culture entered stationary phase (Fig. 3A). σ^B was also activated in the Obg^- culture; however, a longer incubation period was required for its onset (Fig. 3B). Presumably, the delayed σ^B activation in the Obg-depleted culture is due to the growth-inhibited culture requiring more time to reach the point where ATP levels fall sufficiently to activate σ^B. As was the case with azide treatment, the stationary-phase induction of σ^B in the Obg-depleted cells was approximately one-half of that seen in the control culture (Fig. 3). We have found that Obg-depleted cells, pulse labeled with ^{35}S-labeled methionine-cysteine under conditions similar to those used in our experiments, incorporate only 60% of the label which is incorporated by an Obg^+ culture (data not shown). Thus, although we cannot exclude the possibility that the reduced level of σ^B activation represents a residue role for Obg in the ATP-responsive pathway, it is more likely to be the result of a general effect of Obg depletion on the biosynthetic capacity of *B. subtilis*. The activation of σ^B in an Obg-depleted culture by conditions that trigger the ATP-responsive pathway, but not by those that induce the stress-dependent pathway, indicates that Obg's role in σ^B activation is largely limited to the stress activation process.

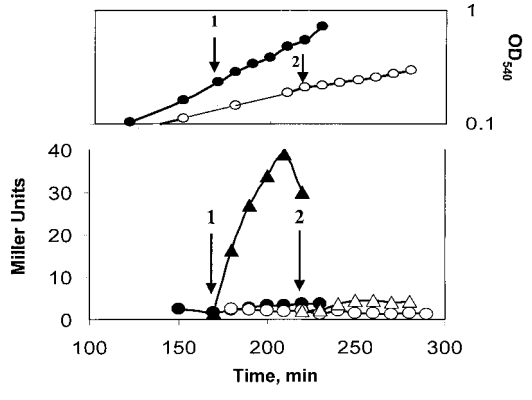

FIG. 2. Effect of Obg depletion on ethanol induction of σ^B. (Top panel) Growth of BSJ-6 (P_{spac}::*obg*) in LB with (●) or without (○) IPTG (100 μM). The arrows represent the points during growth when ethanol (4% final concentration) was added to the IPTG-induced (1) and the $IPTG^-$ (2) cultures. The curves display the untreated cultures; the ethanol-treated cultures had their growth reduced by approximately 50% (not shown). (Bottom panel) *ctc*::*lac* expression in BSJ-6. Samples of the cultures represented in the top panel were analyzed for σ^B-dependent β-galactosidase activity. The arrows depict the times of ethanol addition in the $IPTG^+$ (1) and $IPTG^-$ (2) cultures. Closed symbols represent $IPTG^+$ cultures with (▲) or without (●) ethanol; open symbols depict the $IPTG^-$ culture with (△) or without (○) ethanol.

Obg effects on σ^B are independent of Spo0A. Obg is essential for both growth and sporulation (21, 31, 32). During studies of the sporulation requirement for Obg, it was discovered that Obg is needed, either directly or indirectly, for the phosphorylation reaction that activates the transition-phase regulatory protein Spo0A (32). Given that both the activation of Spo0A and the stress induction of σ^B require Obg, we asked whether the stress activation of σ^B could be a Spo0A-mediated process. Wild-type and *spo0A*::*cat* strains of *B. subtilis* were exposed to ethanol stress and examined for σ^B activation. σ^B induction occurred regardless of the presence or absence of Spo0A (Fig. 4). Thus, the need for Obg in σ^B activation is not likely to be due to an Obg effect on Spo0A.

Obg interacts with RsbT and RsbW in the yeast dihybrid system. Knowing that high-molecular-weight complexes containing multiple Rsb proteins and unknown additional components are readily detectable in *B. subtilis* extracts (10) and that our initial screening of the *B. subtilis* library had not been

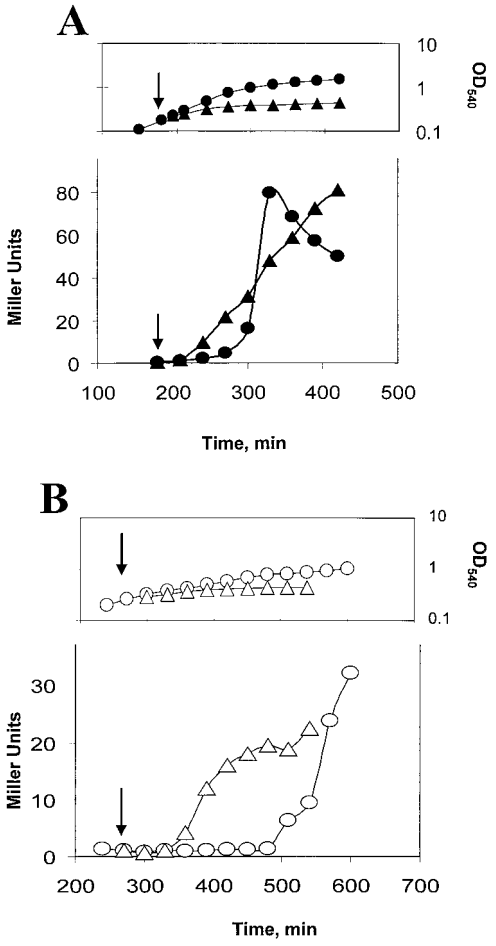

FIG. 3. ATP-responsive induction of σ^B in Obg-depleted cells. BSJ-6 (P_{spac}::*obg*) was grown in the presence (A) or absence (B) of IPTG. The top portions of panels A and B illustrate the growth of the cultures without (○ and ●) or in the presence of 2 mM sodium azide (△) and ▲), which was added at the times indicated by the arrows. The lower portions of panels A and B display σ^B-dependent β-galactosidase activity of the cultures represented in the panels above them, with the arrows depicting the time of sodium azide addition. The triangles represent azide-treated cultures; the circles represent untreated cultures.

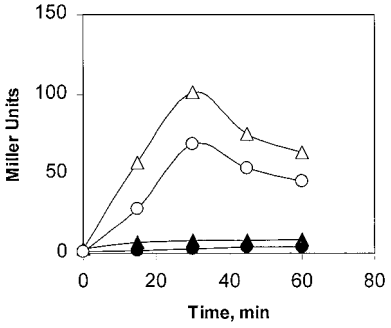

FIG. 4. Activation of σ^B in Spo0A$^-$ *B. subtilis*. *B. subtilis* BSA46 (wild type, circles) and BSJ-17 (*spo0A*::*cat*, triangles) were grown without stress in LB or exposed to ethanol (4%) during an early stage of exponential growth. Samples were taken from control (solid symbols) and ethanol-treated (open symbols) cultures at the times indicated and analyzed for σ^B-dependent (*ctc*::*lacZ*) β-galactosidase activity.

exhaustive, we revisited the yeast dihybrid system to test whether Obg could interact with additional Rsb components. Specifically, we paired both the entire *obg* gene and the *obg* fragment which had interacted with RsbX with other *rsb* genes. *rsbS* was not included in the analyses due to its ability to activate Gal4-dependent promoters independently when fused to Gal4-BD.

No interactions were detected between either the Obg fusion and RsbR, -U, or -V (i.e., yeast strains expressing the fusion pairs failed to activate the *lacZ* reporter gene and turn blue in X-Gal [5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid] filter assays [36]); however, we observed reporter gene activity when the carboxy-terminal fragment of Obg was paired with RsbW or when the entire *obg*::Gal4-AD fusion was paired with either RsbW or RsbT. The RsbX-Obg interaction, although obvious when RsbX was paired with the Obg fragment, was barely detectable when RsbX was paired with the full-length Obg fusion protein. The relative reporter gene activity for each of the reactive pairings is given in Table 3.

The level of reporter gene activity in the yeast dihybrid system can be a reflection of the affinity of the interacting proteins for each other (11). Based on reporter gene activity, relative to the vector pairings, the interaction between RsbT and the full-length Obg protein is approximately 2.5-fold that observed in a similar assay where RsbT was paired with RsbU, the phosphatase that it activates (36). The interaction between RsbW and the full-length Obg is more than three times the reporter gene response seen with RsbW paired with its antagonist RsbV (36). The degree of reporter gene activation that occurs in yeast cells when Obg is paired with RsbW or RsbT indicates that significant interactions are possible between these proteins. Although the relevance of these interactions in yeast cells to σ^B control in *B. subtilis* is unclear, the fact that they occur suggest that RsbT and RsbW, as well as RsbX, are possible targets for Obg-essential steps in the stress activation of σ^B.

Obg's essential role in σ^B activation is independent of RsbX. Obg was initially identified as a potential σ^B regulator on the basis of an interaction between its carboxy terminus and RsbX in the yeast dihybrid system. RsbX is a negative regulator of the stress-inducible pathway for σ^B activation (3, 15, 17, 30, 33, 35). Thus, if Obg's essential role in σ^B activation entails an effect on RsbX, we would expect this to involve a lifting of RsbX's negative control. If this is so, Obg should not be essential for the stress activation of σ^B in the absence of RsbX.

The loss of RsbX normally causes a toxic activation of σ^B (3,

TABLE 3. Interactions of Obg and Rsb proteins by yeast two-hybrid system[a]

BD fusion	AD fusion (ratio)	
	Obg′	Obg
Vector, pAS2-1	1	1
X	1.88	1.15
T	0.525	4.41
W	5.52	33.86

[a] The horizontal rows represent the Gal4 DNA BD vector (pAS2-1) either without insert DNA (row 1) or with the *sigB* gene depicted by a single letter (e.g., X = *rsbX*). The vertical rows depict the AD vector (pACT-2) encoding either the 285- to 328-amino-acid fragment of Obg, originally identified in the library screen (Obg′) or else intact Obg. Intersecting rows represent reporter gene activity (i.e., β-galactosidase expression) in a yeast strain cotransformed with both plasmids indicated by row. β-Galactosidase assays were performed on two clones from each pairing in duplicate. The results are presented as a ratio of the Rsb protein-AD fusion value from the indicated pairing divided by the value obtained from the AD fusion pairing with the pAS2-1 vector.

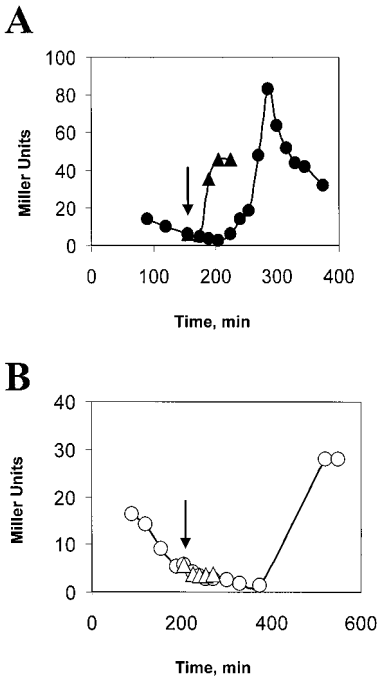

FIG. 5. Effects of Obg on activation of σ^B in RsbX$^-$ *B. subtilis*. BSJ-10 (*P*$_{spac}$::*obg rsbX*::*spec rsbU194VA*) was grown in LB with (A) or without (B) IPTG (0.1 mM). As in Fig. 2, cells were treated with ethanol (4%) at the times indicated by the arrows either during growth (A) or after the culture slowed due to Obg depletion (B). Symbols: triangles, ethanol-treated cultures; circles, untreated cultures.

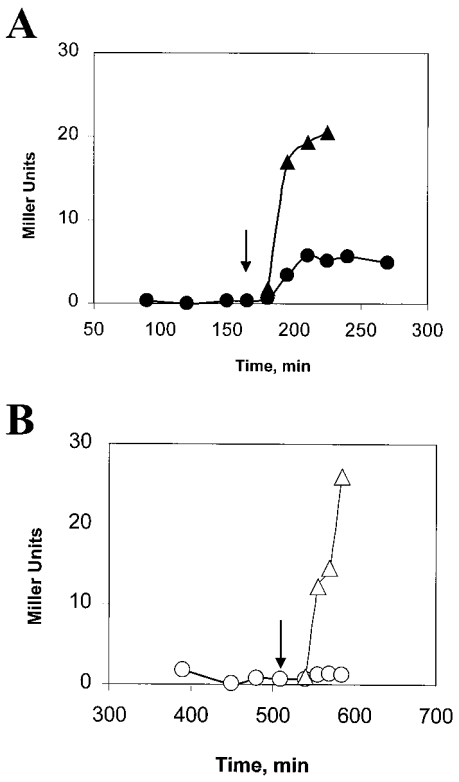

FIG. 6. Activation of σ^B by RsbT overexpression in Obg-depleted *B. subtilis*. BSJ-13 (*P*$_{xyl}$::*obg P*$_{spac}$::*rsbT*) was grown in LB with or without xylose (0.5%) to maintain Obg levels (A) or to deplete the cell of Obg (B), respectively. At the times indicated by the arrows, IPTG (1 mM) was added to the exponentially growing cells (A), and the culture that had slowed due to Obg depletion (B) to induce *P*$_{spac}$ upstream of *rsbT*. Samples were taken from IPTG-induced (▲) and △) and control (● and ○) cultures and were analyzed for σ^B-dependent β-galactosidase activity as in Fig. 2.

15, 17, 33); however, there are several *B. subtilis* strains in which the loss of RsbX is tolerated due to suppressor mutations in either RsbT, -U, or -V, that reduce their activities as positive regulators (30). We placed an inducible *obg* operon into one of these strains, XS15 (*rsbU194VA rsbX*::*spec*), and examined the effects of Obg depletion on ethanol-induced σ^B activity in this RsbX$^-$ background. As was the case in the RsbX$^+$ strains, σ^B activity was induced after both ethanol treatment, and entry into stationary phase when Obg was present (Fig. 5A), but increased only upon entry into stationary phase in the culture depleted of Obg (Fig. 5B). Thus, Obg is needed for the stress activation of σ^B in the absence of RsbX. This result does not exclude the possibility that Obg can modulate RsbX activity, but it does demonstrate that Obg provides an essential function for σ^B activation aside from any putative RsbX interaction.

RsbT can activate the σ^B stress pathway in the absence of Obg. Stress is believed to set in motion a process (Fig. 1) whereby RsbT frees itself from its inhibitor and then binds and activates RsbU, the RsbV-P phosphatase (41). RsbV then interacts with RsbW to free σ^B (2, 6, 9). There are several points at which an Obg-dependent function might be needed in this process. To determine whether Obg is required for the activation of RsbT or a downstream event, we took advantage of the observation that the induced expression of RsbT, in the absence of a corresponding synthesis of its inhibitor (RsbS), can drive activation of σ^B in unstressed bacteria (18, 28). If the essential Obg-dependent function is limited to the activation of RsbT, then the induced synthesis of RsbT should activate σ^B in Obg-depleted cells. Conversely, if Obg has a critical role in RsbT's ability to activate RsbU, RsbU's capacity to dephosphorylate of RsbV-P, or RsbV's ability to displace σ^B from RsbW, then providing additional RsbT should not activate σ^B in Obg-depleted cells.

To independently control both the levels of Obg and the induction of *rsbT*, we constructed a *B. subtilis* strain in which *obg* expression was driven from a xylose-inducible promoter (*P*$_{xyl}$::*obg*), and an IPTG-inducible promoter was placed immediately upstream of *rsbT* in the *sigB* operon. Growth and stress induction of this strain is xylose dependent due to Obg depletion in the absence of the *P*$_{xyl}$ inductant. When IPTG was added to cultures of this strain, σ^B was activated regardless of whether or not Obg had been depleted (Fig. 6). We conclude that Obg's essential role in activation of σ^B is upstream of RsbT's activation of RsbU in the stress pathway and is likely in the activation of RsbT itself.

DISCUSSION

The general stress regulon of *B. subtilis* is induced when environmental stress communicates with a kinase/phosphatase pathway to trigger the activation of the σ^B transcription factor (41). Neither the stress-generated signal that activates σ^B nor the component within the pathway that initially responds to the signal are known. Previous studies indicated that the known σ^B regulators are insufficient to detect and respond to stress and that the inducing signal is likely to be novel and *Bacillus* specific (29). This suggested that additional *B. subtilis* gene products are needed to communicate the presence of stress to the

σ^B activation cascade. In the current study, we attempted to identify such gene products by using the yeast dihybrid system to isolate *B. subtilis* proteins that could interact with important members of the σ^B activation cascade and, as such, might be involved in this signaling. As is evident from our results, the dihybrid selection system readily detects peptides that can interact with Rsb fusion proteins to activate the yeast selection system. Although a few of these associations are clearly biologically relevant, i.e., known Rsb-Rsb interactions were detected, most of the isolates likely represent fortuitous interactions between domains on the fusion proteins. Fortunately, access to the *B. subtilis* genome database and our ability to readily alter the expression of the genes for these putative interactors allowed us to test whether the abundance or absence of their products could affect σ^B stress activation and also allowed us to put aside unpromising candidates. By this approach, *obg* was detected in our current screening as a gene whose product influences σ^B activity. If *B. subtilis* is depleted of Obg, stress activation of σ^B, but not its ATP-responsive activation, is blocked. Overexpression of Obg from a high-copy-number plasmid had no effect on σ^B activity (data not shown).

Obg is the second gene in the operon that encodes Spo0B, a critical protein in the phosphorelay which modulates the activity of the sporulation-transition state regulatory protein Spo0A (31). Obg is essential for both *B. subtilis* growth and sporulation (31, 32). It is a GTP binding protein (39) whose explicit function is unknown; however, there is evidence that Obg activity influences the initiation of chromosome replication (21) and the phosphorylation state of the postexponential-phase gene regulator, Spo0A (32). It is unclear whether Obg's activities in either of these processes is related to its effects on σ^B activity. We note however, that the Obg-dependent activation of Spo0A is not needed for the σ^B activation process, since stress activation of σ^B occurs in the absence of Spo0A function.

Obg is a member of a unique family of small GTP binding proteins that have been identified in diverse organisms from bacteria and mammals (reviewed in reference 25). In bacteria, the Obg subfamily is speculated to monitor the state of intracellular GTP levels and to serve as a switch to promote growth when bound to GTP, but not when associated with GDP (25). The actual targets for this switch protein are unknown. Our yeast dihybrid data suggest that Obg may directly interact with a number of the σ^B Rsb regulators; however, even if these interactions ultimately prove to be biologically relevant, the Rsb proteins cannot be Obg's only targets. Obg is essential for growth and sporulation, while neither the Rsb proteins nor σ^B are essential for either process. Perhaps Obg, as a general sensor of intracellular GTP levels and a regulatory switch, has several targets within *B. subtilis*, allowing it to coordinate diverse functions in response to nucleotide changes.

Although it is clear that Obg is essential for stress activation of σ^B, it remains possible that the apparent interaction of Obg with particular Rsb proteins is accidental and that the requirement for Obg in σ^B stress activation is indirect. The putative RsbT-Obg interaction is, however, intriguing given that one function of the Obg class of proteins is intracellular signaling and that stress activation of RsbT fails to occur in its absence. Determining whether Obg directly communicates the existence of environmental stress to RsbT or is only indirectly involved in this process will likely require in vitro biochemical analyses.

ACKNOWLEDGMENTS

We thank Alan Grossman and Wolfgang Schulmann for strains and plasmids and Jim Hoch for advice and unpublished data.

This work was supported by NIH grant GM48220.

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