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The Growth-Promoting and Stress Response Activities of the *Bacillus subtilis* GTP Binding Protein Obg Are Separable by Mutation▽

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Bacillus subtilis **Obg is a ribosome-associating GTP binding protein that is needed for growth, sporulation, and induction of the bacterium's general stress regulon (GSR). It is unclear whether the roles of Obg in sporulation and stress responsiveness are direct or a secondary effect of its growth-promoting functions. The present work addresses this question by an analysis of two *obg* alleles whose phenotypes argue for direct roles for Obg in each process. The first allele [*obg*(*G92D*)] encodes a missense change in the protein's highly conserved "obg fold" region. This mutation impairs cell growth and the ability of Obg to associate with ribosomes but fails to block sporulation or the induction of the GSR. The second *obg* mutation [*obg*(Δ*22*)] replaces the 22-amino-acid carboxy-terminal sequence of Obg with an alternative 26-amino-acid sequence. This Obg variant cofractionates with ribosomes and allows normal growth but blocks sporulation and impairs the induction of the GSR. Additional experiments revealed that the block on sporulation occurs early, preventing the activation of the essential sporulation transcription factor Spo0A, while inhibition of the GSR appears to involve a failure of the protein cascade that normally activates the GSR to effectively catalyze the reactions needed to activate the GSR transcription factor (σ^B).**

Obg, a *Bacillus subtilis* GTP binding protein, was the first recognized member of a subfamily of GTP binding proteins that are conserved in both prokaryotic and eukaryotic cells (7, 8, 12). *B. subtilis obg* is the downstream component of a bicistronic operon that encodes an essential sporulation gene (*spo0B*) as its upstream member (45). Obg is essential for *B. subtilis* growth, sporulation, and physical stress activation of the bacterium's general stress regulon (GSR) (31, 40, 45, 46).

Genetics studies have also revealed a growth-essential role for the Obg orthologs of *Escherichia coli*, *Caulobacter crescentus*, and *Vibrio* and *Streptomyces* species (25, 34, 35, 39, 43). In vitro, purified *B. subtilis* and *C. crescentus* Obgs display high GTP/GDP dissociation exchange rates (K_d, ~1.5 s^{-1}) but relatively low GTP hydrolysis rates (half-life, ~23 min) (32, 52). These properties suggested that Obg proteins are less likely to function as GTPases but instead might serve as monitors of intracellular GTP/GDP ratios, promoting growth or differentiation, in response to the particular nucleotides that are bound to them (32, 35, 52).

The mechanism by which Obg promotes growth is not clear; however, a ribosome-associated process may be involved. *B. subtilis* Obg specifically binds to L13, a protein component of the 50S ribosomal subunit, in an affinity blot assay and cofractionates with ribosomes in a GTP-dependent manner during gel filtration or velocity centrifugation (42, 58). The Obg orthologs of *E. coli*, *C. crescentus*, and *Vibrio harveyi* have also been shown to bind to 50S ribosomal subunits (15, 33, 43, 55). The association of Obg with ribosomes may, in part, be related to a role in facilitating ribosome maturation. Putative ribosome assembly intermediates accumulate at the expense of mature 50S ribosome subunits in a number of bacterial strains that are deficient in Obg (15, 25, 39). Consistent with a possible role for Obg in ribosome maturation, overexpression of the homologue of Obg in *E. coli* (ObgE) was found to suppress the loss of an RNA methyltransferase (RrmJ) that participates in late 50S ribosome subunit assembly (44).

Apart from a possible role in ribosome maturation, Obg has been implicated in modulation of the "stringent response," a process that normally downregulates growth-promoting operons (e.g., ribosome-encoding genes) in response to nutritional stress (13). The stringent response is effected by the regulatory nucleotide (p)ppGpp (guanosine tetraphosphate) (13). *B. subtilis* Obg, overexpressed and purified from *E. coli*, was isolated with ppGpp in its nucleotide binding site (11). In *E. coli* and *Vibrio* species, ppGpp levels are controlled by RelA, a ppGpp-synthetase, and SpoT, a ppGpp synthetase and hydrolase (13). The Obgs of both *E. coli* and *V. cholerae* interact with SpoT in the yeast two-hybrid assay (25, 37), In addition, the homologue of Obg in *E. coli* copurifies and coimmunoprecipitates with SpoT (25). *V. cholerae*'s Obg has been found to be no longer essential for growth in a $RelA^-$ background (37). This suggests that, at least in *V. cholerae*, the essential function of Obg may involve stringent response repression, presumably by regulating SpoT activity. Obg's growth-essential role(s) in other bacteria likely include additional functions. Obg remains essential in $RelA^-$, $SpoT^-$ (25), and in $RelA^-$ *B. subtilis* (S. Kuo, unpublished data).

In addition to ribosome-associated processes, a potential involvement of Obg in *E. coli* chromosome replication was uncovered when a transposon insertion, isolated on the basis of

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heightened sensitivity to replication inhibitors, was found to lie in the 3′ end of *obgE* (21). The *obgE*::Tn strain was viable but abnormally sensitive to an inhibitor (hydroxyurea) of ribonucleotide reductase. Additional experiments suggested that this phenotype is a reflection of an undefined role of ObgE in replication checkpoint signaling, a process in which the failure of one DNA replication fork triggers the arrest of other forks and the inhibition of new replication initiations (21).

Like its roles in the previously described phenomena, the contributions of Obg to *B. subtilis* sporulation and stress response induction are unclear. In the case of sporulation, the Obg-dependent function is required early. Obg-depleted strains placed under sporulation conditions fail to effectively phosphorylate a gene activator protein (Spo0A) that is the key initiator of the sporulation gene program (46). A similar depletion of Obg in strains subjected to physical stress (e.g., ethanol) prevents the activation of the bacterium's GSR (40).

The diverse phenotypes associated with *obg* mutations are a curiosity. Do they reflect distinct roles for Obg in multiple processes or are they the secondary consequences of a basic underlying defect in Obg-deficient strains? In the case of *B. subtilis*, is the failure to initiate sporulation or trigger the GSR a reflection of a direct role for Obg in each of these responses or an indirect effect of Obg's growth-promoting function? In an attempt to better understand the role(s) of Obg in these processes, we sought to isolate mutations in *obg* that selectively affected one or the other of these activities. Two mutations with novel phenotypes were isolated and characterized. One mutation [*obg*(*G92D*)] is a missense change at amino acid 92. This mutation inhibits bacterial growth and the ability of Obg to associate with ribosomes but has minimal or no effect on the mutant strain's ability to sporulate or activate the GSR. A second mutation, a substitution of the 22-amino-acid Obg carboxy terminus with a heterologous 26-amino-acid sequence, does not inhibit growth or ribosome association but blocks sporulation at its outset and impairs stress regulon induction. This apparent separation of the growth promotion and stress response induction activities of Obg by mutation argues that Obg likely has separate roles to play in each of these processes and that the failure of *B. subtilis* to properly initiate stress responses in the absence of Obg is not merely a consequence of a growth defect.

MATERIALS AND METHODS

Bacterial strains, plasmids, and mutant isolation. All plasmids and strains and their relevant genotypes are listed in Table 1. Except where indicated, the *B. subtilis* strains used in the present study are derivatives of BSA46 (4). Plasmid pSK100 was created by cloning a 1.8-kbp HindIII-PstI DNA fragment containing the entire *obg* gene plus 500 bp of upstream sequence, a PstI-SalI *spc* cassette from pDG1726 (24), and a 500-bp SalI-SacI DNA fragment downstream of *obg* into pUC19 (38). pSK100 was subjected to random mutagenesis by a one-step error-prone rolling-circle amplification (RCA) using a TempliPhi 100 DNA amplification kit from Amersham Biosciences (Piscataway, NJ) (22). Products from the RCA mutagenesis were transformed into BSA46 with transformants selected on the basis of the insert-encoded antibiotic resistance. Transformants were screened for small colony and temperature sensitivity phenotypes. Strains with growth defect ("pinpoint" colonies) were streaked on Luria-Bertani (LB) plates (38) without spectinomycin to verify that the apparent growth defect was not due to mutations in the *spc* resistance gene. The *obg* regions of these DNAs were then amplified by PCR and sequenced. Each strain isolated in this screening was found to have multiple amino acid changes. After a series of "backcross" transformations into the wild-type parental strain, two amino acid changes—G→D(92) and R→P(346)—remained associated with the small colony phenotype. Each mutation was individually introduced into *obg* in pSK100 by site-directed mutagenesis (GeneTailor; Invitrogen, Carlsbad, CA), giving rise to pSK101 (G92D mutation) and pSK102 (R346P mutation). After transformation into BSA46, only the transformants receiving the *obg*(*G92D*) allele displayed the "pinpoint" colony phenotype. A representative clone (BSKG92D) bearing the *obg*(*G92D*) mutation was picked for further study.

Plasmids pSK103 and pSK104 were created in a manner similar to pSK100. They differ in the oligonucleotide primers that were used to amplify the 1.8-kbp HindIII-PstI fragments containing *obg*. The primer used at the 3′ end of *obg*, amplified and cloned into pSK103, generated an *obg* variant ending 22 codons before its normal carboxy terminus. When this fragment was joined to the spectinomycin-resistant (Spc^r) DNA element, the resulting *obg* reading frame substitutes 22 amino acids of the Obg sequence with 26 amino acids encoded by the adjoining DNA. The *obg* allele in pSK104 has a 15-bp deletion immediately upstream of the 3′ stop codon, thereby encoding an Obg truncated by 5 amino acids. When each of these plasmids was linearized and transformed into BSA46, only pSK103 gave Spc^r colonies. A representative clone (BSK22/26) was picked, and the presence of the *obg*(Δ*22*) allele was verified by PCR amplification and sequencing of the *obg* region.

pSK105 and pSK106 were formed by using *obg* regions amplified from strains containing either the *obg*(*G92D*) or the *obg*(Δ*22*) allele. The amplified DNAs contain 700 bp upstream of *obg*, including the operon's (*spo0B*) promoter. The resulting 2.0-kbp HindIII-PstI pieces encoding either *obg*(*G92D*) or *obg*(Δ*22*), the PstI-SalI *spc* cassette from pDG1726, and a 700-bp SalI-SacI DNA fragment downstream of *obg* were cloned into pUC19 to create plasmids pSK106 or pSK105, respectively. Both plasmids were transformed into BSA46 as circular DNA to isolate Spc^r clones in which the plasmid had integrated by "single-site" recombination. The resulting strains, BSK90 and BSK95, are merodiploid for *obg*, containing both wild-type *obg* and either *obg*(*G92D*) or *obg*(Δ*22*), respectively, under the control of their normal promoter (P_{spo0B}).

Plasmid pSK130 is a 0.6-kbp NcoI-BamHI piece containing the *spo0B* gene in-frame with the GAL4 binding domain in pAS2-1. Plasmids pSK125 and pSK127 were created by cloning a 1.3-kbp BamHI-XhoI piece encoding *obg*(*G92D*) and *obg*(Δ*22*), respectively, in-frame with the GAL4 activation domain in pACT2. The presumptive in-frame fusions for all of the plasmids were verified by DNA sequencing.

B. subtilis strains BSK101 and BSK102 are BSH113 (*spoIIGB*::*lacZ*) (26) transformed with either ObgG92D or Obg22/26 chromosomal DNA, respectively. The integrated fusion separates the full-length SpoIIGB gene from its promoter and results in a strain that is blocked at stage II of sporulation. Transformants were selected on the basis of Spc^r, linked to *obg* alleles. BSK103, BSK104, BSK105, and BSK106 were similarly created by transforming ObgG92D and Obg22/26 chromosomal DNAs into BSA419 (40) (P_{SPAC}::*rsbT*, i.e., BSK103 and BSK104) or BSA115 (P_{SPAC}::*rsbW313*, i.e., BSK105 and BSK106) (48).

Sporulation assay and staining. Sporulation assays (36) were performed on *B. subtilis* strains inoculated into Difco sporulation medium (DSM) and incubated with aeration for 24 h at 37°C. Culture samples were diluted 1/10 into 2 ml of minimal medium salt base. Chloroform (100 μl) was added and, after vortexing, the cultures were incubated for 30 min at 80°C. Dilutions of treated and untreated cultures were plated on DSM to determine the percentage of resistant cells (spores).

BSH113, BSK100, and BSK101 carry a disruption of the *spoIIG* operon that prevents sporulation from proceeding beyond stage II. To visualize whether the *obg* mutations would allow progression to this early stage in the process, cells from isolated colonies that had formed on Difco sporulation agar after 18 h at 37°C were resuspended in 2 μl of H_2O on a microscope slide and mixed with a solution of FM4-64 (final concentration, 5 μg/ml; Invitrogen). Cells were viewed with an Olympus BX-50 fluorescence microscope, and images were captured by an Orca ER digital camera with MetaVue imaging software (Universal Imaging Corp., Dowingtown, PA). The FM4-64 was observed with an Olympus filter set UMWG (510-550 exciter filter, 515-590 barrier filter).

Centrifugation assays. To examine Obg-ribosome associations, *B. subtilis* mutant and wild-type strains grown to an optical density at 540 nm (OD_{540}) of ~0.7 in 1 liter of LB medium were harvested on ice, washed with a low-salt buffer (10 mM Tris [pH 8.0], 50 μM EDTA, 1.5 mM $MgCl_2$), and resuspended in 5 ml of the same buffer. After disruption in a French pressure cell, debris was removed by centrifugation (5,000 × g for 10 min). Then, 1 ml of the resulting crude lysate was layered on a 9-ml, 10 to 30% sucrose gradient prepared with 10 mM guanylimidodiphosphate (GIDP) in low-salt buffer. Gradients containing identical samples were centrifuged (37,000 rpm in a Sorvall TH641 swinging-bucket rotor) for 5 h at 4°C. Next, 0.5-ml fractions were collected from the gradients, precipitated with 2 volumes of ethanol, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting.

TABLE 1. Plasmids and strains used in this study

B. subtilis strain or plasmid	Relevant genotype or features[a]	Source, construction, or reference
Plasmids		
pDG1726	Spc^r	24
pSK100	Ap^r Spc^r (500 bp); *spo0B obg*::*spc*::*pheB* (500 bp)	This study
pSK101	Ap^r Spc^r (500 bp); *spo0B obg*(*G92D*)::*spc*::*pheB* (500 bp)	This study
pSK102	Ap^r Spc^r (500 bp); *spo0B obg*(*R346P*)::*spc*::*pheB* (500 bp)	This study
pSK103	Ap^r Spc^r (500 bp); *spo0B obg*(Δ*22*)::*spc*::*pheB* (500 bp)	This study
pSK104	Ap^r Spc^r (500 bp); *spo0B obg*(Δ*5*)::*spc*::*pheB* (500 bp)	This study
pSK105	Ap^r Spc^r (700 bp); *spo0B obg*(*G92D*)::*spc*::*pheB* (700 bp)	This study
pSK106	Ap^r Spc^r (700 bp); *spo0B obg*(Δ*22*)::*spc*::*pheB* (700 bp)	This study
pACT-2	Ap^r vector with GAL4 AD	Clontech, Mountain View, CA
pAS2-1	Ap^r vector with GAL4 BD	Clontech, Mountain View, CA
pUV31	Ap^r protein fusion of RsbX with GAL4 BD	50
pUV46	Ap^r protein fusion of RsbW with GAL4 BD	50
pUV76	Ap^r protein fusion of RsbT with GAL4 BD	50
pUV126	Ap^r protein fusion of RsbR with GAL4 BD	50
pUV145	Ap^r protein fusion of RsbU with GAL4 BD	50
pSK125	Ap^r protein fusion of ObgG92D with GAL4 AD	This study
pSK127	Ap^r protein fusion of ObgΔ22 with GAL4 AD	This study
pSK130	Ap^r protein fusion of Spo0B with GAL4 BD	This study
pJMObg	Ap^r protein fusion of Obg with GAL4 AD	40
B. subtilis strains		
BSA46	*trpC2 SP*β *ctc*::*lacZ*	4
SMY	*trpC*	Laboratory strain
BSKG92D	*trpC2 obg*(*G92D*)::*spc SP*β *ctc*::*lacZ*	Linearized pSK101→BSA46
BSK22/26	*trpC2 obg*(Δ*22*) (3′ 22-amino-acid truncation/26 random addition)::*spc SP*β *ctc*::*lacZ*	Linearized pSK103→BSA46
BSK90	*trpC2 aph3′5″*/P_{spo0B} *spo0B obg*(*G92D*) *spc pheB SP*β *ctc*::*lacZ*	pSK105→BSA46
BSK95	*trpC2 aph3′5″*/P_{spo0B} *spo0B obg*(Δ*22*) *spc pheB SP*β *ctc*::*lacZ*	pSK106→BSA46
BSH113	*trpC* JH642(pSGMU31ΔZ) *spoIIGB*::*lacZ*	26
BSK100	*trpC* JH642(pSGMU31ΔZ) *obg*(*G92D*)::*spc spoIIGB*::*lacZ*	BSKG92D→BSH113
BSK101	*trpC* JH642(pSGMU31ΔZ) *obg*Δ*22*::*spc spoIIGB*::*lacZ*	BSK22/26→BSH113
BSA419	*trpC2* P_{SPAC}::*rsbT SP*β *ctc*::*lacZ*	40
BSK102	*trpC2* P_{SPAC}::*rsbT SP*β *ctc*::*lacZ obg*(*G92D*)::*spc*	BSKG92D→BSA419
BSK103	*trpC2* P_{SPAC}::*rsbT SP*β *ctc*::*lacZ obg*(Δ*22*)::*spc*	BSK22/26→BSA419
BSA115	*trpC2 rsbU*::*aphA3* P_{B}*28*::P_{SPAC} *rsbW313 pTet-I SP*β *ctc*::*lacZ*	48
BSK104	*trpC2 rsbU*::*aphA3* P_{B}*28*::P_{SPAC} *rsbW313 pTet-I SP*β *ctc*::*lacZ obg*(*G92D*)::*spc*	BSKG92D→BSA115
BSK105	*trpC2 rsbU*::*aphA3* P_{B}*28*::P_{SPAC} *rsbW313 pTet-I SP*β *ctc*::*lacZ obg*(Δ*22*)::*spc*	BSK22/26→BSA115

[a] AD, activation domain; BD, DNA binding domain; Ap^r, ampicillin resistance.

To assess the ribosome composition, extracts prepared as described above from wild-type and mutant Obg samples were sedimented in two-channel Epon-charcoal centerpieces at 4°C and 12,000 rpm in a Beckman XLA analytical ultracentrifuge. Two sets of samples were prepared, with concentrations corresponding to absorbances of 1.1 OD_{280} and 1.1 OD_{260}, respectively. Separate runs were made at each wavelength, with approximately 100 scans taken for each experiment. The data were analyzed by using the enhanced van Holde-Weischet method (16) as implemented in UltraScan (17).

Yeast two-hybrid analysis. The yeast two-hybrid plasmid system and the appropriate yeast strains were obtained using the Matchmaker two-hybrid system from Clontech Laboratories, Inc. (Mountain View, CA). Cloning, transformations, and assays were performed according to protocols provided by Clontech. BD::Obg fusions were found to activate the reporter system independently of the GAL4 activator domain. Therefore, all experiments involving Obg fusions needed to be done with Obg fused to the GAL4 activation domain.

β-Galactosidase assays. *B. subtilis* strains to be tested for σ^B-dependent reporter gene activity (*ctc*::*lacZ*) were inoculated into LB medium and grown to an OD_{540} of ~0.4 at 37°C. Cultures to be induced by IPTG (isopropyl-β-D-thiogalactopyranoside) were split, and IPTG was added to one culture (1 mM final concentration). Samples were collected every 10 min for 1 h. Cultures to be induced by ethanol were split, and ethanol was added to one culture (4% final concentration). Samples were collected every 10 min. To assay *spoIIG*::*lacZ*, cultures of BSH113, BSK100, and BSK101 were grown in DSM until the end of exponential phase (OD_{540} of ~1.0). Samples were then collected at hourly intervals and assayed for β-galactosidase, as described by Kenney and Moran (29).

General methods. Transformations of *E. coli* and *B. subtilis* were performed by standard methods (38, 57). Western blot analyses of Obg, RsbR, RsbU, and σ^B were done as previously described using mouse polyclonal anti-Obg antibody and monoclonal antibodies to RsbR, RsbU, and σ^B (19, 42). Sequencing of DNA was performed by The University of Texas Health Science Center at San Antonio Center for Advanced DNA Technologies.

RESULTS

Isolation of an *obg* mutation impairing growth. To isolate *obg* alleles whose products impair growth, a plasmid (pSK100) encoding wild-type *obg* plus 500 bp of upstream DNA, a Spc^r cassette, and 500 bp of downstream DNA was mutagenized in vitro by error-prone RCA and transformed into wild-type *B. subtilis* (BSA46). Spc^r transformants were screened for impaired growth ("pinpoint" colony morphology) at 37°C and/or temperature sensitivity when replica plated at 50°C. Five clones were isolated, with multiple amino acid changes. Subsequent analyses (see Materials and Methods) revealed that an aspartate substitution present at a highly conserved glycine residue [*obg*(*G92D*)] in each *obg* variant (Fig. 1) is sufficient to confer the growth impaired plate phenotype. A strain (BSKG92D)

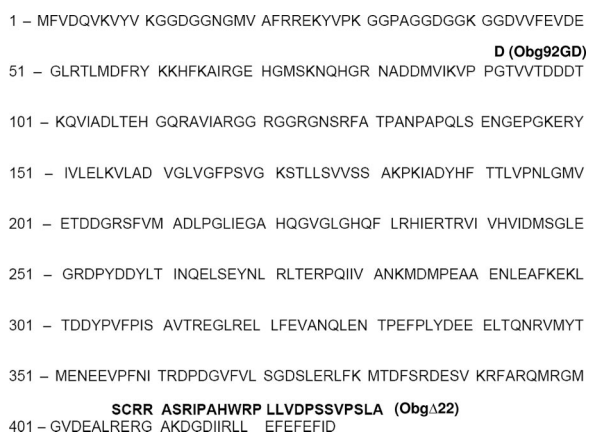

FIG. 1. Mutations of *B. subtilis* Obg protein sequence. The 428-amino-acid *B. subtilis* Obg protein has three domains (11, 54): (i) a glycine-rich N-terminal region. the "Obg fold" (amino acids 1 to 158), which is of unknown function but is conserved among members of the Obg subfamily, (ii) a GTP binding domain consisting of amino acids 159 to 342, and (iii) a TCS domain (amino acids 343 to 428), unique to *B. subtilis* Obg and named for the three protein families in which the domain is found (ThrRS, GTPase, and SpoT). The Obg92GD mutation is a missense change (GGC to GAC) at nucleotide 92. ObgΔ22 is a substitution of 22 amino acids (NH-RERGAKDGDIIRLLEFEFEFID-COOH) at the C-terminal end of Obg for the 26-amino-acid sequence (NH-SCRRASRIPAHWRPLLVDPSSVPSLA-COOH).

containing an *obg* allele with this single change was selected for further characterization.

BSKG92D displays impaired growth at all temperatures. Its doubling time in liquid culture (LB medium) at 37°C is approximately twice that of its wild-type parental strain (i.e., 41 min versus 21 min) (data not shown) and, unlike the parental strain, BSKG92D fails to form colonies at 50°C. To determine whether the growth defect in BSKG92D is a consequence of the *obg*(*G92D*) allele's product failing to provide a growth-promoting function or the variant Obg initiating a process that is inhibitory to growth, an *obg*/*obg*(*G92D*) merodiploid strain was constructed. Wild-type *B. subtilis* (BSA46) was transformed with an integrating plasmid carrying the *obg*(*G92D*) allele and its promoter (pSK105). The growth properties of the resulting *obg* merodiploid strain were indistinguishable from those of the wild-type parental strain (data not shown). Thus, the growth-impaired phenotype of the *obg*(*G92D*) allele is recessive to the wild-type *obg* allele, arguing that the growth defect is due to an impaired Obg function rather than the acquisition of a toxic activity by Obg. This impairment is not, however, a consequence of failure of the mutant gene product to accumulate. Western blot analyses (data not shown) revealed similar Obg protein levels in strains that express either *obg*(*G92D*) or the wild-type allele.

Isolation of a mutation in the Obg carboxy terminus. The Obg NH terminus is important for Obg's growth-promoting functions. The growth-inhibiting mutations isolated both in the present study and a previous study (31) lie in this conserved region. The carboxy terminus of Obg may play a more significant role in other Obg functions. A transposon insertion that altered the carboxy terminus of *E. coli* Obg did not disrupt growth but instead interfered with chromosome replication fork control (21). To explore the possibility that the carboxy terminus of *B. subtilis* Obg might contribute in a novel way to the activities of Obg, a series of sequential (5-amino-acid) deletions of the Obg carboxy terminus were planned. The technique involved the creation of a DNA element similar to that used in the rolling-circle mutagenesis experiment, with the *obg* portion amplified using oligonucleotide primers that terminated *obg* at sites upstream of its normal terminus. When these DNAs were transformed into *B. subtilis*, the DNA fragment with even the smallest *obg* deletion (5 amino acids) failed to yield viable Spc^r transformants. In the course of these experiments, however, a viable transformant was isolated with 22 amino acids of the Obg carboxy terminus replaced with 26 amino acids encoded by the sequence between *obg* and the *spc* cassette within the cloned DNA (Fig. 1). Given the finding that an amino acid substitution at the carboxy terminus of Obg in *E. coli* could result in a novel phenotype, we chose to characterize this *obg* variant. Unlike the amino-terminal mutation (BSK92GD), a *B. subtilis* strain (BSK22/26) carrying the *obg* allele with the carboxy-terminal substitutions formed normal-size colonies on LB agar and had a doubling time in liquid media that was similar to that of the parental strain (data not shown). Thus, although a 5-amino-acid deletion of the Obg carboxy terminus does not appear to be tolerated, the 26/22 substitution does not compromise the growth essential function(s) of Obg.

Effects of *obg*(*G92D*) and *obg*(Δ*22*) mutations on Obg ribosome association and structure. A common property of the characterized Obg proteins is their ability to cofractionate with ribosomes (15, 33, 42, 43, 55, 58). The cosedimentation of *B. subtilis* Obg with ribosomes during velocity centrifugation analysis is enhanced by the presence of GTP or a nonhydrolyzable GTP analog (e.g., GIDP) (58). To investigate whether either of the *obg* mutations influences the ability of Obg to interact with ribosomes, extracts were prepared from *B. subtilis* strains carrying the wild-type or mutant *obg* alleles. These were then fractionated by centrifugation in the presence of GIDP. Figure 2 illustrates the result of this analysis, in which samples from fractions obtained from the centrifugation runs were subjected to SDS-PAGE and either stained with Coomassie blue (Fig. 2A) or probed with anti-Obg antibody in a Western blot analysis (Fig. 2B). Both the wild-type and the *obg*(Δ*22*) products were found to sediment predominantly in the ribosome-containing fractions (Fig. 2B1 and B2); however, the ObgG92D protein was largely displaced from the ribosomes and remained near the top of the gradient (Fig. 2B3). Thus, the *obg* mutation that results in growth impairment also compromises the mutant Obg's ability to maintain an association with ribosomes in the presence of GIDP.

Previous studies of Obg-depleted *E. coli* and *C. crescentus* demonstrated significant changes in ribosome profiles with decreased levels of mature 70S ribosomes and the appearance of putative ribosomal precursor forms (15, 25, 39). Given that one of our *obg* mutants [*obg*(*G92D*)] showed impaired growth and altered Obg-ribosome association, we thought it possible that the status of its ribosomes could also be affected. To examine this possibility, the ribosome profiles found in extracts of wild-type *B. subtilis* and the two *obg* mutant strains were analyzed by analytical ultracentrifugation. As can be seen in Fig. 3, the bulk of the ribosome fractions sedimented in all three extracts as mature 70S particles; however, there was a decrease in the

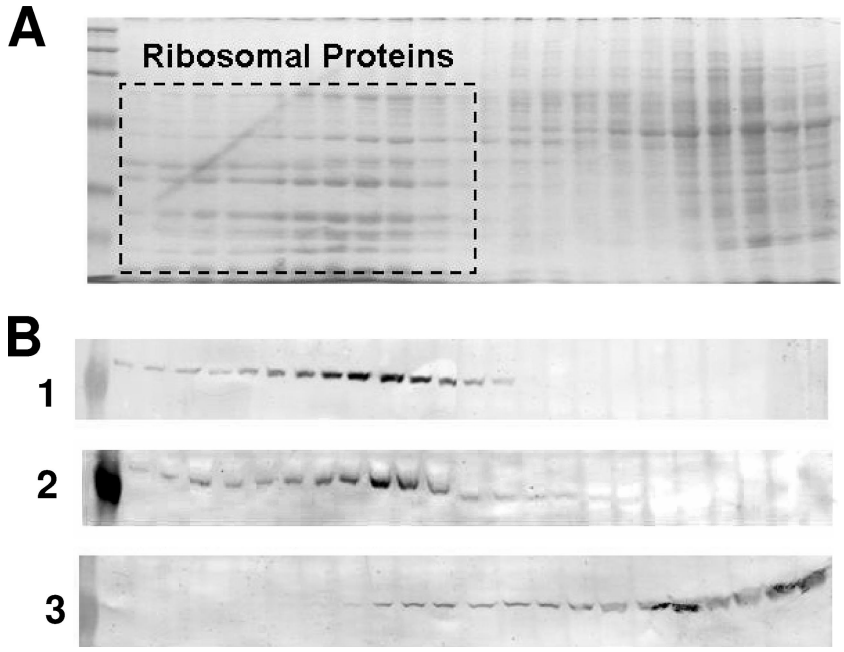

FIG. 2. Sedimentation analysis of ObgG92D and Obg22/26. Crude extracts of wild-type BSA46, ObgG92D, and Obg22/26 were prepared as described and subjected to centrifugation through a 10 to 30% sucrose gradient containing 10 μM GIDP. Fractions were collected from the bottom of the tube (fraction 1) and analyzed by SDS-PAGE. (A) The protein profile of a representative extract was visualized by Coomassie blue staining. The characteristic cluster of low-molecular-mass ribosomal proteins in fast-sedimenting fractions is bracketed in the Coomassie blue-stained gels. (B) Western blot analyses using polyclonal antibodies against Obg are presented for BSA46 (panel 1), ObgΔ22 (panel 2), and ObgG92D (panel 3). Fraction numbers are indicated at the top of the figure.

relative abundance of the 70S peak and a "shoulder" of 30S and 50S material in the extract from the *obg*(*G92D*) strain. This altered profile is relatively modest compared to that seen in extracts prepared from Obg-depleted strains; however, given that the BSK92GD strain is still viable, albeit growth impaired, it would be anticipated that any deleterious effect that the *obg*(*G92D*) mutation might have on the growth-essential functions of Obg would be incomplete. Taken together, these findings argue that the *obg*(*G92D*) mutation, but not the *obg*(Δ*22*) mutation, impairs growth, reduces the association of the mutant Obg protein with ribosomes, and causes a decrease in the percentage of 70S ribosomes seen in extracts prepared from cells grown at 37°C.

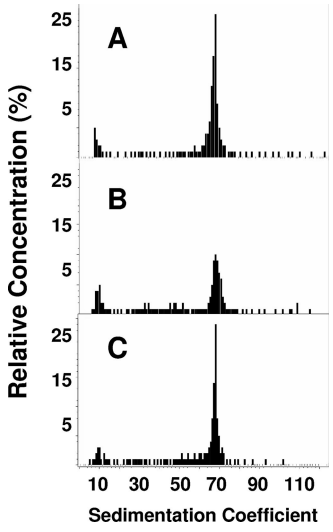

FIG. 3. Ribosome sedimentation in *obg* mutant strains. Wild-type and *obg* mutant extracts were sedimented at 4°C and 12,000 rpm in a Beckman XLA analytical ultracentrifuge. The histograms illustrate the percentage of the protein (OD_{280}) in wild-type (A), *obg*(*G92D*) mutant (B), and *obg*(Δ*22*) mutant (C) extracts that sedimented with the indicated sedimentation coefficients (Svedberg).

Effect of *obg* mutations on *B. subtilis* sporulation. Aside from an essential role in growth, Obg is also critical for *B. subtilis* sporulation (31, 46). Obg has been shown to be needed at the earliest stages of sporulation. In Obg-depleted strains, the master regulator of sporulation (Spo0A) fails to be activated by the phosphorelay that regulates sporulation induction (46). It is unknown whether the role of Obg in this process is direct or indirect. To determine whether either of the *obg* mutations isolated in the present study affected sporulation, *B. subtilis* strains carrying these mutations were grown and allowed to sporulate in a sporulation-inducing medium (DSM). The "sporulated cultures" were then subjected to chloroform and heat treatment as described in Materials and Methods to kill the residual vegetative cells. All of the untreated cultures had similar numbers of CFU after an overnight incubation in DSM (ca. 4×10^8 CFU/ml). We found that 37% of the cells in a wild-type (Obg^+) parental (BSA46) culture and 34% of the cells in the *obg*(*G92D*) culture were resistant to the heat-chloroform treatment, while only 0.02% of the cells in the *obg*(Δ*22*) culture survived. Thus, although the *obg*(*G92D*) mutation compromises growth, it has little effect on sporulation. In contrast, the

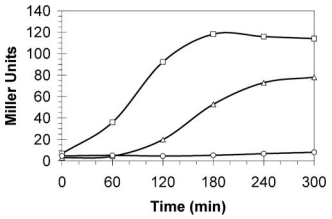

FIG. 4. *spoIIG* transcriptional activation of Obg mutants. BSH113 (*spoIIGB*::*lacZ*) (□), BSK100 [*spoIIGB*::*lacZ obg*(*G92D*)] (△), and BSK101 [*spoIIGB*::*lacZ obg*(Δ*22*)] (○) were grown in DSM until logarithmic growth ceased. Samples were then collected at hourly intervals and analyzed for $P_{spoIIGA}$-dependent β-galactosidase, as described in Materials and Methods.

obg(Δ*22*) mutation has little effect on growth but profoundly impairs sporulation.

Previous studies had revealed a role for Obg in the activation of the Spo0A transcription factor (46). To determine whether the *obg*(Δ*22*) mutation might also be blocking a process required for Spo0A activation, both of the *obg* mutations were placed into a *B. subtilis* strain with a reporter gene fused to a Spo0A-dependent promoter (*spoIIG*::*lacZ*) (29). Wild-type and *obg* mutant, *spoIIG*::*lacZ* strains were allowed to enter sporulation in liquid DSM and monitored for P_{spoIIG}-dependent β-galactosidase activity. As illustrated in Fig. 4, the *obg*(Δ*22*) strain failed to induce the *spoIIG* promoter, while the strain carrying the *obg*(*G92D*) mutation had approximately half the reporter gene activity seen in the wild-type strain. The failure of the *obg*(Δ*22*) strain to activate *spoIIG* transcription is consistent with a block in sporulation prior to the activation of Spo0A. The *obg*(*G92D*) strain ultimately forms spores at a frequency similar to that of the wild-type strain (34% versus 37%). Thus, it is likely that the reduced Spo0A-dependent reporter gene activity seen in this strain reflects an impaired biosynthetic potential rather than a specific sporulation defect. The growth rate of the *obg*(*G92D*) strain, like its *spoIIG*::*lacZ* expression, is half that of the Obg^+ strain.

obg(Δ*22*)'s block at the onset of sporulation can also be visualized by fluorescence microscopy. The *spoIIG*::*lacZ* cassette disrupts the *spoIIG* operons of *B. subtilis* strains that carry them. As a consequence, these strains enter sporulation but are unable to progress beyond the stage at which asymmetric septa form within the developing cell (stage II). Wild-type and *obg* mutant *spoIIG*::*lacZ* strains were incubated overnight in DSM, treated with a fluorescent membrane stain (FM4-64), and examined by fluorescence microscopy. As seen in Fig. 5, the strains with either the wild-type *obg* (Fig. 5A) or the *obg*(*G92D*) (Fig. 5B) alleles display the terminal two-septum phenotype of sporulation arrest at stage II, while the *obg*(Δ*22*) mutant (Fig. 5C), consistent with a block at stage 0, lacks septa. These results demonstrate that the *B. subtilis* strain with the growth-inhibiting *obg*(*G92D*) mutation is relatively unimpaired in its ability to induce sporulation, while the strain with the *obg*(Δ*22*) allele is unable to initiate an early sporulation event that is necessary for the activation of Spo0A.

Effects of *obg* mutations on activation of the σ^B transcription factor. σ^B, the general stress response transcription factor, is activated when the bacterium encounters physical (e.g., heat shock) or nutritional (e.g., carbon and PO_4 starvation) stress

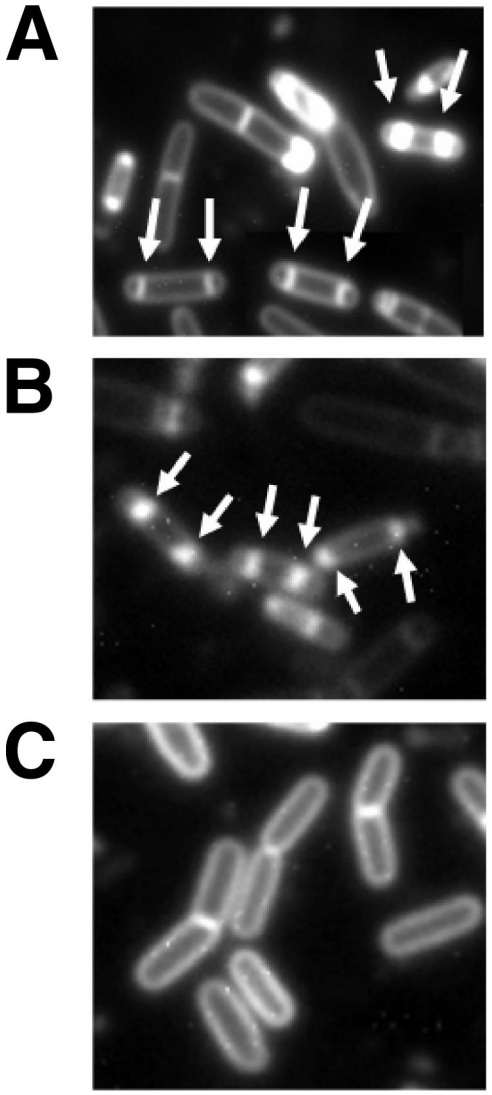

FIG. 5. FM4-64 staining of Obg mutants in σ^E mutant cells. (A) BSH113, (B) BSK100 [*obg*(*G92D*)], and (C) BSK101 [*obg*(Δ*22*)] cells were cultured for 18 h on Difco sporulation agar at 37°C. Bacteria were suspended in water, stained with FM4-64, and observed by fluorescence microscopy. Arrows indicate the double septa in both BSH113 and BSK100.

(49). Each of these types of stress (physical versus nutritional) activates σ^B via specific pathways that come together in a reaction that catalyzes the release of σ^B from an anti-σ^B inhibitor (RsbW) (Fig. 6A). In previous studies, Obg-depleted *B. subtilis* could no longer activate σ^B in response to environmental stress but retained the ability to activate σ^B via the nutritional stress pathway (40). To determine whether one or both of the *obg* mutations might affect σ^B activation, strains that carried them were subjected to a physical stress (4% ethanol) and analyzed for σ^B activity levels using a *lacZ* reporter gene under the control of a σ^B-dependent promoter (*ctc*::*lacZ*). Figure 7A illustrates the reporter gene activity at intervals after ethanol treatment. The *obg*(*G92D*) strain displayed approximately half the activity of the wild type, while the *obg*(Δ*22*) strain had less than 20% of the wild-type's σ^B reporter activity.

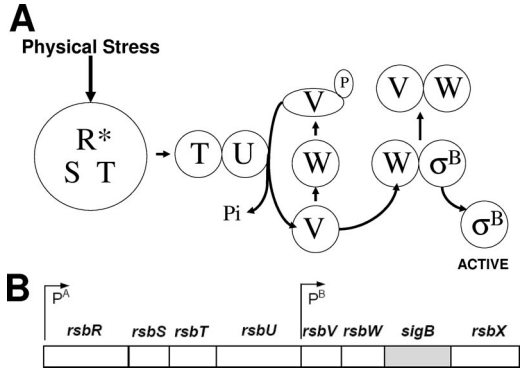

FIG. 6. Model of σ^B regulation and σ^B operon. (A) σ^B is present in prestressed cells but held inactive in a complex with an anti-σ^B protein, RsbW (6, 18). σ^B is released from its anti-sigma factor, RsbW, by the binding of the anti-sigma factor antagonist, RsbV, to RsbW (18). In unstressed cells RsbV is phosphorylated by RsbW and inactive (3, 18, 51). Physical and nutritional stress activate novel phosphatases to dephosphorylate and reactivate RsbV-P. RsbU, the phosphatase that responds to physical stress, requires an additional protein, RsbT, for activity. RsbT is both a kinase and an RsbU activator. In unstressed *B. subtilis* cells, RsbT is held inactive by RsbS (56) in a complex with RsbR and a family of homologous proteins (RsbRB, RsbRC, and RsbRD) that facilitate the RsbT-RsbS interactions (1, 2, 23, 30). Upon exposure to physical stress (e.g., ethanol or osmotic shock), RsbT becomes empowered to phosphorylate RsbR and RsbS, freeing itself to interact with RsbU, to induce the dephosphorylation of RsbV-P. Once dephosphorylated, RsbV can sequester RsbW, freeing σ^B to activate the GSR (14, 30, 56). Negative regulation is reestablished by RsbX, a phosphatase that dephosphorylates RsbR-P and RsbS-P, allowing RsbR/S to again sequester RsbT (14, 49, 56). Nutritional stress (e.g., glucose or phosphate limitation) generates an unknown signal to activate a separate phosphatase (RsbP/Q) that is able to dephosphorylate RsbV-P (10, 28, 47, 51). (B) σ^B (*sigB*) is coexpressed in an eight-gene operon (*rsbR*, *rsbS*, *rsbT*, *rsbU*, *rsbV*, *rsbW*, and *rsbX*) from a promoter (P^A) likely recognized by σ^A-containing RNA polymerase (27, 41, 53). A σ^B-dependent promoter (P^B) within the operon upregulates the distal four genes upon σ^B activation (5, 9).

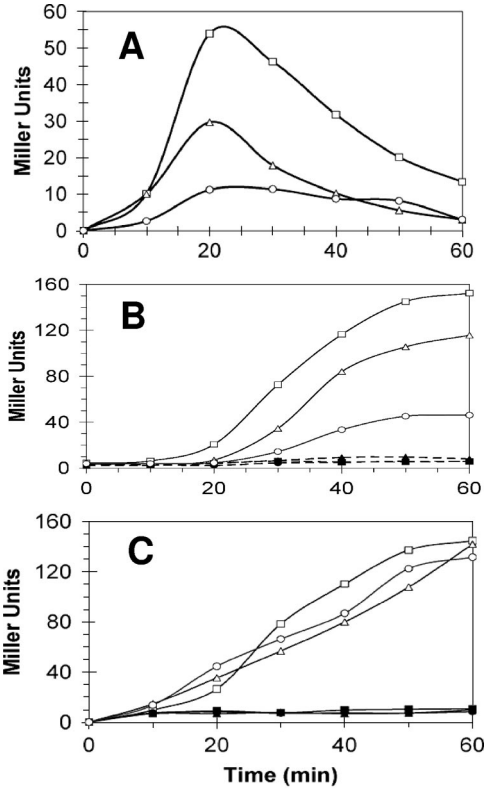

FIG. 7. Activation of σ^B in Obg mutants. (A) BSA46 (□), BSKG92D (△), and BSK22/26 (○) were grown in LB medium until reaching an OD_{540} of ~0.4 and then treated with ethanol. Samples were collected at 10-min intervals and assayed for σ^B-dependent β-galactosidase activity. (B) BSA419 (P_{SPAC} *rsbT*) (squares) and the *obg* variants of this strain, BSK102 [*obg*(*G92D*)] (triangles) and BSK103 [*obg*(Δ*22*)] (circles), were grown in LB medium until reaching an OD_{540} of 0.4. Cultures were then split, and 1 mM IPTG was added to half of the cultures (open symbols), with the other half of the cultures uninduced (solid symbols). Samples were taken and assayed as for panel A. (C) BSK115 (P_{B}*28*::P_{SPAC} *rsbW313*) (diamonds) and the congenic *obg* mutant strains BSK104 [*obg*(*G92D*)] (triangles) and *obg*(Δ*22*) (squares) were grown in LB medium to an OD_{540} of 0.4. Cultures were split, induced, and analyzed as for panel B.

The reduced level of reporter gene activity in the *obg*(*G92D*) strain is similar to the reduced level seen with the *spoIIG*::*lacZ* system and may reflect the strain's impaired growth rate. In contrast, given that the *obg*(Δ*22*) strain's growth is comparable to that of the parent strain, its reduced level of σ^B activation is more likely due to a specific defect in this process. This defect is presumably due to a deficit Obg function. A merodiploid strain (BSK95) containing both wild-type and *obg*(Δ*22*) alleles activates σ^B to an extent similar to that of a wild-type strain after ethanol stress (results not shown).

In order to better define the point in σ^B's physical stress pathway at which the *obg* mutation interferes with σ^B activation, σ^B was artificially induced at different points in the pathway. Figure 6A depicts a model of σ^B activation by physical stress. As described in the figure legend, stress allows the release of a positive regulator (RsbT) from an inhibitory complex which, in turn, triggers downstream events, leading to σ^B's release from its primary inhibitor RsbW. To determine whether Obg22/26 reduced σ^B activation at a point upstream of RsbT release, an IPTG-inducible promoter (P_{SPAC}) was inserted into the *sigB* operon (Fig. 6B) immediately upstream of *rsbT* (40). Induction of this promoter allows for enhanced RsbT levels and activation of σ^B by a process dependent on the downstream members of the pathway (Fig. 6A). Wild-type and *obg* mutant strains carrying P_{SPAC} upstream of *rsbT* were induced with IPTG and monitored for reporter gene activity. Western blot analyses of the induced cultures (data not shown) demonstrated that representative gene products (i.e., RsbU and SigB) downstream of the P_{SPAC} promoter were similarly induced relative to an upstream gene product (RsbR) by the addition of IPTG. Although induction of the *sigB* operon is similar in the wild-type and *obg* mutant strains, the activation of σ^B itself is not (Fig. 7B). The differences in reporter gene activity between the wild-type and mutant *obg* strains are very similar to that seen when σ^B is induced by stress itself in strains with these *obg* alleles. In this case, the levels of reporter gene activity in the *obg*(*G92D*) and *obg*(Δ*22*) strains were ca. 75 and 25% that of the Obg^+ strain by 60 min postinduction. This result argues that the Obg-dependent process in σ^B activation that is compromised by the *obg*(Δ*22*) mutation is downstream of the release of RsbT from the complex which holds it inactive in the absence of stress.

TABLE 2. Interactions of Obg or Obg mutants and Rsb proteins or Spo0B evaluated by a yeast two-hybrid system[a]

BD fusion	AD fusion (ratio)		
	Wild-type Obg	ObgG92D	ObgΔ22
pUV31 (X)	1.96	1.41	1.30
pUV75 (T)	4.54	1.21	1.03
pUV130 (W)	34.32	34.13	9.70
Spo0B	0.87	0.19	0

[a] The rows represent the GAL4 DNA binding vector (pAS2-1) with a fusion either the *rsb* gene depicted by a single letter (e.g., X = RsbX or *spo0B*). Columns represent the activation domain (AD) vector (pACT-2) encoding either to indicate wild-type Obg or the Obg mutant allele. The values represent reporter gene activity (i.e., β-galactosidase expression) in yeast strains (Y180) cotransformed with both plasmids. The results are presented as a ratio of the Rsb protein-activation domain fusion value from the indicated pairing divided by the value obtained from the activation domain fusion paired with the pAS2-1 vector (i.e., the pairing of pUV31 [*rsbX*] with the wild-type Obg gene gave an activity level that was approximately twice that of its pairing with the activation domain vector alone).

The inability of overexpressed RsbT to significantly induce σ^B activity in the *obg*(Δ*22*) strain suggests that either the RsbT-dependent process involved in σ^B activation is compromised in this strain or that σ^B-dependent transcription itself is inhibited. To explore these possibilities, the Obg mutations were placed into a strain (BSA115) with a null (frameshift) mutation in the principal σ^B inhibitor (RsbW) and replacement of the operon's internal P_B promoter with P_{SPAC} (48). The addition of IPTG to such a strain triggers the production of σ^B, without the inhibitor (RsbW) that normally restricts its activity. Induction of these strains with IPTG results in similar levels of reporter gene activity regardless of which *obg* allele that it carries (Fig. 7C). This result implies that the *obg*(Δ*22*) mutation does not inherently restrict σ^B's activity but rather the RsbT-dependent process that activates σ^B.

Yeast two-hybrid interactions. The level of reporter gene activity in the yeast two-hybrid system can be a reflection of the affinity of interacting proteins for each other (20). Studies of Obg interactions using the yeast two-hybrid system demonstrated interactions between Obg and several regulators that control the activation of σ^B (40). The yeast two-hybrid system was therefore used to investigate whether the Obg mutations might have altered the previously reported interactions between Obg and several Rsb proteins. In addition to the Rsbs, a possible interaction between Obg and Spo0B was also tested. *spo0B*, a gene whose product is critical to the phosphorelay that controls the phosphorylation state of Sp0A, is cotranscribed with *obg* (45). Given that the phosphorylation state of Spo0A is an apparent target for the role of Obg in sporulation induction (46), a direct interaction between these proteins seemed plausible. Evidence for such an interaction was not, however, detected in the yeast system. Based on reporter gene activity, the Obg-Spo0B pairing (Table 2) did not exceed the background level of the vector alone with Obg. If an interaction between Spo0B and Obg exists, it may require additional factors or modifications that are absent in the yeast system.

Earlier yeast studies detected interactions between Obg and the σ^B regulators RsbX, RsbT, and RsbW, with the RsbW interaction being the strongest (40). When the mutant *obg* alleles were paired with these *rsb* genes, both mutant Obgs interacted with RsbX to a degree that was similar to that of the wild-type Obg, but their reporter gene expression levels with RsbT were approximately one-quarter of that seen in a similar pairing of RsbT with the wild-type *obg* allele (Table 2). Although similar in their interactions with RsbT, the two *obg* mutants differed in their pairing with RsbW. In this case, the *obg*(*G92D*) allele displayed the strong response seen with the wild-type *obg* allele (~34X background), while the *obg*(Δ*22*) allele's response was less than one-third of that (Table 2).

Given that the interaction between ObgG92D and RsbT was reduced to a level similar to that seen with the *obg*(Δ*22*) allele without a comparable change in σ^B activation, it is unclear whether the changes seen in Obg22/26's interactions with RsbT or RsbW are indicative of its compromised activity in σ^B induction. Nevertheless, both proteins are components of the pathway that are downstream of RsbT release and, as such, they could be potential sites for an Obg-dependent interaction that is altered by the *obg*(Δ*22*) mutation.

DISCUSSION

The Obg protein of *B. subtilis* is necessary for growth, sporulation, and the ability of physical stress to activate the bacterium's GSR (31, 40, 45, 46). Sporulation and stress induction are just two of several diverse processes for which Obg and its orthologs are needed in a number of bacterial species (15, 21, 25, 34, 35, 39, 43). A requirement for Obg in growth and ribosome maturation raises the possibility that the primary role of Obg is associated with growth and ribosome biogenesis and that the other phenotypes attributed to Obg deficiencies are indirect consequences of the underlying growth defect. The data presented here argue against this notion. The two *obg* alleles that are described appear to separate the growth-promoting function of Obg from that needed for sporulation and stress response induction.

The *obg*(*G92D*) mutation replaces a glycine residue that is conserved in *obg* orthologs with a charged aspartic acid molecule. This is similar to a previously described mutation in which glycine at position 97 was changed to glutamic acid (31). In both instances, strains that carried the Obg variants displayed a growth-impaired phenotype and an inability to grow at elevated temperatures. It is unclear whether the temperature sensitivity of these strains is due to a thermal instability of the Obg proteins themselves or an inability of cells lacking the activity compromised by these mutations to survive at elevated temperatures. Both the *obg*(*G92D*) and the *obg*(*G97E*) mutations lie in a highly conserved amino-terminal region of *obg* that in the crystal structures of Obg proteins from *B. subtilis* and *Thermus thermophilus* form a handle-like projection from the body of the protein (11, 54). In this regard, it is interesting that the ObgG92D protein displayed impaired ribosome association during the centrifugation experiments. It is possible that this Obg "handle" is the portion of the protein involved in tethering Obg to ribosomes. Although the ObgG92D protein failed to cofractionate with ribosomes during the centrifugation experiment, it is likely to still be able to interact with ribosomes to at least some degree in vivo. Obg-depleted cultures have marked defects in ribosome maturation, with a significant decrease in the abundance of 70 S particles concomitant with the appearance of putative 50S subunit intermediate particles (15, 25, 39, 42). Although the ribosome profile

of the *obg*(*G92D*) mutant did display a shift from that of the wild-type strain with an increase in 50S components at the expense of the 70S peak, this shift was relatively modest. This would argue that the ObgG92D protein can still interact sufficiently with ribosomes to adequately provide most of the functions required for ribosome maturation. It is not clear whether this small effect of the *obg*(*G92D*) mutation on ribosome maturation or another process (i.e., an effect on control of the stringent response, etc.) is responsible for halving the growth rate of strains that carry it. Regardless of the basis of ObgG92D's effect on growth, the mutation did not prevent the induction of sporulation and the GSR. The level of sporulation gene transcription (i.e., *spoIIG*) and that of a GSR reporter gene (i.e., *ctc*) is approximately half that seen in a wild-type strain. This effect on transcription is comparable to the effect of the *obg*(*G92D*) allele on growth itself, suggesting that these processes are only indirectly affected by this particular *obg* mutation and the result of the compromised metabolic fitness of the strains that carry it.

The defects in sporulation and GSR induction in the *obg*(Δ*22*) strain are more likely to be direct consequences of the *obg* mutation itself. The *obg*(Δ*22*) allele has little effect on growth or the ability of the mutant protein to associate with ribosomes in a centrifugation assay but does have a marked effect on sporulation and induction of the GSR. The *obg*(Δ*22*) allele carries an alternative fortuitous 26-amino-acid sequence, in lieu of the 22 amino acids that normally comprise the carboxy-terminal region of Obg. The possibility that the carboxy terminus of Obg could have unique contributions was suggested by the phenotype of an *E. coli obgE* allele in which a transposon insertion replaced the 9 amino acids that normally lie at the protein's carboxy terminus with a transposon-encoded 66-amino-acid extension (21). *E. coli* strains with this *obg* allele are viable but appear to be compromised in DNA replication fork control and exhibit enhanced sensitivity to hydoxyurea. In contrast to the *E. coli* strain, *B. subtilis* with the *obg*(Δ*22*) mutation did not display an enhanced sensitivity to hydroxyurea in the range from 0.025 to 0.2 M (data not shown). It is unclear whether this difference in hydroxyurea sensitivity is a reflection of novel features of each of the *obg* alleles or of the bacteria themselves.

Our attempts to examine the role of the carboxy-terminal region of Obg in *B. subtilis* by a sequential deletion analysis were unproductive. Even the smallest deletion (i.e., 5 amino acids) failed to yield viable transformants when the mutation was passed into *B. subtilis*. The ability of strains with the *obg*(Δ*22*) allele to be viable and grow normally argues that either the loss of the carboxy-terminal sequence rendered the mutant gene product liable to rapid turnover or the carboxy terminus of Obg plays a structural, rather than a sequence-specific, role in the protein's activity. Regardless of the basis of this phenomenon, the isolation of an *obg* allele that appears to affect stress signaling without conferring an obvious growth defect argues that the role of Obg in these processes is unlikely to be an indirect consequence of a growth-essential function.

It had previously been demonstrated that depletion of Obg results in a block in sporulation at the level of phosphorylation of the master regulatory protein Spo0A (46). Given that depletion of Obg also arrests growth, it could be argued that the failure to phosphorylate Spo0A is an indirect consequence of growth arrest. The failure of our Obg22/26 strain to activate Spo0A-dependent transcription while unimpaired in growth argues for a direct role for Obg in the phosphorylation and activation of Spo0A. Such a role should not be unexpected, given that *obg* is itself cotranscribed with *spo0B*, a key component of the phosphorelay that is responsible for Spo0A regulation (45). A simple model might envision Obg directly interacting with Spo0B, to modulate Spo0B's activity in the phosphorelay; however, our attempt to demonstrate a potential interaction between Obg and Spo0B in the yeast two-hybrid system was not successful. If *obg* actually influences the activity of Spo0A's phosphorelay, it may affect another point in the relay, or if it does interact with Spo0B, this interaction might require protein modifications or factors that are missing in the yeast system.

The inhibition of physical stress induction of the GSR by the *obg*(Δ*22*) allele differs from the effect of Obg depletion on this process. In an earlier study, interrupting Obg synthesis appeared to block GSR induction at a point upstream of the release of the RsbT activator from its inhibitors (40), while in the present study the ObgΔ22 protein caused an inhibition that was downstream of RsbT release. It is possible that Obg plays a role in each of these processes. In the earlier experiment the effect of Obg depletion on GSR induction was tested in a *B. subtilis* strain in which the only source of Obg was expressed from an IPTG-inducible promoter. Withholding IPTG eventually causes this strain to cease growth, at which point the strain was subjected to a GSR-inducing stress. There may have been sufficient Obg still present in the depletion study to provide the function needed downstream of RsbT release but insufficient activity to drive the upstream process. Given that growth had ceased in that strain, the upstream process that was inhibited by the loss of *obg* might be associated with the growth-promoting activity of Obg. If this is so, the phenotype of the *obg*(Δ*22*) allele may more accurately define the role of Obg in GSR activation.

The *obg*(Δ*22*) allele displayed an altered interaction in the yeast two-hybrid system (Table 2) with two σ^B regulators that lie in the pathway downstream of RsbT release; however, the relationship between these changes and the *obg*(Δ*22*) phenotype is unclear. Assuming that the *obg*(Δ*22*) mutation hinders the ability of RsbT to catalyze downstream events, a possible modulation of RsbT's activity by Obg, which is compromised by the *obg*(Δ*22*) mutation, is not implausible. However, if such a change is reflected in the reduced ability of ObgΔ22 to interact with RsbT, we need to assume that the basis for this reduced interaction is distinct from that which lessens the interactions between Obg92GD and RsbT but has no obvious consequences on σ^B activation. In contrast, the interaction between Obg and RsbW is uniquely compromised by the *obg*(Δ*22*) mutation (Table 2). The interaction between Obg and RsbW is the strongest of any of the interactions between Obg and the GSR regulators. As such, RsbW may be the most likely candidate Rsb protein with which Obg may actually interact in vivo. RsbW is both the RsbV kinase and the direct inhibitor/binding partner of the transcription factor (σ^B) responsible for GSR induction (Fig. 6). If Obg directly interacts with RsbW to modulate the activity state of σ^B, it presumably could accomplish this either by altering the RsbW-dependent phosphorylation and inactivation of RsbV or the ability of

RsbW to sequester σ^B. Either of these events would represent an unanticipated point of control on this system.

We describe here mutations that separate the growth promotion and stress response activities of Obg. The existence of such mutations argues that the contribution of Obg to stress induction and sporulation is not an indirect consequence of its growth-promoting activities but instead reflects direct participation of Obg in these and presumably other cellular processes. Direct participation of Obg in promoting entry into alternative developmental and stress response pathways would be consistent with its originally hypothesized role as a nucleotide sensor and contributor to the regulatory decisions that are made when cells experience changing environments. It will be interesting to see how it fulfills this role in the diverse processes that it affects.

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