Associations between *Bacillus subtilis* σ^B regulators in cell extracts

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The general stress regulon of *Bacillus subtilis* is induced by the activation of the σ^B transcription factor. Activation of σ^B occurs as a consequence of the dephosphorylation of its positive regulator RsbV by one of two phosphatases that respond to either physical or nutritional stress. The physical stress phosphatase (RsbU) requires a second protein (RsbT) for activity. Stress is thought to initiate a process that triggers the release of RsbT from a large inhibitory complex composed of multiple copies of two protein species, RsbR (and/or its paralogues) and RsbS. The stress-derived signal driving RsbT release is unknown, but it fails to develop in *B. subtilis* lacking either ribosome protein L11 or the ribosome-associated protein Obg. RsbR, RsbS, RsbT, Obg and ribosomes elute in common high-molecular-mass fractions during gel-filtration chromatography of crude *B. subtilis* extracts. This paper reports the investigation of the basis of this coelution by the examining of associations between these proteins in extracts prepared from wild-type and mutant *B. subtilis*, and *Escherichia coli* engineered to express RsbR, RsbS and RsbT. Large RsbR/RsbS complexes, distinct from ribosomes, were detected in extracts of both *B. subtilis* and *E. coli*. In *E. coli*, high-molecular-mass forms of RsbS were less abundant when RsbR was absent, but in *B. subtilis*, only when both RsbR and its principal paralogues were missing from the extract was this form less abundant. This finding is consistent with the notion that the RsbR paralogues, present in *B. subtilis* but not *E. coli*, can substitute for RsbR in such complexes. RsbT was not bound to RsbR/RsbS in any extract that was examined, including one prepared from a *B. subtilis* strain with an RsbS variant (RsbS59SA) that is believed to continuously associate with RsbT. The high-molecular-mass forms of RsbT were found to be Triton-sensitive and independent of any other *B. subtilis* protein for their formation. These probably represent RsbT aggregates. The data suggest that the contribution of ribosomes/Obg to σ^B activation does not involve formation of a stable association between these proteins and the Rsb complex. In addition, the binding of RsbT to RsbS/RsbR appears to be more labile than the binding between the previously analysed Rsb proteins which form inhibitory complexes. This, and the apparent proclivity of RsbT to aggregate, suggests an inherent instability in RsbT which may play a role in its regulation.

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INTRODUCTION

The general stress regulon (GSR) of *Bacillus subtilis* is a collection of more than 200 genes whose expression is enhanced following exposure to physical (e.g. heat shock, osmotic shock, ethanol) or nutritional (e.g. azide treatment, glucose or phosphate limitation) stress (Hecker *et al.*, 1996; Petersohn *et al.*, 1999; Price *et al.*, 2001; Voelker *et al.*, 1994). Induction of the GSR is triggered by the activation of the σ^B transcription factor, an RNA-polymerase-binding protein that directs the resulting holoenzyme to GSR promoters (Benson & Haldenwang, 1992, 1993a, b; Boylan *et al.*, 1992,

1993). σ^B is constitutively coexpressed with seven of its principal regulators (Regulator of Sigma B: *rsbR*, *rsbS*, *rsbT*, *rsbU*, *rsbV*, *rsbW* and *rsbX*) in an operon that is probably recognized by the cell's housekeeping σ factor, σ^A (Kalman *et al.*, 1990; Wise & Price, 1995). An internal σ^B -dependent promoter upregulates the expression of the operon's four downstream genes (*rsbV*, *rsbW*, *sigB* and *rsbX*) during periods of σ^B activity (Benson & Haldenwang, 1992; Boylan *et al.*, 1992, 1993; Kalman *et al.*, 1990).

RsbV and RsbW are the primary σ^B regulators. As illustrated in Fig. 1, RsbW is a σ^B -binding protein, able to sequester σ^B into an association that prevents it from joining RNA polymerase (Benson & Haldenwang, 1993b; Boylan *et al.*, 1993). RsbV is an antagonist to the RsbW- σ^B complex

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Fig. 1. Model of σ^B activation. σ^B is held inactive in unstressed *B. subtilis* as a complex with an anti- σ^B protein, RsbW (W). σ^B is freed from RsbW when a release factor, RsbV (V), binds to RsbW. In unstressed *B. subtilis*, RsbV is inactive due to an RsbW-catalysed phosphorylation (V-P). Physical stress activates an RsbV-P phosphatase, RsbU (U), which reactivates RsbV. RsbT (T) is the RsbU activator. In unstressed *B. subtilis*, RsbT is believed to be bound to a negative regulator, RsbS (S), in a large complex composed of RsbR and a family of paralogous proteins (R^*) that are thought to facilitate the RsbR/T interactions. Upon exposure to stress, RsbT phosphorylates and inactivates RsbS, freeing itself to activate the RsbU phosphatase. Obg, a ribosome-associated GTPase, and a ribosome-mediated process play essential, but unknown, roles in the activation of RsbT. RsbS-P is dephosphorylated and reactivated by a phosphatase, RsbX (X), that is encoded by one of the genes downstream of the *sigB* operon's σ^B -dependent promoter. RsbX levels become elevated when σ^B is active. This may facilitate the dephosphorylation of RsbS-P and the return of RsbT to an inactive complex with RsbS. Nutritional stress activates a separate pathway in which a novel RsbV-P phosphatase (P) and an associated protein (Q) dephosphorylate RsbV. The nutritional stress signal is unknown, but the triggering of this pathway coincides with a drop in ATP and is inhibited in *RelA*⁻ *B. subtilis*. The model is based on the references given in the text.

(Benson & Haldenwang, 1993b; Dufour & Haldenwang, 1994). RsbW forms mutually exclusive complexes with either RsbV or σ^B (Delumeau *et al.*, 2002; Dufour & Haldenwang, 1994). The ability of RsbV to compete for RsbW is dependent on RsbV's phosphorylation state (Dufour & Haldenwang, 1994). RsbW is both a binding protein and an RsbV-specific kinase. In unstressed *B. subtilis*, RsbW-dependent phosphorylation of RsbV inactivates RsbV as a potential σ^B release factor (Alper *et al.*, 1996; Dufour & Haldenwang, 1994). The dephosphorylation and reactivation of RsbV-P are effected by two stress-activated phosphatases, RsbP and RsbU. Each of these enzymes responds to a particular class of stress: RsbP to nutritional stress and RsbU to physical stress (Kang *et al.*, 1996; Vijay *et al.*, 2000; Voelker *et al.*, 1995a, b, 1996; Yang *et al.*, 1996).

The nutritional stress phosphatase (RsbP) is cotranscribed with a predicted hydrolase (RsbQ) that is needed for RsbP's activity (Brody *et al.*, 2001; Vijay *et al.*, 2000). The metabolic

inducer of RsbP/Q is unknown, but the conditions that trigger its activation are associated with a decrease in ATP levels, suggesting that changes in nucleotide pools may be involved in the activation process (Voelker *et al.*, 1996; Zhang & Haldenwang, 2003).

The phosphatase that responds to physical stress (RsbU) requires an additional protein, RsbT, for activity (Yang *et al.*, 1996). A current model envisions RsbT to be held inactive in unstressed *B. subtilis* in a complex with its binding partner RsbS. Exposure to physical stress enables RsbT to phosphorylate RsbS. This results in the release of RsbT and its activation of RsbU (Akbar *et al.*, 1997; Chen *et al.*, 2003; Voelker *et al.*, 1995a, b; Yang *et al.*, 1996). RsbS/RsbT interactions are believed to be modulated by RsbR and a family of related proteins (Akbar *et al.*, 1997, 2001; Chen *et al.*, 2003; Gaidenko *et al.*, 1999). It has been recently demonstrated that RsbR, and by inference its paralogues, can self-associate into large-molecular-mass complexes ($\sim 10^6$ Da) that can incorporate RsbS and RsbT (Chen *et al.*, 2003). These complexes may represent the normal state of the RsbR, -S and -T proteins in unstressed *B. subtilis*. RsbR, like RsbS, can be phosphorylated by RsbT (Gaidenko *et al.*, 1999). *In vitro*, RsbR is necessary for RsbS to bind and be phosphorylated by RsbT (Chen *et al.*, 2003). The phosphorylation state of RsbR does not affect the binding of RsbT to RsbS in the RsbR/RsbS complex, but does influence the ability of RsbT to phosphorylate RsbS, with RsbR-P facilitating the phosphorylation reaction (Chen *et al.*, 2003). Inhibition of RsbT is re-established through the activity of RsbX, an RsbS-P phosphatase that reactivates RsbS and allows it to again sequester RsbT (Voelker *et al.*, 1995a, b; Yang *et al.*, 1996).

Aside from RsbR, -S, and -T, there is evidence that a ribosome-associated process may contribute to stress activation of σ^B . σ^B fails to be induced by physical stress in *B. subtilis* strains that are either missing ribosome protein L11 or deficient in the ribosome-associated GTP-binding protein Obg (Scott & Haldenwang, 1999; Zhang *et al.*, 2001).

We previously noted that RsbR, -S and -T, as well as Obg, cofractionate with ribosomes during gel-filtration chromatography of crude *B. subtilis* extracts (Scott *et al.*, 2000). Obg was subsequently shown to bind to ribosome protein L13 in an affinity blot assay (Scott *et al.*, 2000). Thus, Obg's cofractionation with ribosomes probably represents a direct interaction between these two entities; however, even though RsbT can interact with Obg in the yeast dihybrid assay (Scott & Haldenwang, 1999), the significance of RsbT, as well as RsbR and RsbS, in the ribosome-containing fractions is unknown. In the present work we explore the possible associations that exist between the Rsb proteins, Obg and ribosomes in extracts of wild-type and mutant *B. subtilis*, and *Escherichia coli* engineered to express RsbR, RsbS and RsbT. The data are consistent with Obg, but not RsbR, RsbS or RsbT, being ribosome-associated. The ribosome/Obg contributions to triggering stress induction

of RsbT apparently do not involve interactions that persist in crude extracts. Additionally, and unlike the inactivating complexes that form between other σ^B regulators (e.g. RsbW- σ^B , RsbV-RsbW) which are readily identified in crude extracts, we find no evidence for persistent associations between the RsbR-RsbS complex and RsbT. Instead, RsbT appears to form Triton-sensitive aggregates in crude extracts, suggesting an inherent instability of free RsbT which may be related to its role as a stress-sensitive activator.

METHODS

Bacterial strains and plasmids. All strains and their relevant genotypes are listed in Table 1. Except where indicated, the *B. subtilis* strains used in this study are derivatives of PY22.

BSJ93, a strain carrying the *rsbS*_{59SA} mutation, was made as follows. PCR-based oligonucleotide mutagenesis (Woodbury *et al.*, 2004) was used to change codon 59 of *rsbS* in the plasmid pRT-2 (Smirnova *et al.*, 1998) from TCA (Ser) to GCA (Ala), creating pJM63. The presence of *rsbS*_{59SA} in pJM63 was confirmed by DNA sequencing. pJM63 encodes a Kan^R cassette flanked on one side by a 520 bp DNA segment encoding the region upstream of the *sigB* operon and on the other by *P_A rsbR rsbS_{59SA} rsbT*. Transformation of *B. subtilis* with linearized pJM63, followed by selection for Kan^R, allowed the isolation of clones in which the Kan^R cassette entered the *B. subtilis* chromosome by homologous recombination between the chromosome and the

sequences bracketing Kan^R. To facilitate the identification of clones in which *rsbS*_{59SA} was transferred with Kan^R to the recipient, pJM63 was transformed into a *B. subtilis* strain (XS352) (Smirnova *et al.*, 1998) with a deletion extending from codon 25 of the 121 codon *rsbS* gene to codon 17 of the 133 codon *rsbT* gene. Kan^R clones containing RsbS and RsbT (detected by Western blot) should have acquired the plasmid-encoded *rsbS*_{59SA} allele. BSJ93 was one such transformant. BSJ93 synthesizes RsbS and RsbT and is unable to activate σ^B following physical stress (the *rsbS*_{59SA} phenotype; Kang *et al.*, 1996).

BSK5 *rsbR* Δ 2 was constructed as follows. A 502 bp *EcoRI* segment internal to *rsbR* in pRS11 (*P_A rsbR-S*; Smirnova *et al.*, 1998) to create pJM49. This deletion terminates the synthesis of RsbR at codon 92 of the 274 codon *rsbR* gene. Using a strategy similar to that described above, BSK5 was identified following transformation of wild-type *B. subtilis* (BSA46) with linearized pJM49, yielding Kan^R clones which were screened by Western blot for the absence of RsbR but normal levels of the downstream *rsbS* gene product.

BSJ39 (RsbS⁻) was created using plasmid p Δ S25. p Δ S25 consists of a PCR fragment that begins approximately 200 bp upstream of the *sigB* operon's transcriptional start site and ends at the fourth codon of RsbS, joined to a second fragment that extends from 10 codons before the *rsbS* carboxy terminus to the end of *rsbT*, cloned in pRT-2. Kan^R clones arising from transformation of linearized p Δ S25 into a wild-type *B. subtilis* strain (BSA46) were screened for the anticipated high σ^B activity (*ctc::lacZ*) associated with the loss of RsbS on LB plates with X-Gal. Putative RsbS⁻ clones were then screened by Western blot for the presence of RsbR and T, and the absence of RsbS.

Table 1. Strains and plasmids

Strain or plasmid	Relevant genotype or features	Source, construction or reference
Plasmids		
pUK19	<i>Ap^R Kan^R</i>	Ju <i>et al.</i> , 1998
pUR1	<i>Ap^R Kan^R 520 bp upstream of sigB</i>	Smirnova <i>et al.</i> , 1998
pDRNT	<i>Ap^R Spc^R P_A rsbR rsbS rsbT</i>	Woodbury <i>et al.</i> , 2004
pDRS	<i>Ap^R Spc^R P_A rsbR rsbS</i>	This study
pKAT-1	<i>Ap^R Kan^R P_A rsbT</i>	This study
pSK-X	<i>Ap^R rsbS rsbT</i>	This study
pJM63	<i>Ap^R Kan^R P_A rsbR rsbS_{59SA} rsbT</i>	This study
pJM49	<i>Ap^R Kan^R P_A rsbRΔ2 rsbS</i>	This study
p Δ S25	<i>Ap^R Kan^R P_A rsbR rsbT</i>	This study
Strains		
<i>B. subtilis</i>		
PY22	<i>trpC2</i>	Benson & Haldenwang, 1992
BSA46	<i>trpC2 SPβ ctc::lacZ</i>	Benson & Haldenwang, 1992
XS332	<i>trpC2 aph(3')(5'')/sigB rsbT_{63Qterm} SPβ ctc::lacZ</i>	Smirnova <i>et al.</i> , 1998
XS352	<i>trpC2 aph(3')(5'')/sigB ΔrsbST SPβ ctc::lacZ</i>	Smirnova <i>et al.</i> , 1998
BSJ39	<i>trpC2 aph(3')(5'')/sigB rsbSΔ2</i>	p Δ S25 $\rightarrow$ BSA46
BSJ93	<i>trpC2 aph(3')(5'')/sigB rsbS_{59SA}</i>	pJM63 $\rightarrow$ XS352
BSK5	<i>trpC2 rsbRΔ2</i>	pJM49 $\rightarrow$ BSA46
PB629	<i>rsbΔR₁ ykoBΔ1::kan yojHΔ1::erm yqhAΔ1::spc</i>	Akbar <i>et al.</i> , 2001
<i>E. coli</i>		
TG-2		Sambrook <i>et al.</i> , 1989
ECW ₁	<i>P_A rsbR rsbS rsbT</i>	pDRNT $\rightarrow$ TG2
ECW ₂	<i>P_A rsbR rsbS</i>	pDRS $\rightarrow$ TG2
ECW ₃	<i>P_A rsbS rsbT</i>	pSK-X $\rightarrow$ TG2
ECW ₄	<i>P_A rsbT</i>	pKAT1 $\rightarrow$ TG2

E. coli (TG-2) strains expressing various combinations of *rsbR*, *-S* and/or *-T* were constructed using pUC19-based plasmids. ECW₁ carries pDRNT and expresses *rsbR*, *-S* and *-T*. ECW₂ expresses *rsbR* and *-S*. It carries pDRS, a plasmid derived from pDRNT by cutting with *Nde*I and *Sph*I to remove *rsbT* and recircularizing after treatment with mung bean nuclease to 'blunt' the ends. ECW₄ expresses *rsbT* from plasmid pKAT-1. pKAT-1 is pUK19 bearing the *sigB* operon's *P_A* promoter, amplified by PCR from PY22 as a 376 bp *Eco*RI/*Bam*HI fragment (−315 to +60) and joined to a 450 bp *Bam*HI/*Sph*I fragment encoding *rsbT* (36 bp upstream of *rsbT* to 12 bp downstream of *rsbT*). ECW₃ expresses *rsbS* and *rsbT* from pSK-X. To make pSK-X, *rsbS* and *rsbT* that had been amplified by PCR as a 715 bp *Hind*III/*Xba*I DNA fragment (23 bp upstream of *rsbS* to 23 bp downstream of *rsbT*) were cloned into pUC19. In plasmids pDNRT, pDRS and pKAT-1, the *rsb* genes are expressed from the *sigB* operon's *P_A* promoter, which is recognized in *E. coli*, and in pSK-X they are expressed from *P_{lac}*. Bacteria were routinely grown in LB at 37 °C.

Gel-filtration analysis. Gel-filtration chromatography was performed as described previously (Scott *et al.*, 2000). One litre of *B. subtilis* or *E. coli* culture (OD₅₄₀, 0.4) was harvested on ice, washed with a low-salt buffer [10 mM Tris (pH 8.0), 50 µM EDTA, 1.5 mM MgCl₂, 1 mM DTT, 0.03 % phenylmethyl sulfonyl fluoride (PMSF)] and resuspended in 5 ml of the same buffer. Following disruption in a French pressure cell, debris was removed by centrifugation (5000 g for 10 min), and the resulting crude lysate loaded onto a 500 ml Sephacryl S-300 column equilibrated with the same buffer. Five millilitre fractions were collected, precipitated with 2 vols of ethanol and analysed by SDS-PAGE (13.5 % acrylamide) and Western blot. If a second round of gel filtration was to be used, the fractions containing the large-molecular-mass complexes were pooled and dialysed into buffer [10 mM Tris (pH 8.0), 50 mM KCl] containing either 10 mM magnesium acetate or no added Mg²⁺. The dialysed fractions were reapplied to the Sephacryl column, equilibrated with the buffer used for dialysis and fractionated/analysed as before.

Velocity-sedimentation analysis. *B. subtilis* grown and harvested, as in the gel-filtration analysis, was resuspended in sedimentation buffer [20 mM Tris (pH 7.5), 10 mM MgCl₂, 0.5 mM EDTA, 1 mM dithiothreitol, 0.1 M NH₄Cl, 0.03 % PMSF], disrupted by passage through a French pressure cell, and centrifuged (8000 g, 15 min) to remove debris. Protease inhibitors were added to the following final concentrations: pepstatin, 1 µM; leupeptin, 1 µM; N-α-tosyl-L lysine chloromethyl ketone (TLCK), 100 µM; antitrypsin, 2 µg ml^{−1}. One hundred microlitres of extract (OD₂₈₀, 200) was layered onto 9 ml 10–30 % sucrose gradients prepared in the sedimentation buffer. Gradients containing identical samples were centrifuged (37 000 r.p.m. in a Sorvall TH641 swinging bucket rotor) for 1.5, 3 and 5 h. Fractions (0.5 ml) were collected from the gradients, precipitated with 2 vols of ethanol and analysed by SDS-PAGE and Western blot.

To analyse the high-molecular-mass complexes that were identified by gel filtration, 4 ml of pooled Sephacryl column fractions was precipitated with (NH₄)₂SO₄ (0.56 g ml^{−1}), resuspended in 0.5 ml of sedimentation buffer without PMSF, and dialysed overnight against 1 l of this buffer. Two hundred microlitres of dialysed extract was layered onto sucrose gradients and analysed as above.

General methods. Transformations of *B. subtilis* and *E. coli* were performed by standard methods (Sambrook *et al.*, 1989; Yasbin *et al.*, 1973). Western blot analyses were done as previously described, using mouse monoclonal antibodies against the Rsb proteins and mouse polyclonal anti-Obg antibody (Dufour *et al.*, 1996; Scott *et al.*, 2000).

RESULTS

Fractionation of wild-type *B. subtilis* extracts

A number of the proteins that are required for stress-dependent regulation of σ^B elute in high-molecular-mass fractions during gel-filtration chromatography of crude *B. subtilis* extracts (Scott *et al.*, 2000). Most of the RsbR, approximately half of the RsbS, and all of the detectable RsbT and Obg are present in fractions that also contain the bulk of the extracts' ribosomal proteins (Fig. 2a, lanes 2–10). To investigate whether this coelution represents a physical association between these proteins, gel-filtration fractions were pooled, and subjected to further analyses.

As a test of the dependence of the high-molecular-mass forms of Obg, RsbR, RsbS and RsbT on intact ribosomes, the ribosome component in the gel-filtration fractions was disrupted by dialysis against a buffer lacking Mg²⁺, a cation essential for ribosome integrity (Spirin, 1990). As a control, a sample was also dialysed against a similar buffer with 10 mM MgCl₂. The fractions dialysed against the Mg²⁺-containing buffer retained their original elution profile when chromatographed on a second gel-filtration column, i.e. Obg, ribosomes and Rsb proteins coeluted in the fast-exiting fractions (data not shown). In contrast, the sample that had been dialysed into magnesium-free buffer displayed an altered pattern (Fig. 2b), with the ribosomal proteins dispersed throughout the fractions. Consistent with Obg's presence in the higher-molecular-mass fractions being a consequence of ribosome association, the Obg protein shifted to a lower-molecular-mass position in the elution profile. However, despite the disassociation of ribosomes, RsbR and RsbS persisted in the original high-molecular-mass fractions. Thus, the presence of RsbR and RsbS in the high-molecular-mass fractions is not the result of a ribosome association. RsbT was not detected in the fractions from the second gel-filtration column, perhaps due to its dispersal among many fractions or its degradation.

In an alternative analysis, the high-molecular-mass fractions from the original gel-filtration column were concentrated by (NH₄)₂SO₄ precipitation, dialysed into 10 mM MgCl₂ buffer and examined by velocity centrifugation. Samples of this material, as well as unfractionated whole-cell lysates, were layered in triplicate onto sucrose gradients and subjected to high-speed centrifugation. Representative gradients were removed at intervals throughout the centrifugation run and, following fractionation, analysed by SDS-PAGE and Western blot. As illustrated in Fig. 3, the velocity fractionation of both a whole-cell lysate (Fig. 3i) and the fast-eluting gel-filtration fractions (Fig. 3ii) resulted in the separation of the RsbR/RsbS proteins and the ribosomes during sedimentation. The RsbR/RsbS proteins sedimented more slowly than ribosomes, consistent with the sedimentation profile reported for the self-assembling RsbR/RsbS complex (Chen *et al.*, 2003). RsbT's sedimentation through the gradient was distinct from that of either the ribosomes or the putative RsbR–RsbS complex.

Fig. 2. Gel-filtration chromatography of *B. subtilis* extracts. (a) *B. subtilis* (PY22) was harvested in exponential phase (OD_{540} 0.4). Extracts were prepared as described in Methods and loaded onto a Sephacryl S-300 column. Samples of the protein-containing fractions were fractionated by SDS-PAGE. The protein profiles in each fraction were visualized by Coomassie-blue staining (upper panel) and Western blot (lower panel) using antibodies against RsbR, -S and -T and Obg as probes. Numbers at the top of the panel indicate fraction numbers with fraction 1 being the earliest-eluting fraction. The characteristic cluster of low-molecular-mass ribosomal proteins in the fast-eluting fractions is bracketed in the upper panel. The positions of Obg, RsbR, -S and -T are indicated in the lower panel, which also includes protein molecular-mass markers (Bio-Rad) in the first and last lanes. (b) Fractions 2–7 of the gel-filtration experiment illustrated in (a) were dialysed into buffer without Mg^{2+} and subjected to a second round of gel filtration using the same Sephacryl S-300 column that had been equilibrated with Mg^{2+} -free buffer. The analyses of the indicated fractions are as in (a).

Fig. 3. Sedimentation analyses of *B. subtilis* extracts. Triplicate samples of a crude extract from wild-type *B. subtilis* were layered onto 10–30 % sucrose gradients and subjected to centrifugation at 37 000 r.p.m. in a Sorvall TH641 rotor for 1·5 (a), 3 (b) or 5 (c) hours. Fractions were collected from the bottom of the tube (fraction 1) and analysed as in Fig. 2. The top three gels in (i) depict Coomassie-blue staining, illustrating protein fractionation with sedimentation of the low-molecular-mass proteins of the ribosomes in the lead fractions. Below, the stained gels are samples from the same fractions analysed by Western blot using antibodies against the proteins indicated in the figure as probes. (ii) depicts triplicate samples of the fraction 2–7 material from the gel-filtration column in Fig. 2(a), concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation, centrifuged through sucrose gradients and analysed by Western blot for the sedimentation profile of RsbR, -S, -T and Obg. (iii) depicts triplicate samples of a crude extract from *B. subtilis* strain BSJ93 (*rsbS_{93SA}*) centrifuged through sucrose gradients and analysed by Western blot for the positions of RsbR, -S and -T in the gradient profile.

Additionally, RsbT's sedimentation pattern varied, depending upon whether the material analysed was pooled gel-filtration fractions or crude cell extract. The bulk of the RsbT in the whole-cell extract (Fig. 3i-c) remains in the uppermost portions of the gradient following centrifugation, apparently unassociated with fast-sedimenting components, while in the Sephacryl-fractionated sample, a second peak of RsbT is seen, trailing the ribosomal proteins but ahead of the putative RsbR/RsbS complex (Fig. 3ii).

Although Obg can specifically bind to ribosomal protein L13 (Scott *et al.*, 2000), it too separated from the ribosomes during centrifugation. In other studies, we observed that GTP or nonhydrolysable GTP analogues (i.e. GIDP) can stabilize the association of Obg with ribosomes (S. Zhang, & W. Haldenwang, unpublished results). To ask whether the apparent Obg/ribosome complex that eluted from the Sephacryl column represents this GTP-stabilizable complex, the sedimentation analysis of the putative Obg/ribosome

Fig. 4. Effects of GIDP on sedimentation of Obg with ribosomes. Pooled, early-eluting fractions (Fig. 2a, fractions 2–7) of a crude *B. subtilis* extract separated by Sephacryl chromatography concentrated and dialysed as in Fig. 3(ii) were subjected to centrifugation through 10–30% sucrose gradients that were either unsupplemented (a, b) or contained 10 μ M GIDP (c). The gradients were centrifuged for 5 h and analysed as in Fig. 3. (a) Coomassie-blue-stained gel; (b, c) Western blot analysis (anti-Obg, -RsbR, -RsbS and -RsbT antibody probes).

complex was repeated incorporating a non-hydrolysable GTP analogue (10 μ M GIDP) in the sucrose gradient. In the presence of the GIDP, Obg sedimented with the ribosome components (Fig. 4c). Apparently, nucleotide-bound Obg is the form which most avidly binds ribosomes, or the ribosomes themselves are altered by the nucleotide to stabilize Obg binding.

The data argue that Obg is ribosome-associated but RsbR, RsbS and RsbT are not part of an Obg/ribosome complex. Based on the velocity-sedimentation analysis, the presence of RsbT in the high-molecular-mass gel-filtration fractions appears to be independent of either RsbR or RsbS. The coincident elution of RsbR and RsbS in the high-molecular-mass gel-filtration fractions persisted in the centrifugation analysis, arguing for their presence in a common complex in these fractions, probably the large RsbR/RsbS multimer that is reported to self-assemble *in vitro* (Chen *et al.*, 2003). RsbT is believed to be sequestered into the RsbR/RsbS

complex in unstressed *B. subtilis* and released following stress-induced phosphorylation of RsbS. Although the nature of the high-molecular-mass form of RsbT is uncertain, it is formally possible that the RsbT remaining near the top of the velocity gradient could represent RsbT that was originally bound to RsbR/RsbS but was released from the complex as a consequence of the phosphorylation of RsbS during extract preparation or analysis. Such a circumstance was proposed to explain the failure of RsbT to cosediment with RsbR/RsbS in complexes prepared from purified components (Chen *et al.*, 2003). In an attempt to circumvent this possibility, extracts were prepared from a *B. subtilis* strain with a mis-sense mutation in *rsbS* (*rsbS59SA*) which removes the target residue for phosphorylation by RsbT. *B. subtilis* strains carrying this *rsbS* allele are not stress-activable and, by the current model, should have RsbT permanently sequestered in the inactivating complex. As can be seen in Fig. 3iii, the presence of the mutant *rsbS* allele had no effects on RsbT's sedimentation profile. It remained in the slow-sedimenting fraction, free from the RsbR/RsbS complex. Thus, it is unlikely that the failure of RsbT to cofractionate with RsbR–RsbS is due to the phosphorylation of RsbS.

Gel-filtration profiles of Obg, RsbR, RsbS and RsbT in mutant *B. subtilis*

To better characterize the nature of the high-molecular-mass forms of RsbR, RsbS and RsbT that are found in *B. subtilis* extracts, cell lysates from *B. subtilis* mutants lacking RsbR, RsbS, RsbT, or RsbR and its three most homologous paralogues YkoB, YojH and YqhA (Akbar *et al.*, 2001) were fractionated by gel filtration and analysed by Western blot (Fig. 5a–d). The abundance of RsbS in the high-molecular-mass fractions decreased only slightly, relative to its abundance in low-molecular-mass fractions, in the RsbR single mutant, but more dramatically in the mutant lacking multiple RsbR-like proteins. In contrast, the RsbT remained exclusively in the high-molecular-mass form regardless of the presence or absence of either RsbR or RsbS. These findings are consistent with the notion that RsbS, but not RsbT, is present in the high-molecular-mass fractions due to its association with RsbR, and that the RsbR paralogues can substitute for RsbR in such a complex. As would be expected from the current model, the absence of RsbT had no obvious effect on the abundance of the high-molecular-mass RsbR/RsbS complex and the loss of RsbS did not prevent the accumulation of the putative RsbR multimer. These results reinforce the notion that RsbT is not part of a stable complex with RsbR and RsbS in *B. subtilis* extracts. If the complex was initially present, it was unable to persist during the fractionation protocols that we employed.

Formation of Rsb complexes in *E. coli*

To better judge the *Bacillus*-specific factors that contribute to the high-molecular-mass forms of RsbR, RsbS and RsbT, combinations of these proteins were expressed from

Fig. 5. Gel-filtration analyses of *B. subtilis* extracts lacking Rsb proteins. *B. subtilis* strains lacking one or more σ^B regulatory genes were grown, fractionated on Sephacryl S-300 and analysed as in Fig. 2(a). (a) BSK5 ($RsbR^-$); (b) PB629 ($RsbR^-$, $YkoB^-$, $YojH^-$, $YqhA^-$); (c) XS332 ($RsbT^-$); (d) BSJ39 ($RsbS^-$).

plasmid-encoded genes in *E. coli*. Extracts prepared from *E. coli* carrying *rsbR*, *rsbS* and *rsbT*, fractionated by gel filtration and analysed by Western blot gave a profile (Fig. 6b) that was virtually indistinguishable from that seen in *B. subtilis* (Fig. 2b). As was also seen in *B. subtilis* (Fig. 5), the absence of RsbT had little effect on the formation of the high-molecular-mass form of RsbR/RsbS (Fig. 6c), but unlike the case in *B. subtilis* the high-molecular-mass

form of RsbS was totally dependent on RsbR (Fig. 6d). Apparently the RsbR paralogues, present in *B. subtilis* but absent in *E. coli*, can substitute for RsbR and allow formation of a high-molecular-mass complex that can incorporate RsbS. As was also seen in *B. subtilis*, a high-molecular-mass form of RsbT occurs independently of RsbR and RsbS. The presence of this form of RsbT in *E. coli* extracts argues that it is independent of *Bacillus*-specific factors (Fig. 6e).

Fig. 6. Gel-filtration chromatography of Rsb proteins in *E. coli* extracts. Crude extracts were prepared from *E. coli* strains carrying plasmids that allowed the expression of (a, b) *rsbR*, *rsbS* and *rsbT* (ECW₁), (c) *rsbR* and *rsbS* (ECW₂), (d) *rsbS* and *rsbT* (ECW₃) or (e) *rsbT* (ECW₄). Extract samples were fractionated on a Sephacryl S-300 column and analysed as in Fig. 2. (a) Coomassie-blue-stained gel; (b–e) Western blot analysis of the indicated fractions using antibody probes for the proteins depicted in each blot.

The failure of RsbT's large-molecular-mass form to be dependent on *Bacillus*-specific factors raises the possibility that it represents an inherent property of RsbT itself. During purification for biochemical studies and antibody

production, RsbT, unlike the other Rsb proteins, showed a marked tendency to aggregate in the absence of detergents (A. Dufour & W. G. Haldenwang, unpublished results). If the RsbT found in the gel-filtration fractions represents a

Fig. 7. Gel-filtration chromatography of Triton-treated *E. coli* extracts. Triton X-100 was added to crude extracts prepared from *E. coli* strains ECW₁ (*rsbR*, *rsbS*, *rsbT*) or ECW₄ (*rsbT*) to a final concentration of 0.5%. The extracts were then fractionated by gel filtration with buffer containing 0.1% Triton X-100. Fractions were collected. Successive fractions were pooled and analysed as in Fig. 2. (a, b) ECW₁ Coomassie-blue-stained gel and Western blot, respectively; (c) ECW₄ Western blot.

similar aggregation of free RsbT, its fractionation properties would be predicted to be affected by non-ionic detergents. We explored this possibility by running the gel-filtration fractionation of the *E. coli* extracts in the presence of 0.1% Triton. The putative RsbR/RsbS complex was unaffected by the detergent treatment, while the RsbT shifted to a low-molecular-mass form that eluted in fractions that overlapped those containing the low-molecular-mass forms of RsbR and RsbS (Fig. 7a). The presence of all three of these proteins in some of the fractions is probably coincidental. Unlike the high-molecular-mass RsbR/RsbS complex, in which RsbR and RsbS abundance is seen to 'peak' in the same fractions, the peak amounts of each of these proteins are displaced one from another in the lower-molecular-mass elution profile. The independence of RsbT from RsbR

and RsbS in the Triton-treated extract is reinforced by the observation that a Triton-treated extract from an *E. coli* strain that expresses *rsbT* alone has an elution profile similar to that of RsbT in a strain expressing all three proteins (Fig. 7b).

DISCUSSION

In previous work, a portion of the σ^B regulatory proteins RsbR and RsbS, and all of the RsbT, present in *B. subtilis* extracts were observed to coelute with the extract's ribosome/Obg population during gel-filtration chromatography (Scott *et al.*, 2000). In light of the necessary, but undefined, roles that the ribosome and Obg play in the stress activation of σ^B , this coincident elution merited

further study. The fractionation analyses reported in the present work demonstrate that the ribosome/Obg, RsbT and RsbR/RsbS complexes are independent components of the high-molecular-mass gel-filtration fractions. Velocity-gradient analyses allowed the separation of each of these as distinct entities. Additional support for the high-molecular-mass forms of RsbR and RsbS being unrelated to ribosome-association comes from the persistence of these forms under buffer conditions that caused the disassembly of ribosomes and the release of Obg. Although ribosomes and Obg contribute necessary inputs to the stress-activation process, these apparently do not involve long-lived associations that can be detected as complexes in crude extracts.

Recent biochemical experiments have shown that RsbR can form high-molecular-mass complexes which incorporate RsbS (Chen *et al.*, 2003). It is thought that formation of such a complex is a prerequisite for RsbS to be able to sequester and inactivate RsbT. The high-molecular-mass RsbR/RsbS association that we observed in fractionated *B. subtilis* and in *E. coli* extracts probably represents this complex. As was seen in the biochemical experiments, RsbR is the essential element in its formation (i.e. the high-molecular-mass complex of RsbR occurs in the absence of RsbS, but the high-molecular-mass RsbS complex requires RsbR) and based on its formation when RsbR and RsbS are expressed in *E. coli* no additional *Bacillus*-specific proteins are needed. In *Bacillus*, in which a family of RsbR paralogues are thought to be able to substitute for RsbR (Akbar *et al.*, 2001), the loss of RsbR itself has only a modest effect on the presence of a high-molecular-mass complex that incorporates RsbS. Only when RsbR and several RsbR paralogues are deleted does the abundance of the RsbS-containing complex show a noticeable decline.

A curiosity in the present study is the failure of RsbT to be part of the RsbR/RsbS complex. A favoured model for the stress-activation mechanism envisions RsbT as unavailable to activate the RsbU phosphatase due to its sequestration into the complex with RsbR/RsbS. This would be similar to the inactivation of σ^B by its sequestration in an RsbW/ σ^B complex or the inactivation of RsbW by its binding to RsbV. Both of these complexes are readily discerned in fractionations of crude *B. subtilis* extracts (Dufour & Haldenwang, 1994), yet the association of RsbT with its putative inactivating complex is not evident. This is true even in extracts prepared from a *B. subtilis* strain with an altered RsbS (RsbS59SA) that should not release RsbT (Kang *et al.*, 1996). Instead, two forms of RsbT were found in crude extracts: a low-molecular-mass form, observed when crude extracts were fractionated by velocity-gradient analysis, and a high-molecular-mass form, found following gel filtration, which, by virtue of its detergent sensitivity, probably represents aggregated RsbT.

In other stress-responsive systems, protein denaturation and chaperone-assisted protein-folding contribute to stress signalling and gene regulation (Narberhaus, 1999). It is plausible, although quite speculative, that the denaturation

of RsbT, reflected in its apparent aggregation in crude extracts, could have regulatory significance. If RsbT is readily given to misfolding and aggregation, the RsbR/RsbS complex could not only serve as an inactivating RsbT-binding complex, but also play a positive role as an RsbT-chaperone complex, stabilizing and releasing a properly folded RsbT in response to a stress-generated trigger.

In an earlier analysis of the role of RsbS in σ^B regulation, it was reported that an *rsbS* allele (*rsbS*_{59SD}), whose product mimics phosphorylated RsbS, was more effective in allowing RsbT to activate σ^B than was a deletion of *rsbS* (Kang *et al.*, 1996). We have confirmed this result and, in addition, note that the *rsbS*_{59SD} allele is codominant with its wild-type counterpart. We also found that the *rsbS* allele (*rsbS*_{59SA}), whose product should sequester RsbT into stable inactive complexes (Kang *et al.*, 1996; Yang *et al.*, 1996), is recessive to the wild-type *rsbS* allele (A. Reeves, S. Zhang & W. Haldenwang, unpublished results). These phenotypes are unusual for an inhibitory system, where the capacity for negative control would be expected to be dominant. Although more complex models can be envisioned to explain these results, the dominance of wild-type *rsbS* over *rsbS*_{59SA}, and *rsbS*_{59SD} over the wild-type allele, could be simply explained if RsbS, when altered due to phosphorylation following stress or the *rsbS*_{59SD} mutation, has a positive influence on RsbT activity. Detailed mutational analyses of *rsbR*, *rsbS* and *rsbT* could reveal the validity of this notion.

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