

Catalytic Function of an α/β Hydrolase Is Required for Energy Stress Activation of the σ^B Transcription Factor in *Bacillus subtilis*

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The general stress response of *Bacillus subtilis* is controlled by the σ^B transcription factor, which is activated in response to diverse energy and environmental stresses. These two classes of stress are transmitted by separate signaling pathways which converge on the direct regulators of σ^B , the RsbV anti-anti- σ factor and the RsbW anti- σ factor. The energy signaling branch involves the RsbP phosphatase, which dephosphorylates RsbV in order to trigger the general stress response. The *rsbP* structural gene lies downstream from *rsbQ* in a two-gene operon. Here we identify the RsbQ protein as a required positive regulator inferred to act in concert with the RsbP phosphatase. RsbQ bound RsbP in the yeast two-hybrid system, and a large in-frame deletion in *rsbQ* had the same phenotype as a null allele of *rsbP*—an inability to activate σ^B in response to energy stress. Genetic complementation studies indicated that this phenotype was not due to a polar effect of the *rsbQ* alteration on *rsbP*. The predicted *rsbQ* product is a hydrolase or acyltransferase of the α/β fold superfamily, members of which catalyze a wide variety of reactions. Notably, substitutions in the presumed catalytic triad of RsbQ also abolished the energy stress response but had no detectable effect on RsbQ structure, synthesis, or stability. We conclude that the catalytic activity of RsbQ is an essential constituent of the energy stress signaling pathway.

In *Bacillus subtilis* and related bacteria, a general stress response controlled by the σ^B transcription factor confers multiple stress resistance on nongrowing cells (reviewed in references 17 and 26). The activity of σ^B is governed by a signal transduction pathway with two distinct branches. One branch is specific for energy stresses, such as carbon, phosphorus, or oxygen limitation, and the other is specific for environmental stresses, such as acid, ethanol, heat, or salt stress (20, 41, 43, 44). According to the model shown in Fig. 1, each branch terminates with a differentially regulated serine phosphatase: RsbU in the environmental signaling branch and RsbP in the energy signaling branch. When activated by its particular class of stress, either RsbU or RsbP engages the common regulators RsbV and RsbW. RsbV and RsbW together control σ^B activity via a partner-switching mechanism in which alternate protein-protein interactions are governed by the phosphorylation state of RsbV (3, 8, 13, 41, 42, 44).

The means by which energy and environmental stress signals enter their respective branches of the pathway are unknown, and it is clear that additional signaling components remain to be identified (1, 33, 41, 45). Here we focus on the energy signaling branch. The amino-terminal half of the RsbP phosphatase contains a Per-ARNT-Sim (PAS) domain (41), similar to those found in a wide variety of proteins involved in sensing fluctuations in redox, light, or oxygen (38). In some proteins, such PAS domains function by binding a ligand or a chromophore and in others by controlling protein-protein interac-

tions with other proteins. As part of a search for new elements of the energy signaling branch, we report here that RsbP interacts with RsbQ, a positive regulator essential for energy stress activation of σ^B . Moreover, our genetic analysis indicates that the predicted catalytic activity of RsbQ is required for energy stress signaling in *B. subtilis*.

MATERIALS AND METHODS

Bacterial strains and genetic methods. *Escherichia coli* DH5 α (Gibco BRL, Gaithersburg, Md.) was host for all plasmid constructions, while *E. coli* BL21(DE3)pLysS (Novagen, Madison, Wis.) was the expression host for protein purification. Standard recombinant DNA methods were as described by Sambrook et al. (32). *B. subtilis* strains used are shown in Table 1. *B. subtilis* PB2 and its derivatives were made competent by standard methods (12). We used the four-primer method of site-directed mutagenesis (18) to make three separate alterations within the cloned *rsbQ* gene. To make the large, in-frame deletion of *rsbQ* (*rsbQ* Δ 2), we removed 546 bp from within the 807-bp coding region, deleting triplets 38 to 219. To make the missense mutations, triplet 96 was changed from serine (TCC) to alanine (GCC) to yield *rsbQS96A* and triplet 247 was changed from histidine (CAT) to alanine (GCA) to yield *rsbQH247A*. Each PCR-mutagenized product was cloned into the pCP115 integration vector (27), yielding pMB56 (*rsbQ* Δ 2), pMB58 (*rsbQH247A*), and pMB59 (*rsbQS96A*). We then introduced the deletion or missense mutations into the homologous copy of *rsbQ* on the *B. subtilis* chromosome by means of a two-step allele replacement method (35). Substitution of the *rsbQ* Δ 2 allele was confirmed by PCR amplification. Substitution of the missense mutations was confirmed by chromosomal sequencing.

β -Galactosidase accumulation assays. All cultures were grown in a shaking water bath at 37°C. For energy stress experiments, cells were either grown into stationary phase in buffered Luria broth (LB) lacking salt (9) or subjected to carbon and energy starvation in a minimal salt medium (5) containing 0.05% glucose. For environmental stress experiments, cells were grown to early logarithmic phase in buffered LB lacking salt. At this point, either NaCl (0.3 M final concentration) or ethanol (4% [vol/vol] final concentration) was added to one of two parallel cultures. For all experiments, samples were collected at the times indicated and treated as described by Miller (22). Cells were washed with Z buffer and permeabilized using sodium dodecyl sulfate and chloroform. Protein concentrations were determined using the Bio-Rad Protein Assay reagent (Bio-Rad, Richmond, Calif.). Activity was defined as $\Delta A_{420} \times 1,000$ per minute per milligram of protein.

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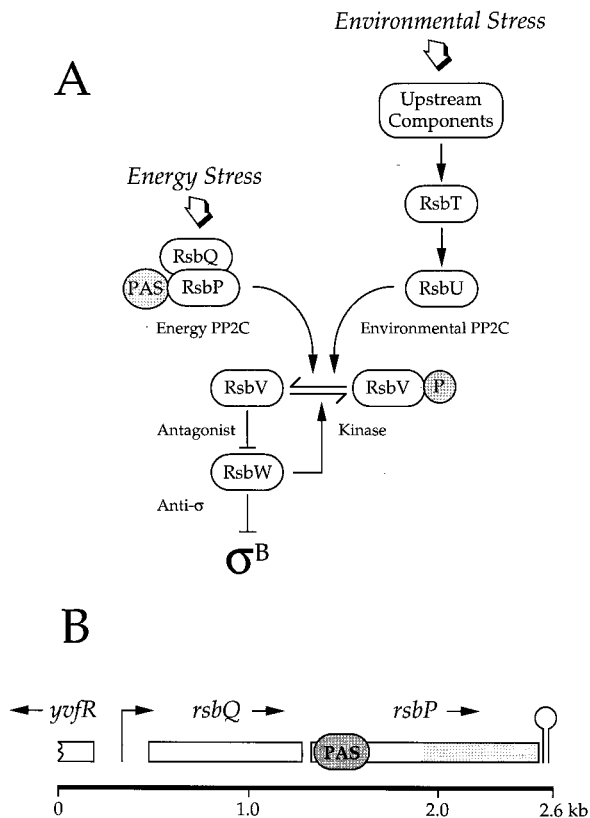


FIG. 1. Model of the signal transduction network that activates the general stress transcription factor σ^B . (A) Two signaling pathways converge on RsbV-P, the antagonist form found in unstressed cells. The energy stress branch terminates with the RsbP phosphatase (Energy PP2C), which contains a PAS domain in its amino-terminal region (41). In contrast the environmental stress branch terminates with the RsbU phosphatase (Environmental PP2C), which is activated by RsbT in response to upstream signaling elements (2, 20, 42–44). When triggered by stress, either the RsbP or the RsbU phosphatase removes the serine phosphate from RsbV-P. Dephosphorylated RsbV then binds the RsbW anti- σ factor, forcing it to release σ^B , which can then activate its target genes (3, 8, 13). We show here that RsbQ (formerly called YvfQ) is an essential component of the energy stress branch and propose that it acts in concert with the RsbP phosphatase. (B) Genetic organization of the *rsbQ-rsbP* operon. The *rsbQ* and *rsbP* reading frames are denoted by open rectangles above the kilobase scale. The regions indicated within *rsbP* code for the PAS domain (PAS) and the PP2C phosphatase domain (shaded). Promoter activity (right-angled arrow) has been proximately located between 0 and 0.7 kb, and a potential terminator sequence (stem-loop) lies downstream from *rsbP*. Energy signaling does not require increased transcription from this promoter, suggesting that signaling is accomplished by a posttranslational mechanism (41).

Yeast two-hybrid analysis. We used the Matchmaker Two-Hybrid System (Clontech, Palo Alto, Calif.) to detect possible interactions among RsbP, RsbQ, RsbT, and RsbU. The *rsbQ* and *rsbP* reading frames were fused to either the GAL4 DNA binding domain in plasmid pGBT9 or the GAL4 activation domain in plasmid pGAD424, as previously described for *rsbT* and *rsbU* (44). These constructs were then paired in yeast SFY526 cells by selecting double transformants on minimal plates. Transcriptional activation of the *lacZ* reporter gene in these transformants was determined qualitatively using a colony lift filter assay according to the manufacturer's protocol.

Overexpression of *rsbQ* and *rsbP* products in *B. subtilis*. We used PCR to amplify the individual *rsbQ* and *rsbP* reading frames along with their predicted ribosomal binding sites, placing them under control of the isopropyl- β -D-thio-

lactopyranoside (IPTG)-inducible Pspac promoter in the multicopy expression vector pDG148 (36). Plasmid pMB64, carrying *rsbQ*, was transformed into *B. subtilis* PB198 (wild-type) and PB567 (*rsbPA1::spc*). Plasmid pKV2, carrying *rsbP*, was transformed into PB198 and PB605 (*rsbQ Δ 2*). These strains also carried a σ^B -dependent *ctc-lacZ* fusion in single copy at the *amyE* locus (10) to permit measurement of σ^B activity. Expression from the plasmid was induced by adding IPTG (1 mM final concentration) to cultures in the early logarithmic phase of growth.

Construction of overexpression clones and purification of His-tagged proteins from *E. coli*. To purify the wild-type, S96A, and H247A forms of RsbQ, we fused the appropriate coding region to a hexahistidine tag in the pET15b expression vector (Novagen). Hexahistidine-tagged proteins were purified from *E. coli* BL21(DE3)pLysS extracts on nickel-nitrilotriacetic acid metal affinity columns (Qiagen, Valencia, Calif.) according to the manufacturer's protocol.

Trypsin proteolysis. Purified wild-type and mutant RsbQ proteins were subjected to limited trypsin proteolysis as follows. Trypsin (L-1-tosylamido-2-phenyl ethyl chloromethyl ketone [TPCK]-treated type XIII from bovine pancreas; Sigma, St. Louis, Mo.) was used to digest a 500-fold-molar excess of the target protein in a buffer containing 25 mM Tris (pH 8.0), 50 mM NaCl, and 1 mM CaCl₂. Digestions were performed at 25°C and were stopped at 2-min intervals by adding phenylmethylsulfonyl fluoride (1 mM final concentration), removing the mixtures to ice, and then freezing them at -80°C. Samples were electrophoresed on sodium dodecyl sulfate (SDS)-15% polyacrylamide gels and were visualized by Coomassie blue staining.

Detection of RsbQ by Western blotting. Polyclonal antibody was raised by injecting rabbits with the purified, His-tagged RsbQ. To test antibody specificity, extracts of PB2 and PB604 (*rsbQ Δ 2*) were prepared from early-stationary-phase cells. For determining RsbQ levels in vivo, strains PB2, PB633 (*rsbQS96A*), and PB631 (*rsbQH247A*) were grown in buffered LB lacking salt, and samples were taken at the indicated times during the logarithmic and stationary growth phases. Cells were harvested by centrifugation, washed in 1 ml of sonication buffer (10 mM Tris, pH 7.4, and 5 mM MgCl₂), and resuspended in a minimal volume (50 to 100 μ l) of the same buffer containing 10 μ g of lysozyme. Following a 10-min incubation at 37°C, cells were broken by sonication and centrifuged to remove cell debris from the extracts. Approximately 100 μ g of total cell protein per lane (as determined by Bio-Rad Protein Assay) was separated on an SDS-12% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Bio-Rad). Blots were blocked and washed in phosphate-buffered saline-Tween (PBST) (10 mM Na₂HPO₄, pH 7.2; 137 mM NaCl; 2.7 mM KCl; 3% [vol/vol] Tween 20) containing 5% (wt/vol) nonfat dry milk (BLOTTO) and were then exposed to the rabbit polyclonal anti-RsbQ antiserum for 1 h at 25°C. Following four washes with BLOTTO and PBST, blots were incubated at 25°C for 1 h with the secondary antibody, a goat anti-rabbit immunoglobulin G peroxidase conjugate (Sigma). Washes were repeated as before, with the addition of a final wash in PBS. Bound antibody was detected using the ECL Plus Western blotting detection kit (Amersham Pharmacia Biotech, Piscataway, N.J.) according to the manufacturer's instructions.

RESULTS

rsbQ is required to activate σ^B in response to energy stress.

As shown in Fig. 1B, *rsbQ* is located in an operon together with *rsbP*, the structural gene for the serine phosphatase which activates σ^B under conditions of energy stress. Although the promoter for the *rsbQP* operon has not been located precisely, fusion analysis found that energy signaling does not require increased operon transcription, leading to the notion that signaling is largely accomplished via a posttranslational mechanism (41). Moreover, because a similar arrangement of *rsbQ* and *rsbP* homologues is also found in *Streptomyces coelicolor*, we inferred that both gene products might affect a common cellular process (41). To determine whether RsbQ is involved in σ^B regulation, we made a large in-frame deletion within the *rsbQ* coding region and substituted it for the chromosomal copy via a two-step allele replacement procedure. The resulting null mutant also contained a single-copy transcriptional fusion between the σ^B -dependent *ctc* promoter and an *E. coli lacZ* reporter gene to provide an assay for σ^B activity.

As shown in Fig. 2, the strain bearing the *rsbQ Δ 2* null allele

TABLE 1. *Bacillus subtilis* strains

Strain	Genotype	Reference or construction ^a
PB2	<i>trpC2</i>	Wild-type Marburg strain
PB198	<i>amyE::ctc-lacZ trpC2</i>	10
PB567	<i>rsbPΔ1::spc amyE::ctc-lacZ trpC2</i>	41
PB604	<i>rsbQΔ2 trpC2</i>	pMB56→PB2 ^b
PB605	<i>rsbQΔ2 amyE::ctc-lacZ trpC2</i>	pDH32-ctc (10)→PB604
PB606	<i>amyE::ctc-lacZ trpC2</i> pMB64	pMB64→PB198
PB607	<i>amyE::ctc-lacZ trpC2</i> pKV2	pKV2→PB198
PB631	<i>rsbQH247A trpC2</i>	pMB58→PB2 ^b
PB632	<i>rsbQH247A amyE::ctc-lacZ trpC2</i>	pDH32-ctc→PB631
PB633	<i>rsbQS96A trpC2</i>	pMB59→PB2 ^b
PB634	<i>rsbQS96A amyE::ctc-lacZ trpC2</i>	pDH32-ctc→PB633
PB716	<i>rsbQΔ2 amyE::ctc-lacZ trpC2</i> pKV2	pKV2→PB491
PB719	<i>rsbPΔ1::spc amyE::ctc-lacZ trpC2</i> pMB64	pMB64→PB567

^a Arrow indicates transformation from donor to recipient.

^b Two-step allele replacement (35).

was unable to induce expression of the reporter fusion in two different energy stress assays: entry into stationary phase in buffered LB (Fig 2A) and glucose limitation in a minimal medium (Fig. 2B). In contrast, the *rsbQΔ2* null mutant retained the ability to respond to signals of environmental stress. Logarithmically growing cells were challenged by the addition of either 0.3 M salt (Fig. 2C) or 4% ethanol (Fig. 2D). Response to ethanol stress was similar in the mutant and wild-type strains, whereas response to salt stress was decreased

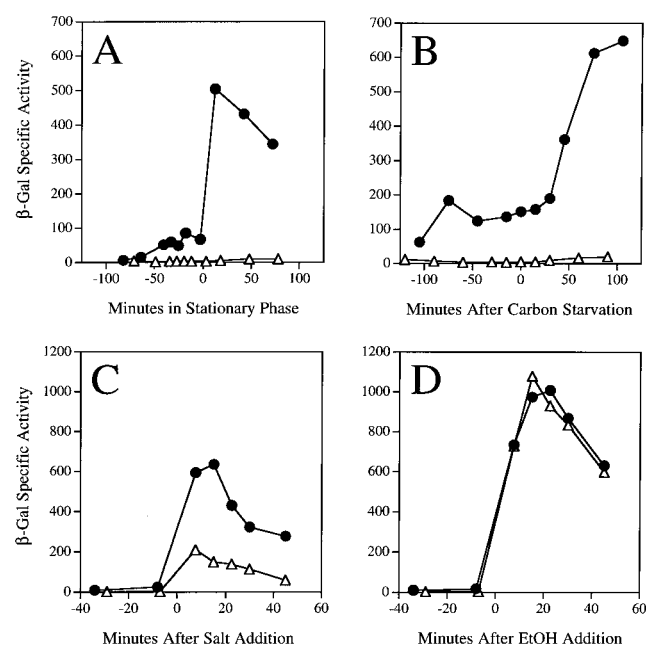


FIG. 2. RsbQ function is required for energy stress signaling. Effect of the *rsbQΔ2* allele on β -galactosidase accumulation from a σ^B -dependent transcriptional fusion in response to entry into stationary phase in buffered LB medium (A), carbon and energy starvation in a minimal medium containing 0.05% glucose (B), addition of 0.3 M NaCl to logarithmically growing cells in buffered LB (C), or addition of 4% ethanol (EtOH) to logarithmically growing cells in buffered LB (D). Time zero indicates when the stress was imposed on wild-type PB198 (●) and on the PB605 mutant bearing the *rsbQΔ2* allele (Δ).

about threefold by the *rsbQΔ2* null allele. Although it appears that *rsbQ* plays a role in response to salt stress, perhaps due to an effect on the levels of σ^B in unstressed cells, *rsbQ* function is clearly not required for the environmental stress response as it is for the energy stress response.

The phenotype elicited by the *rsbQΔ2* null allele was essentially the same as that previously reported for the *rsbPΔ1::spc* null allele (41). This raised the possibility that the in-frame deletion in *rsbQ* was polar on *rsbP* gene expression. To address this concern, we performed a complementation experiment in the *rsbQ* null background. σ^B activation in response to stationary-phase stress was restored by insertion of a single copy of *rsbQ*⁺ at the *thr* locus, in *trans* to the *rsbQΔ2* allele at the *rsbQ* locus (data not shown). We therefore conclude that *rsbQ* encodes a positive regulator which plays an essential role in transmitting energy stress signals to σ^B .

Activation of σ^B by RsbP requires RsbQ function in vivo. In the parallel environmental signaling pathway (Fig. 1), the RsbU phosphatase is activated by direct protein-protein interaction with RsbT, the product of the gene immediately upstream from *rsbU*. RsbT therefore functions as a regulatory subunit of the RsbU environmental signaling phosphatase (21, 44). To probe whether a similar relationship exists between RsbQ and the RsbP energy-signaling phosphatase, we first asked if the two proteins could interact to activate the yeast

TABLE 2. Activation of the yeast two-hybrid system by RsbQ paired with RsbP, RsbT, or RsbU^a

Fusion protein in pGAD424	Activation result for different fusion proteins in pGBT9			
	RsbQ (0) ^b	RsbP (+)	RsbT (0)	RsbU (0)
RsbQ	ND	++	ND	0
RsbP	++	ND	0	0
RsbT	ND	+	ND	++
RsbU	0	ND	++	ND

^a ++, strong blue color in colony lift assay; +, weak blue; 0, no blue detected; ND, not done.

^b The symbol shown in parentheses indicates the basal activation elicited by the protein of interest that was fused to the DNA binding domain carried by pGBT9 and tested in the absence of a companion activating domain carried by pGAD424.

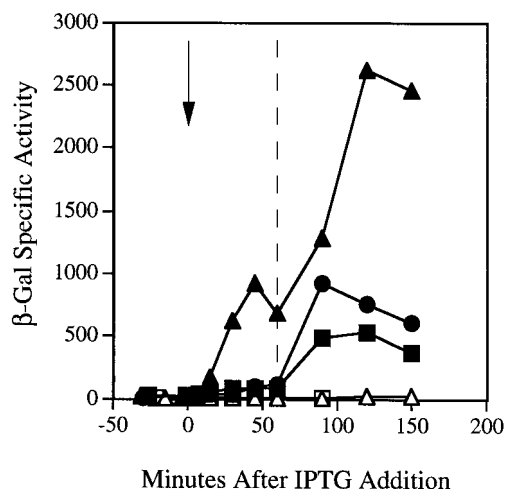


FIG. 3. Overexpression of either RsbP or RsbQ cannot compensate for the loss of the other. At time zero (arrow) IPTG was added to logarithmically growing cells to induce overexpression of either *rsbP* (carried in pKV2) or *rsbQ* (carried in pMB64). Samples were periodically removed for assay of β -galactosidase activity both before and after cells had entered stationary phase in buffered LB medium (indicated by the dashed line at 60 min). ●, wild-type PB198; ■, wild-type PB606 with *rsbQ* overexpressed; ▲, wild-type PB607 with *rsbP* overexpressed; □, PB719 mutant bearing the *rsbPΔI::spc* allele with *rsbQ* overexpressed; △, PB716 mutant bearing the *rsbQΔ2* allele with *rsbP* overexpressed.

two-hybrid system. As shown in Table 2, RsbQ and RsbP together activated transcription of the yeast reporter fusion. Moreover, this interaction was specific in that there was no detectable activation when RsbQ was paired with RsbU or when RsbP was paired with RsbT.

We next used overexpression experiments to further characterize the relationship between RsbQ and RsbP. We moved each gene into a multicopy plasmid that placed its expression under control of the LacI-repressible, IPTG-inducible Pspac promoter. pMB64 (bearing the *rsbQ* coding region) was introduced into the wild-type strain and also into a strain harboring the *rsbPΔI::spc* null allele, whereas pKV2 (bearing the *rsbP* coding region) was introduced into the wild-type strain and also into a strain harboring the *rsbQΔ2* null allele. To permit measurement of σ^B activity, these four strains each contained a single-copy *ctc-lacZ* reporter fusion.

As shown in Fig. 3, overexpression of *rsbQ* during early logarithmic growth did not immediately induce expression of the reporter fusion in wild-type cells. Induction occurred only when cells encountered the energy stress of stationary phase, and the magnitude of this induction was reduced compared to the wild-type control in which *rsbQ* was not overexpressed. Moreover, overexpression of *rsbQ* could not compensate for the loss of *rsbP* function in the strain bearing the *rsbPΔI::spc* null allele. The phenotype manifested in this latter strain was the same as that of the *rsbP* null mutant without overexpression of *rsbQ*: *ctc-lacZ* was not induced by energy stress. The failure of *rsbQ* overexpression to induce σ^B activity in logarithmically growing cells stands in marked contrast to the results obtained by Yang et al. (44) in their study of the environmental stress pathway. These authors found a strong, *rsbU*-dependent

induction of σ^B activity immediately upon overexpression of the *rsbT* regulator. From the experiments shown in Fig. 3, we conclude that RsbQ does not act as a regulatory subunit for its cognate phosphatase, as does RsbT.

We then performed the reciprocal experiment to determine the dependence of *rsbP* upon *rsbQ*. As shown in Fig. 3, overexpression of *rsbP* during early logarithmic growth caused a rapid and significant increase in σ^B activity in wild-type cells. This activation occurred well before the energy stress of stationary phase, whereupon a further increase in σ^B activity could be seen. However, this result required the presence of at least one copy of *rsbQ*. In the absence of *rsbQ* function, overexpression of *rsbP* had no apparent effect. The phenotype manifested in this experiment was therefore the same as that of the *rsbQΔ2* null mutant without overexpression of *rsbP*: *ctc-lacZ* was not induced by energy stress. We conclude that overexpression of either *rsbQ* or *rsbP* is not sufficient to compensate for the loss of the other regulator.

Serine 96 and histidine 247 of RsbQ are important for energy stress signaling. In order to suggest the in vivo function of RsbQ, we first considered the Cluster of Orthologous Groups of proteins (COG) to which it belonged. The COG database (<http://www.ncbi.nlm.nih.gov/COG>) is a phylogenetic classification of proteins encoded by 44 complete microbial genomes representing 30 lineages, and it allows detection of both close and distant relationships (37). RsbQ (formerly called YvfQ) is a member of COG 0596—predicted hydrolases or acyltransferases (α/β hydrolase superfamily). Examples of the α/β hydrolase superfamily are widely distributed and manifest unusually diverse catalytic activities, functioning variously as proteases, lipases, peroxidases, epoxide hydrolases, dehalogenases, and esterases (23, 25). While their primary amino acid sequences are not highly conserved, members of the α/β hydrolase superfamily share a common three-dimensional core structure containing the catalytic domain. Within this domain is a catalytic triad in an invariant order: nucleophilic residue/acidic residue/histidine residue (23, 25). The residues comprising this triad can be broadly separated in the primary sequence but are brought together in the tertiary structure.

Comparison of the predicted RsbQ sequence to well-characterized α/β hydrolases yielded good candidates for the catalytic triad residues. Based on the alignments shown in Fig. 4, we chose serine 96 as the potential catalytic nucleophile for RsbQ and histidine 247 as the potential catalytic histidine. In contrast, the assignment of aspartate 219 as the acidic residue was less certain due to other nearby candidates. We therefore focused our analysis on serine 96 and histidine 247.

If serine 96 and histidine 247 comprised part of a catalytic triad in RsbQ and if the enzymatic activity of RsbQ was important for its signaling function, we would predict that changing either residue would affect σ^B activation in response to energy stress. We therefore constructed two mutant versions of *rsbQ*, one in which an alanine replaced serine 96 (RsbQS96A) and another in which an alanine replaced histidine 247 (RsbQH247A). These mutant versions were substituted for the wild-type copy of *rsbQ* on the chromosome. The resulting mutant strains also contained a *ctc-lacZ* reporter fusion to measure σ^B activity. As shown in Fig. 5, replacement of either serine 96 or histidine 247 with alanine completely abolished induction of the reporter fusion in response to energy stress.



FIG. 4. Partial alignment of RsbQ with well-characterized members of the α/β hydrolase superfamily. The crystal structures have been solved and the residues comprising the catalytic triads have been suggested or established for BpoA2, a bromoperoxidase from *Streptomyces aureofaciens* (16); DhlA, a haloalkane halohydrolyase from *Xanthobacter autotrophicus* (14, 28, 29, 39, 40); and EchA, an epoxide hydrolase from *Agrobacterium radiobacter* (24, 31). RsbQ and these other family members are aligned by the Reverse Position Blast algorithm (4) against the consensus for the α/β superfamily found in the Conserved Domain Database, maintained by the National Library of Medicine (www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). RsbQ was 23.5% identical to the α/β hydrolase fold consensus over a 226-residue overlap ($E = 3 \times 10^{-15}$). The two regions surrounding the proposed catalytic triad residues are shown here. Residues in boldface indicate the positions conserved in at least three of the four proteins. (A) Residues surrounding the proposed nucleophile S96 of RsbQ, shown here in larger font together with the S \rightarrow A substitution. The catalytic nucleophiles in the other proteins and in the consensus are also identified by the larger font. (B) Residues surrounding the proposed acidic residue D219 and the proposed histidine residue H247 of RsbQ, shown here in larger font together with the H \rightarrow A substitution at H247. The catalytic acidic and histidine residues in the other proteins and in the consensus are also identified by the larger font.

These results suggest that the catalytic activity of RsbQ is required for energy stress signaling. However, to strengthen this conclusion, it was necessary to test the possibility that the S96A and H247A alterations might only indirectly perturb catalytic activity as a result of their primary effects on RsbQ structure, synthesis, or stability.

The S96A and H247A alterations do not detectably affect RsbQ structure or accumulation. To address the possibility

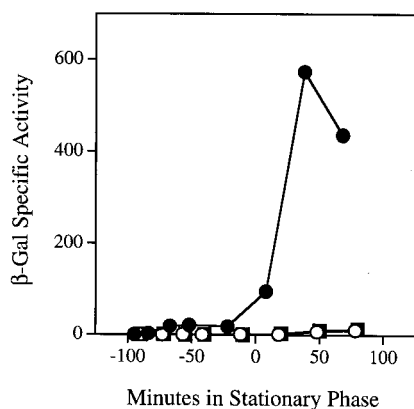


FIG. 5. Residues S96 and H247 of RsbQ are required for energy stress signaling. β -Galactosidase accumulation from a σ^B -dependent transcriptional fusion as cells were grown to stationary phase in buffered LB medium. ●, wild-type PB198; ○, PB634 mutant bearing the *rsbQ*S96A allele; ■, PB632 mutant bearing the *rsbQ*H247A allele.

that the S96A and H247A alterations might affect RsbQ structure, we used limited trypsin proteolysis to compare the conformation of mutant and wild-type RsbQ proteins. His-tagged proteins were overexpressed in *E. coli* and purified as described in Materials and Methods. As shown in Fig. 6, the mutant proteins were not substantially altered in their trypsin susceptibility relative to the wild type. This implies that the native conformation of RsbQ was not materially disrupted by the S96A or H247A substitutions.

To determine whether the S96A and H247A substitutions

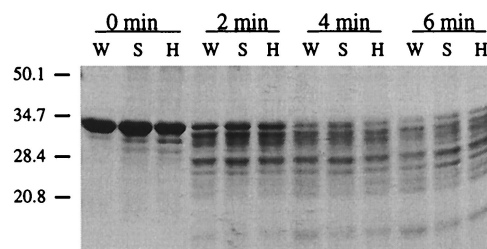


FIG. 6. The S96A and H247A substitutions do not affect trypsin susceptibility of RsbQ. His-tagged versions of wild-type RsbQ (W), the S96A substitution (S), and the H247A substitution (H) were purified by metal affinity chromatography and subjected to limited trypsin digestion. Samples were removed at 2-min intervals and digestion was terminated by addition of phenylmethylsulfonyl fluoride. Protein fragments were separated by SDS-polyacrylamide gel electrophoresis and stained with Coomassie blue. The mobilities of the marker protein are given on the left, labeled with their masses (in kilodaltons).

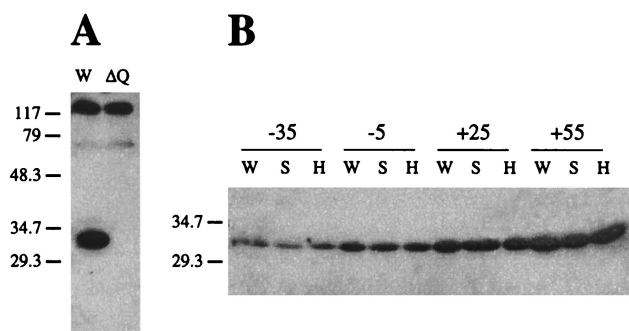


FIG. 7. The S96A and H247A substitutions do not affect steady-state levels of RsbQ in vivo. Polyclonal antibody raised against purified RsbQ was used to probe Western blots of whole-cell extracts separated by SDS-polyacrylamide gel electrophoresis. (A) The anti-RsbQ antibody detected three clear signals in the wild-type PB2 extract (W). One of these signals was absent in the extract from the PB604 mutant bearing the *rsbQ* Δ 2 allele (Δ Q); this signal had the mobility expected for RsbQ (29.9 kDa). Marker protein masses (in kilodaltons) are shown on the left. (B) The wild-type PB2 (W), the PB633 mutant bearing the *rsbQS96A* allele (S), and the PB631 mutant bearing the *rsbQH247A* allele (H) were grown in buffered LB medium. The time before or after entry into stationary phase at which each group of three samples was taken is indicated in minutes at the top. At each time point, the anti-RsbQ antibody detected a signal of similar strength in all three strains. This signal increased in magnitude (relative to total cell protein) as cells entered stationary phase. Only that portion of the gel containing the RsbQ signal is shown; mobilities of the 29.3- and 34.7-kDa marker proteins are given on the left.

affected synthesis or stability of the mutant proteins, we used Western blotting experiments to compare steady-state, in vivo levels of mutant and wild-type RsbQ. Polyclonal antibody against RsbQ was raised by injecting rabbits with the His-tagged protein purified from *E. coli*; the specificity of this antibody is shown in Fig. 7A. When tested against a whole-cell extract of wild-type cells that had been subjected to SDS-polyacrylamide gel electrophoresis, the anti-RsbQ antibody detected three proteins with distinctly different mobilities. One of these proteins had the mobility expected for RsbQ and was missing from an extract of the *rsbQ* Δ 2 null mutant. Figure 7B shows that the signals from this protein were the same in the wild type and the two substitution mutants at multiple points in the growth curve. We infer from these results that the alterations in the mutant proteins had no significant effect on synthesis or stability of RsbQ in vivo.

From the sum of these analyses, we conclude that the loss of energy stress signaling caused by the RsbQS96A and RsbQH247A substitutions most likely resulted from altered catalysis and not from a disruption of protein structure, synthesis, or stability. We therefore propose that RsbQ possesses an enzymatic activity that plays an essential role in the energy stress signaling pathway which activates the general stress response.

DISCUSSION

Bacteria use a variety of mechanisms to sense and respond to impending energy stress. These include control of individual enzyme activities by energy charge (6) as well as control of regulatory proteins by reaction centers which most frequently sense oxygen tension or electron flux (7, 38). Some of these

reaction centers are provided by PAS domains, and in the best-characterized examples the PAS domain binds a chromophore specific for the parameter sensed, such as heme in the oxygen sensors FixL and Dos (11, 15) and flavin adenine dinucleotide in the redox sensors NifL and Aer (30, 34). In eukaryotic signaling pathways, PAS domains are also known to mediate ligand binding or protein-protein interactions (38), but these aspects of PAS function have not yet been demonstrated in prokaryotic systems.

Here we report that RsbQ is a positive regulator required for energy stress activation of the σ^B transcription factor. Moreover, RsbQ interacts in the yeast two-hybrid system with the RsbP energy-signaling phosphatase, which contains a PAS domain in its amino-terminal region (41). We presently have no additional biochemical evidence to corroborate this interaction, nor have we detected a hypothetical ligand or chromophore which the PAS domain of RsbP might bind. However, we have shown that the predicted catalytic activity of RsbQ is important for its signaling role, and on the basis of our genetic analysis we can propose a model of RsbQ function.

We first considered the possibility that RsbQ might hydrolyze a small molecule and thereby produce a direct activator of the RsbP phosphatase. However, simply overexpressing RsbQ in growing cells did not trigger the energy stress response (Fig. 3), so this model would also require that RsbQ activity is somehow regulated by energy stress. Such energy stress regulation was not apparent even when the cells that were overexpressing RsbQ subsequently entered stationary phase. In contrast, overexpressing the RsbP phosphatase in growing cells did immediately induce a mock energy stress response, and this response was intensified upon subsequent challenge with an authentic energy stress. We interpret these results to indicate that in unstressed cells the RsbP phosphatase has a low intrinsic activity which normally sets the steady-state level of functioning σ^B , a necessary feature of its autocatalytic induction mechanism (19). Overexpression of RsbP would rapidly increase the total amount of this intrinsic phosphatase activity, and, therefore, of functioning σ^B . Moreover, we presume that RsbP activity in vivo is stimulated by energy stress (41). Therefore, induction of σ^B activity would occur upon energy stress in wild-type cells, in which RsbP levels are normal, and also in cells in which RsbP levels have been artificially elevated.

We infer that the catalytic activity of RsbQ is required for RsbP phosphatase activity—both for the low intrinsic activity as well as for the activity stimulated by energy stress (Fig. 3). We therefore propose that the target of the RsbQ activity is RsbP itself. In this view, the RsbP phosphatase would be synthesized in an inactive form and converted to an active form by RsbQ. RsbQ could conceivably modify the covalent structure of RsbP by means of its presumed hydrolytic or acyltransferase activity or perhaps modify the hypothetical chromophore which binds the PAS domain of RsbP.

The relationship of the RsbQ and RsbP energy stress regulators is clearly different from that of the RsbT and RsbU environmental stress regulators. RsbT lies directly on the signaling pathway, and it passes the environmental stress signal to the RsbU phosphatase via a specific protein-protein interaction (21, 44). In contrast, RsbQ does not appear to lie directly on the energy signaling pathway but instead acts catalytically to render the RsbP phosphatase competent to receive the signal.

This difference in the relationship between the terminal regulators of the energy and environmental branches suggests that the upstream sections of these two signaling pathways will also prove to function by distinctly different mechanisms.

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