Phosphorylation and RsbX-Dependent Dephosphorylation of RsbR in the RsbR-RsbS Complex of Bacillus subtilis

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In Bacillus subtilis, the general response to stress is controlled by the activity of the alternative sigma factor, σB (7, 13, 15, 16, 24, 35), which, when active, associates with RNA polymerase and allows the transcription of a set of genes conferring multiple resistances on the cell (23–25). In the absence of stress, the activity of σB is inhibited by the formation of a complex with RsbW (5), an anti-sigma factor which is also a kinase for RsbV (5, 6, 11, 12). A partner-switching mechanism involving these three proteins, RsbV, RsbW, and σB, has been shown to control the activity of σB (3). A variety of stresses triggers the release of σB from RsbW, which then forms an alternative complex with the newly dephosphorylated RsbV. The phosphorylation state of RsbV is therefore the key factor for the regulation of σB. It is controlled by the balance between the kinase activity of RsbW and the activities of two specific phosphatases for phosphorylated RsbV (RsbY-P), namely, RsbP (32) and RsbU (34, 36).

In this study with purified proteins, we used mutant RsbR to analyze the role of its phosphorylatable threonine residues. The results show that the phosphorylation of either of the two RsbT-phosphorylatable threonine residues (T171 and T205) in RsbR enhanced the kinase activity of RsbT towards RsbS. However, it appeared that RsbT preferentially phosphorylates T171. We also present in vitro evidence that identifies RsbX as a potential phosphatase for RsbR T205.

In the sigB operon, upstream of rsbT and rsbU, the rsbR and rsbS genes have been shown to be involved in the environmental stress response (2, 14, 19). It has recently been shown that RsbR and RsbS form a high-molecular-mass complex (~1 MDa) in vitro, and there is evidence for its existence in vivo (9). Unlike its constituent individual proteins, the RsbR-RsbS complex is capable of binding to RsbT, forming a stable complex (RsbR-RsbS-RsbT) in which RsbT is trapped and unable to associate with and activate RsbU. The kinase activity of RsbT towards RsbS is counterbalanced by the phosphatase activity of RsbX, the phosphatase for RsbS-P (9). It has also been observed that if RsbR is phosphorylated by RsbT before the formation of the complex with RsbS, then the kinase activity of RsbT towards RsbS in the RsbR-P-RsbS complex is increased and the balance between the kinase activity of RsbT and the phosphatase activity of RsbX tilts in favor of RsbT (9). Under these conditions, RsbS-P accumulates, and RsbT, which has no affinity for the RsbR-P-RsbS-P complex, is released and can associate with and activate RsbU. It has therefore been suggested that a key step in the transduction of the signal is the phosphorylation of RsbR. RsbR can be phosphorylated by RsbT on two threonine residues, T171 and T205, and genetic studies have revealed that these two residues have an important role in the activation of σB (2, 14). These residues are highly conserved in RsbR homologues in close relatives and also in three paralogs encoded by the B. subtilis genome, namely, YkoB, YojH, and YqhA (1).

In this paper we report the effect of the phosphorylation of each of the threonine residues of RsbR in the RsbR-RsbS complex on the kinase activity of RsbT towards RsbS. We have also discovered that RsbX, which has been described as a specific phosphatase for RsbS (36), is also a phosphatase for phosphorylated T205 of RsbR in the RsbR-RsbS complex.

MATERIALS AND METHODS

Site-directed mutagenesis of RsbR. Site-directed mutagenesis of RsbR was carried out by the method described in reference 17, in which Pfu DNA polymerase (Stratagene) was used in PCR. Two overlapping DNA fragments were amplified by PCR from genomic DNA (B. subtilis SG38) with two sets of primers. The first fragment was amplified with a forward primer corresponding to the 5′.
end of the rsbR gene and an added NdeI site and a reverse, internal primer carrying the mutated gene codon; the second fragment was amplified with a forward primer overlapping the 3' end of the first amplified fragment and carrying the same mutated codon and a reverse primer corresponding to the 3' end of the rsbR gene, which also adds a BamHI site. A final PCR was carried out with the two end primers and the two fragments generated from the first PCR as the DNA template. The fragment was further digested by NdeI and BamHI and cloned into pET-11a (Novagen). The resulting DNA fragments were cloned into pET-11a, and the presence of the different mutations was confirmed by sequencing.

**Purification of the proteins and gel filtration experiment.*** *Escherichia coli* BL21 (DE3) was used for the overexpression of all proteins, which was induced consisting of 2 complex and for complexes made of mutant RsbRs and RsbS. Each complex RsbR was measured as described previously (9) for the wild-type RsbR-RsbS high-molecular-weight complexes by the use of a gel electrophoresis (PAGE) and stained with Coomassie blue.

This technique was also applied to isolate complexes of RsbS and mutated versions of RsbR used in this study. Since the complexes are composed of an uncertain number of units (between 8 and 10) believed to be three RsbR molecules complexed with one RsbS molecule, the mass concentration of the complex was converted to a molar concentration by using the molecular weight of one unit.

**Enzymatic measurements.*** The kinase activity of RsbT towards RsbS and RsbR was measured as described previously (9) for the wild-type RsbR-RsbS complex and for complexes made of mutant RsbRs and RsbS. Each complex consisted of 2 μM RsbS was incubated with 0.2 μM RsbT at 30°C in phosphorylation buffer (50 mM Tris-HCl [pH 7.5], 50 mM KCl, 2 mM MgCl₂, 1 mM diithiothreitol) supplemented with 0.1 mM ATP and 20 μCi of [γ-32P]ATP. At time intervals, reactions were stopped by adding 3X SDS loading buffer and heating the mixtures for 3 min at 95°C. Samples were loaded onto SDS-15.5% PAGE gels, and the gels were exposed to a phosphor screen. The bands were quantified by using the Image Gauge program, version 3.3 (FLA-3000 phosphorimager; Fujifilm).

**Dephosphorylation reactions were carried out essentially as described in reference 22.** Complexes of RsbS and RsbR-P and mutant RsbR-P proteins were incubated with phosphatases in dephosphorylation buffer (50 mM Tris-HCl [pH 7.5], 50 mM KCl, 10 mM MgCl₂, and 1 mM MnCl₂). The reactions were performed at 30°C and stopped at intervals by mixing samples with loading buffer containing 8 M urea, 5% β-mercaptoethanol, 10 mM Tris-HCl (pH 8.5), and 1% bromophenol blue. Samples were subjected to alkaline urea-PAGE. Gels were prepared with 200 mM Tris-HCl buffer (pH 8.9)-4% acrylamide-8 M urea for the stacking gel and with 8% acrylamide-4 M urea for the separating gel. This technique was used to denature the RsbR-RsbS complex before electrophoresis, because the complex is too large to allow it to enter a usual native gel. Gel electrophoresis was conducted in Tris-glycine buffer (pH 8.5) at 150 V for 3 h at room temperature. Protein bands were visualized by Coomassie blue staining, and purified RsbR was used as a marker. Gels were scanned (Adobe Photoshop 5.0), and the intensities of the bands were measured using Scion Image Beta 4.02 software.

**RESULTS**

**Electrophoretic mobilities of mutant RsbR**s. *We introduced mutations in the rsbR sequence (i) to convert the threonine residues at positions 171 and 205 to alanine to prevent phosphorylation and (ii) to convert the same residues to aspartic acid to mimic the phosphorylated forms. The resulting altered RsbR proteins, RsbR<sup>T171A</sup>, RsbR<sup>T205A</sup>, RsbR<sup>T171D</sup>, and RsbR<sup>T205D</sup>, were purified in the same way as RsbR (9), and their electrophoretic mobilities were analyzed by native gel electrophoresis (Fig. 1A). When compared to RsbR (for alanine substitutions) or RsbR-P (for aspartic acid substitutions), only the electrophoretic mobilities of RsbR<sup>T171A</sup> (Fig. 1A, lane 2) and RsbR<sup>T205D</sup> (Fig. 1A, lane 9) differed significantly from that of wild-type RsbR, possibly because of a conformational change in RsbR as a result of these mutations. RsbR<sup>T171A</sup> was also affected in its ability to act as a coantagonist with RsbS in binding to RsbT (see Fig. 2 and Table 1). We therefore made an additional mutant RsbR in which we replaced T171 with an asparagine residue (RsbR<sup>T171N</sup>). Electrophoretic mobilities of RsbR<sup>T205A</sup> and RsbR<sup>T205D</sup> after phosphorylation by RsbT were identical to that of RsbR-P.

We noted that most of our preparations of RsbR-P also showed a minor band that migrated to the same position as RsbR<sup>T205D</sup> (Fig. 1B). Given that RsbR is phosphorylatable at two threonines, we had previously assumed that the two bands corresponded to monophosphorylated and diphosphorylated RsbR. The present result (Fig. 1) leads to a different interpretation: the faster-running band might correspond to the phosphorylation only of T171, and the slower-running band might correspond to the phosphorylation only of T205. Indeed, RsbR<sup>T171D</sup> and RsbR<sup>T205D</sup>-P (Fig. 1A) have the same mobility as most of RsbR-P (Fig. 1B), whereas RsbR<sup>T205D</sup> has the same mobility as the minor band in RsbR-P. It is therefore possible that RsbT phosphorylates RsbR mainly (-90%) at T171 and to only a slight extent (-10%) at T205. This conclusion is reinforced by the results obtained with the mimics of a doubly phosphorylated RsbR protein, RsbR<sup>T171P</sup>-P and RsbR<sup>T205P</sup>-P, which migrate faster than wild-type RsbR-P (Fig. 1A), suggesting again that only one threonine residue of RsbR is phosphorylated. The same conclusion is also in accordance with the results of mass spectroscopic analysis of wild-type RsbR-P, which clearly showed the presence of only one...
phosphate group per mole (results not shown). However, we cannot discount the possibility that the phosphorylation of RsbR might be different when RsbR is in the high-molecular-weight complex with RsbS (9).

RsbR-RsbS-RsbT complex formation with mutated versions of RsbR. The role of the RsbR-RsbS complex is to trap RsbT prior to stress (9). We first checked the ability of the RsbR mutants to form a high-molecular-weight complex with RsbS only. All the RsbR mutants, including their phosphorylated counterparts, could form high-molecular-weight complexes with RsbS (data not shown). We then investigated the ability of all the complexes containing RsbS and either the mutant RsbRs or their phosphorylated forms to bind to RsbT. The absence or presence of RsbT in the complexes was analyzed by SDS-PAGE of the first fractions of the Superose-12 gel filtration column (data not shown). We used the mutant versions of RsbR described above in RsbT kinase assays. Complexes for all of the altered RsbRs with RsbS were obtained and purified by gel filtration and then mixed with a standardized amount of RsbT and radiolabeled with ATP. The results revealed that the kinase activity of RsbT towards RsbS was enhanced when RsbS was complexed with the phosphorylated proteins RsbR-P, RsbRT205A-P, and RsbRT171N-P (Fig. 3). All these versions of RsbR are monophosphorylated, and therefore we conclude that, whichever threonine residue is phosphorylated in RsbR, be it T171 or T205, the same enhanced activity of RsbT towards RsbS is observed. This finding also implies that the phosphorylation of one threonine residue of RsbR is sufficient to affect the rate of phosphorylation of RsbS by RsbT.

We performed similar analyses with complexes that mimic diphosphate RsbRs, i.e., RsbRT205D-P, RsbRT171D-P, and RsbRT171D,T205D. These data revealed that the addition of two negative charges to RsbR abolished the enhancement of the activity of RsbT (data not shown), which was even slower than in the unphosphorylated RsbR-RsbS complex. This double modification of RsbR also prevents the RsbR-RsbS complex from trapping RsbT (Fig. 2), which suggests either that RsbT is repelled by these additional negative charges or that the phosphorylation of the two threonines affects the structure of RsbR-RbsS in such a way that it no longer binds to RsbT.

**TABLE 1. Presence or absence of binding to RsbT in different combinations of complexes of RsbS and RsbR or RsbR mutants**

| Complex                  | Binding to RsbT
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<tr>
<td>RsbR-RsbS</td>
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<tr>
<td>RsbR–P–RsbS</td>
<td>+</td>
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<tr>
<td>RsbRT171A–RsbS</td>
<td>–</td>
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<tr>
<td>RsbRT171N–RsbS</td>
<td>–</td>
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<tr>
<td>RsbRT171P–RsbS</td>
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<td>RsbRT205A–P–RsbS</td>
<td>+</td>
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<tr>
<td>RsbRT171P–P–RsbS</td>
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<tr>
<td>RsbRT171D–P–P–RsbS</td>
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<td>RsbRT205D–P–P–RsbS</td>
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*a Experimental procedures were as described for Fig. 2.

**Effect of the phosphorylation of RsbR on the kinase activity of RsbT.** It was previously demonstrated that the kinase activity of RsbT towards RsbS was enhanced by a factor of about 5 when RsbR in the RsbR-RsbS complex was phosphorylated (9). To establish whether the phosphorylation of one threonine residue was sufficient for this effect (and if so, which one), we used the mutant versions of RsbR described above in RsbT kinase assays. Complexes for all of the altered RsbRs with RsbS were obtained and purified by gel filtration and then mixed with a standardized amount of RsbT and radiolabeled with ATP. The results revealed that the kinase activity of RsbT towards RsbS was enhanced when RsbS was complexed with the phosphorylated proteins RsbR-P, RsbRT205A-P, and RsbRT171N-P (Fig. 3). All these versions of RsbR are monophosphorylated, and therefore we conclude that, whichever threonine residue is phosphorylated in RsbR, be it T171 or T205, the same enhanced activity of RsbT towards RsbS is observed. This finding also implies that the phosphorylation of one threonine residue of RsbR is sufficient to affect the rate of phosphorylation of RsbS by RsbT.

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**Phosphorylation rates of RsbR T171 and T205 in the RsbR-RsbS complex.** We have demonstrated that when RsbR is not in a complex with RsbS, RsbT preferentially phosphorylates T171 of RsbR (Fig. 1). As the formation of the RsbR-RsbS complex might affect the structure of RsbR, we wondered if the formation of the RsbR-RsbS complex might affect the preference of RsbT for T171 over T205. We therefore used complexes formed with RsbS and RsbRT171N or RsbRT205A, which left only one threonine available for phosphorylation, T205 or T171, respectively. Figure 4A shows that the rates of phosphorylation of the two threonines of RsbR, T171 and T205, were almost identical to and only slightly lower than that of wild-type RsbR.

We then asked if the rate of phosphorylation of one threonine residue of RsbR could be affected by the phosphorylation state of the other. The results (Fig. 4B) showed that in complexes formed with RsbRT205D, the rate of phosphorylation of T171 was almost identical to that observed in complexes.
formed with wild-type RsbR. However, with complexes formed with RsbR<sup>T171N</sup>-P, the phosphorylation of RsbR T205 by RsbT appeared to be delayed. The proximity of these two residues may explain this delay.

The C-terminal domain of RsbR is similar in sequence to the whole of SpoIIAA. RsbR T205 corresponds to S58 of SpoIIAA (the phosphorylatable residue in SpoIIAA), and RsbR T171 corresponds to H24. The solution structure of SpoIIAA has been solved by nuclear magnetic resonance (21), and it has been found that H24 and S58 face each other. It has recently been shown by <sup>1</sup>H nuclear magnetic resonance pH titration of H24 in SpoIIAA that H24 interacts with the phosphate group of S58-P (10). As previously suggested (14), if the overall structure of the C-terminal domain of RsbR is comparable to that of SpoIIAA, an interaction between RsbR T205 and T171 is highly probable. This could explain the negative effect on the phosphorylation rate of T205 in RsbR<sup>T171N</sup>-P, which could be reinforced by the two negative charges on a phosphothreonine residue. The single negative charge of an aspartic acid might only partially mimic phosphorylation. The inhibition of the phosphorylation of T205 by T171-P is consistent with our previous conclusion that RsbR is phosphorylated mainly on one residue, T171.

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Dephosphorylation of RsbR-P by RsbX in the RsbR-RsbS complex. RsbR can be phosphorylated by RsbT (14), but to date no phosphatase for RsbR has been reported. Here we present evidence that RsbR in the RsbR-RsbS complex can be dephosphorylated by RsbX, the phosphatase for RsbS. The RsbR<sup>T171N</sup>-P-RsbS complex was mixed with RsbX, and samples of the reaction were analyzed by urea-alkaline gel electrophoresis. The intensity of the band corresponding to RsbRT171N-P gradually diminished, while a band corresponding to RsbR T171N appeared (Fig. 5A). The experiment was repeated with purified RsbR<sup>T171N</sup>-P alone (i.e., not complexed with RsbS), but no dephosphorylation was observed (data not shown). Hence, the dephosphorylation of T205 of RsbR by RsbX can occur only when RsbR is in a complex with RsbS. The fact that RsbX has previously been reported to be unable...
to dephosphorylate RsbR (14) can perhaps be explained by the need for RsbR to be in a complex with RsbS for RsbX to be active. Control experiments performed with either RsbU or the catalytic domain of SpoIIE as the phosphatase and using the RsbRT171N-P–RsbS complex as the substrate failed to produce the unphosphorylated form of RsbRT171N (data not shown). This result demonstrates, first, that the appearance of a new band was not due to the instability of the phosphorylated form of RsbR, and second, that the dephosphorylation of T205 of RsbR was due to RsbX specifically.

From these experiments, a turnover number of 0.08 \( \times 10^{-2} \) s\(^{-1}\) can be derived for the RsbX dephosphorylation activity towards T205 of RsbR (Fig. 5A), which is much lower than that of other PP2C phosphatases of \( B. subtilis \), such as RsbU or SpoIIE. Under the same conditions (buffer, Mg\(^{2+}\) and Mn\(^{2+}\) concentrations, and temperature), the turnover number for RsbU, the phosphatase for RsbV-P, in the presence of an equimolar amount of its activator RsbT reaches 6 \( \times 10^{-2} \) s\(^{-1}\) (O. Delumeau, M. D. Yudkin, and R. J. Lewis, unpublished data) and that for SpoIIE, the phosphatase for SpoIIAA-P, is 7 \( \times 10^{-2} \) s\(^{-1}\) (22). The rate of dephosphorylation of T205 of RsbR by RsbX is therefore 75 times lower than that of SpoIIE and activated RsbU. However, the in vitro phosphatase activity of purified RsbX towards RsbRT205-P (turnover number, 0.08 \( \times 10^{-2} \) s\(^{-1}\)) is more comparable to the activity of inactivated RsbU towards RsbV-P (0.3 \( \times 10^{-2} \) s\(^{-1}\)) than to that of RsbU activated by a molar equivalent of RsbT (O. Delumeau, M. D. Yudkin, and R. J. Lewis, unpublished data). To determine whether phosphorylation of the other threonine, T171, affected the rate of dephosphorylation of T205, we used the RsbRT171D-P–RsbS complex, but we found that the rate of dephosphorylation was increased only by a factor of 2 (data not shown). Given that RsbT activates RsbU by more than 20-fold, we are inclined to think that a 2-fold increase in RsbX activity is probably not physiologically significant.

We then assayed the dephosphorylation of T171-P using the RsbRT205A-P–RsbS complex. No dephosphorylation of T171-P was observed, either in the presence of RsbX (Fig. 5B) or with a large amount of RsbU and its activator RsbT (data not shown). We conclude that only T205-P in RsbR can be dephosphorylated by RsbX and that no phosphatase tested so far can dephosphorylate T171-P.

We exploited the fact that RsbX can dephosphorylate T205-P of RsbR to test whether RsbX could dephosphorylate RsbR-P in a phosphorylated RsbR-RsbS complex. This complex was prepared by incubating RsbR–RsbS with RsbT and [\( \gamma^{32}\)P]ATP, leading to phosphorylation of both RsbR and RsbS, before purifying the complex by gel filtration and mixing it with RsbX. While RsbX was active towards RsbS-P, whose band disappeared in less than 2 min, the intensity of the band corresponding to RsbR-P remained constant throughout the experiment (Fig. 5C). The fact that RsbX could not dephosphorylate RsbR-P, even in a complex with RsbS, suggests that the phosphorylation site of RsbR in the RsbR-RsbS complex is predominantly T171 and not T205.

**DISCUSSION**

This study shows that the phosphorylation of either of the two threonines (T171 or T205) of RsbR has a positive effect on the phosphorylation rate of RsbS by RsbT and therefore is expected to have a positive effect on \( \sigma^{\text{H}} \) activity in vivo. It has been shown previously that RsbT, a positive regulator of \( \sigma^{\text{H}} \), is trapped by the RsbR–RsbS complex unless RsbS is phosphorylated (9). The role of RsbX in the control of \( \sigma^{\text{H}} \) activity can therefore be seen as twofold: first, it takes part in the trapping of RsbT by forming a complex with the antagonist RsbS, and second, it exerts an inhibition on the kinase activity of RsbT towards RsbS. This inhibition, although not completely, is probably sufficient to keep RsbT trapped by the RsbR–RsbS complex (9). The phosphorylation of either of the two threonine residues of RsbR abolishes the inhibition of RsbT kinase activity. However, this study suggests that RsbT appears to phosphorylate preferentially T171 of RsbR, although predictions based on alignments of the C-terminal domain of RsbR with the three other members of the STAS family (4) of \( B. subtilis \) (SpoIIAA, RsbV, and RsbS) had suggested that T205 of RsbR is the preferred phosphorylation site, as this residue corresponds to the phosphorylatable serines of SpoIIAA (S58), RsbS (S59), and RsbV (S56). The fact that the preferred phosphorylation site is RsbR T171 contrasts with our discovery that RsbX, the known phosphatase for RsbS, dephosphorylates T205-P, and not T171-P, of RsbR. Whether this phosphatase activity is effective in vivo needs further investigation.

RsbX is overexpressed following the imposition of stress, and it plays a role in restricting the activity of \( \sigma^{\text{H}} \) and curtailing its activation after stress (18, 30, 33). In our study, RsbX phosphatase activity appeared to be very weak, and it would certainly need to be enhanced in vivo to play a physiological role. It has been proposed that RsbX is activated by stress signals, as the cellular levels of RsbX are insufficient to limit \( \sigma^{\text{H}} \)
activation (28, 29). The phosphatases involved in the stress response, RsbU, RsbP, and RsbX, in addition to the related phosphatase SpoIE, are extremely specific for their substrates and do not cross talk, despite the obvious sequence similarities between their substrates (31, 35). RsbU, RsbP, and SpoIE are regulated by a variety of mechanisms, and perhaps RsbX is also under some means of control, the biochemical basis for which remains unknown.

Taken together, the results presented in this study suggest that phosphorylation of RsbR by a stress-dependent mechanism could be the most upstream event in the environmental stress-signaling pathway defined by the Rsb proteins. The facts that the preferred phosphorylation site for RsbT is RsbR T171 and that the dephosphorylation by RsbX occurs only on T205 of RsbR also suggest either that there is an additional, unknown protein kinase which phosphorylates T205 of RsbR upon stress or that additional components of the stress-signaling pathway modify the preference of RsbT so that it phosphorylates T205 of RsbR in the case of stress. For example, the presence of paralogs of RsbR, YkoB and YojH, in RsbR-RsbS complexes purified in vivo (C.-C. Chen, et al., unpublished data) might affect this preference and/or allow interactions with other signaling partners. For instance, Obg, an essential GTP-binding protein, has been shown to be important in σB activation by stress, and a possible interaction between Obg and RsbT has been revealed by the yeast two-hybrid system (26, 27, 31).

The results of this in vitro study suggest a biochemical mechanism by which the stress signal might enter the σB activation pathway. The early event would be the stress-dependent phosphorylation of RsbR in the RsbR-RsbS complex, which would relieve the inhibition exerted on RsbT by RsbR and allow RsbT to phosphorylate RsbS efficiently. Once RsbS is phosphorylated, RsbT could bind to and activate RsbU, the positive regulator of σB activity. Our results predict that, if RsbT is the only kinase for RsbR, then the phosphorylation of the latter should occur on the T171 residue. How RsbX, for which we have demonstrated a phosphatase activity towards T205-P of RsbR in the RsbR-RsbS complex, acts to fulfill its role as the negative feedback phosphatase is still unclear. Nevertheless, a recent independent study of the system in vivo (20) confirms our in vitro results in three respects: that the phosphorylation of RsbR is crucial for the transduction of the signal, that RsbR is phosphorylated mainly at T171, and that RsbR dephosphorylation is dependent on RsbX.

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REFERENCES


