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A New Model of How Environmental Signals Control the General Stress Response in *Bacillus subtilis*

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In nature, microorganisms encounter a variety of different environmental conditions and their environment can change rapidly, especially on a microscopic scale. Therefore, effective stress responses are the key to how well microorganisms can survive. Under a specific stress condition, microorganisms can survive by turning on the protection mechanism to directly counter the current stress. But if this specific stress is growth limiting, the cell cannot respond well to future stresses because its ability to synthesize new proteins is also limited. In order to have more chance to survive in the unpredictable future stress condition, growth-limited microorganisms also need to change their cellular machinery to be resistant against a variety of stresses, and this is the so-called general stress response that is superimposed on the specific stress response (5, 6, 9).

The σ^B is an alternative transcription factor controlling general stress response in *Bacillus subtilis* (3, 10). In *B. subtilis* and its relative bacteria, such as *Listeria monocytogenes* and *Staphylococcus aureus*, the general stress resistance contributes the bacterial survival under stress conditions and the pathogenesis (7, 11). By activation of σ^B, *B. subtilis* changes the gene expression profile which increases the expression of more than 200 genes.

In the current model shown in Figure 1, two different types of stress signals, energy stress signals, such as carbon limitation and stationary phase, and environmental stress signals, such as heat shock and high salt, are transferred and activate σ^B through independent signal transduction branches (9). Among them, the environmental stress response branch has a unique partner-switching module that has the RsbU phosphatase as a target protein (12) and additional RsbR family as regulators (1, 4).

In the current model, RsbS is the sole antagonist and RsbRA (formerly RsbR) only facilitate the phosphorylation of RsbS by the RsbT kinase. The function of RsbRB (formerly YkoB), RsbRC (YojH), and RsbRD (YqhA) is uncertain and assumed it is similar to RsbRA function from the sequence homology. If this model is true, the alanine mutation on serine 59, the phosphorylation site, of RsbS will abolish any phenotype of RsbR family mutation. But the result shows that the strain has $RsbS_{S59A}$ and the triple deletion of RsbRA, RsbRB, and RsbRD has the similar phenotype of triple deletion of RsbR family (Figure 2). This result invalidates the current model for the environmental stress branch of σ^B dependent general stress response. With considering the requirement of RsbS (7, 12) and RsbR family members (1) for wild type σ^B activation by environmental stresses, this result suggests RsbR family members are co-antagonists that

function with the RsbS antagonist to negatively regulate the environmental stress response. Recently, Chen *et al.* (2) also demonstrated that RsbS alone cannot inhibit the activation of RsbT toward RsbU and it needs RsbRA for its function in vitro using an independent biochemical method.

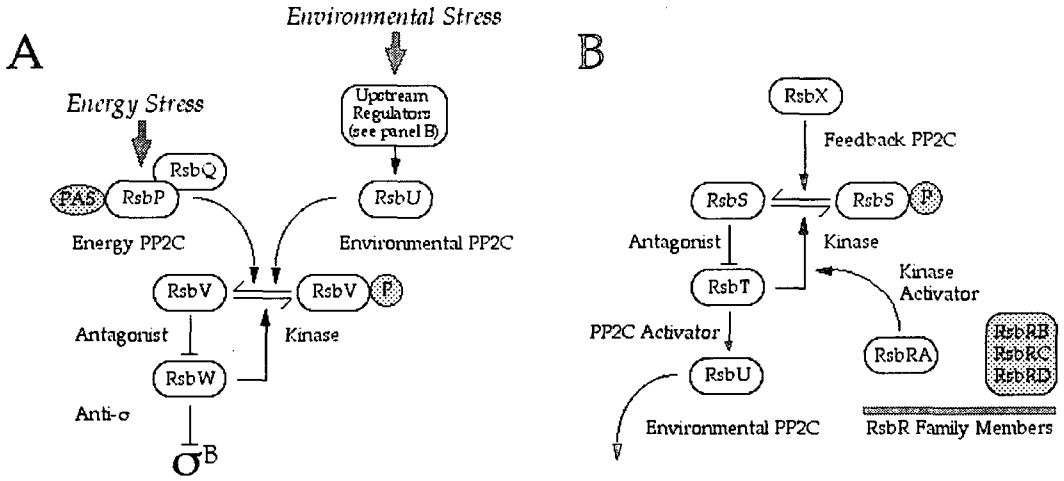

Figure 1. Current model of the σ^B signal transduction network (9). (A) Two signaling pathways converge on the RsbV antagonist and the RsbW anti-σ factor, the direct regulators of σ^B activity. The energy signaling pathway terminates with the RsbP phosphatase (Energy PP2C), which contains a PAS domain important for energy stress sensing. By contrast, the environmental signaling pathway terminates with the RsbU phosphatase (Environmental PP2C), which is activated by upstream signaling elements. RsbV-P is the antagonist form in unstressed cells. When activated by stress, either RsbP or RsbU can dephosphorylate RsbV-P, allowing RsbV to bind and inactivate the RsbW anti-σ factor. (B) In the environmental signaling branch RsbS and RsbT are paralogs of RsbV and RsbW, respectively. RsbS is the antagonist form in unstressed cells, and it binds and inactivates the RsbT switch protein/kinase. Following stress, RsbT phosphorylates RsbS, releasing RsbT to bind and activate the RsbU phosphatase. RsbRA (formerly RsbR) is a positive regulator which enhances RsbT kinase activity. RsbRA also acts negatively with its paralogs RsbRB (formerly YkoB), RsbRC (YojH), and RsbRD (YqhA); the mechanism of this negative action is unknown and is the focus of this study.

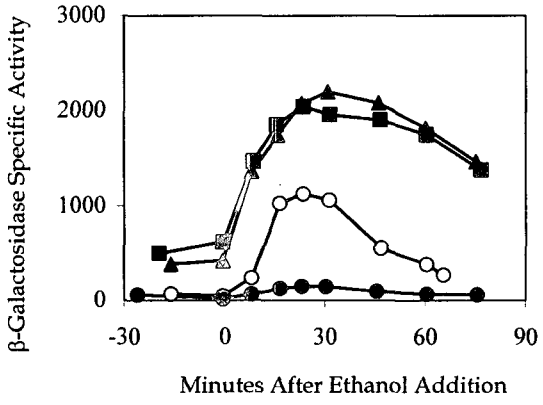

Figure 2. RsbS cannot fulfill its antagonist function in the absence of RsbR family members. The effects of the RsbS S59A alteration and the *rsbRA*, *rsbRB*, and *rsbRC* triple deletion were measured using a σ^B-dependent *ctc-lacZ* transcriptional fusion present in single-copy on the *B. subtilis* chromosome. Cells were grown to early logarithmic phase in buffered Luria broth lacking salt (BLB) and stressed by ethanol addition at time zero. Samples were taken at the indicated times and assayed for β-galactosidase activity as described in Materials and Methods. (○) wild type (PB198); (●) *rsbS*S59A (PB470); (▲) *rsbRAΔ*1 *rsbRBΔ*1::*kan rsbRDΔ*1::*spc* (PB530); and (■) *rsbRAΔ*1 *rsbRBΔ*1::*kan rsbRDΔ*1::*spc rsbS*S59A (PB742).

Since all RsbR family members are similar in sequence, it is tested how many RsbR family member is needed for their co-antagonist function. The alone strains which has deletion of the other three RsbR family members are made and tested σ^B activation by environmental stresses. The result shows the presence of any single co-antagonist is sufficient to decrease σ^B activity from the deletion of all RsbR family members and render it controllable by an environmental stress signal (Figure 3). This result furthermore suggests that not only RsbRA but also any RsbR family member can be the co-antagonist and they are nearly interchangeable co-antagonist functions under these experimental conditions.

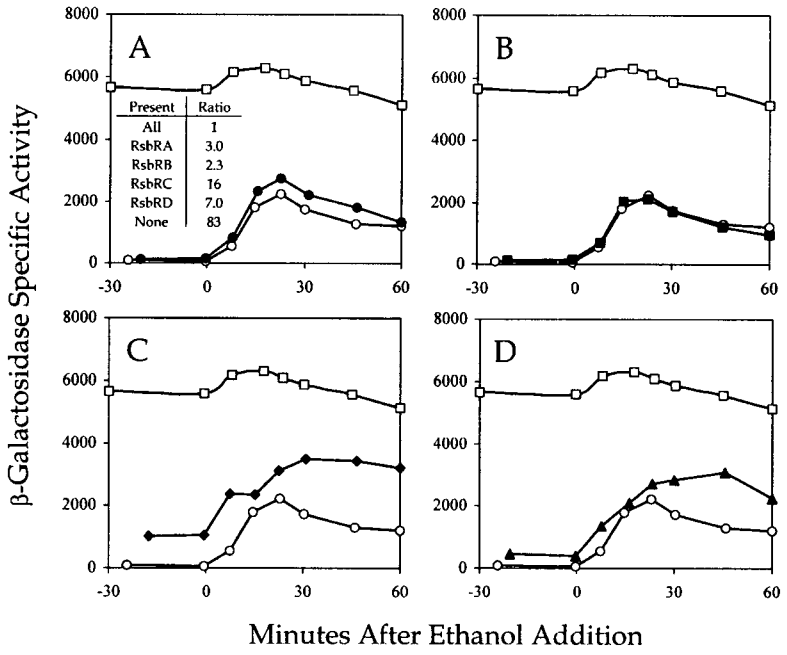

Figure 3. RsbR family members have a redundant role in environmental signaling. The environmental stress response of strains bearing only one member of the RsbR family was measured using a σ^B-dependent *ctc-lacZ* transcriptional fusion, as described in the Fig. 2 legend. In panels A-D the wild type strain (PB198) is indicated by (○) and the *rsbRA*, *rsbRB*, *rsbRC*, *rsbRD* quadruple mutant (PB629) is indicated by (□). Panel A, (●) RsbRA alone (PB804); Panel B, (■) RsbRB alone (PB639); Panel C (◆) RsbRC alone (PB530); Panel D, (▲) RsbRD alone (PB653). The table inset within Panel A shows σ^B activity in unstressed cells bearing only one or another member of the RsbR family; the average of two independent experiments is expressed as a ratio, with the activity of the wild type strain (All) taken as 1.

This functional association between RsbR family members and RsbS parallels a physical partnership. A two-step purification of RsbRA using Ni affinity chromatography and gel filtration from cell extracts finds it associated with RsbRB and RsbS in a complex which is more than 670 kDa complex (Figure 4). As an independent approach, immuno-precipitation with RsbRB antibody verifies this complex (Figure 5). This method is different from previous two-step purification method in several points: (i) a different bait protein; (ii) a different protein purification method; and (iii) using wild type cell extracts without any modification. These results propose that the RsbS antagonist and the RsbR family co-antagonists are binding each other and function together as σ^B regulators in this experiment condition.

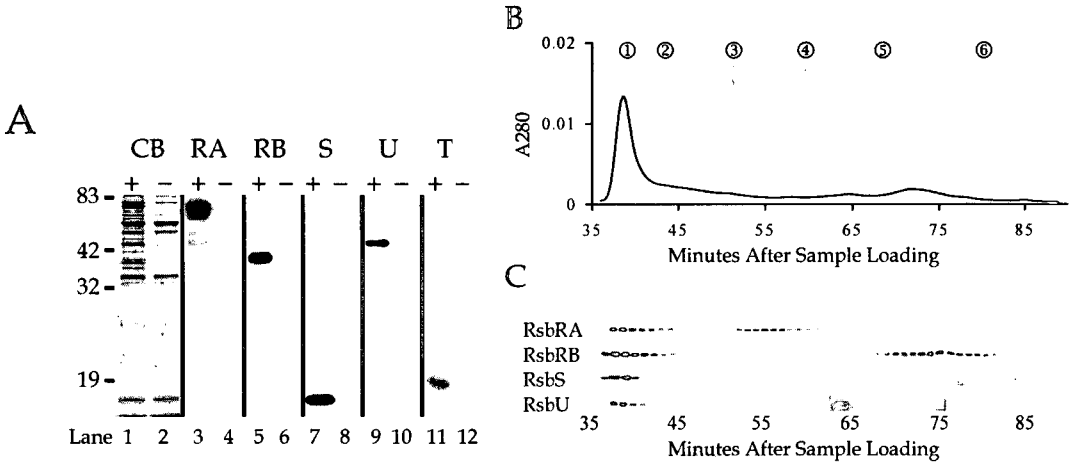

Figure 4. Purification of RsbRA and associated proteins from cell extracts. Strain PB593 was engineered to encode at its *rsbRA* locus an allele that added a combined hexahistidine-*myc* tag to the amino-terminal end of RsbRA. (A) After nickel affinity chromatography, purified proteins were analyzed by SDS-PAGE and Western blotting. Two different cell extracts were used: PB593 which bore the tagged RsbRA (+) and the PB2 wild type control with no tagged protein (-). Lanes 1 and 2 show the gel stained with Coomassie blue (CB) and lanes 3-12 show the Western blots with specific antibodies: lanes 3 and 4, anti-RsbRA (RA); lanes 5 and 6, anti-RsbRB (RB); lanes 7 and 8, anti-RsbS (S); lanes 9 and 10, anti-RsbU (U), lanes 11 and 12, anti-RsbT (T). Exposure times were 10 sec for the RsbRA, RsbRB and RsbS blots and 120 sec for the RsbU and RsbT blots. Positions of the molecular mass standards are indicated on the left (in kDa). (B) RsbRA-containing fractions from the nickel affinity column were applied to a Sephacryl S-300 sizing column. Arrows above the A_{280} trace indicate the elution volumes of the molecular mass standards: 1, thyroglobulin, 669 kDa; 2, ferritin, 416 kDa; 3, catalase, 219 kDa; 4, aldolase, 176 kDa; 5, bovine serum albumin, 66 kDa; and 6, carbonic anhydrase, 29 kDa. (C) Fractions from the Sephacryl column shown in (B) were analyzed by SDS-PAGE and Western blotting with anti-RsbRA, RsbRB, RsbS, RsbU, or RsbT antibody. The RsbT signal was barely detectable, so this blot is not shown.

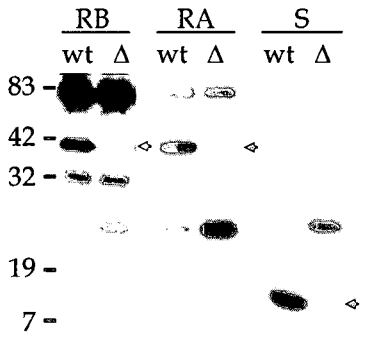

Figure 5. Co-immune precipitation of RsbRB, RsbRA and RsbS. Proteins were precipitated from cell extracts using anti-RsbRB rabbit antibody and protein-G agarose conjugate. The precipitated proteins were analyzed by SDS-PAGE and Western blotting. Positions of molecular mass standards are indicated on the left (in kDa). For each specific antibody, the proteins precipitated from two different cell extracts are shown: wt indicates the PB2 wild type and Δ indicates the PB545 control bearing a null *rsbRB* allele. Lanes 1 and 2, anti-RsbRB rabbit antibody (RB); lanes 3 and 4, anti-RsbRA mouse antibody (RA); lanes 5 and 6, anti-RsbS mouse antibody (S). Each pair of lanes contains one specific signal for the protein detected, shown by the arrows on the right, and also non-specific signals for protein-G and the IgG light and heavy chains. No specific signals were detected using anti-RsbT or anti-RsbU antibody (not shown).

We conclude that the RsbR family proteins serve as redundant co-antagonists necessary for RsbS antagonist function (Figure 6). Moreover, the finding a multicomponent complex containing the RsbRA

and RsbRB co-antagonists together with the RsbS antagonist propose that this complex serves as a machine to transmit stress signals to σ^B, and that the properties of the complex may contribute to environmental stress sensing.

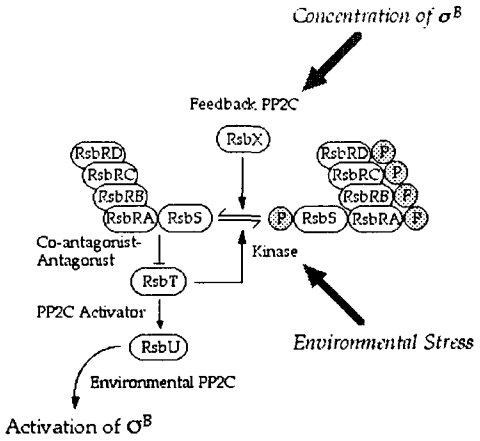

Figure 6. New model of the environmental branch of the σ^B signal transduction network. RsbRA, RsbRB, RsbRC, and RsbRD are redundant co-antagonists that function with the RsbS antagonist to counter the positive role of the RsbT switch protein/kinase. We propose that the RsbR co-antagonists and the RsbS antagonist form a complex in vivo. In this complex, the RsbR family members and RsbS act via two mechanisms: they (i) bind RsbT in an inactive complex and (ii) shield the RsbU phosphatase. Following environmental stress, the RsbT kinase phosphorylates the RsbR co-antagonists and RsbS, releasing RsbT and uncovering the RsbU phosphatase, promoting the activating interaction of RsbT with RsbU. The RsbX feedback phosphatase serves to return the system to its prestress condition.

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