JOURNAL OF BACTERIOLOGY, Dec. 1996, p. 7020–7023 Vol. 178, No. 23
0021-9193/96/$04.00+0
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The Yeast Two-Hybrid System Detects Interactions between *Bacillus subtilis* σ^B Regulators

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Received 5 July 1996/Accepted 24 September 1996

σ^B, the general stress response σ factor of *Bacillus subtilis*, is regulated by the products of seven genes (*rsbR*, *S*, *T*, *U*, *V*, *W*, and *X*) with which it is cotranscribed. Biochemical techniques previously revealed physical associations among RsbW, RsbV, and σ^B but failed to detect interactions of RsbR, S, T, U, or X with each other or RsbV, RsbW, or σ^B. Using the yeast two-hybrid system, we have now obtained evidence for such interactions. The yeast reporter system was activated when RsbS was paired with either RsbR or RsbT, RsbR was paired with RsbT, and RsbV was paired with either RsbU or RsbW. In addition, $RsbW_2$ and $RsbR_2$ dimer formation was detected. RsbX failed to show interactions with itself or any of the other *sigB* operon products.

σ^B is the general stress response σ factor of *Bacillus subtilis* (5, 8, 23). A drop in the bacterium's intracellular ATP or exposure to environmental stress (e.g., heat shock or ethanol) activates σ^B by triggering its release from an inhibitory complex with an anti-σ^B protein (1, 6, 8, 9, 26). The anti-σ^B protein, RsbW, forms mutually exclusive complexes with either σ^B or an alternative protein, RsbV (6). RsbW is a protein kinase as well as a σ^B-RsbV-binding protein (9). Conditions which favor RsbW's binding to σ^B (high ATP and absence of stress) empower RsbW to phosphorylate RsbV and convert it into a form (RsbV-P) which can no longer compete with σ^B for RsbW binding (1, 9). A drop in intracellular ATP or environmental stress activates the dephosphorylation of Rsb-P, the formation of stable RsbW-RsbV complexes, and the release of σ^B (25).

σ^B, RsbV, and RsbW are encoded within an eight-gene operon that is expressed constitutively from a σ^A-dependent promoter (15, 27). An internal σ^B-dependent promoter enhances expression of the four downstream genes (*rsbV*, *rsbW*, *sigB*, and *rsbX*) during periods of σ^B activity (4, 15). RsbX and the products of at least three of the four upstream genes (*rsbR*, *rsbS*, *rsbT*, and *rsbU*) are involved in stress-dependent σ^B activation (16, 19, 22, 26).

We had previously used gel filtration chromatography and immunoprecipitation techniques to establish that RsbW physically associates with RsbV and σ^B (6, 10, 22). The participation of the other *sigB* operon gene products in a common σ^B activation pathway suggested that these proteins were also likely to interact; however, the techniques which showed the RsbW-σ^B and RsbW-RsbV interactions failed to convincingly establish complex formation between RsbR, RsbS, RsbT, RsbU, or RsbX and each other or RsbV, RsbW, or σ^B (10). Assuming that some of the Rsb associations might be too tenuous to be detected by in vitro analyses, we applied the yeast dihybrid activator system (12, 13) to analyze potential interactions between these proteins. This involved cloning the coding sequences of each of the *sigB* operon products into specialized plasmids to create translational fusions between these proteins and the separated modular domains (DNA binding and transcriptional activation) of the yeast transcriptional activator Gal4. If the proteins fused to these domains can interact within the yeast cell, they bring together the two domains, which then activate a target promoter fused to an appropriate reporter gene (e.g., *lacZ*). The system is believed to be especially useful for detecting weak and transient protein interactions.

The dihybrid plasmid system and the appropriate yeast strains were obtained as the Matchmaker Two-Hybrid System from Clontech Laboratories, Inc. (Palo Alto, Calif.). The clonings, transformations, and assays were performed according to the protocols provided by Clontech. The fragments of each of the eight *sigB* operon genes, as well as the plasmids used in this study, are listed in Table 1. Each fragment was cloned into both the DNA binding domain vector (pAS2-1) and the activation domain vector (pACT2) and tested for its ability to activate expression of the reporter gene in the absence of a second plasmid. Synthesis of the hybrid protein in yeast cells was verified with monoclonal antibodies specific for the *B. subtilis sigB* operon products (10).

Most of the hybrid proteins were incapable of activating the yeast reporter system without a second hybrid protein. The RsbS::BD (Gal4 DNA binding domain) fusion was an exception. This fusion activated $GAL1_{UAS}$-$GAL1_{TATA}$-*lacZ* to high levels when paired with the activator domain vector lacking an insert (Table 2) or in the absence of a second plasmid (data not shown). Fortunately, the RsbS::AD (Gal4 activator domain) fusion failed to independently activate the reporter system. Thus, an assay with an RsbS::AD fusion and the other *sigB* products joined to the BD was possible.

An additional complication developed when yeast was cotransformed with *rsbW* and *sigB* hybrids. *rsbW* or *sigB* in either domain vector could be transformed into yeast and yield normal-sized colonies; however, cotransformation with *sigB*- and *rsbW*-containing plasmids resulted in very small yeast colonies. Rare colonies of normal size were obtained, but these had variable reporter gene activity. We assumed that the RsbW and σ^B fusions were causing secondary effects when present together in yeast cells and chose not to attempt a characterization of RsbW-σ^B interactions by this means.

The interactions that we did analyze are illustrated in Table 2. If paired plasmids activated the *lacZ* reporter gene, the relative strength of that interaction (judged by color develop-

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TABLE 1. Plasmids and cloned DNAs

Plasmid	Relevant characteristics[a]	Construction, source, or reference
pACT2	Vector with the AD of GAL4, Ap^r	Clontech Laboratories, Inc.
pAS2-1	Vector with the BD of GAL4, Ap^r	Clontech Laboratories, Inc.
pBluescriptKS$^+$	Ap^r	20
pRSETB	Ap^r	Invitrogen Corp.
pRSETBW	*rsbW* in pRSETB, Ap^r	865-bp *Eco*RI fragment of pW313 cloned into pRSETB digested with the same enzyme
pUS19rsbXBam	*rsbX* in pUS19, Ap^r	788-bp *Sau*3A fragment of pML7 (7) cloned into pUS19 (4) digested with *Bam*HI
pUV31	Protein fusion of RsbX with the BD of GAL4, Ap^r	790-bp *Bam*HI fragment of pUS19rsbXBam cloned into pAS2-1 digested with *Bam*HI
pUV36	Protein fusion of RsbX with the AD of GAL4, Ap^r	790-bp *Bam*HI fragment of pUS19rsbXBam cloned into pACT2 digested with *Bam*HI
pUV41	Protein fusion of RsbV with the BD of GAL4, Ap^r	430-bp *Eco*RI-*Sna*BI fragment of pV312 (3) cloned into pAS2-1 digested with *Eco*RI-*Sma*I
pUV46	Protein fusion of RsbW with the BD of GAL4, Ap^r	850-bp *Eco*RI-*Pvu*II fragment of pW313 (3) cloned into pAS2-1 digested with *Eco*RI-*Sma*I
pUV51	Protein fusion of RsbW with the AD of GAL4, Ap^r	850-bp *Nco*I-*Pvu*II fragment of pRSETBW cloned into pACT2 digested with *Nco*I-*Sma*I
pUV56	Protein fusion of SigB with the BD of GAL4, Ap^r	800-bp *Bam*HI-*Pst*I fragment of pDAG3 (1) cloned into pAS2-1 cut with the same enzymes
pUV62	*sigB* in pBluescriptKS$^+$, Ap^r	800-bp *Bam*HI-*Pst*I fragment of pDAG3 (1) cloned into pBluescriptKS$^+$ cut with the same enzymes
pUV70	Protein fusion of RsbS with the AD of GAL4, Ap^r	610-bp *Bam*HI-*Eco*RI pAL290 (10) cloned into pACT2 cut with the same enzymes
pUV71	Protein fusion of RsbS with the BD of GAL4, Ap^r	770-bp *Bam*HI-*Pst*I fragment of pAL290 (10) cloned into pAS2-1 cut with the same enzymes
pUV76	Protein fusion of RsbT with the BD of GAL4, Ap^r	400-bp *Bam*HI-*Pst*I fragment of pAL294 (10) cloned into pAS2-1 cut with the same enzymes
pUV85	*rsbR* in pBluescriptKS$^+$, Ap^r	820-bp *Bam*HI-*Hin*dIII fragment of pAL285 (10) cloned into pBluescriptKS$^+$ cut with the same enzymes
pUV126	Protein fusion of RsbR with the BD of GAL4, Ap^r	830-bp *Bam*HI-*Sal*I fragment of pUV85 cloned into pAS2-1 cut with the same enzymes
pUV134	Protein fusion of RsbR with the AD of GAL4, Ap^r	830-bp *Bam*HI-*Xho*I fragment of pUV85 cloned into pACT2 digested with the same enzymes
pUV136	Protein fusion of SigB with the AD of GAL4, Ap^r	800-bp *Bam*HI-*Eco*RI fragment of pUV62 cloned into pACT2 cut with the same enzymes
pUV145	Protein fusion of RsbU with the BD of GAL4, Ap^r	1,000-bp *Sma*I-*Sal*I fragment of a PCR product generated with the primers RsbU5MM[b] and RsbU3MM[b] cloned into pAS2-1 digested with the same enzymes
pUV151	*rsbT* in pBluescriptKS$^+$, Ap^r	450-bp *Bam*HI-*Hin*dIII fragment of pAL294 cloned into pBluescriptKS$^+$ cut with the same enzymes
pUV166	Protein fusion of RsbT with the AD of GAL4, Ap^r	450-bp *Bam*HI-*Xho*I fragment of pUV151 cloned into pACT2 cut with the same enzymes
pUV172	Protein fusion of RsbS with the AD of GAL4, Ap^r; low-level expression of the fusion protein	770-bp *Bam*HI-*Bgl*II fragment of pUV70 cloned into pGAD424 cut with the same enzymes
pUV187	Protein fusion of RsbU with the AD of GAL4, Ap^r	1,000-bp *Sma*I-*Eco*RI fragment of a PCR product generated with the primers RsbU5MM[b] and RsbU3MM[b] cloned into pACT2 digested with the same enzymes
pUV201	Protein fusion of RsbR with the AD of GAL4, Ap^r; low-level expression of the fusion protein	830-bp *Bam*HI-*Bgl*II fragment of pUV134 cloned into pGAD424 cut with the same enzymes
pUV207	Protein fusion of RsbV with the AD of GAL4, Ap^r	350-bp *Bam*HI-*Xho*I fragment of a PCR product generated with the primers RsbV5MM[b] and RsbV3MM[b] cloned into pACT2 digested with the same enzymes

[a] AD, activation domain; BD, DNA-binding domain.
[b] The PCRs were performed with the following primers: RsbV3MM, 5′-AGAGAGAGGATCCGTATGAATATAAATGTTGATGTG-3′; RsbV3MM, 5′-AGAGAGCTCGAGTCGACTCATTGCACTCCACCTTC-3′; RsbU5MM, 5′-AGAGCCCGGGGGTGGATTTTAGGGAGGTT-3′; RsbU3MM, 5′-AGAGGATTCGTCGACGTTAAACCTTTCTCCG-3′.

ment of the yeast colonies in the presence of X-Gal [5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside]) is represented by the number of plus signs. The β-galactosidase activity (Miller units) of reacting clones was measured in a liquid assay and indicated in Table 1. Liquid β-galactosidase assays were performed only on vector control cotransformants and experimental cotransformant colonies that turned blue on X-Gal.

The strongest responses were obtained when yeast cells were transformed with plasmids containing *rsbW* fused to both of the Gal4 domains or when the transformants carried *rsbS* in the binding domain vector. Presumably, the *rsbW* fusions bring together both of the Gal4 domains as $RsbW_2$ chimeric protein dimers. Dimerization of RsbW, as well as RsbS and RsbR, was previously suggested by their gel filtration profiles (10). The high background of the RsbS::BD fusion (28.8 U) limited our ability to judge whether RsbS dimers could occur in this system. The 25% increase in β-galactosidase activity accompanying the introduction of RsbS::AD into the RsbS::BD strain is

TABLE 2. Activation of *lacZ* reporter gene by *sigB* operon product-Gal4 chimerase[a]

Activation domain fusion	Binding domain fusion							
	Vector pAS2-1	R	S	T	U	V	W	σ^B	X
Vector pACT2	W, 0.13	W, 0.09	++++, 28.80	W, 0.13	W, 0.07	W, 0.08	++, 1.67	W, 0.05	W, 0.04
R	W, 0.10	+++, 4.59	+++++, 49.20	++, 0.83	W	W	++, 1.56	W	W
S	W, 0.12	+++, 6.00	++++, 35.50	++++, 14.30	W	W	++, 1.50	W	W
T	W, 0.16	+/−, 0.20	++++, 33.12	++, 0.90	+, 0.29	W	W	W	W
U	W, 0.09	W	++++, 26.30	++, 0.70	+, 0.28	W	++, 1.58	W	W
V	W, 0.14	W	++++, 30.20	W	++, 0.84	W	+++, 6.50	W	W
W	W, 0.17	W	++++, 31.70	W	W	++, 1.85	+++++, 42.87	ND	W
σ^B	W, 0.13	W	++++, 24.50	W	W	W	ND	W	W
X	W, 0.15	W	++++, 29.00	W	W	W	W	W	W

[a] The vertical rows represent yeast clones carrying the Gal4 DNA binding domain vector pAS2-1 either without insert DNA (row 1) or with the *sigB* gene depicted by the single letter (e.g., R-*rsbR*) translationally fused to the domain. The horizontal rows represent the Gal4 activation domain vector pACT2 with or without *sigB* genes as described for the binding domain fusions. Intersecting rows depict the reporter gene ($Gal1_{UAS}$-$GAL1_{TATA}$-*lacZ*) activity in yeast cells cotransformed with both of the plasmids indicated by the rows. The W or plus marks illustrate the colony color after freeze-thawing of the colonies and exposure to X-Gal. W indicates that the colony remained white, while "+" represents blue colonies, with the intensity of the color approximated by the number of plus symbols (e.g., +, light blue; ++++, dark blue). The numbers in the columns provide a quantitative measurement of *lacZ* activity in clones carrying the vectors alone, a vector paired with its counterpart carrying a *sigB* fusion protein, or those plasmid pairings that gave a blue colony phenotype. The assays were performed on 1 ml of yeast culture growing exponentially in a synthetic complete medium (SD) (minus Trp and Leu) (21). Cells were washed, resuspended in 200 μl of Z buffer, and subjected to two cycles of freeze-thawing in liquid N_2. The resulting permeabilized cells were analyzed for β-galactosidase as described by Miller (18). The values given represent (culture optical density at 420 nm × 1,000)/(culture optical density at 600 nm × time of assay) and were calculated from duplicate assays of at least four independent clones of each plasmid pair. The standard deviations for each datum point were approximately 10 to 20% of the value illustrated. ND, not determined.

not compelling and is, in fact, similar to the deviations (10 to 20%) which we typically observed for a given strain from experiment to experiment. Our only evidence for RsbS dimerization remains the gel filtration data (10). The dihybrid system did, however, support the notion of RsbR dimerization. Reporter gene activity in yeast cells carrying *RsbR* in both Gal4 vectors was more than 40-fold higher than that seen in control transformations of *rsbR* fused to one domain and paired with the second domain vector without an insert (Table 2). Gel filtration of RsbV also indicated that this protein might exist as a homodimer when not bound to RsbW (9); however, we saw no evidence for dimerization of RsbV in the present study. Fusion of *rsbV* in both domains gives only background levels of reporter gene activity (Table 2). The RsbV homolog, SpoIIAA, appears to be monomeric but assumes a configuration that chromatographs as a protein of higher molecular mass (17). It is likely that the free form of RsbV-P behaves similarly and chromatographs as an apparent during gel filtration, when it is actually a monomer with aberrant filtration properties.

A strong activation response also occurred when RsbS was paired with RsbR or RsbT and RsbV was paired with RsbW. A smaller activation was detected when RsbU was paired with RsbT. This latter response was still fivefold over the control levels and probably reflects an association between these two proteins. An interaction between RsbS and RsbT was not apparent in the RsbS::BD and RsbT::AD pairing but was evident in an RsbT::BD and RsbS::AD pairing. The high RsbS-BD background likely masked the activation in the former circumstance.

Two pairings gave a significant activation only when one member was fused to a particular Gal4 domain. RsbU::AD paired with RsbV::BD gave a response that was 10-fold higher than control levels, but an RsbV::AD-RsbU::BD interaction was not evident. Similarly, the RsbR::AD-RsbT::BD pair, but not the reciprocal fusions, gave a reaction almost 10-fold over control levels. The reason for the RsbU-RsbV and RsbR-RsbT pairings failing to activate $Gal1_{UAS}$-$Gal1_{TATA}$-*lacZ* when present in the alternative fusion domains is unclear, but the failure may be due to differences in the accessibility of interacting domains in a particular chimera.

Although associations predicted from the dihybrid interactions must be taken as tentative, it is reassuring that several of the interactions suggested by the dihybrid system are also supported by biochemical (i.e., RsbR-RsbR, RsbW-RsbW, RsbV-RsbW) (6, 9, 10) or genetic (i.e., RsbS-RsbT) (16, 19) data or are hypothesized to take place based on homologies with the σ^F system (i.e., RsbU-RsbV versus SpoIIE-SpoIIAA) (2, 11). The interactions that we detect in the yeast system, along with those suggested by previous studies (6, 9, 10), are illustrated in Fig. 1. The figure depicts which Rsb proteins appear to associate one with another and is not meant to imply that all of the associations occur simultaneously to form a single large complex within *B. subtilis*. Proteins that we believe interact with each other, based on the dihybrid data or previous gel filtration studies (6, 9, 10), are represented by adjoining circles. All of the proteins except for RsbX appear to associate with at least one other known σ^B regulator. The associations of RsbR, S, T, and U with the proteins illustrated in Fig. 1 are plausible. Each of these proteins is believed to participate in a common pathway which conveys stress-dependent signals for σ^B activation (10, 16, 26). Stress activation of σ^B involves the dephosphorylation of RsbV-P (25). This reactivates RsbV to bind to RsbW and free σ^B. Based on homologies with a phosphatase (SpoIIE) that reactivates the RsbV homolog in the σ^F system, RsbU is thought to be the RsbV-activating phosphatase (2). As such, it is reasonable that RsbU should be found to interact with RsbV and communicate with at least one of the gene products needed for stress induction (i.e., RsbT). In vitro evidence for RsbU being an RsbV-P phosphatase that is stimulated by RsbT was recently described by C. W. Price (University of California at Davis) at the 12th International Spores Conference (5 to 8 June 1996, Madison, Wis.). Our current data do not establish whether the associations of RsbR, S, T, and U, which are illustrated in Fig. 1, can result in a complex of the four gene products in *B. subtilis* or separate transient pairings of these proteins which might occur in the course of passing a stress activation signal to RsbU. Our previous gel filtration experiments detected a high-molecular-mass aggregate (600 kDa) that included much of the cell's detectable RsbR, S, and T as well as a significant, but smaller, portion of the cell's RsbU (10). It is possible that this aggregate repre-

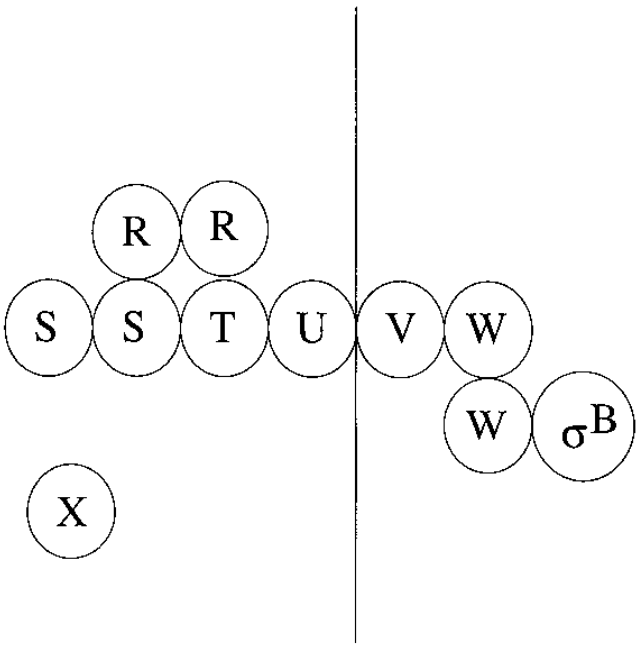

FIG. 1. Predicted interactions between σ^B regulators. The dihybrid activation studies and/or previous gel filtration studies suggest that RsbR, RsbS, and RsbW can form homodimers. Dihybrid-dependent reporter gene activation with paired proteins in either domain suggests binding of RsbR with RsbS and RsbT, RsbT with RsbS and RsbU, and RsbV with RsbW. The possible RsbS-RsbT interaction had been predicted by genetic studies (16), and the RsbV-RsbW interaction was previously seen in biochemical analyses (6). Interactions of RsbR, RsbS, RsbT, and RsbU with at least one other member of this group are in keeping with genetic data demonstrating that all four proteins are part of the σ^B stress activation pathway (10, 16, 19, 22). The RsbU-RsbV interaction is consistent with the notion that RsbU is a stress-activated RsbV-P phosphatase (2, 11), while the RsbT-RsbU interaction suggests that RsbT may transmit the stress activation signal to RsbU. The RsbW-σ^B interaction was previously demonstrated biochemically (6). We have no evidence for RsbX associating or interacting with σ^B or any of its regulators; however, genetic studies show that RsbX is a negative regulator of the σ^B stress-activated pathway (3, 15, 22). It is unknown whether the associations between multiple proteins depicted in this figure can occur simultaneously or whether they represent independent single interactions between each of the proteins. The RsbW-RsbV and the RsbW-σ^B interactions are, for example, known to be mutually exclusive.

sents a persistent complex of RsbR, S, T, and U, associated with other cell components. Alternatively, only single associations might occur between each of these proteins. In such a model, RsbS would associate with either RsbR or RsbT, and RsbT would associate with either RsbR, RsbS, or RsbU, etc. Our failure to detect associations between RsbX and the other σ^B regulators may reflect the true state of RsbX. Although RsbX is a negative regulator of the stress activation pathway (3, 14, 15), we can observe stress induction of σ^B in the absence of RsbX (24). Thus, RsbX may not interact with the components of the stress induction pathway. Our current view is that RsbX does not participate in the σ^B induction process but rather limits the degree to which the activation process can proceed. Presumably, the increases in RsbX, which parallel the increase in σ^B activity, can downregulate σ^B activation. We are now turning to genetic means to better characterize the interactions between the σ^B regulators during stress.

This work was supported by NIH grant GM48220. U. Voelker was the recipient of a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft during a portion of this project.

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