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Parallel pathways of repression and antirepression governing the transition to stationary phase in *Bacillus subtilis*

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The **AbrB** protein of the spore-forming bacterium *Bacillus subtilis* is a repressor of numerous genes that are switched on during the transition from the exponential to the stationary phase of growth. The gene for **AbrB** is under the negative control of the master regulator for entry into sporulation, **Spo0A~P**. It has generally been assumed that derepression of genes under the negative control of **AbrB** is achieved by **Spo0A~P**-mediated repression of *abrB* followed by rapid degradation of the **AbrB** protein. Here, we report that **AbrB** levels do decrease during the transition to stationary phase, but that this decrease is not the entire basis by which **AbrB**-controlled genes are derepressed. Instead, **AbrB** is inactivated by the product of a uncharacterized gene, *abbA* (formerly *yzkF*), whose transcription is switched on by **Spo0A~P**. The *abbA* gene encodes an antirepressor that binds to **AbrB** and prevents it from binding to DNA. Combining our results with previous findings, we conclude that **Spo0A~P** sets in motion two parallel pathways of repression and antirepression to trigger the expression of diverse categories of genes during the transition to stationary phase.

sporulation | transcription

Bacteria ordinarily spend a relatively brief period of their existence in the exponential phase of growth (1–3). Nutrients become limiting, or other adverse environmental changes take place as cells reach a high population density, causing growth to slow and the bacteria to enter stationary phase. Coping with the transition to stationary phase involves dramatic changes in gene expression in which suites of genes are switched on that enable the cells to adapt to unfavorable circumstances. These changes are governed by signal transduction pathways that sense the onset of adverse circumstances and respond by activating (or inactivating) global regulatory proteins. One such global regulator is the general stress response transcription factor σ^S , which helps govern the transition to stationary phase in *Escherichia coli* (4, 5). In the spore-forming bacterium *Bacillus subtilis*, the subject of this investigation, the transition to stationary phase is principally governed by five regulatory proteins, **CodY** (6), σ^B (7, 8), σ^H (9), **Spo0A~P** (10), and **AbrB** (11).

How **CodY** helps to govern the transition to stationary phase is well understood. Its activity as a repressor depends on either of two cofactors, GTP or a branched chain amino acid (12, 13). In the absence of either ligand, **CodY**'s ability to bind DNA is impaired. Thus, because GTP or branched chain amino acid levels drop during nutrient limitation, repression is relieved and genes under the control of **CodY** are derepressed. Our understanding of how σ^B , σ^H , and **Spo0A**-controlled gene expression is coupled to the exit from the exponential phase of growth is less complete. The σ^B factor, for example, is activated by convergent pathways that sense, in an as yet undefined way, the lack of certain nutrients and the presence of certain kinds of physical-chemical signals (14). **Spo0A**, a member of the response regulator family of phosphoproteins, is activated by phosphorylation in response to nutrient limitation via a multicomponent phosphorelay (15). The phosphorelay is initiated by several kinases

that are thought to recognize intra- or extracellular signals. When phosphorylated, **Spo0A~P** acts as an activator or repressor of ≈ 120 genes under its direct control, including genes required for sporulation (16). However, how phosphorelay-mediated phosphorylation of **Spo0A** is coupled to a drop in nutrient availability has not been elucidated.

The fifth transcriptional control protein, the “transition-state regulator” **AbrB** and the focus of this report, has been of interest for almost 40 years (17–19). Yet little is known about the mechanisms that govern the derepression of **AbrB**-controlled genes at the end of the exponential phase of growth. The gene for **AbrB** was discovered because of the observation that mutations at the *abrB* locus suppressed some of the phenotypes characteristic of *spo0A* and other mutants blocked in the initiation of sporulation. However, how **AbrB** acted was mysterious for many years (20). An important clue came from studies of two promoters that depended on **Spo0A~P** for their activation (21, 22). In both cases, an *abrB* mutation was found to bypass the dependence on *spo0A*, and in one case, it resulted in constitutive transcription. These findings indicated that **AbrB** is likely a repressor that is present in vegetatively growing cells and is inactivated or eliminated by the action of **Spo0A** at the end of the exponential phase of growth. Indeed, subsequent biochemical work confirmed that **AbrB** is a DNA-binding protein that acts by repressing target genes (23, 24). The further demonstration that **Spo0A~P** directly represses *abrB* (25) led to the view that derepression of genes under **AbrB** control is mediated by a **Spo0A~P**-imposed block in *abrB* transcription combined with rapid depletion of **AbrB** protein by degradation (24, 26, 27).

As we report here, **AbrB** levels do decrease as cells transition from exponential growth to stationary phase, but this drop in **AbrB** levels is not the sole basis for the derepression of genes under its control. Instead, **AbrB** is inactivated by the product of a previously uncharacterized gene, *yzkF* (for which we introduce the name *abbA* for antirepressor of *abrB*) that is directly switched on by **Spo0A~P** (16, 28). We show that *abbA* encodes an **AbrB**-binding protein that forms a complex with the repressor and prevents it from adhering to DNA. Thus, the derepression of some or all genes under the negative control of **AbrB** involves the **Spo0A~P**-induced synthesis of an antirepressor. A parallel thereby emerges between **AbrB** and the **SinR** repressor of *B.*

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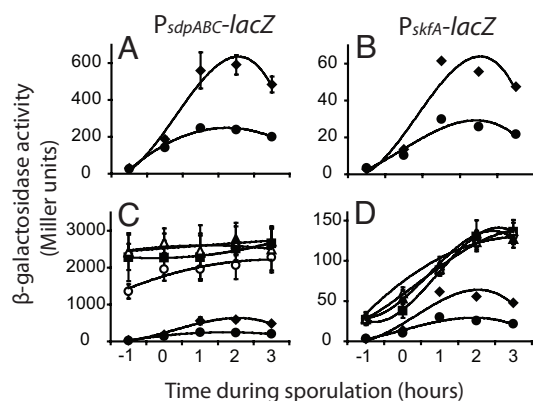


Fig. 1. Two cannibalism operons are under the indirect control of Abba. (A and B) Abba is required for maximal expression of the *sdpABC* and *skfABCDE* operons. (A) Strains harbored *amyE::P_{sdpABC}-lacZ*, and were either wild-type (◆; RL4264), or mutant for *abbA* (●; AB149). (B) Strains harbored *amyE::P_{skfA}-lacZ*, and either were wild-type (◆; RL3554), or mutant for *abbA* (●; AB148). (C and D) An *abrB* mutation is epistatic to the effect of an *abbA* mutation on expression of the *sdpABC* and *skfABCDE* operons. (C) Strains harbored *amyE::P_{sdpABC}-lacZ*, and were wild-type (◆; RL4264), mutant for *abbA* (●; AB149), harbored the overexpression construct *P_{hyperspank}-abbA* (○; AB151), mutant for *abrB* (■; AB183), mutant for both *abbA* and *abrB* (▲; AB184), or mutant for *abrB* and harbored *P_{hyperspank}-abbA* (△; AB182). (D) Strains harbored *P_{skfA}-lacZ*, and were either wild-type (◆; RL3554), mutant for *abbA* (●; AB148), harbored *P_{hyperspank}-abbA* (○; AB150), mutant for *abrB* (■; AB188), mutant for both *abbA* and *abrB* (▲; AB189), or mutant for *abrB* and harbored *P_{hyperspank}-abbA* (△; AB190). Cells were grown in liquid DS medium; hour 0 of sporulation was the end of the exponential phase of growth. Expression of *P_{hyperspank}-abbA* was induced by the addition of 1 mM (final concentration) IPTG to the medium.

subtilis, which is also inactivated by an antirepressor (SinI) whose synthesis is induced by Spo0A~P (29–31).

Results

The Cannibalism Operons *sdp* and *skf* Are Under the Indirect Control of Abba. The starting point for this investigation was the phenomenon of cannibalism in which cells that have activated Spo0A in response to nutrient limitation produce a toxin and a killing factor that kill sibling cells that have not activated the response regulator (32, 33). Colonies of cells that exhibit cannibalism are delayed in sporulation. It is presumed that nutrients released by the dead cells delay sporulation by reversing or slowing the activation of Spo0A in the toxin- and killing factor-producing cells. The toxin and the killing factor are produced under the direction of operons called *sdpABC* (hereafter simply *sdp*) and *skfABCDE* (hereafter simply *skf*), respectively. Both operons are under the direct negative control of AbrB (34). Colonies of cells mutant for *sdp* or *skf* are mutant for cannibalism and exhibit an accelerated sporulation phenotype. A previous survey of members of the Spo0A regulon for genes involved in cannibalism revealed an uncharacterized open-reading frame *abbA* (*yzkF*), which when mutant caused accelerated sporulation (16).

We asked whether the cannibalism phenotype of an *abbA* mutant was due to impaired expression of the *sdp* and *skf* operons. To do this, we examined the effect of an *abbA* mutation (Δ *abbA*) on the expression of *lacZ* fused to the promoters for *sdp* (*P_{sdp}-lacZ*) and *skf* (*P_{skf}-lacZ*) during sporulation in DS medium. The results show that deletion of *abbA* led to decreased transcription from both promoters (Fig. 1 Upper), thereby providing an apparent explanation for the cannibalism mutant phenotype of an *abbA* mutant.

Next, we determined the effect of overproducing Abba on the expression of *sdp* and *skf*. To do this, we constructed a fusion of the *abbA* gene to the IPTG-inducible promoter *P_{hyperspank}* and

examined the effect of inducing this construct on the expression of *P_{sdp}-lacZ* and *P_{skf}-lacZ* during sporulation. We observed that expression of both genes was markedly elevated when *abbA* was overexpressed. Strikingly, the patterns of expression we observed were similar to those seen in an *abrB* mutant (Fig. 1 Lower).

AbrB directly represses both *sdp* and *skf*, and deleting *abrB* results in a dramatic increase in expression from both operons (34, 35). Could Abba act by relieving AbrB-mediated repression of these operons? To test this hypothesis, we compared *P_{sdp}-lacZ* and *P_{skf}-lacZ* expression in an *abrB* mutant, an *abrB abbA* double mutant, and an *abrB* mutant that also contained *P_{hyperspank}-abbA*. We observed that removing or overexpressing *abbA* had no effect on *P_{sdp}-lacZ* and *P_{skf}-lacZ* expression in the absence of AbrB (Fig. 1, Lower). In other words, the effect of the *abrB* mutation was epistatic to that of the *abbA* mutation. These results suggest that Abba and AbrB act in the same pathway to control *sdp* and *skf*, and that Abba acts upstream of AbrB. This observation raised the possibility that Abba might be involved in other AbrB-regulated pathways as well.

We note that the *abbA* mutation only partially impaired expression of the *sdp-lacZ* and *skf-lacZ* fusions. If Abba acts upstream of AbrB to reverse the effects of the repressor, then why did the *abbA* mutation not block expression of the *lacZ* fusions completely? In other work, we have found that a second, independently acting pathway contributes to the transcription of *sdp* (unpublished results). Similarly, *skf* is controlled by two pathways, one involving Spo0A indirectly via its induction of *abbA* and one involving Spo0A directly in which Spo0A~P binds to and activates the *skf* promoter (34). Henceforth, and for simplicity, we will simply consider the contribution of Abba to the transcription of *sdp*, *skf* and other operons under the negative control of AbrB.

Many Genes Under the Positive Control of Abba Are Repressed by AbrB. To determine whether Abba was dedicated to cannibalism or governed a wider set of genes, we performed transcriptional profiling experiments that compared an *abbA* mutant strain (Δ *abbA*) to a strain that overexpressed Abba (*P_{hyperspank}-abbA*). Our analysis was carried out with RNA from cells growing exponentially in LB medium.

As a first step, we confirmed that *sdp* and *skf* were both up-regulated when Abba was overexpressed, which gave us confidence that our findings represented members of the Abba regulon (Table 1). Further analysis of our data revealed that Abba promotes the transcription of genes beyond those involved in cannibalism. Strikingly, genes known to be repressed by AbrB represented the vast majority of the Abba regulon (Table 1). Eleven of the 23 genes that we found to be controlled by Abba had been reported to be either directly or indirectly (via the gene for σ^W) repressed by AbrB (36–38). Recently, nine additional Abba-controlled genes have been identified as being under the negative control of AbrB (M. A. Strauch, personal communication). Interestingly, the gene that was controlled most strongly by Abba, *lip*, was not known to be a direct target of AbrB, but, as shown below, the promoter region for *lip* contains a binding site for AbrB. The remaining three genes whose expression was stimulated by overproduction of Abba are not well characterized. It will be interesting to see whether they too are direct or indirect targets of AbrB.

Abba Blocks the Binding of AbrB to DNA. The results so far are consistent with the idea that Abba acts by inhibiting the ability of AbrB to bind to DNA. To investigate this possibility, we carried out EMSA using purified His₆-Abba, purified His₆-AbrB, and radiolabeled DNAs that contained the promoters for *comK*, *sdp*, *skf*, or *lip*. The promoter regions of *comK*, *sdp*, and *skf* have been shown to be bound by AbrB *in vitro* (35, 39), and

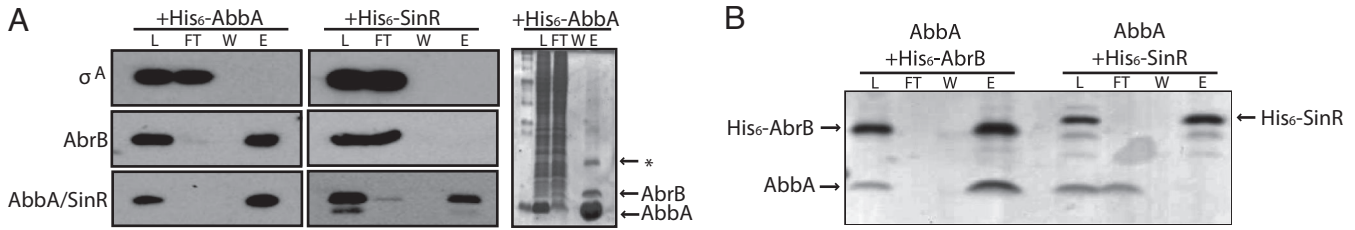


Fig. 3. AbbA binds AbrB. **A** shows affinity chromatography of AbrB using immobilized His₆-AbbA. Lysates prepared from exponentially growing wild-type cells (PY79) were incubated with purified His₆-AbbA (Left and Right) or His₆-SinR (Center) and applied to a Ni²⁺-NTA-agarose column. The presence of AbrB, σ^A , His₆-AbbA and His₆-SinR was monitored in the load (L), flow-through (FT), wash (W) and eluate (E) by immunoblotting using specific antibodies (Left and Center) or Coomassie staining (Right). Benchmark prestained protein ladder (Invitrogen) is shown in the far left lane of the Coomassie-stained gel (Right). Elution fractions were concentrated 2.5-fold relative to the load. **B** shows affinity chromatography of AbbA using immobilized His₆-AbrB. Purified AbbA was incubated with purified His₆-AbrB or His₆-SinR and applied to Ni²⁺-NTA-agarose. The presence of AbbA, His₆-AbrB, and His₆-SinR was detected in the load (L), flow-through (FT), wash (W) and elution (E) fractions by Coomassie staining. Elution fractions were concentrated 10-fold relative to the load.

control, and Spo0A levels were also monitored and seen to increase after exponential phase as expected (Fig. 4). Our results reveal that AbrB levels decreased several fold during the period when derepression of *sdp-lacZ* was occurring (Fig. 4). Thus, decreasing AbrB could contribute to the derepression of *sdp-lacZ*, augmenting the effect of AbbA.

We wondered whether AbbA was contributing to the drop in AbrB levels. To investigate this, we compared AbrB levels in an *abbA* mutant strain (Δ *abbA*) to those in a strain that overexpressed *abbA* (*P_{hyperspank}-abbA*) during growth in LB medium. Overexpression of AbbA did not lead to a decrease in AbrB levels [supporting information (SI) Fig. S1].

Discussion

The principal contribution of this work is the elucidation of a previously unknown mechanism by which genes under the negative control of AbrB are derepressed during the transition to stationary phase. It has generally been assumed that derepression is achieved by Spo0A~P-mediated repression of the *abrB* gene combined with depletion of AbrB protein by degradation. Indeed, earlier findings, which are confirmed here, do show a reduction in AbrB levels during the transition from growth to stationary phase. This reduction may contribute to the derepression of AbrB-controlled genes (see below), but it is not the exclusive basis for the derepression of genes and operons,

such as *skf* and *sdp*, that are rapidly switched on as cells exit exponential phase. Instead, AbrB is inactivated by the action of an anti-repressor AbbA that binds to AbrB and thereby prevents the repressor from adhering to operator sites in target genes.

Does AbbA contribute to the derepression of all AbrB-controlled genes? Our evidence showed that AbbA reversed the binding of AbrB to four target genes, *sdp*, *skf*, *comK*, and *lip*. In addition, we identified 23 genes that were up-regulated by the overproduction of AbbA. A high proportion of these genes were identified as targets of AbrB. Nonetheless, AbrB is known to control many genes that did not appear in our microarray analysis (M. A. Strauch, personal communication). AbrB has various affinities for the operators of genes it controls (40), and AbbA may only be capable of removing AbrB from operators for which it has a relatively low affinity. If this is the case, then the intracellular drop in AbrB levels or other unknown mechanisms might account for the derepression of remaining targets that have a high affinity for the repressor and are not derepressed by the action of AbbA alone. Alternatively, the conditions we used for our transcriptional profiling analysis might not have allowed us to detect all of the members of the AbbA regulon. Our analysis was carried out by using cells in the exponential phase of growth, and AbbA is normally produced during sporulation. Thus, it is possible that AbbA contributes to the derepression of genes that are under the negative control of AbrB but that are not expressed during exponential phase growth because of a direct requirement for Spo0A or other postexponential phase transcription factors. In sum, the simplest interpretation of our results is that AbbA contributes to the derepression of all AbrB-controlled genes. Conceivably, however, AbbA governs the derepression of only a subset of AbrB-controlled genes, and depletion of AbrB or a yet-to-be-discovered mechanism or both is responsible for relieving repression of the remaining members of the AbrB regulon.

How might repression of *abrB* by Spo0A~P contribute to the derepression of AbrB-controlled genes? We note from our immunoblot analysis that the level of AbrB decreased slowly and steadily from mid exponential phase growth into stationary phase. We therefore posit that Spo0A~P-mediated repression of *abrB* and induction of *abbA* is a mechanism to adjust the ratio of AbbA to AbrB under different growth states. Thus, in exponential phase growth in rich medium when Spo0A~P levels are expected to be at their lowest, the ratio of AbrB to AbbA would be at its highest, ensuring maximal repression of target genes. Conversely, during the transition to stationary phase, when Spo0A~P levels begin to rise, the ratio of AbrB to AbbA would decrease, facilitating antirepression of AbrB and derepression of target genes.

The discovery that the action of AbrB is reversed by an antirepressor reveals an interesting parallel to the circuitry that

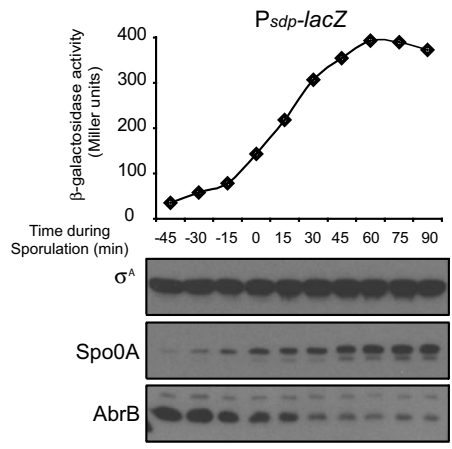


Fig. 4. AbrB levels decrease as cells exit the exponential phase of growth. Upper shows the activation of *sdp* at the end of stationary phase. The strain harbored *amyE::P_{sdp}-lacZ*, and was otherwise wild-type (\blacklozenge ; RL4264). Lower shows the kinetics of AbrB and Spo0A accumulation in cells taken from the same culture as followed by immunoblot analysis. σ^A was used as a loading control. Cells were grown in liquid DS medium; hour 0 of sporulation was the end of the exponential phase of growth.

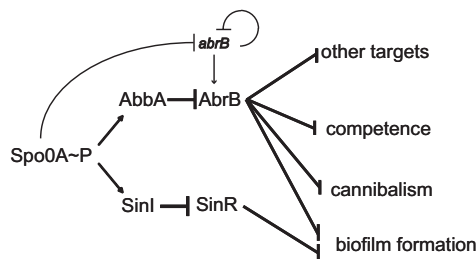


Fig. 5. Spo0A~P controls two parallel pathways of repression and antirepression during the transition to stationary phase. The SinR repressor is largely dedicated to genes involved in biofilm formation whereas AbrB controls a wide variety of genes, including genes for biofilm formation, competence and cannibalism. The AbrB and SinR repressors are each subject to antirepression by AbbA and SinI, respectively, whose synthesis is induced by Spo0A~P. Also shown is that *abrB* is subject to repression by Spo0A~P and autorepression.

governs biofilm formation. Recent work has shown that genes involved in the production of the extracellular matrix are governed by two parallel pathways of repression mediated by the SinR and AbrB repressors (29, 30, 38). SinR is largely dedicated to the control of genes involved in biofilm formation whereas AbrB has a broad spectrum of targets and is not restricted to genes governing the production of extracellular matrix. Relief from SinR-mediated repression is known to be achieved by the action of an antirepressor, SinI, which binds to and blocks the action of SinR. SinI is produced under the direct control of Spo0A~P. Likewise, relief from AbrB-mediated repression, as we have now shown, is mediated in part by AbbA, whose synthesis is also under the direct positive control of Spo0A~P. Therefore, the regulatory logic governing biofilm formation is that of two parallel pathways of repression and antirepression, both of which are set in motion by the same master regulatory protein Spo0A~P (Fig. 5). Of course, the AbrB circuit has the additional feature that Spo0A~P also represses the gene for the AbrB repressor.

The combination of Spo0A~P inducing the synthesis of an antirepressor of AbrB and repressing the gene for AbrB conforms to a network motif known as a coherent feed-forward loop [specifically, a type 3 loop (41)]. Theoretical analysis indicates that coherent feed-forward loops display a delay in a response to input signal (increasing Spo0A~P) and thus are insensitive to fluctuations in signal levels. In addition, the type 3 loop has the property that once a response has been achieved (AbrB inactivation) it persists after the input signal is removed (decreasing Spo0A~P). Therefore, derepression of genes under AbrB control might be expected to require continuously rising levels of Spo0A~P but once AbrB is inactivated, expression of target genes should persist even if Spo0A~P levels subsequently drop.

In closing, we note that the *abbA* gene is found exclusively in the genomes of bacteria that have *abrB*, a finding in keeping with the idea that AbbA is a dedicated antirepressor for AbrB (Fig. S2). Interestingly, whereas AbrB is conserved in all *Bacillus*, *Clostridium*, *Geobacillus* and *Listeria* species examined, AbbA is only present in a subset of these species (those of *Bacillus* and *Geobacillus*). In AbrB-containing species that lack AbbA, an as-yet-unknown mechanism must be responsible for derepression of target genes. Especially interesting is the case of *Listeria monocytogenes*, which lacks both AbbA and Spo0A. Therefore, the mechanism(s) that governs relief from AbrB-mediated repression in *L. monocytogenes* must be unrelated to those that operate in *B. subtilis*. It may be that AbrB first arose as a general regulator of stationary phase and that AbbA appeared later as a sporulation-specific antagonist of AbrB in certain spore-forming species. Perhaps rapid inactivation of AbrB in AbbA-containing species is beneficial because it creates a variety of

options in stationary phase, as exemplified by cannibalism, competence and biofilm formation in *B. subtilis*, before the cells commit to the costly and time-consuming process of sporulation.

Materials and Methods

Strain Construction. All strains used are listed in Table S1.

General Methods. Media, culture conditions, preparation of competent cells, and assays of β -galactosidase activity were as described (42, 43).

Transcriptional Profiling Assay. RNA was isolated from midexponential phase cultures grown in LB medium with IPTG added to a final concentration of 1 mM. RNA was isolated by using the hot acid/phenol method (44). The strains used were AB141 (Δ *abbA::tet*) and AB147 (*thrc::PhyB_{sp}pank⁻abbA*). RNA was prepared and hybridized as described (29). Oligoarrays were as described (9). Images were processed and analyzed with GenePix 4.0 software (Axon Instruments). We included only genes for which there was a 2.5-fold change in expression in at least three of our five replicates.

EMSA. Radiolabeled probes were generated by PCR (with the primers listed in Table S2) by using PY79 chromosomal DNA and the following primer combinations: ECH343/ECH344 (*PcomK*) ECH337/ECH338 (*Psdp*), ECH339/ECH340 (*Pskf*) and AVB041/AVB042 (*Plip*). Each probe was 5' end labeled with 10 mCi of [γ -³²P]-ATP (NEG002A, New England Nuclear) by using polynucleotide kinase (New England Biolabs). Various concentrations of His₆-AbrB, His₆-AbbA or BSA were added to \approx 100 nM radiolabeled probe. Binding reactions were carried out in 30- μ l volumes including binding buffer [20 mM Tris-HCl (pH 8.0), 100 mM KCl, 5 mM MgCl₂, 0.5 mM DTT, 10% glycerol, 0.05% Nonidet P-40] containing 25 mg ml⁻¹ poly dI-dC, at 37°C for 20 min. A 6% polyacrylamide TAE gel was loaded with 10 μ l of each binding reaction and resolved for 2.5 h at 50 V.

Protein Expression Constructs. To generate plasmids for the expression of an N-terminal 6-histidine translation fusion to AbbA (pAB113) and unmodified AbbA (pAB111), PCR products of the *abbA* ORF inclusive of the stop codon were generated by using the primers AVB032/AVB026 (AVB032 contained a sequence to add 6 histidine residues to the N terminus of AbbA) and AVB015/AVB026, respectively. The PCR products were cloned into the NdeI and XhoI sites of plasmid pET21b (Novagen). The plasmids were then transformed into *E. coli* BL21(DE3) RIL-CodonPlus cells (Stratagene). Plasmids for the expression of His₆-AbrB and His₆-SinR (pEH213 and pDP90, respectively) have been described (30, 38).

Protein Purification. His₆-AbbA, His₆-AbrB and His₆-SinR were purified as described (38). To purify unmodified AbbA, BL21(DE3) pAB111 was grown at 30°C in 1 liter of LB supplemented with 100 μ g ml⁻¹ ampicillin until OD₆₀₀ reached \approx 0.5. IPTG was added to a final concentration of 1 mM and the culture grown for 2 h at 30°C. The cells were harvested, resuspended in 30 ml of binding buffer [50 mM Tris (pH 7.5), 25 mM NaCl] and lysed by sonication. The lysate was centrifuged at 32,000 \times g and the supernatant was loaded onto a HiTrap Q Sepharose Fast Flow (GE Healthcare) anion exchange column that had been equilibrated with binding buffer. The column was washed with 50 ml of binding buffer, and then washed again with 10 ml of binding buffer containing 250 mM NaCl. A stepwise elution was performed with 4-ml aliquots of 50 mM Tris (pH 7.5) supplemented with 350, 400, 450, 500, and 550 mM NaCl. Purity was assessed by separating load, flow-through, wash and elutions by 15% PAGE.

Copurification of AbrB with His₆-AbbA. Overnight cultures of PY79 grown at 22°C were diluted into 50 ml of LB medium and grown until midexponential phase. Harvested cells were resuspended in 5 ml of sucrose buffer (500 mM sucrose, 20 mM MgCl₂, 10 mM KPO₄, and 0.1 mg ml⁻¹ lysozyme) and incubated at 37°C for 20 min. The cells were pelleted, resuspended in 5 ml of binding buffer [25 mM Tris (pH 8.0), 100 mM KCl, 5 mM MgCl₂, 0.2 mM DTT, and 10% (vol/vol) glycerol], and lysed by sonication. Lysate was cleared of debris as described above.

The cleared lysate was incubated with highly purified His₆-AbbA or His₆-SinR (20 μ M final concentration of peptide) for 1 h at 4°C (final volume, 5 ml). Each binding reaction was placed onto 500 μ l (bed volume) of Ni²⁺-NTA-agarose, washed with 50 ml of binding buffer containing 20 mM imidazole, and eluted with 2 ml of binding buffer containing 500 mM imidazole. Equal volumes of the various fractions were loaded onto a 15% polyacrylamide gel and AbrB, His₆-AbbA, His₆-SinR and σ^A were detected both by immunoblotting by using specific antisera and by Coomassie staining.

Copurification of AbbA with His₆-AbrB. Highly purified AbbA (2 μ M) was added to binding buffer as above and incubated with highly purified His₆-AbrB or His₆-SinR (20 μ M) (for a total volume of 1 ml) at 37°C for 10 min. One hundred microliters (bed volume) of Ni²⁺-NTA agarose was added to each binding reaction and incubated for 10 min at room temperature. The Ni²⁺-NTA agarose was pelleted, and the supernatant removed and set aside. Each reaction was then washed with 30 ml of binding buffer containing 20 mM imidazole, and eluted at 50°C with 100 μ l of SDS sample buffer. AbbA, His₆-AbrB, and His₆-SinR were detected in the various fractions by Coomassie staining.

Immunoblot Analysis. Cells were collected in 1-ml aliquots and resuspended in an equal volume of lysis buffer [20 mM Tris (pH 8.0), 100 mM NaCl]. Lysozyme was

added to each sample to a final concentration of 0.1 mg ml⁻¹ and incubated at 37°C for 15 min. Protein concentration was determined with Coomassie Plus Bradford kit (Pierce), and equal amounts of protein from each sample was loaded onto a 15% polyacrylamide gel. AbrB, Spo0A and σ^A levels were followed by immunoblot analysis by using specific antibodies. Anti-AbbA and anti-AbrB antibodies were raised in rabbits by using purified *B. subtilis* His₆-AbbA and His₆-AbrB proteins, respectively (Cocalico Biologicals). The anti-SinR antibodies were obtained from F. Chu (Harvard University, Cambridge, MA).

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