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Cell

A Three-Protein Signaling Pathway Governing Immunity to a Bacterial Cannibalism Toxin

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SUMMARY

We describe a three-protein signal-transduction pathway that governs immunity to a protein toxin involved in cannibalism by the spore-forming bacterium *Bacillus subtilis*. Cells of *B. subtilis* enter the pathway to sporulate under conditions of nutrient limitation but delay becoming committed to spore formation by killing nonsporulating siblings and feeding on the dead cells. Killing is mediated by the exported toxic protein SdpC. We report that extracellular SdpC induces the synthesis of an immunity protein, SdpI, that protects toxin-producing cells from being killed. SdpI, a polytopic membrane protein, is encoded by a two-gene operon under sporulation control that contains the gene for an autorepressor, SdpR. The autorepressor binds to and blocks the promoter for the operon. Evidence indicates that SdpI is also a signal-transduction protein that responds to the SdpC toxin by sequestering the SdpR autorepressor at the membrane. Sequestration relieves repression and stimulates synthesis of immunity protein.

INTRODUCTION

Microbial cells respond to stress by inducing the expression of an appropriate suite of adaptive (stress-response) genes that help the cells cope with adverse environmental circumstances. Generally, the response to adverse conditions is rapid and reversible and involves the coordinate induction of the stress-response genes. Under certain conditions, certain microbes respond to stress in a more elaborate manner, an extreme example of which is endospore formation in *Bacillus subtilis*. Endospore (or more simply, spore) formation, which is triggered by nutrient limitation, is a complex developmental process that involves the expression of more than 500 genes over the course of 6 to 8 hr (Britton et al., 2002; Eichenberger et al., 2004; Fujita et al., 2005; Molle et al., 2003; Steil et al., 2003). The process culminates in the formation of a resting cell that is capable of resisting environmental extremes and remaining dormant for long periods of time. It might be expected that the decision to commit to spore formation is not taken lightly since converting a cell into a spore requires a large investment of time and energy and because the process becomes irreversible after about 2 hr (Dworkin and Losick, 2005; Parker et al., 1996). Indeed, evidence indicates that, under conditions of high cell population density (colonies), the bacterium forestalls committing itself for as long as possible by a process of cannibalism (Gonzalez-Pastor et al., 2003), a central aspect of which is the subject of this report.

The master regulator for entry into sporulation is the response regulator Spo0A, which is activated by phosphorylation (Burbulys et al., 1991). Spo0A is activated in response to nutrient limitation, but only about half of the cells in the population activate the master regulator under nutrient-limiting conditions (Chung et al., 1994; Fujita et al., 2005; Gonzalez-Pastor et al., 2003). Evidence indicates that activation of Spo0A is subject to a bistable switch and that the switch is mediated by a positive-feedback loop involving the gene for Spo0A and genes for proteins that govern its phosphorylation (Veening et al., 2005). The generation of a mixed population of cells in which Spo0A is active (Spo0A-ON) or in which it is not (Spo0A-OFF) is the basis for cannibalism (Figure 1A). In Spo0A-ON cells, the master regulator switches on the transcription of two operons, *skfA-H* and *sdpABC*, that are responsible, respectively, for the production and export of a peptide-antibiotic-like killing factor and a protein toxin that kill Spo0A-OFF siblings (Figure 1A). Nutrients released from the dead cells slow down or prevent Spo0A-ON cells from reaching the stage of development when sporulation becomes irreversible. Thus, by killing their Spo0A-OFF

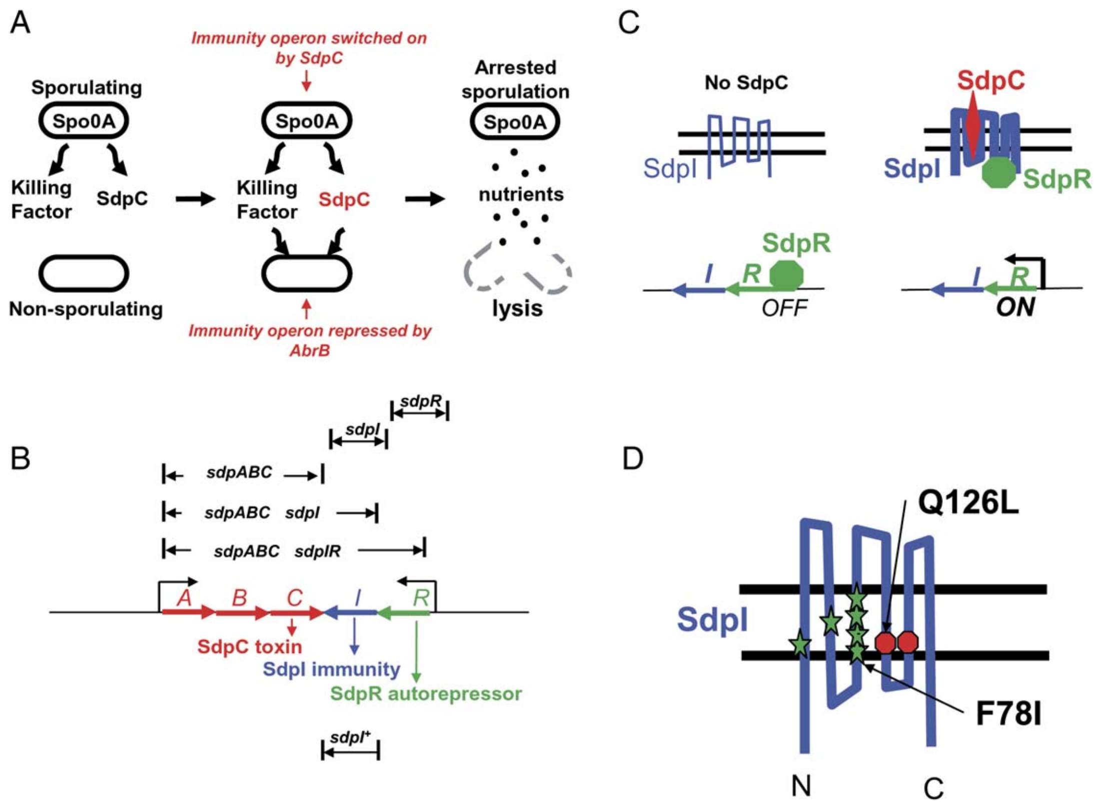

Figure 1. Cannibalism and the Induction of Immunity Protein

(A) A model for cannibalism depicting a cell in which Spo0A is activated (Spo0A-ON in the text) and a cell in which it is not (Spo0A-OFF). Spo0A-ON cells produce and export a peptide killing factor and the SdpC toxin, which kill Spo0A-OFF siblings. The dead cells release nutrients that arrest sporulation by Spo0A-ON cells.

(B) The panel depicts the convergent *sdpABC* and *sdpRI* operons and identifies genes encoding toxin, immunity protein, and autorepressor. The bent arrows represent promoters. The lines above the operons denote the deletions used in this study. The line below the operon indicates the *sdpI*-containing DNA segment used for complementation.

(C) Sequestration model for how extracellular SdpC induces expression of the *sdpRI* immunity operon. The thick arrow on the right denotes high-level transcription, and the thick symbol for SdpI on the right indicates its presence in high abundance.

(D) Amino acid substitutions of SdpI that rendered expression of the *sdpRI* operon independent of (green stars) or refractory to (red octagons) SdpC. The SdpC-independent amino acid substitutions were I6T, I50T, F78I, M84T, L85S, and I98K, and the refractory substitutions were Q126L and S156T.

siblings, Spo0A-ON cells delay becoming committed to spore formation.

How do Spo0A-ON cells avoid killing themselves? In the case of the killing factor, the answer is evidently simple. The *skfA-H* operon contains the genes for the biosynthesis of the inferred, peptide-like antibiotic as well as the genes for an export pump that transports the killing factor out of the producing cells and prevents self-killing (Gonzalez-Pastor et al., 2003). Thus, Spo0A switches on both the production of the killing factor and the protective export pump. Since Spo0A-OFF cells do not express *skfA-H*, they produce neither killing factor nor the pump that confers resistance to it (Gonzalez-Pastor et al., 2003).

In contrast, the basis for self-resistance to the toxin has been mysterious. The toxin (SdpC*) is a 63 amino acid protein that is derived from the C-terminal portion of the product of *sdpC*, the third member of the three-gene *sdpABC* operon (Figure 1B). All three genes are needed for production and export of the toxin (hereafter simply referred to as SdpC). A striking feature of SdpC is that it is an intercellular, signaling protein that induces the expression of an adjacent, convergent, two-gene operon herein referred to as *sdpRI* (Figure 1B; formerly *yvaZ*- *yvbA*). In earlier work, we speculated that SdpC exerts its toxic effects by inducing the expression of *sdpRI* in target cells (Gonzalez-Pastor et al., 2003). Our current results, however, lead us to the conclusion that *sdpRI* is an immunity operon and that its induction in response to SdpC protects Spo0A-ON cells from the toxic effects of the protein. Here we present evidence that SdpI is an immunity protein and that its synthesis is induced by a remarkably simple signal-transduction system consisting of SdpC, SdpI, and SdpR. We argue that SdpC is both a toxin and a ligand; that SdpI is both an immunity protein and a receptor/signal-transduction protein; and, finally, that SdpR is an autorepressor. Evidence indicates that induction of the operon is achieved by membrane sequestration of the

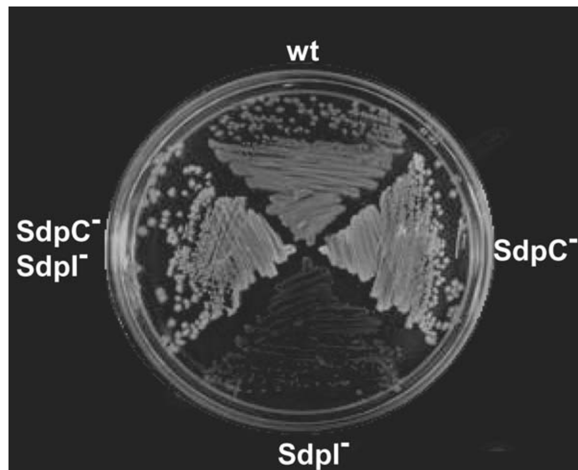

Figure 2. SdpI Is Required for Immunity to the SdpC Toxin
Strains were wild-type (PY79) or mutant for SdpC toxin (Δ*sdpABC*; strain EH273), SdpI immunity protein (Δ*sdpI*; strain RL2937), or both (Δ*sdpABC* Δ*sdpI*; strain EH274). Deletions are described in Figure 1B and the Experimental Procedures. Growth was for 16 hr at 37ºC on solid DS medium (Harwood and Cutting, 1990).

SdpR autorepressor in a manner that depends both on SdpC and SdpI (Figure 1C). Finally, we show that expression of *sdpRI* is blocked in Spo0A-OFF cells by the action of a repressor known as AbrB. Thus, Spo0A-ON cells avoid self-killing by inducing the synthesis of the SdpI immunity protein in response to the SdpC toxin/ligand. In contrast, Spo0A-OFF cells are unable to induce SdpI synthesis due to the action of the AbrB repressor and hence succumb to the toxin (Figure 1A).

RESULTS

SdpI Confers Immunity to the SdpC Toxin

We began our investigation by building an insertion/deletion mutation of the downstream member of the *sdpRI* operon, *sdpI* (Figure 1B). On solid sporulation medium (DS), the *sdpI* mutant grew as small transparent colonies (Figure 2). Poor growth was dependent upon SdpC because a strain deleted for both *sdpABC* and *sdpI* (created by means of a single deletion that removed all four genes; Figure 1B) grew normally and exhibited the accelerated sporulation phenotype characteristic of *sdpABC* mutants (Figure 2). Thus, the absence of SdpC was epistatic to the absence of SdpI. The simplest interpretation of these results is that SdpI is an immunity protein that protects cells from the toxic effects of SdpC.

To quantify the effects of SdpC and SdpI and to show that SdpC acts in an extracellular manner, we performed competition experiments with mixtures of wild-type and mutant bacteria. Wild-type cells (that had been tagged with *lacZ*) were mixed with an equal number of cells that were mutant for SdpC alone (as a result of an *sdpABC* deletion) or both SdpC and SdpI (as a result of a single deletion removing both the *sdpABC* and *sdpRI* operons; see Figure 1B), and the cell mixture was plated on solid DS medium. After 24 hr, we determined a competitive index, which was the ratio of the mutant to wild-type ($LacZ^+$) cells divided by the initial ratio of mutant to wild-type cells. Thus, if a mutant strain were as fit as the wild-type, the expected ratio would be 1. We found that a strain that lacked SdpC but could produce the immunity protein SdpI was not at a competitive disadvantage to the wild-type (line 1, Table 1). However, a strain that was unable to produce both SdpC and SdpI had a markedly low index (line 2, Table 1). Moreover, the low competitive index of the mutant lacking SdpC and SdpI was reversed by a construct (Figure 1B) in which *sdpI* was under the control of an inducible promoter and was inserted into the chromosome at the *amyE* locus (line 4, Table 1).

Table 1. Competition Experiments with LacZ-Tagged Bacteria

lacZ-Containing Strains (Relevant Genotype)	Test Strain (Relevant Genotype)	Competitive Index	p Value
wt	Δ*sdpABC*	1.493	0.283
wt	Δ*sdpABC* Δ*sdpRI*	0.040	0.024
Δ*sdpABC*	Δ*sdpABC* Δ*sdpRI*	1.120	0.133
wt	Δ*sdpABC* Δ*sdpRI* *amyE*::$P_{hy\text{-}spac}$-*sdpI*	2.389	0.734
wt	Δ*sdpABC* Δ*sdpIR* *amyE*::$P_{hy\text{-}spac}$-*sdpI* ^{Q126L}	2.049	0.038

The *lacZ*-containing strains were wt (CDE816) and Δ*sdpABC* (CDE817; see Table S1). The test strains were Δ*sdpABC* (EH273), Δ*sdpABC* Δ*sdpRI* (EG494), Δ*sdpABC* Δ*sdpRI amyE*::$P_{hy\text{-}spac}$-*sdpI* (CDE235), and Δ*sdpABC* Δ*sdpIR amyE*::$P_{hy\text{-}spac}$-*sdpI*^{Q126L} (CDE541; see Table S1). The competitive index (CI) was the ratio of the number of $LacZ^-$ cells (test strain) to $LacZ^+$ cells arising after growth in solid DS medium divided by the input ratio of cells from the two strains. Student's t test was used for statistical analyses.

Because a strain harboring a deletion (Figure 1B) that removed both *sdpABC* and *sdpRI* was at a strong competitive disadvantage (line 2, Table 1), we conclude that sensitivity to SdpC did not require SdpR or any other product of the two operons. Because the strain used in the complementation experiment (line 4, Table 1) was likewise lacking both operons, we also conclude that SdpI was both necessary and sufficient to confer immunity to SdpC. In sum, the results are consistent with the idea that SdpC is an exported, toxic protein against which SdpI confers immunity.

SdpR Represses the *sdpRI* Operon

It was known that SdpC, acting extracellularly, activates transcription of *sdpRI*, but the transcription factor responsible for controlling the operon was not identified. We now present evidence that SdpR, a member of the ArsR family

of transcription factors, is an autorepressor that is responsible for inhibiting transcription from *sdpRI*. Transcription was monitored using *lacZ* fused to the promoter region for the operon, P_{sdpRI}-*lacZ*. The results of Figure 3 show that a mutant lacking SdpC (as a result of a deletion removing both *sdpABC* and *sdpI*; Figure 1B) was strongly impaired in the expression of the P_{sdpRI}-*lacZ* construct. However, a mutant that was lacking SdpC as well as SdpR (as a result of a deletion removing both the *sdpABC* and *sdpRI* operons; Figure 1B) exhibited a high level of P_{sdpRI}-*lacZ* expression (Figure 3). That is, P_{sdpRI}-*lacZ* was expressed in an SdpC-independent manner in the absence of SdpR. These results are consistent with the idea that SdpR is responsible for repressing *sdpRI* and that the SdpC toxin somehow counteracts or inactivates SdpR and thereby induces the operon.

SdpR Binds to the Promoter Region of the *sdpRI* Operon

The promoter for the *sdpRI* operon is contained within a 447 bp DNA segment spanning the region between *sdpR* and the gene immediately upstream (Gonzalez-Pastor et al., 2003). To localize the promoter precisely, we carried out primer extension analysis to map the 5′ end of RNA originating from the operon (Figure 4A). The results of Figure 4A show that upstream of the 5′ terminus (putative start site) were sequences that exhibited a three-out-of-six match (TTGttt) to the canonical −35 sequence (TTGACA) and a perfect match (TATAAT) to the canonical −10 sequence for promoters recognized by σ^A-containing RNA polymerase.

To determine whether SdpR interacts with the promoter region, we performed electrophoretic mobility shift assays using purified SdpR that had been tagged at its C terminus with six histidine residues (SdpR-His_6) and DNA corresponding to a 418 bp DNA segment containing the *sdpRI* promoter (P_{sdpRI}). As a negative control, we used a similarly sized DNA from within the open-reading frame for *sdpC*. The results of Figure 4B show that SdpR-His_6 retarded the electrophoretic mobility of the P_{sdpRI}–containing DNA but not that of the control DNA. The existence of two retarded species suggests that SdpR-His_6 binds to a minimum of two sites in the promoter region.

Finally, we carried out DNase I footprinting to localize the binding sites for SdpR. A 108 bp DNA probe corresponding to the region upstream and inclusive of the predicted start codon for *sdpR* was incubated with various concentrations of SdpR-His_6 and then treated with DNase I. Upon subjecting the products of enzyme treatment to gel electrophoresis, we observed two regions of protection: a 7 bp region located immediately upstream of the transcriptional start site (position +1) and a 25 bp region located between positions +5 and +29 (Figure 4A). Contained within the two regions were four similar direct repeat sequences centered at positions -4 (5′-TGAAAAT-3′), +13 (5′-TACAAAT-3′), +21 (5′-TCTAA AT-3′), and +29 (5′-TCTAAAT-3′). Other members of the ArsR family are known to bind to DNA as homodimers (Busenlehner et al., 2003), and the results of our electrophoretic mobility shift and DNase I footprinting assays suggest that SdpR binds to the *sdpRI* promoter region as two dimeric complexes. The extensive overlap of the SdpR binding sites with P_{sdpRI} suggests that the autorepressor blocks transcription by interfering with the binding of RNA polymerase to the promoter.

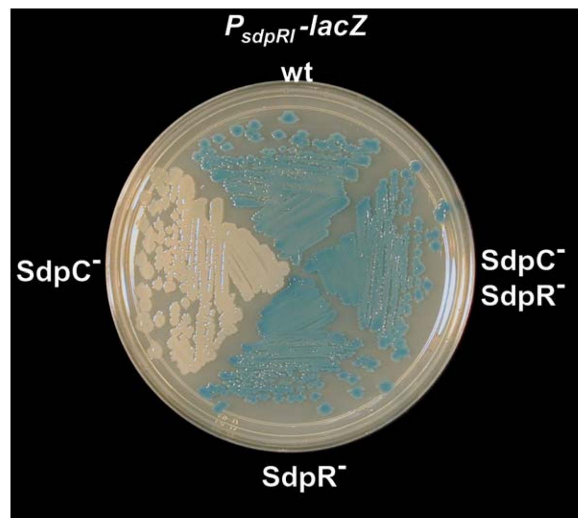

Figure 3. SdpR Is Required for Repression of the *sdpRI* Immunity Operon

All strains contained P_{sdpRI}-*lacZ* and were otherwise wild-type (wt; strain CDE304) or mutant for the SdpC toxin and the SdpI immunity protein (Δ*sdpABC* Δ*sdpI*; strain CDE569), the SdpR autorepressor (Δ*sdpR*; strain CDE362), or all three (Δ*sdpABC* Δ*sdpIR*; strain CDE312). Growth was for 16 hr at 37ºC on solid LB medium (Harwood and Cutting, 1990) containing X-Gal.

The SdpI Immunity Protein Is Required for the Transcriptional Response to SdpC

How does extracellular SdpC toxin relieve SdpR-mediated repression of the *sdpRI* operon? A clue came from the observation that a mutant lacking the SdpI immunity protein was blocked in the expression of the *sdpRI* operon (see Figure S1 in the Supplemental Data available with this article online). Two possible explanations for this observation were that SdpI is required for the production of SdpC or that SdpI is involved in the transcriptional response to the toxin.

To investigate the possibility that SdpI is required for SdpC production, we carried out a series of extracellular complementation experiments each involving three parallel streaks of cells on solid sporulation medium in which the two flanking streaks were from a reporter strain that lacked the *sdpABC* operon and contained the P_{sdpRI}-*lacZ* fusion construct. The results of Figure 5 (top set of streaks) show that induction of P_{sdpRI}-*lacZ* was readily detected when the streak in the middle (the producer strain) was generated with wild-type cells or with cells of an *sdpI* mutant. In contrast and as a control, no induction was detected when the middle streak was generated with cells of an *sdpABC* mutant. We conclude that SdpI is not required for the production or export of the SdpC toxin.

Next we asked whether SdpI was required for the response to SdpC by creating a P_{sdpRI}-*lacZ*-bearing reporter

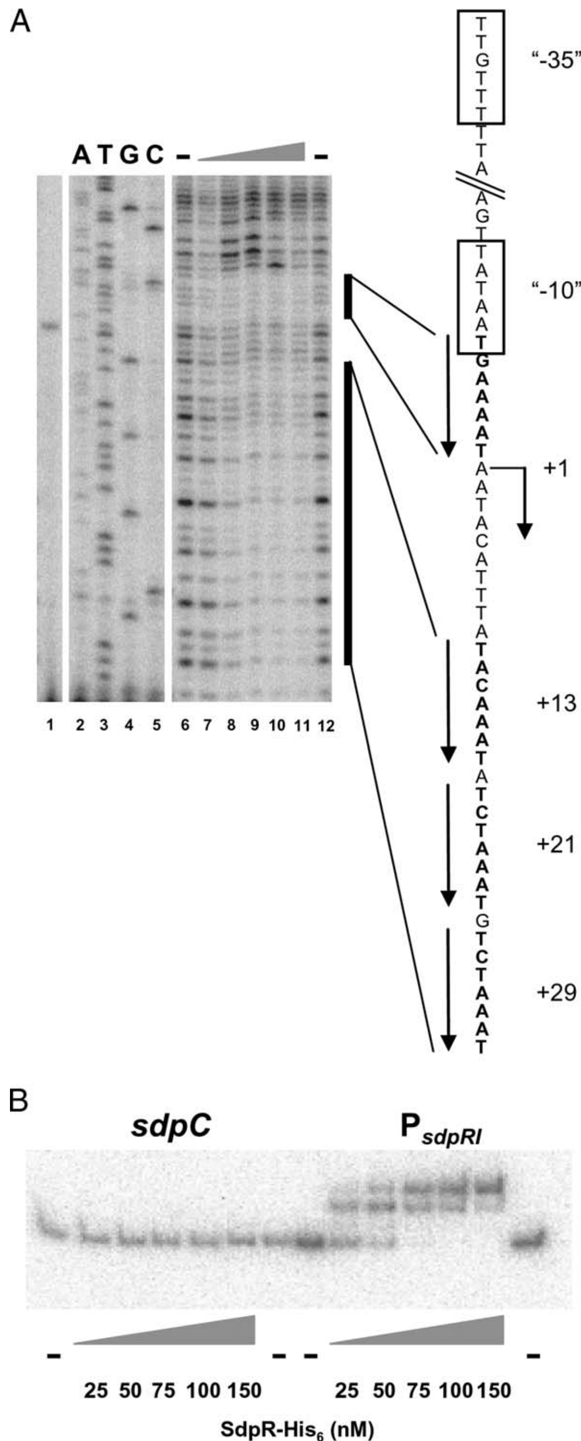

Figure 4. Identification of SdpR Binding Sites within the *sdpRI* Promoter Region

(A) Identification of the *sdpRI* transcriptional start site and SdpR binding sites within the P_{sdpRI} promoter region. Lane 1 in (A) shows the results of a primer-extension assay performed to determine the transcription start site of the *sdpRI* promoter. RNA isolated from early-stationary-phase *B. subtilis* cells was used as a template for extension of the radiolabeled primer ECH253. The same primer was also used to generate dideoxy-sequencing ladders that were terminated with ddATP (lane 2), ddTTP (lane 3), ddGTP (lane 4), and ddCTP (lane 5). Lanes 6–12 show the results of a DNase I protection assay performed with radiolabeled DNA and increasing concentrations of SdpR-His_6: 0 nM (lanes 6 and 12), 5 nM (lane 7), 50 nM (lane 8), 100 nM (lane 9), 300 nM (lane 10), and 500 nM (lane 11). The annotated DNA sequence shown to the right of (A) is complementary to the sequence shown in lanes 2–5. The putative −10 and −35 promoter elements are marked with solid black boxes. Predicted recognition sequences for SdpR-His_6 are denoted by black arrows and a number indicating the position of the center of the sequence relative to the transcription start site.

(B) Electrophoretic mobility shift assays shown in (A) contain ^{32}P-labeled DNA corresponding to a DNA fragment internal to the *sdpC* open reading frame or to DNA contained within the *sdpR* promoter region. Increasing concentrations of purified SdpR-His_6 ranging from 0 nM to 150 nM were included in the indicated reactions.

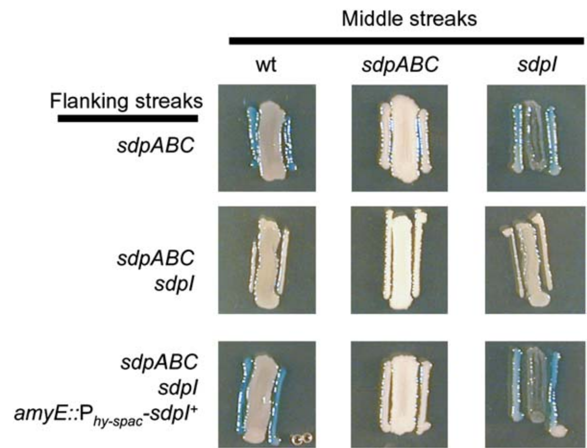

Figure 5. The SdpI Immunity Protein Is Required for the Induction of P_{sdpRI}-*lacZ* in Response to Extracellular SdpC

The central streaks were wild-type (wt; PY79) or mutant for *sdpABC* (SdpC$^-$; EH273) or mutant for *sdpI* (SdpI$^-$; RL2937). (Note that the central streaks of SdpC-producing cells in the right-hand column were undergoing lysis due to the absence of SdpI.) The flanking streaks were strains that were mutant for *sdpABC* (CDE568), mutant for *sdpABC* and *sdpI* (CDE569), or mutant for *sdpABC* and *sdpI* and containing the complementation construct *amyE::*$P_{hy\text{-}spac}$-*sdpI*$^+$ (CDE571). All of the flanking strains contained the reporter P_{sdpRI}-*lacZ*. Growth was at 37ºC for 24 hr on solid DS medium containing X-Gal and IPTG.

strain that lacked both *sdpABC* and *sdpI*. When this reporter strain (flanking streaks) was tested against the indicated producer strains (middle streaks), little or no expression of P_{sdpRI}-*lacZ* was detected (Figure 5, center set of streaks). (Since killing requires higher concentrations of toxin than induction does, the gaps between the streaks allowed induction to be studied without extensive killing of the cells lacking SdpI [left- and right-hand images].)

Finally, we created a P_{sdpRI}-*lacZ*-bearing reporter strain that lacked both *sdpABC* and *sdpI* but additionally harbored an inducible copy of *sdpI* that had been inserted into the chromosome at the *amyE* locus (*amyE*::$P_{hy\text{-}spac}$-*sdpI*). The results of Figure 5 (bottom set of streaks) show that the presence of the *amyE*::$P_{hy\text{-}spac}$-*sdpI* construct restored the

capacity of the reporter strain to respond to SdpC. In toto, the results indicate that SdpI is involved in the transcriptional response to the toxin.

SdpI Is Both an Immunity Protein and a Signal-Transduction Protein

SdpI is predicted to be an integral membrane protein with multiple membrane-spanning segments (Figure 1C). Two models for how it could mediate the transcriptional response to SdpC are that (1) SdpI is a transporter that pumps SdpC into the cell where the toxin directly or indirectly antagonizes the SdpR autorepressor and (2) SdpI is a signal-transduction protein that acts as a receptor for SdpC on the outside surface of the cell, transducing a signal that triggers events inside the cell that inactivate the SdpR autorepressor. To distinguish between these models, we sought mutants of SdpI that would constitutively activate transcription of the *sdpRI* operon in the absence of SdpC. We reasoned that if SdpI is a receptor/signal-transduction protein, then it should be possible to obtain mutants that lock the protein in an activated state in the absence of the SdpC ligand, but that if SdpI acts by transporting SdpC into the cell where it antagonizes the SdpR autorepressor, then it should not be possible to obtain such mutants.

Accordingly, we performed localized mutagenesis on *sdpI* and screened for mutants that allowed P_{sdpRI}-*lacZ* to be expressed in the absence of SdpC. We readily obtained several amino acid substitution mutants of SdpI that partially bypassed the requirement for SdpC in the expression of P_{sdpRI}-*lacZ* (e.g., $SdpI^{F78I}$; Figure S2). Most of the substitutions were found to cluster in the first three, putative transmembrane segments of SdpI (Figure 1D). The simplest interpretation of these results is that that SdpI normally acts as a receptor for SdpC and that SdpI responds to the SdpC ligand by directly or indirectly inhibiting the repressor activity of SdpR.

Mutants of SdpI Blocked in Signal Transduction

We also sought mutants of SdpI that retained the capacity to confer immunity but failed to induce transcription of *sdpRI* in response to SdpC. To do this, we performed localized mutagenesis on an IPTG-inducible copy of *sdpI* and screened for mutants that were unable to induce expression of P_{sdpRI}-*lacZ*. Mutants that failed to induce P_{sdpRI}-*lacZ* were then subjected to a secondary screen by streaking on solid DS medium to identify those that maintained the capacity to protect against killing by SdpC. We obtained two such mutants, one of which, the amino acid substitution mutant $SdpI^{Q126L}$ (Figure 1D), was characterized further. Figure S1 shows that an inducible copy of the mutant gene ($sdpI^{Q126L}$) was unable to respond to extracellular SdpC in inducing the expression of P_{sdpRI}-*lacZ.* The $sdpI^{Q126L}$ mutation was recessive as evidenced by the fact that a strain containing both *sdpI* and $sdpI^{Q126L}$ was fully capable of inducing expression of P_{sdpRI}-*lacZ* (data not shown). To quantify the capacity of resistance of $SdpI^{Q126L}$ to confer immunity to SdpC, we created a strain that contained an inducible copy of $sdpI^{Q126L}$ and that lacked both the *sdpABC* and the *sdpRI* operons. Next, this $SdpI^{Q126L}$-producing strain was mixed with an equal number of wild-type cells. The results of Table 1 show that the $SdpI^{Q126L}$-producing strain was not at a competitive disadvantage to the SdpC-producing, wild-type cells.

In toto, these results indicate that $SdpI^{Q126L}$ had retained the capacity to confer immunity to SdpC but was unable to induce a transcriptional response in the presence of the toxin. In effect, $SdpI^{Q126L}$ exhibited the opposite phenotype of $SdpI^{F78I}$ (above), which was capable of stimulating transcription from the *sdpRI* promoter in the absence of SdpC. On the basis of the phenotypes of both kinds of mutants, we conclude that SdpI has two separable functions: it protects cells against the toxic effects of SdpC, and it responds to SdpC by inducing the *sdpRI* operon and thus the synthesis of additional immunity and repressor protein.

SdpR-GFP Is Sequestered to the Membrane in an SdpC- and SdpI-Dependent Manner

How do SdpI and SdpC prevent repression by SdpR? The answer emerged from experiments in which we visualized the subcellular localization of SdpR using a functional fusion of the autorepressor to the green fluorescent protein (GFP). Production of the SdpR-GFP fusion was placed under the control of the normal promoter for the *sdpRI* operon. The results of Figure 6A show that, in otherwise wild-type cells, the fusion protein was localized to the cytoplasmic membrane (left-hand image). In SdpC mutant cells, in contrast, only a faint signal could be detected (middle image), which upon image enhancement was seen as being diffusely distributed throughout the cytoplasm (right-hand image). The simplest interpretation of these results is that SdpC is responsible for causing SdpR-GFP to become sequestered at the membrane. In the absence of SdpC, SdpR-GFP was not sequestered and hence was free to repress transcription. Because production of SdpR-GFP was under the control of the *sdpRI* promoter, this repression would have resulted in a low level of synthesis of the fusion protein, accounting for the faint signal observed in the mutant cells.

To uncouple the synthesis of both SdpR-GFP and SdpI from autoregulation, we constructed a strain in which the expression of *sdpR-gfp* was under the control of a xylose-inducible promoter and the only copy of *sdpI* was under the control of an IPTG-inducible promoter. The results of Figure 6B show that SdpR-GFP was localized to the membrane of cells of this strain that had been treated with xylose and IPTG. In inducer-treated cells of an otherwise identical strain that was mutant for SdpC, SdpR-GFP was uniformly distributed in the cytoplasm (Figure 6C, left-hand image).

To investigate whether membrane sequestration was dependent upon SdpI, we created a strain in which SdpR-GFP was under the control of the xylose-inducible promoter and that was mutant for SdpI and wild-type for SdpC. Once again, SdpR-GFP was found to be diffusely localized throughout the cytoplasm (Figure 6B, middle image). We conclude that membrane sequestration depends both on the toxin/ligand SdpC and the immunity/signal-transduction protein SdpI.

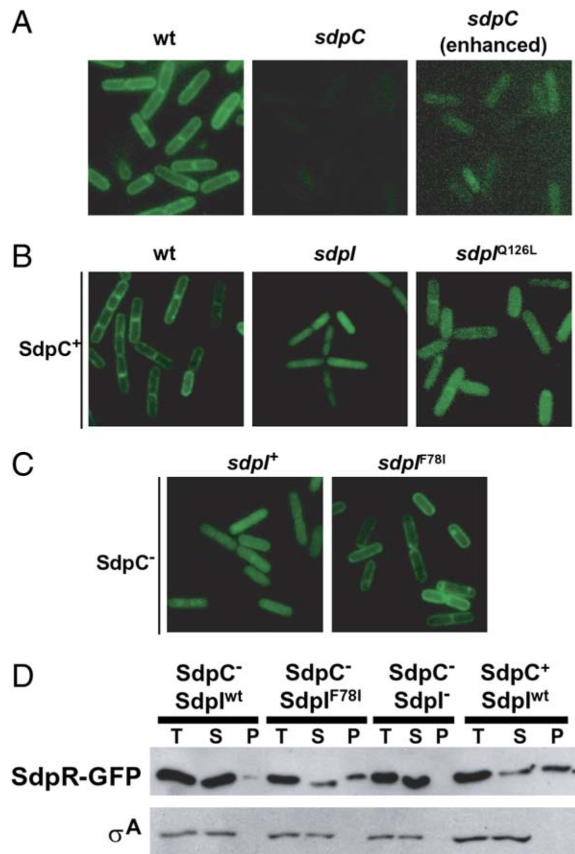

Figure 6. SdpI and SdpC Sequester SdpR-GFP to the Membrane

(A) Shown are cells in which a functional SdpR-GFP fusion was produced under the control of its native *sdpRI* promoter (P_{sdpRI}). The top panel shows, from left to right, fluorescent images of $SdpC^+$ cells (strain EH390), cells mutant for SdpC (Δ*sdpC*; EH391), and an artificially enhanced micrograph of the middle image.

(B) Shown are cells in which SdpR-GFP was produced under the control of a xylose-inducible promoter and that were producing wild-type SdpI (Δ*sdpI amyE::*$P_{hy\text{-}spac}$-*sdpI*; EH398), were lacking SdpI (Δ*sdpI*; EH393), or were producing the $SdpI^{Q126L}$ mutant protein (Δ*sdpI amyE::*$P_{hy\text{-}spac}$-*sdpI*^{Q126L}; CDE622).

(C) Shown are cells in which SdpR-GFP was produced under the control of a xylose-inducible promoter and that were lacking SdpC. Additionally, the cells in the image on the left were producing wild-type SdpI (Δ*sdpABC* Δ*sdpI amyE::*$P_{hy\text{-}spac}$-*sdpI*; EH394), and those on the right were producing the $SdpI^{F78I}$ mutant protein (Δ*sdpABC* Δ*sdpI amyE::*$P_{hy\text{-}spac}$-*sdpI*^{F78I}; EH395).

(D) Shown is an immunoblot of proteins from cells grown on solid DS medium. T, S, and P represent total, soluble, and pellet fractions, respectively. All four strains produced SdpR-GFP under the control of xylose. The phenotypes of the strains with respect to SdpI and SdpC are indicated at the top of the figure. The strains used for this experiment (from left to right on the gel: EH394, EH395, EH404, and EH398) are as described above, with the exception of EH404 (*thrC::*P_{xyl}-*sdpR-gfp sdpABCI::kan*). The immunoblot was probed with antibodies against the cytoplasmic protein σ^A and GFP.

Based on these results, we predicted that the constitutively active mutants of SdpI (e.g., $SdpI^{F78I}$), which are capable of activating P_{sdpRI}-*lacZ* in the absence of SdpC, would sequester SdpR-GFP to the membrane in an SdpC-independent manner. Conversely, mutants of SdpI that provide immunity against SdpC but fail to activate P_{sdpRI}-*lacZ* in its presence (e.g., $SdpI^{Q126L}$) would be blocked in membrane sequestration. To test these predictions, we created constructs in which the *sdpI*^{F78I} and *sdpI*^{Q126L} alleles were under the control of the IPTG-inducible promoter. Figure 6C (right-hand panel) shows that SdpR-GFP was localized to the membrane in cells of an $SdpI^{F78I}$-producing strain that was mutant for SdpC. Conversely, Figure 6B (right-hand panel) shows that SdpR-GFP was diffusely distributed in the cytoplasm of an $SdpI^{Q126L}$-producing strain that was $SdpC^+$.

As a biochemical test of the idea that the SdpR autorepressor is sequestered in an SdpI-dependent manner, we carried out subcellular fractionation experiments in which SdpR-GFP-producing cells were disrupted and soluble proteins were separated from insoluble material (the pellet) by centrifugation (Figure 6D). We monitored the presence of SdpR-GFP in the soluble and pellet fractions with material from cells of a strain engineered to produce the constitutively active mutant form of the immunity protein $SdpI^{F78I}$ and with material from cells of a strain of an SdpI null mutant. SdpR-GFP was detected by immunoblot analysis using anti-GFP antibodies. Strikingly, SdpR-GFP was present in the pellet fraction from cells of the constitutively active $SdpI^{F78I}$ mutant, but little or no SdpR-GFP was in the pellet fraction of the SdpI null mutant. As a control, immunoblot analysis with antibodies directed against the RNA polymerase subunit σ^A showed that the presence of this cytoplasmic protein was restricted to the soluble fraction. The results of Figure 6D also show that SdpR-GFP was detected in the pellet fraction of cells producing wild-type SdpI and that the level of the signal was stronger in the presence of SdpC than in its absence. Finally, an experiment with cells producing the mutant form ($SdpI^{Q126L}$) of the signal-transduction protein that was unable to sequester SdpR-GFP revealed little or no SdpR-GFP in the membrane-containing pellet fraction (data not shown).

sdpRI Is under the Indirect Control of Spo0A

A final question was why cells that have not activated Spo0A are killed by the SdpC toxin. That is, why are they unable to induce the immunity operon? A simple hypothesis was that the *sdpRI* operon (like the *sdpABC* operon) is under Spo0A control, thereby ensuring that only cells that have entered the pathway to sporulate will be able to produce the SdpI immunity protein. In confirmation of this hypothesis, the results of Figure 7 show that, in the absence of SdpC and SdpR, P_{sdpRI}-*lacZ* was induced in liquid sporulation medium at the start of sporulation and that this induction was dependent upon Spo0A. The dependence on Spo0A was, however, indirect, as P_{sdpRI}-*lacZ* was expressed at a high level during both growth and sporulation in cells of an AbrB mutant and in cells doubly mutant for Spo0A and AbrB (Figure 7 and Figure S3). The gene for AbrB is known to be under the direct negative control of Spo0A, and AbrB is known to be a repressor of certain genes that are switched on at the onset of sporulation (Strauch et al., 1989, 1990).

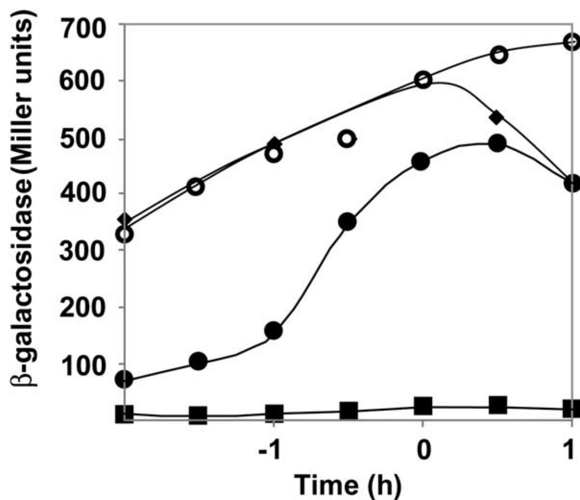

Figure 7. The *sdpRI* Operon Is under the Indirect Control of Spo0A via the AbrB Repressor

All strains harbored *amyE::P$_{sdpRI}$-lacZ* and carried a single deletion of *sdpABC* and *sdpIR* (Figure 1B). The strains were wild-type for *spo0A* and *abrB* (EG504) (closed circles), mutant for *spo0A* (EH253) (closed boxes), mutant for *abrB* (EH254) (closed diamonds), or mutant for both *spo0A* and *abrB* (EH255) (open circles). Cells were grown in liquid DS medium to induce sporulation; the initiation of sporulation is hour 0. The β-galactosidase activity units were calculated as Miller units (Harwood and Cutting, 1990; Miller, 1972).

DISCUSSION

A Three-Protein Signal-Transduction System

We have elucidated a remarkably simple signal-transduction system that is composed of a ligand, SdpC; a receptor/signal-transduction protein, SdpI; and an autorepressor, SdpR. We propose that the ligand SdpC interacts at the membrane with SdpI, an inferred polytopic membrane protein, to induce a conformational change in the receptor/signal-transduction protein. This conformational change, in turn, allows SdpI to bind SdpR and thereby capture the autorepressor at, and on the cytoplasmic face of, the membrane (Figure 1C). Sequestration in a complex with SdpI at the membrane directly or indirectly prevents SdpR from binding at the promoter for the *sdpRI* operon, thereby relieving repression and stimulating the synthesis of SdpR and SdpI. At the heart of our model is a heteromeric complex composed of SdpC, SdpI, and SdpR (Figure 1C), but we do not know whether these are the only proteins in the complex and whether SdpI directly contacts SdpC and SdpR.

Underscoring the economy of this simple system, two of its components have a second function. Thus, SdpC is the ligand that induces *sdpRI* and also a toxin that kills target cells during cannibalism. The mechanism by which this 63 residue peptide acts is unclear, but a possible clue is the presence of a putative hydrophobic α helix that resembles a transmembrane segment. It is therefore attractive to imagine that SdpC acts by inserting itself into the membrane of target cells where it could, for example, cause the membrane to become leaky. Also, and in keeping with the idea that SdpC is a toxin, the peptide's amino acid sequence weakly resembles that of certain antimicrobial peptides of insects known as defensins (Lehane et al., 1997).

SdpI also does double duty in that it is both a signal-transduction protein and an immunity protein. That SdpI has two distinct functions is supported by the isolation of mutants that separate one function from the other. Thus, the mutant protein SdpI^{Q126L} was capable of conferring immunity to SdpC but failed to trigger induction of the *sdpRI* immunity operon in the presence of the toxin/ligand. Conversely, mutants like SdpI^{F78I} were capable of causing membrane sequestration of the SdpR autorepressor and transcription of *sdpRI* in the absence of SdpC. The simplest interpretation of our results is that SdpC forms a complex with SdpI in the membrane and that this SdpC-SdpI complex serves both to neutralize the toxic effects of SdpC and to induce the expression of *sdpRI*.

The SdpR autorepressor belongs to the ArsR/SmtB family of repressors, whose prototypical member, ArsR, inhibits the transcription of genes involved in resistance to arsenate (Busenlehner et al., 2003). Expression of these genes is induced by arsenate, which acts by binding to a metal binding domain in the protein. In contrast to ArsR and other family members, SdpR lacks the conserved amino acids found in metal-responsive members of the family (Busenlehner et al., 2003). Instead, our evidence indicates that induction of its target, *sdpRI*, is achieved by sequestration of the autorepressor in a complex with SdpI.

Repressor Regulation by Membrane Sequestration

Regulation of a bacterial repressor by membrane sequestration is an unusual mode of gene control in bacteria, but two precedents are known. One is PutA, a repressor of genes involved in proline uptake and utilization in *Salmonella typhimurium* (Ostrovsky de Spicer et al., 1991). In the absence of proline, PutA binds to and represses *putA* and *putP*, which are required for utilization and transport of proline, respectively (Hahn et al., 1988). In the presence of proline, however, PutA redistributes itself to the membrane, losing its ability to bind to DNA (Ostrovsky de Spicer and Maloy, 1993; Ostrovsky de Spicer et al., 1991). Genetic and biochemical evidence indicate that DNA binding and membrane localization are mutually exclusive (Muro-Pastor et al., 1997; Ostrovsky de Spicer and Maloy, 1993). A second is the *E. coli* repressor Mlc, which is sequestered at the membrane by the glucose transporter IICB when it is phosphorylated and actively importing glucose (Lee et al., 2000; Tanaka et al., 2000). Mlc is unable to repress target genes in a transporter-independent manner when it is artificially tethered to the membrane (Bohm and Boos, 2004; Tanaka et al., 2004).

Thus, in these examples, simple sequestration at the membrane is evidently sufficient to block repressor activity. It will be interesting to determine whether this is also the case for SdpR or whether the interaction of SdpR with SdpI contributes to preventing the autorepressor from blocking the *sdpRI* promoter.

Just-in-Time Regulation

We view the SdpC-SdpI-SdpR system as a just-in-time regulatory circuit. In the absence of the toxin/ligand SdpC, the *sdpRI* operon is held at low (basal) level of expression by the autorepressor SdpR, and hence only a small number of SdpI molecules are produced (left side of Figure 1C). When SdpC appears in the medium, it binds to the SdpI signal-transduction protein, triggering sequestration of SdpR (right side of Figure 1C). This derepresses the operon, leading to the synthesis of more SdpI molecules and more SdpR molecules. As long as sufficient SdpC is present to bind to all of the SdpI molecules, newly synthesized SdpR molecules are sequestered at the membrane, leading to continued high-level transcription. However, when excess, unligated SdpI molecules start to accumulate, free SdpR molecules begin to accumulate in the cytoplasm and inhibit further transcription. Thus, just-in-time regulation explains how SdpC-producing cells make only as much SdpI immunity protein as they need.

The Immunity Operon Is Subject to an AND Gate

Just-in-time regulation leaves unanswered the question of why during cannibalism cells that have not activated Spo0A are killed by the toxin. The discovery that the *sdpRI* operon is also under the indirect control of Spo0A via the repressor AbrB provides a simple answer. Thus, in cells that have not activated Spo0A, the operon is held inactive by the AbrB repressor. Conversely, in cells that have activated the master regulator for sporulation, Spo0A represses the gene for AbrB, thereby releasing the operon from repression. The immunity operon is therefore subject to an AND Gate in which expression requires both the presence of the toxin/ligand SdpC and the absence of the repressor AbrB.

We have elucidated an intricate series of regulatory interactions that explain how and whether cells respond to the cannibalism toxin SdpC. Cells that have activated Spo0A switch on both the operon (*sdpABC*) that is responsible for the production and export of the SdpC as well as the immunity operon (*sdpRI*) that protects the producing cells from the toxin. Induction of the immunity operon is subject to two control mechanisms, which together ensure that the operon is expressed in toxin-producing cells and not in sibling cells that have not activated Spo0A (Figure 1A). One control mechanism is a signal-transduction pathway that induces synthesis of the SdpI immunity protein in a just-in-time manner via the action of the autorepressor SdpR. The other control mechanism involves the repressor AbrB, which blocks the induction of the immunity operon in cells that have not activated Spo0A even when those cells are challenged with the SdpC toxin/ligand. Thus, whether or not a cell activates Spo0A in response to conditions of nutrient limitation has a profound influence on whether that cell will kill its non-sporulating siblings or whether it will itself be cannibalized.

EXPERIMENTAL PROCEDURES

Strain Construction

Strains are otherwise isogenic derivatives of the wild-type *B. subtilis* strain PY79 (Youngman et al., 1984) and are listed in Table S1. *B. subtilis* competent cells were prepared by the one-step method previously described (Wilson and Bott, 1968). The P_{sdpRI}-*lacZ* reporter at *thrC* was constructed by PCR amplifying the P_{sdpRI} region using CDEP128 and CDEP129. The resulting PCR product was digested with EcoRI and BamHI and cloned into pDG1663 (Guerout-Fleury et al., 1996) digested with the same sites to create pCE111. The *sdpR-gfp* fusion under the control of the P_{sdpRI} promoter (pEH210) was constructed by PCR amplifying P_{sdpRI}-*sdpR* using CDEP128 and ECH302. The *gfp* gene was PCR amplified using OMF10 and OMF11, and the resulting products were digested with EcoRI and XbaI (P_{sdpRI}-*sdpR*) and XbaI and BamHI (*gfp*). The digested products were then cloned by three-way ligation into pDG1731, which had been digested with EcoRI and BamHI, to create pEH210. A plasmid containing a xylose-inducible promoter that could be integrated into the *thrC* locus (pEH211) was created by cloning the HindIII-EcoRI fragment from pDR150 (gift of David Rudner) into pDG1664 (Guerout-Fleury et al., 1996), which had also been digested with HindIII and EcoRI. The *sdpR-gfp* fusion under the control of a xylose-inducible promoter (pEH212) was constructed by PCR amplifying *sdpR-gfp* from pEH210 using ECH301 and OMF11. The resulting products were digested with HindIII and BamHI. The digested products were then cloned into pEH211, which had also been digested with HindIII and BamHI to create pEH212.

Deletion Mutations

We used the long-flanking homology polymerase chain reaction (LFH-PCR) technique for creating deletion mutations (Wach, 1996) using the primers listed in Table S2. The deletion/insertion *sdpABC::kan* was constructed by PCR amplifying the 5′-flanking region of *sdpABC* with ECH278 and ECH279, while the 3′-flanking region was amplified using ECH280 and ECH281. The deletion/insertion *sdpABCI::kan* was constructed by PCR amplifying the 5′-flanking region of *sdpABCI* using ECH278 and ECH279, while the 3′-flanking region was amplified using ECH288 and ECH289. The deletion/insertion *sdpI::tet* was constructed by PCR amplifying the 5′-flanking region of *sdpI* with EG_SDPI-1 and EG_SDPI-2, while the 3′-flanking region was amplified using EG_SDPI-3 and EG_SDPI-4. The resulting PCR products were then used as primers to amplify the kanamycin-resistance cassette from the plasmid pDG780 or the tetracycline-resistance cassette from the plasmid pDG1515 (Guerout-Fleury et al., 1995) as previously described (Wach, 1996). The PCR products were then transformed into PY79 as previously described. The *sdpR::tet* mutation was similarly constructed. It is insertion of the tetracycline-resistance cassette from pDG1515 that replaces the entire open reading frame of *sdpR*. The mutants were confirmed by PCR.

Medium Supplements

Antibiotics were used at the following concentrations: chloramphenicol, 5 μg/ml; erythromycin plus lincomycin, 1 μg/ml and 25 μg/ml; kanamycin, 5 μg/ml; spectinomycin, 100 μg/ml; tetracycline, 10 μg/ml; ampicillin, 100 μg/ml. The β-galactosidase chromogenic indicator 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) was used at a concentration of 100 μg/ml. Xylose was used at a final concentration of 0.1% (w/v). IPTG was used at a final concentration of 1 mM.

Localized Mutagenesis of *sdpI*

Localized mutagenesis was performed by PCR using primers CDEP133 and CDEP134 to amplify the inducible copy of $sdpI^+$ integrated at *amyE* CDE254 using Expand polymerase according to the manufacturer's instructions. The resulting PCR product was concentrated and transformed into the strain CDE569, which contains a deletion of *sdpABCI* and the P_{sdpRI}-*lacZ* reporter integrated at *thrC*. Mutants that increased expression of the P_{sdpRI}-*lacZ* reporter were then backcrossed to confirm that the mutation was linked to *sdpI*. These mutants were then sequenced by PCR amplifying the *sdpI* mutant alleles using primers CDEP133 and CDEP134. The resulting PCR products were then sequenced using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (PerkinElmer) and either primer CDEP137 or CDEP138 according to the manufacturer's instructions.

Competition Assays

Solid Difco sporulation (DS) medium was inoculated with approximately 5×10^8 cells per ml of a 1:1 mixture of a wild-type strain marked with *lacZ* (*amyE::*P_{cons}*-lacZ*) and mutant strains. The colony-forming units and relative percentage represented by each strain were determined by direct plating of the inocula. After 24 hr, the cells were collected with 5 ml liquid DS medium, and serial dilutions were plated on LB + X-Gal medium to determine the ratio of mutant to wild-type. The competitive index (CI) was calculated as (mutant recovered/wild-type recovered)/(mutant inoculated/wild-type inoculated). Student's t test was used for statistical analyses.

Primer Extension

Total RNA was prepared as previously described from PY79 cells grown in DS medium until 1 hr past the start of stationary phase (Britton et al., 2002). The 5′ end of a DNA oligonucleotide primer (ECH253) was radioactively labeled using polynucleotide kinase (PNK) and 40 μCi of [γ-^{32}P]ATP (NEG002A, New England Nuclear). This end-labeled primer was annealed to 20 μg of RNA in the presence of 1× first-strand buffer (Invitrogen SuperScript First-Strand Synthesis System for RT-PCR) in a total reaction volume of 10 μl. The reaction mixture was incubated at 95ºC for 1 min, at 70ºC for 2 min, and finally on ice for 15 min. The extension reaction was performed using 5 μl of the above annealing reaction, 10 mM DTT, 1 mM dNTPs (250 μM for each dNTP), 1× first-strand buffer, and 10 units of SuperScript II RNase H^- Reverse Transcriptase. The reaction mixture was incubated for 45 min at 44ºC, after which 5 μl of formamide loading buffer (80% deionized formamide, 10 mM EDTA, 0.001% [w/v] xylene cyanol FF, 0.001% [w/v] bromophenol blue) was added to stop the reaction.

Purification of SdpR-His$_6$

The entire *sdpR* ORF except its stop codon was amplified from *B. subtilis* PY79 chromosomal DNA by PCR using primers ECH218 and ECH219. This DNA fragment was cloned into the NdeI and XhoI sites of pET21b (Invitrogen) to create pEH201. pEH201 was subsequently transformed into *E. coli* BL21(DE3) RIL-CodonPlus cells. This strain carries a plasmid that contains genes coding for three rare tRNAs recognizing arginine, isoleucine, and leucine to allow for optimal expression of heterologous proteins.

One liter of culture was grown in LB broth supplemented with 100 μg/μl^{-1} ampicillin at 30ºC until the OD_{600} reached 0.5. At this point, IPTG was added to a final concentration of 1 mM, and the culture was allowed to grow for an additional 3 hr at 30ºC. The cells were then harvested and resuspended in 40 ml of lysis/binding buffer (20 mM Tris-HCl [pH 8.0], 20% [v/v] glycerol, 5 mM imidazole) and lysed by sonication. The lysate was centrifuged at 30,000 × g to separate cellular debris from soluble material. One milliliter of Ni^{2+}-NTA agarose beads (QIAGEN) was added to the cleared lysate, which was subsequently rotated for 1 hr at 4ºC. The beads were then separated from the liquid by centrifugation at 5000 × g and resuspended in 2 ml of binding buffer. The beads were then washed with 2 ml of binding buffer five times (10 ml total). The bound protein was eluted from the beads with 2 ml of elution buffer (20 mM Tris [pH 8.0], 20% glycerol, 400 mM imidazole), and the eluate was dialyzed against 1 liter of TGE buffer (50 mM Tris [pH 8.0], 50% [v/v] glycerol, 5 mM EDTA) overnight at 4ºC. The dialyzed protein was aliquoted and stored at −80ºC.

Electrophoretic Mobility Shift Assay

DNA probes corresponding to the *sdpRI* promoter (P_{sdpRI}) and to a portion of the *sdpC* ORF were generated by PCR using *B. subtilis* PY79 chromosomal DNA and primer pairs ECH245/ECH246 (P_{sdpRI}) and ECH239/ECH240 (*sdpC* ORF). These probes were gel purified and subsequently 5′-end labeled using PNK and 10 μCi of [γ-^{32}P]ATP (NEG002A, New England Nuclear). DNA binding reactions were conducted in a total volume of 30 μl containing 10 mM Tris (pH 8.0), 50 mM NaCl, 1 mM EDTA, 5% (v/v) glycerol, 1 mM DTT, 50 μg ml^{-1} bovine serum albumin (BSA), 25 μg/ml^{-1} polydeoxyinosinicdeoxycytidylic acid (poly dI-dC), 2000 cpm/ml^{-1} radioactive probe, and various amounts of SdpR-His$_6$. The reactions were incubated for 20 min at room temperature. Ten microliters of each reaction was loaded onto a 6% polyacrylamide TBE gel and electrophoresed for 1 hr at 200V. Radioactive species were detected by autoradiography after exposure overnight to a Fujifilm Phosphoimaging Plate.

DNase I Footprint Assay

The 5′ end of the DNA oligonucleotide primer ECH253 was radioactively labeled using PNK and 70 mCi of [γ-^{32}P]ATP. A DNA fragment corresponding to the *sdpRI* promoter region (P_{sdpRI}) was generated by PCR using *B. subtilis* PY79 chromosomal DNA, ECH254, and radiolabeled ECH253. This PCR product was subsequently gel purified, and the amount of incorporated radioactivity was quantified with a scintillation counter. DNA binding reactions were conducted in 100 μl of footprinting buffer (20 mM Tris [pH 8.0], 5 mM $MgCl_2$, 5 mM $CaCl_2$, 100 μM DTT, 100 μM EDTA, 50 μg/ml^{-1} BSA, 5 μg/ml^{-1} poly dI-dC) with 30,000 cpm of radiolabeled PCR probe and various concentrations of SdpR-His$_6$. After a 15 min incubation period at room temperature, 0.04 units of DNase I was added to each DNA binding reaction. The digestion was allowed to proceed for 30 s at room temperature before 25 μl of stop solution (1.5 M sodium acetate [pH 5.3], 20 mM EDTA, 400 mg/ml^{-1} glycogen) was added to terminate the reaction. The reaction products were then precipitated with ethanol and resuspended in 8 ml of formamide running buffer. Four microliters of each sample was loaded onto an 8% sequencing gel (SequaGel Sequencing System, National Diagnostics) and subjected to electrophoresis for 2 hr at 35 mW. Radioactive species were detected by autoradiography after exposure overnight to a Fujifilm Phosphoimaging Plate.

Microscopy

Cells growing on DS agar were suspended in 1× PBS containing the membrane stain FM4-64 (Molecular Probes) at a concentration of 1 μg/ml. When necessary, IPTG and xylose were added to the media at final concentrations of 1 mM and 0.1% (w/v), respectively. The concentrated cell suspension (3 μl) was placed on a microscope slide. Cells were immobilized using a freshly prepared poly-L-lysine-treated coverslip. The equipment used and the capture and analysis of the images were done as previously described (Fujita and Losick, 2002).

Immunoblot Analysis of SdpR-GFP Localization

Overnight cultures were diluted to an OD_{600} of 0.5 and plated onto solid DS media supplemented with 1 mM IPTG and 0.1% (w/v) xylose. These plates were incubated for 4.5 hr at 37ºC, after which time the confluent "lawns" of bacteria were harvested. The cells were then protoplasted in 10 mM KH_2PO_4/K_2HPO_4 (pH 6.8) containing 0.5 M sucrose and 20 mM $MgCl_2$ essentially as described (Harwood and Cutting, 1990). Protoplasts were lysed by suspension in ice-cold 10 mM KH_2PO_4/K_2HPO_4 (pH 6.8) containing 250 mM EDTA (pH 8.0) followed by sonication. Soluble material was separated from the insoluble material by ultracentrifugation at 100,000 × g for 1 hr at 4ºC. After electroblotting, proteins were detected by incubating in a 1:10,000 dilution of either α-σ^A or α-GFP antibodies followed by incubation in a 1:10,000 dilution of goat anti-rabbit IgG (H+L)-HRP conjugate from Bio-Rad.

Supplemental Data

Supplemental Data include Supplemental References, two tables, and three figures and can be found with this article online at http://www.cell.com/cgi/content/full/124/3/549/DC1/.

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