Evidence for a novel protease governing regulated intramembrane proteolysis and resistance to antimicrobial peptides in *Bacillus subtilis*

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Evidence is presented that the activation of the RNA polymerase σ factor σ^W in *Bacillus subtilis* by regulated intramembrane proteolysis is governed by a novel, membrane-embedded protease. The σ^W factor is activated by proteolytic destruction of the membrane-bound anti-σ^W factor RsiW in response to antimicrobial peptides and other agents that damage the cell envelope. RsiW is destroyed by successive proteolytic events known as Site-1 and Site-2 cleavage. Site-2 cleavage is mediated by a member of the SpoIVFB-S2P family of intramembrane-acting metalloproteases, but the protease responsible for Site-1 cleavage was unknown. We have identified a previously uncharacterized, multipass membrane protein called PrsW (annotated YpdC) that is both necessary and sufficient (when artificially produced in an unrelated host bacterium) for Site-1 cleavage of RsiW. PrsW is a member of a widespread family of membrane proteins that includes at least one previously known protease. We identify residues important for proteolysis and a cluster of acidic residues involved in sensing antimicrobial peptides and cell envelope stress.

[*Keywords*: Signal transduction; Site-1 cleavage; ECF σ factor; antimicrobial peptides]

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Cells respond to their environment by means of systems that transduce a signal from outside the cell into a transcriptional response inside the cell. One broad category of signal transduction systems is known as regulated intramembrane proteolysis (RIP) and involves the cleavage of a protein from within the plane of the membrane to affect the release or activation of a transcription factor (Brown and Goldstein 1997; Brown et al. 2000). Here we are concerned with a particular example of RIP involving a member of the extracytoplasmic function (ECF) family of alternative σ factors in *Bacillus subtilis* known as σ^W, which is activated in response to antimicrobial peptides and other agents that cause damage to the cell envelope (Pietiainen et al. 2005; Butcher and Helmann 2006).

The signal transduction system governing the activation of σ^W represents a type of RIP involving a member of the SpoIVFB-S2P family of proteases. SpoIVFB-S2P family members are intramembrane-acting, zinc metalloproteases and are found in a wide range of organisms, including many kinds of bacteria as well as flies and mammals (Lewis and Thomas 1999; Rudner et al. 1999; Yu and Kroos 2000). In such systems, activation of the transcription factor is governed by two, successive proteolytic events known as Site-1 and Site-2 cleavage (Rawson et al. 1997; Sakai et al. 1998). Regulation is exerted principally at Site-1 cleavage with Site-2 cleavage, the step involving intramembrane proteolysis, generally (but not always) (Chen et al. 2005, 2006) occurring as a passive consequence of the first cleavage event. In some cases the two cleavage events take place on the same substrate, whereas in at least one case, Site-1 cleavage takes place on one protein and Site-2 cleavage on a second protein.

The founding member of the SpoIVFB-S2P family of proteases is the *B. subtilis* multipass, membrane protein SpoIVFB, which is responsible for activating the sporulation-specific transcription factor σ^K by catalyzing the proteolytic processing (Site-2 cleavage) of its inactive proprotein precursor, pro-σ^K (Fig. 1; Cutting et al. 1990, 1991b; Rudner et al. 1999; Yu and Kroos 2000). The conversion of pro-σ^K to mature σ^K is governed by a signal transduction pathway in which a signaling protein, the serine protease SpoIVB, cleaves SpoIVFA (Site-1 proteolysis), which (together with another protein called BofA) inhibits the SpoIVFB processing enzyme (Cutting

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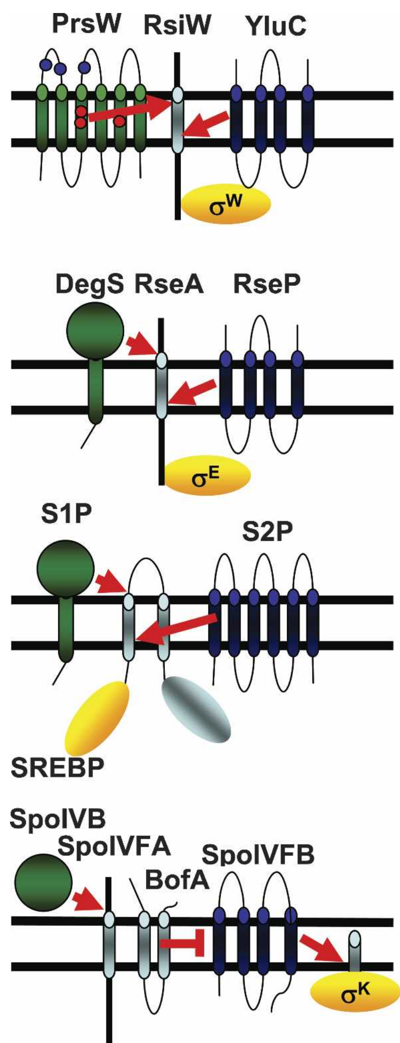

Figure 1. Examples of regulated intramembrane proteolysis and a model for the role of PrsW. Shown in green are proteases for Site-1 cleavage, the substrates are light blue, the proteases for Site-2 cleavage are dark blue, and transcription factors activated or released as a consequence of Site-2 cleavage are yellow. Red arrows point to the sites of cleavage on the substrates. See the text for details. Shown as green circles are the amino acid substitutions that rendered PrsW constitutively active (D23G, E28A, and E95K), and shown as red circles are residues conserved among family members at which substitutions (E75A, E76A, and H175A) rendered the protein inactive.

et al. 1991a; Wakeley et al. 2000; Hoa et al. 2002; Rudner and Losick 2002; Dong and Cutting 2003; Zhou and Kroos 2005; Campo and Rudner 2006). Thus, Site-1 cleavage of SpoIVFA relieves inhibition of SpoIVFB, thereby triggering Site-2 cleavage of pro-σ^K (Kroos et al. 2002; Rudner and Losick 2002; Dong and Cutting 2003). The σ^K signal transduction system is therefore a cascade in which Site-1 and Site-2 cleavages take place on different substrates. In contrast is the RIP signal transduction system governing the expression of genes involved in cholesterol biosynthesis in mammals, in which cleavage within the plane of the membrane was first recognized (Wang et al. 1994). In this system, a subtilisin-like, serine protease called S1P causes Site-1 cleavage of the Sterol Response Element-Binding Protein (SREBP), a transcriptional regulatory protein that is tethered in the Golgi apparatus by transmembrane segments (Sakai et al. 1998; Brown and Goldstein 1999). Site-1 cleavage of SREBP takes place in the lumen of the Golgi (Rawson et al. 1997; Zelenski et al. 1999). This proteolysis, in turn, triggers Site-2 cleavage of the remaining portion of SREBP by the Site-2 protease S2P, resulting in the release from the membrane of a fragment of SREBP containing the DNA-binding domain of the regulatory protein, which then enters the nucleus (Wang et al. 1994; Brown and Goldstein 1999).

A third example of RIP involving a SpoIVFB-S2P-related protease involves members of the ECF family of alternative σ factors, for which σ^E of *Escherichia coli* is the best-studied example (Fig. 1). Here, the substrate for Site-1 and Site-2 cleavage is a membrane-bound anti-σ factor (RseA) that tethers σ^E to the cytoplasmic membrane. In response to extracytoplasmic signals (the presence of unfolded, outer membrane β-barrel proteins in the periplasm), a serine protease in the periplasm known as DegS carries out Site-1 cleavage of the RseA anti-σ factor (Ades et al. 1999; Alba et al. 2002; Walsh et al. 2003; Wilken et al. 2004). The resulting remnant of RseA becomes a substrate for a Site-2 protease (YaeL, which is also referred to as RseP) (Alba et al. 2002; Kanehara et al. 2002, 2003), which, in turn, renders the remaining N-terminal portion of RseA a substrate for further degradation by ClpXP (Flynn et al. 2004). As a consequence of these events, the anti-σ factor is destroyed and σ^E is free to activate transcription.

Like σ^E of *E. coli*, σ^W of *B. subtilis* is controlled by a membrane-bound anti-σ factor RsiW (Fig. 1; Cao et al. 2002b; Schobel et al. 2004; Yoshimura et al. 2004). The Site-2 protease for RsiW is called YluC and is closely related to RseP of *E. coli* (Schobel et al. 2004). However, up until now the protease responsible for Site-1 cleavage of RsiW has remained elusive. *B. subtilis* has homologs of *E. coli* DegS, but evidence indicates that none of these are involved in Site-1 cleavage (Schobel et al. 2004). Also, *B. subtilis* lacks a periplasm, and hence, the mechanisms by which Site-1 cleavage is achieved and by which extracytoplasmic signals are sensed are likely to be different than the mechanisms for the σ^E ECF factor. Here we present evidence indicating that the product of a previously uncharacterized gene (annotated *ypdC*) is a novel protease that is responsible for Site-1 cleavage of the RsiW anti-σ factor. The *ypdC* gene product (hereafter referred to as PrsW for protease responsible for activating σ^W) is not a serine protease and is unrelated to other proteases known to be responsible for Site-1 cleavage. Instead, PrsW is a member of a novel and widespread family of proteins that includes at least one other known protease. Our analysis leads us to propose that PrsW is responsible for sensing antimicrobial peptides that damage the cell membrane and other agents that cause cell envelope stress, and to suggest a mechanism by which it does so.

Results

Isolation of mutants resistant to the cannibalism toxin SdpC

Our discovery of a previously uncharacterized gene involved in the activation of σ^W arose during the course of studying cannibalism in *B. subtilis*. Cannibalism is a phenomenon in which cells at the start of sporulation, which is triggered by nutrient limitation, kill sibling cells that have not entered the pathway to sporulate (Gonzalez-Pastor et al. 2003). The nutrients released by this killing arrest the sporulating cells prior to the stage of development at which spore formation becomes irreversible. Cannibalism is therefore a means by which *B. subtilis* delays becoming committed to spore formation. Killing is mediated in part by a protein toxin called SdpC. Sporulating cells, which produce the cannibalism toxin, are protected from self-killing (suicide) by an immunity protein called SdpI. Cells that are mutant for the SdpI immunity protein are sensitive to, and killed by, the SdpC toxin (Ellermeier et al. 2006). Thus, as seen in Figure 2, the absence of SdpI (SdpI$^-$) caused lethality in cells grown on solid Difco Sporulation (DS) medium. This lethality depended on SdpC in that cells lacking both the SdpI immunity protein and the SdpC toxin were fully viable (SdpI$^-$ SdpC$^-$ in Fig. 2). Instead, colonies of the double mutant (in which cannibalism was abrogated) sporulated faster than colonies of the wild type as evidenced by the greater whiteness and opacity of the SdpI$^-$ SdpC$^-$ mutant colonies. The requirement for the SdpI immunity protein in protecting against killing is also seen in the competition experiment of Table 1, in which cells mutant for SdpC alone or for both SdpC and SdpI were challenged with wild-type cells that had been tagged with LacZ. A comparison of line B with line C shows that survival against toxin-producing wild-type cells required SdpI.

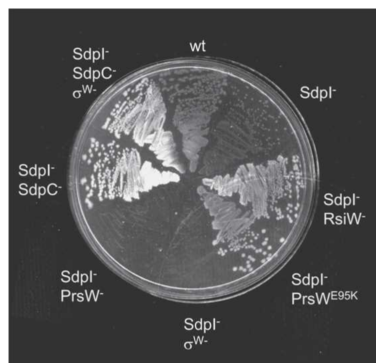

Figure 2. Isolation of mutants resistant to the cannibalism toxin SdpC. Strains were wild type (PY79) or mutant for the SdpI immunity protein (Δ*sdpI*; strain EG441), mutant for SdpI and RsiW (Δ*sdpI* and Δ*rsiW*; strain CDE562), mutant for SdpI and producing the missense mutant protein PrsW^{E95K} (Δ*sdpI* Δ*prsW* and *thrC*::*PprsW*^{E95K}; strain CDE423), mutant for SdpI and PrsW (Δ*sdpI* and Δ*prsW*; strain CDE407), mutant for SdpI and σ^W (Δ*sdpI* and Δ*sigW*; strain CDE432), and mutant for the SdpC toxin and the SdpI immunity (Δ*sdpABCIR*; strain EG494), or SdpC, SdpI, and σ^W (Δ*sdpABCIR* and Δ*sigW*; strain CDE433). Growth was for 16 h at 37°C on solid DS medium (Harwood and Archibald 1990). Note that mutants lacking SdpC and SdpI (or SdpC, SdpI, and σ^W) were not only unimpaired in their growth but also produced whiter and more opaque colonies than the wild type due to accelerated sporulation (lack of cannibalism).

During the course of studying cannibalism in colonies growing on solid medium, we noticed that suppressor mutants arose that appeared to be resistant to SdpC in the absence of SdpI. As a starting point for this investigation, we isolated and characterized 15 such suppressor mutant derivatives of an *sdpI* mutant. Evidence indicated that none of the suppressor mutants were blocked in the production of the SdpC toxin (data not shown). Evidently, then, they had acquired mutations that protected them against the toxin.

We mapped the suppressor mutations by generating transposon insertions in the suppressor strains and then identifying insertions linked to the suppressor mutations (see Materials and Methods). We mapped the mutations to *rsiW* (*ybbM*), *ysdB*, and the previously uncharacterized gene *prsW* (*ypdC*). Eight were recessive mutations in *rsiW* (as they could be complemented by wild-type *rsiW*), six were recessive mutations in *ysdB* (as they could be complemented by wild-type *ysdB*), and one that caused a glutamate-to-lysine substitution at residue 95 was a missense mutation (*prsW*^{E95K}) in *prsW*. As we explain below, *prsW*^{E95K} is a dominant (gain-of-function) mutation. RsiW is an anti-σ factor that inhibits σ^W, a member of the extracytoplasmic family of σ factors (ECF), and null mutations in *rsiW* are known to cause σ^W to be active constitutively (Turner and Helmann 2000; Cao et al. 2002a; Schobel et al. 2004). It has also been reported that null mutations in *ysdB* cause σ^W to be active constitutively, but the mechanism of this activation is not known (Turner and Helmann 2000; Cao et al. 2002b). PrsW is predicted to be a multipass membrane protein and is of unknown function.

In light of these findings, we created insertion/deletion mutations for all three genes in which the open reading frames were replaced with a drug-resistance gene (see Materials and Methods). Henceforth the experiments presented were based on these three null mutations as well as the missense mutation *prsW*^{E95K}. As expected and consistent with the results presented above, the *rsiW*::*spec* or *ysdB*::*erm* insertion/deletion mutations suppressed the growth defect caused by an *sdpI* mutation (Fig. 2; Table 1, lines E and F, respectively). (Note also in Fig. 2 that mutant cells lacking both the RsiW anti-σ factor and the SdpI immunity protein [and hence in which cannibalism was arrested] exhibited accelerated sporulation as evidenced from the greater whiteness and opacity of the SdpI$^-$ RsiW$^-$ double mutant as compared with the wild type.) In contrast, the *prsW* insertion/deletion mutation (*prsW*::*erm*) exacerbated the growth defect of an *sdpI* mutant (Fig. 2; Table 1, line H). Thus, the null mutant phenotype of *prsW* was opposite to that of the *prsW*^{E95K} missense mutation, which exhibited heightened resistance to the SdpC toxin in cells lacking the SdpI immunity protein (Fig. 2; Table 1, line G). Also,

Table 1. *Competition experiments with LacZ-tagged strains*

Line	Reference strain (phenotype)[a]	Test strain (phenotype)[b]	Competitive index[c]	p-value[d]
A	wild type	wild type	1.05	0.13
B	wild type	SdpC$^-$	1.49	0.28
C	wild type	SdpC$^-$ SdpI$^-$	0.04	0.02
D	SdpC$^-$	SdpC$^-$ SdpI$^-$	1.12	0.13
E	wild type	SdpC$^-$ SdpI$^-$ RsiW$^-$	1.51	0.11
F	wild type	SdpC$^-$ SdpI$^-$ YsdB$^-$	0.21	0.01
G	wild type	SdpC$^-$ SdpI$^-$ PrsW^{E95K}	2.3	0.9
H	wild type	SdpC$^-$ SdpI$^-$ PrsW$^-$	0.003	0.014
I	wild type	SdpC$^-$ SdpI$^-$ σ^{W-}	0.007	0.003

[a]The strains were wild type (CDE816) and Δ*sdpABC* (CDE817) (see Supplementary Table S1)
[b]The strains were SdpC$^-$ (EH273 Δ*sdpABC*), SdpC$^-$ SdpI$^-$ (EG494 Δ*sdpABC* Δ*sdpRI*), SdpC$^-$ SdpI$^-$ RsiW$^-$ (CDE631 Δ*sdpABC* Δ*sdpRI* Δ*rsiW*), SdpC$^-$ SdpI$^-$ YsdB$^-$ (CDE458 Δ*sdpABC* Δ*sdpRI* Δ*ysdB*), SdpC$^-$ SdpI$^-$ PrsW^{E95K} (CDE430 Δ*sdpABC* Δ*sdpRI prsW*^{E95K}), SdpC$^-$ SdpI$^-$ PrsW$^-$ (CDE406 Δ*sdpABC* Δ*sdpRI* Δ*prsW*), and SdpC$^-$ SdpI$^-$ σ^{W-} (CDE433 Δ*sdpABC* Δ*sdpRI* Δ*sigW*) (see Supplementary Table S1).
[c]The competitive index 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.
[d]The two-tailed Student's t-test was used for statistical analyses. A p-value of <0.05 was considered significant.

prsW^{E95K} was dominant to the wild-type allele as judged from the construction of a partially diploid strain harboring both *prsW*$^+$ and *prsW*^{E95K} (data not shown). Evidently, then, *prsW*^{E95K} is a gain-of-function mutation.

Because RsiW is known to be an anti-σ factor for σ^W and because YsdB is also known to act negatively on the ECF σ factor (in an unknown manner), we inferred that activation of σ^W was responsible for conferring resistance to the SdpC toxin in cells lacking the SdpI immunity protein. As a test of this inference, we introduced a *sigW*-null mutation into a mutant lacking SdpI but producing SdpC and into a mutant lacking both SdpI and SdpC. As can be seen in Figure 2, the combined absence of σ^W and SdpI caused exquisite sensitivity to SdpC: Almost no growth was seen in toxin-producing cells (Fig. 2;Table 1, line I), whereas growth was normal in cells unable to produce toxin (Fig. 2). Conversely, when SdpI was present, the absence of σ^W in a toxin-producing mutant caused no measurable growth defect (data not shown). Similar results were obtained by Butcher and Helmann (2006), who found that σ^W is responsible for conferring resistance to the SdpC toxin in cells lacking the SdpI immunity protein.

Taken together, our results and those of Butcher and Helmann (2006) suggest the following scenario. The SdpI immunity protein ordinarily suffices to protect against the SdpC toxin (Butcher and Helmann 2006; Ellermeier et al. 2006). However, when the immunity protein is absent, a gene or genes under the control of σ^W can also confer protection against the toxin under conditions in which the ECF σ factor is active due to the absence of RsiW or YsdB.

PrsW is required for the activation of σ^W

It is known that the gene for σ^W is itself under σ^W control and hence that *lacZ* fused to the promoter for *sigW* (P_{sigW}-*lacZ*) can be used as a reporter for σ^W activity. Indeed, in conformation of previous findings, the results of Figure 3A and Table 2 show that our newly constructed *rsiW*- and *ysdB*-null mutations caused increased expression of P_{sigW}-*lacZ* in a manner that was dependent on σ^W (Fig. 3A; Table 2; Turner and Helmann 2000; Schobel et al. 2004). In light of the results presented above, we wondered whether PrsW is also required for the activation of σ^W. Accordingly, we introduced a deletion of *prsW* into a strain containing P_{sigW}-*lacZ* and found that the absence of PrsW caused a marked decrease in P_{sigW}-*lacZ* expression, which was similar to that observed with a *sigW* deletion (Fig. 3A; Table 2). The block in P_{sigW}-*lacZ* expression was due to the absence of PrsW because the effect of the mutation was complemented by the introduction of a wild-type copy of *prsW* at the *thrC* locus (Fig. 3B; Table 2). In sharp contrast to the results with the deletion mutation, the presence of the *prsW*^{E95K} allele resulted in enhanced expression of P_{sigW}-*lacZ*. In toto, these results suggest that PrsW is required for the activation of σ^W and that the gain-of-function mutant PrsW^{E95K} is locked in a state that causes constitutive activation of σ^W.

Next, we asked whether PrsW exerts its affect by antagonizing RsiW, the anti-σ factor for σ^W. The results of Figure 3A and Table 2 show that P_{sigW}-*lacZ* expression in an *prsW rsiW* double mutant was similar to that observed in cells mutant for *rsiW* alone (Fig. 3A; Table 2). Thus, an *rsiW*-null mutation is epistatic to a *prsW*-null mutation. We conclude that in the absence of the anti-σ factor RsiW, σ^W activity does not depend on PrsW.

PrsW is required for Site-1 cleavage of RsiW

As in the case of σ^E of *E. coli*, activation of σ^W is known to be mediated by proteolytic destruction of its anti-σ factor RsiW in two steps (Fig. 1). First, the extracellular region of RsiW is cleaved by an unknown protease in a process known as Site-1 cleavage in *E. coli*. This protease is DegS; however, none of the *B. subtilis* DegS homologs are required for Site-1 cleavage of RsiW (Schobel et al.

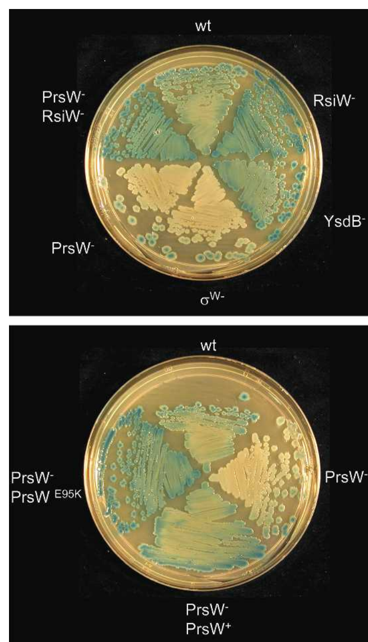

Figure 3. PrsW is required for σ^W activity only in the presence of the anti-σ factor RsiW. All strains contained P_{sigW}-*lacZ*. Growth was for 16 h at 37°C on solid LB medium (Harwood and Cutting 1990) containing X-Gal. (*A*) σ^W-directed transcription is PrsW dependent. Shown are strains that were wild type (wt; strain CDE717) or mutant for the RsiW anti-σ factor (Δ*rsiW*; strain CDE718), for the YsdB membrane protein (Δ*ysdB*; strain CDE719), for the σ^W ECF σ factor (Δ*sigW*; strain CDE720), for the PrsW; strain CDE721), or for both the PrsW protease and the RsiW anti-σ factor (Δ*prsW*, Δ*rsiW*; strain CDE727). (*B*) $PrsW^{E95K}$ is a dominant, gain-of-function mutant. Shown are strains that were wild type (wt; strain CDE717) or mutant for the PrsW protease (Δ*prsW*; strain CDE721), and complementing the *prsW* deletion with wild-type PrsW (Δ*prsW thrC*::*prsW*+; strain CDE739) or complementing the *prsW* deletion with the mutant form of $PrsW^{E95K}$ (Δ*prsW thrC*::$prsW^{E95K}$; strain CDE740).

2004). Next, RsiW undergoes cleavage at an intramembrane site known as Site 2 by a member of the SpoIVFB-S2P family of proteases (Fig. 1). The *B. subtilis* protease responsible for Site-2 cleavage is YluC (corresponding to RseP of *E. coli*) (Schobel et al. 2004).

In light of these considerations, we wondered whether PrsW was responsible for Site-1 cleavage. To investigate this, we created a construct in which an IPTG-inducible copy of *rsiW* was fused to the coding sequence for three copies of the epitope tag Flag (3X-Flag-RsiW). We then induced the synthesis of the Flag-tagged anti-σ factor in exponential phase cells and monitored its processing before and after a shift from pH 7.0 to pH 9.0 by the addition of 24 mM NaOH—a previously described, convenient procedure for inducing degradation of RsiW and activating of σ^W (Schobel et al. 2004). As expected, the results of Figure 4 show that 3X-Flag-RsiW disappeared following the treatment with base. Next, we introduced the *prsW*-null mutation into the 3X-Flag-RsiW-producing strain. The results of Figure 4 show that in the absence of PrsW full-length 3X-Flag-RsiW remained present after treatment with base to shift the pH to 9.0. Destruction of the anti-σ factor was, however, restored, when a wild-type copy of *prsW* was introduced into the *prsW* mutant strain at the *thrC* locus.

As a further test of the idea that PrsW was required for Site-1 cleavage, we introduced a mutation of the gene (*yluC*) for the Site-2 protease into cells producing the Flag-tagged anti-σ factor. The results of Figure 4 show (in confirmation of previously reported results) that the absence of YluC led to the accumulation of a truncated form of the anti-σ factor (Schobel et al. 2004). However, when the cells were mutant both for PrsW and YluC, only the full-length RsiW could be seen (Fig. 4). We conclude that PrsW is needed for Site-1 cleavage and that Site-2 cleavage depends on the prior action of PrsW.

The results presented above indicated that $PrsW^{E95K}$ is a gain-of-function mutant in which the protein is locked in a state that causes constitutive activation of σ^W. If this interpretation is correct and PrsW is responsible for Site-1 cleavage, then the gain-of-function mutant should cause cleavage of the anti-σ factor in a manner that does not depend on an increase in pH. In confirmation of this expectation, the results of Figure 4 show that in $PrsW^{E95K}$-producing cells, the Flag-tagged anti-σ factor could not be detected even when the pH was not raised. Furthermore, when these cells also harbored a mutation in *yluC*, the product of Site-1 cleavage was seen to accumulate both in the presence and in the absence of treatment with base (Fig. 4). In sum, these results are consistent with the idea PrsW is directly or indirectly responsible for Site-1 cleavage of RsiW.

PrsW is similar to a newly recognized family of proteases

The simplest interpretation of our results so far is that PrsW is itself directly responsible for Site-1 cleavage of

Table 2. *Activation of* σ^W *is dependent on PrsW*

Strain (relevant genotype)[a]	β-galactosidase[b]	Standard deviation[c]
Wild type	16.9	0.53
Δ*rsiW*	50.8	5.53
Δ*ysdB*	28.2	6.17
Δ*sigW*	2.10	0.30
Δ*prsW*	2.29	0.33
Δ*prsW* Δ*rsiW*	45.7	6.76
Δ*prsW thrC*::*prsW*+	12.9	2.42
Δ*prsW thrC*::$prsW^{E95K}$	41.6	1.99
Δ*prsW thrC*::$prsW^{H175A}$	1.48	0.06
Δ*prsW thrC*::$prsW^{E75A\ E76A}$	1.60	0.10

[a]All strains contain *amyE*::P_{sigW}-*lacZ* (*cat*) and were wild type (CDE717) and Δ*rsiW* (CDE718) Δ*ysdB* (CDE719), Δ*sigW* (CDE720), Δ*prsW* (CDE721), Δ*prsW* Δ*ypdC* (CDE727) Δ*prsW thrC*::*prsW*+ (CDE739), Δ*prsW thrC*::$prsW^{E95K}$ (CDE741), Δ*prsW thrC*::$prsW^{H175A}$ (CDE763), and Δ*prsW thrC*::$prsW^{E75A\ E76A}$ (CDE764) (see Supplementary Table S1).
[b]The β-galactosidase activity units were calculated as Miller Units as previously described (Miller 1972; Harwood and Cutting 1990). Cells were grown in liquid DS medium to an OD_{600} of 0.8; the average of three samples is shown.
[c]Standard deviation, $n = 3$.

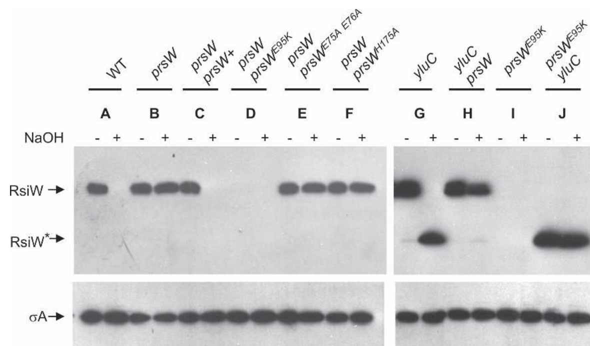

Figure 4. PrsW is required for Site-1 cleavage of the anti-σ^{W} factor RsiW. The strains contained RsiW that had been tagged with three copies of Flag (3X-Flag-RsiW), the gene for which was under the control of an IPTG-inducible promoter. Immunoblot analysis was carried out with antibodies against the cytoplasmic protein σ^{A} or antibodies against Flag. The capital letters designate pairs of lanes in which the cells of the same strain had (+) or had not (−) been treated with 24 mM NaOH as described in Materials and Methods. (*A*) Wild type (strain CDE743). (*B*) Δ*prsW* (strain CDE744). (*C*) Δ*prsW thrC*::*prsW*$^{+}$ (strain CDE745). (*D*) Δ*prsW thrC*::*prsW*^{E95K} (strain CDE746). (*E*) Δ*prsW thrC*::*prsW*$^{E75A\ E76A}$ (strain CDE769). (*F*) Δ*prsW thrC*::*prsW*^{H175A} (strain CDE768). (*G*) Δ*yluC* (strain CDE802). (*H*) Δ*prsW* Δ*yluC* (strain CDE820). (*I*) *prsW*^{E95K} (strain CDE819.) (*J*) *prsW*^{E95K} Δ*yluC* (strain CDE824). RsiW denotes full-length 3X-Flag-RsiW while RsiW* refers to the site-1 cleavage product of 3X-Flag-RsiW.

RsiW. Standard BLAST analysis failed to reveal any similarity to known proteases. Instead, BLAST analysis revealed that PrsW belongs to a family of membrane proteins found in Gram-positive and Gram-negative bacteria as well as some Archaea and has been identified as COG2339 (cluster of orthologous groups). We were, however, able to find similarity to a family of putative proteases COG1266 using the recently reported algorithm HHpred. HHpred performs a PSI-BLAST and then generates a hidden Markov model (HMM) which it uses to perform a pair-wise comparison with a database of HMMs (Soding et al. 2005). Members of the COG1266 family are found in a wide range of organisms, including yeast, mammals, plants, and a large number of Gram-positive and Gram-negative bacteria. Importantly, one member of the family, RCE1, is known to be a protease. RCE1 is a CAAX prenyl endopeptidase in *Saccharomyces cerevisiae* and is involved in removing the C-terminal AAX motif from phenylated proteins (Boyartchuk et al. 1997; Boyartchuk and Rine 1998). Members of the family are characterized by two adjacent and highly conserved glutamic acid residues and a conserved histidine residue (Pei and Grishin 2001), features that are present in PrsW (Supplementary Fig. S1). These conserved residues were also found to be essential to proteolytic activity of RCE-1 (Dolence et al. 2000).

To investigate the significance of these results, we created a double mutant with loss-of-side-chain substitutions of the two, conserved glutamic acid residues (E75A and E76A) and a single mutant of a conserved histidine (H175A). We then introduced the mutant genes into the chromosome at the *thrC* locus of a strain lacking *prsW* and containing the reporter P_{sigW}-*lacZ*. The results of Figure 5 and Table 2 show that the block in P_{sigW}-*lacZ* expression caused by the *prsW*-null mutation was complemented by a wild-type copy of *prsW* at *thrC* but not by alleles producing the mutant proteins PrsW$^{E75A\ E76A}$ or PrsW^{H175A}. These results are consistent with the hypothesis that PrsW is a protease that is directly responsible for mediating Site 1 cleavage.

As a further test of this hypothesis, we used Flag-tagged RsiW to monitor degradation of the anti-σ factor in cells producing the putative, active-site mutants of PrsW following treatment with base. The results of Figure 4 show that whereas wild-type PrsW was capable of supporting destruction of 3X-Flag-RsiW, the PrsW$^{E75A\ E76A}$ and PrsW^{H175A} mutant proteins were not.

Finally, in other work (data not shown), we found that activation of σ^{W} was blocked in merodiploid strains producing both wild-type PrsW and either the PrsW$^{E75A\ E76A}$ or the PrsW^{H175A} mutant proteins. The simplest interpretation of these results is that PrsW$^{E75A\ E76A}$ and PrsW^{H175A} are catalytically inactive and trap RsiW in a complex in which the anti-σ factor is protected from undergoing proteolysis.

PrsW causes cleavage of RsiW in E. coli

As a test of the idea that PrsW is directly responsible for Site-1 cleavage of RsiW, we engineered cells of *E. coli* to produce 3X-Flag-RsiW and PrsW. The results of Figure 6 show that the Flag-tagged protein underwent cleavage at Site-1 in *E. coli* but not at Site-2 (at least not efficiently), as judged by the accumulation of the Site-1 cleavage product. The results also show that elimination of the Flag-tagged anti-σ factor was dependent on PrsW, with either the wild-type protein or the constitutively active

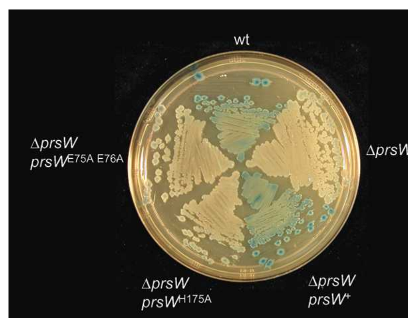

Figure 5. Mutants of the putative active site for the PrsW protease block activation of σ^{W}. Strains contained P_{sigW}-*lacZ* and growth was for 16 h at 37°C on solid LB medium (Harwood and Cutting 1990) containing X-Gal. Shown is a strain that was wild type for *prsW* (wt; CDE717), a strain that harbored a *prsW* deletion mutation (Δ*prsW*; strain CDE721) and strains that harbored a *prsW* deletion and also contained at the *thrC* locus a wild-type copy of *prsW* (Δ*prsW thrC*::*prsW*$^{+}$; strain CDE739), the mutant allele *prsW*$^{E75A\ E76A}$ (Δ*prsW thrC*::*prsW*$^{E75A\ E76A}$; strain CDE763), or the mutant allele *prsW*^{H175A} (Δ*prsW thrC*::*prsW*^{H175A}; strain CDE764).

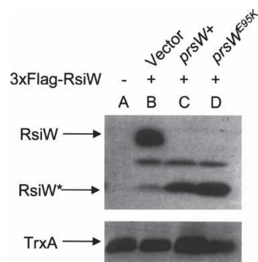

Figure 6. PrsW is the only *B. subtilis* protein needed for Site-1 cleavage of RsiW in *E. coli*. The strains were grown in the presence of IPTG and arabinose to induce synthesis of 3X-Flag-RsiW and PrsW, respectively. The strains were *E. coli* (strain DH5α) (*A*), P_{lac}-*3x-flag-rsiW*$^{+}$ pBAD33 (strain CDE747) (*B*), P_{lac}-*3x-flag-rsiW*$^{+}$ P_{bad}-*prsW*$^{+}$ (strain CDE748) (*C*), and P_{lac}-*3x-flag-rsiW*$^{+}$ P_{bad}-*prsW*^{E95K} (strain CDE749) (*D*). The immunoblot was probed with antibodies against the cytoplasmic protein TrxA and Flag. Vector refers to the pBAD construct without a *prsW* insert.

mutant protein PrsW^{E95K} causing proteolysis. In contrast, the catalytically inactive mutant PrsW$^{E75A\ E76A}$ was unable to cause processing in *E. coli* (data not shown). Interestingly, and in contrast to the results seen in *B. subtilis*, proteolysis in the engineered *E. coli* cells did not require a pH shift, a point to which we return in the Discussion. In sum, these results show that no *B. subtilis* protein is required for Site-1 cleavage other than PrsW, a finding that reinforces the view that PrsW is directly responsible for processing of RsiW.

PrsW is required for activation of σ^{W} in response to cell envelope stresses

Our data have shown that PrsW is required for activation of σ^{W}, evidently by directly cleaving the anti-σ^{W} factor RsiW. Others have demonstrated that σ^{W} activity is induced by a variety of cell envelope stresses, including treatment with antibiotics (vancomycin and penicillin) and detergents (Triton X-100) (Cao et al. 2002b). We wanted to determine if PrsW was required to respond to these other stress-causing agents. To test this we performed diffusion assays by placing disks containing various compounds on a lawn of cells harboring a P_{sigW}-*lacZ* reporter. As reported previously, the cell envelope stress-inducing compounds ampicillin and Triton X-100 caused activation of the P_{sigW}-*lacZ* fusion construct (as seen from the blue halos around the disks), whereas the antibiotic chloramphenicol, a protein synthesis inhibitor, did not (Supplementary Fig. S2). As expected, we observed no induction of P_{sigW}-*lacZ* in the absence of σ^{W} (Supplementary Fig. S2). Importantly, we also observed impaired induction in the absence of PrsW (Supplementary Fig. S2). Thus activation of σ^{W} by cell envelope stress depends on PrsW.

YsdB is not required for responding to cell envelope stresses

As noted above, σ^{W} is subject to negative regulation by the anti-σ factor RsiW and by YsdB, an apparent integral membrane protein of unknown function. We wondered whether, like the PrsW protease, YsdB was required for responding to cell envelope stress. Accordingly, we monitored expression of the P_{sigW}-*lacZ* reporter in response to treatment with Triton X-100 in the presence and absence of YsdB. The results of Supplementary Figure S2 show that the basal level of P_{sigW}-*lacZ* expression was, as expected, enhanced by the absence of YsdB but that treatment with Triton X-100 caused a pronounced further increase in the level of expression of the reporter construct (Supplementary Fig. S2). Thus, YsdB is not required for sensing cell envelope stress. Instead, and as discussed below, we propose that YsdB normally serves to create a negative feedback loop that dampens (down-regulates) σ^{W}-directed gene expression following exposure of cells to stress.

Amino acid substitutions causing PrsW to be constitutively active are clustered in a patch of acidic residues

The simplest interpretation of our results so far is that PrsW is itself responsible for sensing cell envelope stress. To gain insight into the sensing mechanism, we sought to obtain additional mutants of the protease that, like E95K, caused it to be constitutively active. Accordingly, we isolated eight independent mutations that rendered PrsW constitutively active in a genome-wide screen for spontaneous mutants that were resistant to the SdpC toxin. (A special feature of this additional screen was the use of cells that were engineered to overproduce the anti-σ factor RsiW, which eliminated the otherwise high background of loss-of-function mutations in *rsiW* and *ysdB*.) All eight mutants were found to harbor amino acid substitutions at one of three acid residues—D23, E28, or E95—causing a change to a neutral or positively charged amino acid (data not shown). Based on predictions of the bioinformatics program TMHMM (transmembrane HMM) (Sonnhammer et al. 1998; Krogh et al. 2001), we believe these residues are located on the extracytoplasmic face of PrsW (Fig. 1), placing them in a position to act as a sensor for cell envelope stress.

Discussion

A novel protease is responsible for Site-1 cleavage of the RsiW anti-σ factor

The principal contribution of this investigation is the discovery of a strong candidate, the multipass membrane protein PrsW, for the protease that is responsible for Site-1 cleavage of the anti-σ^{W} factor RsiW. The principal evidence for this is that (1) a PrsW-null mutant was blocked in Site-1 cleavage of RsiW; (2) a gain-of-function mutant of PrsW was constitutively active for Site-1 cleavage; (3) PrsW is similar to a newly recognized family of proteins that includes a known protease (RCE1 in yeast, which removes the C-terminal three amino acids from prenylated proteins) (Boyartchuk et al. 1997; Pei

and Grishin 2001); and (4) PrsW was the only *B. subtilis* protein necessary for proteolysis of RsiW when the two proteins were artificially produced in *E. coli*. On the basis of this evidence, it is probable that PrsW is directly responsible for Site-1 cleavage of RsiW. We cannot, however, exclude the possibility that PrsW activates an unknown protease that in turn acts on RsiW, or that PrsW induces a conformational change in the anti-σ factor that renders it susceptible to cleavage by an unknown protease. If so, then a close relative of this hypothetical, unknown protease must be present in *E. coli* and must be capable of recognizing the *B. subtilis* RsiW protein. Because *E. coli*, a Gram-negative bacterium, is unrelated to *B. subtilis*, a Gram-positive bacterium, and because *E. coli* lacks the σ^W system, including an ortholog of the RsiW anti-σ factor, we consider this scenario unlikely.

Assuming that PrsW is indeed the protease for Site-1 cleavage of RsiW, then our discovery reinforces the view that Site-1 proteases differ markedly from one regulatory system to another. Thus, PrsW exhibits little or no similarity to DegS, which is responsible for activation of σ^E in *E. coli*, or to SpoIVB, which is believed to cleave SpoIVFA, an inhibitor of the Site-2 protease SpoIVFB in *B. subtilis*, or to PerP, which cleaves the polar localization factor PodJ in *Caulobacter* or to the Site-1 protease for SREBP and ATF6 in mammalian cells (Sakai et al. 1998; Ades et al. 1999; Hoa et al. 2002; Chen et al. 2006). DegS, SpoIVB, and S1P are serine proteases but otherwise exhibit little similarity to each other, PerP is an aspartyl protease, and PrsW is an apparent glutamic acid metalloprotease (Sakai et al. 1998; Ades et al. 1999; Hoa et al. 2002; Chen et al. 2006). In contrast, Site-2 proteases all appear to be related to each other. Thus, the Site-2 proteases for the σ^W (YluC), σ^E (RseP), pro-σ^K (SpoIVFB), and SREBP's ATF6 (S2P) regulatory systems all belong to the same family of membrane-embedded, zinc metalloproteases (Rawson et al. 1997; Rudner et al. 1999; Hoa et al. 2002; Kanehara et al. 2002; Schobel et al. 2004; Chen et al. 2005). Interestingly, and ironically, the active site of PrsW appears to be located within the membrane (Fig. 1A), suggesting that like Site-2 proteases PrsW cleaves RsiW from within the plane of the membrane. If so, then this cleavage is expected to occur near the outer edge of the membrane so that the transmembrane segment can subsequently undergo a second, Site-2 cleavage event.

As in almost all other related examples of RIP, activation of σ^W appears to be governed at the stage of Site-1 cleavage. We base this conclusion on two lines of evidence. First, Schobel et al. (2004) have shown that a truncated form of the RsiW anti-σ causes σ^W to be constitutively active in a YluC-dependent manner. Second, we have described the isolation of gain-of-function mutants of PrsW that also cause σ^W to be constitutively active. Together these results suggest that Site-2 cleavage of RsiW is a passive response to the primary cleavage event at Site-1, which generates the appropriate substrate for the Site-2 protease YluC.

If activation of σ^W is in fact governed at the stage of Site-1 cleavage, then a likely candidate for the sensor that detects cell envelope stress is PrsW itself. A possible clue as to how PrsW might sense stress comes from the observation that all three of the constitutively active mutants of PrsW isolated in our work were substitutions of negatively charged residues on extracytoplasmic loops of the protease with neutral or positively charged residues (Fig. 1). It is therefore tempting to speculate that this patch of residues is a receptor site for antimicrobial peptides such as SdpC, in which electrostatic contacts are made with a complementary cluster of positively charged amino acids. We further speculate that treatment with base and Triton X-100 causes unfolding of proteins in the cell envelope, revealing peptide domains that are detected by PrsW. Finally, we return to the unexpected observation of Figure 6 that PrsW caused degradation of the anti-σ^W factor RsiW in *E. coli* in a manner that did not depend on treatment of *E. coli* with base or other stress-causing agents. In light of our proposal that PrsW is a sensor for certain peptides, we speculate that PrsW interacts with, and responds to, a protein or peptide that is present in the cytoplasmic membrane or periplasm of the heterologous host bacterium.

A precedent for the idea that PrsW is a peptide-sensing protein and that sensing is mediated by an acidic patch of amino acids comes from work with the *Salmonella typhimurium*, two-component regulatory system PhoPQ (Bader et al. 2005). The PhoPQ system is known to control gene expression in manner that is influenced by antimicrobial peptides produced in macrophages (Bader et al. 2005). Responsiveness to these peptides has been attributed to an acidic patch on the periplasmic face of the sensor kinase PhoQ (Bader et al. 2005). Other work indicates that the same region of PhoQ additionally (or alternatively) serves in the recognition of Mg^{++}, which inhibits PhoQ activity and is held at a low concentration in macrophages (Chamnongpol et al. 2003).

Finally, we note that orthologs of PrsW are present in *Streptococcus pneumoniae* and *Listeria monocytogenes* (Supplementary Fig. S1). Conceivably, in these pathogenic bacteria, PrsW plays an analogous role in sensing and mounting a response to antimicrobial peptides produced by the host organism.

A YsdB-mediated, negative feedback loop

Our investigation also provides a basis for speculating on the function of *ysdB*, a σ^W-controlled gene mutants of which were known to exhibit enhanced, basal levels of σ^W-directed gene expression (Turner and Helmann 2000; Cao et al. 2002b). We have shown that induction of σ^W-controlled gene expression remains responsive to cell envelope stress even in the absence of YsdB. Therefore, YsdB is not itself an essential part of the stress-sensing mechanism for σ^W. Instead, we propose that σ^W-directed synthesis of YsdB constitutes a negative feedback loop that dampens the expression of the σ^W regulon following the activation of σ^W in response to conditions of cell envelope stress. Like PrsW, YsdB is an integral membrane protein, and it is tempting to speculate that YsdB

directly interacts with and inhibits the activity of PrsW. Alternatively, YsdB could bind to RsiW and thereby protect it from PrsW-mediated cleavage. If our speculation that YsdB mediates a negative feedback loop is correct, then YsdB plays an analogous role to that of RseB, which is produced under the control of the ECF σ factor σ^E in *E. coli* and inhibits σ^E activation by binding to the anti-σ factor RseA (Missiakas et al. 1997; Collinet et al. 2000; Grigorova et al. 2004).

Role of σ^W in cannibalism

Our findings also provide insights into the role of σ^W in cannibalism. Results presented here and those of Butcher and Helmann (2006) show that σ^W-directed gene expression helps to confer resistance to the cannibalism toxin SdpC in the absence of SdpI. Butcher and Helmann (2006) have also shown that resistance to SdpC is mediated by two σ^W-controlled transcription units, *yknWXYZ* and *yfhL*. The *yknWXYZ* operon encodes an apparent ABC transporter similar to other ABC transporters that provide resistance to cationic antimicrobial peptides (Diaz et al. 2003; Butcher and Helmann 2006). Interestingly, the product of *yfhL* is a paralog of SdpI, a finding that suggests that YfhL functions in a similar manner to the immunity protein (Butcher and Helmann 2006).

If σ^W can help protect cells against the toxic effects of SdpC, then why does the ECF σ factor not counteract the effects of the SdpC toxin during cannibalism? Both SdpC and the SdpI immunity protein are produced by cells that have activated the master regulator for sporulation Spo0A (Spo0A-ON cells) (Ellermeier et al. 2006). In contrast, cells that fail to activate Spo0A (Spo0A-OFF cells) succumb to the toxin because they are prevented from producing SdpI due to the action of AbrB, a repressor that is only found in Spo0A-OFF cells (Ellermeier et al. 2006). Recently, it was shown that like the gene for SdpI, the gene for σ^W is subject to repression by AbrB (Qian et al. 2002). Thus, and pleasingly, the presence of the AbrB repressor in Spo0A-OFF cells blocks genes involved in two different pathways for protecting against the toxic effects of SdpC. This explains why Spo0A-OFF cells are exquisitely sensitive to the cannibalism toxin and Spo0A-ON cells are not.

Materials and methods

Strain construction

Strains are otherwise isogenic derivatives of the wild-type *B. subtilis* strain PY79 (Youngman et al. 1984) and are listed in Supplementary Table S1. *B. subtilis* competent cells were prepared by the one-step method previously described (Wilson and Bott 1968). The P_{sigW}-*lacZ* reporter at *amyE* was constructed by PCR-amplifying the P_{sigW} region using CDEP275 and CDEP276. The resulting PCR product was digested with EcoRI and BamHI and cloned into pDG1661 (Guerout-Fleury et al. 1996) digested with the same sites to create pCE144. The clones of *prsW*+ and *prsW*^{E95K} at *thrC* were constructed by PCR-amplifying either *prsW*+ or *prsW*^{E95K} using CDEP165 and CDEP166. The resulting PCR was digested with EcoRI and BamHI and cloned into pDG1731 (Guerout-Fleury et al. 1996) digested with the same sites to create pCE113 (*prsW*+) and pCE117 (*prsW*^{E95K}). The plasmids utilized to express *prsW* in *E. coli* were constructed by PCR-amplifying either *prsW*+ or *prsW*^{E95K} using CDEP312 and CDEP313. The resulting PCR was digested with KpnI and SalI and cloned into pBAD33 digested with the same sites to create pCE154 (*prsW*+) and pCE155 (*prsW*^{E95K}). The plasmid for expressing 3X-Flag-RsiW in *B. subtilis* was constructed by PCR-amplifying *rsiW* using CDEP252 and CDEP246. The resulting PCR product was then PCR-amplified using CDEP253 and CDEP246. The resulting PCR product was digested with HindIII and SphI and cloned into pDP111 (gift of D. Kearns, Harvard University) to create pCE153.

Deletion mutations

We used the long-flanking homology PCR (LFH-PCR) technique for creating deletion mutations (Wach 1996) using the primers listed in Supplementary Table S2. The deletion/insertion *prsW*::*erm* was constructed by PCR-amplifying the 5′-flanking region of *prsW* with CDEP167 and CDEP168, while the 3′-flanking region was amplified using CDEP169 and CDEP170. The deletion/insertion *ysdB*::*kan* was constructed by PCR-amplifying the 5′-flanking region of *ysdB* using CDEP149 and CDEP175, while the 3′-flanking region was amplified using CDEP176 and CDEP152. The deletion/insertion *rsiW*::*spec* was constructed by PCR-amplifying the 5′-flanking region of *rsiW* with CDEP205 and CDEP206, while the 3′-flanking region was amplified using CDEP207 and CDEP208. The deletion/insertion *yluC*::*spec* was constructed by PCR-amplifying the 5′-flanking region of *yluC* with CDEP209 and CDEP210, while the 3′-flanking region was amplified using CDEP211 and CDEP212. The resulting PCR products were then used as primers to amplify the kanamycin resistance cassette from the plasmid pDG780, the erythromycin resistance cassette from pDG646, or the spectinomycin resistance cassette from the plasmid pDG1726 (Guerout-Fleury et al. 1995) as previously described (Wach 1996). The PCR products were then transformed into PY79 as previously described. The mutants were confirmed by PCR.

Construction of PrsW active-site mutants by site-directed mutagenesis

Mutants of the YydC active site were constructed using the QuickChange site-directed mutagenesis kit (Stratagene). Mutagenesis was carried out according to the manufacturer's instructions using the following primer pairs: CDEP306 and CDEP307 (*ypcC*^{E75AE76A}) and CDEP314 and CDEP319 (*prsW*^{H175A}). The sequence of the plasmids was confirmed by sequencing using CDEP165 and CDEP166 as described below. The resulting plasmids pCE156 (*ypcC*^{E75AE76A}) and pCE158 (*prsW*^{H175A}) were transformed into *B. subtilis* PY79.

Medium supplements

Antibiotics were used at the following concentrations: 5 µg/mL chloramphenicol, 1 µg/mL and 25 µg/mL erythromycin plus lincomycin, 5 µg/mL kanamycin, 100 µg/mL spectinomycin, 10 µg/mL tetracycline, and 100 µg/mL ampicillin. 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. IPTG was used at a final concentration of 1 mM.

Isolation of sdpI *suppressor mutations resistant to SdpC*

To isolate mutations resistant to SdpC in the absence of SdpI, we plated 100 µL of an overnight culture of 15 independent colonies of an *sdpI::tet* mutant strain onto DSM. The plates were incubated for 3 d at 37°C, after which one SdpC-resistant mutant was isolated and then subjected to mapping. Mapping of the SdpC-resistant mutants was performed by transforming SdpC-resistant mutants with pIC333 and generating a random transposon library as previously described (Steinmetz and Richter 1994). DNA from the transposon library was pooled and transformed into an sdpI mutant, and selecting for the transposon was followed by screening for resistance to SdpC. The linked Tn10-spec were then cloned and sequenced as previously described (Steinmetz and Richter 1994).

The *prsW* mutants were sequenced by PCR-amplifying the *prsW* mutant alleles using primers CDEP167 and CDEP170. The resulting PCR products were then sequenced using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) and either primer CDEP165 or CDEP166, according to the manufacturer's instructions.

Competition assays

Solid DS medium was inoculated with ~5×10^8 cells per milliliter 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 h, the cells were collected with 5 mL of 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). The Student's *t*-test was used for statistical analyses.

Immunoblot analysis of 3X-Flag-RsiW

Overnight cultures grown at 22°C in LB were diluted 1:100 in LB + 1 mM IPTG and grown until an OD_{600} of 0.8. After pelleting 1 mL of cells, the pellets were lysed by resuspending in 250 µL of TE (pH 8.0) and 10 µg/mL lysozyme and, were incubated at 37°C for 10 min; 250 µL of 2× loading buffer was added and the samples were heated for 10 min at 65°C. Proteins were electrophoresed on a 12% SDS–polyacrylamide gel. The proteins were then blotted onto nitrocellulose, and the proteins were detected by incubating in a 1:10,000 dilution of either α-σ^A or α-Flag antibodies (Sigma) followed by incubation in a 1:10,000 dilution of goat anti-rabbit IgG (H + L)-HRP conjugate from Bio-Rad.

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