Activation of the Extracytoplasmic Function $\sigma$ factor $\sigma^P$ by $\beta$-lactams in *Bacillus thuringiensis* requires the site-2 protease RasP

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Abstract

Bacteria can utilize alternative σ factors to regulate sets of genes in response to changes in the environment. The largest and most diverse group of alternative σ factors are the Extracytoplasmic Function (ECF) σ factors. σ^P is an ECF σ factor found in *Bacillus anthracis*, *B. cereus*, and *B. thuringiensis*. Previous work showed σ^P is induced by ampicillin, a β-lactam antibiotic, and required for resistance to ampicillin. However, it was not known how activation of σ^P is controlled or what other antibiotics may activate σ^P. Here we report that activation of σ^P is specific to a subset of β-lactams and σ^P is required for resistance to these β-lactams. We demonstrate that activation of σ^P is controlled by the proteolytic destruction of the anti-σ factor, RsiP, and that degradation of RsiP requires multiple proteases. Upon exposure to β-lactams, the extracellular domain of RsiP is cleaved by an unknown protease, which we predict cleaves at site-1. Following cleavage by the unknown protease, the N-terminus of RsiP is further degraded by the site-2 intramembrane protease, RasP. Our data indicate that RasP cleavage of RsiP is not the rate-limiting step in σ^P activation. This proteolytic cascade leads to activation of σ^P which induces resistance to β-lactams likely via increased expression of β-lactamases.

Importance

The discovery of antibiotics to treat bacterial infections has had a dramatic and positive impact on human health. However, shortly after the introduction of a new antibiotic bacteria often develop resistance. The bacterial cell envelope is essential for cell viability and is the target of many of the most commonly used antibiotics including β-lactam antibiotics. Resistance to β-lactams is often dependent upon β-lactamases. In *B. cereus*, *B. thuringiensis* and some *B. anthracis* strains the expression of some β-lactamases is inducible. This inducible β-lactamase expression is controlled by activation of an alternative σ factor called σ^P. Here we show that β-lactam antibiotics induce σ^P activation by degradation of the anti-σ factor RsiP.
Introduction

The bacterial cell envelope is essential for cell viability and is the target of many of the most commonly used antibiotics including β-lactams like penicillins, penems, and cephalosporins. These are broad-spectrum antibiotics that target peptidoglycan biosynthesis by inhibiting the transpeptidase activity of penicillin-binding proteins. This results in decreased and/or altered crosslinking of peptidoglycan which leads to cell envelope damage and subsequent cell lysis and death (1, 2).

Members of the *Bacillus cereus* group, including *Bacillus thuringiensis* and *Bacillus cereus* and some strains of *Bacillus anthracis*, are highly resistant to β-lactam antibiotics (3–6). This resistance is due in part to expression of at least two β-lactamases (3, 5). The expression of these β-lactamases is induced by ampicillin and is dependent upon the alternative σ factor, σ^P_. σ^P_ belongs to the Extracytoplasmic Function (ECF) family of alternative σ factors (5).

Bacteria often utilize alternative σ factors to regulate subsets of genes required for survival in specific environmental conditions or stress responses. ECF σ factors are the largest and most diverse group of alternative σ factors and represent the “third pillar” of bacterial signal transduction (7, 8). ECF σ factors belong to the σ^70 family but unlike the “housekeeping” σ factor, σ^70, ECF σ factors contain only region 2 and region 4.2 of σ^70 which recognize and bind to the -10 and -35 regions of promoter sequences, respectively (8, 9). In addition, unlike σ^70, ECF σ factors are generally held inactive by anti-σ factors until bacteria encounter an inducing signal (10, 11). Upon induction, ECF σ factors are released from their cognate anti-σ factors to promote transcription of specific stress-response genes.

The ECF σ factors have been subdivided into more than 40 distinct groups with ECF01 being the best studied [Reviewed in (7, 11, 12)]. σ^P_ belongs to the ECF01 family which includes members like σ^E_ and σ^W_ from *E. coli* and *Bacillus subtilis*, respectively. The activities of the ECF01 family are inhibited by their cognate transmembrane anti-σ factors (8, 13). To activate ECF01 σ factors, the anti-σ factors must be destroyed via a proteolytic cascade (14, 15).
example, the *E. coli* anti-σ factor, RseA, is degraded in response to outer membrane stress leading to σ^E_ activation (16, 17). DegS, a serine protease, cleaves the anti-σ factor RseA at site-1 (14, 18, 19). After site-1 cleavage, the conserved site-2 protease, RseP, cleaves RseA within the membrane leading to increased σ^E_ activity (14, 20, 21). Similarly, the σ^W_ anti-σ factor, RsiW, from *B. subtilis* is proteolytically degraded by site-1 and site-2 proteases. In the case of RsiW, the site-1 protease is PrsW, a metalloprotease unrelated to DegS. PrsW cleaves RsiW in response to antimicrobial peptides, vancomycin and pH change (22–24). RsiW is further processed by the conserved site-2 protease RasP, a homolog of RseP (15).

The closely related ECF30 family member, σ^V_ from *B. subtilis*, is activated by lysozyme (25–29). Activation of σ^V_ differs from σ^E_ and σ^W_ activation in that σ^V_ is not controlled by a dedicated site-1 protease but instead utilizes signal peptidases (30, 31). Signal peptidases are essential proteases which are required to cleave substrates secreted from the general secretion or twin arginine secretion systems (32–34). The anti-σ factor, RsiV binds to lysozyme which allows signal peptidase to cleave RsiV at site-1 (30, 31). This allows the site-2 protease, RasP, to cleave RsiV, leading to σ^V_ activation (35).

Previous studies found σ^P_ is induced by ampicillin and its activity is required for resistance to ampicillin (5). The activity of σ^P_ is inhibited by the transmembrane anti-σ factor, RsiP (5, 6). However, whether σ^P_ is activated specifically by ampicillin or more generally by cell wall stress is not known. In *B. subtilis*, activation of σ^V_ is specific to lysozyme (26, 27), while activation of σ^W_, σ^X_ and σ^M_ is in response to more general cell envelope stress (9, 36, 37). Here, we show σ^P_ is activated by a specific subset of β-lactams and this activation occurs via regulated intramembrane proteolysis of the anti-σ factor, RsiP.
Results

A subset of β-lactams induces σP activation.

Previously, Koehler and colleagues demonstrated that ampicillin induces expression of the β-lactamase encoded by *bla1* (*hd73_3490*) in a σP-dependent manner in *B. thuringiensis* and *B. cereus* (5). Activation of some ECF σ factors is highly specific to an inducing signal, while others are activated by more general cell envelope stress. Thus, we sought to determine the specificity of σP activation using *B. thuringiensis* as a model system.

Like many ECF σ factor systems, σP is required for its own transcription (5). To monitor σP activation, we fused the σP promoter (PsigP) to the *lacZ* reporter gene and integrated this construct into the genome of *B. thuringiensis* (THE2549 *thrC*:PsigP-*lacZ*). We tested several classes of β-lactams and cell wall-targeting antibiotics for their ability to induce expression of PsigP-*lacZ*. We observed wide zones of PsigP-*lacZ* induction around cefoxitin and cefmetazole (Fig. 1). We detected fainter zones of induction in the areas around cephalothin and cephalexin (Fig. 1). Very faint zones of induction were present in the cells around ampicillin and methicillin (Fig.1). Interestingly, we did not observe this induction surrounding the β-lactams, cefoperazone and piperacillin or antibiotics that target other steps in cell wall biosynthesis including, ramoplanin, phosphomycin, nisin, bacitracin, and vancomycin (Fig. 1). We also tested compounds that do not target peptidoglycan biosynthesis including kanamycin, polymyxin B and erythromycin and saw no induction of PsigP-*lacZ* (Fig.1).

To quantify the levels of β-lactam induction, we tested eight β-lactams for their ability to activate the PsigP-*lacZ* fusions using a β-galactosidase assay. Mid-log cells were incubated in the presence of various concentrations of ampicillin, cefoxitin, cefmetazole, cephalothin, methicillin, cephalexin, cefoperazone, and cefsulodin for 1 hour at 37°C. We observed dose-dependent induction with a subset of these β-lactams (Fig. 2A-B). Interestingly, ampicillin, methicillin and cephalexin showed low levels of PsigP-*lacZ* induction when spotted onto a lawn of cells (Fig. 1) but strongly induced PsigP-*lacZ* in liquid assays (Fig. 2A-B), a point we will return to.
later. In contrast, neither cefoperazone nor cefsulodin were able to induce on the plates or in liquid (Fig. 1 and 2B). This confirms our observation that a sub-set of β-lactams induce σP activation.

We found that deletion of the sigPrsiP genes blocked expression of PsigP-lacZ in the presence of β-lactams (Fig. 1 and Fig. 2C) demonstrating σP is required for induction of PsigP-lacZ in response to β-lactams. When we introduced a low copy plasmid containing PsigPrsigP′rsiP′ into the ΔsigPrsiP mutant (ΔsigPrsiP/pSigPRsiP), we restored the induction of PsigP-lacZ in response to cefoxitin (Fig. 2C). Taken together, these data suggest a subset of β-lactam antibiotics activate σP.

σP and Bla1 are involved in resistance to some β-lactams.

To determine the impact of σP on resistance to β-lactams, we measured the minimal inhibitory concentration (MIC) of wild type and a ΔsigPrsiP mutant for several β-lactams. We found the wild type was greater than 100-fold more resistant to ampicillin, methicillin, and cephalothin than was the ΔsigPrsiP mutant (Table 1). Wild type was 16 to 50-fold more resistant to cefmetazole, cefoxitin and cephalexin than the mutant (Table 1). There was little or no difference in resistance to piperacillin, cefoperazone and cefsulodin which also failed to activate σP (Table 1 and Fig. 1). We also demonstrate that complementing the ΔsigPrsiP mutant with a plasmid carrying PsigP-sigP′-rsiP′ restored resistance to ampicillin and cefoxitin (Table 2). For reasons that remain unclear, strains containing plasmids including empty vector have slight increases in β-lactam resistance. However, this does not impact the observation that the presence of PsigP-sigP′-rsiP′ restored resistance to ampicillin and cefoxitin.

Since σP was shown to control expression of hd73_3490 (referred to hereafter as bla1), which encodes a β-lactamase, we sought to determine if this gene played a role in resistance to β-lactams. We made a deletion of bla1 and determined the MIC of ampicillin and cefoxitin for this strain. The bla1 mutant was 8 to 16-fold more sensitive to ampicillin, ~5 fold more sensitive
to methicillin but no more sensitive to cefoxitin than wild type (Table 2). This contrasts with the
sigP mutant which is greater than 1000-fold more sensitive to ampicillin, 600-fold more sensitive
to methicillin and ~25-fold more sensitive to cefoxitin than the wild type (Table 2). This suggests
that Bla1 plays a more important role in resistance to ampicillin and methicillin than to cefoxitin.
Furthermore, our data suggests that while Bla1 contributes to β-lactam resistance, additional σP
regulated genes must also contribute to β-lactam resistance.

When we tested various β-lactams for induction of P_{sigP-lacZ} on X-gal plates, we did not
consistently observe a strong zone of induction surrounding ampicillin and methicillin (Fig. 1).
We hypothesized this weak induction zone was because the wild type efficiently produced β-
lactamases which degraded the inducer (ampicillin and methicillin). Thus, we were unable to
observe the increased production of β-galactosidase. To test this hypothesis, we determined the
effect of a Δbla1 mutant on σP activation. We found that in the Δbla1 mutant, ampicillin and
methicillin produced more distinct zones of induction (Fig. 1). However, all other induction zones
of the Δbla1 mutant were similar to wild type. Thus, in the absence of Bla1 which degrades
ampicillin and methicillin, we detected greater induction of P_{sigP-lacZ} expression. Taken
together, these observations suggest that the weak ampicillin induction of P_{sigP-lacZ} on plates is
in part due to the efficient degradation of the inducer by β-lactamases.

RsiP is degraded in response to cefoxitin in a dose-dependent manner.

The anti-σ factors of other ECF01 family members are degraded which leads to the
activation of their cognate σ factors (7, 14, 15). We sought to determine if β-lactams activate σP
by inducing degradation of RsiP. To investigate this, we constructed a strain with an
anhydrotetracycline-inducible copy of green fluorescent protein (GFP) fused to the N-terminus
of RsiP (GFP-RsiP). The inducible promoter allows us to uncouple expression of RsiP from
induction of σP. The GFP-RsiP fusion allows us to follow the fate of the cytoplasmic portion of
RsiP. Expression of GFP-RsiP complements an rsiP null mutation (Fig. S1) and localizes to the
membrane (Fig. S2). We then induced the synthesis of GFP-RsiP in exponential phase cells and monitored its processing before and after treatment with cefoxitin. We chose to utilize cefoxitin for these experiments because cefoxitin induces $\sigma^p$ activation over a wide concentration range and the $\Delta\text{sigPrsiP}$ mutant strain grows at most of these concentrations (Fig. 2A and Table 1). Cell pellets were then lysed by sonication and western blots were performed using anti-RsiP antisera against the extracellular portion of RsiP or anti-GFP antisera which detects GFP fused to the intracellular portion of RsiP.

When cells producing GFP-RsiP were grown in the absence of cefoxitin we detected full length GFP-RsiP at the expected size of ~60 kD using anti-RsiP anti-sera. This band was absent in the empty vector control (Fig. 3A). When cells were incubated with cefoxitin (5 µg/ml) for various times we found that the level of full-length GFP-RsiP decreased over time (Fig. 3A and Fig. S3A). We observed loss of GFP-RsiP by 30 minutes to 1-hour post exposure to cefoxitin (Fig. 3A and Fig. S3A). This suggests GFP-RsiP is likely degraded in the presence of cefoxitin.

We also tested the effect of cefoxitin concentration on GFP-RsiP levels by incubating cells with a range of cefoxitin concentrations (0-500 µg/mL) for one hour. We found increasing concentrations of cefoxitin resulted in greater decrease of full length GFP-RsiP (Fig. 3B and Fig. S3B). We obtained comparable results when we blotted for the N-terminal domain using anti-GFP antisera (Fig. S4). These data suggest activation of $\sigma^p$ occurs via loss of RsiP in a cefoxitin dose-dependent manner.

**RasP is necessary for $\sigma^p$ activation.**

Both $\sigma^E$ and $\sigma^W$ are activated by regulated intramembrane proteolysis of their cognate anti-$\sigma$ factors. Proteolysis of these anti-$\sigma$ factors requires multiple proteases, including the highly-conserved site-2 protease, RseP and RasP, respectively (14, 15). We hypothesize that activation of $\sigma^p$ requires multiple proteases including the conserved site-2 protease RasP to
degrade RsiP. To test this we used BLAST to identify a putative membrane embedded metalloprotease, HD73_4103, which is 76% similar and 60% identical to B. subtilis RasP and is hereafter referred to as RasP (Fig. S5) (38–43). To determine if RasP was required for σ^p activation, we generated a strain containing a deletion of rasP and the P_{sigP-lacZ} reporter. In the absence of RasP, we do not detect increased expression of P_{sigP-lacZ} reporter in response to cefoxitin (Fig. 2C). In MIC experiments, we found that, similar to the ΔsigPrsiP mutant, the ΔrasP mutant was more sensitive to ampicillin and cefoxitin (Table 2). We found both resistance to β-lactams and induction of P_{sigP-lacZ} could be complemented when a plasmid expressing rasP^+ was introduced into the ΔrasP mutant (Fig. 2C and Table 2). These data suggest RasP is required for σ^p activation.

**RasP is required for degradation of RsiP.**

To determine if RasP is required for degradation of RsiP, we expressed the GFP-RsiP fusion in both wild type and a ΔrasP mutant. We treated cells with 5 µg/mL cefoxitin for various lengths of time from 0 to 180 minutes (Fig. 4 and Fig. S6). In wild type, we observed loss of full-length RsiP over time (Fig. 4 and Fig. S6). In contrast, we observed loss of full-length GFP-RsiP and the accumulation of a smaller ~35kD band in the ΔrasP mutant (Fig. 4 and Fig. S6). This suggests RasP is required for complete degradation of RsiP. Since a truncated product accumulates in the ΔrasP mutant, RasP is likely required for site-2 cleavage and an unidentified protease is required for cleavage at site-1.

**Mutations in rsiP result in constitutive sigP expression.**

To further characterize the σ^p signal transduction system, we isolated mutants which resulted in constitutive expression of P_{sigP-lacZ}. We selected for mutants with increased resistance to cefoxitin by plating cultures of the wild type P_{sigP-lacZ} strain (THE2549) on LB cefoxitin 200 µg/ml agar. At this concentration of cefoxitin, wild type B. thuringiensis fails to...
grow. These strains were tested for $P_{\text{sigP}}$-lacZ expression in the absence of cefoxitin by streaking on LB X-gal. We isolated 8 independent mutants with increased resistance to cefoxitin that have constitutive $P_{\text{sigP}}$-lacZ expression. We hypothesized these strains harbored mutations in $rsi\text{P}$. We PCR amplified and sequenced the $\text{sigP}$ and $rsi\text{P}$ genes from the constitutive mutants. The 8 constitutive mutants contained mutations in different regions of the $rsi\text{P}$ gene that resulted in C-terminal truncations of RsiP (Fig. S7). We selected four $rsi\text{P}$ mutants for further study. We found that each mutant strain showed increased $P_{\text{sigP}}$-lacZ expression even in the absence of $\beta$-lactams (Fig. 5). When a wild type copy of $rsi\text{P}$ ($p\text{SigPRsiP}$) was introduced to each of these mutants, $P_{\text{sigP}}$-lacZ expression was no longer constitutive but was induced in the presence of cefoxitin (Fig. S8). This indicates that the $rsi\text{P}$ mutations were responsible for the increased $P_{\text{sigP}}$-lacZ expression.

In the $\sigma^V$ and $\sigma^W$ systems, RasP cleaves the anti-$\sigma$ factors RsiW and RsiV within the transmembrane domain to activate the cognate $\sigma$ factors (15, 35). The RsiP transmembrane is predicted to be residues 54-71 based on TMHMM (44). Two of the four RsiP truncations produce proteins with the transmembrane domain intact while the remaining RsiP truncations lack the transmembrane domain. Since RasP is known to cleave proteins within the transmembrane domain we hypothesized that those truncations which still contain a transmembrane domain would require RasP in order to activate $\sigma^P$. To test this, we introduced the $\Delta$rasP mutation into each of the $rsi\text{P}$ mutants. In the absence of RasP, strains containing truncations which have a transmembrane domain ($RsiP^{1-220}$, $RsiP^{1-80}$; Fig. 4 and Fig. S7) no longer constitutively activate $\sigma^P$ (Fig. 5). However, the strains with the $rsi\text{P}$ truncation lacking the transmembrane domain ($RsiP^{1-16}$, $RsiP^{1-61}$) constitutively activate $\sigma^P$ even in the absence of RasP ($RsiP^{1-16}$, $RsiP^{1-61}$; Fig. 4 and S5). Thus, RasP is required for $\sigma^P$ activation when the transmembrane domain of RsiP is intact, consistent with the role of RasP as a site-2 protease.
RasP cleaves within the transmembrane domain of RsiP and is not the regulated step in $\sigma^p$ activation.

In the case of $\sigma^W$ and $\sigma^V$ the rate-limiting step in $\sigma$ factor activation is site-1 cleavage (15, 35). Since the identity of the site-1 protease is not currently known we sought to determine if RasP cleavage of RsiP is a rate-limiting step in $\sigma^P$ activation. To test this, we constructed truncations of GFP-RsiP that lack the extracellular portion of RsiP. One truncation includes the transmembrane domain (gfp-rsiP\textsuperscript{1-72}) and one truncation lacks the transmembrane domain (gfp-rsiP\textsuperscript{1-53}). We expressed the truncated GFP-RsiP proteins in wild type and $\Delta$rasP backgrounds and exposed these strains to cefoxitin (5 µg/ml). In wild type strains we found both GFP-RsiP\textsuperscript{1-72} and GFP-RsiP\textsuperscript{1-53} were degraded (Fig. 6 and Fig. S9). However, in the $\Delta$rasP mutant the GFP-RsiP\textsuperscript{1-72} accumulated, while the GFP-RsiP\textsuperscript{1-53} was degraded (Fig. 6 and Fig. S9). These data indicate that GFP-RsiP\textsuperscript{1-72} requires RasP for degradation while GFP-RsiP\textsuperscript{1-53} does not. One possible interpretation is that GFP-RsiP\textsuperscript{1-72} is not produced or localized properly to the membrane. Thus, we confirmed that GFP-RsiP\textsuperscript{1-72} localizes to the membrane by fluorescent microscopy (Fig. S2). This suggests the RasP cleavage site of RsiP occurs within the transmembrane domain between amino acids 53 and 72. The presence or absence of cefoxitin had no effect on the degradation (Fig. 6 and Fig. S9). Since GFP-RsiP\textsuperscript{1-72} is constitutively degraded we conclude GFP-RsiP\textsuperscript{1-72} mimics the site-1 cleavage product and that RasP activity is not induced by cefoxitin. This suggests that RasP cleavage of RsiP is not the regulated step in $\sigma^P$ activation and that site-1 cleavage is the step that is controlled by the presence of $\beta$-lactams.

Discussion

Many ECF $\sigma$ factors are induced in response to extracytoplasmic stressors and initiate transcription of a subset of genes to modulate the cell’s response to these stresses. ECF $\sigma$ factors can respond to signals such as misfolded periplasmic protein, antimicrobial peptides, or...
lysozyme. The ECF σ factors encoded in highly related organisms can vary widely. For example, *B. subtilis* encodes 7 ECF σ factors, while *B. thuringiensis* encodes 15 predicted ECF σ factors. The only ECF σ factor these organisms share in common is σ^M (45). Thus, there is a variability in how bacteria utilize ECF σ factors to respond to stress. Ross *et al.* demonstrated the novel ECF σ factor, σ^P, is induced in the presence of ampicillin and initiates transcription of β-lactamases (5). Here we demonstrated σ^P responds specifically to a subset of β-lactams, while other β-lactams and cell wall targeting antibiotics fail to induce σ^P activation. We also showed σ^P confers varying degrees of resistance to these β-lactam antibiotics. We found that σ^P was not required for resistance to other cell wall antibiotics including vancomycin, nisin, bacitracin suggesting specificity in resistance to β-lactams and not a general cell envelope stress response.

For ECF σ factors to be activated, their cognate anti-σ factors must be inactivated. This can be accomplished via various mechanisms including: a conformational change of the anti-σ, partner switching where an anti-anti-σ factor frees the σ factor form the anti-σ factor, or proteolytic destruction of the anti-σ factor (Ho and Ellermeier, 2012; Helmann, 2016). The anti-σ factors RseA in *E. coli* as well as RsiW and RsiV in *B. subtilis* are degraded sequentially by regulated intramembrane proteolysis. Each of these anti-σ factors requires a different family of proteases to cleave the anti-σ factor at site-1 (14, 22, 30, 47, 48) while site-2 cleavage is carried out by the conserved site-2 protease (14, 15, 35). We hypothesize that σ^P is activated in a similar manner. Our data indicate σ^P is released from RsiP by proteolytic degradation when β-lactams are present. We found RasP is required for activation of σ^P. We also observe that a RasP degradation product approximately the size of our predicted RasP substrate accumulates in a ΔrasP mutant. This indicates RasP is required for degradation of RsiP. Our data also suggest, similar to other anti-σ factors, site-2 cleavage of RsiP is not the rate-limiting step since the C-terminal RsiP truncations are constitutively degraded and lead to constitutive σ^P activation in the absence of β-lactams. Thus, we hypothesize that RasP is required for site-2 cleavage of
RsiP and an as yet unidentified protease is required to initiate degradation of RsiP by cleaving RsiP at site-1. We hypothesize, that like other ECF σ factors activated by regulated intramembrane proteolysis, site-1 cleavage of RsiP is likely the rate-limiting step in σP activation.

Our data suggest a subset of β-lactams induce σP activation. We found, in addition to ampicillin, σP is activated by cefoxitin, cefmetazole, cephalothin, cephalaxin, and methicillin; but not by piperacillin, cefoperazone, cefsulodin, or antibiotics that target other steps in peptidoglycan biosynthesis. This raises the question: what is the signal for σP activation? The β-lactams could be sensed directly or indirectly. For example RsiV directly senses lysozyme and degradation of RsiV is rapid (31). In contrast activation of σE is indirect and due to buildup of products that occur when the outer membrane is damaged (31, 49). Our data suggest RsiP degradation is a relatively slow process. One possible interpretation of this is that β-lactam induced PG damage must accumulate to induce RsiP degradation. We hypothesize that the β-lactams we tested have different affinities for PBPs and this affinity may explain why some β-lactams induce σP while others do not. In other organisms, including Streptococcus pneumoniae, B. subtilis and E. coli, β-lactams can differentially target PBPs (50–52). This raises the possibility that activation of σP could be the result of inhibition of specific PBPs. Unfortunately, at this time we do not know which PBPs are targeted by the different β-lactams in B. thuringiensis. Thus, the precise mechanism and signal responsible for σP activation remain to be clearly defined.

Materials and Methods

Media and Growth Conditions

All B. thuringiensis strains are isogenic derivatives of AW43, a derivative of B. thuringiensis subspecies kurstaki strain HD73 (53). All strains and genotypes can be found in Table 3. All B. thuringiensis strains were grown in or on LB media at 30°C unless otherwise
specified. Cultures of *B. thuringiensis* were grown with agitation in a roller drum. Strains containing episomal plasmids were grown in LB containing chloramphenicol (cam, 10 µg/ml) or erythromycin (erm, 10 µg/ml). *E. coli* strains were grown at 37°C using LB-ampicillin (amp, 100 µg/ml) or LB-cam (10 µg/ml) media. To screen for threonine auxotrophy, *B. thuringiensis* strains were patched on minimal media plates without or with threonine (50 µg/ml) (54, 55). 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. Anhydrotetracycline (ATc, Sigma) was used at a concentration of 100 ng/ml.

**Strain and Plasmid Construction.**

All plasmids are listed in Table 4 which includes information relevant to plasmid assembly. Plasmids were constructed by isothermal assembly (56). Regions of plasmids constructed using PCR were verified by DNA sequencing. The oligonucleotide primers used in this work were synthesized by Integrated DNA Technologies (Coralville, IA) and are listed in Table S1. All plasmids were propagated using OmniMax 2-T1R as the cloning host and passaged through the non-methylating *E. coli* strain INV110 before being transformed into a *B. thuringiensis* recipient strain.

To construct deletion mutants, we cloned 1 kb DNA upstream and 1 kb downstream of the site of desired deletion using primers listed in Table S1 onto the temperature sensitive pMAD plasmid (erythromycin-resistant) between the BglII and EcoRI sites (57).

Complementation constructs were constructed in pAH9 which is an *E. coli*-Gram positive shuttle vector with a pE194 origin of replication (58). Chromosomal DNA including the promoter sequence was cloned for $P_{\text{sigP}}-\text{sigP}'\text{rsiP}^+$ and cloned into pAH9 digested with EcoRI and HindIII while $\text{rasP}$ was cloned downstream of the $P_{\text{sarA}}$ promoter from *Staphylococcus aureus* by digesting with EcoRI and KpnI. In *B. thuringiensis* $P_{\text{sarA}}$ has moderate constitutive expression.
To generate strains containing the \textit{sigP} promoter fused to the \textit{lacZ} reporter integrated into the chromosome, we constructed a number of intermediate vectors. To switch the antibiotic resistance of the temperature-sensitive pMAD vector, we constructed pTHE946 which contains the \textit{E. coli} origin (ColE1 ori) of replication, \textit{erm}-resistance gene (for selection in Gram-positives), \textit{amp}-resistance gene (for selection in \textit{E. coli} strains) and the temperature-sensitive origin (pE194 ori) from pMAD (7.3 kb Stul, BamHI fragment) as well as the conjugation origin of transfer and \textit{cam}-resistance gene from pRPF185 (SmaI, BamHI fragment). The \textit{thrC} (primers 2917, 2918) and \textit{thrB} (primers 2919, 2920) genes were cloned into the Scal, Sall digested pTHE946 plasmid (lacking \textit{erm}^R and \textit{amp}^R genes) to generate a vector (pTHE948) which can integrate into the \textit{thrC} operon. A promoterless \textit{lacZ} fragment (primers 2922, 2923) was added between the \textit{thrC} and \textit{thrB} genes of pTHE948 (XhoI, SbfI) to generate pTHE950. This plasmid (XhoI, NotI digested) was used to clone the \textit{sigP} promoter (primers TE2929, 2930) to generate the \textit{P}_{\textit{sigP}}-\textit{lacZ} promoter fusion (pTHE949).

\textbf{\textit{B. thuringiensis} DNA Transformation.}

Plasmids were introduced into \textit{B. thuringiensis} by electroporation (59, 60). Briefly, recipient cells were grown to late-log phase at 37°C. For each transformation, cells (1.5 ml) were pelleted by centrifugation (9,000 x g) and washed twice in room temperature sterile water. After careful removal of all residual water, 100 µl of sterile 40% PEG 6000 (Sigma) was used to gently resuspend cells. Approximately 2-10 µl of unmethylated DNA (>50 ng/µl) was added to cells and transferred to a 0.4 cm gap electroporation cuvette (Bio-Rad). Cells were exposed to 2.5kV for 4-6 msec. LB was immediately added and cells were incubated at 30°C for 1-2 hours prior to plating on selective media.

\textbf{Construction of deletions or promoter-\textit{lacZ} fusions in \textit{B. thuringiensis}}
To generate unmarked mutants and $\text{thrC}::P_{\text{sigP}}$-$\text{lacZ}$ strains, we used plasmid vectors containing the temperature-sensitive origin of replication (pE194 ori) from the pMAD plasmid (57). At permissive temperatures (30°C), pMAD replicates episomally as a plasmid. At non-permissive temperatures (42°C) pMAD must integrate into the chromosome via homologous recombination otherwise the plasmid will be lost to segregation and the strain will become sensitive to erythromycin. Plasmids were transformed into a $B.\text{thuringiensis}$ recipient strain and selected for on LB-erm agar at 30°C. To select for the integration of the deletion plasmid into the recipient strain genome, plasmid-containing bacteria were grown at 42°C on LB-erm plates. The plasmid-integrated strain was then struck on LB agar at 30°C twice. Individual colonies were patched on LB and LB-erm agar to identify the erm-sensitive bacteria which had lost the deletion plasmid by segregation. To verify each deletion, genomic DNA was isolated from each strain candidate and PCR was used to verify the deletion. Integration of $P_{\text{sigP}}$-$\text{lacZ}$ fusion into the $\text{thrC}$ operon results in threonine auxotrophy and can be identified by lack of growth on minimal media plates without threonine.

**Zones of Inhibition and Zones of Induction**

To determine the zones of inhibition and induction by various antibiotics, we first washed mid-logarithmically grown cells in fresh LB. Washed cells were diluted 1:100 in molten LB agar containing X-gal (100 µg/ml) and poured into empty 100 mm Petri dishes. Sterile cellulose disks (8 mm) were saturated with different antibiotics and allowed to dry for greater than 10 minutes. After each antibiotic disk was placed on the solidified agar, plates were incubated at 30°C overnight before observing.

**β-Galactosidase Assays**

To quantify expression from the $\text{sigP}$ promoter, we measured the β-galactosidase activity of cells containing a $P_{\text{sigP}}$-$\text{lacZ}$ promoter fusion. Overnight cultures were diluted 1:50 in
fresh LB media and incubated for 3 hours at 30°C. One ml of each subculture was pelleted (9,000 x g), washed (in LB broth) and resuspended in 1 ml LB broth lacking or including specified antibiotics. After 1 hour of incubation at 37°C, 1 ml of each sample was pelleted and resuspended in 200 µl of Z-buffer. Cells were permeabilized by mixing with 16 µl chloroform and 16 µl 2% sarkosyl (26, 61). Permeabilized cells (100 µl) were mixed with 10 mg/ml ortho-Nitrophenyl-β-Galactoside (ONPG, RPI, 50 µl) and OD$_{405}$ was measured over time using an Infinite M200 Pro plate reader (Tecan). β-Galactosidase activity units (µmol of ONP formed min$^{-1}$) X 10$^3$/ (OD$_{600}$ X ml of cell suspension) were calculated as previously described (62). Experiments were performed in triplicate with the mean and standard deviation shown.

**Minimum Inhibitory Concentration Assay**

To determine the minimum inhibitory concentration (MIC) for various antibiotics, we diluted overnight cultures of bacteria (washed in LB) 1:1000 in media containing two-fold dilutions of each antibiotic. All MIC experiments were performed in round-bottom 96-well plates. Each experiment was performed in triplicate and allowed to incubate for 24 hours at 37°C before observing growth or no growth.

**Immunoblot Analysis**

Samples were electrophoresed on a 15% SDS polyacrylamide gel and proteins were then blotted onto a nitrocellulose membrane (GE Healthcare, Amersham). Nitrocellulose was blocked with 5% Bovine Serum Albumin (BSA) and proteins were detected with either 1:10,000 anti-GFP or 1:5,000 anti-RsiP$^{76-275}$ anti-sera. Streptavidin IR680LT (1:10,000) was used to detect two biotin-containing proteins, PycA (HD73_4231) and AccB (HD73_4487), which serve as loading controls (63, 64). To detect primary antibodies, the blots were incubated with 1:10,000 Goat anti-Rabbit IR800CW (Li-Cor) and imaged on an Odyssey CLx Scanner (Li-Cor).
or Azure Sapphire (Azure Biosystems). All immunoblots were performed a minimum of three times with a representative example shown.

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Table 1. \( \Delta \text{sigPrsiP} \) is more sensitive to \( \beta \)-lactams than WT

|                  | WT\(^1\)  | \( \Delta \text{sigPrsiP} \)\(^1\) | Fold Difference |
|------------------|-----------|-----------------------------------|-----------------|
| Ampicillin       | 6000 +/- 0| 1.67 +/- 0.5                      | 3592            |
| Cefoxitin        | 200 +/- 0 | 20 +/- 0                          | 10              |
| Methicillin      | 666 +/- 115| 1 +/- 0                          | 666             |
| Piperacillin     | 5 +/- 0   | 1.25 +/- 0                        | 4               |
| Cephathoin       | 88 +/- 25 | 0.25 +/- 0                        | 350             |
| Cephalaxin       | 200 +/- 0 | 4 +/- 0                          | 50              |
| Cefmetazole      | 44 +/- 13 | 2.8 +/- 1.1                      | 16              |
| Cefoperazone     | 5 +/- 2   | 4 +/- 0                          | 1.25            |
| Cefsulodin       | 400 +/- 0 | 400 +/- 0                        | 1               |

\(^1\) MIC of antibiotic concentrations reported as \( \mu \text{g/ml} \)
Table 2. RasP is required for resistance to β-lactams.

| Genotype   | Vector | Ampicillin $^1$ | Cefoxitin $^1$ | Methicillin$^{1,2}$ |
|------------|--------|----------------|----------------|---------------------|
| WT         | Empty  | 8000 +/- 0     | 200 +/- 0      | 666.7 +/- 115       |
| ΔsigPrsiP  | Empty  | 2 +/- 0        | 20 +/- 0       | 1 +/- 0             |
| ΔsigPrsiP  | pSigP  | 6666 +/- 3011  | 100 +/- 0      | n.d.                |
| ΔrasP      | Empty  | 6.7 +/- 2.1    | 20 +/- 0       | n.d.                |
| ΔrasP      | pRasP  | 6333 +/- 1966  | 133 +/- 57.7   | n.d.                |
| Δbla1      | Empty  | 400 +/- 0      | 200 +/- 0      | 125 +/- 50          |

$^1$ MIC of antibiotic concentrations reported as µg/ml

$^2$ n.d. = not determined
Table 3. Strains

**B. thuringiensis**

| Strain | Description | Reference |
|--------|-------------|-----------|
| AW43   | B. thuringiensis serovar kurstaki HD73 cured of both pAW63 and pHT73, NaI<sup>R</sup> | (53) |
| THE2549| AW43 thrC::P<sub>sigP-lacZ</sub> | This study |
| EBT140 | AW43 thrC::P<sub>sigP-lacZ</sub> ΔrasP | This study |
| EBT232 | AW43 thrC::P<sub>sigP-lacZ</sub> ΔsigPrsiP | This study |
| EBT215 | AW43 thrC::P<sub>sigP-lacZ</sub> dra1 | This study |
| EBT360 | AW43 thrC::P<sub>sigP-lacZ</sub> / pAH9 P<sub>ter-gfp-rsiP</sub> | This study |
| EBT366 | AW43 thrC::P<sub>sigP-lacZ</sub> ΔrasP / pAH9 P<sub>ter-gfp-rsiP</sub> | This study |
| EBT510 | AW43 thrC::P<sub>sigP-lacZ</sub> ΔrasP / pAH9 P<sub>ter-gfp-rsiP</sub> | This study |
| EBT516 | AW43 thrC::P<sub>sigP-lacZ</sub> / pAH9 P<sub>ter-gfp-rsiP</sub> | This study |
| EBT518 | AW43 thrC::P<sub>sigP-lacZ</sub> / pAH9 P<sub>ter-gfp-rsiP</sub> | This study |
| EBT533 | AW43 thrC::P<sub>sigP-lacZ</sub> ΔsigPrsiP / pAH9 P<sub>sigP-sigP-rsiP</sub> | This study |
| EBT175 | AW43 thrC::P<sub>sigP-lacZ</sub> ΔsigPrsiP / pAH9 | This study |
| EBT176 | AW43 thrC::P<sub>sigP-lacZ</sub> ΔsigPrsiP / pAH9 rasP | This study |
| EBT238 | AW43 thrC::P<sub>sigP-lacZ</sub> ΔsigPrsiP / pAH9 P<sub>sigP-sigP-rsiP</sub> | This study |
| EBT251 | AW43 thrC::P<sub>sigP-lacZ</sub> ΔsigPrsiP / pAH9 | This study |
| THE2642 | AW43 thrC::P<sub>sigP-lacZ</sub> rsiP<sup>1-16</sup> | This study |
| THE2637 | AW43 thrC::P<sub>sigP-lacZ</sub> rsiP<sup>1-61</sup> | This study |
| THE2628 | AW43 thrC::P<sub>sigP-lacZ</sub> rsiP<sup>1-80</sup> | This study |
| THE2602 | AW43 thrC::P<sub>sigP-lacZ</sub> rsiP<sup>1-220</sup> | This study |
| THE2605 | AW43 thrC::P<sub>sigP-lacZ</sub> ΔrasP rsiP<sup>1-16</sup> | This study |
| EBT133 | AW43 thrC::P<sub>sigP-lacZ</sub> ΔrasP rsiP<sup>1-61</sup> | This study |
| EBT148 | AW43 thrC::P<sub>sigP-lacZ</sub> ΔrasP rsiP<sup>1-80</sup> | This study |
| EBT116 | AW43 thrC::P<sub>sigP-lacZ</sub> ΔrasP rsiP<sup>1-220</sup> | This study |
| EBT567 | AW43 thrC::P<sub>sigP-lacZ</sub> rsiP<sup>1-16</sup> / pAH9 P<sub>sigP-sigP-rsiP</sub> | This study |
| EBT566 | AW43 thrC::P<sub>sigP-lacZ</sub> rsiP<sup>1-61</sup> / pAH9 P<sub>sigP-sigP-rsiP</sub> | This study |
| EBT565 | AW43 thrC::P<sub>sigP-lacZ</sub> rsiP<sup>1-80</sup> / pAH9 P<sub>sigP-sigP-rsiP</sub> | This study |
| EBT564 | AW43 thrC::P<sub>sigP-lacZ</sub> rsiP<sup>1-220</sup> / pAH9 P<sub>sigP-sigP-rsiP</sub> | This study |
| EBT168 | AW43 thrC::P<sub>sigP-lacZ</sub> / pAH9 P<sub>sigP-sigP-rsiP</sub> | This study |
| EBT169 | AW43 thrC::P<sub>sigP-lacZ</sub> pAH9 | This study |
| EBT563 | AW43 thrC::P<sub>sigP-lacZ</sub> rsiP<sup>1-16</sup> / pAH9 | This study |
| EBT562 | AW43 thrC::P<sub>sigP-lacZ</sub> rsiP<sup>1-61</sup> / pAH9 | This study |
| EBT561 | AW43 thrC::P<sub>sigP-lacZ</sub> rsiP<sup>1-80</sup> / pAH9 | This study |
| EBT560 | AW43 thrC::P<sub>sigP-lacZ</sub> rsiP<sup>1-220</sup> / pAH9 | This study |
| UM20   | AW43 / pAH13 | This study |
| EBT587 | AW43 thrC::P<sub>sigP-lacZ</sub> rsiP<sup>1-80</sup> / pAH9 P<sub>ter-gfp-rsiP</sub> | This study |

**E. coli**

F<sup>+</sup> [proAB<sup>+</sup> lac<sup>R</sup> lacZ<sub>M15</sub> Tn<sub>10(TeI<sup>R</sup>)</sub> Δ(ccdAB)] mcrA
<sup>Δ(mrr-hsdRMS-mcrBC)</sup> φ80(lacZ)ΔM15 Δ(lacZYA-argF) U169
endA1 recA1 supE44 thi-1 gyrA96 relA1 tonA panD

OmniMax 2-T1R

INV110
endA1 rpsL, thr, leu, thi, lacY, galK, galT, ara, tomA, tsx, dam, dcm, supE44, Δ(lac-proAB), [F' traD36, proAB, lacIqZΔM15]

### Table 4  Plasmids

| Plasmid  | Relevant features | Parent vector | Digest       | Insert primers | Reference       |
|----------|-------------------|---------------|--------------|----------------|-----------------|
| pMAD     | ori-pE194ts       |               |              |                |                 |
| pAH9     | ori-pE194 P_sarA-mcherry |               |              |                |                 |
| pAH13    | P_b1-gfp         |               |              |                |                 |
| pRAN332  | P_b1-gfp         |               |              |                |                 |
| pEBT4    | ori-pE194ts, ΔblaP | pMAD          | BglII, EcoRI | 3832-3833; 3834-3835 | This study      |
| pEBT5    | ori-pE194ts, ΔrasP | pMAD          | BglII, EcoRI | 3632-3633; 3634-3635 | This study      |
| pEBT6    | ori-pE194ts, ΔsigPrsiP | pMAD          | BglII, EcoRI | 3776-3777; 3778-3779 | This study      |
| pEBT13   | P_b1-gfp-rsiP    | pAH9          | HindIII, EcoRI | 3838-3839     | This study      |
| pCE630   | P_b1-gfp-rsiP1-72 | pAH9          | HindIII, EcoRI | 3838-4258     | This study      |
| pCE632   | P_b1-gfp-rsiP1-53 | pAH9          | HindIII, EcoRI | 3838-4259     | This study      |
| pTHE960  | P_sigP-sigP-rsiP+ | pAH9          | HindIII, EcoRI | 3774-3775     | This study      |
| pIA02    | P_sarA-rasP+     | pAH9          | EcoRI, KpnI  | 3744-3745     | This study      |
| pTHE946  | pE194ts          | pMAD          | BamHI, Stul  |                | This study      |
| pTHE948  | pE194ts, 'thrC thrB' | pTHE946    | Scal, Sall   | 2917-2918; 2919-2920 | This study      |
| pTHE950  | pE194ts, 'thrC lacZ thrB' | pTHE948    | XhoI, SbfI   | 2922-2923     | This study      |
| pTHE949  | pE194ts, 'thrC P_sarA-lacZ thrB' | pTHE950    | XhoI, Sall   | 2929-2930     | This study      |

This study
Figure Legends

Figure 1. Expression of sigP is specifically induced by β-lactams. All the strains contained P_{sigP}-lacZ in either wild type (THE2549), ΔsigPrsiP (EBT232) or ΔblaI (EBT215) background. Mid-log cells were washed and diluted 1:100 in molten LB agar containing X-gal (100 µg/ml) and poured into empty 100 mm Petri dishes. Filter disks containing Cef (1 µl of 5 mg/ml cefoxitin), Bac (1 µl of 50 mg/ml bacitracin), Nis (3 µl of 100 mg/ml nisin), Vanc (1 µl of 10 mg/ml vancomycin), Cmet (1 µl of 5 mg/ml cefmetazole), Poly (1 µl of 50 mg/ml polymyxin B), Kan (1 µl of 10 mg/ml kanamycin), Pip (1 µl of 5 mg/ml piperacillin), Cthi (1 µl of 50 mg/ml cephalothin), Ramo (1 µl of 25 mg/ml ramoplanin), Cper (1 µl of 25 mg/ml cefperazone), Phos (1 µl of 100 mg/ml phosphomycin), Amp (2 µl of 200 mg/ml ampicillin), Clex (1 µl of 50 mg/ml cefalexin), Erm (1 µl of 5 mg/ml erythromycin), and Meth (2 µl of 100 mg/ml methicillin) were then placed on the top agar and incubated for 16 hours at 30°C.

Figure 2. Expression of P_{sigP}-lacZ is dose-dependent and dependent upon σ^P and RasP.
A. B. thuringiensis with a transcriptional fusion P_{sigP}-lacZ (THE2549) was grown overnight at 30°C and subcultured in LB and grown to OD_{600} ~0.8 before being incubated with varying concentrations of β-lactams (0, 0.0625, 0.125, 0.25 0.5, 1, and 2 µg/ml) for 1 hour. Cells were collected and resuspended in Z-buffer. B. B. thuringiensis with a transcriptional fusion P_{sigP}-lacZ (THE2549) was grown overnight at 30°C and subcultured in LB and grown to OD_{600} ~0.8 before being incubated with varying concentrations of β-lactams (0, 0.0625, 0.125, 0.25 0.5, 1, and 2 µg/ml) for 1 hour. Cells were collected and resuspended in Z-buffer. C. All strains contain P_{sigP} lacZ and the genotype and plasmid noted: wild type/Vect (EBT169); sigP/Vect (EBT251); ΔsigPrsiP/pSigPRsiP (EBT238); ΔrasP/Vect (EBT175); rasP/pRasP (EBT176). Strains were grown to mid-log then treated with cefoxitin 5 µg/ml (5) or untreated (0) and incubated for 1
hour. β-Galactosidase activity was calculated as described in the material and methods. These experiments were done in triplicate and standard deviation is represented by error bars.

**Figure 3. RsiP levels decrease in the presence of cefoxitin.** *B. thuringiensis* expressing tetracycline-inducible *gfp-rsiP* (EBT360) or empty vector (EV; EBT169) was subcultured 1:50 into LB supplemented with ATc (50 ng/ml). At mid-log cells were incubated with A. 5 µg/ml of cefoxitin for various times (0, 15, 30, 60, 120, or 180 minutes) or B. increasing concentrations of cefoxitin (0, 0.05, 0.5, 5, 50, 500 µg/ml) for 1 hour. The immunoblot was probed with anti-RsiP (α-RsiP76-275). Streptavidin IR680LT was used to detect HD73_4231 (PycA homolog) which served as a loading control (69, 70). The color blot showing both anti-RsiP and Streptavidin on a single gel is Fig. S3.

**Figure 4. RsiP degradation is dependent upon the site-2 protease RasP.** *B. thuringiensis* wild type (EBT360) or ΔrasP (EBT366) containing a tetracycline-inducible copy of *gfp-rsiP* were subcultured 1:50 into LB supplemented with ATc (50 ng/mL). At mid-log cultures were incubated for 1 hour untreated (-) or treated (+) with cefoxitin (5 µg/ml) at 37°C. The immunoblot was probed with anti-GFP antisera. EV is wild type with pAH9 (EBT169) and GFP is wild type with pAH13 (UM20). Streptavidin IR680LT was used to detect HD73_4231 (PycA homolog) which served as a loading control (63, 64). The color blot showing both anti-GFP and Streptavidin on a single gel is Fig. S6.

**Figure 5. Truncations of RsiP lead to constitutive σ^p^ activation.** To determine if RasP was required for ampicillin-inducible P_{sigP-lacZ} expression we assayed β-galactosidase activity of *B. thuringiensis* with a transcriptional fusion P_{sigP-lacZ} and different *rsiP* truncation mutants (WT, THE2549; RsiP^{1-220}, THE2602; RsiP^{1-80}, THE2628; RsiP^{1-61}, THE2637; RsiP^{1-16}, THE2642) and
a ΔrasP deletion (WT, EBT140; RsiP\textsuperscript{1-220}, EBT116; RsiP\textsuperscript{1-80}, EBT148; RsiP\textsuperscript{1-61}, EBT133; RsiP\textsuperscript{1-508}, THE2605). Cells were grown overnight at 30°C and subcultured in LB and grown to OD\textsubscript{600} of ~0.8 before being incubated with cefoxitin (5 μg/ml) for 1 hour. The experiment was performed in triplicate and standard deviation is represented by error bars.

**Figure 6. Truncation of RsiP results in constitutive degradation in a RasP-dependent manner.** *B. thuringiensis* containing a tetracycline-inducible copy of *gfp-rsiP*, *gfp-rsiP\textsuperscript{1-72} (rsiP without the extracellular domain)*, or *gfp-rsiP\textsuperscript{1-53} (rsiP without the transmembrane and extracellular domains)* were constructed in either wild type (*rasP\textsuperscript{+}*) or a ΔrasP mutant strain [(GFP-RsiP· wild type (*rasP\textsuperscript{+}*) ; EBT360), (GFP-RsiP ΔrasP; EBT366), (GFP-RsiP\textsuperscript{1-53} wild type (*rasP\textsuperscript{+}*) ; EBT518), (GFP-RsiP\textsuperscript{1-53} ΔrasP; EBT510), (GFP-RsiP\textsuperscript{1-72} wild type (*rasP\textsuperscript{+}*) ; EBT516), (GFP-RsiP\textsuperscript{1-72} ΔrasP; EBT533)]. Strains were subcultured 1:50 into LB supplemented with ATc (100 ng/ml) and grown to mid-log then incubated for 2 hours untreated (-) or treated (+) with cefoxitin (5 μg/ml) at 37°C. The immunoblot was probed with anti-GFP antisera. Streptavidin IR680LT was used to detect HD73_4231 (PycA homolog) which served as a loading control (63, 64). The color blot showing both anti-GFP and Streptavidin on a single gel is Fig. S9.

**Supplemental Figure 1. GFP-RsiP is functional.** *B. thuringiensis rsiP\textsuperscript{1-80} (THE2628) containing tetracycline-inducible gfp-rsiP (EBT587) or empty vector (EBT561) were plated on LB Xgal without or with ATc (50 ng/ml).

**Supplemental Figure 2. GFP-RsiP localizes to the membrane.** *B. thuringiensis* expressing tetracycline-inducible gfp-rsiP (EBT360), tetracycline inducible gfp-rsiP\textsuperscript{1-72} (EBT533), or empty vector (EBT169) were subcultured 1:50 with ATc 100 ng/mL and grown at 30°C to late log phase. 2 μL were spotted on a 1% agarose pad for immobilization and imaged for GFP.
localization. Phase-contrast and fluorescence micrographs were recorded on an Olympus BX60 microscope with a 100 UPlanApo objective (numerical aperture, 1.35). For the GFP micrographs a filter set from Chroma Technology Corp (catalog no. 41017) was used. The GFP filter consists of a 450- to 490-nm excitation filter, a 495-nm dichroic mirror (long pass), and a 500- to 550-nm emission filter. Micrographs were captured with a Hamamatsu Orca Flash 4.0 V2 complementary metal oxide semiconductor (CMOS) camera.

**Supplemental Figure 3. RsiP levels decrease in the presence of cefoxitin.** *B. thuringiensis* expressing tetracycline-inducible *gfp-rsiP* (EBT360) or empty vector (EBT169) was subcultured 1:50 into LB supplemented with ATc (50 ng/ml). At mid-log cells were incubated with (A) 5 µg/ml of cefoxitin for various times (0, 15, 30, 60, 120, or 180 minutes) or (B) increasing concentrations of cefoxitin (0, 0.05, 0.5, 5, 50, 500 µg/ml) for 1 hour. The immunoblot was probed with antisera against RsiP (α-RsiP\textsuperscript{76-275}) followed by goat-anti-rabbit IgG IR800cw (Green). EV is wild type with pAH9 (EBT169) and GFP is wild type with pAH13 (UM20). Streptavidin IR680LT (Red) was used to detect HD73_4231 (PycA homolog) which served as a loading control (63, 64).

**Supplemental Figure 4. RsiP levels decrease in the presence of cefoxitin.** *B. thuringiensis* expressing tetracycline-inducible *gfp-rsiP* (EBT360), empty vector (EBT169) or GFP alone (UM20) were subcultured 1:50 into LB supplemented with ATc (50 ng/ml). At mid-log cells were incubated with increasing concentrations of cefoxitin (0, 0.05, 0.5, 5, 50, 500 µg/ml) for 1 hour. The immunoblot was probed with antisera against GFP (α-GFP) followed by goat-anti-rabbit IgG IR800cw (Green). Streptavidin IR680LT (Red) was used to detect HD73_4231 (PycA homolog) which served as a loading control (63, 64). The western is shown in (A) Black and white or (B) color.
Supplemental Figure 5. Amino acid alignment of RasP. An alignment of *B. subtilis* RasP and *B. thuringiensis* HD73_4301. The active site is marked by a red box.

Supplemental Figure 6. RsiP degradation is dependent upon the site-2 protease, RasP. *B. thuringiensis* containing a tetracycline-inducible copy of gfp-rsiP; wild type (EBT360) or ΔrasP (EBT366) were subcultured 1:50 into LB supplemented with ATc (50 ng/mL). At mid-log cultures were incubated for 1 hour untreated (-) or treated (+) with cefoxitin (5 μg/ml) at 37°C. The immunoblot was probed with antisera against GFP (anti-GFP) followed by goat-anti-rabbit IgG IR800cw (Green). EV is wild type with pAH9 (EBT169) and GFP is wild type with pAH13 (UM20). Streptavidin IR680LT (Red) was used to detect HD73_4231 (PycA homolog) which served as a loading control (63, 64).

Supplemental Figure 7. RsiP mutants result in constitutive σ^Pactivity. An alignment of RsiP mutants that result in nonsense, frameshift or point mutations. Amino acid residues in red are indicative of the change in amino acid sequence due to mutations. The mutation rsiP^{1-232} was isolated 3 independent times while rsiP^{1-61} and rsiP^{1-15} were isolated twice each. The red box indicates the predicted transmembrane domain.

Supplemental Figure 8. Complementation of RsiP mutants. *B. thuringiensis* containing either Empty Vector (wild type, EBT169; rsiP^{1-220}, EBT564; rsiP^{1-81}, EBT565; rsiP^{1-61}, EBT566; rsiP^{1-16}, EBT567) or Pₜₚ-rsiP (wild type, EBT168; rsiP^{1-220}, EBT563; rsiP^{1-81}, EBT562; rsiP^{1-61}, EBT561; rsiP^{1-16}, EBT560) were grown overnight and spotted onto LB X-gal (100 μg/ml) plates (A) lacking cefoxitin or (B) containing cefoxitin (5 μg/ml).
Supplemental Figure 9. Truncation of RsiP results in constitutive degradation in a RasP-dependent manner. B. thuringiensis containing a tetracycline-inducible copy of gfp-rsiP, gfp-rsiP\textsuperscript{1-72} (rsiP without the extracellular domain), or gfp-rsiP\textsuperscript{1-53} (rsiP without the transmembrane and extracellular domains) were constructed in either wild type of a ΔrasP mutant strain [(GFP-RsiP-wild type (ras\textsuperscript{P}); EBT360), (GFP-RsiP ΔrasP; EBT366), (GFP-RsiP\textsuperscript{1-53} wild type (ras\textsuperscript{P}); EBT518), (GFP-RsiP\textsuperscript{1-53} ΔrasP; EBT510), (GFP-RsiP\textsuperscript{1-72} wild type (ras\textsuperscript{P}); EBT516), (GFP-RsiP\textsuperscript{1-72} ΔrasP; EBT533)]. Strains were subcultured 1:50 into LB supplemented with ATc (100 ng/ml) and at mid-log were incubated for 2 hours untreated (-) or treated (+) with cefoxitin (5 μg/ml) at 37°C. The immunoblot was probed with antisera against GFP (anti-GFP) followed by goat-anti-rabbit IgG IR800cw (Green). Streptavidin IR680LT (Red) was used detect HD73_4231 (PycA homolog) which served as a loading control (63, 64).

Supplemental Table 1. Oligonucleotides.
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A

\[ \text{\( \beta \)-galactosidase Activity} \]

- Ampicillin
- Cefoxitin
- Cefmetazole
- Cephalothin

B

\[ \text{\( \beta \)-galactosidase Activity} \]

- Methicillin
- Cephalexin
- Cefoperazone
- Cefsulodin

C

\[ \text{\( \beta \)-galactosidase Activity} \]

| Cefoxitin µg/ml | Vect. | Vect. pSigPRsiP | Vect. pRasP |
|----------------|-------|----------------|------------|
| 0              |       |                |            |
| 5              |       |                |            |
| WT             |       |                |            |
| \( \Delta \text{sigPrsiP} \) |       |                |            |
| \( \Delta \text{rasP} \)      |       |                |            |
