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Sensing Extracellular Stress: Key Features of the Anti-Sigma Factor RsiV in Sensing Lysozyme

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SENSING EXTRACELLULAR STRESS: KEY FEATURES OF THE ANTI-SIGMA FACTOR RSIV IN SENSING LYSOZYME

by

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Abstract: *Bacillus subtilis* is a soil dwelling organism which uses alternative extracytoplasmic function (ECF) sigma factors that respond to environmental stressors encountered by a cell. Upon encountering a signal, ECF sigma factors are activated and bind RNAP to initiate transcription of genes that confer resistance to stressors that threaten cellular integrity [5]. One such factor, sigmaV (σ^V), is required by *B. subtilis* for resistance to lysozyme. In the absence of lysozyme, σ^V is inhibited by the anti-sigma factor, RsiV, which is a transmembrane protein that has an extracellular lysozyme sensing domain. RsiV binds lysozyme, allowing for regulated intramembrane proteolysis (RIP) of the anti-sigma factor. The rate limiting step in degradation of RsiV is site-1 cleavage, which is performed by signal peptidase. Once RsiV is degraded it releases σ^V, allowing it to bind RNAP and transcribe lysozyme resistance genes. We sought to understand how RsiV avoids signal peptidase cleavage in the absence of lysozyme. To investigate this, we fused various lengths of RsiV to GFP in order to identify portions of RsiV needed to shield it from signal peptidase cleavage in the absence of lysozyme. We also defined the transmembrane domain of RsiV using the substituted cysteine accessibility method (SCAM). These experiments contribute to our investigation of a putative amphipathic helix with protective properties from signal peptidase.

Introduction: Sigma factors (σ) are an essential component of RNA polymerase (RNAP). Some bacteria possess multiple σ factors that allow the cell to selectively transcribe genes whose products contribute to the cell's survival. The RNAP core itself is unable to bind DNA and needs a σ factor to make a holoenzyme. This holoenzyme is then able to locate the promoter region of a gene and begin synthesis of a few nucleotides before the σ dissociates [3, 8, 13, 14]. In order to respond rapidly to an environment in constant flux bacteria employ multiple mechanisms, of which extracytoplasmic function sigma factors (σ^{ECF}) are prominently used. Generally speaking, σ^{ECF} activate the transcription of genes used to protect cellular envelope integrity [8, 14, 15]. The σ^{ECF} factors are related to σ^{70}, which is the σ factor required for expression of housekeeping genes in bacteria [15].

In the absence of extracellular signal, the activity of some σ^{ECF} are inhibited by an anti-sigma factor. In order to function, the σ^{ECF} must be released from the anti-sigma factor. One mechanism for release is the regulated intramembrane proteolysis (RIP) of the anti-sigma factor [5, 10, 12]. This is a multi-step process that is initiated by a site-1 protease cleaving the anti-σ factor in the extracellular domain. This creates a substrate for the site-2 protease to cleave within the transmembrane domain of the anti-σ factor [10]. The remaining anti-sigma factor is then degraded by intracellular protease, thus freeing the σ^{ECF} to bind RNAP [5, 10].

One common cell envelope stressor is the hydrolytic enzyme, lysozyme, which is part of the innate immune system. In susceptible bacteria, lysozyme is capable of cleaving the β-(1,4)-glycosidic bond between *N*-acetylmuramic acid (MurNAc) and *N*-acetyl-glucosamine (GlcNAc) found in the peptidoglycan (PG) backbone [2, 9]. In several pathogenic organisms, lysozyme induces an ECF sigma factor, σ^V [1, 2, 5, 11]. The model organism, *B. subtilis*, also uses σ^V to

transcribe genes conferring resistance to lysozyme [4, 5, 8-10, 16]. Here, σ^V is encoded in an operon with three other genes: *rsiV* (the σ^V anti-sigma factor), *oatA* (*O*-acetyltransferase) and *yrhK* (unknown function) [5, 9, 16]. In the absence of lysozyme, the activity of σ^V is inhibited by the anti-sigma factor, RsiV.

We previously demonstrated that RsiV acts as a receptor for lysozyme [6, 7]. RsiV is a single-pass transmembrane protein that, upon lysozyme binding, becomes susceptible to RIP cleavage by site-1 and site-2 proteases (SipS or SipT and RasP, respectively). These events release σ^V into the cytoplasm where it will bind RNAP and begin transcription of lysozyme resistance genes [5-7]. One such gene induced in *B. subtilis* is *oatA*, whose protein product causes *O*-acetylation of MurNAc on the bacteria's peptidoglycan [1, 4, 5, 9, 11]. In several organisms σ^V also induces the *dlt* operon, causing D-alanylation of cell wall teichoic acids [1, 4, 11]. These pathways are thought to increase lysozyme resistance by blocking lysozyme access to the β-(1,4)-glycosidic bonds or repelling it away due to a likeness in charge of the PG, respectively.

It is generally thought that signal peptidases constitutively cleave their substrates. Yet RsiV is not immediately cleaved by signal peptidase in the absence of lysozyme. To better understand how RsiV protects itself from signal peptidase cleavage in the absence of lysozyme, we sought to define the extracellular region surrounding the RsiV signal peptidase cleavage sequence. By fusing various lengths of RsiV to GFP, we have identified a region sufficient for RsiV protection against signal peptidase. Since the signal peptidase motif is near the RsiV transmembrane (TM) domain, we also determined where the extracellular domain begins using the substituted cysteine accessibility method (SCAM). These experiments have contributed to our hypothesis that the extracellular region surrounding the signal peptidase cleavage site contains an amphipathic helix which conceals that sequence until RsiV binds lysozyme.

Results

RsiV amino acids 1-86 sufficiently protect against signal peptidase cleavage in the absence of lysozyme. As previously mentioned, RsiV is not immediately cleaved by signal peptidase in the absence of lysozyme. This suggests the presence of a protective region within the anti-σ factor. To help define these regions, we fused various lengths of RsiV to GFP. Constructs were made by PCR amplification using isolated *B. subtilis* PY79 DNA and a plasmid containing *gfp* as templates (see Materials and Methods). The resulting fragments were then assembled by Gibson assembly into pDR111, placing the *rsiV-gfp* fusion under the control of an IPTG inducible promoter and can integrate at the *amyE* loci of *B. subtilis*. Once confirmed by sequencing, the plasmids were introduced into *B. subtilis* Δ*sigVrsiV::kan*. The resulting strains were grown to mid-log phase in the presence of 1mM IPTG. The culture supernatant and cell pellets were collected for western blot analysis using anti-GFP antibody (1:10,000; anti-rabbit secondary antibody). We predicted GFP to be cleaved by signal peptidase and secreted into culture media if the RsiV-GFP fusion lacked sufficient protective features to avoid signal peptidase cleavage. Conversely, if the RsiV-GFP fusion has these protective qualities then we expect the protein to remain intact and associated with the cell pellet.

We detected GFP in the culture supernatant and lysed cells when it was fused right after the RsiV signal peptidase motif (i.e. $RsiV^{1-66}$; Figure 1). Similar results were seen when we fused GFP 10 amino acids downstream of said motif (i.e. $RsiV^{1-76}$). However, when GFP was fused 20

and 30 amino acids downstream of RsiV signal peptidase cleavage site, no cleavage products were found ($RsiV^{1-86}$ and $RsiV^{1-96}$, respectively). These data suggest that amino acids 1-86 are sufficient for protecting RsiV from signal peptidase in the absence of lysozyme.

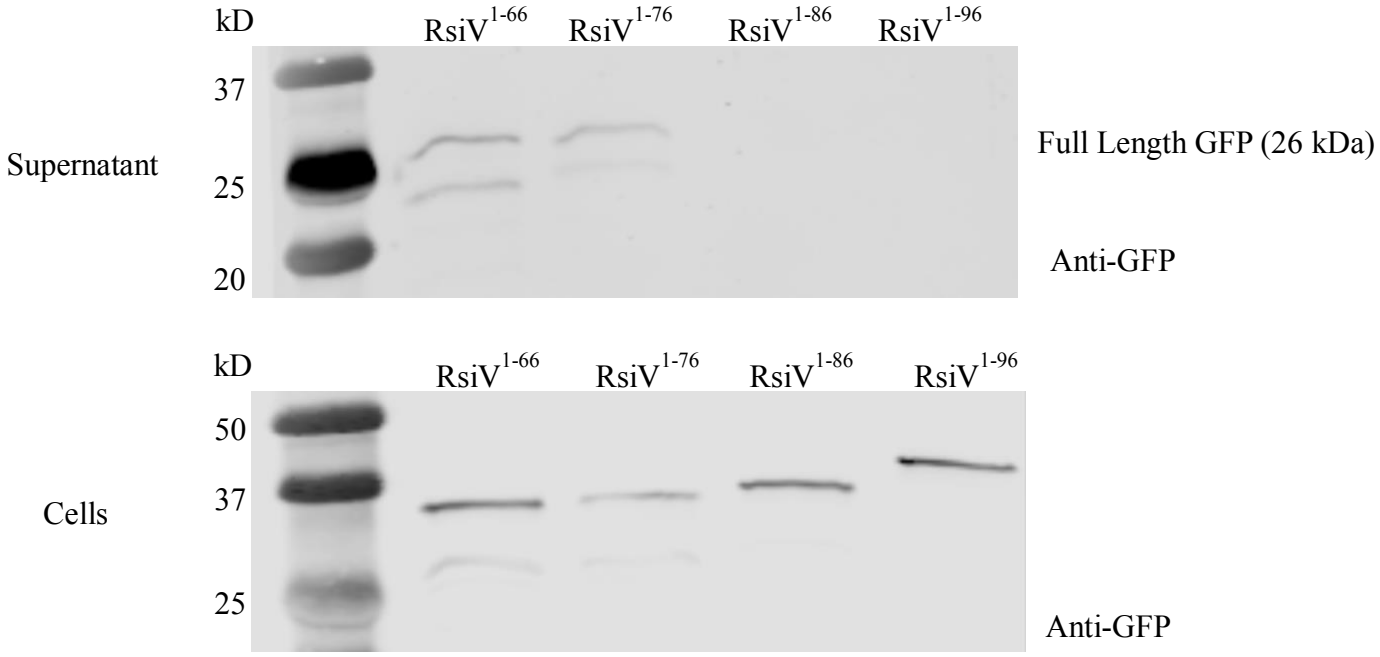

Figure 1: RsiV amino acids 1-86 are sufficient for protection from signal peptidase. RsiV-GFP fusion proteins from strains LTL423-LTL426. Unwashed, mid-log cell pellets and concentrated supernatants were used for western analysis. Expected bands: 1-66 RsiV-GFP ~36 kDa; 1-76 RsiV-GFP ~37 kDa; 1-86 RsiV-GFP ~39 kDa; 1-96 RsiV-GFP ~40 kDa.

The RsiV extracellular domain begins around amino acid 60.

We hypothesized that RsiV undergoes a conformational change upon binding to lysozyme exposes the signal peptidase cleavage motif, which is in close proximity to the TM domain. Defining the start of the RsiV extracellular domain would help aid in experimental planning and data interpretation. We used a substituted cysteine accessibility method (SCAM) to define the start of the extracellular domain of RsiV (wild-type RsiV lacks cysteine residues). Cysteine point mutations were introduced into the predicted transition region using site-directed PCR mutagenesis of *B. subtilis* PY79 with 6x histidine tag and an A66W point mutation for template (see Materials & Methods; Figure 2). For the reasons described above, Gibson assembly of

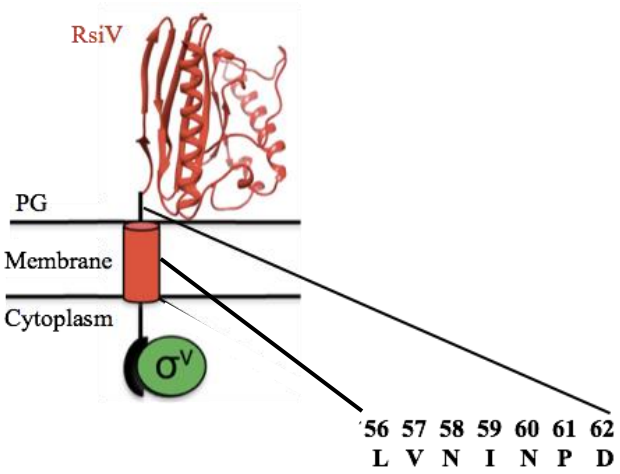

Figure 2: RsiV amino residues point mutated to cysteine. Shown in green is σ^V and RsiV is red. Our SCAM experiment incorporated 7 amino acids in which we thought the transition from TM to extracellular domains would occur.

each PCR mutagenesis product onto pDR111 was conducted, followed by vector transformation into *B. subtilis* PY79 Δ*sigVrsiV::kan*. These strains were then grown to mid-log phase before being labeled with *N*α-(3-Maleimidylpropionyl)Biocytin (MPB), after which cells were lysed and RsiV purified using a nickel resin that bound to the 6x-histidine tag on RsiV.

To ensure (1) each strain was able to express RsiV upon IPTG induction and (2) purified RsiV was isolated in similar amounts, we ran a western blot using an anti-RsiV antibody (1:10000 dilution; anti-rabbit secondary antibody). This antibody was raised against amino acids 59-285 and thus bound to each mutant RsiV regardless of the introduced cysteine or bound MPB (Figure 3, top). We expected to see similar band intensities if strains expressed RsiV at similar levels, as seen in Figure 3. Therefore, the absence of fluorescence for some strains in the Streptavidin western blot cannot be explained by a decreased or inability to express the point-mutated RsiV (Figure 3, bottom).

The MPB label does not cross membranes and, therefore, was expected to label only cysteines residing on the extracellular side of RsiV. To identify which cysteines were labeled we used to streptavidin-IRDYExxx which will bind to the biotinylated cysteine residues of RsiV with high specificity (IRDye 800CW; 1:10000 dilution). To make certain MPB labeled cysteines properly we used the following positive and negative controls, respectively: RsiV with a cysteine located far out in the extracellular domain and an RsiV containing no cysteine residues. We found that the no cysteine RsiV was not labeled while the positive control A167C did label with MPB (Figure 3, "A167C" and "No Cys").

We then compared the ability of residues 59-62 to label with MPB when they were mutated to cysteine. We found that residues 56-59 (i.e. L56C, V57C, N58C, and I59C) did not label, suggesting that these residues resided in within the transmembrane domain of RsiV. On the other hand, we did detect labeling with the N60C, P61C, and D62C mutant proteins which suggests that they were likely on the extracellular side of RsiV. Taken together these data indicate that the RsiV extracellular domain begins around amino acid N60.

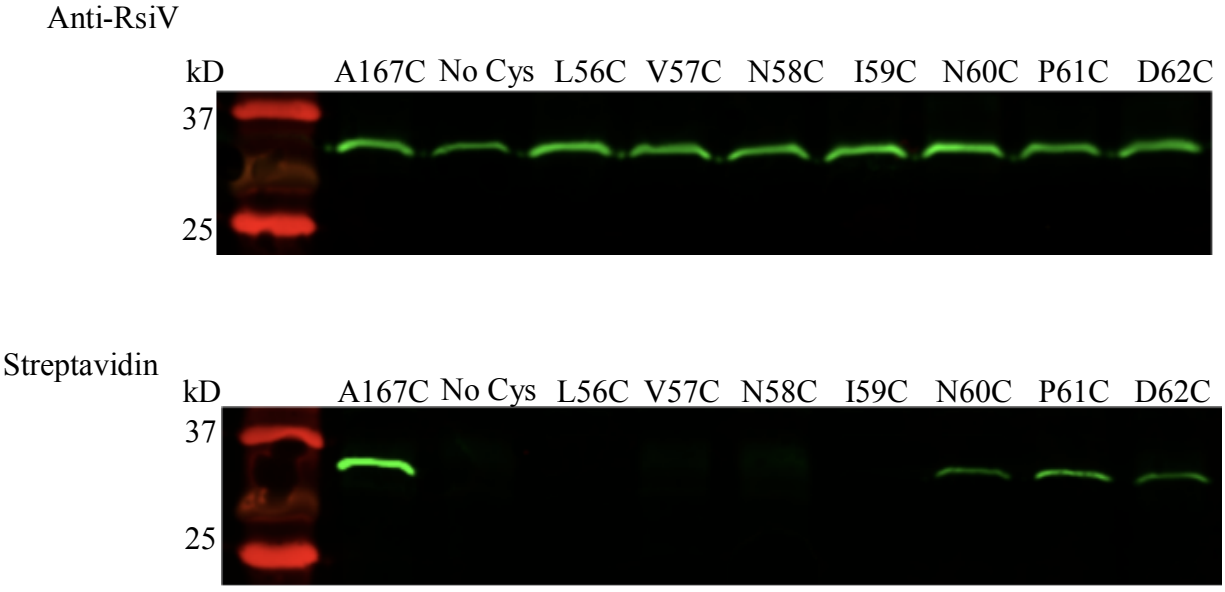

Figure 3: RsiV transition from TM to extracellular domains. Westerns of a SCAM protocol using *B. subtilis* strains LTL279, LTL281, LTL301, LTL315, LTL325, LTL354, LTL369, LTL370, and LTL371 (see Materials and Methods). MPB labeled RsiV was resuspended in 2x sample buffer + BME. Westerns were developed using LI-COR using IR channels 700 and 800. The RsiV protein is approximately 33 kD.

Discussion

Does RsiV have an amphipathic helix with protective properties?

Previously, we aligned ~200 homologs of RsiV to identify highly conserved residues in the extracellular region surrounding the signal peptidase cleavage site (Figure 4). The alignment shows conserved hydrophobic and hydrophilic residues occurring at regular intervals. This suggests the presence of a putative amphipathic helix. Using a helical wheel projector, we observed the presence of a putative amphipathic helix (Figure 4, upper left). One experiment done to investigate this helix was a modified SCAM protocol. We have observed that a $RsiV^{A66W}$ protein is not cleaved by signal peptidase in the presence of lysozyme. Thus, we made cysteine point mutations into a $RsiV^{A66W}$ background in the proposed amphipathic helix region. The SCAM protocol was then used with RsiV in the presence and absence of lysozyme. This has shown us which residues are likely to reside in the cell's membrane before and after RsiV binds to lysozyme. The data from this experiment is consistent with how we expect an amphipathic helix to be labeled by MPB (data not shown).

We also think Figure 1 suggests that the putative amphipathic helix blocks signal peptidase cleavage in the absence of lysozyme. Based on this and our predicted location of the helix, we are making in-frame deletions of 67-76, 67-86, and 67-96 and 86-96. We plan to test these RsiV deletion mutants in a ß-galactosidase assay where σ^V activity is measured in the absence of lysozyme. This assay will indirectly tell us if RsiV mutant is undergoing RIP (i.e. higher σ^V activity correlates to RsiV being cleaved). We predict this experiment to further narrow down which portion of RsiV is necessary for protection from signal peptidase in the absence of lysozyme.

In all, we propose a more refined model for how signal peptidase cleavage of RsiV controls $-\sigma^V$ activation (Figure 5). We hypothesize that in the absence of lysozyme an amphipathic helix sits in the membrane, effectively masking the signal peptidase cleavage site. Once lysozyme binds to RsiV we predict that the helix moves out of the membrane allowing signal peptidase to cleave its substrate and initiate the RIP process and thus σ^V activation.

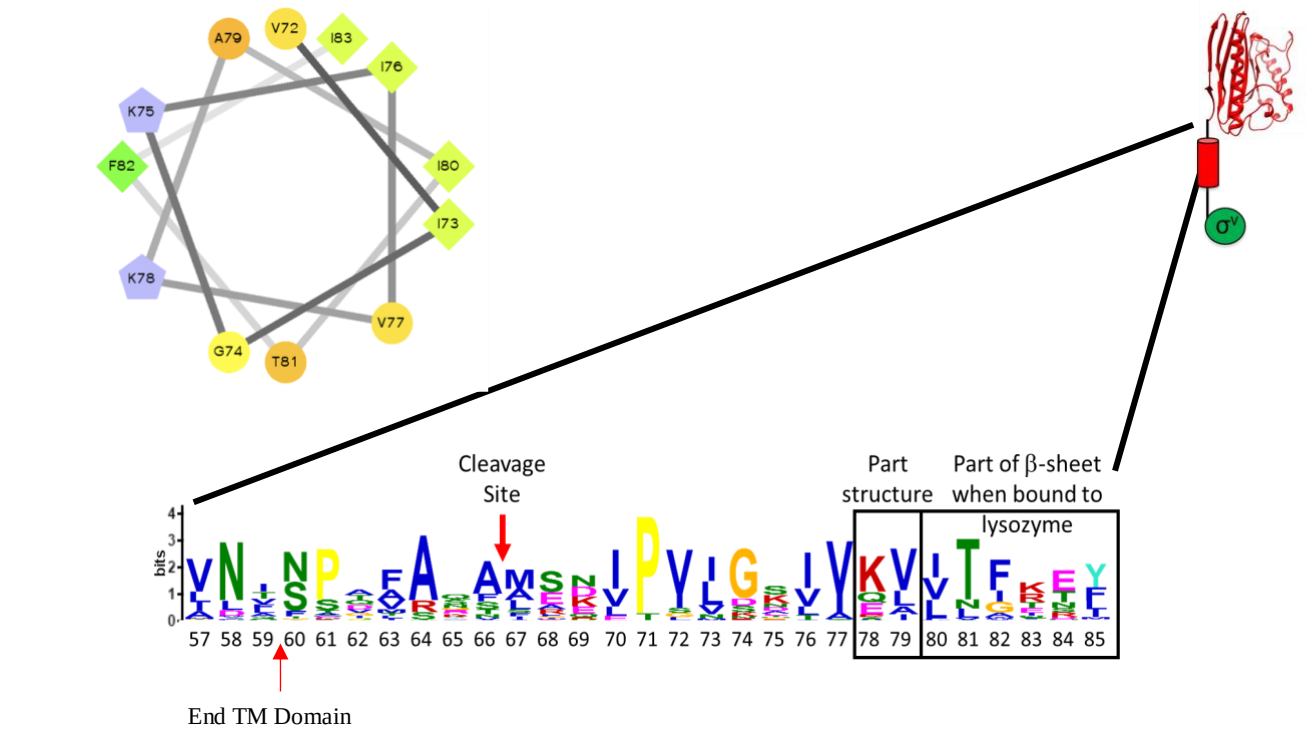

Figure 4: Sequence alignment indicates a putative amphipathic helix. Sequencing of all gram positive organisms known to contain RsiV homologues in their genomes using Clustal Omega database. Highly conserved amino residues have a large font than those with less conservation. A wheel projection indicates a possible amphipathic helix in the extracellular region adjacent to the TM domain.

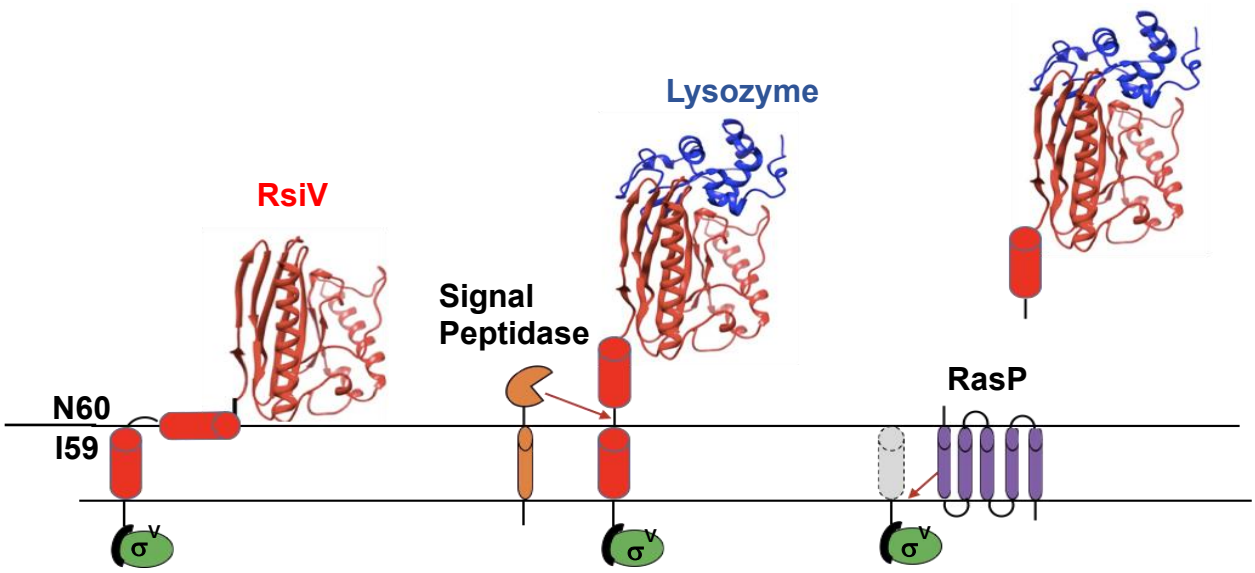

Figure 5: Revised model for regulated intramembrane proteolysis of RsiV. σ^V is shown in green and is bound to RsiV in the absence of lysozyme (blue). Lysozyme is bound by the RsiV (red) extracellular sensing domain, shifting the amphipathic helix as a result. Signal peptidase (orange) now has access to its cleavage site on RsiV. Removal of the RsiV extracellular domain creates a proper substrate for site-2 protease, RasP (purple), to cleave in an intramembrane fashion.

Acknowledgments
The author would like to thank Craig D Ellermeier*, Lincoln Lewerke, (University of Iowa) and the rest of the Ellermeier lab for mentorship, guidance, and helpful suggestions.

*Primary Investigator

Materials & Methods

Strains:

Strains	Genotype	Reference
B. subtilis		
CDE1563	PY79 *ΔsigVrsiV::kan*	[5]
LTL315	PY79 *amyE::* P_{hs}*-6xhis -rsiV*$^{A167C, A66W}$*(spec) ΔsigVrsiV::kan*	
LTL279	PY79 *amyE::* P_{hs}*-6xhis -rsiV*^{A66W}*(spec) ΔsigVrsiV::kan*	
LTL325	PY79 *amyE::* P_{hs}*-6xhis -rsiV*$^{L56C, A66W}$*(spec) ΔsigVrsiV::kan*	
LTL369	PY79 *amyE::* P_{hs}*-6xhis -rsiV*$^{V57C, A66W}$*(spec) ΔsigVrsiV::kan*	
LTL370	PY79 *amyE::* P_{hs}*-6xhis -rsiV*$^{N58C, A66W}$*(spec) ΔsigVrsiV::kan*	
LTL354	PY79 *amyE::* P_{hs}*-6xhis -rsiV*$^{I59C, A66W}$*(spec) ΔsigVrsiV::kan*	
LTL301	PY79 *amyE::* P_{hs}*-6xhis -rsiV*$^{N60C, A66W}$*(spec) ΔsigVrsiV::kan*	
LTL371	PY79 *amyE::* P_{hs}*-6xhis -rsiV*$^{P61C, A66W}$*(spec) ΔsigVrsiV::kan*	
LTL281	PY79 *amyE::* P_{hs}*-6xhis -rsiV*$^{D62C, A66W}$*(spec) ΔsigVrsiV::kan*	
LTL423	PY79 *amyE::* P_{hs}*-6xhis -rsiV*$^{1-66}$*-GFP (spec) ΔsigVrsiV::kan*	
LTL424	PY79 *amyE::* P_{hs}*-6xhis-rsiV*$^{1-76}$*-GFP (spec) ΔsigVrsiV::kan*	
LTL425	PY79 *amyE::* P_{hs}*-6xhis -rsiV*$^{1-86}$*-GFP (spec) ΔsigVrsiV::kan*	
LTL426	PY79 *amyE::* P_{hs}*-6xhis-rsiV*$^{1-96}$*-GFP (spec) ΔsigVrsiV::kan*	
E. coli **Omnimax**		
CDEE2	F= *proAB lacI*^{q} *lacZ* M15 Tn*10*(Tet^{r}) *ccdAB mcrA* (*mrr-hsdRMS-mcrBC*) 80*lacZ* M15 (*lacZYA-argF*) U169 *endA1 recA1 supE44 thi-1 gyrA96 relA1 tonA panD*	Invitrogen
Plasmids		
pDR111	amyE P_{hs} specR ampR P_{hs}	[7]
pLL169	pDR111 *amyE* P_{hs} *specRampR* P_{hs}*-6xhis -rsiV*$^{A167C, A66W}$	
pLL158	pDR111 *amyE* P_{hs} *specRampR* P_{hs}*-6xhis -rsiV*^{A66W}	
LTL323	pDR111 *amyE* P_{hs} *specRampR* P_{hs}*-6xhis -rsiV*$^{L56C, A66W}$	
pLL187	pDR111 *amyE* P_{hs} *specRampR* P_{hs}*-6xhis -rsiV*$^{V57C, A66W}$	
pLL194	pDR111 *amyE* P_{hs} *specRampR* P_{hs}*-6xhis -rsiV*$^{N58C, A66W}$	
pLL180	pDR111 *amyE* P_{hs} *specRampR* P_{hs}*-6xhis -rsiV*$^{I59C, A66W}$	
pLL161	pDR111 *amyE* P_{hs} *specRampR* P_{hs}*-6xhis -rsiV*$^{N60C, A66W}$	
pLL188	pDR111 *amyE* P_{hs} *specRampR* P_{hs}*-6xhis -rsiV*$^{P61C, A66W}$	
pLL160	pDR111 *amyE* P_{hs} *specRampR* P_{hs}*-6xhis -rsiV*$^{D62C, A66W}$	
pLL197	pDR111 *amyE* P_{hs} *specRampR* P_{hs}*-6xhis -rsiV*$^{1-66}$*-GFP*	
pLL194	pDR111 *amyE* P_{hs} *specRampR* P_{hs}*-6xhis -rsiV*$^{1-76}$*-GFP*	
pLL195	pDR111 *amyE* P_{hs} *specRampR* P_{hs}*-6xhis -rsiV*$^{1-86}$*-GFP*	
pLL196	pDR111 *amyE* P_{hs} *specRampR* P_{hs}*-6xhis -rsiV*$^{1-96}$*-GFP*	

CDE Oligo Number	Primer Sequence	Primer Description
Cysteine Mutations		
CDEP3195	ccgaattagcttgcatgctattttatcgaatataacgttctcc	VP2 RsiV pDR111; end primer
CDEP3629	gtgagcggataacaattaagct taaggaggctttcttaatg caccatcaccatcaccat	Clone 6xhis RsiV into pDR111; start primer
CDEP1561	atcaacccggacgcc gct cag tgg atgtcaaagatccct	Fwd RsiV site drected AQA/ AQW A66W
CDEP1562	agggatctttgacatccactgagcggcgtccgggttgat	Rev RsiV site drected AQA/ AQW A66W
CDEP3609	gagcggcgtccgggcagatattaacaagcgcagtg	Rev RsiV N60C
CDEP3610	cactgcgcttgttaatatctgcccggacgccgctc	Fwd RsiV N60C
CDEP3611	gcggcgtccgggttgatacaaacaagcgcagtgaataa	Rev RsiV N58C
CDEP3612	ttattcactgcgcttgtttgtatcaacccggacgccgc	Fwd RsiV N58C
CDEP3613	gcagctattttattcactgcgtgtgttaatatcaacccggacgc	Rev RsiV L56C
CDEP3614	gcgtccgggttgatattaacacacgcagtgaataaaatagctgc	Fwd RsiV L56C
CDEP3623	gacatccactgagcggcgcacgggttgatattaacaag	Rev RsiV D62C A66W
CDEP3624	cttgttaatatcaacccgtgcgccgctcag tgg atgtc	Fwd RsiV D62C A66W
CDEP3764	attttattcactgcgctttgtaatatcaacccggacgcc	Rev RsiV V57C
CDEP3765	ggcgtccgggttgatattacaaagcgcagtgaataaaat	Fwd RsiV V57C
CDEP3766	ttcactgcgcttgttaattgcaacccggacgccgctcag	Fwd RsiV I59C
CDEP3767	ctgagcggcgtccgggttgcaattaacaagcgcagtgaa	Rev RsiV I59C
CDEP3897	gcgcttgttaatatcaactgcgacgccgctcagtggatg	Fwd RsiV P61C A66W
CDEP3898	catccactgagcggcgtcgcagttgatattaacaagcgc	Rev RsiV P61C A66W
RsiV-GFP Fusion		
CDEP3865	aacaattaagcttaaggaggctttcttaatggataagagattacagc	PY79 RsiV 5' Clone RsiV-GFP
CDEP3866	agttcttctcctttgctcattcgaatataacgttctccca	PY79 RsiV 3' Clone RsiV-GFP
CDEP3867	gagaacgttatattcgaatgagcaaaggagaagaact	GFP 5' template pCM11
CDEP3868	ccaccgaattagcttgcatgggatcctttgtagagctcat	GFP 3' template pCM11
CDEP3925	agttcttctcctttgctcatagcctgagcggcgtccgg	PY79 RsiV 3' for RsiV(1-66)-GFP
CDEP3926	ccggacgccgctcaggctatgagcaaaggagaagaact	GFP 5' template pCM11 for RsiV(1-66)-GFP
CDEP3927	agttcttctcctttgctcatgattttgccgatgacagg	PY79 RsiV 3' for RsiV(1-76)-GFP
CDEP3928	cctgtcatcggcaaaatcatgagcaaaggagaagaact	GFP 5' template pCM11 for RsiV(1-76)-GFP
CDEP3929	agttcttctcctttgctcattttgatttcaataaaggt	PY79 RsiV 3' for RsiV(1-86)-GFP
CDEP3930	acctttattgaaatcaaaatgagcaaaggagaagaact	GFP 5' template pCM11 for RsiV(1-86)-GFP
CDEP3931	agttcttctcctttgctcatgacatcaatgcttgattg	PY79 RsiV 3' for RsiV(1-96)-GFP
CDEP3932	caatcaagcattgatgtcatgagcaaaggagaagaact	GFP 5' template pCM11 for RsiV(1-96)-GFP

Primers: Table 2 (above). Note: all PCR amplification products (either cysteine mutated or RsiV-GFP PCR products) were cloned onto a pDR111 vector digested with HindIII-HF and SpHI. For fusion proteins – all PCR templates containing (1) pCM111 used CDEP3868 as the 3' end primer or (2) *B. subtilis* PY79, a prototrophic derivative of B. *subtilis* strain 168, used CDEP3865 as the 5' end primer [7].

Q5TD PCR: Each reaction template was denatured at 94ºC for 30, then for 10 seconds. Annealing was at 60ºC for 20 seconds with -1ºC per cycle. Extension occurred at 68ºC for 1 min. This was then repeated for 10 cycles, starting from 10 seconds of 94ºC denaturing. Next, reactions were denatured at 94ºC for 10 seconds, then annealed at 50ºC for 20 seconds. Extension occurred at 68ºC for 30 seconds. These three steps were repeated for 25 cycles. Reactions were held at 69ºC for 5 minutes, followed by PCR termination.

Cysteine Point Mutations: Into 22 µl of dH_2O, we put 1 µl of *B. subtilis* PY79 with 6x histidine tag and an A66W point mutation for template. We combined Q5 Master Mix, dH_2O, *B. subtilis* PY79 with 6x histidine tag and an A66W point mutation template, with forward primer CDEP3195 or CDEP3629 in separate PCR tubes in a 1:0.9:0.04:0.04:0.04 fashion, respectively. All reactions underwent Q5TD PCR amplification (see above). Using purified DNA bands, we conducted Gibson assembly using a pDR111 vector, as previously described [17]. Gibson assembly reactions were then transformed into *E. coli* omnimax and placed into recovery media for 1 hour. LB transformation media was then plated onto LB-Amp100 and grown overnight at 37ºC. Plasmid preps followed by restriction digest with HindIII-HF & SphI checked for presence of plasmid insert. After getting desired plasmid construct, we transform it into *B. subtilis* CDE1563 by putting an isolated colony of CDE1563 into 1x MC and was incubated until cells were in log phase. We then placed a 1/100 volume of eluted plasmid prep into competent CDE1563 and let the mixture incubated for ~1-2 hours before plating onto LB-Spectinomycin100. Plates were then grown overnight at 37ºC.

Substituted Cysteine Accessibility Mutagenesis (SCAM): We started 5 mL LB liquid media ON cultures of strains that expressed a RsiV containing a cysteine residue. Overnights were then subculture 1:100 into 50 mL LB liquid media with 1 mM IPTG. Flasks were then grown shaking at 37ºC until they reached an OD 600 of ~0.8 (approx. 3 hours). We then pelleted 15 mL of culture at 5000 g for 10 min at room temperature. was removed and the cell pellet was resuspended in 500 µl Protoplast buffer (0.4M Sucrose, 10mM Potassium Phosphate, 15mM MgCl2) + MPB (100 µg/mL) and left to be biotinylated for 30 minutes. Labeling was quenched by adding 5 µl β-Mercaptoethanol. Cells were pelleted (13,000 xg for 1 minute) and supernatant removed before resuspension with 500 µl of wash Protoplast + BME (20 mM). Cell were then spun down (13,000 x g for 1 minute). Small scale protein purification was performed by lysing the cell with 500 µl of 3 mM TritonX-100 (TX100) and sonicating. Cell lysates were then spun at 15,000 x g for 10 minutes and the clarified lysate was then added to 50 µl of HisPur Ni-NTA Resin that was washed twice with 500 µl 3 mM TX100 lysis buffer (50 mM NaH_2PO_4, 250 mM NaCl, 10 mM imidazole, pH 8.0; spun for 30 seconds at 2,000 x g). The lysate was then rocked at 4ºC for 30 minutes before being spun down for 30 seconds at 2,000 x g. The supernatant was removed and the resin washed 3 times with 500 µl of 0.3 mM TX100 wash buffer (50 mM NaH_2PO_4, 250 mM NaCl, 20 mM imidazole, pH 8.0; spun for 30 seconds at 2,000 x g). Resin-bound protein was then eluted with 50 µl of 0.03 mM TX100 elution buffer (50 mM NaH_2PO_4, 250 mM NaCl, 250 mM imidazole, pH 8.0) before being spun at 2,000 x *g* for 30 seconds. Elution buffer was removed and added to 50 µl of 2x sample buffer + BME (40 mM). Our western blot analysis used 1) 1:10,000 Anti-RsiV primary antibody + 1:10,000 Tween 20 [Polyoxyethylenesorbitan Monolaurate] (blocked using 5% nonfat dried milk in 1x PBS) with anti-rabbit secondary antibody (1:10,000) or 2) Streptavidin IR dye 800 (1:10,000, blocked in 5% Albumin, Bovine Fraction V (BSA) in 1x PBS, covered) + 1:10,000 Tween 20 [Polyoxyethylenesorbitan Monolaurate]. The blots were imaged using a LI-COR Odyssey CLx.

RsiV-GFP Fusion Constructs: We amplified the desired portion of RsiV from *B. subtilis* PY79 template and GFP from pCM111 template. Gibson assembly and transformation into *E. coli* omnimax or *B. subtilis* strain PY79 Δ*sigVrsiV::kan* were done as described above.

RsiV-GFP Construct Western Blot Cell Preparation: Cells were grown overnight in 5 mL LB at 37ºC. The overnight cultures were diluted 1/100 into 5 mL fresh liquid LB + 1mM ITPG and grown shaking to mid log (~3 hours) at 37ºC. 1.5 mL cells were pelleted (13,000g for 2 min) and 500 µl of supernatant was saved from each strain before discarding the rest. Pellets were resuspended in 150 µl liquid LB. To lyse cells 150 µl of 2x sample buffer + BME was added to the pellets and samples were sonicated, as described above. The proteins in the supernatant were concentrated using a Methanol Chloroform Extraction (see below). Western blot analysis was done using Anti-GFP primary antibody (1:2,000; blocked in 5% nonfat dried milk in 1x PBS) and Anti-rabbit secondary antibody (1:10,000). The blots were imaged using a LI-COR Odyssey CLx.

Methanol-Chloroform Extraction: Proteins in the supernatant were concentrated by mixing equal volumes of culture supernatant and MeOH along with ¼ volume of $CHCl_3$ and vortexing vigorously. Mixtures were then centrifuged at 15,000g for 5 minutes. The top phase was removed without disrupting the interface and discard. 500 µl MeOH was added and the samples vortexed. Samples were then centrifuged at 15,000g for 2 minutes. Supernatant was removed, discarded, and the pellet left to dry before resuspension in 75 µl 2x Sample buffer + BME and heating at 90°C for 8 minutes to resuspend the pellet.

References

1. Bernard, E., Rolain, T., Courtin, P., Guillot, A., Langella, P., Hols, P., & Chapot-Chartier, M.-P. (2011). Characterization of *O*-Acetylation of *N*-Acetylglucosamine: A Novel Structural Variation of Bacterial Peptidoglycan. *The Journal of Biological Chemistry*, *286*(27), 23950–23958
2. Callewaert, L., & Michiels, C. W. (2010). Lysozymes in the animal kingdom. *Journal of Biosciences*, *35*(1), 127-160.
3. Feklistov A, Sharon BD, Darst SA, Gross CA. (2014). Bacterial σ factors: a historical, structural, and genomic perspective. Annu Rev Microbiol, 68, 357–376.
4. Guariglia-Oropeza, V., & Helmann, J. D. (2011). Bacillus subtilis σV Confers Lysozyme Resistance by Activation of Two Cell Wall Modification Pathways, Peptidoglycan O-Acetylation and d-Alanylation of Teichoic Acids. *Journal of Bacteriology*, *193*(22), 6223–6232
5. Hastie, J. L., Williams, K. B., & Ellermeier, C. D. (2013). The Activity of σV, an Extracytoplasmic Function σ Factor of Bacillus subtilis Is Controlled by Regulated Proteolysis of the Anti-σ Factor RsiV. *Journal of Bacteriology*, *195*(14), 3135–3144.
6. Hastie, J. L., Williams, K. B., Sepúlveda, C., Houtman, J. C., Forest, K. T., & Ellermeier, C. D. (2014). Evidence of a Bacterial Receptor for Lysozyme: Binding of Lysozyme to the Anti-σ Factor RsiV Controls Activation of the ECF σ Factor σV . *PLoS Genetics*, *10*(10), e1004643.
7. Hastie, J. L., Williams, K. B., Bohr, L. L., Houtman, J. C., Gakhar, L., & Ellermeier, C. D. (2016). The Anti-sigma Factor RsiV Is a Bacterial Receptor for Lysozyme: Co-crystal Structure Determination and Demonstration That Binding of Lysozyme to RsiV Is Required for σV Activation. *PLoS Genetics*, *12*(9), e1006287.
8. Helmann, J. D. (2016). *Bacillus subtilis* extracytoplasmic function (ECF) sigma factors

and defense of the cell envelope. *Current Opinion in Microbiology*, *30*, 122–132.
9. Ho, T. D., Hastie, J. L., Intile, P. J., & Ellermeier, C. D. (2011). The Bacillus subtilis Extracytoplasmic Function σ Factor σV Is Induced by Lysozyme and Provides Resistance to Lysozyme. *Journal of Bacteriology*, *193*(22), 6215–6222.
10. Ho, T. D., & Ellermeier, C. D. (2012). Extra Cytoplasmic Function σ Factor Activation. *Current Opinion in Microbiology*, *15*(2), 182–188.
11. Laaberki, M.-H., Pfeffer, J., Clarke, A. J., & Dworkin, J. (2011). *O*-Acetylation of Peptidoglycan Is Required for Proper Cell Separation and S-layer Anchoring in *Bacillus anthracis*. *The Journal of Biological Chemistry*, *286*(7), 5278–5288.
12. Lal, M., & Caplan, M. (2011). Regulated Intramembrane Proteolysis: Signaling Pathways and Biological Functions. *Physiology*, *26*(1), 34-44.
13. Mascher, Thorsten (2013). Signaling diversity and evolution of extracytoplasmic function (ECF) σ factors. *In Current Opinion in Microbiology*, 16(2), 2013, 148-155.
14. Paget, M. S. (2015). Bacterial Sigma Factors and Anti-Sigma Factors: Structure, Function and Distribution. *Biomolecules*, *5*(3), 1245–1265.
15. Staroń, A., Sofia, H. J., Dietrich, S., Ulrich, L. E., Liesegang, H., & Mascher, T. (2009). The third pillar of bacterial signal transduction: classification of the extracytoplasmic function (ECF) σ factor protein family. *Molecular Microbiology*, *74*(3), 557-581.
16. Zellmeier, S., Hofmann, C., Thomas, S., Wiegert, T., & Schumann, W. (2005). Identification of sigma (V)-dependent genes of Bacillus subtilis. *FEMS Microbiology Letters*, *253*(2), 221-229.
17. Gibson, D. G., Young, L., Chuang, R., Venter, J. C., Hutchison, C. A., & Smith, H. O. (2009). Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nature Methods*, *6*(5), 343-345. doi:10.1038/nmeth.1318