Spring 2010

SdpAB are required for post-translational modification of SdpC

Tiara G. Perez Morales
University of Iowa

SDPAB ARE REQUIRED FOR POST-TRANSLATIONAL MODIFICATION OF SDPC

by

Tiara G. Perez Morales

A thesis submitted in partial fulfillment
of the requirements for the Master of
Science degree in Microbiology
in the Graduate College of
The University of Iowa

May 2010

Thesis Supervisor: Assistant Professor Craig D. Ellermeier

Graduate College
The University of Iowa
Iowa City, Iowa

CERTIFICATE OF APPROVAL

_____

MASTER'S THESIS

_____

This is to certify that the Master's thesis of

Tiara G. Perez Morales

has been approved by the Examining Committee
for the thesis requirement for the Master of Science
degree in Microbiology at the May 2010 graduation.

Thesis Committee:_____
Craig D. Ellermeier, Thesis Supervisor

_____
David S. Weiss

_____
John R. Kirby

ABSTRACT

Bacillus subtilis is a Gram-positive spore-forming soil bacterium. Under environmental stress conditions such as starvation, *B. subtilis* enters the pathway of sporulation. Earlier work demonstrated that *B. subtilis* can delay sporulation by undergoing cannibalism. Sporulating cells secrete toxins that kill nearby siblings, thus allowing cells to feed on the released contents. One of these toxins, SdpC, is encoded by the *sdpABC* operon. To uncover the requirements for SdpC toxic activity during cannibalism, all proteins in the *sdpABC* operon were analyzed. We report that mutations of SdpC which block signal peptidase cleavage also block toxin production. In addition, production and secretion of SdpC do not require SdpA and SdpB. Our results indicate that SdpC secretion is indispensable for induction of the immunity operon *sdpRI*. Furthermore, SdpC secreted from an *sdpAB* strain does not fully induce *sdpRI* expression and has decreased toxicity to cells that are sensitive to wild type SdpC. Lastly, differences in SdpC mobility are observed in the presence of SdpA and SdpB. Thus, we propose that SdpA and SdpB may function by post-translationally modifying SdpC into the active form of the toxin.

TABLE OF CONTENTS

LIST OF TABLES

Table

LIST OF FIGURES

Figure

LIST OF ABBREVIATIONS

AMP	antimicrobial peptide
DSM	Difco sporulation media
EMSA	electro mobility shift assay
GLA	γ-carboxylated glutamic acid
GLU	glutamic acid
IPTG	Isopropyl-β,D-thiogalactopyranoside
IMS	imaging- mass spectrometry
KO	vitamin K 2,3, epoxide
LB	Luria Bertani
MALDI	matrix assisted laser desorption ionization
MS	mass spectrometry
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
VKD-γ carboxylase	vitamin K dependent gamma carboxylase
X-GAL	5-bromo-4-chloro-3-indolyl-β,D-galactopyranoside

CHAPTER I: INTRODUCTION

In the environment, microorganisms constantly face competition for available nutrients. Thus, they have evolved different mechanisms for survival in the absence of these nutrients. In times of severe nutrient limitation the Gram-positive soil bacterium *Bacillus subtilis* will undergo sporulation. The entry into sporulation is controlled by the master regulator Spo0A. The phosporylation of Spo0A occurs via a phosphorylation cascade which is initiated via the activity of one of five sensor kinases (KinABCDE) (*Burbulys, D. et al 1991*). Spo0A-P can then activate an intricate regulatory cascade that will lead ultimately to spore formation.

Endospore formation is characterized by the formation of an asymmetric cell division septum which separates the cell into two unequally sized compartments: the larger cell becomes the mother cell and the small cell becomes the forespore. Subsequent regulation between these two compartments is necessary for the activation of genes involved in production of a mature spore. Once formed spores can survive a wide range of environmental stresses including heat, desiccation and irradiation, remaining dormant until conditions are favorable for growth.

Once the asymmetric cell division septum has formed the process of sporulation becomes irreversible *(Parker et al 1996, Dworkin and Losick 2005)*. Work by Chung et al demonstrated that not all cells within the population activate Spo0A, leading to two subpopulations of cells, SpoOA-ON and SpoOA-OFF (*Chung et al 1994)*. The Spo0A-ON population would be able to activate downstream genes required for sporulation. How would cells be able to prevent sporulation when nutrient starvation is only temporary? Interestingly, recent work showed that SpoOA-ON cells could delay entry to sporulation by cannibalizing a

portion of its population (*Gonzalez-Pastor et al. 2003, Ellermeier et al. 2006*). SpoOA-ON cells achieve cannibalism by inducing expression of two operons, *skfABCDEFGH* and *sdpABC,* that produce toxins. These toxins kill the non-sporulating cells of the population and Spo0A-ON cells feed on their released contents which lead to a delay in sporulation (*Gonzalez-Pastor et al. 2003, Ellermeier et al. 2006*). Although the basis of cannibalism is understood, very little is known about how these toxins are produced.

The *skfABCFEGH* operon is responsible for producing the sporulating killing factor SkfA. This operon is under direct control of Spo0A-P and indirect control by repression of AbrB *(Fujita et al, 2005* and *Chen et al 2006).* SkfA was first identified as an antimicrobial peptide (AMP) that inhibited activity of the rice pathogen *Xanthomonas oryzae* pv. *oryzae (Lin et al 2001).* Later work suggested that SkfA is a small toxic molecule exported during cannibalism by Spo0A-ON cells (*Gonzalez-Pastor et al., 2003)*. Production, export and immunity to SkfA all appear to be encoded by the *skfABCFEGH* operon (*Gonzalez-Pastor et al., 2003)*. SkfB is predicted to be involved in production of SkfA while SkfC may be required for its C-terminal proteolysis (*P. Dorrestein personal communication)*. The proteins SkfEF have homology to ABC transporters, which suggested these are involved in transport/immunity of SkfA (*Gonzalez-Pastor et al., 2003)*. Lastly, SkfG has unknown function, while SkfH is suggested to be involved in disulfide bond formation in SkfA (*P. Dorrestein personal communication).* While production/export of SkfA appears to be a complex process, it is known that its toxic activity is lower than its counterpart toxic protein encoded in *sdpABC* (*Gonzalez-Pastor et al., 2003)*.

Regulation of the *sdpABC* operon occurs indirectly by Spo0A-P via repression of AbrB *(Fujita et al, 2005)*. Direct repression of AbrB to the *sdpABC* operon was observed using a *lacZ* transcriptional fusion to the *sdp* promoter

(Strauch et al 2007). Absence of AbrB induced high levels of *sdp* expression. In addition, electromobility shift assays (EMSA) showed direct binding of AbrB to the promoter region of *sdpABC (Strauch et al 2007)*. Thus, production of cannibalistic toxin in *sdpABC* is controlled by repression of AbrB.

The mechanism of cannibalism by *sdpABC* takes place similarly to the *skfABCEFGH* operon. Spo0A-ON cells induce *sdpABC* expression, thus producing a toxin that kills Spo0A-OFF cells to delay commitment to sporulation. SdpA and SdpB are proteins that share very little similarity to any known genes. However, SdpC has been examined as a secreted protein in former studies (*Linde et al. 2003)*. SdpC possess a N-terminal signal peptide sequence. Work by Linde et al demonstrated SdpC was secreted via the Sec Pathway (*Linde et al. 2003)*. Moreover, analysis of the 5 signal peptidases encoded in *B. subtilis* indicated that SipS/T were the major proteases involved in SdpC processing. While SdpC was originally identified as a signaling molecule, further analysis suggested SdpC was the cannibalistic toxin present in this operon (*Gonzalez-Pastor et al., 2003* and *Ellermeier et al. 2006).* Additionally, it was demonstrated that extracellular SdpC induced expression of the immunity operon *sdpRI* (*Gonzalez-Pastor et al., 2003* and *Ellermeier et al. 2006).*

The *sdpRI* operon encodes SdpR, an autorepressor, and SdpI, which confers resistance towards SdpC (*Ellermeier et al. 2006*). Expression of *sdpRI* is also controlled indirectly by Spo0A via the transition state regulator AbrB (*Ellermeier et al. 2006 and Banse et al. 2008)*. SdpI acts as a signal transduction protein and confers resistance to SdpC. When no toxin is present small amounts of SdpI and SdpR are made. However, in the presence of the toxin the operon is induced and SdpR is sequestered to the membrane by the immunity protein SdpI (*Ellermeier et al. 2006*).

Although SdpC acts as an antimicrobial peptide (AMP), it is not understood what is required for its toxic activity or how it is recognized by the immunity protein. We hypothesize that SdpAB may affect production or activity of SdpC. We sought to determine the role of SdpAB in production of the SdpC toxin. We will present evidence that SdpC is required for *sdpRI* induction and SdpAB act to increase the activity of the toxin. In this work, we have determined that SdpC is responsible for toxicity and that is dependent upon expression of SdpAB. SdpAB are not required for production or secretion of SdpC but rather for post-translational modification of SdpC. Further analysis of these proteins should reveal the mechanism by which SdpAB modify SdpC toxicity.

CHAPTER II: MATERIALS AND METHODS

Media and Bacterial Growth

Overnight cultures of *B. subtilis* were grown in Luria-Bertani (LB) broth at 30°C. Samples used for SdpC immunoblot analysis were grown in Difco sporulation broth medium (DSM) supplemented with 1mM Isopropyl-β,D-thiogalactopyranoside (IPTG). P_{sdpRI}-*lacZ* phenotypic analyses were performed in solid LB medium containing IPTG, 100 µg/ml of β-galactosidase indicator 5-bromo-4-chloro-3-indolyl-β,D-galactopyranoside (X-Gal) and solid DSM medium with 1mM IPTG.

Strain Construction

Strains listed in Table 1 are isogenic derivatives of wild type *B. subtilis* PY79 and the protease minus strain RL1056. *B. subtilis* transformations were performed by the one-step method *(Wilson and Bott, 1968).*

Construction of SdpC Signal Peptide Cleavage Mutant

The SdpC signal peptide cleavage site mutant was constructed using the QuickChange site-directed mutagenesis kit (Stratagene). Mutagenesis was carried out according to the manufacturer's instructions using the following primer pairs CDEP640 and CDEP641 ($sdpC^{T30H}$). The sequence of the plasmids was confirmed by sequencing using CDEP269 and CDEP270. The resulting plasmid pCE260 was transformed into *B. subtilis* PY79. All primers are listed in Table 2.

Immunoblot Analysis of SdpC

Cell cultures were grown on Luria Bertani (LB) medium at room temperature. 1:100 sub-cultures were grown in DS broth supplemented with 1mM $Ca(NO_3)_2$, 1 mM $FeSO_4$, 10 mM $MnCl_2$ and 1mM IPTG at 37°C for 4 hours. After pelleting 1 ml of cells, the supernatants were methanol chloroform extracted

and resuspended in Laemmli buffer (Bio-Rad). The pellets were lysed by resuspending in 500ul lysis buffer (0.5M EDTA, 0.1M NaCl; pH7.5) and 10 ug/ml lysozyme and incubated at 37°C for 10 min. Followed incubation, the pellet was methanol chloroform extracted and resuspended in Laemmli buffer (Bio-Rad). Samples were heated for 10min at 65°C and electrophoresed on a 15% SDS Polyacrylamide gel (Bio-Rad). The proteins were then blotted onto nitrocellulose and the proteins were detected by incubating in a 1:3,000 dilution of anti-SdpC antibodies *(Linde et al 2003)* followed by incubation in a 1:10,000 dilution of goat anti-rabbit IgG (H+L)-HRP conjugate from BioRad.

β-galactosidase activity assays

Cultures were grown in LB broth 30°C overnight. 40ul spots of overnight culture were inoculated on DSM supplemented with 1 mM IPTG and grown at 37°C for 4 hours. Cells were scraped off the plates and resuspended in Z buffer containing β-mercaptoethanol and measured for OD_{600}. β-galactosidase activity was measured at OD_{405} for 1 hour using kinetic analysis *(Harwood and Cutting, 1990* and *Slauch and Silhavy 1991*).

Sub-cellular localization of SdpC

Culture was grown in DS broth with supplements at 37°C for 4 hours. Supernatant samples were collected and 500ul were concentrated 10 fold using a methanol chloroform extraction followed by resuspension in Laemmli buffer (Bio-Rad). The cell wall fraction was obtained by resuspending the cell pellets in 60mM Tris-Cl with 20mM $MgCl_2$ and 0.5M sucrose pH 8.0 and 10 ug/ml lysozyme and incubated at 37°C for 10 min *(Harwood and Cutting, 1990)*. The protoplasts were pelleted and the supernatant was removed and concentrated using methanol chloroform extraction. The protoplasts were resuspended in lysis buffer (0.5M EDTA, 0.1M NaCl; pH7.5) and lysed by sonication. The soluble material (cytosol) was separated from the insoluble material (membranes) by

ultracentrifugation at 100,000 x g for one hour at 7ºC. All samples were subject to immuno blot analysis as described.

SdpC concentration

One liter of culture was grown in DS broth with supplements at 37°C for 4 hours. Cultures were centrifuged to collect supernatants. Supernatants were subject to 20% and 60% ammonium sulfate cuts followed by centrifugation 12000 x g for 15 minutes at 10ºC. Pellets were resuspended in 0.25M NaOAc, pH4.5 and ultracentrifuged at 100,000 x g for ½ hour at 7ºC to remove debris. Samples were then separated by ion exchange chromatography using HiTrap Capto MMC 1 ml column (GE Healthcare). Resulting peaks were analyzed by immunoblot analysis using antibodies against SdpC. Samples with the highest SdpC concentration were methanol chloroform extracted and resuspended in Laemmli buffer (Bio-Rad) and stored at -20ºC until used for *in situ* assays or immunoblot analysis.

***In situ* assay**

Samples were concentrated as described above and separated by 15% SDS-PAGE (Bio-Rad). The poly acrylamide gel was washed in sterile water for 3 hours and placed in a sterile petri dish to dry for 20 minutes. The gels were overlaid with either 0.7% LB top agar with 1mM IPTG containing approximately 10^6 cells from SdpC sensitive cells or SdpI expressing cells grown for 3 hrs in LB broth with 1mM IPTG. Plates were incubated overnight at 30°C.

Table 1. Bacterial Strains

Strain	Parent	Genotype	Reference /Source
PY79	PY79	Prototrophic derivative of *B. subtilis* 168	(Youngman et al., 1984)
EG441	PY79	*sdpI*::*tet*	
EH273	PY79	*sdpABC*::*kan*	
EH274	PY79	*sdpABCI*::*kan*	
EG494	PY79	*sdpABCIR*::*tet*	(Gonzalez-Pastor et al., 2003)
CDE304	PY79	*thrC*::P_{sdpRI}-*lacZ* (*mls*)	
CDE433	PY79	Δ*sigW*::*kan* Δ*sdpABCIR*::*tet*	Ellermeier and Losick 2007
TPM663	RL1056	*sdpABC::kn*	This work
TPM706	RL1056	*sdpABC::kn amyE::*P_{hs}*-sdpC (spec)*	This work
TPM721	PY79	*pyrD::*P_{sdpR}*-lacZ (kan) sdpABC::cat*	This work
TPM725	PY79	*pyrD::*P_{sdpR}*-lacZ (kan) sdpABC::cm amyE:*P_{hs}*-sdpC (spec)*	This work
TPM829	RL1056	P_{hs}*-sdpAB (cat) sdpC::tc amyE::*P_{hs}*-sdpC (spec)*	This work
TPM830	PY79	*pyrD::*P_{sdpRI}*-lacZ (kn)* P_{hs}*-sdpAB (cat) sdpC::tet*	This work
TPM831	PY79	P_{hs}*-sdpAB (cat) sdpC:tet*	This work
TPM832	PY79	*pyrD::*P_{sdpRI}*-lacZ (kan)* P_{hs}*-sdpAB (cat) sdpC::tet amyE::*P_{hs}*-sdpC (spec)*	This work
TPM1002	RL1056	*sdpABC::kan amyE::*P_{hs}*-sdpC*^{T30H} *(spec)*	This work
TPM1003	RL1056	P_{hs}*-sdpAB (cat) sdpC::tet amyE::*P_{hs}*-sdpC*^{T30H} *(spec)*	This work
TPM1004	PY79	*pyrD:*P_{sdpRI}*-lacZ (kan)* P_{hs}*-sdpAB (cat) sdpC:tc amyE::*P_{hs}*-sdpC*^{T30H} *(spec)*	This work
TPM1005	PY79	*pyrD:*P_{sdpRI}*-lacZ (kan) sdpABC::cat amyE::*P_{hs}*-sdpC*^{T30H} *(spec)*	This work

Table 2. Primers

Primer Name	Use	Sequence 5'-3'
CDEP124	Clone *sdpAB*	aaaagcttggaggtaatctacatcaaa
CDEP125	Clone *sdpAB*	aagcatgcagttatttctccattatcta
CDEP126	Clone *sdpC*	aagcatgctagataatggagaaataact
CDEP127	Clone *sdpC*	aagcatgcagacactcaattataatgga
CDEP566	Clone *sdpA*	aagcatgcttataggtgtcaatataacc
CDEP567	Clone *sdpB*	aaaagctttaaggaggatttaagtatgaagatattaaatag
CDEP504	Clone *sdpC-6xHis*	aagcatgcttaatggtgatggtgatggtgttgatgaatcaattttag
CDEP128	P_{sdpRI}-*lacZ* Fusion	acgtcagaattcggacatcatcgtcagaggatcaa
CDEP129	P_{sdpRI}-*lacZ* Fusion	tccagcaagcttctccttgttggatctgatatagctt
CDEP640	$SdpC^{T30H}$ Mutant	ttcattagtaggactctctaaggagtcaagtcattctgctaaagaaa accatacatt
CDEP641	$SdpC^{T30H}$ Mutant	aatgtatggtttttctttagcagaatgacttgactccttagagagtccta ctaatgaa
CDEP269	Sequencing SdpC mutant	cgttttttatgcagcaatggc
CDEP270	Sequencing SdpC mutant	gtacgtacgatctttcagccg

CHAPTER III: RESULTS

Mature SdpC localizes to the Cell Wall and Supernatant

We began investigating the production of SdpC by first determining the subcellular localization of SdpC using antibodies which recognize SdpC *(Linde et al 2003)*. Cell cultures were grown as described in materials and methods. The cells were subsequently fractionated into culture supernatant, cell wall, membrane and cytosolic fractions. The protein samples were then separated by SDS-PAGE and immunoblot analysis. As seen in Figure 1, we observed the appearance of two bands, SdpC and SdpC*, which localized to the supernatant fraction of *sdpABC*$^{+}$ cells. Both SdpC species were observed in the cell wall fraction as well. A small amount of SdpC could be detected in the membrane fraction and was larger than the other two species of SdpC (pre-SdpC). We did not detect significant levels of SdpC in the cytosolic fractions.

Signal Peptide Cleavage is required for secretion of SdpC

It was previously reported that SdpC was secreted via the Sec pathway and either of the signal peptidases SipS and SipT were required for processing of SdpC in *E.coli* (*Linde et al. 2003a* and *Linde et al. 2003b)*. Our data show the appearance of two bands which react with anti-SdpC antibodies present in both the cell wall and supernatant fractions. The molecular weight of the SdpC where signal peptide sequence has been cleaved is about 17 kDa. Based upon the predicted molecular weight, the smaller of the two species corresponds to the mature form of SdpC. Although unlikely we sought to determine if the larger species was pre-SdpC by blocking signal peptide cleavage of SdpC. A double mutant of SipS/SipT is lethal in *B. subtilis (Tjalsma et al 1998)*. Therefore, to

determine if signal peptide cleavage is required for secretion of SdpC we constructed a point mutation (*sdpC*^{T30H}) in the -3 position of the predicted signal peptide cleavage site which should block cleavage by the *B. subtilis* signal peptidases SipS/T *(Antelmann et al 2001).* We then fractionated cells as previously described and compared the localization of SdpAB^{+}C^{T30H} to SdpABC+ by immunoblot analysis. Results in Figure 1 show that the SdpC^{T30H} detected is about 22 kDa, which corresponds to the predicted molecular weight of full length SdpC (pre-SdpC). In addition, we observe that SdpC^{T30H} remained localized to cell membrane and very little SdpC^{T30H} could be detected in the culture supernatant or cell wall fractions. Based on these results we conclude that the SdpC^{T30H} is produced but is unable to be cleaved by *B. subtilis* signal peptidases. We also conclude that neither of the two bands observed in the cell wall and supernatant fractions of an *sdpABC*+ correspond to the full length SdpC protein.

SdpC Secretion is required for

Induction of P*sdpRI*

Our data suggested that SdpC signal peptide cleavage is essential for its secretion. To determine if SdpC secretion is required for induction of P*sdpRI* we tested the ability of SdpC^{T30H} to induce expression of a P*sdpRI*-*lacZ* reporter fusion. We compared cells expressing one of the following; *sdpAB*$^{+}$*C*^{T30H}, *sdpC*^{T30H}, *sdpABC*$^{+}$, and *sdpC*$^{+}$. As previously described cells that produce SdpABC^{+} fully induced expression of the *sdpRI* operon (*Ellermeier et al. 2006)*. Cells that produce SdpC^{+} in the absence of SdpAB are unable to fully induce *sdpRI*. Results in Figure 2A show that cells expressing SdpAB^{+}C^{T30H} have markedly reduced induction of *sdpRI* in comparison to WT. Furthermore, cells that only express SdpC^{T30H} are insufficient to induce expression of P*sdpRI* when compared to SdpAB^{+}C^{T30H}. These results are consistent that SdpAB are important for SdpC induction of *sdpRI* operon.

We also assayed the levels of β-galactosidase to quantify results observed on plates. In agreement with our earlier data, we observed that P_{sdpRI}-*lacZ* expression is decreased approximately 20 fold when cells are producing $SdpAB^{+}C^{T30H}$ (Figure 2B). In the absence of SdpAB the $SdpC^{T30H}$ mutant exhibits an even further decrease in β-galactosidase activity compared to both SdpC+ cells. Taken together, we conclude that signal peptide cleavage and thus secretion of SdpC is essential for induction of the *sdpRI* operon.

SdpAB required for maximal induction of P_{sdpRI}

Previous work demonstrated that SdpC was required for expression of the s*dpRI* operon; however the roles for SdpAB have not been determined (*Gonzalez-Pastor et al. 2003* and *Ellermeier et al. 2006)*. Here we present evidence that SdpAB are required for induction of the *sdpIR* operon. Since *sdpABC* are encoded in a single operon we began our investigation by constructing strains capable of expressing different combinations of *sdpABC*. We determined the effect of combinations of these genes on expression of *sdpRI* using a P_{sdpRI}-*lacZ* reporter fusion.

We found that cells expressing *sdpABC*+ were able to fully induce expression of *sdpRI* (Figure 3A). As previously reported a deletion of the *sdpABC* genes blocked induction of P_{sdpRI}-*lacZ* expression. We found that cells producing only SdpAB do not induce expression from *sdpRI* operon. This result suggests that SdpAB are not sufficient for P_{sdpRI} induction and are consistent with previous observations that absence of SdpC blocked induction of P_{sdpRI}-*lacZ* (*Ellermeier et al. 2006)*. However cells expressing only *sdpC*+ show a significant decrease in expression of the P_{sdpRI} operon (Figure 3A). From these results, we conclude that SdpC is essential for *sdpRI* signaling and that SdpAB are required for maximum *sdpRI* expression in an SdpC dependent manner.

We quantified these results by isolating cells from DS agar plates and performed β-galactosidase assays. As expected, maximal expression P_{sdpRI}-*lacZ* is observed when cells are producing SdpABC (Figure 3B). We also observed that in agreement with our previous results expression of P_{sdpRI}-*lacZ* decreases in the absence of SdpABC, SdpAB, or SdpC (Figure 3B). These data suggests that SdpABC are all required for induction of the *sdpRI* operon.

SdpAB alter mobility of SdpC

Our data suggested that SdpAB require the presence of SdpC to induce expression of the *sdpRI* operon, suggesting SdpAB affect SdpC function. Thus we compared the production of SdpC from cells expressing *sdpABC*$^+$, *sdpC*$^+$, and Δ*sdpABC.* We prepared samples of culture supernatants and whole cell extracts as described in the materials and methods. The samples were separated by SDS-PAGE and probed with anti-SdpC antibodies.

When immunoblot analysis was performed on strains expressing *sdpABC*$^+$, we observed the appearance of a band of ~17 kDa range (Figure 4). The signal peptide cleavage product of SdpC is predicted to be 17 kDa as previously observed in *E. coli* experiments and could likely correspond to the band observed *(Linde et al 2003)*. In addition, cells expressing *sdpABC*$^+$ produced a second band in the 19kDa region (SdpC*). Both of these SdpC species were present in the supernatant and whole cell extracts (Figure 4). Neither band was present in a cell lacking the *sdpABC* genes suggesting both bands are in fact related to SdpC.

Interestingly we found that cells which express only the *sdpC* gene produce a single band (~17 kDa) which is consistent to the signal peptide cleavage product. This species was again observed in both the supernatants and whole cells of *B. subtilis* cells (Figure 4). These results lead us to conclude that SdpAB are not required for secretion of SdpC as SdpC is present in both the

whole cell extracts and culture supernatants. We also observed that SdpC protein levels remain similar in both fractions in the presence and absence of SdpAB, suggesting SdpAB are not required for transcription, translation or stability of SdpC. The appearance of a second species of SdpC in an SDS denaturing gel raised the possibility that SdpC is post-translationally modified in the presence of SdpAB.

SdpAB are required for activity

of the SdpC toxin

Our data suggest that SdpAB are required for maximal induction of P_{sdpRI} and production of a modified form of SdpC, SdpC*. We sought to determine if SdpAB were required for the toxin activity of SdpC. We concentrated the supernatants from strains expressing *sdpABC*$^{+}$, *sdpC*$^{+}$ and Δ*sdpABC* as described in materials and methods. As seen in panel 5A, SdpC is present in the culture supernatants of strains producing *sdpABC*$^{+}$ and *sdpC*$^{+}$ but is absent from the Δ*sdpABC* culture supernatant.

To determine if the culture supernatant samples had toxin activity we performed an *in situ* assay *(Wu et al 2005).* The samples were separated by SDS-PAGE and overlaid with cells that are sensitive to SdpC (Δ*sigW* Δ*sdpABCIR)*. We observed two zones of inhibition in gels overlaid with SdpC sensitive cells. One zone of inhibition was present between 17-19 kDa and is present only in the *sdpABC*+ producing samples (Figure 5B). A second zone of inhibition was observed around 5 kDa and was present in all samples, even those from a Δ*sdpABC* strain. This suggests that the 5 kDa species is independent of SdpABC (Figure 5B).

We had previously demonstrated that SdpI provides resistance to the SdpC killing factor (*Ellermeier et al. 2006)*. To further confirm that the zone of inhibition observed between 17-19 kDa is due to SdpC, we performed an *in situ*

assay using cells expressing the immunity protein SdpI. As seen in Figure 5C, when the gel is overlaid with cells expressing SdpI, the 17-19 kDa zone of inhibition is lost. This suggests that SdpI provides immunity against this species, which is consistent with this species being SdpC. In contrast, the 5 kDa zone appears independent of the presence or absence of SdpI. These results suggest that 17-19 kDa zone of inhibition is SdpC and requires SdpAB for the toxic activity observed.

Figure 1. Secreted mature SdpC localizes to the Cell Wall and Supernatant. Cells were grown in liquid DS medium and fractioned as described in materials and methods. From left to right (S) supernatant, (CW) cell wall, (M) membrane and (C) cytosol. The strains used for this experiment *sdpABC+* (TPM829; P_{hs}-*sdpAB (cat) sdpC::tc amyE::*P_{hs}-*sdpC spec)* and *sdpAB*$^{+}$*C*^{T30H} (TPM1013; P_{hs}-*sdpAB (cat) sdpC::tc amyE::*P_{hs}-*sdpC*^{T30H} *spec)*.

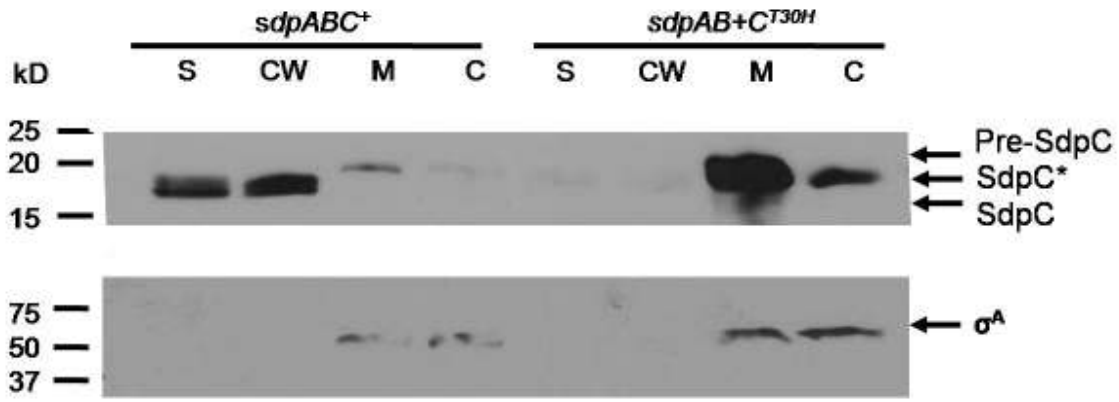

sdpABC+
sdpAB+CT30H
kD
S
CW
M
C
S
CW
M
C
25
20
15
Pre-SdpC
SdpC*
SdpC
75
50
37
σA

Figure 2. SdpC Secretion is Required for Induction of P*sdpRI* . (A) The effect of SdpC signal peptidase cleavage on expression of P_{sdpRI}-*lacZ.* All strains contained P_{sdpRI}-*lacZ* and the relevant genotypes of the strains with respect to SdpABC are noted. The strains used for this experiment contain *pyrD::*P_{sdpRI}-*lacZ*$^+$ and have the following genotypes SdpABC$^+$ (TPM832; P_{hs}-*sdpAB*$^+$ Δ*sdpC::tet amyE::*P_{hs}-*sdpC*$^+$), SdpC$^+$ (TPM 725; *sdpABC::cm amyE:*P_{hs}-*sdpC (spec*)), SdpAB$^+$C^{T30H} (TPM1004; P_{hs}-*sdpAB (cat) sdpC::tet amyE::*P_{hs}-*sdpC*^{T30H}) and SdpC^{T30H} (TPM1005; Δ*sdpABC amyE::*P_{hs}-*sdpC*^{T30H}). Growth was for 16 hours at 37°C on solid LB medium *(Harwood, 1990)* containing X-Gal and IPTG. (B) β-galactosidase assays on cells expressing different combinations of SdpABC using the same strains noted above. Cells were spotted on solid DS media + IPTG and grown for 4 hours. The cells were harvested and the β-galactosidase activity determined *(Harwood et al 1990 and Slauch et al 1991)*. Bars are the average of three experiments and the error bars denote standard deviation of the mean.


SdpABC+
SdpC^{T30H}
SdpC+
SdpABC^{T30H}

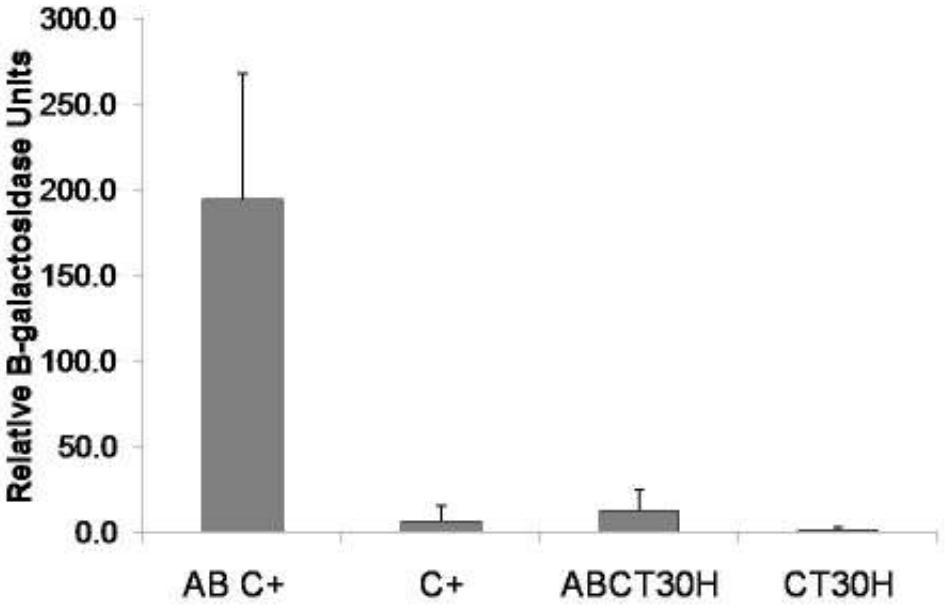

Relative B-galactosidase Units
300.0
250.0
200.0
150.0
100.0
50.0
0.0
AB C+
C+
ABCT30H
CT30H

Figure 3. SdpAB required for maximal induction of the *sdpRI* operon. (A) The effect of different combinations of SdpABC on expression of P_{sdpRI}-*lacZ.* All strains contained P_{sdpRI}-*lacZ* and the relevant genotypes of the strains with respect to SdpABC are noted. The strains used for this experiment contain *pyrD::*P_{sdpRI}-*lacZ*$^+$ and have the following genotypes SdpABC$^+$ (TPM832; P_{hs}-*sdpAB*$^+$ Δ*sdpC::tet amyE::*P_{hs}-*sdpC*$^+$), SdpC$^+$ (TPM 725; *sdpABC::cm amyE:*P_{hs}-*sdpC (spec*)), *sdpAB* (TPM830; P_{hs}-*sdpAB (cat) sdpC::tet*) and ∆*sdpABC* (TPM 721; Δ*sdpABC*). Growth was for 16 hours at 37° C on solid LB medium *(Harwood and Cutting, 1990*) containing X-Gal and IPTG. (B) β-galactosidase assays on cells expressing different combinations of SdpABC using the same strains noted above. Cells were spotted on solid DS media + IPTG and grown for 4 hours. The cells were harvested and the β-galactosidase activity determined *(Harwood and Cutting, 1990* Bars are the average of three experiments and the error bars denote standard deviation of the mean.


SdpABC+
SdpC+
SdpABC-
SdpAB+

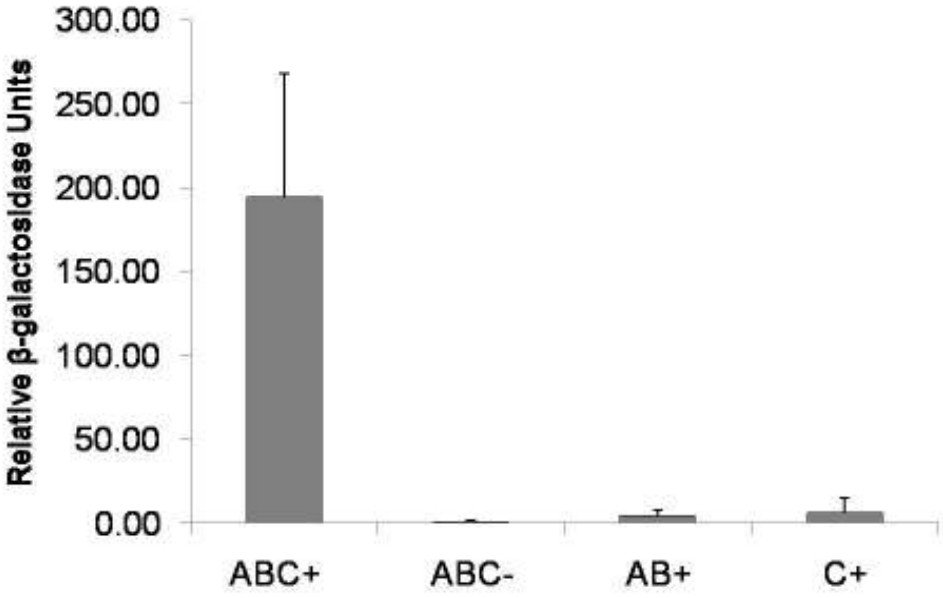

Relative β-galactosidase Units
300.00
250.00
200.00
150.00
100.00
50.00
0.00
ABC+
ABC-
AB+
C+

Figure 4. SdpAB alter mobility of SdpC. Differences in SdpC mobility in the presence and absence of SdpAB. The relevant genotypes of the strains with respect to SdpABC are indicated at the top of the figure. The strains used for this experiment on the gel are *sdpABC*$^{+}$ (TPM829), *sdpC*$^{+}$ (TPM 706) and Δ*sdpABC* (TPM 663). Samples were prepared as described in materials and methods.

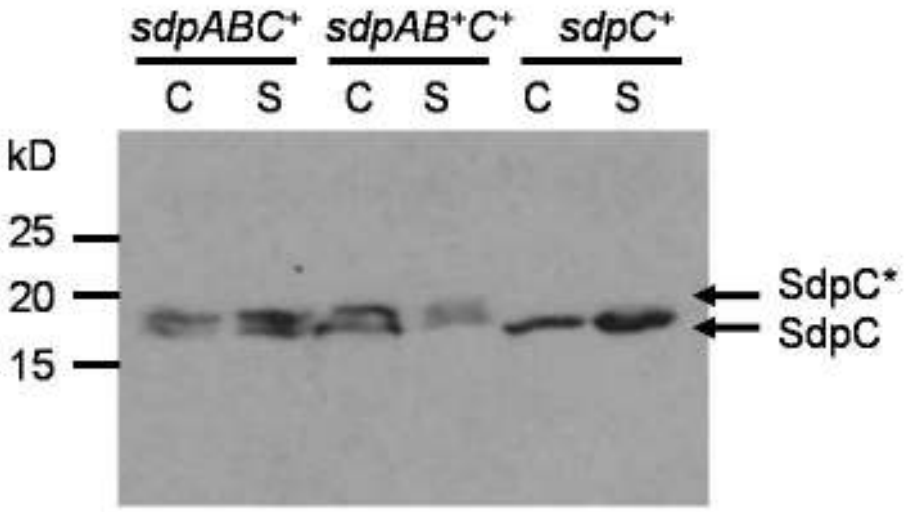
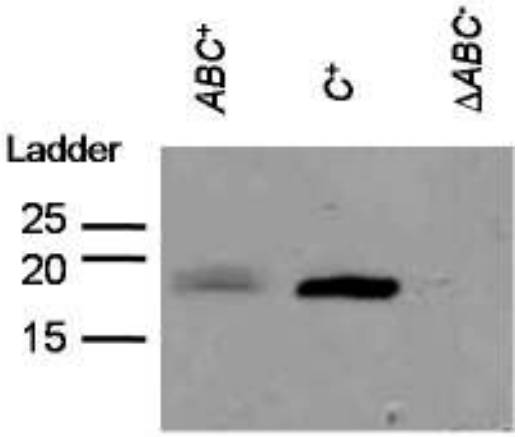
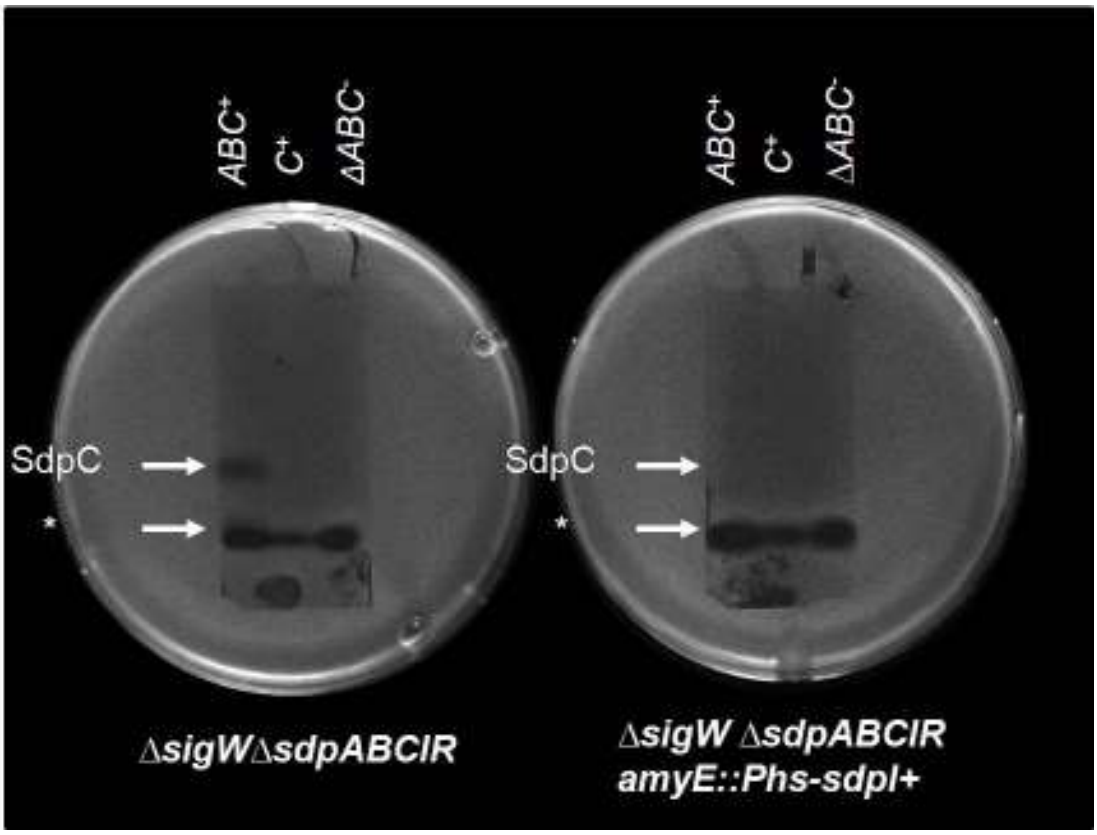

sdpABC+
sdpAB+C+
sdpC+
C
S
C
S
C
S
kD
25
20
15
SdpC*
SdpC

Figure 5. SdpC toxic activity requires SdpAB and immunity is dependent on SdpI. (A) Immunoblot of concentrated proteins from culture supernatants of cells grown in liquid DS medium. The relevant genotypes of the strains with respect to *sdpABC* are indicated at the top of the figure. The strains used for this experiment (from left to right) on the gel are *sdpABC*$^{+}$ (TPM829), *sdpC*$^{+}$ (TPM 706) and Δ*sdpABC* (TPM 663). Samples were separated on a 15% SDS-PAGE and probed with polyclonal anti-SdpC antibodies. (B) In situ assay to detect killing. Samples were separated on a 15% SDS-PAGE, washed with water and overlaid with LB top agar +IPTG containing either Δ*sigW* Δ*sdpABCIR* cells (CDE433) or (C) Δ*sigW* Δ*sdpABCIR amyE::*P_{hs}*-sdpI* (TPM758) and incubated at 30°C overnight. The SdpC arrow points to a band which only kills cells lacking SdpI. The "*" arrow denotes a band which is present in supernatants from strains which have been deleted for *sdpABC* and kills all cells including those expressing SdpI.


ABC+
C+
ΔABC-
Ladder
25
20
15


ABC+
C+
ΔABC-
SdpC
*
ΔsigWΔsdpABCIR
ABC+
C+
ΔABC-
SdpC
*
ΔsigW ΔsdpABCIR
amyE::Phs-sdpI+

CHAPTER IV: DISCUSSION

Cannibalism in *B. subtilis* is a mechanism of last resort to postpone commitment to sporulation under nutrient limiting conditions (*Gonzalez-Pastor et al. 2003, Ellermeier et al. 2006*). Toxin production and immunity are only expressed by a subpopulation of cells that has activated Spo0A (Spo0A-ON). This ensures that the Spo0A-OFF population will be sensitive to the toxin and killed. Thus, allowing a portion of the cells to survive and delay sporulation. In this work we have described the requirements for production of toxic SdpC during cannibalism in *B. subtilis.* Earlier evidence pointed out that SdpC had dual functions; a toxin and an inducer of the immunity operon (*Ellermeier et al. 2006)*. We propose that SdpC is the toxin and its secretion is essential for induction of the *sdpRI* operon. Analysis of our data suggests that SdpAB function by post-translationally modifying SdpC**.** We do not know if this post-translational modification occurs before or after cleavage by signal peptidases SipS/T. Once SdpC has been cleaved, it will be released extracellularly and act upon neighboring non-sporulating cells (Figure 6).

Post-translational modification of AMPs is not an unknown concept in *B. subtilis.* For instance, Subtilosin A was discovered as an antimicrobial peptide with activity against *Listeria monocytogenes.* Its production, export, and immunity towards Subtilosin A, requires the expression of the *albABCDEFG* operon *(Zheng et al 1999 and Babasaki et al 1985).* Post-translational modification of Subtilosin A involves cyclization and inter-residue linkages *(Zheng et al 1999, Marx et al 2001 and Kawulka et al 2004)*. Thus, AMP production is a complex mechanism that becomes unique to the environmental requirements of bacteria.

What types of post-translational modification SdpAB may be involved?

SdpB as a VKD γ-carboxylase

Because these proteins have no homology to any known bacterial proteins, there is no obvious predicted function for these proteins. SdpA shows nearly no homology to any other proteins. In contrast SdpB shows distant homology to the human vitamin K-dependent γ-carboxylase protein domain (simply VKD γ-carboxylase) *(Schultz 2004)*. This protein is essential in humans and is dependent upon the vitamin K cycle. Vitamin K is utilized as a co-factor for the process to occur *(Shah et al 1971)*. In humans VKD γ-carboxylase has dual roles, it possess both carboxylase and epoxidase activity. Carboxylation involves addition of a carboxyl group to several glutamic (Glu) residues resulting in γ-carboxylated glu residues (Gla). In this case, VKD γ-carboxylation occurs at Glu residues in human clotting factors. At the same time, the reduced vitamin KH_2 is converted to vitamin K 2,3 epoxide (KO) by the VKD γ-carboxylase *(Dowd et al 1995)*. In the end, clotting factors will contain several Gla residues that allow binding to Ca^{+2} and thus interact and tighten disrupted membranes *(Stenflo et al 1974)*.

The presence of the VKD γ-carboxylase proteins has recently been reported for other organisms *(Schultz 2004)*. One of these VKD γ-carboxylases was found in *Conus* snails *(Czerwiec et al 2002)*. VKD γ-carboxylation was first described as one of the post-translational modifications required to activate small peptides known as conotoxins *(McIntosh et al 1984)*. Biochemical work shows that the majority of the conotoxins studied attack the nervous system of their prey, usually affecting ion channels and causing paralysis *(Gray et al 1981* and

Cruz et al 1985). To date, most of the VKD γ-carboxylases characterized have very unique roles in each organism.

In prokaryotes, Rishavy et al 2005 suggested the presence of a VKD carboxylase in *Leptospira interrogans* using bioinformatics. In addition, they expressed the protein in microsomes and tested carboxylase and epoxidase activity by labeled CO_2 incorporation and KO formation. Their work suggested that the *L. interrogans* ortholog did not possess carboxylase activity but retained epoxidase activity *(Rishavy et al 2005)*. Yet, this work focused primarily to define the enzymatic activity of the VKD γ-carboxylase and no targets for post-translational modification were investigated.

There are 10 Glu residues present in the sequence of SdpC. Of these, 9 appear after the signal peptide sequence cleavage site. An attractive hypothesis would be that SdpB may function by post-translationally modifying SdpC via VKD γ-carboxylation. .In addition, this would be a novel post translational modification not seen in bacteria. Further biochemical studies should provide insight as whether SdpC possess Gla residues in its mature sequence and validate the role of SdpB.

SdpB as a protease

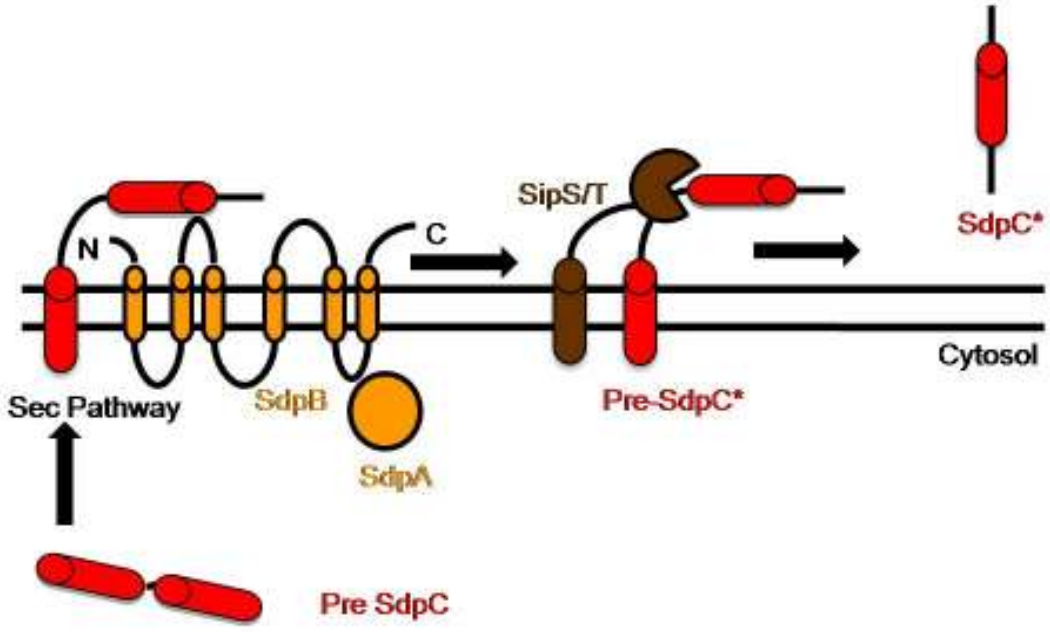
The full-length SdpC protein is 203 amino acids with an approximate molecular weight of 22 kDa. Once the signal peptide sequence has been cleaved, the molecular weight of the protein is predicted to be 17 kDa. As seen in our protein immunoblot analysis, SdpC is present in this region in wild-type conditions (Figure 4). Previous work by Gonzalez-Pastor et al identified SdpC as a 63 amino acid secreted peptide that stimulated expression from the *sdpRI* operon. This peptide would be a cleavage product that occurs at the S140 residue. This SdpC species of about 5 kDa has not been detected in our assays. A simple explanation is that our polyclonal SdpC antibodies do not recognize an

epitope in the 5 kDa species. However if this 5 kDa species is produced, one hypothesis would be that the 17 or 19 kDa is processed into a smaller active peptide. Although, it would not correlate with killing of SdpC sensitive cells by a 17-19 kDa band observed in our *in situ* assay.

Work in progress by the Dorrestein Lab suggests SdpC is a smaller peptide of approximately 42 amino acids in length. The new species of SdpC was detected by matrix-assisted laser desorption ionization – imaging mass spectrometry (MALDI-IMS) *(P. Dorrestein direct communication).* It possesses an S-S disulfide bond between C141 and C147 and two cleavage sites. These correspond to the first cleavage at S140 and a second cleavage event at S181 residue. Thus, this would suggest that the post-translational modification of SdpC occurs by internal proteolytic cleavage. However, this does not explain why our toxic SdpC peptide is larger.

Within the SdpC sequence, there is a predicted intramembrane domain located before the cleavage sites. One possibility would be that the active portion of SdpC resides in the last 63 or 42 residues and the intramembrane domain is required for cell binding. We are currently studying this possibility by performing site directed mutagenesis in both predicted cleavage site S140 and S181. We will determine its effects in the presence and absence of SdpAB. In addition, we are making SdpC truncations that lack the last 42 or 63 residues to observe differences in toxicity. An additional approach will be by analyzing purified SdpC via mass spectrometry (MS). This will aid to resolve if there any specific residues with post-translational modifications. Subsequent mutations in these residues will show if these are important for SdpC activity. In all, how post-translational modification of SdpC occurs will be a novel mechanism described in bacteria.

Figure 6. SdpC toxin production model. SdpC is secreted via the Sec Pathway. During secretion it is post-translationally modified by SdpAB. Finally, SipS/T cleave the N-terminal signal peptide sequence of SdpC and is secreted extracellular.


SipS/T
SdpC*
N
C
Cytosol
Sec Pathway
SdpB
Pre-SdpC*
SdpA
Pre SdpC

REFERENCES

Antelmann, H., H. Tjalsma, B. Voigt, S. Ohlmeier, S. Bron, J. M. van Dijl, and M. Hecker. 2001. A proteomic view on genome-based signal peptide predictions. Genome Res. **11**:1484-1502

Babasaki K., Takao T., Shimonishi Y., Kurahashi K. (1985). Subtilosin A, a new antibiotic peptide produced by Bacillus subtilis 168: isolation, structural analysis, and biogenesis. *J Biochem*. (**3**):585-603.

Banse, A.V., Chastanet, A. Rahn-Lee, L., Hobbs, E.C., and Losick, R. (2008) Parallel pathways of repression and antirepression governing the transition to stationary phase in *Bacillus subtilis. PNAS* **105**:15547-15552

Bott, K. F., and Wilson, G.A. (1968). Metabolic and nutritional factors influencing the development of competence for transfection of *Bacillus subtilis*. *Bacteriol. Rev.* **32**:370-378.

Burbulys, D., Trach, K.A. and, Hoch, J.A. (1991). Initiation of sporulation in *Bacillus subtilis* is controlled by a multicomponent phosphorelay. *Cell*, **64** p. 545-552

Chen G., Kumar A., Wyman T.H., Moran C.P. Jr. (2006). Spo0A-dependent activation of an extended-10 region promoter in *Bacillus subtilis*. *J Bacteriol* **188**: 1411–1418

Chung, J.D., Stephanopoulos, G., Ireton, K., and Grossman, A.D. (1994) Gene expression in single cells of *Bacillus subtilis*: evidence that a threshold mechanism controls the initiation of sporulation. *J Bacteriol* **176:** 1977–1984

Cruz L.J., Gray W.R., Olivera B.M., Zeikus R.D., Kerr L., Yoshikami D., Moczydlowski E. (1985). *Conus geographus* toxins that discriminate between neuronal and muscle sodium channels. *J. Biol. Chem.* **260**: 9280-9288

Czerwiec, E., Begley, G.S., Bronstein, M., Stenflo, J., Taylor, K.L.,Furie, B.C. and Furie, B. (2002) Expression and characterization of recombinant vitamin K-dependent γ-glutamyl carboxylase from an invertebrate, *Conus textile*. *Eur J Biochem* **269**:6162–6172.

Dowd P., Hershline R., Ham S.W., Naganathan, S. *(1995)* Vitamin K and energy transduction: a base strength amplification mechanism. *Science* **269**:1684–1691

Dworkin J, and Losick R. (2005). Developmental commitment in a bacterium. *Cell*. **121**:401-9

Ellermeier C.D., Hobbs E.C., Gonzalez-Pastor J.E., Losick R. (2006). A three-protein signaling pathway governing immunity to a bacterial cannibalism toxin. *Cell*. **124**:549-59.

Fujita, M., Gonzalez-Pastor, J.E., and Losick, R. (2005). High- and low-threshold genes in the Spo0A regulon of *Bacillus subtilis*, *J. Bacteriol.* **187**: 1357–1368

Gray W.R., Luque A., Olivera B.M., Barrett J., Cruz L.J. (1981). Peptide toxins from *Conus geographus* venom. J Biol Chem. **256**:4734–4740.

Gonzalez-Pastor, J.E., Hobbs, E.C., and Losick R. (2003). Cannibalism by sporulating bacteria, *Science* **301**:510–513.

Harwood C.R., Cranenburgh R. (2008). *Bacillus* protein secretion: an unfolding story. *Trends Microbiol*. **2**:73-9.

Kawulka K.E., Sprules T., Diaper C.M., Whittal R.M., McKay R.T., Mercier P., Zuber P., Vederas J.C. (2004). Structure of subtilosin A, a cyclic antimicrobial peptide from *Bacillus subtilis* with unusual sulfur to alpha-carbon cross-links: formation and reduction of alpha-thio-alpha-amino acid derivatives. *Biochemistry.* **43**:3385-95.

Linde, D., Volkmer-Engert, R., Schreiber, S. and Müller, J.P. (2003a). Interaction of the *Bacillus subtilis* chaperone CsaA with the secretory protein YvaY. *FEMS Microbiol. Lett.* **226**: 93–100

Linde, D., Marischen, L., and Müller J.P. (2003b) Characterisation of preYvaY export reveals differences in the substrate specificities of *Bacillus subtilis* and *Escherichia coli* leader peptidases. *FEMS Microbiol. Lett.* **227:**149-156

Marx, R., Stein, T., Entian, K-D, and Glaser S.J. (2001) Structure of *Bacillus subtilis* peptide antibiotic Subtilosin A determined by 1H-NMR and matrix assisted laser desorption/ionization time-of-flight mass spectrometry. *J Protein Chem* **20:**501-506

McIntosh, J.M., Olivera, B.M., Cruz, L.J. and Gray, W.R. (1984) γ-Carboxyglutamate in a neuroactive toxin. *J Biol Chem* **259**:14343–14346.

Parker, G.F., Daniel, R.A., and Errington, J. (1996). Timing and regulation of commitment to sporulation in *Bacillus subtilis. Microbiology* **142:** 3445-3552.

Rishavy M.A., Hallgren K.W., Yakubenko A.V., Zuerner R.L., Runge K.W., Berkner K.L. (2005). The Vitamin K dependent Carboxylase has been acquired by *Leptospira* Pathogens and shows altered activity that suggests a role other than protein carboxylation *J Biol Chem* **280**:34870–34877

Schultz, J. (2004) HTTM, a horizontally transferred transmembrane domain. *Trends Biochem Sci.* **29**:4-7

Shah, D. V., Suttie, J. W. (1971). Mechanism of Action of Vitamin K: Evidence for the conversion of a Precursor Protein to Prothrombin in the Rat. *PNAS* **68**: 1653-1657

Slauch JM, Silhavy TJ. (1991) Genetic fusions as experimental tools. *Methods Enzymol*. **204**:213-48

Stenflo J., Fernlund P., Egan W., Roespstorff P. (1974). Vitamin K dependent modifications of glutamic acid residues in prothrombin. *PNAS* **71**:2730

Strauch, M. A., B. G. Bobay, J. Cavanagh, F. Yao, A. Wilson, and Y. Le Breton. 2007. Abh and AbrB control of *Bacillus subtilis* antimicrobial gene expression. *J. Bacteriol*. **189**:7720-7732.

Tjalsma H., Bolhuis A., van Roosmalen M.L., Wiegert T., Schumann W., Broekhuizen C.P., Quax W.J., Venema G., Bron S., van Dijl J.M. (1998). Functional analysis of the secretory precursor processing machinery of *Bacillus subtilis*: identification of a eubacterial homolog of archaeal and eukaryotic signal peptidases. *Genes Dev.* **15**:2318-31.

Wu S.*,* Jia S.*,* Sun D.*,* Chen M., Chen X., Zhong J., Huan L. (2005)*.* Purification and characterization of two novel antimicrobial peptides subpeptin JM-A and subpeptin JM4-B produced by *Bacillus subtilis* JM4*. Curr. Microbiol* **51***:*292–*296.*

Zheng, G., Yan, L.Z., Vederas, J.C., and Zuber, P. (1999). Genes of the *sbo-alb* locus of *Bacillus subtilis* are required for production of the antilisterial bacteriocin subtilosin. *J. Bacteriol.* **181**: 7346-7355

Zheng G., Hehn R., Zuber P. (2000). Mutational analysis of the *sbo-alb* locus of *Bacillus subtilis*: identification of genes required for subtilosin production and immunity. *J Bacteriol.* **182**:3266-73.