Production of the Cannibalism Toxin SDP Is a Multistep Process That Requires SdpA and SdpB

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During the early stages of sporulation, a subpopulation of *Bacillus subtilis* cells secrete toxins that kill their genetically identical siblings in a process termed cannibalism. One of these toxins is encoded by the *sdpC* gene of the *sdpABC* operon. The active form of the SDP toxin is a 42-amino-acid peptide with a disulfide bond which is processed from an internal fragment of pro-SdpC. The factors required for the processing of pro-SdpC into mature SDP are not known. We provide evidence that pro-SdpC is secreted via the general secretory pathway and that signal peptide cleavage is a required step in the production of SDP. We also demonstrate that SdpAB are essential to produce mature SDP, which has toxin activity. Our data indicate that SdpAB are not required for secretion, translation, or stability of SdpC. Thus, SdpAB may participate in a posttranslation step in the production of SDP. The mature form of the SDP toxin contains a disulfide bond. Our data indicate that while the disulfide bond does increase activity of SDP, it is not essential for SDP activity. We demonstrate that the disulfide bond is formed independently of SdpAB. Taken together, our data suggest that SDP production is a multistep process and that SdpAB are required for SDP production likely by controlling, directly or indirectly, cleavage of SDP from the pro-SdpC precursor.

In the environment, microorganisms face constant competition for nutrients. In times of severe nutrient limitation, the Gram-positive soil bacterium *Bacillus subtilis* initiates sporulation. Sporulation is an energetically costly process which becomes irreversible after the asymmetric septum is formed (1). *B. subtilis* can delay the commitment to sporulation by inducing cannibalism, a process by which the sporulating cells in the population kill the non-sporulating cells (2, 3). There are two toxins responsible for cannibalism: SDP and SKF (2, 3). These toxins have antimicrobial activity against other bacteria, including *Xanthomonas oryzae*, *Listeria monocytogenes*, and *Staphylococcus aureus* (4–6). SKF is produced by the *skfABCEFGH* operon, while SDP is produced by the *sdpABC* operon. Expression of both operons is controlled by the master regulator of sporulation, Spo0A, which when phosphorylated can repress expression of *abrB*, a negative regulator of *skfABCEFGH* and *sdpABC* (7, 8). Since *AbrB* negatively regulates expression of *sdp* and *skf*, the expression of both toxin-encoding operons increases during early stationary phase upon entry into sporulation (2). However, these toxins are produced only by a subset of *B. subtilis* cells, as activation of Spo0A is subject to a bistable regulatory mechanism (9). While the mechanism of SKF killing is unknown, the SDP toxin appears to kill sensitive cells by disrupting the proton motive force (10).

Antimicrobial peptides (AMPs) can be ribosomally or nonribosomally synthesized. Nonribosomally synthesized AMPs are generated from protein complexes that build, modify, and release an active peptide. The mycosubtilin AMP produced by *B. subtilis* is a nonribosomally synthesized β -amino-fatty-acid-linked cyclic heptapeptide which is produced by the products of the *fenF-mycABC* operon (11–13). Ribosomally synthesized AMPs often require posttranslational modification in order to produce an active form of the toxin. For example, production of subtilisin A requires the *albA* and *albF* genes for modification of subtilisin A (14, 15).

SKF is a ribosomally synthesized 26-amino-acid peptide encoded by *skfA* (2, 6). SKF is a posttranslationally modified cyclic

peptide with disulfide and thioether bonds (6). Several genes in the *skf* operon have been proposed to be involved in the posttranslational modifications of SKF (6). It was recently demonstrated that SkfB is a 4Fe-4S cluster containing the radical S-adenosylmethionine (SAM) enzyme, which is required for formation of a thioether bond in SKF (16).

SDP is a 42-amino-acid, ribosomally synthesized AMP which contains a disulfide bond between two cysteine residues located at the N terminus (6). The active form of SDP is derived from an internal fragment of the full-length pro-SdpC protein (6) (Fig. 1). Although the mature form of SDP has been determined, little is known about the factors required to process pro-SdpC into the active SDP peptide. The pro-SdpC form is a 203-amino-acid protein secreted via the general secretory pathway (17). Signal peptidases SipS and/or SipT can cleave pro-SdpC to SdpC^{33–203} when expressed in *Escherichia coli* (17). However, it was not known what role signal peptide cleavage plays in SDP production. *sdpA* and *sdpB* are genes located in an operon with *sdpC*, but it is not known if they are required for the production of the toxin SDP (2).

Here, we provide evidence to show that SDP production requires multiple steps, including signal peptide cleavage of pro-SdpC, which creates the SdpC^{33–203} protein, formation of disulfide bonds in SdpC^{33–203}, and processing of SdpC^{33–203} into mature SDP (Fig. 1). We also provide evidence that SdpAB are essential for the production of active SDP toxin and are presumably required for processing SdpC^{33–203} into mature SDP.

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FIG 1 SDP toxin production model. (A) Detectable forms of SdpC. pro-SdpC¹⁻²⁰³ contains an N-terminal signal peptide sequence. SdpC³³⁻²⁰³ is secreted, and the signal peptide is removed by signal peptidase. The disulfide bond between amino acid residues 141 to 147 is noted. SDP is produced from residues 141 to 181. The active toxin is secreted and has a disulfide bond between amino acid residues 141 to 147. (B) SDP production requires multiple steps. In the cytosol, full-length SdpC (pro-SdpC¹⁻²⁰³) is secreted via the Sec pathway. Following secretion, the signal peptidases SipS and SipT cleave the N-terminal signal peptide sequence of SdpC (17), and disulfide bond formation occurs independently of SdpAB. Finally, posttranslational cleavage of SdpC occurs via SdpAB to produce a 42-amino-acid SDP that will be secreted extracellularly as an active SDP peptide.

MATERIALS AND METHODS

Bacterial strains and growth. All strains used in the study are isogenic derivatives of PY79, a prototrophic derivative of *B. subtilis* strain 168, and are listed in Table 1 (18). Strains were routinely grown in Luria-Bertani (LB) broth and Difco sporulation medium (DSM) at 37°C, except for cultures grown overnight, which were grown at 30°C (19). Antibiotics were used at the following concentrations: chloramphenicol, 10 µg/ml; erythromycin plus lincomycin, 1 µg/ml and 25 µg/ml, respectively; kanamycin, 5 µg/ml; spectinomycin, 100 µg/ml; tetracycline, 10 µg/ml; and ampicillin, 100 µg/ml. The β-galactosidase chromogenic indicator 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) was used at a concentration of 100 µg/ml. Isopropyl β-D-thiogalactopyranoside (IPTG) was used at a final concentration of 1 mM. Bacterial strains were constructed by transformation of relevant genomic or plasmid DNA into *B. subtilis* competent cells prepared by the one-step method previously described (20).

Construction of plasmids. All DNA oligomers and plasmids used in this study are listed in Tables S1 and S2 in the supplemental material. The IPTG-inducible *P_{hs}-sdpC* integrated at *amyE* was constructed by PCR by amplifying *sdpC* from *B. subtilis* using oligonucleotides CDEP126 and CDEP127. The resulting PCR product was digested with HindIII and SphI, cloned into pDR111 (21), and digested with the same enzymes to create pCE106. The IPTG-inducible *P_{hs}-sdpA*, *P_{hs}-sdpB*, and *P_{hs}-sdpAB* genes were constructed by PCR by amplifying *sdpA* (CDEP124 and CDEP566), *sdpB* (CDEP567 and CDEP125), or *sdpAB* (CDEP124 and CDEP125) from *B. subtilis*. The resulting PCR products were digested with HindIII and SphI, cloned into pDP150 (22), and digested with the same enzymes to create pCE216 (*sdpA*), pCE315 (*sdpB*), and pTP092 (*sdpAB*). The sequence of the resulting plasmids was confirmed by se-

quencing (Iowa State University) and transformed into the wild-type (WT) *B. subtilis* strain PY79.

A Gateway destination vector was constructed to build N-terminal *gfp-sdpA* fusions (Invitrogen). This was generated by cloning the RfA cassette (Invitrogen) into pCE236 (pDR111-GFP; where GFP is green fluorescent protein), which had been digested with SphI and EcoRI and blunt ended with Klenow (NEB) to generate pJH183. N-terminal GFP-tagged SdpA (GFP-SdpA) was constructed by PCR by amplifying *sdpA* from *B. subtilis* by using oligonucleotides CDEP890 and CDEP566 and cloning into pEntrD-TOPO, resulting in pDT001. To construct a plasmid producing GFP-SdpA, *sdpA*⁺ was moved from pDT001 onto pCE291 by using LR Clonase II (Invitrogen), resulting in plasmid pDT002.

Site-directed mutagenesis of SdpC. Site-directed mutagenesis of pCE106 (*P_{hs}-sdpC*) was performed using the QuikChange site-directed mutagenesis kit (Agilent Technologies) according to the manufacturer's instructions. The SdpC signal peptide cleavage site mutant (*sdpC*^{T30H} mutant) was constructed using primer pairs CDEP640 and CDEP641 to generate plasmid pCE260. The SdpC disulfide bond single mutants were constructed using the following oligonucleotide pairs: CDEP912 and CDEP913 (*sdpC*^{C141A} mutant) and CDEP892 and CDEP893 (*sdpC*^{C147A} mutant). The *sdpC*^{C141A C147A} mutant was constructed by site-directed mutagenesis of pTP085 with CDEP1247 and CDEP1248 to generate pTP091. The resulting plasmids pTP085 (*sdpC*^{C141A} mutant), pTP076 (*sdpC*^{C147A} mutant), and pTP091 (*sdpC*^{C141A C147A} mutant) were confirmed by sequencing and transformed into *B. subtilis* PY79.

β-Galactosidase activity assays. Cultures were grown overnight in LB broth at 30°C, and 40 µl was spotted onto LB agar supplemented with 1 mM IPTG. Plates were incubated at 37°C for 4 h. Samples were harvested from the plates and resuspended in 1 ml of Z buffer (60 mM Na₂HPO₄, 40

TABLE 1 Strain list

Strain	Genotype	Reference
PY79	Prototrophic derivative of <i>B. subtilis</i> 168	18
EH273	Δ <i>sdpABC::kan</i>	3
CDE433	Δ <i>sigW::kan</i> Δ <i>sdpABCIR::tet</i>	6
TPM727	<i>pyrD::P_{sdpRI}-lacZ (kan) thrC::P_{hs}-sdpB (mls) sdpABC::cat</i>	
TPM758	Δ <i>sigW::kan</i> Δ <i>sdpABCIR::tet amyE::P_{hs}-sdpI (spec)</i>	
TPM1005	<i>pyrD::P_{sdpRI}-lacZ (kan) Δ<i>sdpABC::cat amyE::P_{hs}-sdpC^{T30H} (spec)</i></i>	
TPM1112	<i>pyrD::P_{sdpRI}-lacZ (kan) Δ<i>sdpABC::cat amyE::P_{hs}-sdpC^{C147A} (spec)</i></i>	
TPM1158	<i>pyrD::P_{sdpRI}-lacZ (kan) Δ<i>sdpABC::cat amyE::P_{hs}-sdpC^{C141A} (spec)</i></i>	
TPM1207	<i>pyrD::P_{sdpRI}-lacZ (kan) Δ<i>sdpABC::cat amyE::P_{hs}-sdpC^{C141A C147A} (spec)</i></i>	
TPM1349	<i>pyrD::P_{sdpRI}-lacZ (kan) Δ<i>sdpABC::cat</i></i>	
TPM1352	<i>pyrD::P_{sdpRI}-lacZ (kan) Δ<i>sdpABC::cat amyE::P_{hs}-sdpC (spec)</i></i>	
TPM1357	<i>pyrD::P_{sdpRI}-lacZ (kan) Δ<i>sdpABC::cat amyE::P_{hs}-sdpC (spec) thrC::P_{hs}-sdpB (mls)</i></i>	
TPM1359	<i>pyrD::P_{sdpRI}-lacZ (kan) Δ<i>sdpABC::cat amyE::P_{hs}-sdpC (spec) thrC::P_{hs}-sdpA (mls)</i></i>	
TPM1361	<i>pyrD::P_{sdpRI}-lacZ (kan) Δ<i>sdpABC::cat thrC::P_{hs}-sdpA (mls)</i></i>	
TPM1476	<i>pyrD::P_{sdpRI}-lacZ (kan) thrC::P_{hs}-sdpAB (mls) Δ<i>sdpABC::cat amyE::P_{hs}-sdpC (spec)</i></i>	
TPM1502	<i>pyrD::P_{sdpRI}-lacZ (kan) thrC::P_{hs}-sdpAB (mls) Δ<i>sdpABC::cat amyE::P_{hs}-sdpC^{T30H} (spec)</i></i>	
TPM1505	<i>pyrD::P_{sdpRI}-lacZ (kan) thrC::P_{hs}-sdpAB (mls) Δ<i>sdpABC::cat amyE::P_{hs}-sdpC^{C141A} (spec)</i></i>	
TPM1506	<i>pyrD::P_{sdpRI}-lacZ (kan) thrC::P_{hs}-sdpAB (mls) Δ<i>sdpABC::cat amyE::P_{hs}-sdpC^{C141A C147A} (spec)</i></i>	
TPM1507	<i>pyrD::P_{sdpRI}-lacZ (kan) thrC::P_{hs}-sdpAB (mls) Δ<i>sdpABC::cat amyE::P_{hs}-sdpC^{C147A} (spec)</i></i>	
TPM1510	<i>pyrD::P_{sdpRI}-lacZ (kan) thrC::P_{hs}-sdpAB (mls) Δ<i>sdpABC::cat</i></i>	
TPM1713	<i>pyrD::P_{sdpRI}-lacZ (kan) P_{hs}-sdpABC (cat) amyE::P_{hs}-GFP (spec)</i>	
TPM1444	<i>pyrD::P_{sdpRI}-lacZ (kan) Δ<i>sdpABC::cat thrC::P_{hs}-sdpBC mls amyE::P_{hs}-gfp-sdpA (spec)</i></i>	
TPM1438	<i>pyrD::P_{sdpRI}-lacZ (kan) Δ<i>sdpABC::cat thrC::P_{hs}-sdpB mls amyE::P_{hs}-gfp-sdpA (spec)</i></i>	
TPM1432	<i>pyrD::P_{sdpRI}-lacZ (kan) Δ<i>sdpABC::cat amyE::P_{hs}-gfp-sdpA (spec)</i></i>	
TPM1349	<i>pyrD::P_{sdpR}-lacZ (kan) Δ<i>sdpABC::cat</i></i>	

mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, 50 mM β -mercaptoethanol [pH 7.0]), and the optical density at 600 nm (OD₆₀₀) was determined. Lysozyme (10 μ g) was added to samples and incubated for 30 min at 37°C (19). Cell lysates were added to 96-well plates with 10 mg/ml ortho-nitrophenyl- β -galactoside (ONPG), and activity of β -galactosidase was measured every 2 min at an OD₄₀₅ for 40 min total. Data were analyzed as previously described (23).

SDP-mediated killing assay. Reporter cells which lack the ability to produce the SDP toxins, SDP-sensitive (Δ *sigW* Δ *sdpABCIR*; CDE433) or SDP-resistant (Δ *sigW* Δ *sdpABCIR amyE::P_{hs}-sdpI*; TPM758) cells, were grown to an OD₆₀₀ of 0.8 in LB broth with 1 mM IPTG. The reporter cells (10⁶) were inoculated into LB agar (0.7%)–1 mM IPTG, which was poured into plates and allowed to solidify. A culture of the SDP-producing strains grown overnight was subcultured 1:100 and grown in LB agar–1 mM IPTG for 4 h at 37°C. A total of 20 μ l of SDP-producing cells was spotted onto plates containing either SDP-sensitive (Δ *sigW* Δ *sdpABCIR*; CDE433) or SDP-resistant (Δ *sigW* Δ *sdpABCIR amyE::P_{hs}-sdpI*; TPM758) cells. Plates were incubated overnight at 37°C, and the zone of inhibition was determined.

Subcellular fractionation of cells. Cultures grown overnight were subcultured 1:100 in liquid DSM supplemented with 1 mM IPTG and grown for 4 h at 37°C. The cultures were separated into whole-cell and supernatant fractions by centrifugation. The supernatants were concentrated by methanol-chloroform extraction (24). Briefly, 2 ml of supernatant was mixed with 2 ml of 95% methanol and 500 μ l of chloroform. The samples were centrifuged at 13,000 $\times$ g for 10 min. The aqueous layer was removed, and 2 ml of 95% methanol was added. The samples were vortexed and centrifuged at 13,000 $\times$ g for 10 min. Precipitated extracts containing protein were resuspended in 100 μ l sample buffer (65.8 mM Tris-HCl [pH 6.8], 2% SDS, 26.3% [wt/vol] glycerol, 0.01% bromophenol blue, and 5% β -mercaptoethanol) with or without β -mercaptoethanol (Bio-Rad). The whole-cell pellets were lysed by being resuspended in 500 μ l lysis buffer with 10 μ g/ml lysozyme and incubated at 37°C for 10 min. The whole-cell lysates were methanol-chloroform extracted and resuspended in 100 μ l of 2 $\times$ sample buffer.

For determining membrane and cytosolic localization, whole-cell pellets were resuspended in 300 μ l of protoplast buffer (1 M sucrose and 60 mM Tris-Cl with 0.04 M MgCl₂). Lysozyme (20 μ g/ml) was added and incubated for 20 min at 37°C to degrade the peptidoglycan. Samples were centrifuged for 10 min at 5,000 $\times$ g. The supernatant which contained the cell wall fraction was removed and concentrated by methanol-chloroform precipitation as described above. The protoplasts were resuspended in 500 μ l lysis buffer (0.5 M EDTA, 0.1 M NaCl [pH 7.5]) and sonicated. Samples were then ultracentrifuged at 7°C for 1 h at 100,000 $\times$ g to separate the membrane from cytosol components. The supernatant at this step represented the cytoplasmic components, and the samples were concentrated using methanol-chloroform precipitation. The membrane or insoluble fractions were resuspended in 2 $\times$ sample buffer.

Immunoblot analysis of SdpC. Samples were heated for 10 min at 65°C and electrophoresed on a TGX Any kD SDS-polyacrylamide gel (Bio-Rad). The proteins were then transferred onto nitrocellulose and detected by being incubated with a 1:3,000 dilution of anti-SdpC antibodies (17), a 1:10,000 dilution of anti-GFP antibodies, or a 1:15,000 dilution of anti- σ^A antibodies, followed by incubation with a 1:10,000 dilution of goat anti-rabbit IgG (H+L)-horseradish peroxidase (HRP) conjugate from Bio-Rad.

In situ assay to monitor SDP. Cultures grown overnight were subcultured 1:100 in liquid DSM with 1 mM IPTG at 37°C for 4 h. Culture supernatants (25 ml) were concentrated by methanol-chloroform extraction and resuspended in 200 μ l 2 $\times$ sample buffer with β -mercaptoethanol. Samples were stored at –20°C until they were used for *in situ* assays (25). Concentrated supernatants were separated by a TGX 10% SDS-polyacrylamide gel (Bio-Rad). The polyacrylamide gel was washed in sterile water for 3 h and placed in a sterile petri dish to dry for 20 min. The gels were overlaid with LB agar (0.7%)–1 mM IPTG containing either 10⁶ SDP-sensitive (Δ *sigW* Δ *sdpABCIR*; CDE433) or SDP-resistant (Δ *sigW* Δ *sdpABCIR amyE::P_{hs}-sdpI*; TPM758) cells. Plates were covered in Parafilm and incubated overnight at 30°C.

Mass spectrometry analysis of strains to detect SDP. Each strain was inoculated on LB agar supplemented with 1 mM IPTG and then incubated

at 28°C for 5 days. The bacteria were then collected and spotted onto a matrix-assisted laser desorption ionization (MALDI) target plate and were mixed approximately 1:1 with a saturated solution of universal MALDI matrix in 78% acetonitrile containing 0.1% trifluoroacetic acid (TFA). The sample was dried and subjected to the Microflex MALDI-time of flight (MALDI-TOF) mass spectrometer (BrukerDaltonics). Mass spectra were obtained with FlexControl by scanning from m/z 400 to 10,000, and the resulting mass spectrometry data were analyzed by FlexAnalysis software (6).

RESULTS

Signal peptide cleavage is required for SDP activity. Previous studies identified the mature form of SDP as a secreted 42-amino-acid peptide with a disulfide bond which is processed from pro-SdpC, a 203-amino-acid protein (6). In earlier work, it was determined that pro-SdpC could be secreted via the general secretory pathway and required the secretion chaperone CsaA (17, 26). In addition, the signal peptidases SipS/T were shown to be required for efficient pro-SdpC cleavage when expressed in *E. coli* (17). However, these experiments were performed in the absence of *sdpAB* expression and did not address the role of pro-SdpC processing in the production of SDP. To determine if pro-SdpC cleavage was required for SDP production, we sought to block this processing. *B. subtilis* strains lacking both SipS and SipT are non-viable (27); thus, to determine if pro-SdpC cleavage by signal peptidases is required for SDP production, we constructed an SdpC mutant (SdpC^{T30H}) which we predicted to be not cleaved by signal peptidases. Signal peptidase cleavage of pro-SdpC is predicted to occur between residues A32 and K33 (28). The presence of a histidine residue at the −3 residue of the putative cleavage site rarely if ever occurs in *B. subtilis* signal peptides, suggesting that mutation of T30 to histidine would result in a form of SdpC which cannot be efficiently processed by *B. subtilis* SipT or SipS (29).

We performed immunoblot analysis using anti-SdpC antibodies on samples prepared from cells expressing the *sdpABC* operon under sporulating conditions. We separated the cellular proteins into supernatant, cell wall, membrane, and cytoplasmic fractions. Using anti-SdpC antibodies, we could detect an ~17-kDa protein, which corresponds to the approximate size of SdpC^{33–203}, in the supernatant and cell wall fractions of the cell (Fig. 2A). When we compared the SdpC^{T30H} (*sdpABC*^{T30H}) signal peptide mutant to wild-type SdpC, we observed a higher-molecular-mass form of SdpC (22 kDa), which remained membrane associated (Fig. 2A). Cells producing SdpC^{T30H} produced a reduced amount of SdpC^{33–203} which remained cell wall associated, and no SdpC^{33–203} was detected in the culture supernatant (Fig. 2A). This is consistent with the idea that the SdpC^{T30H} mutant blocks signal peptide cleavage. This suggests that SdpC^{33–203} is secreted and likely requires signal peptidase to process the pro-SdpC into mature SdpC^{33–203}.

To assay SDP toxin activity, we spotted cultures onto soft agar containing SDP-sensitive (Δ *sdpABCIR* Δ *sigW*) cells. This strain lacks the ability to produce the SDP toxin as well as both SdpI and σ^W , which induce independent mechanisms of resistance to SDP (3, 30, 31). We found that cells expressing SdpABC were able to induce a zone of inhibition when spotted on SDP-sensitive cells (Fig. 2C). In comparison, we found that cells producing SdpABC^{T30H} created a smaller zone of inhibition than the WT when spotted on SDP-sensitive cells (Fig. 2C).

SDP also induces expression of the *sdpRI* immunity operon (3). We tested the effect of the SdpC^{T30H} protein on expression of

FIG 2 Signal peptide cleavage is required for full secretion and activity of SDP. (A) SdpC subcellular localization. The relevant genotypes of the strains with respect to SdpABC are noted as ABC⁺ (TPM1476) and ABC^{T30H} (TPM1502). Cultures were fractionated into supernatant (S), cell wall (CW), membrane (M), and cytoplasm (C) as described in Materials and Methods. SdpC was detected by immunoblotting using anti-SdpC antibodies. (B) The effect of different combinations of SdpC^{T30H} on expression of *P*_{*sdpRI*}-*lacZ*. The relevant SdpABC phenotypes are indicated in the figure, and all strains contain *P*_{*sdpRI*}-*lacZ* (*pyrD::P*_{*sdpRI*}-*lacZ*⁺). The relevant genotypes of the strains with respect to SdpABC are noted as ABC⁺ (TPM1476), C⁺ (TPM1352), ABC^{T30H} (TPM1502), and C^{T30H} (TPM1005). (C) SDP zones of inhibition on SDP-sensitive cells. The relevant genotypes of the strains with respect to SdpABC are noted as ABC⁺ (TPM1476), C⁺ (TPM1352), ABC^{T30H} (TPM1502), and ABC^{T30H} (TPM1005). SDP-producing cultures were spotted on LB soft agar containing IPTG and SdpI[−] (SDP sensitive; CDE433 Δ *sigW* Δ *sdpABCIR* mutant). Plates were incubated at 37°C overnight.

*P*_{*sdpRI*}-*lacZ*. We found that cells producing SdpABC^{T30H} showed an ~10-fold decrease in induction of *P*_{*sdpRI*}-*lacZ* compared to cells expressing SdpABC (Fig. 2B). Taken together, these results suggest that signal peptide cleavage of SdpC is an essential step required for SDP production.

SdpAB are required for SDP activity. Since *sdpABC* reside in a single operon, we sought to determine the contribution of SdpAB to SDP production by constructing strains capable of expressing different combinations of the *sdpABC* genes from an IPTG-inducible promoter. We determined the effect of different combinations of the *sdpABC* genes on expression of *sdpRI* by monitoring a *P*_{*sdpRI*}-*lacZ* reporter fusion. We found that cells expressing *sdpABC*⁺ were able to fully induce expression of *sdpRI* (Fig. 3A). As previously reported, a deletion of the *sdpABC* genes blocked *P*_{*sdpRI*}-*lacZ* induction (Fig. 3A) (3). We observed that cells producing only SdpAB were unable to induce expression from the *sdpRI* operon (Fig. 3A). This result is consistent with previous observations that the absence of SdpC alone blocked induction of *P*_{*sdpRI*}-

FIG 3 SdpAB are required for induction of the *sdpRI* operon and SDP toxicity. (A) The effect of different combinations of SdpABC on expression of *P_{sdpRI}-lacZ*. The relevant SdpABC phenotypes are indicated in the figure, and all strains contain *P_{sdpRI}-lacZ* (*pyrD::P_{sdpRI}-lacZ*⁺). The relevant genotypes of the strains with respect to SdpABC are noted as ABC⁺ (TPM1476), A⁺ (TPM1361), B⁺ (TPM727), C⁺ (TPM1352), AC⁺ (TPM1359), BC⁺ (TPM1357), AB⁺ (TPM1510), and SdpABC⁻ (TPM1349). The β-galactosidase activity was assayed as described in Materials and Methods, and assays were performed in triplicate. The averages and standard deviations are shown. (B) SDP zones of inhibition on SDP-sensitive and SDP-resistant cells. All strains contained *P_{sdpRI}-lacZ*, and the relevant genotypes of the strains with respect to SdpABC are described above. Cultures were spotted on LB soft agar containing IPTG and either SdpI⁻ (SDP-sensitive; CDE433) or SdpI⁺ (SDP-resistant; TPM758) cells and incubated at 37°C overnight. (C) In-gel SDP peptide zone of inhibition on SDP-sensitive cells and SDP-resistant cells (SdpI⁺). The culture supernatants were prepared as described in Materials and Methods. Relevant SdpABC phenotypes are indicated in the figure as follows: ABC⁺ (TPM1476), C⁺ (TPM1352), AC⁺ (TPM1359), BC⁺ (TPM1357), and SdpABC⁻ (TPM1349). The gels were overlaid with LB soft agar and IPTG and contain 10⁶ SdpI⁻ (SDP-sensitive; CDE433) or SdpI⁺ (SDP-resistant; TPM758) cells. The plates were incubated overnight at 30°C.

lacZ (3). The expression of *sdpC*⁺ alone, however, was not sufficient to induce expression of *P_{sdpRI}-lacZ* (Fig. 3A). Expression of either *sdpAC*⁺ or *sdpBC*⁺ was not sufficient to increase expression of *P_{sdpRI}-lacZ* (Fig. 3A).

We next tested if SdpAB were required for SDP toxin production. We found that strains expressing *sdpABC*⁺ created a zone of inhibition when plated on SDP-sensitive (*ΔsigW ΔsdpABCIR*) cells which lack the immunity protein SdpI (Fig. 3B). This zone was absent in strains that do not express *sdpABC* (Fig. 3B). Production of SdpI in the *ΔsigW ΔsdpABCIR* strain is sufficient to provide immunity against SDP, as cells expressing *sdpABC*⁺ were unable to inhibit growth of the SdpI-producing strain (Fig. 3B) (3). Cells expressing either *sdpA*⁺, *sdpB*⁺, or *sdpC*⁺ individually were unable to produce a zone of inhibition on SDP-sensitive cells (Fig. 3B). Similar to the effect on induction of the *P_{sdpRI}-lacZ* fusion, cells expressing *sdpAB*⁺, *sdpAC*⁺, and *sdpBC*⁺ did not pro-

FIG 4 Secretion of SdpC^{33–203} does not require SdpAB. SdpC secretion is shown in the presence of different constructs of SdpABC. The relevant phenotypes of the strains with respect to SdpABC are indicated at the top of the figure. All strains contain *P_{sdpRI}-lacZ* (*pyrD::P_{sdpRI}-lacZ*⁺). The relevant genotypes of the strains are as follows: ABC⁺ (TPM1476), AC⁺ (TPM1359), BC⁺ (TPM1357), C⁺ (TPM1352), and SdpABC⁻ (TPM1349). Cultures were separated into supernatants and pellets as described in Materials and Methods. Samples were separated by SDS-PAGE, and SdpC was detected by immunoblotting using 1:3,000 rabbit anti-SdpC antibodies. Anti-σ^A antibodies were used to detect σ^A as the cytoplasmic control.

duce any detectable toxin activity (Fig. 3B). From these results, we conclude that in addition to expression of *sdpC*, expression of *sdpAB* is also required for both induction of *sdpRI* expression and SDP toxin activity.

To determine the relative size of the toxin being produced and demonstrate that the SDP toxin activity was in the culture supernatants, we performed an *in situ* assay (25). Culture supernatant samples were concentrated and then separated on an SDS-PAGE gel. The gel was then overlaid with SDP-sensitive (*ΔsigW ΔsdpABCIR*) or SDP-resistant (*ΔsigW ΔsdpABCIR amyE::P_{hs}-sdpI*⁺) cells. We observed a zone of inhibition present around the 5-kDa size range from the supernatants of cells producing SdpABC (Fig. 3C). This zone of inhibition is absent in cells producing SdpC, SdpAC, or SdpBC (Fig. 3C). The zone of inhibition produced by SdpABC strains was absent in gels overlaid with SDP-resistant cells (Fig. 3C). This suggests that SdpABC are required for production of the 5-kDa SDP toxin.

Export and secretion of SdpC^{33–203} does not require SdpAB. We reasoned that SdpAB could affect SDP production by altering export of SdpC^{33–203}. We used immunoblot analysis to determine the effect of SdpAB on SdpC^{33–203} export and secretion. Samples of culture supernatants and whole-cell extracts were prepared as described in Materials and Methods. The samples were separated by SDS-PAGE and probed with anti-SdpC antibodies (17). Samples were also probed with anti-σ^A antibody as a cytoplasmic and loading control. When immunoblot analysis was performed on strains expressing *sdpABC*⁺, we observed a predominant band, with an approximate size of ~17 kDa (Fig. 4), corresponding to SdpC^{33–203}. This band was absent in cells lacking the *sdpABC* genes, suggesting it is SdpC^{33–203}. We observed that SdpC^{33–203} protein levels were similar in the whole-cell pellets of all the strains, suggesting that SdpAB are not required for production of SdpC^{33–203}. Similarly, the levels of SdpC^{33–203} in the culture supernatants were not altered by the presence or absence of either SdpA, SdpB, or SdpAB (Fig. 4). Since export into supernatant still oc-

FIG 5 SdpAB are required for production of the 42-amino-acid SDP toxic peptide. Detection of SDP using mass spectrometry analysis from *B. subtilis* strains expressing different combinations of *sdpABC*. The relevant genotypes of the strains with respect to *sdpABC* are indicated at the sides of the figure: ABC⁺ (TPM1476), A⁺ (TPM1361), B⁺ (TPM727), C⁺ (TPM1352), AC⁺ (TPM1359), BC⁺ (TPM1357), AB⁺ (TPM1510), and SdpABC[−] (TPM1349). Samples were prepared as described in Materials and Methods.

curs, these results lead us to conclude that SdpAB are not essential for proper SdpC^{33–203} export.

SdpAB are required for SDP production. Our data indicate that cells producing SdpC in the absence of SdpAB do not exhibit SDP toxin activity. We hypothesized this could be due to either production of inactive SDP or failure to produce the SDP peptide. Therefore, we sought to detect SDP in the supernatants of cells expressing different combinations of *sdpABC*⁺ using MALDI-TOF mass spectrometry as previously described (6). We found that the 42-amino-acid peptide of SDP was observed in cells producing SdpABC (Fig. 5) (6). However, we were unable to detect a peptide in cells not producing SdpABC (Fig. 5). Similarly, the SDP peptide was not observed in cells expressing *sdpA*⁺, *sdpB*⁺, or *sdpC*⁺ individually or in combinations of *sdpAB*⁺, *sdpAC*⁺, or *sdpBC*⁺ (Fig. 5). These results suggest that expression of all three genes of the *sdpABC* operon is required for production of the toxic 42-amino-acid peptide SDP.

An SDP disulfide bond is not essential for activity. Although the mature form of SDP contains an intramolecular disulfide bond between C141 and C147 (6), the importance of the disulfide bonds for SDP activity is not known. Each of the cysteine residues was mutated individually and simultaneously to alanine residues. The ability of the resulting SdpC mutant protein to induce *P_{sdpRI}-lacZ* expression in the presence of SdpAB was determined. We found that cells producing SdpAB with SdpC^{C141A}, SdpC^{C147A}, or SdpC^{C141A C147A} resulted in an approximate 7-fold decrease in *P_{sdpRI}-lacZ* expression compared to expression resulting from cells using wild-type SdpC (Fig. 6A).

To determine if disulfide bond formation was essential for SDP toxin activity, we performed spot assays as previously described. Cells that express *sdpABC*⁺ produce a zone of inhibition when spotted in a lawn of SDP-sensitive cells (Fig. 6B). We observed that when either SdpC^{C141A}, SdpC^{C147A}, or SdpC^{C141A C147A} were pro-

FIG 6 SDP disulfide bond formation is not essential for SDP activity and is independent of SdpAB. (A) β-Galactosidase activity of SdpC cysteine mutants in the presence and absence of SdpAB. All strains contain *P_{sdpRI}-lacZ* (*pyrD*::*P_{sdpRI}-lacZ*⁺). The figures are labeled for their relevant *sdp* genotypes and are as follows: ABC⁺ (TPM1476), C⁺ (TPM1352), ABC^{C141A} (TPM1505), C^{C141A} (TPM1158), ABC^{C147A} (TPM1507), C^{C147A} (TPM1112), ABC^{C141A C147A} (TPM1506), and C^{C141A C147A} (TPM1207). The β-galactosidase activity assays were performed in triplicate as described in Materials and Methods. The averages and standard deviations are shown. (B) Toxic effect of SDP cysteine single and double mutants on SDP-sensitive (SdpI[−]) cells (CDE433) and SDP-resistant (SdpI⁺) cells (TPM758). The figures are labeled for their relevant *sdp* genotypes and are as follows: ABC⁺ (TPM1476), C⁺ (TPM1352), ABC^{C141A} (TPM1505), C^{C141A} (TPM1158), ABC^{C147A} (TPM1507), C^{C147A} (TPM1112), ABC^{C141A C147A} (TPM1506), and C^{C141A C147A} (TPM1207). (C) SdpC^{33–203} disulfide bond formation in the presence and absence of SdpAB. Whole-cell cultures were prepared as described in Materials and Methods. Final samples were resuspended in 2× sample buffer with (+) or without (−) β-mercaptoethanol. The figures are labeled with their relevant *sdp* genotypes, as follows: ABC⁺ (TPM1476) and C⁺ (TPM1352).

duced in the presence of SdpAB, there was killing of SDP-sensitive cells, but the zones of inhibition were smaller than those produced when wild-type SdpC was used (Fig. 6B). These results suggest that the disulfide bond in SDP is required for maximum SDP activity but is not essential for toxin activity.

SdpC disulfide bond formation occurs independently of SdpAB. Our data suggest that SdpAB most likely affect SDP production posttranslationally. Our previous results show that disulfide bond formation is not essential, as cells expressing SdpABC^{C141A C147A} retain some SDP activity. One hypothesis is that SdpAB are involved in disulfide bond formation and thus are required for SDP activity. To test this, we compared the ability of cells producing either SdpC^{C141A}, SdpC^{C147A}, or SdpC^{C141A C147A} to induce *P_{sdpRI}-lacZ* expression in the presence and absence of

SdpAB. We found that cells producing only SdpC were also unable to induce $P_{sdpRI-lacZ}$ expression (Fig. 6A). Cells producing SdpC^{C141A}, SdpC^{C147A}, or SdpC^{C141A C147A} alone were unable to induce expression of $sdpRI$ (Fig. 6A). Similarly, SdpC^{C141A}, SdpC^{C147A}, and SdpC^{C141A C147A} were dependent upon SdpAB for production of toxin activity, as cells producing these SdpC mutants in the absence of SdpAB were unable to produce a zone of inhibition (Fig. 6B). These results suggest that SdpAB have an activity that is independent of SDP disulfide bond formation.

To further confirm that disulfide bond formation occurred independently of SdpAB, we resuspended whole-cell pellets in the presence or absence of the reducing agent, β -mercaptoethanol. We observed a 17-kDa band corresponding to SdpC^{33–203} when the cell pellets from cells expressing SdpABC were resuspended in sample buffer with β -mercaptoethanol (Fig. 6C). However, in the absence of β -mercaptoethanol, SdpC^{33–203} migrates slower and thus appears larger, ~20 kDa (Fig. 6C). This is consistent with altered mobility due to reduction of the disulfide bond by the β -mercaptoethanol. We observed similar migration patterns of SdpC^{33–203} in cells producing only SdpC and in cells producing SdpABC (Fig. 6C). This suggests that SDP disulfide bond formation occurs in an SdpAB-independent manner and likely prior to processing of SdpC^{33–203} into SDP.

SdpA is a cytoplasmic protein. Based upon sequence analysis, SdpB is suggested to be a multipass membrane protein; however, the localization of SdpA is unclear. To determine where SdpA localizes, we constructed and expressed a GFP-SdpA fusion in *B. subtilis*. We found that the GFP-SdpA fusion protein could complement a strain lacking SdpA for expression of a $P_{sdpRI-lacZ}$ transcriptional fusion (see Fig. S1A in the supplemental material). We also determined that GFP-SdpA complemented a strain lacking SdpA for toxin production (Fig. S1B). Although GFP-SdpA complemented to a slightly lower level than wild-type SdpA for both $P_{sdpRI-lacZ}$ and toxin production, our data indicate that the GFP-SdpA fusion was functional. We then performed subcellular localization experiments and determined that the majority of GFP-SdpA was cytosolic, although a small portion was found in the insoluble fraction, suggesting that at some level it may also associate with the membrane (see Fig. S1C).

DISCUSSION

Production of SDP requires multiple steps. SDP is a 42-amino-acid antimicrobial peptide that is derived from the internal cleavage of SdpC (6). Our evidence suggests that production of mature SDP requires multiple processing events. First, pro-SdpC is secreted via the general secretory pathway. Subsequently, pro-SdpC is processed by signal peptidases, most likely SipS and/or SipT, which results in production of SdpC^{33–203} (Fig. 1) (17). A disulfide bond is formed in SdpC^{33–203} between cysteine residues C141 and C147 (6). This disulfide bond is formed independently of SdpAB, and we hypothesize that it requires one of the known disulfide bond isomerases in *B. subtilis*, BdbB and/or BdbC (32). Our work provides evidence that the disulfide bond is not essential for SDP toxic or signaling activities (Fig. 5). We hypothesize that an SDP disulfide bond confers increased stability and/or activity. Finally, SdpC^{33–203} is then processed by unknown proteases to produce mature SDP (Fig. 1). These proteases remove the N-terminal amino acids 33 to 140 and the C-terminal amino acids 182 to 203 of SdpC. In principle, there should be equal molar amounts of both the N-terminal peptide, SdpC^{33–140}, and the C-terminal pep-

tide, SdpC^{182–203}, for every molecule of SDP present. However, we did not detect any of the predicted cleavage products by immunoblotting. This could be due to either (i) the absence of antibody epitopes on these peptides, (ii) peptides which are rapidly degraded, or (iii) low-occurrence events which we cannot detect. We were also unable to detect the production of peptides corresponding to SdpC^{182–203} (2.1 kDa) using mass spectrometry, which should have detected peptides below 10 kDa. This raises the possibility that the resulting cleavage products may be rapidly degraded. Our work shows that SdpAB are required to produce SDP from SdpC^{33–203}; however, the mechanism by which they function is still unclear.

Possible roles for SdpA and SdpB. The *sdpABC* operon is a unique set of genes which has homologs in a very limited number of sequenced bacterial genomes, including *Stigmatella aurantiaca*, *Myxococcus xanthus*, *Streptomyces* sp. MG1, and *Bacillus clausii*. SdpA is predicted to be a 158-amino-acid protein with no homology to any proteins of known function, although our data suggest that it is a primarily a cytoplasmic protein (see Fig. S1C in the supplemental material). There are several models to address SdpA function in the production of SDP. Secretion of SdpC in the absence of SdpAB requires the cytosolic chaperone CsaA (26). It was shown that CsaA binds several regions of pro-SdpC (26). It is possible that SdpA could act as an alternate chaperone to aid in proper export of SdpC. However, our data suggest that the absence of SdpAB does not block export of SdpC. It is also possible that SdpA could act in a complex with SdpB since the absence of either protein results in very similar phenotypes: no toxin activity and decreased expression of $P_{sdpRI-lacZ}$.

Unlike SdpA, SdpB shares homology to a family of proteins which are distantly related to the human enzyme vitamin K-dependent gamma carboxylase (VKD- γ -carboxylase) (33, 34). In humans, VKD- γ -carboxylases change glutamic acid residues in blood clotting factors into γ -carboxylated glutamic acid (35). VKD- γ -carboxylase requires epoxidation of vitamin K for this modification (36). The SdpB homology to VKD- γ -carboxylases is restricted mostly to the N-terminal portion of VKD- γ -carboxylases specifically from amino acids 13 to 283 (34). This region has been identified by bioinformatics as a horizontally transferred transmembrane domain and is found in a range of bacteria. VKD- γ -carboxylase homologs are present in the marine mollusk *Conus textile*, which produces numerous posttranslationally modified small peptides known as conotoxins (33). Some of these peptides contain γ -glutamic acid residues, and the presence of a putative VKD- γ -carboxylase suggests a possible role for VKD- γ -carboxylase homologs in modifying some of these conotoxins (33). This raises the intriguing possibility that SdpB could perform a similar function in SDP production. pro-SdpC has 10 glutamic acid residues; however, there are no glutamic acid residues present in the mature form of SDP.

Although the most closely related SdpB homologs are encoded in an operon with SdpA and SdpC homologs, there are more distant SdpB homologs present in other bacteria which lack clear SdpA and SdpC homologs. Only the SdpB homolog from *Leptospira borgpetersenii* has been studied (37). Experimental data suggested that the *Leptospira* homolog has an unregulated epoxidase activity but no detectable carboxylase activity (37). This led the authors to suggest that *Leptospira* may encode an enzyme with an unknown enzymatic activity (37). We hypothesize that SdpB encodes an enzyme required for SdpC processing. The most direct

model would be that SdpB acts directly as the protease responsible for one or more of the cleavage events required for production of mature SDP. However, SdpB may also function as an enzyme in a less direct manner. For example, SdpB may posttranslationally modify SdpC, and this modification could then allow SdpC to be cleaved by other proteases. Thus, SdpB would be required for the initial step of SdpC processing, although not directly cleaving SdpC.

We have identified several steps required for the production of SDP. In addition, we have identified two proteins, SdpA and SdpB, which are essential for the production of the antimicrobial peptide SDP. SdpAB are required for the production of SDP, but the precise functions of SdpAB are still unknown. Further studies are needed to resolve these hypotheses.

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