Investigations into the Mechanism of Action of Sublancin

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Abstract

Antimicrobial resistance is a global threat that poses a rising concern. One underlying challenge is the limited number of targets in bacteria affected by the current pool of antibiotics. To potentially help find new targets, we studied a member of the class of antimicrobial natural products named glycocins. We examined the mode of action of sublancin, which contains an unusual and essential glucosylated Cys residue, by monitoring macromolecular synthesis. Sublancin negatively affected DNA replication, transcription, and translation without affecting cell wall biosynthesis. In addition, we confirmed that the presence of the PTS sugar glucose in the medium negatively impacted antimicrobial activity of sublancin. Additionally, sublancin analogues carrying different sugars retained their antimicrobial activity regardless of which sugar was attached to the peptide or the carbon source used. These data suggest a novel mechanism upstream of transcription and translation and are consistent with previous studies suggesting that the glucose uptake system is involved.

Graphical Abstract

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Supporting Information

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Supporting Figures S1–S10 and Table S1, including macromolecular synthesis assays, ¹⁴C-glucose incorporation assays, sublancin–sugar analogue activity analysis, schematic diagrams, HPLC traces, and HRMS spectra (PDF)

The authors declare no competing financial interest.

Keywords

RiPP; bacteriocin; glycocin; glycopeptide; antibiotic

Ribosomally synthesized and post-translationally modified peptides (RiPPs) are a rapidly emerging class of natural products.^{1,2} One class of RiPPs are the glycocins (glycopeptide bacteriocins). Glycocins are characterized by one or more conjugated sugar moieties and two disulfide bonds that stabilize the final product.³ Sublancin, produced by *Bacillus subtilis* 168, is a member of the glycocin family (Figure 1a) and displays antimicrobial activity against a subset of Gram-positive bacteria, including methicillin resistant *Staphylococcus aureus* (MRSA).^{4,5} The production of sublancin begins with translation of the *sunA* gene encoded on the *SPβ* prophage.⁵ A glycosyltransferase, encoded by *sunS*, then attaches glucose to Cys22 of the precursor peptide SunA.⁴ Two disulfide bonds are subsequently generated by two thiol–disulfide oxidoreductases encoded by *bdbA* and *bdbB*.⁶ The mature bioactive peptide is produced after the removal of a leader peptide by the protease domain of a bifunctional enzyme encoded by *sunT*, which also transports the peptide out of the producing cell.

The solution phase NMR structure of sublancin has been elucidated (Figure 1b),⁷ but the mechanism by which sublancin kills target bacteria remains elusive. Previous studies have shown that the phosphoenolpyruvate:sugar phosphotransferase system (PTS) of *B. subtilis* plays a critical role in sensitivity to sublancin, and deletion of the *ptsGHI* operon results in resistance to sublancin.⁸ In addition, the presence of PTS sugars in LB medium decreased sensitivity to sublancin.⁸ In this study, we investigated inhibition imposed by sublancin on the synthesis of macromolecules in target cells as well as the bioactivity of different sugar analogues of sublancin against sublancin sensitive strains.

Sublancin was previously shown not to affect the integrity of the bacterial membrane by measuring propidium iodide uptake as well as performing LIVE/DEAD BacLight bacterial cell viability assays on *B. subtilis* 168 *SPβ*.⁸ This strain has the *SPβ* prophage removed such that sublancin immunity genes are not present. Furthermore, individual gene deletions of this strain were investigated in prior studies as discussed above.⁸ Here, we applied macromolecular synthesis (MMS) assays by monitoring the incorporation of radioactive precursors involved in the cell wall, DNA, RNA, and protein synthesis pathways in *B. subtilis* 168 *SPβ*.

RESULTS AND DISCUSSION

The minimum inhibitory concentrations (MICs) were first determined by the broth dilution method. The MIC of sublancin against *B. subtilis* 168 *SPβ* was 0.312 μ M. The MICs of antibiotics used as controls in this study, including vancomycin, ciprofloxacin, rifampicin, and chloramphenicol, were 2.5, 0.312, 1.25, and 10 μ M, respectively. ¹⁴C-*N*-acetyl-D-glucosamine (GlcNAc), ¹⁴C-thymidine, ³H-uridine, and a ¹⁴C-labeled mixture of L-amino acids were used as the precursors to track the incorporation into the cell wall, DNA, RNA, and proteins, respectively. Each of the radiolabeled precursors was added into cell culture in the presence of sublancin. At set time points, the macromolecules were isolated using

established protocols, and the incorporation of radioactivity into macromolecules was determined.⁹ The final radioactivity was normalized by correction to the OD₆₀₀ of untreated cell culture to account for cell death and cell growth inhibition caused by antibiotic treatment. The normalized incorporation of radioactivity for each macromolecular synthesis pathway was then plotted as a function of time for three independent replicates (Figure 2 and Figures S1–S4).

DNA synthesis was disrupted significantly even at 0.5× MIC of sublancin (Figure 2a). The effect was similar to that observed with the positive control ciprofloxacin. In contrast, the disruption of radiolabel incorporation by the negative control rifampicin was significantly smaller. Thus, sublancin significantly inhibits DNA biosynthesis in *B. subtilis* 168 SPβ. The effects of sublancin on disrupting RNA and protein biosynthesis pathways were also significant. Sublancin at 2× MIC interrupted ³H-uridine incorporation slightly less than the positive control rifampicin, and the inhibition was much stronger than the negative control ciprofloxacin at 2× MIC (Figure 2b). Similar results were observed for amino acid incorporation (Figure 2c). Therefore, sublancin directly or indirectly disrupts macromolecule synthesis pathways, including DNA, RNA, and protein biosynthesis.

In contrast, the effect of sublancin on cell wall synthesis at early time points was comparable to the negative control ciprofloxacin and considerably less than the positive control vancomycin under identical conditions. After 60 min, the effect of sublancin on cell wall synthesis was greater than that of the negative control but still remained considerably lower than the positive control (Figure 2d).

The glucose PTS was previously proposed as either the target or potential entry site of sublancin in target cells.⁸ To further investigate this hypothesis, we measured incorporation of radiolabeled ¹⁴C-glucose into *B. subtilis* 168 SPβ cells grown in LB medium in the presence of 0.1 and 0.01% glucose (Figure 3 and Figures S5 and S6). After OD₆₀₀ adjustment, the sublancin-treated samples clearly incorporated less radioactivity into macromolecules than control samples, an observation consistent with sublancin reducing/preventing glucose uptake. Interestingly, the reduction in radioactivity at all three time points was much more significant in 0.01% glucose-containing medium than in 0.1% glucose-containing medium (Figure S7). This observation is consistent with potential competition between glucose and sublancin for the PTS. Higher concentrations of glucose would lower the effectiveness of sublancin to prevent glucose uptake. These data are also consistent with glucose at even higher concentrations, decreasing the antimicrobial activity of sublancin.⁸

Previous studies have shown that the glycosyl transferase SunS displays considerable promiscuity with regards to the nucleotide sugar substrate and can be used to install different sugars on Cys22 of SunA.⁴ In addition, all sugar-modified peptides were amenable to oxidative folding, producing various sublancin analogues with the correct disulfide connectivity.⁴ The consequences of these substitutions with respect to antimicrobial activity have not been investigated in much detail. Here, we investigated these previously reported analogues of sublancin carrying *N*-acetylglucosamine (GlcNAc), galactose (Gal), and mannose (Man) (Figure 1c). Surprisingly, the MIC values of these sublancin analogues against *B. subtilis* SPβ were less variable than anticipated (Table 1). The biological activity

of the analogues was also evaluated against *B. halodurans* C-125, resulting again in antimicrobial activity regardless of the sugar that was installed (Figure S8).

As noted, glucose is able to protect sensitive *Bacillus* cells from the effect of sublancin.⁸ Therefore, to evaluate the effect of different sugar sources, *B. halodurans* C-125 cells were spotted on M9 minimal media agar plates supplemented with a single carbon source (Glc, GlcNAc, Man, or Gal) and treated with sublancin and its analogues. Only glucose was able to suppress the antimicrobial activity of sublancin and its analogues (Table 2 and Figure S8). These compounds were also tested against four different sublancin-resistant *B. halodurans* C-125 mutant strains that contain mutations in the PTS with nisin used as a positive control.⁸ None of the sublancin analogues were active against the resistant strains, showing that the resistant mutations in the glucose PTS also affect sublancin analogues carrying nonglucose sugars. Collectively, these studies suggest that the analogues interact with the same target as wild-type sublancin and are not redirected to new PTS targets specific for other sugars when those sugars are installed on Cys22.

In summary, this study demonstrates that sublancin treatment affects DNA replication and transcription and RNA translation. Although inhibited, protein and RNA biosynthesis are likely not direct targets of sublancin because if they were, one would not expect strong inhibition of upstream DNA biosynthesis (e.g., rifampicin does not strongly inhibit DNA biosynthesis). The incorporation of precursors into the cell wall was also not affected by sublancin. Thus, while DNA biosynthesis cannot be ruled out at present, other macromolecular synthesis pathways appear not to be the direct target of sublancin. Instead, the collective observations of the effects of sublancin on macromolecular biosynthesis are most consistent with another upstream central process being negatively affected, which leads to subsequent disruption of DNA, RNA, and protein biosynthesis. Previously, the glucose PTS system was implicated on the basis of resistance mutants and transcriptional analysis.⁸ Furthermore, the presence of glucose in the media antagonizes the activity of sublancin, and conversely, sublancin decreases the amount of glucose uptake. These observations are consistent with, but do not prove, a competition between glucose and sublancin for the glucose PTS. Importantly, sublancin cannot simply inhibit the function of the glucose PTS because that would not lead to cell death under the growth conditions used, and it would also not explain the downregulation of the glucose PTS upon sublancin treatment observed in transcriptional profiling.⁸ Instead, these observations may be explained if sublancin resulted in a currently still unknown deleterious gain-of-function of the glucose PTS.

Our studies also show that the C2 hydroxyl of Glc can be replaced by an acetylated amine and that the stereochemistry at C2 and C4 of the hexose installed on the peptide is not critical, even though the presence of a sugar is required for sublancin activity.¹⁰ The results with resistant mutant strains suggest that the glucose PTS remains important for the activity of these analogues carrying different sugars. This suggestion is further supported by the observation that only addition of glucose to the medium, and not other sugars, decreased the activity of sublancin and its analogues. Thus, the involvement of the glucose PTS in the mode of action of sublancin appears now firmly established, but the exact mechanism of its activity requires further investigation.

It is interesting to compare the results of this study with those on glycocin F, produced by *Lactobacillus plantarum*. This glycocin carries two GlcNAc moieties, one on the equivalent loop connecting the two disulfide-linked helices and one on the C-terminal tail.^{11,12} Its activity can be counteracted by adding GlcNAc to the growth medium, but not other sugars,¹¹ and recent studies showed that it is the GlcNAc on the interhelical loop that appears responsible for its activity.^{13,14} A main difference in addition to glycocin F carrying two GlcNAc and sublancin only one Glc is that glycocin F is bacteriostatic, and sublancin is bactericidal.^{8,11} Glycocin F is thought to interact with the GlcNAc PTS through a currently unknown mechanism with the C-terminal GlcNAc possibly responsible for its specificity.^{3,13} Thus, glycocins have some commonalities in their activities but also many differences that future studies may be able to unravel. The glycocins are part of an increasing group of compounds that target sugar transfer systems,^{15–17} but the exact molecular details for these compounds are still poorly understood.

EXPERIMENTAL SECTION

Sample Preparation and Strain Growth Conditions.

Sublancin was isolated as previously described.¹⁰ The structure and the purity of the peptide were confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and high performance liquid chromatography (HPLC) using a Shimadzu Prep-instrument. MALDI-TOF MS was performed at the Mass Spectrometry Laboratory of the School of Chemical Sciences at UIUC using a Bruker Daltonics UltrafleXtreme MALDI TOF/TOF instrument. *B. subtilis* SP β was grown in refined LB at 37 °C. Refined LB was prepared as described previously.¹⁸

Antimicrobial Activity Assay for MIC Determination.

A 96-well microtiter plate was prepared as mentioned previously.¹⁸ *B. subtilis* SP β was grown in refined LB at 37 °C overnight. The culture was diluted to 10⁵ CFU/mL in refined LB media in each well. Sublancin was dissolved in water and serially diluted to obtain working concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, 0.19, and 0.09 μ M. OD₆₀₀ readings were obtained using a Synergy H4 Hybrid Multi-Mode Microplate BioTek plate reader. Two negative controls, cell-free untreated refined LB medium, and untreated cell culture, were used in this experiment.

Macromolecular Synthesis Assays.

Radiolabel incorporation experiments were carried out similarly as previously described.⁹ *B. subtilis* SP β cells were inoculated into refined LB medium and incubated at 37 °C until the OD₆₀₀ reached 0.4. Cells were centrifuged for 10 min at 4500g at room temperature. The cell pellet was then resuspended in prewarmed refined LB medium at 37 °C to a final cell density of OD₆₀₀ 0.2. Five sets of tubes were prepared, with each set containing eight tubes (see Figure S9). Then, 0.6 mL of cell culture at OD₆₀₀ 0.2 was distributed into each of the first set of tubes. These were used for the radioactivity assays. The same cells were also distributed to each of the other four sets of tubes. These tubes were used to measure the OD₆₀₀ at each time point to allow for normalization. A final concentration of 0.8 μ Ci/mL radiolabeled precursors was added to the first set of tubes. All tubes were incubated for 15

min, and 0.1 mL of radioactive culture was removed from the first set of tubes and transferred into tubes containing 1 mL of 10% TCA. The radioactivity in the macromolecular fraction from these tubes was used as the $t = 0$ time points on the graph. All tubes were then treated with either sublancin or control at 0.5 $\times$, 1 $\times$, 2 $\times$, or 4 $\times$ MIC and incubated at 37 °C. At desired time points (20, 40, 60, and 80 min), an aliquot of 0.1 mL of treated radioactive culture was removed and transferred to tubes that contained 1 mL of 10% TCA. The OD₆₀₀ of the nonradioactive cultures was also measured at each time point to normalize the measured radioactivity and minimize effects due to cell death and growth inhibition. The radioactive samples were incubated on ice for 1 h and passed through glass microfiber filters (GE Healthcare 1822025). Each filter was washed with 10 mL of 5% TCA three times followed by 5 mL of 75% ethanol three times. The filters were allowed to air-dry for 10 min and were placed in scintillation fluid. Radioactivity was then determined on a scintillation counter (PerkinElmer Tri-Carb 2910 TR). The radiolabels and their corresponding positive and negative antibiotic controls were: Cell wall: [¹⁴C] *N*-acetyl-D-glucosamine (American Radiolabeled Chemicals, ARC 0105), positive control: vancomycin, negative control: ciprofloxacin. DNA: [¹⁴C] thymidine (American Radiolabeled Chemicals, ARC 1219), positive control: ciprofloxacin, negative control: rifampicin. RNA: [5-³H] uridine (PerkinElmer, NET174250UC), positive control: rifampicin, negative control: ciprofloxacin. Amino acids: [¹⁴C] L-amino acid mixture (PerkinElmer, NEC850E050UC), positive control: chloramphenicol, negative control: ciprofloxacin. All experiments were conducted independently three times.

¹⁴C-Glucose Incorporation.

The procedure was the same as that listed above, except two concentrations (0.1% and 0.01%) of glucose were added in the refined LB medium to grow *B. subtilis* 168 SP β . A final concentration of 0.8 μ Ci/mL [¹⁴C]-glucose (PerkinElmer, NEC042X050UC) was added.

Preparation of Sublancin Analogues.

Sublancin and sublancin analogues were prepared by *in vitro* modification of His₆-SunAXa by SunS to install the sugars.⁴ Oxidative folding afforded the disulfide linkages, and the leader peptide was removed by proteolytic cleavage by Factor Xa. Sugar-modified His₆-SunA Xa was prepared in 100 μ L of 50 mM Tris (pH 7.5), 1 mM MgCl₂, 1 mM TCEP, 5 mM NDP-sugar, 200 μ M His₆-SunA Xa, and 2 μ M His₆-SunS. The reaction was incubated at 25 °C for 12 h. The extent of sugar modification was verified by removing a 5 μ L aliquot of the reaction, quenching with 5% TFA to pH 1–2, desalting using a ZipTip_{C18}, and analysis by MALDI-TOF and ESI Q/TOF MS. Following analysis, the disulfides were formed by addition of Tris (pH 7.5), oxidized glutathione (GSSG), and reduced glutathione (GSH) to final concentrations of 50, 2, and 2 mM, respectively. The total volume of the oxidative folding reaction was 100 μ L, and the reaction was incubated at 25 °C for an additional 12 h. The extent of disulfide formation was monitored by removing a 5 μ L aliquot of the reaction, quenching with 5% TFA to pH 1–2, desalting using a ZipTip_{C18}, and analyzing by MALDI-TOF MS. The leader peptide was proteolytically cleaved by the addition of NaCl and CaCl₂ to 100 and 2 mM, respectively, and the addition of Factor Xa to 0.075 mg/mL. The reaction was incubated at 25 °C for 1 h, and the extent of cleavage was

monitored by MALDI-TOF MS. Disulfide formation was observed as a peak with a –4 Da mass difference compared with material that was not subjected to oxidative folding.

Activity of Sublancin–Sugar Analogues When Sugars Are Added to the Growth Media of *Bacillus* Cells.

For each prepared sublancin analogue, 15 μL of the oxidative folding reactions described above was spotted on an overnight culture of sensitive *B. halodurans* C-125 or sublancin-resistant *B. halodurans* C-125. *B. halodurans* C-125 strains were grown in LB or M9 minimal media, supplemented with various carbon sources, under aerobic conditions at 37 °C for 16 h. Ninety-six-well agar plates were prepared by combining 20 mL of molten LB or M9 minimal medium agar (cooled to 42 °C) with 100 μL of dense overnight culture (approximately 10^8 to 10^9 CFU/mL). The seeded agar was poured into a sterile OmniTray (Nunc) and allowed to solidify at 25 °C for 30 min. An additional 30 mL of molten LB medium was cooled to 42 °C, combined with 150 μL of culture, and poured over the lower solidified agar layer. A sterile 96-well PCR plate was placed in the molten agar upper layer, and the agar was allowed to solidify at 25 °C for 45 min. After sufficient solidification, the 96-well PCR plate was removed. The total 20 μL volume of each concentrated *in vitro* reaction was dispensed into separate newly formed wells. Authentic sublancin standards were spotted in 15 μL volumes at the concentrations indicated. Plates were left at 25 °C for 24 h, and antibacterial activity was qualitatively determined by the presence or absence of growth inhibition.

MIC Determinations of Sublancin Analogues against *B. subtilis* 168 SP β .

Sublancin and sublancin analogues were prepared as mentioned above except the SunS *in vitro* incubation reaction now contained sublancin aglycone core peptide and 1 mM NDP-sugar. Following MALDI-TOF and ESI Q/TOF MS analysis, the disulfides of the modified sublancin and sublancin analogues were formed by addition of Tris (pH 7.5), cystamine, and cysteamine to final concentrations of 50, 10, and 10 mM, respectively. The analogues were purified using a C5 Phenomenex column (10 μm , 100 Å, 250 $\times$ 10 mm) with an Agilent preparative HPLC and eluted at 18.2–18.5 min with a gradient of 2% solvent B to 100% solvent A (solvent A = 0.1% TFA in water, solvent B = 0.1% TFA in 100% MeCN) over 34.0 min at a flow rate of 8.0 mL/min. Prior to determination of MIC values, the purity of samples was checked using a C18 Macherey Nagel column (5 μm , 110 Å, 250 $\times$ 4.6 mm) with an Agilent analytical HPLC system (Figure S10). MIC determinations were performed as described above against sublancin sensitive strain *B. subtilis* SP β . HRMS characterizations of the different sublancin analogues are shown in Figure S10 and Table S1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

CFU	colony-forming unit
ESI	electrospray ionization
Gal	galactose
Glc	glucose
GlcNAc	<i>N</i> -acetylglucosamine
HPLC	high performance liquid chromatography
LB	lysogeny broth
MALDI	matrix assisted laser desorption/ionization
Mann	mannose
MIC	minimum inhibitory concentration
MMS	macromolecular synthesis
MRSA	methicillin resistant <i>Staphylococcus aureus</i>
MS	mass spectrometry
NDP sugar	nucleotide diphospho sugars
NMR	nuclear magnetic resonance
PTS	phosphotransferase system
RiPPs	ribosomally synthesized and post-translationally modified peptides
TCA	trichloroacetic acid
TCEP	tris(2-carboxyethyl)-phosphine
TOF	time-of-flight

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Figure 1.

(A) Structure of sublancin. (B) Three-dimensional solution structure of sublancin. (C) Structures of sugars attached in this work to Cys22 of sublancin.

Figure 2.

Sublancin treatment results in the inhibition of DNA, RNA, and protein biosynthesis without affecting cell wall synthesis. Time dependent incorporation of (A) ^{14}C -thymidine, (B) ^3H -uridine, (C) ^{14}C -L-amino acids, and (D) ^{14}C -N-acetyl-D-glucosamine in *B. subtilis* SP β . Positive controls (green) used were (A) ciprofloxacin, (B) rifampicin, (C) chloramphenicol, and (D) vancomycin. Negative controls were untreated cell culture (black) as well as (A) rifampicin, and (B–D) ciprofloxacin (blue). The data shown are from three independent experiments (Figures S1–S4).

Figure 3.

Incorporation of ^{14}C -glucose in *B. subtilis* SP β after sublancin treatment when (A) 0.01% glucose or (B) 0.1% glucose was added to LB medium. Cell culture without antibiotic treatment was used as control and was set to 100% at each time point. The radioactivity of each antibiotic treated culture was normalized by comparing the OD₆₀₀ of treated and untreated cell culture. The data shown are from three independent experiments (Figures S5 and S6).

Table 1.MICs of Sublancin Analogues against *B. subtilis* SP β

sublancin analogues	MIC (nM)
sublancin ^a	156
sublancin–Glc ^b	156
sublancin–GlcNAc ^b	625
sublancin–Man ^b	312
sublancin–Gal ^b	156

^aPurified from *B. subtilis* 168.^bPrepared *in vitro*.

Table 2.

Activity of Reconstituted Sublancin Analogues against *B. halodurans* C-125 and Sublancin-Resistant *B. halodurans* C-125 Mutants^a

	<i>B. halodurans</i> C-125						sublancin-resistant <i>B. halodurans</i> C-125 ^b			
	glucose M9 ^c	GlcNAc M9	Man M9	Gal M9	LB media	Glc M9	GlcNAc M9	Man M9	Gal M9	LB media
nisin	+	+	+	+	+	-	+	+	+	+
sublancin ^d	-	+	+	+	+	-	-	-	-	-
Sublancin-Glc ^e	-	+	+	+	+	-	-	-	-	-
sublancin-GlcNAc	-	+	+	+	+	-	-	-	-	-
sublancin-Man	-	+	+	+	+	-	-	-	-	-
sublancin-Gal	-	+	+	+	+	-	-	-	-	-

^a Each strain was grown on minimal medium with the indicated sugar as the sole carbon source. Plus (+) indicates antimicrobial activity under the growth conditions; minus (-) indicates no such activity was observed. Nisin was used as a positive control for each condition.

^b The same outcome was observed with four other resistant strains reported previously.⁸

^c Sugar source in M9 minimal medium.

^d Isolated from the producing organism.

^e Prepared enzymatically *in vitro*.