

The Phosphoenolpyruvate: Sugar Phosphotransferase System Is Involved in Sensitivity to the Glycosylated Bacteriocin Sublancin

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The mode of action of a group of glycosylated antimicrobial peptides known as glycocins remains to be elucidated. In the current study of one glycocin, sublancin, we identified the phosphoenolpyruvate:sugar phosphotransferase system (PTS) of *Bacillus* species as a key player in bacterial sensitivity. Sublancin kills several Gram-positive bacteria, such as *Bacillus* species and *Staphylococcus aureus*, including methicillin-resistant *S. aureus* (MRSA). Unlike other classes of bacteriocins for which the PTS is involved in their mechanism of action, we show that the addition of PTS-requiring sugars leads to increased resistance rather than increased sensitivity, suggesting that sublancin has a distinct mechanism of action. Collectively, our present mutagenesis and genomic studies demonstrate that the histidine-containing phosphocarrier protein (HPr) and domain A of enzyme II (PtsG) in particular are critical determinants for bacterial sensitivity to sublancin.

Bacteriocins are ribosomally synthesized peptides produced by a wide range of bacterial species. These bacteriocins endow the producing bacteria with a competitive advantage in their respective niche. Many bacteriocins are heavily posttranslationally processed during their biosynthesis, and these modifications are required for activity (1). Nisin is the best-studied bacteriocin and belongs to the lantibiotic family (2). The mode of action of nisin involves binding to lipid II, which prevents further cell wall synthesis, followed by the formation of pores within the membrane. The leakage of essential metabolites from these cells results in death of the bacteria. Targeting of lipid II by bacteriocins is a common mechanism of action (3–5). Other mechanisms include the targeting of phosphotransferase systems (6, 7), acting as Trojan horses (8, 9), parasitizing iron uptake pathways (10) and causing the collapse of membrane potential, together with leakage of ions and/or a decrease in intracellular ATP concentrations (11). There is much interest in bacteriocins for use in the control of bacterial infections and therefore in their mechanisms of action.

Sublancin is a bacteriocin produced by the Gram-positive soil bacterium *Bacillus subtilis* strain 168. It is capable of killing several species of Gram-positive bacteria, such as *Staphylococcus aureus*, including methicillin-resistant *S. aureus* (MRSA) (12). Sublancin is encoded by the *sunA* gene on the SP β prophage as a prepeptide (13). The genes necessary for the synthesis of sublancin are also included in this region and are expressed from two promoters. The biosynthetic operon is made up of five individual genes, which are responsible for producing active sublancin. The *sunT* gene is responsible for the export of sublancin and cleavage of its leader sequence. Two thiol-disulfide oxidoreductases, encoded by *bdbA* (the only gene of the operon that is dispensable for active sublancin production) and *bdbB*, are responsible for creating the two disulfide bonds of sublancin. These disulfide bonds involve four of the five cysteine residues that are present in the sublancin peptide (14). The fifth cysteine residue undergoes glycosylation by the glucosyltransferase encoded by the *sunS* gene (15). The second promoter drives the expression of a gene encoding the immunity protein SunI, which is also required for the production of active

sublancin by protecting the producing organism from sublancin (16).

Sublancin is one of five bacteriocins that have been described as being S-glycosylated. Glycocin F is produced by *Lactobacillus plantarum* (17), ASM1 is produced by *Lactobacillus plantarum* strain A-1 and is an orthologue of glycocin F with five different residues in the C-terminal tail (18, 19), and the putative products of *Bacillus thuringiensis*, thurandacin A and B, have been produced *in vitro* (20). Sublancin is modified on Cys22, with a β -S-linked glucose (15, 21). For glycocin F, an S-linked N-acetylglucosamine (GlcNAc) is added to the C-terminal cysteine residue, and the other modification is on an internal serine residue with an O-linked GlcNAc (17, 22). Like glycocin F, thurandacin B is glycosylated on two residues: a cysteine residue undergoes S-linked glycosylation, and a serine is modified with an O-linked glucose moiety (20). Judged by homology searches to the bacteriocin sequence or the glucosyltransferase, many other Gram-positive bacteria appear to potentially encode such bacteriocins, with much variation in the sequences among them (15, 17).

The mechanisms by which glycosylated bacteriocins kill sensi-

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TABLE 1 Strains used in this study

Strain	Genotype	Reference
168	Wild-type <i>B. subtilis</i> strain	Laboratory collection
ΔSPβ	Wild-type <i>B. subtilis</i> 168 strain lacking the entire SPβ prophage	14
C-125	Wild-type <i>B. halodurans</i> strain	56
ATCC 6633	Wild-type <i>B. subtilis</i> strain	57
168 deletion collection	Collection of <i>B. subtilis</i> mutants lacking large genomic regions	29
ΔSPβ::cat	Chloramphenicol-selectable ΔSPβ prophage mutant of <i>B. subtilis</i>	14
Δ <i>yvkS-yvkW</i>	<i>B. subtilis</i> Δ <i>yvkS-yvkW</i> ::phleo	This study
Δ <i>ykvY-glcT</i>	<i>B. subtilis</i> Δ <i>ykvY-glcT</i> ::phleo	This study
Δ <i>ptsG-ptsI</i>	<i>B. subtilis</i> Δ <i>ptsG-ptsI</i> ::phleo	This study
ΔSPβ-QB5435	<i>B. subtilis</i> Δ <i>pstG</i> ::cat; QB5435→SPβ	48
ΔSPβ-MZ303	<i>B. subtilis</i> Δ <i>ptsH</i> ::cat; MZ303→SPβ	58
ΔSPβ-GP864	<i>B. subtilis</i> Δ <i>ptsI</i> ::ermC; GP864→SPβ	59
ΔSPβ-QB5407	<i>B. subtilis</i> Δ <i>ccpA</i> ::spec; QB5407→SPβ	60
ΔSPβ-GP202	<i>B. subtilis</i> Δ <i>hprK</i> ::spec; GP202→SPβ	61
ΔSPβ::cm-GP506	<i>B. subtilis</i> <i>ptsH</i> H15A; SPβcm→GP506	62
ΔSPβ::cm-GP507	<i>B. subtilis</i> <i>ptsH</i> S46A; SPβcm→GP576	63

tive cells are currently unknown. Previous work has identified several genes in *B. subtilis* and *S. aureus* that alter sensitivity to sublancin. The *mscL* gene encodes the large mechanosensitive channel, and its deletion confers sublancin resistance in both *S. aureus* and *B. subtilis* (23). The addition of increased amounts of NaCl also results in increased resistance to sublancin, presumably due to the MscL channel being forced closed. This observation has led to speculation as to whether sublancin is able to enter the cell through this channel. Interestingly, since the connection between sublancin and MscL was reported, streptomycin has also been reported to use the MscL channel to enter the cell (24). In *B. subtilis*, the alternative sigma factor σ^W is known to play a role in the resistance to sublancin through its regulation of the *yqeZ-yqfA-yqfB* operon (25). The role these genes play in resistance to sublancin is unknown, but it is likely to be at the cell surface, due to their membrane localization (26).

In this study, we demonstrate that the phosphoenolpyruvate: sugar phosphotransferase system (PTS) of *B. subtilis* plays a major role in its sensitivity to sublancin. In the case of other bacteriocins for which the PTS was found to be involved, the addition of the PTS-requiring sugars resulted in increased sensitivity to the respective bacteriocin. However, for sublancin, the addition of PTS-requiring sugars leads to increased resistance, suggesting that sublancin has a distinct mechanism of action.

MATERIALS AND METHODS

Bacterial growth. See Table 1 for the strains used in this study. *B. subtilis* 168, *B. subtilis* strain ATCC 6633, and *Bacillus halodurans* strain C-125 were grown in lysogeny broth (LB) at 37°C with vigorous shaking (250 rpm) on LB agar plates. *B. subtilis* was also grown on M9 agar plates (as described previously [27] but with the addition of 1.5% final concentration agar) with and without the addition of sugars at a final concentration of 0.3% glucose, 0.4% malate, or 0.4% citrate, as specified below. The LB agar used for sublancin inhibition plate assays did not include NaCl. Antibiotics were used for selection when necessary at the following concentrations: 100 μg/ml spectinomycin, 20 μg/ml kanamycin, 4 μg/ml phleomycin, 5 μg/ml chloramphenicol, and 2 μg/ml erythromycin. Stock sublancin solutions were prepared using phosphate-buffered saline (PBS).

Production and isolation of sublancin 168. The purification of sublancin from its natural producer, *B. subtilis* 168, was performed as previously reported (15).

Strain construction. Chromosomal DNA was prepared from *B. subtilis* 168 using a standard procedure, as described previously (28). Deletion mutants in *B. subtilis* 168 were created as described by Tanaka et al. (29), and the oligonucleotides used are shown in Table S1 in the supplemental material. *B. subtilis* 168 was transformed using PCR products or chromosomal DNA, according to a standard procedure (30).

MIC determination. MICs were determined by the broth dilution method (31). Serial dilutions of sublancin were prepared in sterile deionized water (SDW). Forty-eight-well microtiter plates (Costar; Corning) were utilized for both *B. subtilis* ATCC 6633 and *B. halodurans* C-125. The total volume of culture in each well was 300 μl; the experimental wells contained 30 μl of 10× stock sublancin at defined concentrations and 270 μl of a 1:10 dilution (approximately 1×10^8 CFU ml⁻¹) of a culture of an indicator strain diluted in fresh LB growth medium. In addition, each plate contained several blanks (270 μl of fresh growth medium and 30 μl of SDW) and control wells (270 μl of untreated culture diluted 1:10 and 30 μl of SDW). The optical density at 600 nm (OD₆₀₀) was recorded at hourly intervals from 0 to 6 h, with an additional measurement at 18 h using a BioTek Synergy H4 plate reader. The plates were incubated under vigorous agitation at 37°C. The readings from triplicate experiments were averaged. Growth curves were developed using control (culture and SDW only) readings to ensure sufficient OD changes for accurate inhibition assessment. Curve fits for MIC determination were produced by fitting the data with the Origin8.5 software, using a dose-response curve with the equation $y = A1 + (A2 - A1)/(1 + 10^{[\log_{10}(x) - x]p})$, where p is the variable Hill slope.

Sublancin killing kinetics against sensitive *Bacillus* species. Sensitive cultures were grown to mid-log phase in LB medium, as described above, transferred to 48-well microtiter plates (Costar; Corning), and exposed to sublancin at 1× and 4× the MIC. Immediately after the addition of sublancin, the OD₆₀₀ was determined using a BioTek Synergy H4 plate reader. The cultures were incubated for 6 h, and the OD₆₀₀ was recorded every 30 min. To verify that cells were killed, CFU counting was performed by serial dilution and plating.

Sublancin sensitivity screen of a gene deletion collection of *B. subtilis*. Sublancin-induced growth inhibition assays were performed using the procedure described by Dorenbos et al. (14) but with a modification to enable the screening of large numbers of strains. Overnight cultures of *B. subtilis* mutants and a background control strain were grown in 96-well microtiter plates in a plate shaker at 37°C with shaking at 800 rpm. Bioassay dishes were prepared with LB agar without adding salt. The plates were thoroughly dried before being divided into 48 squares for inoculation. Cotton swabs were dipped into individual wells of the overnight culture before being spread on the appropriate square. The plates were

allowed to dry before spotting 2 μ l of an overnight culture of the *B. subtilis* 168 wild-type strain in the center of each inoculated square. The plates were incubated overnight, and visual analysis was used to determine zones of growth inhibition that were smaller or larger than that of the background strain. Strains with altered zones of inhibition were checked a further three times to ensure that the phenotype was reproducible.

Sublancin sensitivity assay in liquid medium. Overnight cultures of *B. subtilis* grown in LB were diluted 1:100 in the same medium and grown to an OD₆₀₀ of 0.5. The bacteria were then diluted 1:20 in a 96-well microtiter plate before growth was monitored every 10 min in a Synergy 4 BioTek plate reader (at 37°C with shaking). When the bacteria reached an OD₆₀₀ of 0.185 (equivalent to 0.5 for a 1-cm path length), sublancin was added at the desired concentration before resuming the monitoring of growth. Sugars were added at the following final concentrations: 0.3% glucose, 0.4% malate, 0.3% sucrose, 0.3% fructose, 0.3% glycerol, 0.4% citrate, 0.4% galactose, and 0.4% succinate.

Membrane integrity assay. *B. subtilis* was grown to an OD₆₀₀ of 0.5 before purified sublancin was added at different concentrations (100 to 500 nM). As a positive control, nisin was added at 10 nM final concentration, and a negative-control sample contained no bacteriocin. Samples were taken at 30 and 90 min and prepared for LIVE/DEAD BacLight analysis (Molecular Probes, Life Technologies) (32). Samples were monitored by flow cytometry using an Accuri C6. The percentages of cells with intact or reduced membrane integrity were calculated.

Propidium iodide uptake. Membrane integrity was also evaluated by measuring the uptake of propidium iodide (PI). *B. subtilis* ATCC 6633 and *B. halodurans* C-125 cultures were grown to a density of 4×10^6 cells ml⁻¹ and then diluted with fresh LB medium to an OD₆₀₀ of 0.1. Cells were transferred to tubes containing PI (final concentration, 25 μ M; Molecular Probes, Inc., Leiden, the Netherlands), HEPES (1 mM), and sublancin (0, 0.2, 2.0, or 20 μ M) or nisin (0, 0.2, 2.0, or 20 μ M), incubated for 30 min at room temperature (RT), and analyzed. Data acquisition was performed with a BD Biosciences LSR II flow cytometer, using excitation at 488 nm with an argon laser and measurement of emission through a band-pass filter at 695/40 nm. A minimum of 50,000 events were detected for each sample, and the experiments were performed in triplicate. Data analysis to calculate the geometric mean fluorescence intensity (MFI) of gated cell populations was performed using FCS Express 3.00.0311 V Lite stand-alone software.

Generation of stable sublancin-resistant mutants and resistance frequency determination. Genetically stable sublancin-resistant mutants were generated by growing the sublancin-susceptible strains *B. halodurans* C-125 and *B. subtilis* ATCC 6633 in LB, as described above (no additional sugars added), until an OD₆₀₀ of 1.0 was reached (mid-log phase, 1-cm light path). The cultures were plated on agar plates containing 1 \times or 4 \times their respective sublancin MICs. Distinct colonies were observed by 24 h. Resistant colonies were picked, grown in LB, and plated on LB plates containing sublancin at 4 \times the MIC to confirm resistance. This procedure generated genetically stable sublancin-resistant mutants. The number of resistant mutants that emerged from each sublancin-susceptible culture was obtained by generating a serial dilution of each culture. Each dilution was plated on sublancin-containing plates. The total number of cells was determined by plating an appropriate (10^{-5} , 10^{-6} , 10^{-7} , 10^{-8} , or 10^{-9}) dilution of the cultures on nonselective LB agar medium. Colonies from sublancin-containing and nonselective plates were counted after 24 h of incubation. The resistance frequency was determined as the mean number of resistant cells divided by the total number of viable cells per culture.

Single nucleotide polymorphisms detected by whole-genome sequencing. Genomic DNA (gDNA) of the wild-type *B. halodurans* C-125 and four different sublancin-resistant isolates was extracted using an UltraClean microbial DNA isolation kit (Mo Bio). The gDNAs thus obtained were sequenced using a HiSeq 2000 Illumina sequencer, which generated close to 180 million single reads per lane, for an overall coverage of 360 \times for the 5-Mb genomes. All libraries were individually barcoded and constructed with the TruSeq sample prep kits (Illumina). The single

nucleotide polymorphisms (SNPs) and the corresponding genes for resistant *B. halodurans* C-125 were identified. In addition, the wild-type *B. halodurans* C-125 strain was mapped to the published *B. halodurans* C-125 sequence (GenBank accession no. NC_002570.2) with CLC Genomics Workbench (CLC bio), using default parameters. A consensus sequence of the wild-type and reference genomes was obtained and used for SNP detection in sublancin-resistant mutants of *B. halodurans*.

PCR amplification and validation of SNPs in sublancin-resistant *B. halodurans* mutants. PCR validation can serve as an iterative and informative process to modify and optimize the SNP filtering criteria to improve SNP calling (33). Primers flanking SNP-containing genes were synthesized and used for PCR amplification of the respective genes. The mutations reported here were all confirmed by PCR (see Table S2 in the supplemental material).

***B. halodurans* C-125 gene expression profile.** A culture of *B. halodurans* C-125 was grown in LB at 37°C with vigorous shaking until mid-log phase, at which point the culture was split into two 150-ml cultures, with one subjected to a subinhibitory concentration of sublancin (0.5 \times the MIC). RNA isolation was performed using the RNeasy minikit (Qiagen) and subsequent treatment with RNase-free DNase (Qiagen). The RNA was dissolved in RNase-free water and quantified using a NanoDrop 2000c spectrophotometer (Thermo Scientific). For each sample (i.e., with and without sublancin), 20 μ g of total RNA was isolated from three biological replicates. cDNA synthesis was performed using the SuperScript double-stranded cDNA synthesis kit (Invitrogen), as per the manufacturer's instructions (NimbleGen arrays user's guide, version 5.1 [34]) and quantified with the NanoDrop instrument. Total cDNA was labeled overnight with the one-color DNA labeling kit (NimbleGen), as per the manufacturer's instructions. The arrays were scanned using an Axon 4000B array scanner.

A *B. halodurans* C-125 NimbleGen custom array, containing a probe set of 22 unique 45-mer to 60-mer oligonucleotide probes for each of the 4,066 genes of this bacterium, was used. The NimbleScan software (version 2.6.0.0; Roche NimbleGen) was used to generate one normalized value per probe set using the robust multiarray average (RMA) algorithm (background correction, normalization, and summarization; data not logged). The data were then imported into R (35) using the limma package (36) and log₂ transformed. Statistical analysis for differential expression between the mutant and wild-type groups was performed, taking into account the correlation due to processing batch (37, 38). Raw *P* values were corrected for multiple hypothesis testing using the false discovery rate method (39). Annotation for the probe sets was primarily provided by NimbleGen and included BH identification numbers (IDs) (e.g., BH0001), gene names, descriptions, genomic locations, and URL links to NCBI. Entrez Gene IDs and official gene symbols were downloaded from the *B. halodurans* genome record in NCBI (GenBank accession no. NC_002570). For analysis, we filtered genes to identify those that were altered by a ≥ 1.5 -fold change in transcription (upregulation and downregulation). For data mining, we used DAVID bioinformatics resources, consisting of an integrated biological knowledge base and analytical tools that use the results from the statistical analysis to explore and interpret gene regulation data (40).

RESULTS

Sublancin displays submicromolar MICs against *Bacillus* strains. The antibacterial activity of sublancin against selected *Bacillus* strains was first determined by solid agar diffusion assays with sublancin at a range of concentrations (0.097 μ M to 50 μ M). After confirmation that *B. subtilis* ATCC 6633 and *B. halodurans* C-125 were sensitive, the MICs were determined by the broth dilution method (41, 42). A series of dilutions of sublancin (0.097 μ M to 50 μ M) were made and incubated with a defined number of bacterial cells in LB medium. Plates were incubated for 18 to 24 h at 37°C, and growth was assessed by measuring the optical density of each well at 600 nm. The MICs were determined by fitting

the data to a dose-response curve. In liquid cultures, the MICs of sublancin against *B. halodurans* C-125 and *B. subtilis* ATCC 6633 were 0.312 μ M and 0.625 μ M, respectively (see Fig. S1 in the supplemental material).

Bactericidal activity of sublancin. One element for consideration, when trying to understand how an antimicrobial compound functions, is whether it is bactericidal or bacteriostatic. Furthermore, some bacteria are lysed after being killed, others are lysed immediately, and yet others undergo nonlytic death (43, 44). The ability of sublancin to kill or arrest the sensitive *Bacillus* strains was therefore evaluated. *B. subtilis* ATCC 6633 and *B. halodurans* C-125 cultures were grown to mid-log phase, transferred to a 48-well plate, and exposed to sublancin (at 1 \times and 4 \times the MIC). After the addition of sublancin, the OD₆₀₀ was monitored periodically. After a 6-h incubation period, the *B. halodurans* C-125 and *B. subtilis* ATCC 6633 cultures showed a decrease in optical density, suggesting that sublancin has bactericidal activity (Fig. 1). To verify whether the activity of sublancin was bactericidal, CFU were determined by plating, which confirmed the bactericidal activity observed by the OD readings. The decrease in optical density was not nearly as great as the decrease in CFU, which implies that sublancin kills without immediate lysis.

Sublancin does not affect the integrity of the cell membrane. Some bacteriocins destabilize the membrane or form pores (4, 45). Nisin is the prototypical pore-forming bacteriocin, which binds to lipid II within the membrane (2). To determine whether sublancin affects membrane integrity, we challenged cultures of *B. subtilis* strain Δ SP β , *B. subtilis* ATCC 6633, and *B. halodurans* C-125 with several different concentrations of sublancin. We monitored the membrane integrity of *B. subtilis* ATCC 6633 and *B. halodurans* C-125 by flow cytometric analysis, using the cell-impermeable propidium iodide (PI) dye, after a 30-min exposure to sublancin. Our nisin control showed an increase in fluorescence due to membrane permeabilization, but sublancin-treated cells did not, even at concentrations as high as 32 \times the MIC for *B. subtilis* ATCC 6633 and 64 \times the MIC for *B. halodurans* C-125 (Fig. 2). We monitored the membrane integrity of *B. subtilis* Δ SP β with the LIVE/DEAD BacLight bacterial cell viability assay at 30 and 90 min after the addition of sublancin (see Fig. S2 in the supplemental material). At both time points, we found no change in membrane integrity. When the same strain was exposed to nisin as a positive control, a dramatic loss of membrane integrity was seen already after 30 min of incubation. Collectively, these experiments show that sublancin does not affect membrane integrity and likely acts through an alternative mechanism.

Resistance frequency. The manifestation of antibiotic resistance to clinically used antibiotics suggests that resistance is likely to develop against any antibacterial compound. It is useful, however, to analyze the frequency at which resistance to novel antibacterial compounds arises (46). The spontaneous resistance frequency is defined as the number of resistant mutants per total number of viable cells that grow after an established period of time. The resistance frequency of sublancin was determined by plating aliquots of bacterial culture onto agar containing the antibacterial compound at 4 \times the MIC. Aliquots were also plated onto agar plates with no antibiotic to determine the number of viable bacterial cells in the liquid culture. The resistance frequencies determined were relatively high, with resistance frequencies of 10⁻⁵ for *B. halodurans* C-125 and 10⁻⁶ for *B. subtilis* ATCC 6633. To verify that the observed colonies were indeed resistant to the

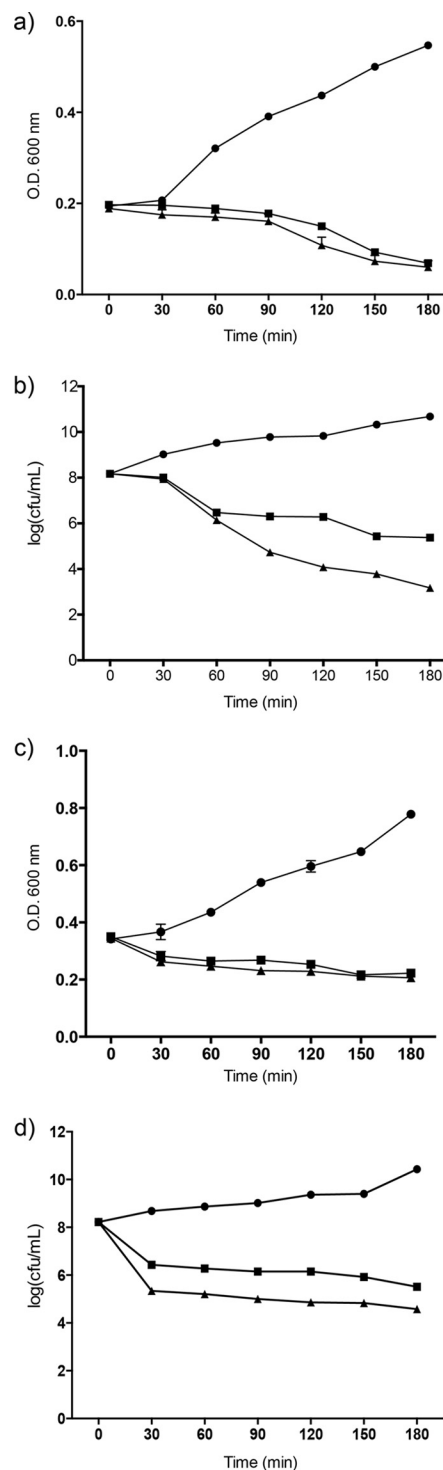


FIG 1 Growth inhibition of *B. subtilis* ATCC 6633 and *B. halodurans* C-125 by sublancin 168. (a) Time-dependent changes in OD₆₀₀ of cultures of *B. subtilis* ATCC 6633 in the absence (circles) or presence of sublancin 168 at 1 \times MIC (squares) and 4 \times MIC (triangles). (b) Aliquots of the cultures in panel a were analyzed for CFU. (c) Time-dependent changes in OD₆₀₀ of cultures of *B. halodurans* C-125. The same symbols are used as in panel a. (d) Aliquots of the cultures in panel c were analyzed for CFU. The mean values of the data from one experiment conducted in triplicate are shown. The data are representative of the results from three independent experiments. The error bars indicate standard deviations. When error bars are not visible, they are smaller than the size of the symbol used.

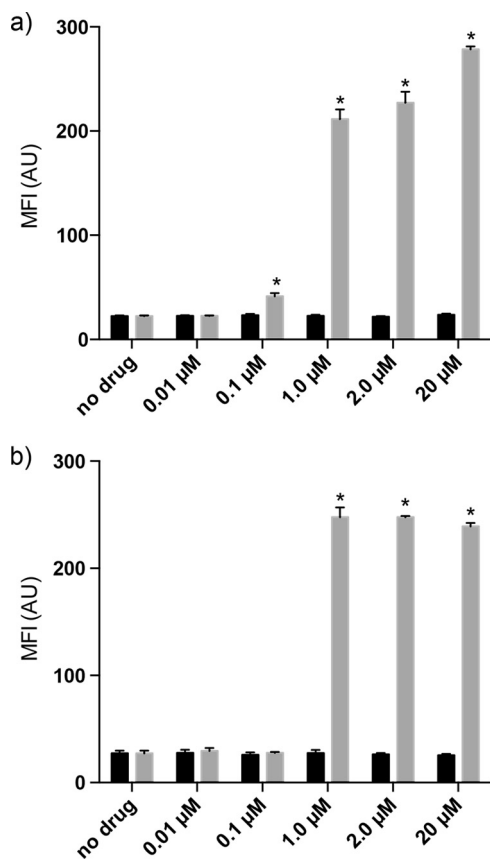


FIG 2 Membrane integrity assays by measuring propidium iodide (PI) uptake. The addition of sublancin at the indicated concentrations (black bars) does not alter the membrane permeability of *B. halodurans* C-125 (a) and *B. subtilis* ATCC 6633 (b). Nisin was used as the positive control (gray bars). *, $P < 0.05$ between nisin (0.1 μM to 20 μM)-treated cells relative to no drug. In all experiments in which the cells were exposed to sublancin, the increase in MFI relative to the control was not statistically significant ($P > 0.05$). The means of the data from one experiment conducted in triplicate are shown. The data are representative of the results from three independent experiments. The error bars indicate standard deviations.

antibiotic, they were subcultured in sublancin-free LB medium and plated on LB agar containing the antibacterial compound at a concentration of $4\times$ the MIC. For both strains, the plated resistant strains grew a full lawn.

Identification of *B. subtilis* chromosomal regions that affect sensitivity to sublancin. We aimed at finding genetic factors that affect sensitivity to sublancin. To do this, we first employed the set of deletion mutants described by Tanaka et al. (29). These mutants were created with a strain in which the prophages of *B. subtilis*, including SP β , had been deleted. Therefore, all mutant strains lack the gene encoding the immunity protein for sublancin, *sunI* (47), making it an ideal collection of mutants for identifying interesting genomic regions with respect to sublancin sensitivity. During the screening, we used LB agar without NaCl, as it was previously shown that *B. subtilis* is more sensitive to sublancin under low-osmosis conditions (23). The strains were plated in duplicate on the LB agar and spotted with 2 μl of an overnight culture of the sublancin-producing strain *B. subtilis* 168. We found *B. subtilis* strain JJS-DIn010, in which *rsiW* and *sigW* are deleted, to have increased sensitivity (i.e., a larger zone of clearing around the pro-

ducing colony) (Fig. 3a). This finding is in concordance with previously reported observations (25), suggesting that our assay was able to identify strains with altered sensitivity. Another strain was identified (*B. subtilis* JJS-DIn042), in which the genes *ykvS*, *ykvT*, *ykvU*, *stoA*, *zosA*, *ykvY*, *ykvZ*, *glcT*, *ptsG*, *ptsH*, and *ptsI* were deleted. JJS-DIn042 was resistant to the effects of sublancin (Fig. 3a) under conditions in which the $\Delta\text{SP}\beta$ strain did not survive. Because of this interesting observation, we investigated this region further by constructing several different individual gene deletion mutants. This approach revealed that only the deletion of the *pts* operon (*ptsGHI*) results in resistance to sublancin (Fig. 3b and Table 2). In contrast, a deletion of *glcT*, which plays a regulatory role in the *pts* operon (48), did not result in sublancin resistance (Fig. 3b). PtsG is the major glucose transporter of the phosphotransferase system (49), and PtsH and PtsI are general components of the sugar transport system that phosphorylates the incoming sugar (50). PtsH is more commonly known as HPr (as referred to here), and PtsI is also called EI. In *B. subtilis*, the PTS transfers a phosphate group from phosphorylated PtsI to HPr, which in turn transfers the phosphate to a variety of different PTS permeases. To utilize glucose, HPr transfers the phosphate to the IIA domain of the sugar permease PtsG. The IIA domain then phosphorylates the IIB domain of PtsG, which in turn transfers the phosphate to the incoming sugar. Last, the phosphorylated sugar moves into glycolysis. It is intriguing that the PTS was identified in our screen for sublancin sensitivity, as the most common PTS substrate is glucose, whereas sublancin is glucosylated. A functional homologue of HPr is present in *B. subtilis*, i.e., Crh. We therefore tested a *crh* deletion mutant in the presence of sublancin, but no change in sensitivity was observed compared to the wild type (data not shown), suggesting that the sensitivity to sublancin is specifically dependent on HPr.

Comparative genomics. Bacteria often acquire stable resistance to antibiotics due to gene mutations. A comparative genomics analysis was therefore performed to identify the mutations that conferred resistance to *B. halodurans* C-125 after exposure to sublancin. The gDNA of the sensitive strain *B. halodurans* C-125 and of four of the spontaneous resistant mutants obtained as described above were extracted and sequenced using a HiSeq 2000 Illumina sequencer. The wild-type *B. halodurans* strain was mapped to the published *B. halodurans* C-125 genome sequence (GenBank accession no. NC_002570.2) to generate a consensus sequence that was used for SNP detection in sublancin-resistant mutants of *B. halodurans*. A comparison of the gDNA of the wild-type sensitive strain with the four sublancin-resistant mutants revealed several mutations. One strain contained three mutations in the intergenic region between the transcriptional antiterminator (locus tag BH0845) and *ptsG* (locus tag BH0844), another strain contained a missense mutation in the gene for mannitol-1-phosphate 5-dehydrogenase (locus tag BH3851), and most interestingly, the three strains that did not have a mutation in the intergenic region mentioned above all had nonsense mutations in the gene for the glucose-specific transporter subunit IIC, which is part of the multidomain protein PtsG (see Table S2 in the supplemental material). The missense mutation prevents the production of PtsG, and the three mutations in the intergenic region between the antiterminator and *ptsG* are predicted to considerably stabilize the structure of the terminator (see Fig. S3 in the supplemental material), thus potentially also preventing *ptsG* transcription. Once more, these

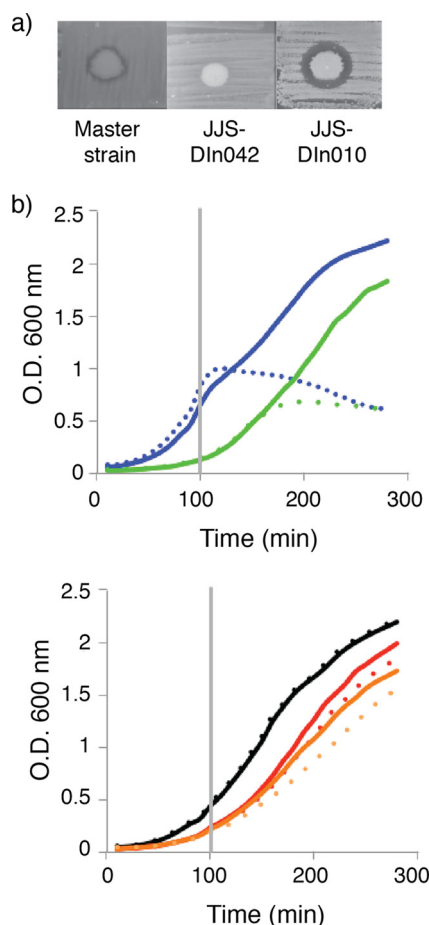


FIG 3 Deletion of the *ptsGHI* operon results in resistance to sublancin. (a) *B. subtilis* strains described by Tanaka et al. (29) were screened for increased and reduced sensitivity to sublancin. The parental strain of the collection is labeled “Master strain.” In JJS-DIn042, the region from *ykvS* to *ptsI* is deleted, and in JJS-DIn010, *rsiW* and *sigW* are deleted. (b) Growth curves of mutant strains with individual deletions of the genes that are responsible for the resistance in strain JJS-DIn042. In the top graph, blue line, *B. subtilis* Δ SP β ; green line, *B. subtilis* Δ SP β -*glcT*. In the bottom graph, black line, *B. subtilis* Δ SP β -*ptsG*; red line, *B. subtilis* Δ SP β -*ptsH*; orange line, *B. subtilis* Δ SP β -*ptsI*; solid lines, no sublancin; dotted lines, 200 nM sublancin added at 100 min, as indicated by the vertical gray line. Deletion of any of the genes within the *ptsGHI* operon results in resistance to sublancin. Deletion of the gene encoding the transcriptional antiterminator *glcT* has no effect on the sensitivity of *B. subtilis* to sublancin. The means of the data from one experiment conducted in triplicate are shown. The data are representative of the results from three independent experiments.

findings point to the PTS being important for sensitivity to sublancin.

Gene expression profile by microarray analysis of *B. halodurans* C-125. Antimicrobial-resistant mutants provide valuable insights, but the information obtained from a resistance phenotype is not always representative of the identity of the target. We therefore also monitored changes in global gene expression upon exposure of *B. halodurans* C-125 to sublancin.

The expression profiles revealed four genes that are part of sulfur metabolism that are highly upregulated (changes from 9- to 14-fold; see Table S3 in the supplemental material). The analysis also revealed upregulated genes involved in transmembrane transporter activities, whereas genes involved in amino sugar and nucleotide sugar metabolism were up- and downregulated. Inter-

estingly, the genes for HPr and for PtsG that were also identified in the set of deletion mutants and in the comparative genomics analysis were downregulated (see Table S3), as was another PTS protein that is homologous to YpqE in *B. subtilis*, a putative phosphotransferase enzyme IIA component (51). These data again suggest that, like in *B. subtilis*, the PTS is involved in the sensitivity of *B. halodurans* toward sublancin.

Addition of PTS sugars to the growth medium results in increased resistance to sublancin. Several bacteriocins have been shown to target PTS proteins as part of their mode of action. In these reported cases, the addition of the relevant sugar resulted in an increased sensitivity to the bacteriocin (6, 7, 11). This effect is due to the elevated uptake of the respective bacteriocins via the PTS. We therefore investigated whether this was also true for sublancin. The PTS is able to use many sugars, employing a different transporter for each sugar, together with the HPr and PtsI proteins. Once the sugar is phosphorylated, it moves into glycolysis at the relevant metabolic junction. To investigate the influence of added sugars, *B. subtilis* Δ SP β cultures were diluted in LB medium (without NaCl) containing different sugars and grown in 96-well microtiter plates with shaking to an OD₆₀₀ of 0.5 before the addition of sublancin at the MIC of 200 nM, as measured for this strain under these conditions. The presence of the PTS sugars glucose, sucrose, and fructose prevented the growth inhibition imposed by sublancin (Fig. 4a), since no significant reduction in the OD was observed. In contrast, the non-PTS sugars citrate, galactose, and succinate had no influence on the activity of sublancin (Fig. 4b). The two exceptions were the non-PTS sugars glycerol and malate. In this respect, it is noteworthy that the glycerol kinase GlpK requires phosphorylation by HPr for glycerol utilization (48, 52). Malate is a preferred carbon source for *B. subtilis* and is known to influence the carbon catabolite repression response (53). In this context, it is noteworthy that a decrease in antimicrobial activity was also reported for glycocin F upon supplementation of the medium with GlcNAc, which is the sugar that is attached to glycocin F at two positions (17).

To further delineate the effects of sugars on sublancin sensitivity, purified sublancin was spotted onto lawns of *B. subtilis* Δ SP β , which were grown on agar plates containing the defined M9 minimal medium supplemented with glucose, malate, or citrate. When glucose was present, the cells were always resistant to sublancin. In contrast, with citrate, a large zone of clearing was observed. In the presence of malate, an intermediate-sized zone of inhibition was observed. This observation underpins the view that

TABLE 2 Resistance phenotype of *B. subtilis* Δ SP β single-gene deletion strains upon exposure to sublancin

Deleted gene	Sublancin resistant or sensitive
<i>ykvS</i>	Sensitive
<i>ykvT</i>	Sensitive
<i>ykvU</i>	Sensitive
<i>stoA</i>	Sensitive
<i>zosA</i>	Inconclusive
<i>ykvY</i>	Sensitive
<i>ykvZ</i>	Sensitive
<i>glcT</i>	Sensitive
<i>ptsG</i>	Resistant
<i>ptsH</i>	Resistant
<i>ptsI</i>	Resistant

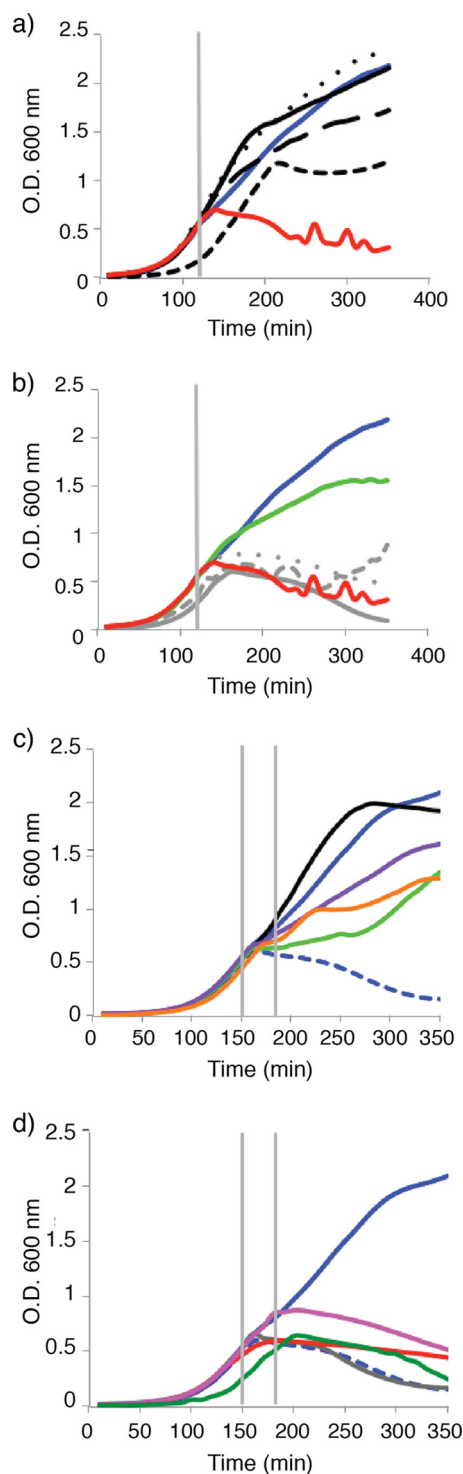


FIG 4 Addition of PTS sugars to LB blocks growth inhibition by subblancin. (a) Growth curves of the *B. subtilis* Δ SP β strain in LB medium with salt with added PTS sugars. Blue line, no addition of subblancin; red line, addition of subblancin; black solid line, addition of 0.3% glucose; black dotted line, addition of 0.3% fructose; black long-dashed line, addition of 0.3% sucrose; black short-dashed line, addition of 0.3% glycerol. Subblancin was added at 120 min, as indicated by the vertical gray line. (b) Growth curve of the Δ SP β strain in LB medium with addition of non-PTS sugars. Blue line, no addition of subblancin; red line, addition of subblancin; green line, addition of 0.4% malate; gray short-dashed line, addition of 0.4% citrate; gray dotted line, addition of 0.4% galactose; gray solid line, addition of 0.4% succinate. (c) Growth curve of the Δ SP β strain with

the carbon source affects the sensitivity to subblancin (see Fig. S4 in the supplemental material).

Since glucose was found to prevent the effect of subblancin, we wondered whether it would be possible to rescue subblancin-treated cells by adding glucose. We therefore grew the bacteria on LB medium (with NaCl) and added subblancin at an OD₆₀₀ of 0.5. The cells were then incubated for 30 min before adding the same PTS and non-PTS sugars as in the previous experiment (Fig. 4c and d). Glucose almost instantaneously rescued the cells from the growth inhibition that subblancin imposed on the cells. Fructose also rescued the cells, but to a smaller extent than glucose. The non-PTS sugar glycerol rescued the cells in a manner similar to that with fructose. Malate was also able to rescue the cells, but this took approximately 100 min following the addition of the sugar, whereas the effect for glycerol and fructose was observed immediately after the addition of the respective sugar. In contrast, the addition of the other non-PTS sugars or sucrose had no effect on the survival of the bacteria.

The observed rescue of growth by addition of the different PTS sugars and glycerol suggests that the PTS transporter is not irreversibly inactivated by subblancin; perhaps instead, subblancin affects the pathway that leads to the phosphorylation of the sugar. The addition of subblancin and the PTS sugar at the same time might result in competition for phosphorylation of the sugar or the glucose on subblancin (or its metabolite). With this in mind, we looked at the phosphorylation sites on the HPr protein.

Phosphorylation of HPr is responsible for sensitivity to subblancin. The HPr protein is phosphorylated on two sites. The first, His15, is phosphorylated by PtsI. HPr then transfers the phosphate group to PtsG, and the phosphate is subsequently used to phosphorylate the incoming sugar. The second, Ser46, is phosphorylated by HPr kinase (HPrK) under conditions of large glycolytic flux. This phosphorylation subsequently allows HPr to form a complex with the catabolite control protein A (CcpA). This HPr-CcpA complex mediates carbon catabolite repression by binding to its cognate operator regions. To link subblancin sensitivity to one of these HPr-mediated processes, we tested two *B. subtilis* Δ SP β strains with point mutations at one of the two HPr phosphorylation sites. As shown in Fig. 5a, *B. subtilis* Δ SP β carrying the S46A point mutation in HPr was fully sensitive to subblancin. In contrast, the strain carrying the H15A point mutation in HPr displayed an increased level of resistance to subblancin. This observation suggests that *hprK* and *ccpA* deletion mutants would remain sensitive to subblancin, since the carbon catabolite-repressing function of HPr is not affected. This prediction was indeed confirmed, as the deletion of either of these two genes had no effect on subblancin sensitivity (Fig. 5b). Also, the addition of glu-

subblancin added at 150 min, followed by the addition of PTS and non-PTS sugars 30 min later, as depicted by the left and right vertical lines, respectively. Blue line, no subblancin; blue dashed line, addition of subblancin; black line, 0.3% glucose; purple line, 0.3% fructose; orange line, 0.3% glycerol; green line, 0.4% malate (final concentration of sugars shown). (d) Growth curve of the Δ SP β strain with subblancin added at 150 min, followed by the addition of PTS and non-PTS sugars 30 min later, as depicted by the left and right vertical lines, respectively. Blue line, no subblancin; blue dashed line, addition of subblancin; gray line, 0.4% citrate; red line, 0.3% sucrose; pink line, 0.4% galactose; green line, 0.4% succinate (final concentration of sugars shown). The means of the data from one experiment conducted in triplicate are shown. The data are representative of the results from three independent experiments.

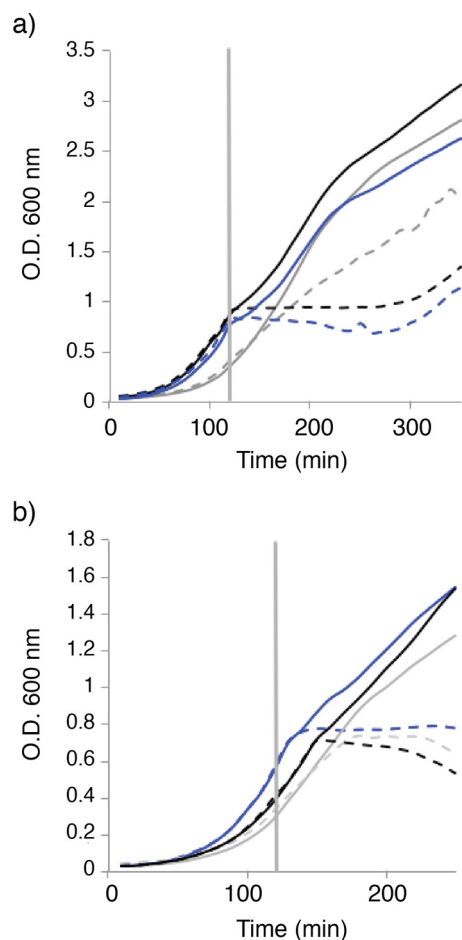


FIG 5 The H15A mutation in HPr results in increased resistance to sublancin. (a) The two phosphorylation sites in the HPr protein were mutated individually to alanine residues. The growth curves of the resulting strains are shown, with 200 nM sublancin added at 120 min, as depicted by the vertical line. Blue line, *B. subtilis* Δ SP β ; gray line, *B. subtilis* Δ SP β -H15A; black line, *B. subtilis* Δ SP β -S46A; solid line, no sublancin; dashed line, with sublancin. (b) Blue line, *B. subtilis* Δ SP β ; gray line, *B. subtilis* Δ SP β -*hprK*; black line, *B. subtilis* Δ SP β -*ccpA*; solid line, no sublancin; dashed line, with sublancin. The Δ *ccpA* and Δ *hprK* mutations have no effect on the sensitivity of the cells to sublancin. Sublancin at 200 nM was added at 120 min. The means of the data from one experiment conducted in triplicate are shown. The data are representative of the results from three independent experiments.

cose to the Δ *ccpA* mutant conferred resistance to sublancin (not shown), providing further evidence that it is not the carbon catabolite-repressing branch of HPr regulation that leads to sublancin sensitivity. Instead, it seems to be the PTS branch involving the H15 phosphorylation site that is responsible for the effects on sublancin sensitivity. However, how phosphorylation of His15 of HPr is exactly tied to sublancin sensitivity is presently unknown.

DISCUSSION

Sublancin is a bacteriocin that was recently found to be glycosylated as part of its maturation process, and this glycosylation is required for its activity (15). We showed in this study that sublancin is bactericidal and that it does not kill by pore formation or disruption of membrane integrity. Four different lines of evidence point toward the phosphoenolpyruvate:sugar phosphotransferase system as a factor affecting the activity of the bacteriocin. Experi-

ments with deletion mutants of *B. subtilis* identified PtsG, HPr, and PtsI, but not GlcT and Crh, as being important for sensitivity to sublancin. In addition, three of four *B. halodurans* sublancin-resistant mutants contained stop codon mutations within the *ptsG* gene, with the fourth resistant strain having three mutations that potentially interfere with *ptsG* expression. The transcriptional profile also indicated a strong downregulation of PTS genes upon exposure to sublancin and, last, the addition of PTS sugars decreased sensitivity to sublancin.

The PTS has been described as affecting sensitivity to other bacteriocins, including dysgalactin and lactococcin A (6, 7, 54). Dysgalactin appears to bind to the glucose and mannose transporters of the PTS (7). Dysgalactin was shown to block the uptake of glucose and the nonmetabolizable analog 2-deoxyglucose; it also perturbs the membrane of the cell, causing the dissipation of membrane potential (7). This activity appears to be different from the mechanism used by sublancin, as the addition of glucose or any other PTS sugar blocked the killing activity of sublancin, and membrane disruption was not observed. Lactococcin A also uses components of the mannose PTS in its mode of action. Lactococcin A binds to the membrane-located complex of the IIC and IID subunits of the mannose transporter (6), resulting in dissipation of the membrane potential (45). Like the observations with dysgalactin, decreased growth rates were observed for cells grown with mannose or glucose as the sole carbon source in the presence of lactococcin A (6). The current study suggests that sublancin is also functioning in a manner different from that of lactococcin A, since the studies with gene deletion strains and spontaneous resistance mutants both point to the phosphorylation components of the PTS, rather than the membrane components, as being key to sublancin sensitivity. When we monitored sublancin susceptibility using M9 minimal medium supplemented with glucose as the single carbon source, cells were completely immune to the effects of sublancin. A third bacteriocin, the circular molecule garvicin ML, requires the maltose-binding protein for activity. Growth in medium in which the sole carbon source is either maltose or galactose again resulted in increased sensitivity to this bacteriocin (55).

Two regions of the *B. subtilis* chromosome that result in resistance to sublancin have now been identified. The first is *mscL*, which encodes a mechanosensitive channel, as described by Kouwen et al. (23) and, in this work, the proteins encoded by the *ptsGHI* operon. Several scenarios may describe the mechanism by which sublancin interacts with the PTS. First, it is intriguing that it is the glucose transporter that was identified, given that sublancin is modified with an S-linked glucose moiety. The glucose moiety on sublancin might be recognized by the transporter to facilitate entry into the cell or potentially to kill it through its interaction with this system. Competition between sublancin and glucose might explain the observed decrease in sensitivity upon the addition of glucose. We note that HPr and domains IIA and IIB of PtsG are located in the cytoplasm (see Table S4 in the supplemental material); hence, for this mechanism to be correct, the glucose on sublancin would have to traverse the transporter or bypass the glucose transporter via the MscL channel. In this respect, it is noteworthy that the other sugars that were able to protect the bacteria from sublancin are either gluconeogenic or feed into glycolysis elsewhere in the pathway, therefore possibly bypassing the need for the glucose transporter. When we tested strains that express variants of the HPr protein with point mutations that re-

move the phosphorylation sites, the mutation that led to increased resistance to sublancin was H15A. The phosphorylation of His15 is responsible for transferring a phosphate group to the incoming PTS sugar. This points to a critical role of phosphorylation in the growth inhibition by sublancin and seems to suggest that sublancin may need to be phosphorylated upon its entry into the cell to exert its bactericidal effect. Interestingly, bacterial growth was rescued when PTS sugars were added to the growth medium 30 min after the challenge with sublancin. This finding implies that either the specific growth-inhibiting mechanism employed by sublancin is reversible or that the addition of the PTS sugars results in the cell using a different biological process that allows survival.

In conclusion, we have shown here that sublancin exerts its bactericidal effects in a novel manner. At present, it is not clear exactly how sublancin interacts with the PTS, and several questions remain for future research. Is there a physical interaction between sublancin and the PTS in the inhibited cells? Is there a link between the PTS and the MscL channel? How does sublancin actually inhibit the growth of the cell? How do the strong structural similarities of glycoicin F and sublancin fit into the mechanism, and what role does the three-dimensional structure of the peptide components of these glycoicins play (21, 22)? In a time in which bacteria are becoming resistant to the antimicrobial compounds that are used in clinical practice, research to understand how infections can be fought in alternative manners is essential. The apparently novel mechanism by which sublancin kills sensitive species of bacteria may therefore offer biological insights for the development of new antimicrobial strategies. Whether the mechanism identified in *Bacillus* is also operational in sensitive pathogens, such as *S. aureus* and *Listeria monocytogenes*, requires further in-depth analyses.

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