The Phosphoenolpyruvate:Sugar Phosphotransferase system is involved in sensitivity to the glucosylated bacteriocin sublancin

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Abstract

The mode of action of a group of glycosylated antimicrobial peptides known as glycocins remains to be elucidated. In the current study on 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 an increased sensitivity, suggesting that sublancin has a distinct mechanism of action. Collectively, our present mutagenesis and genomic studies demonstrate that, in particular, the histidine-containing phosphocarrier protein (HPr) and domain A of enzyme II (PtsG) are critical determinants for the bacterial sensitivity to sublancin.

Antimicrobial Agents and Chemotherapy

Introduction

Bacteriocins are ribosomally synthesised 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 post-translationally 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 formation of pores within the membrane, leading to membrane permeabilization. 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 control of bacterial infections and therefore in their mechanisms of action.

Sublancin is a bacteriocin produced by the Gram-positive soil bacterium *Bacillus subtilis* 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 on the SPβ prophage as a prepeptide by the *sunA* gene (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 disulphide bonds of sublancin. These disulphide bonds involve four of the five cysteine residues that are present in the sublancin peptide (14). The fifth cysteine residue undergoes glucosylation by the glucosyltransferase encoded by the *sunS* gene (15). The second promoter drives expression of a gene encoding the immunity protein SunI that 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 so far been described as being S-glycosylated. Glycocin F is produced by *Lactobacillus plantarum* (17), ASM1 is produced by *Lactobacillus plantarum* 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 glucosylated on two residues; a cysteine residue undergoes S-linked glucosylation, whereas a Ser 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 sequence amongst them (15, 17).

The mechanisms by which glucosylated bacteriocins kill sensitive cells are currently unknown. Previous work has identified several genes in *B. subtilis* and *S. aureus* that alter the 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). 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 localisation (26).

In this study, we demonstrate that the phosphoenolpyruvate:sugar phosphotransferase system (PTS) of *B. subtilis* plays a major role in sensitivity to sublancin. In the case of other bacteriocins where the PTS was found to be involved, 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

For all strains used in this study, see Table 1. *B. subtilis* 168, *B. subtilis* ATCC 6633 and *B. halodurans* C-125 were grown in Lysogeny Broth (LB) at 37 °C with vigorous shaking (250 rpm) and on LB agar plates. *B. subtilis* was also grown on M9 agar plates (M9 as described (27), but with the addition of 1.5 % final concentration agar) with and without the addition of sugars at final concentrations 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: spectinomycin 100 µg/mL, kanamycin 20 µg/mL, phleomycin 4 µg/mL, chloramphenicol 5 µg/mL and erythromycin 2 µg/mL. Stock sublancin solutions were prepared using PBS.

Production and isolation of sublancin 168

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

Minimum inhibitory concentration (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 (Corning Costar) were utilized for both *B. subtilis* ATCC 6633 and *Bacillus halodurans* C-125. The total volume of culture in each well was 300 µL; the experimental wells contained 30 µL of 10x stock sublancin at defined concentrations and 270 µL of a 1-in-10 dilution (approximately 1 x 10^8 colony-forming units (CFU) mL^{-1}) of a culture of indicator strain diluted in fresh LB growth medium. In addition, each plate contained several blanks (270

µL fresh growth medium and 30 µL SDW) and control wells (270 µL of untreated 1-in-10 diluted culture and 30 µL SDW). The optical density at 600 nm ($O.D._{600nm}$) was recorded at hourly intervals from 0 to 6 h with an additional measurement at 18 h using a BioTek Synergy 4H plate reader. Plates were incubated under vigorous agitation at 37 °C. The readings of triplicate experiments were averaged. Growth curves were developed using control (culture and SDW only) readings to ensure sufficient O.D. changes for accurate inhibition assessment. Curve fits for MIC determination were produced by fitting the data with Origin8.5 software using a dose-response curve with the equation: y = A1 + (A2 – A1) / (1 +$10^{(Logx0 - x)p}$), with *p* = 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 (Corning Costar) and exposed to sublancin at 1x and 4xMIC. Immediately after the addition of sublancin, the $O.D._{600nm}$ was determined using a BioTek Synergy H4 plate reader. The cultures were incubated for 6 h and the $O.D._{600nm}$ 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 modification to enable the screening of large numbers of strains. Overnight cultures of *B. subtilis* mutants and 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. Plates were allowed to dry, before spotting 2 µL of an overnight culture of the *B. subtilis* 168 wild-type strain in the centre of each inoculated square. Plates were incubated overnight and visual analysis was used to determine zones of 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 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 $O.D._{600nm}$ 0.5. The bacteria were then diluted 1:20 in a 96-well microtiter plate before growth was monitored in a Synergy4 Biotek plate reader every 10 min (37 °C, with shaking). When the bacteria reached $O.D._{600nm}$ 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: glucose 0.3%, malate 0.4%, sucrose 0.3 %, fructose 0.3%, glycerol 0.3%, citrate 0.4%, galactose 0.4% and succinate 0.4%.

Membrane integrity assay

B. subtilis was grown to $O.D._{600nm}$ 0.5, before purified sublancin was added at different concentrations (100 – 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® *Bac*Light™ 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 x 10^6 cells mL^{-1} and then diluted with fresh LB medium to an $O.D._{600nm}$ of 0.1. Cells were transferred to tubes containing PI (final concentration 25 µM; Molecular Probes Inc., Leiden, NL), HEPES (1 mM), and sublancin (0, 0.2, 2.0, 20 µM) or nisin (0, 0.2, 2.0, 20 µM), incubated for 30 min at RT, and analysed. 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 was detected for each sample, and 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 $O.D._{600nm}$ of 1.0 (mid log phase, 1 cm light path). The cultures were plated on agar plates containing 1x or 4x 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 4xMIC to confirm resistance. This procedure generated genetically stable sublancin 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}, 10^{-9}) dilution of the cultures on non-selective LB agar medium. Colonies from sublancin-containing and non-selective 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 Polymorphism (SNP) 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 HiSeq2000 Illumina sequencer,

which generated close to 180 million single-reads per lane, for an overall coverage of 360x for the 5 MB genomes. All libraries were individually barcoded and constructed with the TruSeq Sample Prep kits (Illumina). The 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 (accession no. NC_002570.2) with CLC Genomics Workbench (CLC bio), using default parameters. A consensus sequence of the wild-type and reference genome 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 herein were all confirmed by PCR (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 sub-inhibitory concentration of sublancin (0.5xMIC). RNA isolation was performed using the RNeasy mini kit (Qiagen) and subsequently treated 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 µg 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) and quantified with NanoDrop. Total cDNA was labeled overnight with the One-Color DNA labeling kit (NimbleGen) as per the manufacturer's instructions. Arrays were scanned using an Axon 4000B array scanner.

A *B. halodurans* C-125 Nimblegen custom array, containing a probe set of 22 unique 45mer-60mer oligonucleotide probes for each of the 4066 genes of this bacterium, was used. NimbleScan software (v 2.6.0.0, Roche NimbleGen) was used to generate one normalized

value per probe set using the RMA algorithm (background correction, normalization and summarization; data not logged). The data were then imported into R (34) using the limma package (35) 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 (36, 37). Raw p-values were corrected for multiple hypotheses testing using the False Discovery Rate method (38). Annotation for the probe sets was primarily provided by Nimblegen and included BH 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 (NC_002570). For analysis, we filtered to identify those genes that were altered by at least a 1.5-fold change in transcription (up-regulation and down-regulation). For data mining, we used DAVID bioinformatics resources that consist of an integrated biological knowledge base and analytic tools that use the results from the statistical analysis to explore and interpret gene regulation data (39).

Results

Sublancin displays sub micromolar MICs against *Bacillus* strains

The antibacterial activity of sublancin against selected *Bacillus* strains was first determined by solid agar diffusion assays containing sublancin at a range of concentrations (0.097 µM – 50 µM). After confirmation that *B. subtilis* ATCC 6633 and *B. halodurans* C-125 were sensitive, the minimum inhibitory concentrations (MICs) were determined by the broth dilution method (40, 41). A series of dilutions of sublancin (0.097 µM – 50 µM) were made and incubated with a defined number of bacterial cells in LB medium. Plates were incubated for 18-24 h at 37 °C, and growth was assessed by measuring the optical density of each well at $O.D._{600nm}$. The MICs were determined by fitting the data to a dose-response curve. The MICs of sublancin against *B. halodurans* C-125 and *B. subtilis* ATCC 6633, in liquid cultures, were 0.312 µM and 0.625 µM, respectively (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 lyse after being killed, others lyse immediately, and yet others undergo non-lytic death (42, 43).

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 (1x and 4x MIC). After the addition of sublancin, the $O.D._{600nm}$ was monitored periodically. After a 6-hour incubation period, the *B. halodurans* C-125 and *B. subtilis* ATCC 6633 cultures showed a decrease in optical density, suggesting sublancin has bactericidal activity (Fig. 1). To verify whether sublancin's activity was bactericidal, CFUs were determined by plating, which confirmed the bactericidal activity observed by O.D. readings. The decrease in optical density was not nearly as large as the decrease in CFUs, 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, 44). 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* ΔSPβ, *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 did not, even at concentrations as high as 32xMIC for *B. subtilis* ATCC 6633 and 64xMIC for *B. halodurans* C-125 (Fig. 2). We monitored the membrane integrity of *B. subtilis* ΔSPβ with the LIVE/DEAD® *Bac*Light™ bacterial cell viability assay at 30 and 90 min after addition of sublancin (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 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 (45).

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 4xMIC. 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 colonies observed were indeed resistant to the antibiotic, they were sub-cultured in sublancin-free LB media and plated on LB agar containing the antibacterial compound at a concentration of 4xMIC. 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 in a strain in which the prophages of *B. subtilis* had been deleted, including SPβ. Therefore, all mutant strains lack the gene encoding the immunity protein for sublancin, *sunI* (46), 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 in low osmotic 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 strain JJS-DIn010, in which *rsiW* and *sigW* are deleted, to have increased sensitivity (i.e. a larger zone of clearing around the producing 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 (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 where the ΔSPβ 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 (47), did not result in sublancin resistance (Fig. 3b). PtsG is the major glucose transporter of the phosphotransferase system (48), and PtsH and PtsI are general components of the sugar transport system that phosphorylates the incoming sugar (49). PtsH is more commonly known as HPr, and we will refer to it as such in this work; 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. For utilization of 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. Lastly, 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 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 *B. halodurans* C-125 and of four of the spontaneous resistant mutants obtained as described above was extracted and sequenced using a HiSeq2000 Illumina sequencer. The wild-type *B. halodurans* strain was mapped to the published *B. halodurans* C-125 genome sequence (accession no. NC_002570.2) to generate a consensus sequence that was used for SNP detection in sublancin-resistant mutants of *B. halodurans*. Comparison of 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 anti-terminator (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 non-sense mutations in the gene for the glucose-specific transporter subunit IIC that is part of the multidomain protein PtsG (Table S2 in the supplemental material). The missense mutation prevents 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 (Fig. S3 in the supplemental material), thus potentially also preventing *ptsG* transcription. Once more, these findings point to the PTS being important for sensitivity to sublancin.

Gene expression profile by microarray analysis of *B. halodurans* C-125

Antimicrobial resistance 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 up-regulated (changes from 9-14 fold, Table S3 in the supplemental material). The analysis also revealed up-regulated genes involved in transmembrane transporter activities, whereas genes involved in amino sugar and nucleotide sugar metabolism were up- and down-regulated. Interestingly, the genes for HPr and for PtsG that were also identified in the set of deletion mutants and in the comparative genomics analysis were down-regulated (Table S3 in the supplemental material) as was another PTS protein that is homologous to YpqE in *B. subtilis*, a putative phosphotransferase enzyme IIA component (50). These data again suggest that, like in *B. subtilis,* the PTS is involved in the sensitivity of *B. halodurans* towards sublancin.

Addition of PTS sugars to the growth media results in increased resistance to sublancin

Several bacteriocins have previously been shown to target PTS proteins as part of their mode of action. In these reported cases, addition of the relevant sugar resulted in an increased sensitivity to the bacteriocin (6, 7, 11). This effect is due to 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* ΔSPβ cultures were diluted in LB media (without NaCl) containing different sugars and grown in 96-well microtiter plates with shaking to an $O.D._{600nm}$ 0.5 before 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 O.D. was observed. In contrast, the non-PTS sugars citrate, galactose and succinate had no influence on sublancin's activity (Fig. 4b). The two exceptions were the non-PTS sugars glycerol and malate. In this respect it is noteworthy that the the glycerol kinase GlpK requires

phosphorylation by HPr for glycerol utilization (48, 51). Malate is a preferred carbon source for *B. subtilis* and is known to influence the carbon catabolite repression response (52). In this context it is noteworthy that a decrease in antimicrobial activity was also reported for glycocin F upon supplementation of the media 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* ΔSPβ, 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 intermediately sized zone of inhibition was observed. This observation underpins the view that the carbon source affects the sensitivity to sublancin (Fig. S4 in the supplemental material).

Since glucose was found to prevent the effect of sublancin, we wondered whether it would be possible to rescue sublancin-treated cells by addition of glucose. We therefore grew the bacteria on LB medium (with NaCl) and added sublancin at $O.D._{600nm}$ 0.5. The cells were then incubated for 30 min before addition of 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 sublancin 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 similar manner to 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 being irreversibly inactivated by sublancin, but perhaps instead sublancin affects the pathway that leads to phosphorylation of the sugar. The addition of sublancin and the PTS sugar at the same time could result in competition for phosphorylation of the sugar or the glucose on sublancin (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 sublancin

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) in 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 sublancin sensitivity to one of these HPr-mediated processes, we tested two *B. subtilis* ΔSPβ strains with point mutations at one of the two HPr phosphorylation sites. As shown in Fig. 5a, *B. subtilis* ΔSPβ carrying the S46A point mutation in HPr was fully sensitive to sublancin. In contrast, the strain carrying the H15A point mutation in HPr displayed an increased level of resistance to sublancin. This observation suggests that *hprK* and *ccpA* deletion mutants would remain sensitive to sublancin, 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 sublancin sensitivity (Fig. 5b). Also, the addition of glucose 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 glucosylated as part of its maturation process and this glucosylation is required for activity (15). We show 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 towards the phosphoenolpyruvate:sugar phosphotransferase system as a factor affecting the activity of the bacteriocin. Experiments with deletion mutants of *B. subtilis* identified PtsG, HPr, and PtsI, but not GlcT and Crh, as 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 down-regulation of

PTS genes upon exposure to sublancin and, lastly, addition of PTS sugars decreased the sensitivity to sublancin.

The PTS has been previously described as affecting sensitivity to other bacteriocins, including dysgalacticin and lactococcin A (6, 7, 53). Dysgalactin appears to bind to the glucose and mannose transporters of the PTS (7). Dysgalacticin was shown to block the uptake of glucose and the non-metabolisable analog 2-deoxyglucose, and also to perturb 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 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 (44). 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 different manner to lactococcin A, since the studies with gene deletion strains and spontaneous resistance mutants both point at the phosphorylation components of the PTS as being key to sublancin sensitivity rather than the membrane components. 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 media where the sole carbon source is either maltose or galactose again resulted in increased sensitivity to this bacteriocin (54).

Two regions of the *B. subtilis* chromosome have now been identified that result in resistance to sublancin. The first being *mscL*, encoding 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 is interacting with the PTS. Firstly, 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 could potentially be recognised by the transporter to facilitate entry into the cell or potentially to kill it through its interaction with this system. A competition between the

sublancin and glucose could explain the observed decrease in sensitivity upon addition of glucose. We note that HPr and domains IIAB of PtsG are located in the cytoplasm (Table S4 in the supplemental material), and 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 lower down 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 remove the phosphorylation sites, the mutation that led to increased resistance to sublancin was H15A. Phosphorylation of His15 is responsible for transferring a phosphate group to the incoming PTS sugar. This points towards 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 in order 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 show that sublancin exerts its bactericidal effects in a novel manner. At present it is not clear how exactly sublancin is interacting 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 is sublancin actually inhibiting growth of the cell? How does the strong structural similarity of glycocin F and sublancin fit into the mechanism and what role does the three-dimensional structure of the peptide components of these glycocins play (21, 22). In a time where bacteria are becoming resistant to the antimicrobial compounds that we currently use 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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789 **Figure Legends**

790

791 **Figure 1. Growth inhibition of *B. subtilis* ATCC 6633 and *B. halodurans* C-125 by**
792 **sublancin 168.** (a) Time-dependent changes in $O.D._{600nm}$ of cultures of *B. subtilis* ATCC
793 6633 in the absence (circles) or presence of sublancin 168 at 1xMIC (squares) and 4xMIC
794 (triangles). (b) Aliquots of the cultures in panel (a) were analysed for colony forming units
795 (CFUs). (c) Time-dependent changes in $O.D._{600nm}$ of cultures of *B. halodurans* C-125. The
796 same symbols are used as in panel (a). (d) Aliquots of the cultures in panel (c) were
797 analysed for CFUs. The mean values of the data from one experiment conducted in
798 triplicate are shown. The data are representative of three independent experiments. Error
799 bars indicate standard deviations. When error bars are not visible, they were a smaller
800 than the size of the symbol used.

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

810 **Figure 3. Deletion of the *ptsGHI* operon results in resistance to sublancin.** (a) The *B.*
811 *subtilis* strains described by Tanaka et al (29) were screened for increased and reduced
812 sensitivity to sublancin. The parental strain of the collection is labelled as 'master strain'. In
813 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. Upper panel - Blue line – *B. subtilis* ΔSPβ, green line - *B. subtilis* ΔSPβ-*glcT.* Lower panel - black line - *B. subtilis* ΔSPβ-*ptsG*, red line - *B. subtilis* ΔSPβ-*ptsH*, orange line *B. subtilis* ΔSPβ-*ptsI*. Solid lines – no sublancin. Dotted lines – 200 nM sublancin added at 100 min as indicated by the vertical grey line. Deletion of any of the genes within the *ptsGHI* operon results in resistance to sublancin. Deletion of the gene encoding the transcriptional anti-terminator *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 those from three independent experiments.

Figure 4. Addition of PTS sugars to LB blocks growth inhibition by sublancin (a) Growth curves of *B. subtilis* ΔSPβ in LB medium with salt with added PTS sugars. Blue line – no addition of sublancin, red line – addition of sublancin, 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. Sublancin was added at 120 min as indicated by the vertical grey line. (b) Growth curve of the ΔSPβ strain in LB medium with addition of non-PTS sugars. Blue line – no addition of sublancin, red line – addition of sublancin, green line – addition of 0.4% malate, grey short dashed line – addition of 0.4 % citrate, grey dotted line – addition of 0.4% galactose, grey solid line – addition of 0.4% succinate. (c) Growth curve of the ΔSPβ strain with sublancin added at 150 min followed by the addition of PTS and non-PTS sugars 30 min later as depicted by the two vertical lines, respectively. Blue line – no sublancin, blue dashed line – addition of sublancin, 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 ΔSPβ strain with sublancin added at 150 min followed by the addition of PTS and

non-PTS sugars 30 min later as depicted by the two vertical lines, respectively. Blue line – no sublancin, blue dashed line – addition of sublancin, grey 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 those from three independent experiments.

Figure 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* ΔSPβ**,** grey line - *B. subtilis* ΔSPβ**-**H15A**,** black line - *B. subtilis* ΔSPβ-S46A. Solid line – no sublancin. Dashed line – plus sublancin. (b) Blue line - *B. subtilis* ΔSPβ**,** grey line - *B. subtilis* ΔSPβ**-***hprK***,** black line - *B. subtilis* ΔSPβ-*ccpA*. Solid line – no sublancin. Dashed line – plus sublancin. The Δ*ccpA* and Δ*hprK* mutations have no effect on the sensitivity of the cells to sublancin. 200 nM sublancin added at 120 min. The means of the data from one experiment conducted in triplicate are shown. The data are representative of those from three independent experiments.

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861 **Table 1. Strains**

Strain	Genotype	Reference
168	Wild-type *B. subtilis* strain	Laboratory Collection
ΔSPβ	Wild-type *B. subtilis* 168 strain lacking the entire SPβ prophage	(14)
C-125	Wild-type *B. halodurans*	(55)
ATCC 6633	Wild-type *B. subtilis*	(56)
168 Deletion collection	Collection of *B. subtilis* mutants lacking large genomic regions	(29)
ΔSPβ::cat	Chloramphenicol selectable ΔSPβ prophage mutant of *B. subtilis*	(14)
Δ*yvkS-yvkW*	*B. subtilis* Δ*yvkS-yvkW*::phleo	This study
Δ*ykvY-glcT*	*B. subtilis* Δ*ykvY-glcT*::phleo	This study
Δ*ptsG-ptsI*	*B. subtilis* Δ*ptsG-ptsI*::phleo	This study
ΔSPβ-QB5435	*B. subtilis* Δ*pstG*::*cat*; QB5435 → SPβ	(47)
ΔSPβ-MZ303	*B. subtilis* Δ*ptsH*::*cat;* MZ303 → SPβ	(57)
ΔSPβ-GP864	*B. subtilis* Δ*ptsI*::*ermC*; GP864 → SPβ	(58)
ΔSPβ-QB5407	*B. subtilis* Δ*ccpA*::*spec*; QB5407 → SPβ	(59)
ΔSPβ-GP202	*B. subtilis* Δ*hprK*::*spec*; GP202 → SPβ	(60)
ΔSPβ::cm-GP506	*B. subtilis ptsH* H15A; SPβcm → GP506	(61)
ΔSPβ::cm-GP507	*B. subtilis ptsH* S46A; SPβcm → GP576	(62)

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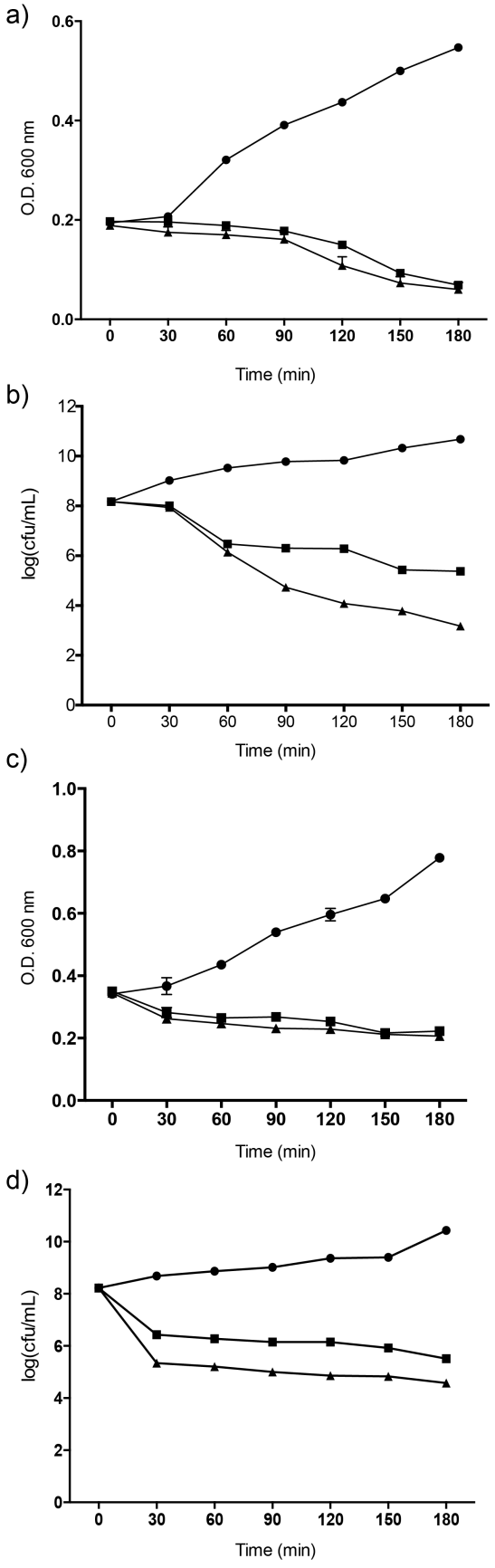
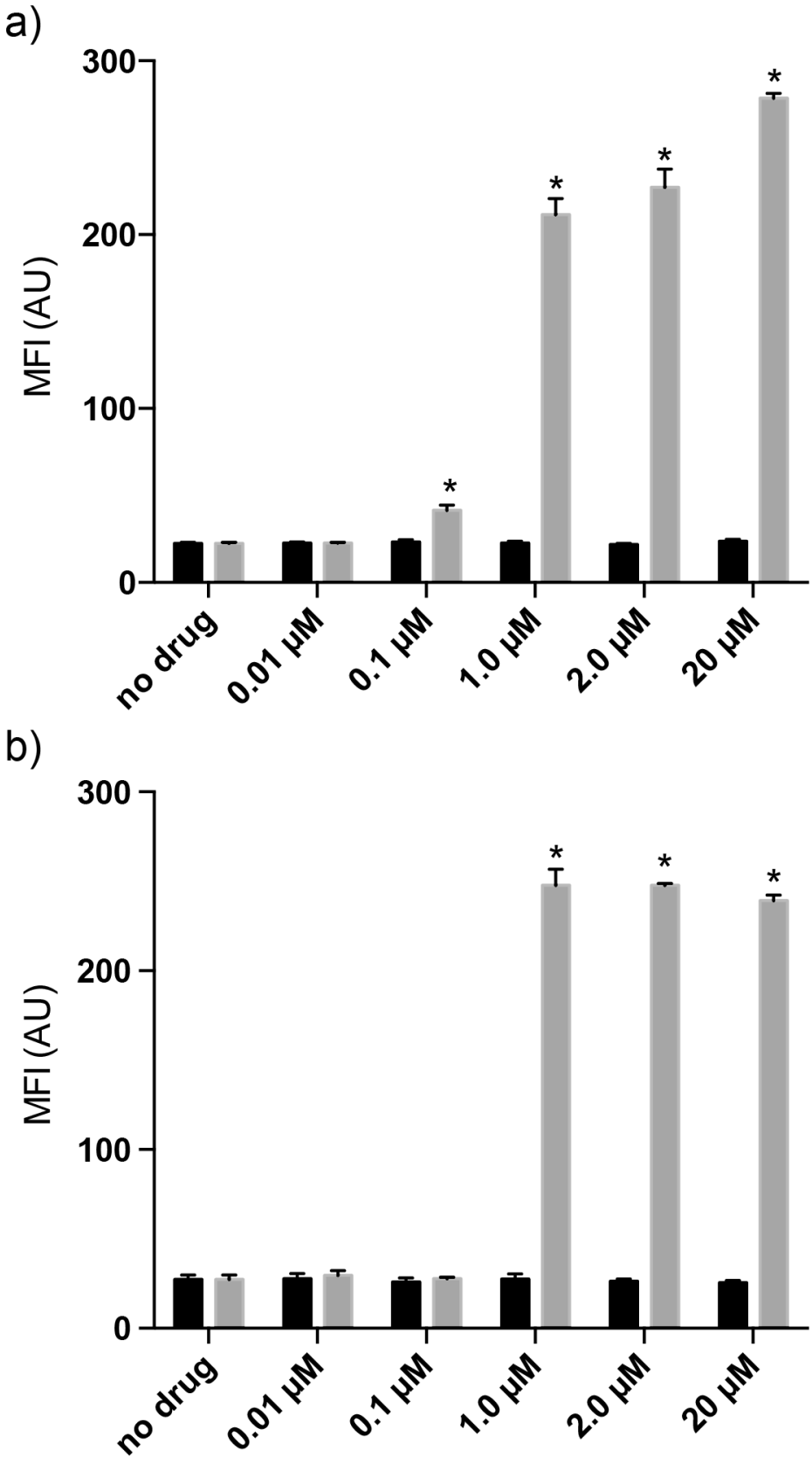
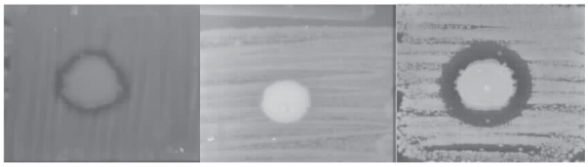
Table 2. Phenotype of single gene deletion strains of *B. subtilis* ΔSPβ upon exposure to sublancin.

Mutation	Sublancin resistant or sensitive
ykvS	Sensitive
ykvT	Sensitive
ykvU	Sensitive
stoA	Sensitive
zosA	Inconclusive
ykvY	Sensitive
ykvZ	Sensitive
glcT	Sensitive
ptsG	Resistant
ptsH	Resistant
ptsI	Resistant





a)



Master strain | JJS-DIn042 | JJS-DIn010

b)