Mechanism of Inhibition of *Bacillus anthracis* Spore Outgrowth by the Lantibiotic Nisin

SUPPORTING INFORMATION

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Table 1. Primer sequences used for mutagenesis

Gene	Desired Mutation	Primer[a]	Primer Sequence (5'→3')[b]
nisA	S5A	nisA-S5AFor	5'-GGTGCATCACCACGCATTACAAGTATTGCGCTATGTACACCCGGTTGTAAAACAAG-3'
		nisA-S5ARev	5'-CCACGTAGTGGTGCGTAATGTTCATAACGCGATACATGTGGGCCAACATTTTGTTC-3'
nisA	M21P	nisA-M21PFor	5'-ACAGGAGCTCTGATGGGTTGTAACCCCAAAACAGCAACTTGTCATTGTAGT-3'
		nisA-M21PRev	5'-TGTCCTCGAGACTACCCAACATTGGGGTTTTGTCGTTGAACAGTAACATCA-3'
nisA	N20PM21P	nisA-N20PM21PFor	5'-AAAACAGGAGCTCTGATGGGTTGTCCCCCCAAAACAGCAACTTGTCATTGTAGT-3'
		nisA-N20PM21PRev	5'-TTTTGTCCTCGAGACTACCCAACAGGGGGGTTTTGTCGTTGAACAGTAACATCA-3'
nisA	M21PK22P	nisA-M21PK22PFor	5'-GGAGCTCTGATGGGTTGTAACCCCCCCACAGCAACTTGTCATTGTAGTAATCAC-3'
		nisA-M21PK22PRev	5'-CCTCGAGACTACCCAACATTGGGGGGGTGTCGTTGAACAGTAACATCATTAGTG-3'

Supplementary Table S1. Primer sequences used for mutagenesis.
[a]Primers were designed in accordance with the QuikChange site-directed mutagenesis protocol by Stratagene (La Jolla, CA) and were synthesized by Integrated DNA Technologies (Coralville, IA). [b]Underlined sequences indicate engineered codon mutations.

Table 2. *E.coli* BL21(DE3) expression plasmids

Plasmid	Relevant Characteristics[a]	Reference
pRSFDuet-1nisAB	MCS1contains polyHis-*nisA*, MCS2 contains*nisB*, Kan^r	(*1*)
pACYCDuet-1nisC	*nisC*, Cam^r	(*1*)
pRSFDuet-1nisAB-S5A	polyHis-*nisA*S5A	This study
pRSFDuet-1nisAB-M21P	polyHis-*nisA*M21P	This study
pRSFDuet-1nisAB-N20PM21P	polyHis-*nisA*N20PM21P	This study
pRSFDuet-1nisAB-M21PK22P	polyHis-*nisA*M21PK22P	This study

Supplementary Table S2. *E. coli* BL21(DE3) expression plasmids. [a]All plasmids contain two multiple cloning sites (MCS) with gene expression under the control of an IPTG-inducible T7 promoter. MCS1 pRSF-Duet-1nisAB installs an N-terminal His_6-tag on the product of *nisA* expression. Neither *nisB* nor *nisC* are tagged allowing for cobalt affinity purification of just the modified prenisin. The amino acid changes of the nisin variants are indicated.

Table 3. Comparison of outgrowth inhibition by nisin and vancomycin			
Antibiotic[a]	Length of *B. anthracis*[b]		
	0 h	5 h	10 h
nisin	1.48 ± 0.14	1.50 ± 0.12	1.46 ± 0.09
vancomycin	1.56 ± 0.18	2.46 ± 0.18*	2.47 ± 0.33*

Supplementary Table S3. Comparison of outgrowth inhibition by nisin and vancomycin. [a]Spores were incubated in BHI with 10 µM of the indicated antibiotic. [b]At indicated time points samples were taken and visualized utilizing DIC microscopy (see Supplementary Figure S7). Size analysis, reported in µm, was performed with SoftWoRX Explorer Suite. n = 30. *Indicates statistically significant observation of longer spores in the listed condition than spores at 0 h and spores at the identical time incubated in the presence of nisin, $P < 0.001$.

Table 4. Comparison of nisin and c-nisin outgrowth inhibition			
Antibiotic[a]	Length of *B. anthracis*[b]		
	0 h	5 h	10 h
nisin	1.59 ± 0.07	1.50 ± 0.06	1.59 ± 0.06
c-nisin	1.56 ± 0.07	8.72 ± 2.88*	7.95 ± 1.18*

Supplementary Table S4. Comparison of nisin and c-nisin outgrowth inhibition. [a]Spores were incubated in BHI with 10 µM of the indicated antibiotic. [b]At indicated time points samples were taken and visualized utilizing DIC microscopy. Size analysis, reported in µm, was performed with SoftWoRX Explorer Suite. n = 30. *Indicates statistically significant observation of longer spores in the listed condition than spores at 0 h and spores at the identical time incubated in the presence of nisin, $P < 0.001$.

Table 5. Comparison of outgrowth inhibition by nisin variants			
Antibiotic[a]	Length of *B. anthracis*[b]		
	0 h	5 h	10 h
nisin	1.65 ± 0.12	1.68 ± 0.10	1.67 ± 0.13
h-nisin	1.67 ± 0.12	1.67 ± 0.14	1.70 ± 0.13
h-nisin S5A	1.68 ± 0.12	1.69 ± 0.12	1.70 ± 0.12
h-nisin N20P/M21P	1.67 ± 0.12	7.04 ± 1.12*	5.91 ± 1.67*
h-nisin M21P/K22P	1.65 ± 0.15	7.76 ± 1.60*	5.45 ± 1.68*

Supplementary Table S5. Comparison of outgrowth inhibition by nisin variants. [a]Spores were incubated in BHI with 10 μM of the indicated antibiotic. [b]At indicated time points samples were taken and visualized utilizing DIC microscopy. Size analysis, reported in μm, was performed with SoftWoRX Explorer Suite. n = 30. *Indicates significantly longer spores in the listed condition than spores at 0 h and spores at the identical time incubated in the presence of nisin, $P < 0.001$.

	IC_{50} and IC_{99} of nisin variants against *Micrococcus flavus*[a]	
Nisin A variant	IC_{50} (nM)[b]	IC_{99} (nM)[c]
nisin	1.74 ± 0.05	3.20 ± 0.31
h-nisin	1.79 ± 0.09	3.74 ± 0.50
h-nisin S5A	1.76 ± 0.07	3.21 ± 0.42
h-nisin N20PM21P	2.03 ± 0.01	5.56 ± 0.01
h-nisin M21PK22P	1.90 ± 0.03	4.58 ± 0.18

	IC_{50} and IC_{99} of nisin variants against *B. anthracis* Sterne[a]	
Nisin A variant	IC_{50}[b] (μM)	IC_{99}[c] (μM)
nisin	0.075 ± 0.005	0.094 ± 0.015
h-nisin N20PM21P	0.123 ± 0.002	0.192 ± 0.003
h-nisin M21PK22P	0.125 ± 0.006	0.196 ± 0.009

Supplementary Table S6. **IC_{50} and IC_{99} of nisin variants against vegetative cells of *Micrococcus flavus* and *B. anthracis* Sterne 7702.** [a] Three independent experiments were performed in triplicate. The values are the averages of three experiments. [b]Defined as the nisin concentration that inhibits the growth of cultures by 50% at 8 h. [c]Defined as the nisin concentration that inhibits the growth of cultures by 99% at 8 h. The potency of the hinge mutants is reduced only 2-fold against vegetative cells and is still in the nM range. In contrast, these nisin analogs were unable to prevent spore outgrowth at a concentration of 10 μM (Fig. 6D of the main text). Wild type nisin A has IC_{50} and IC_{90} values of 0.57 μM and 0.90 μM, respectively, for the inhibition of spore outgrowth of *B. anthracis* Sterne 7702 (*2*). Hence, because treatment of spores with the hinge region mutants at concentrations 10x the IC_{99} for wild type nisin did not show any inhibition of spore outgrowth, we conclude that the native hinge region is critical for inhibiting spore outgrowth, but not for inhibiting growth of vegetative cells.

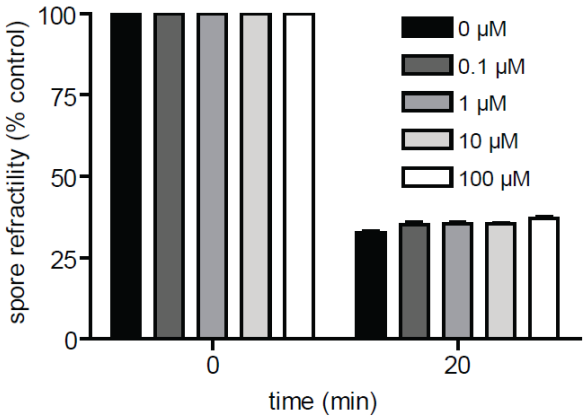
Competition Binding Assay. Competitive binding of nisin and vancomycin was evaluated by determining the interaction of the fluorescently labeled antibiotic with lipid II in the presence of the other unlabeled antibiotic (in 100-fold molar excess) as a competitor. Spores were incubated in either BHI (germinating) or 0.1 M MOPS pH 6.8 (non-germinating) for 60 min at 37 ºC followed by incubation with 0 or 100 µM of the unlabeled antibiotic for 5 min at 37 ºC. The labeled antibiotic was then added to cultures at a final concentration of 1.0 µM. The binding of the labeled antibiotic to the spores was assayed by flow cytometry to observe the reduction in antibiotic-associated increase in fluorescence in the presence of the competing unlabeled antibiotic. Analytical flow cytometry-based assays were carried out using a Beckman Coulter Epics XL-MCL flow cytometer equipped with a 70-µm nozzle, a 633 nm HeNe laser, and a 17-mV output. The band pass filter used for detecting labeled antibiotic binding was 660/20. Spore analysis was standardized for side/forward scatter and fluorescence by using a suspension of fluorescent beads (Beckman Coulter). At least 10,000 events were detected for each experiment (>2,000 events per min). Events were recorded on a log fluorescence scale. Density plots and fluorescence intensity histograms were generated using FCS Express 3.00.0311 V Lite Standalone. Sample debris (as indicated by lower forward and side scatter and a lack of PI staining) represented a small fraction (1 to 2%) of the detected events and was excluded from analysis.

Purification of heterologously expressed nisin and nisin mutants.

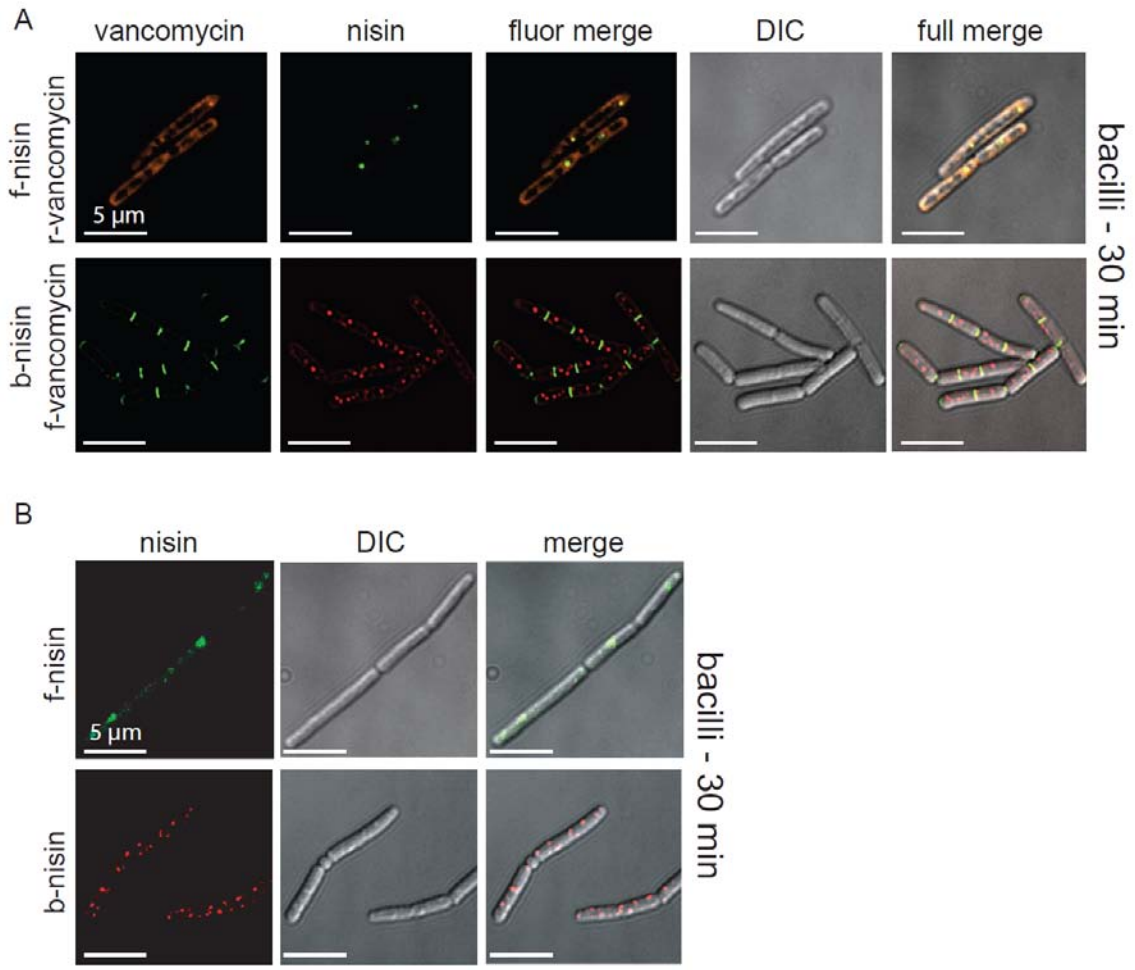
The cell pellet was resuspended in 45 mL of start buffer (20 mM Tris, pH 8.0; 500 mM NaCl), 10% glycerol, and lysed by sonication (35 % amplitude, 4.4 s pulse, 9.9 s pause for total 25 min; Sonics & Materials, Inc.). The sample was centrifuged at 23,700*xg* for 30 min at 4 °C. The supernatant was loaded onto Talon® cobalt affinity resin (Clontech) pre-equilibrated with start buffer. Following loading, the resin was washed twice with 10 mL of start buffer and once with 10 mL of wash buffer (start buffer + 30 mM imidazole). The peptide was eluted from the resin with 8 mL of elution buffer (start buffer + 1 M imidazole).

The pellet from the first sonication and centrifugation was homogenized once more via sonication (35% amplitude, 4.4 s pulse, 9.9 s pause for a total time of 25 min) in start buffer. The cell homogenates were centrifuged at 23,700*xg* for 30 min at 4 °C. The supernatant was loaded onto Talon® cobalt affinity resin for a second round of affinity protein purification as previously described. The pellet was resuspended one more time in 30 mL of denaturing buffer (6 M guanidine hydrochloride; 20 mM NaH_2PO_4; 500 mM NaCl, pH 7.5) and sonicated as before. The insoluble portion was removed by centrifugation at 23,700*xg* for 30 min at 4 °C, and the supernatant was loaded onto Talon® cobalt affinity resin. The resin was washed with denaturing buffer containing 30 mM imidazole and eluted with 8 mL of denaturing buffer containing 1 M imidazole. The eluents from all three cobalt affinity purifications were desalted via RP-HPLC utilizing a C4 semi-preparative column with a linear gradient of 0-100% acetonitrile with 0.1% TFA over 40 min. The fractions containing modified prenisin were lyophilized and analyzed by MALDI-TOF MS. The average yield from this procedure was 20-25 mg of modified NisA from 2 L of culture.

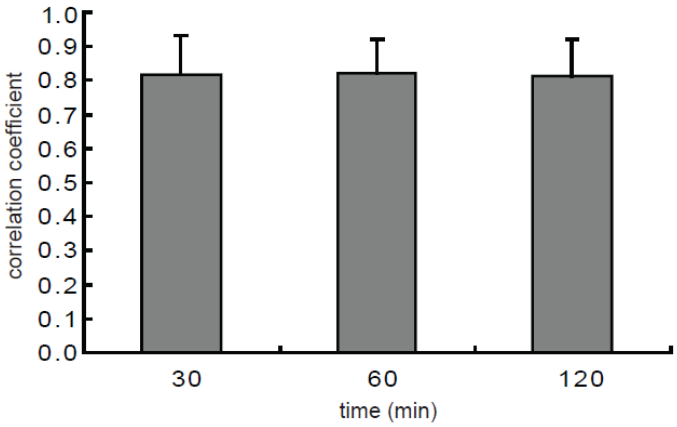
Supplementary Figures



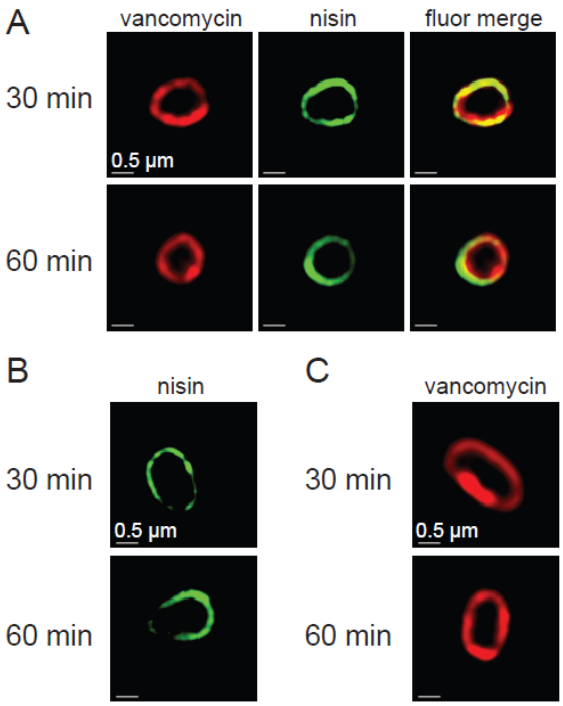
Supplementary Figure S1. Effect of vancomycin on germination. The data are expressed as the percentage of the OD_{600} relative to that of each culture at time zero. Error bars indicate standard deviation.



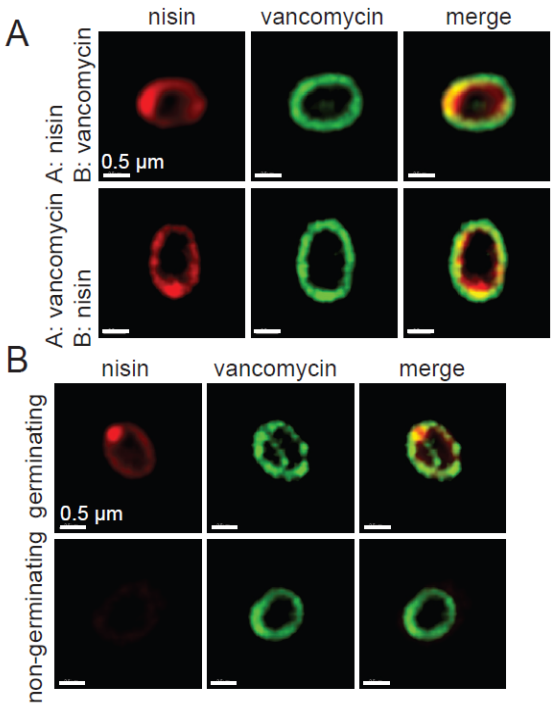
Supplementary Figure S2. Localization of labeled nisin and vancomycin on *B. anthracis* vegetative cells. A. Vegetative cells were incubated in BHI with 0.1 µM f-nisin and rhodamine-vancomycin or 0.1 µM b-nisin and f-vancomycin. At time 30 min, samples were removed and visualized by epi-fluorescence microscopy. For each panel, a single vegetative cell is shown for clarity but the image is representative of all other *B. anthracis* vegetative cells within that sample. Images were obtained with live cells as described in the methods section of the main text except for the images with f-nisin and rhodamine-vancomycin. For these images, samples were removed from *B. anthracis* cultures and fixed by incubation in 4% formaldehyde (Sigma) for 30 min at 37 °C followed by mounting on glass slides in 20% glycerol (Sigma) or Slow-Fade® antifade reagent (Invitrogen) under glass cover slips for epi-fluorescence microscopy. B. Vegetative cells were incubated in BHI with 0.1 µM f-nisin or with 0.1 µM b-nisin.



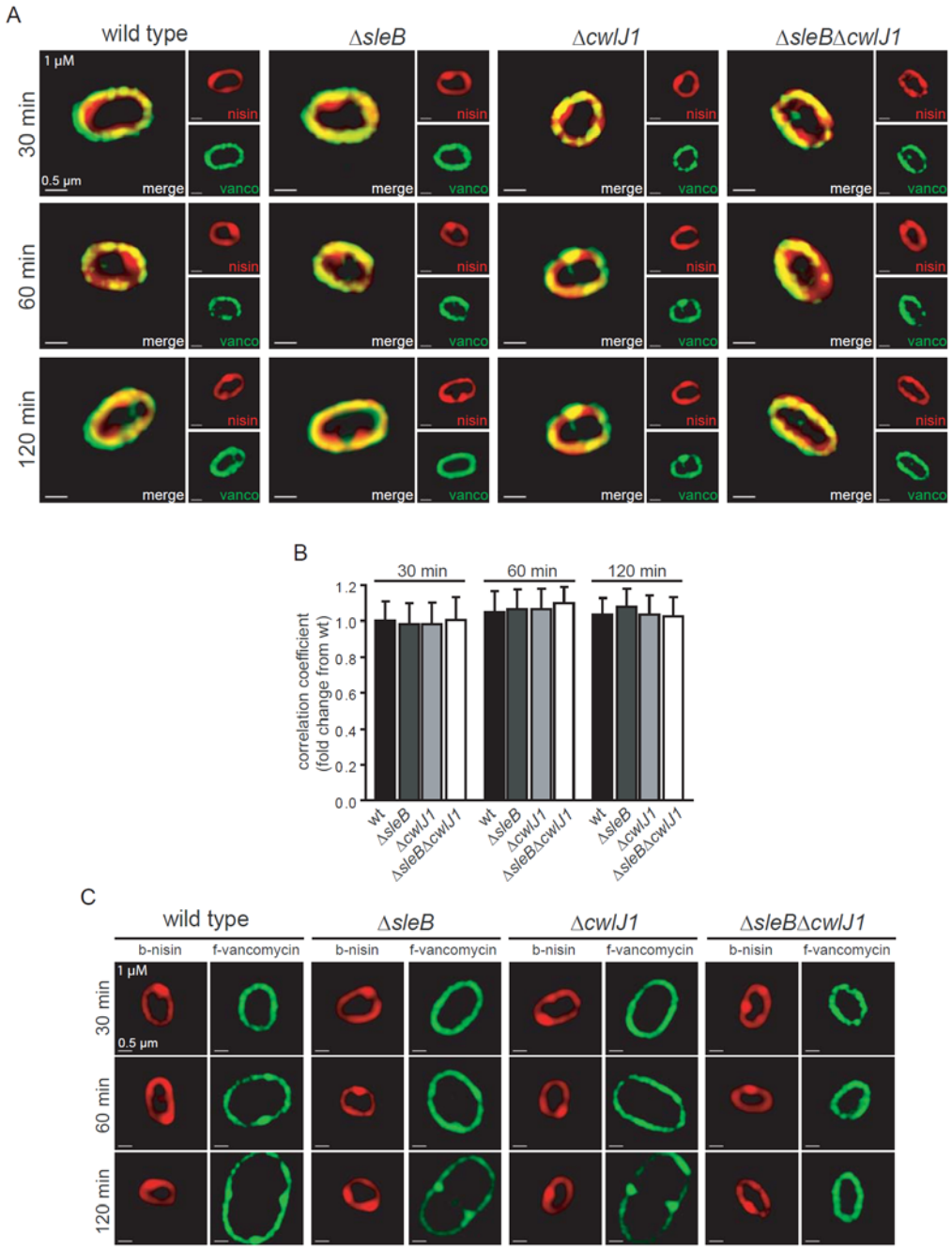
Supplementary Figure S3. Quantification of nisin and vancomycin co-localization in Figure 2A of the main text by Pearson's coefficient analysis. At time 30, 60, and 120 min, samples were removed and visualized by epi-fluorescence microscopy. Images were processed and Pearson's coefficient co-localization analysis of 50 spores per condition was performed using SoftWoRX Explorer Suite. The analysis focused on the question whether vancomycin was localized at sites where nisin was present.



Supplementary Figure S4. Effect of fluorescent label on antibiotic localization. At time 30, 60, and 120 min, samples were removed and visualized by epi-fluorescence microscopy. A. Incubation of germinated spores with f-nisin (green) and b-vancomycin (red) at 1 µM. Co-localization is indicated by yellow in merged images B. Single antibiotic incubation of germinated spores with f-nisin (green) or b-vancomycin (red) at 1 µM. For each panel, a single spore is shown for clarity but the image is representative of all other *B. anthracis* spores within that sample.

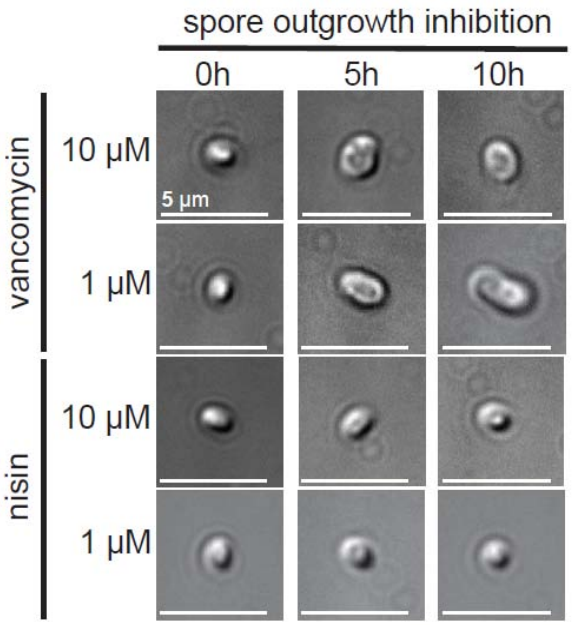


Supplementary Figure S5. Conditional effects on antibiotic localization. Spores were germinated for 30 min followed by the addition of labeled antibiotic A at 1 µM for 5 min and then subsequent addition of labeled antibiotic B for 5 min at 1 µM. Samples were removed and visualized by epi-fluorescence microscopy. Dual antibiotic incubation of germinated spores were performed with b-nisin (red) and f-vancomycin (green). B. Spores were incubated with a germinant (BHI) or without a germinant (0.1 M MOPS pH 6.8) in the presence of both labeled antibiotics. At 30 min, samples were removed and visualized by epi-fluorescence microscopy. For each panel, a single spore is shown for clarity but the image is representative of all other *B. anthracis* spores within that sample.

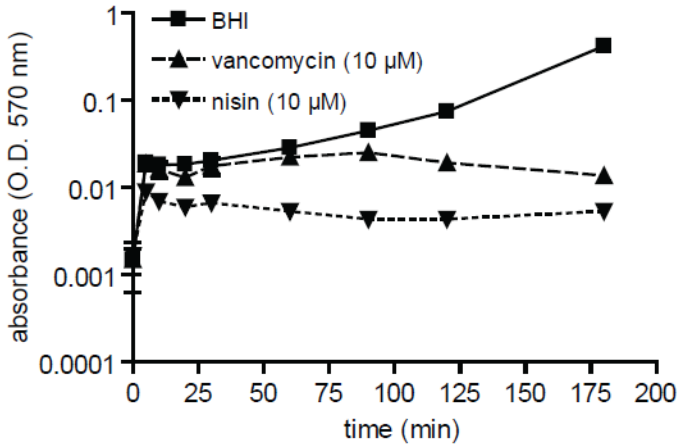


Supplementary Figure S6. Cortex hydrolase mutants do not alter nisin and vancomycin localization. At time 30, 60, and 120 min, samples were removed and visualized by epi-fluorescence microscopy. A. Dual antibiotic incubation of germinated spores with bodipy-nisin (b-nisin, red) and fluorescein-vancomycin (f-vancomycin, green) at 1 μM. Co-localization is indicated by yellow in merged images. B. Images were processed and Pearson's coefficient co-localization analysis of 30 spores per condition was performed using SoftWoRX Explorer Suite. The analysis focused on the question whether vancomycin was present where nisin was present. C. Single antibiotic incubation of germinated spores with b-nisin (red) or f-vancomycin (green) at 1 μM.

A,C. For each panel, a single spore is shown for clarity but the image is representative of all other *B. anthracis* spores within that sample. As expected outgrowth was not observed in the double mutant in panel C. Both the wild type strain and the single mutants appear to start cell division at 120 min. Importantly, the localization of both nisin and vancomycin appears to be identical for the single mutants and the wild type strain. Thus, although the structure of the cell wall is changing during cortex hydrolysis, the accessible D-Ala-D-Ala binding sites for vancomycin do not appear to change much.



Supplementary Figure S7. Lipid II binding is not sufficient for outgrowth inhibition - microscopy. At time zero and 5 and 10 h, samples were removed and visualized by DIC microscopy. For each panel, a single spore is shown for clarity but the image is representative of all other *B. anthracis* spores within that sample.



Supplementary Figure S8. Lipid II binding is not sufficient to disrupt metabolic function during germination. At the indicated times, aliquots were removed from the cultures and were evaluated for oxidative metabolism by measuring spectrophotometrically the production of formazan at 570 nm.

1. Shi, Y., Yang, X., Garg, N., and van der Donk, W. A. (2011) Production of lantipeptides in Escherichia coli, *J. Am. Chem. Soc. 133*, 2338-2341.
2. Gut, I. M., Prouty, A. M., Ballard, J. D., van der Donk, W. A., and Blanke, S. R. (2008) Inhibition of Bacillus anthracis spore outgrowth by nisin, *Antimicrob. Agents Chemother. 52*, 4281-4288.