INSIGHTS INTO THE MODE OF ACTION OF THE TWO-PEPTIDE LANTIBIOTIC HALODURACIN
SUPPORTING INFORMATION

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Figure S1. MALDI-TOF mass spectrum of purified Halα.

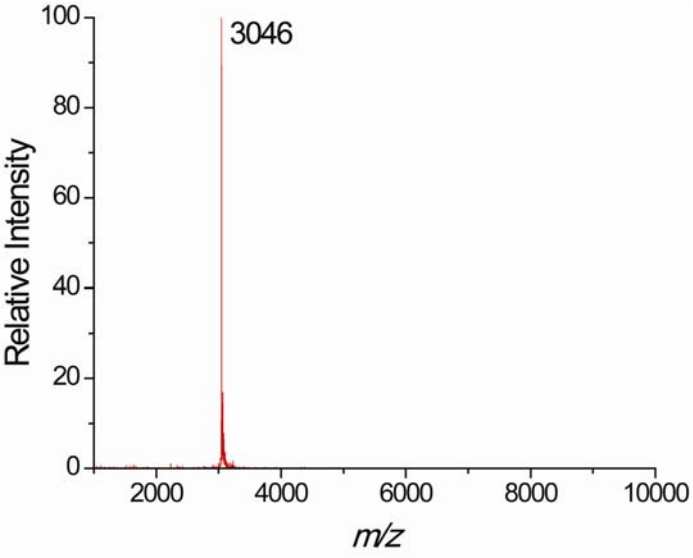


Figure S2. MALDI-TOF mass spectrum of purified Halβ.

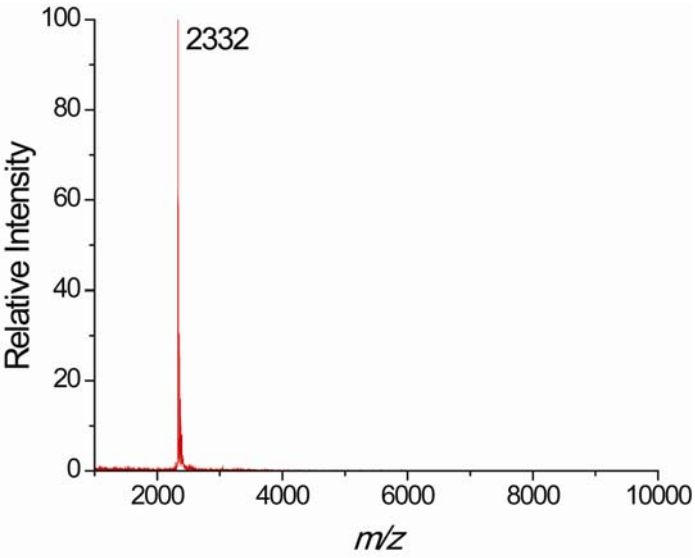


Figure S3. MALDI-TOF mass spectrum of purified nisin.

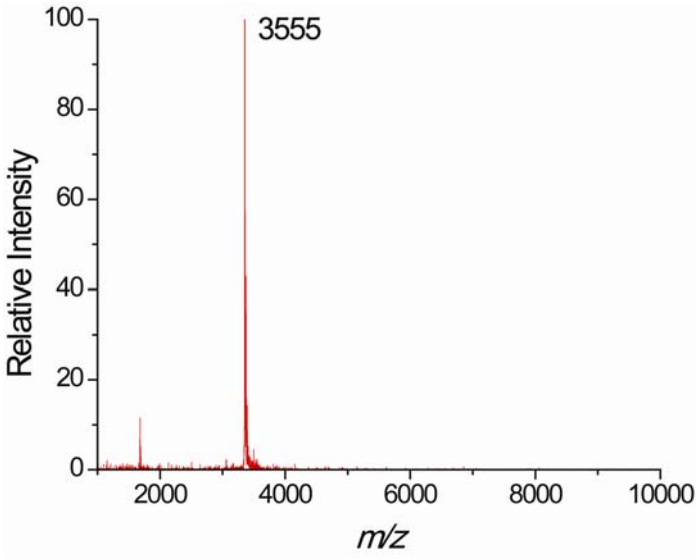


Figure S4. Halα mutants generated for this study. Shaded spheres indicate mutated residues.

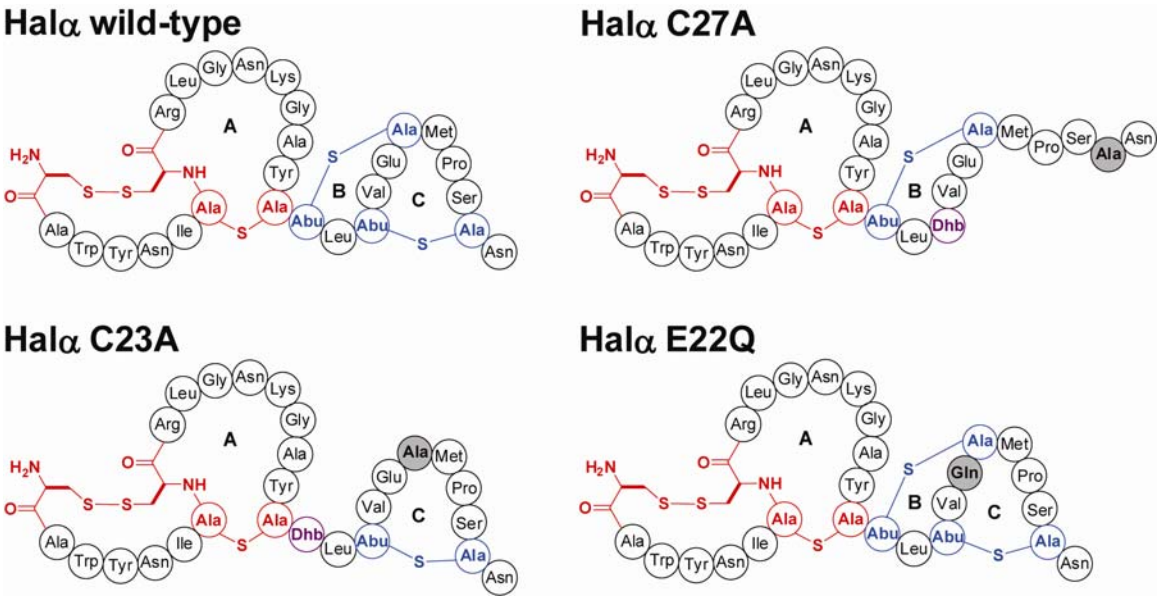


Table S1. Growth conditions for strains used in this study.

Strain	Source	Media	Conditions
Bacillus halodurans C-125	ATCC BAA-125	LB	30 °C, aerobic
Lactoccoccus lactis HP	ATCC 11602	GM17	30 °C, anaerobic
Lactoccoccus lactis 481	CNRZ 481	GM17	30 °C, anaerobic
Lactoccoccus lactis 11454	ATCC 11454	GM17	30 °C, anaerobic
Vancomycin-resistant *Enterococcus faecium*	C33105*	BHI	37 °C, aerobic
Bacillus anthracis Sterne 7702	Gut, I. M. *et al. (1)*	BHI	30 °C, aerobic
Bacillus subtilis	ATCC 6633	LB	30 °C, aerobic
Methicillin-resistant *Staphylococcus aureus*	C5*	BHI	37 °C, aerobic
Staphylococcus aureus	ATCC 12600	BHI	37 °C, aerobic
Staphylococcus epidermidis 15X	Ekkelenkamp. *et al. (2)*	MHB	37 °C, aerobic
Micrococcus luteus	ATCC 4698	LB	37 °C, aerobic
Streptococcus mutans	ATCC 25175	TH	37 °C, aerobic
Lactococcus lactis 117	CNRZ 117	GM17	30 °C, anaerobic
Bacillus subtilis DB104	Kawamura, F. *et al. (3)*	LB	30 °C, aerobic
Bacillus subtilis LH45	Liu, W. *et al. (4)*	LB	30 °C, aerobic
Bacillus cereus Z4222	INRA Z4222	LB	30 °C, aerobic
Bacillus cereus TZ415	INRA TZ415	LB	30 °C, aerobic
Enterobacter cloacae 10-19C	ATCC 29893	LB	37 °C, aerobic
Escherichia coli DH5α	UIUC-CMF	LB	37 °C, aerobic

Notes: ATCC: American Type Culture Collection, CNRZ: National Centre for Zootechnical Research, INRA: Institut National de la Recherche Agronomique, UIUC-CMF: University of Illinois Urbana-Champaign Cell and Media Facility. An * denotes clinical isolates from Carle Foundation Hospital (Urbana, IL).

Figure S5. Inhibition of *Bacillus anthracis* spore outgrowth by Halα, Halβ, or nisin. Scale bars equal 5.0 μm for all images.

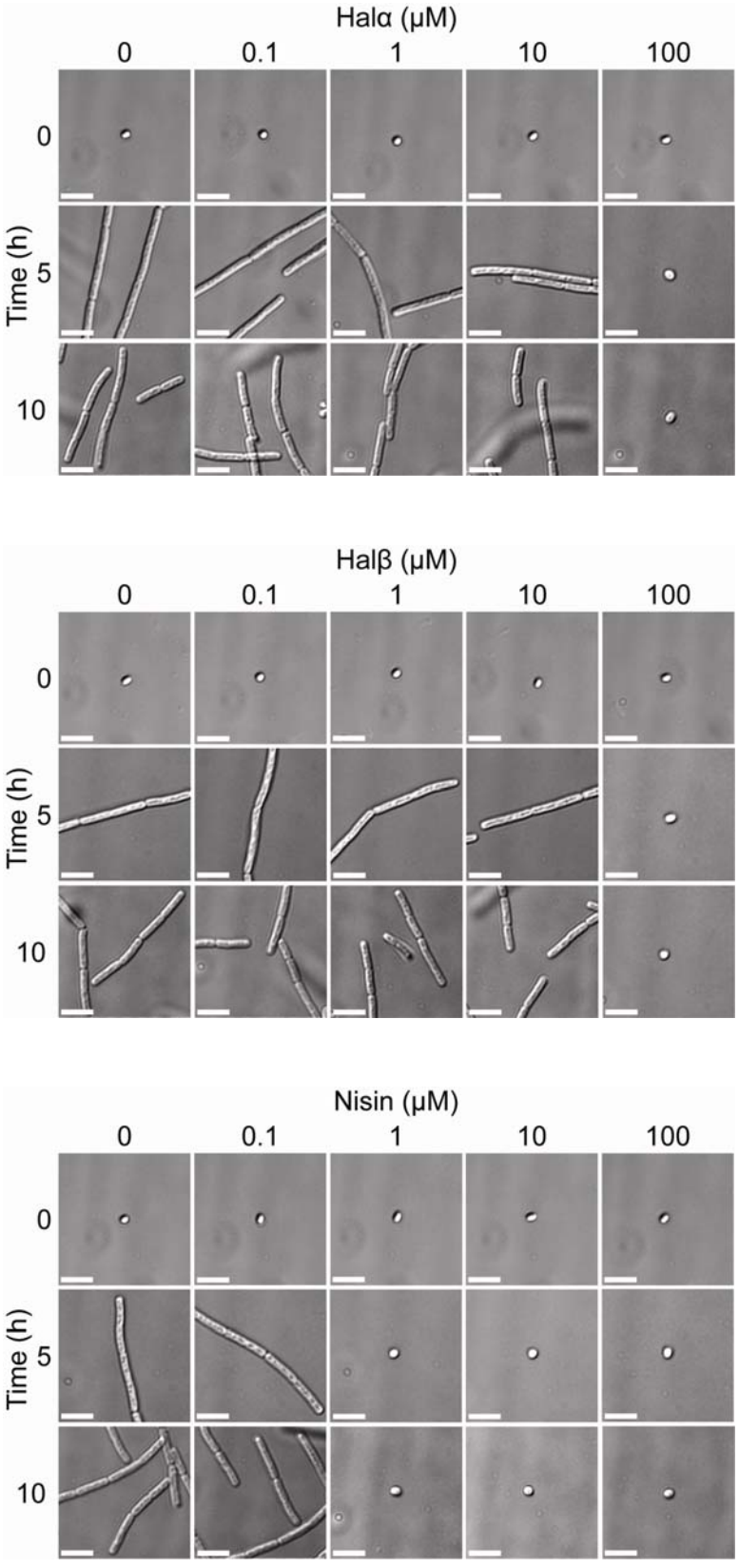
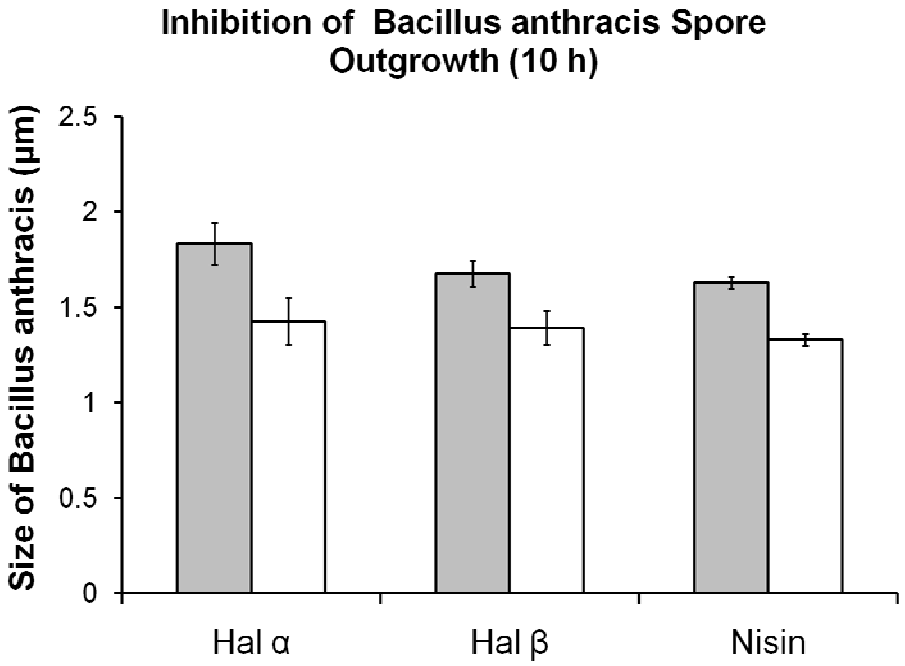


Figure S6. Measured dimensions of *Bacillus anthracis* spores.



Procedures:

Purification of Haloduracin. A culture of *Bacillus halodurans* C-125 was grown in LB under aerobic conditions for 15 h at 37 °C. Aliquots of the culture (300 µL) were removed and plated on Brain Heart Infusion agar plates (15 x 150 mm) and bacteria were grown for 96 h at 30 °C. Agar plates were gently washed with 70% isopropanol in sterile deionized water (v/v) to harvest cells from plates. The cell suspension was incubated for 24 h at 30 °C with vigorous agitation. Cells and debris were removed by centrifugation at 10k x *g* for 30 min at 4 °C and the haloduracin containing supernatant was concentrated via rotary evaporation. The resultant preparation was filtered using a 0.45 µm syringe filter to remove any residual cells or spores. Haloduracin peptides were purified by preparative HPLC performed on a Waters Delta 600 instrument equipped with a Phenomenex Jupiter Proteo C12 column (10 µm, 90 Å, 250 mm x 15 mm) equilibrated in 10% B (A: 0.1% TFA in water, B: 0.0866% TFA in 80% ACN/20% water (v/v)). The crude material was applied to the column and haloduracin peptides were eluted by maintaining the mobile phase at 10% B for 5 min, followed by an increase to 100% B over 50 min with a flow rate of 10.0 mL min^{-1}. Under these conditions, Halα and Halβ eluted at 30.5 and 32 min, respectively. Halα fractions containing trace amounts of Halβ were further purified by lyophilization, resuspension of the peptide mixture in 50 mM TCEP and incubating at RT for 3 h. This material was re-applied to the preparative HPLC column and further purified using the conditions described above. The reduced Halα peptide retention time shifted to 27 min whereas the Halβ retention time remained unchanged at 32 min. All fractions were analyzed by mass spectrometry performed on a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (Applied Biosystems Voyager DE-STR). A 1 µL aliquot of sample was combined with 1 µL of matrix consisting of saturated α-cyano-4-hydroxy-cinnamic acid matrix in 50% ACN/50% water with 0.1% TFA, and the total volume was spotted onto a MALDI target and dried under ambient conditions prior to MS analysis. The purified peptides were

lyophilized to dryness and stored under N_2 at −80 °C until further use (See Supp. Info. Figure S1 and Figure S2). Typical yields for the peptides were 2 – 3 mg Halα and 1.5 – 2 mg Halβ per liter of agar.

Purification of Nisin. A 500 mg sample of Nisaplin (Danisco) was suspended in 5 mL of 25% acetonitrile with 0.1% TFA and was sonicated for 30 min, followed by centrifugation at 15,000 x *g* for 10 min to remove insoluble material. Nisin was purified by preparative HPLC using a Waters Delta 600 instrument equipped with a Waters Delta-Pak C4 column (15 μm, 100 Å, 100 mm x 25 mm) equilibrated in 2% B (A: 0.1% TFA in water, B: 0.0866% TFA in 80% ACN/20% water (v/v)). The soluble material was applied to the column and nisin was eluted by increasing to 100% B over 45 min with a flow rate of 8.0 mL min^{-1}. Nisin eluted at 28 min. All fractions were analyzed by MALDI-TOF mass spectrometry as described above. The purified peptide was lyophilized to dryness and stored under N_2 at −80 °C until further use (See Supp. Info. Figure S3).

Preparation of Halα mutants. Purified HalA1-Xa peptides were dissolved (final concentration 0.3 mg mL^{-1}) in HalM1 assay buffer (50 mM Tris, pH 8.3) containing 10 mM $MgCl_2$, 2.5 mM ATP, and 1 mM TCEP. HalM1 enzyme was added (10 μM final concentration) and reactions were incubated at 25 °C for 3.5 h. At 3.5 h, complete conversion to dehydrated and cyclized product was observed in the soluble fraction as determined by MALDI-TOF MS. Insoluble material (unmodified peptide) was pelleted via centrifugation, and to the supernatant was added NaCl, $CaCl_2$, and Factor Xa protease (final concentrations of 100 mM, 2 mM, and 1.5 μg mL^{-1}, respectively). Cleavage reactions were incubated at 25 °C for 6 h and were quenched with 1% TFA. Reactions were lyophilized to dryness and resuspended in 10% acetonitrile in water (v/v). Halα mutant peptides were purified using preparative HPLC and analyzed by MALDI-TOF MS as described above. Specific activities for Halα mutants were determined against the *L. lactis* HP as described above in combination with wild type Halβ peptide obtained from the producing strain. Both Halα and Halβ wild type peptides (in combination) and nisin standards were used as controls.

Agar diffusion growth inhibition assays. Solid agar diffusion assays were used to assess antimicrobial activity. An overnight culture of indicator strain was grown in appropriate media and under optimal conditions (Table S1). Ninety-six well agar plates were prepared by combining 20 mL of molten media agar (cooled to 42 °C) with 200 μL of dense overnight culture (approx 10^8-10^9 CFU mL^{-1}). The seeded agar was allowed to solidify at 25 °C in a sterile Nunc OmniTray. An additional 20 mL of cooled molten media was combined with 200 μL of culture and poured over the lower solidified agar layer. A sterile 96-well PCR plate was placed in the molten agar upper layer, which was allowed to solidify at 25 °C. After sufficient solidification, the 96-well PCR plate was removed, and 15 μL of peptide solutions at a range of concentrations were separately dispensed into the newly formed wells. For assays with haloduracin peptides, a 1:1 ratio of Halα:Halβ was used. Plates were left at optimal growth temperature (strain dependent) for 15 h and antibacterial activity was qualitatively determined by the presence or absence of growth inhibition.

Construction of the isobologram. Individual peptides were incubated at the concentrations indicated in Figure 2 with the indicator strain *L. lactis* HP and nisin standards were used as controls. Ninety-six well microtiter plates were used for analysis. Serial dilutions of Halα and Halβ peptides were prepared in sterile deionized water (SDW). For assay plates, the total volume in each well was 200 μL; the experimental wells contained 50 μL of diluted Halα and Halβ peptides at defined concentrations and 150 μL of a 1-in-10 dilution (approximately 1 x 10^8 CFU mL^{-1}) of a culture of indicator strain diluted in fresh growth medium. In addition, each plate contained several blank (150 μL fresh growth medium and

50 μL SDW) and control wells (150 μL of untreated 1-in-10 diluted culture and 50 μL SDW). The optical density at 600 nm (OD_{600}) was recorded at hourly intervals from 0 to 5 h with an additional measurement at 18 h. Plates were incubated at 30 °C under anaerobic conditions. The triplicate readings were averaged, and blanks (growth medium and SDW only) were subtracted from these readings.

Stability tests. Stock solutions of nisin, Halα, and Halβ were prepared in 50 mM MOPS, 100 mM NaCl at pH 7.5 with final peptide concentrations of 25 μM. These stocks were divided into 1 mL aliquots in sterile microcentrifuge tubes and were incubated at 25 °C. At the times indicated, an aliquot was analyzed by reverse-phase HPLC (Beckman Coulter System Gold) and MALDI-TOF MS (Applied Biosystems Voyager DE-STR). Peptide aliquots were applied to a Phenomenex Jupiter Proteo C12 column (10 μm, 90 Å, 250 mm x 4.6 mm) equilibrated in 10% B (A: 0.1% TFA in water, B: 0.0866% TFA in 80% ACN/20% water (v/v)). Separation of intact peptide from degradation products was achieved by maintaining the mobile phase at 10% B for 5 min, followed by an increase to 100% B over 50 min with a flow rate of 1.0 mL min^{-1}. Fractions were collected and analyzed by MALDI-TOF MS as described above. Peak area corresponding to the intact peptide was determined via integration and plotted as a percentage relative to the peak area of peptide of day 0 (peak area of intact peptide day *n* / peak area of intact peptide day 0). Agar diffusion growth assays were performed against *L. lactis* HP as described above. For haloduracin samples, 7.5 μL of Halα (day *n*) was combined with 7.5 μL of Halβ (day *n*) and the total 15 μL was dispensed in the well. For nisin samples, 7.5 μL of nisin (day *n*) was combined with 7.5 μL of sterile 50 mM MOPS, 100 mM NaCl at pH 7.5 and the total 15 μL was dispensed in the well. Positive controls included 15 μL of nisin at 20 μM, 10 μM, 1 μM, and 100 nM in sterile buffer.

Sequential binding assays with Halα and Halβ. *L. lactis* HP cultures (in triplicate) were diluted 1-in-10 (approximately 1 x 10^8 CFU mL^{-1}), and 150 μL of each culture was added to 0.6 mL sterile microcentrifuge tubes which contained 50 μL of Halα or Halβ alone (at concentrations of 1 μM, 500 nM, 250 nM, 100 nM, 10 nM, and 0 nM). Tubes were incubated at 25 °C for 20 min prior to centrifugation at 13,000 x *g* for 1 min. The supernatants were removed from each tube and cell pellets were washed twice in fresh GM17 media. Following the second wash, cell pellets were resuspended in 150 μL of fresh GM17. Cells treated with Halα alone were added to microtiter wells, which contained 50 μL of Halβ, and cells treated with Halβ alone were added to microtiter wells that contained 50 μL of Halα (at concentrations of 1 μM, 500 nM, 250 nM, 100 nM, 10 nM, and 0 nM). As controls, Halα and Halβ were used in combination and nisin was used at 1 μM concentrations. The microtiter plates were incubated at 30 °C and the optical density at 600 nm was recorded at hourly intervals from 0 to 5 h with an additional measurement at 18 h using a BioTek Synergy 2. The triplicate readings were averaged, and blanks (150 μL of GM17 media and 50 μL SDW) were subtracted from these readings.

Analysis of inhibition of *Bacillus anthracis* spore outgrowth. Differential interference contrast (DIC) microscopy images were collected as described in *Methods*. Spore dimensions (length and width) were determined with SoftWoRX Explorer Suite. Averages and standard deviations of spore measurements were determined for each set (Figure S6). To determine if the differences in spore dimensions were significant, a Student's t-test was performed. Significance was defined by $P < 0.05$. From this analysis it appears that inhibited spores following Halα treatment are slightly larger than those following nisin or Halβ treatment.

References

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