

# Insights into the Mode of Action of the Two-Peptide Lantibiotic Haloduracin

Trent J. Oman and Wilfred A. van der Donk\*

Department of Chemistry, University of Illinois at Urbana-Champaign and the Howard Hughes Medical Institute, 600 S. Mathews Ave, Urbana, Illinois 61801

The continuing appearance of multidrug-resistant bacteria poses a significant threat to human health and presents an urgent need for the development of new antimicrobial drugs. Lantibiotics, a diverse and promising class of antimicrobial peptides produced by a variety of bacteria, display a broad spectrum of activity (1, 2). Over 50 members have been identified to date, with several displaying potent antimicrobial activity at nanomolar levels against nosocomial pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), and oxacillin-resistant Gram-positives (2, 3). Importantly, development of significant levels of resistance has not been observed for nisin, the best-studied lantibiotic that has been used worldwide against food-borne pathogens for more than four decades (4).

Lantibiotics are ribosomally synthesized as inactive precursors that undergo extensive enzyme-mediated post-translational modifications to their biologically active forms (3). The inactive precursor peptides (LanA) consist of an unmodified N-terminal leader sequence and a C-terminal structural region that is modified to the mature lantibiotic. The molecular details of the function of the leader sequence remain unclear (5); however, in addressed cases it is required for complete maturation of the structural region and for secretion of the bioactive product (3). Modifications observed in all lantibiotics involve the dehydration of select serine and threonine residues to produce dehydroalanine (Dha) and dehydrobutyrine (Dhb) residues, respectively. Following dehydration, an intramolecular Michael-type addition of cysteine thiols onto the unsaturated amino acids generates lanthionine (Lan) and methyllanthionine (MeLan) thioether rings (1).

Two-peptide lantibiotics function through the activities of two peptides whereby each peptide contributes

**ABSTRACT** Haloduracin, a recently discovered two-peptide lantibiotic composed of the post-translationally modified peptides Hal $\alpha$  and Hal $\beta$ , is shown to have high potency against a range of Gram-positive bacteria and to inhibit spore outgrowth of *Bacillus anthracis*. The two peptides display optimal activity in a 1:1 stoichiometry and have efficacy similar to that of the commercially used lantibiotic nisin. However, haloduracin is more stable at pH 7 than nisin. Despite significant structural differences between the two peptides of haloduracin and those of the two-peptide lantibiotic lactacin 3147, these two systems show similarities in their mode of action. Like Ltn $\alpha$ , Hal $\alpha$  binds to a target on the surface of Gram-positive bacteria, and like Ltn $\beta$ , the addition of Hal $\beta$  results in pore formation and potassium efflux. Using Hal $\alpha$  mutants, its B- and C-thioether rings are shown to be important but not required for bioactivity. A similar observation was made with mutants of Glu22, a residue that is highly conserved among several lipid II-binding lantibiotics such as mersacidin.

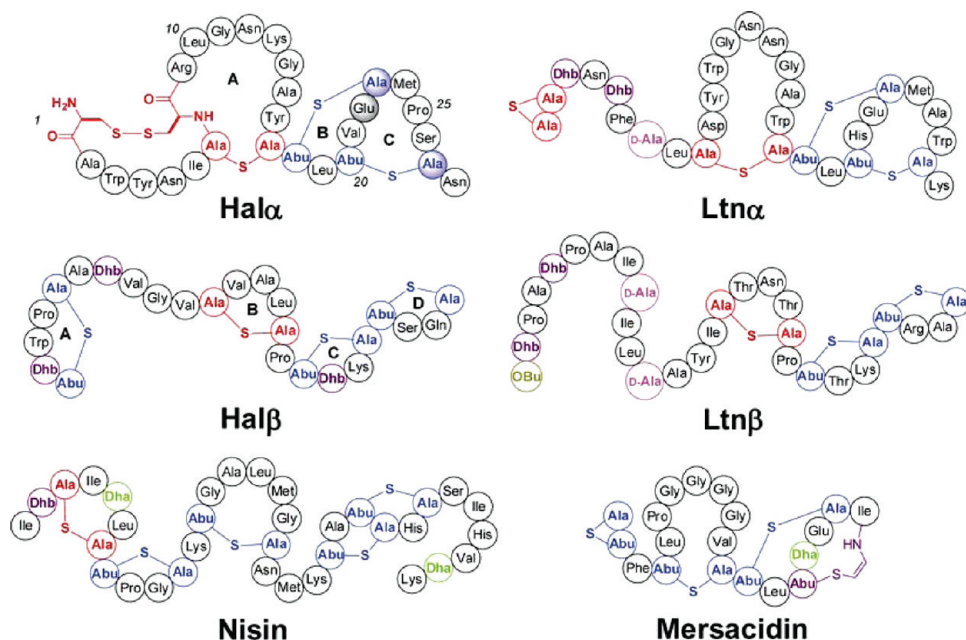
\*Corresponding author,  
vddonk@illinois.edu.

Received for review August 4, 2009  
and accepted August 12, 2009.

Published online August 13, 2009

10.1021/cb900194x CCC: \$40.75

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**Figure 1. Structures of haloduracin, lactacin 3147, nisin, and mersacidin. Shaded circles of Hal $\alpha$  indicate residues that were mutated in this study (Glu22, Cys23, and Cys27). Ala-S-Ala represents lanthionine, Abu-S-Ala represents methyllanthionine, and OBU represents 2-oxobutyryl.**

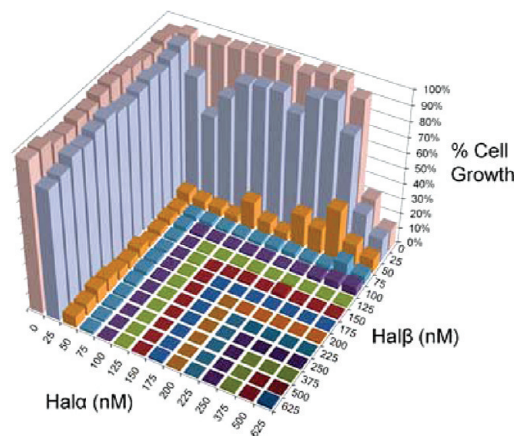
specific roles in antimicrobial activity. In these systems, two prepeptides are ribosomally synthesized as inactive precursors (LanA1 and LanA2), which are both enzymatically modified to their mature, bioactive forms (Lan $\alpha$  and Lan $\beta$ ). For all currently known two-peptide lantibiotics, the dehydration of hydroxyl-amino acids and subsequent cyclization to form (methyl)lanthionine residues results from the actions of a bifunctional modification enzyme termed LanM. In most two-peptide systems, the sequence homology between the LanA1 and LanA2 prepeptides is low; therefore two different bifunctional enzymes (LanM1 and LanM2) each act specifically on only one of the prepeptides (6, 7). The modified LanA1 and LanA2 prepeptides are further processed by a LanT protein, which removes the leader peptides and secretes the final products. The mature peptides of several two-peptide systems share structural and sequence homology with known single-peptide lantibiotics. The mature  $\alpha$ -peptides resemble the globular lantibiotic mersacidin with several fused thioether rings, whereas the mature  $\beta$ -peptides are typically elongated and more flexible (8, 9) (Figure 1).

Both nisin (10) and mersacidin (11) target the membrane-bound cell wall precursor lipid II, resulting

in the inhibition of peptidoglycan biosynthesis (12) and, for nisin but not mersacidin, pore formation (13). Binding to lipid II inhibits peptidoglycan biosynthesis by physical sequestration, preventing the actions of transglycosylase and transpeptidase enzymes that polymerize lipid II and cross-link the glycan chains of the nascent cell wall, respectively (13). In a second mode of action, lipid II is utilized as a docking molecule for the generation of defined and stable pores that result in membrane damage and depolarization and ultimately cell death (14). An additional activity for several lantibiotics including nisin is the ability to prevent spore outgrowth of Gram-positive bacteria (15, 16). A recent study suggested that inhibition of outgrowth is directly related to the

ability of the lantibiotic to induce damage to the membrane of germinating spores, thereby preventing the establishment of oxidative metabolism and a membrane potential (17).

Haloduracin, a two-peptide lantibiotic recently discovered by genome mining, is produced by the Gram-



**Figure 2. Isobologram indicating the concentrations and ratio of Hal $\alpha$  and Hal $\beta$  required to inhibit the growth of the indicator strain *Lactococcus lactis* HP.**

**TABLE 1. Comparison of haloduracin and nisin activities**

Specific activity of haloduracin and nisin assessed by liquid growth inhibition assay					
Strain	Source	Haloduracin		Nisin	
		IC <sub>50</sub> (nM)	MIC (nM)	IC <sub>50</sub> (nM)	MIC (nM)
<i>Lactococcus lactis</i> HP	ATCC <sup>a</sup> 11602	29 ± 21	73.4	14 ± 5	32.2
<i>Lactococcus lactis</i> 481	CNRZ <sup>b</sup> 481	63 ± 24	195	68 ± 6	127
<i>Lactococcus lactis</i> 11454	ATCC 11454	308 ± 13	625	1,759 ± 400	2,800
Vancomycin-resistant <i>Enterococcus faecium</i>	C33105 <sup>c</sup>	265 ± 15	781	4,953 ± 187	12,500
<i>Bacillus anthracis</i> Sterne 7702	Gut <i>et al.</i> <sup>d</sup>	363 ± 54	677	311 ± 216	417
<i>Bacillus subtilis</i>	ATCC 6633	262 ± 54	469	414 ± 165	820
methicillin-resistant <i>Staphylococcus aureus</i>	C5 <sup>e</sup>	2,616 ± 396	4,690	1,459 ± 7	1,560
<i>Staphylococcus aureus</i>	ATCC 12600	1,144 ± 290	1,560	2,205 ± 523	4,690
<i>Staphylococcus epidermidis</i> 15X	Ekkelenkamp <i>et al.</i> <sup>e</sup>	273 ± 23	313	368 ± 152	508
<i>Micrococcus luteus</i>	ATCC 4698	520 ± 17	1,250	266 ± 92	410
<i>Streptococcus mutans</i>	ATCC 25175	1,837 ± 417	2,500	5,394 ± 732	12,500

Activity assessed by agar diffusion growth inhibition assay			
Strain	Source	Haloduracin	Nisin
<i>Lactococcus lactis</i> 117	CNRZ 117	+	+
<i>Bacillus subtilis</i> DB104	Kawamura <i>et al.</i> <sup>f</sup>	+	+
<i>Bacillus subtilis</i> LH45	Liu <i>et al.</i> <sup>g</sup>	+	+
<i>Bacillus cereus</i> Z4222	INRA <sup>h</sup> Z4222	+	+
<i>Bacillus cereus</i> TZ415	INRA TZ415	+	+
<i>Enterobacter cloacae</i> 10-19C	ATCC 29893	–	–
<i>Escherichia coli</i> DH5α	UIUC-CMF <sup>i</sup>	–	–

<sup>a</sup>ATCC: American type Culture Collection. <sup>b</sup>CNRZ: National Centre for Zootechnical Research. <sup>c</sup>Clinical isolates from Carle Foundation Hospital (Urbana, IL). <sup>d</sup>Reference 17. <sup>e</sup>Reference 43. <sup>f</sup>Reference 44. <sup>g</sup>Reference 45. <sup>h</sup>INRA: Institut National de la Recherche Agronomique. <sup>i</sup>UIUC-CMF: University of Illinois Urbana-Champaign Cell and Media Facility.

positive alkaliphilic bacterium *Bacillus halodurans* C-125 (6, 18). The lantibiotic consists of two post-translationally modified peptides, Hal $\alpha$  and Hal $\beta$ . Here we describe studies investigating the mode of action of haloduracin and compare it to nisin, the prototypical lantibiotic.

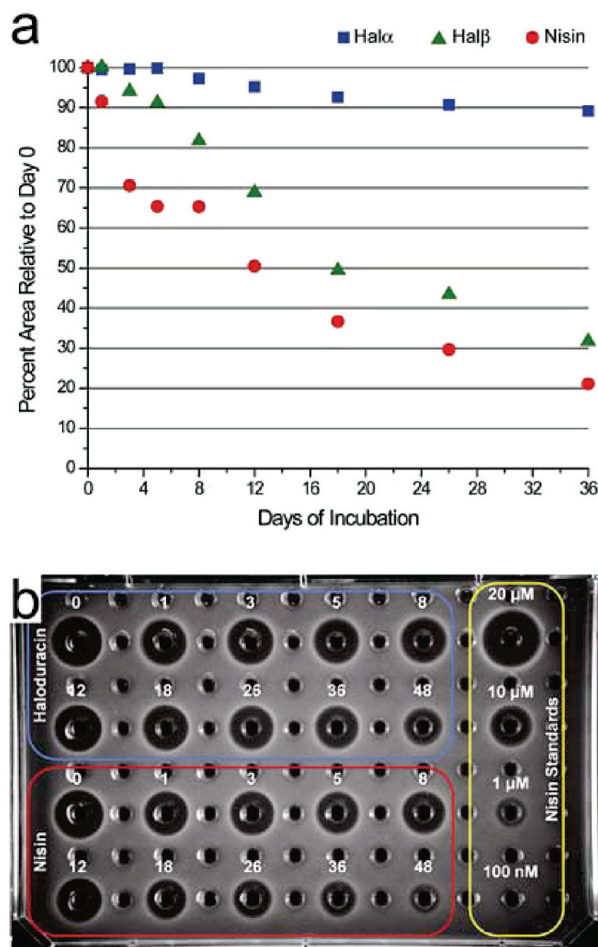
## RESULTS AND DISCUSSION

### Haloduracin Peptides Function Optimally at a 1:1

**Ratio.** Prior to assessing the specific activity of haloduracin against a panel of bacterial strains, the ratio of Hal $\alpha$  and Hal $\beta$  that yields maximum bioactivity was established. For this purpose, the ability of Hal $\alpha$  and Hal $\beta$  to inhibit cell growth at varying concentrations and ratios was examined. As indicated by the resultant isobologram (Figure 2), haloduracin is active at nanomolar

levels against the indicator strain *Lactococcus lactis* HP, and Hal $\alpha$  and Hal $\beta$  display optimal activity at a 1:1 ratio. This ratio was determined as the lowest concentration of individual Hal $\alpha$  or Hal $\beta$  peptide that when combined caused essentially complete growth inhibition (defined as >90%). The optimal synergy of Hal $\alpha$  and Hal $\beta$  at a 1:1 ratio is shared with other two-peptide lantibiotic systems such as staphylococcin C55, plantaricin W, and lactacin 3147 (19–21).

Although the haloduracin peptides are most active in combination, at much higher concentrations both Hal $\alpha$  and Hal $\beta$  displayed independent activity against the highly susceptible *L. lactis* HP strain. Using liquid media growth assays, Hal $\alpha$  and Hal $\beta$  had independent



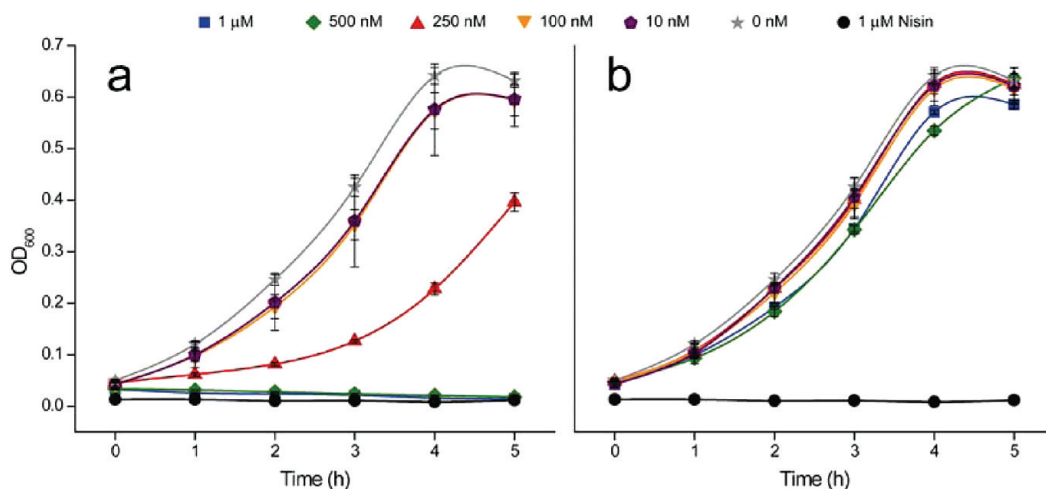
**Figure 3. Haloduracin displays greater stability than nisin at physiological pH. (a)** Assessment of relative stabilities of Hal $\alpha$ , Hal $\beta$ , and nisin at pH 7.5 using HPLC and MS analysis. **(b)** Bioassay against *Lactococcus lactis* HP using the same samples as analyzed by HPLC in panel a. The day of incubation is denoted above each well. Haloduracin: upper left (blue), nisin: lower left (red), nisin standards: right (yellow).

IC<sub>50</sub> values of 1031 ± 13 and 3146 ± 81 nM, respectively, corresponding to a 50- to 100-fold decrease in efficacy as compared to when the peptides were used together (Table 1). Though further experiments will be necessary to fully identify how these peptides are able to possess bioactivity in the absence of their counterpart, we speculate on the basis of the structural similarity of Hal $\alpha$  with mersacidin (Figure 1) that Hal $\alpha$  likely binds to lipid II, thereby preventing peptidoglycan biosynthesis. Hal $\beta$  may exert its independent activity through a mechanism similar to that of nisin in the ab-

sence of lipid II, in which the cationic Hal $\beta$  peptide binds to anionic lipids of the cell membrane and the aggregation of Hal $\beta$  monomers causes the transient formation of pore-like structures in the bacterial cell membrane (13).

**Comparison of the Antimicrobial Potency of Haloduracin and Nisin.** Agar diffusion growth inhibition assays were performed against a panel of Gram-positive and Gram-negative bacteria to obtain qualitative information regarding their sensitivity to treatment with haloduracin and nisin. These preliminary studies provided a binary outcome of either cell growth inhibition or proliferation. Strains that showed susceptibility toward haloduracin and nisin were subjected to growth inhibition assays in liquid media from which quantitative IC<sub>50</sub> values were determined. Haloduracin and nisin shared a similar spectrum of inhibitory activity against a wide range of Gram-positive organisms but lacked potency against the Gram-negative bacteria tested (Table 1). The concentrations of haloduracin and nisin required to inhibit several strains revealed that the effectiveness of the antimicrobial peptides is target-strain-dependent. For example, *L. lactis* HP was quite sensitive to both lantibiotics with IC<sub>50</sub> values in the low-nanomolar range (29 ± 21 and 14 ± 5 nM for haloduracin and nisin, respectively). Some strains were more sensitive to haloduracin than to nisin (vancomycin-resistant *Enterococcus*) and vice versa (*Micrococcus luteus*). However, in most cases, only a 2- or 3-fold difference was observed in the IC<sub>50</sub> values of haloduracin and nisin against a particular strain, and overall, haloduracin and nisin activity was roughly equivalent against the panel of strains examined in this study.

**Enhanced Stability of Haloduracin as Compared with Nisin.** Nisin shows promising activities *in vitro* and in mouse models against several clinically relevant bacteria strains (22); however, its inherent chemical instability (23, 24) and poor solubility at physiological pH have prevented therapeutic development. Nisin is produced by acidophilic *Lactococcus lactis* strains that grow and produce the compound in highest quantities in an acidic environment. Accordingly, nisin has been shown to exhibit highest solubility and stability at low pH (25). A number of degradation products of nisin have been reported (23, 24, 26). In a previous study, nisin stability was systematically monitored in low buffer concentrations over a pH range of 2–8. The results showed that nisin is most stable at pH 3, and its stability was drasti-



**Figure 4.** Sequential activity of haloduracin peptides against *L. lactis* HP. Growth curves are shown for (a) Hal $\alpha$  treatment, washing, and Hal $\beta$  addition and (b) Hal $\beta$  treatment, washing, and Hal $\alpha$  addition. When errors bars are not visible, the error was smaller than the size of the symbol used.

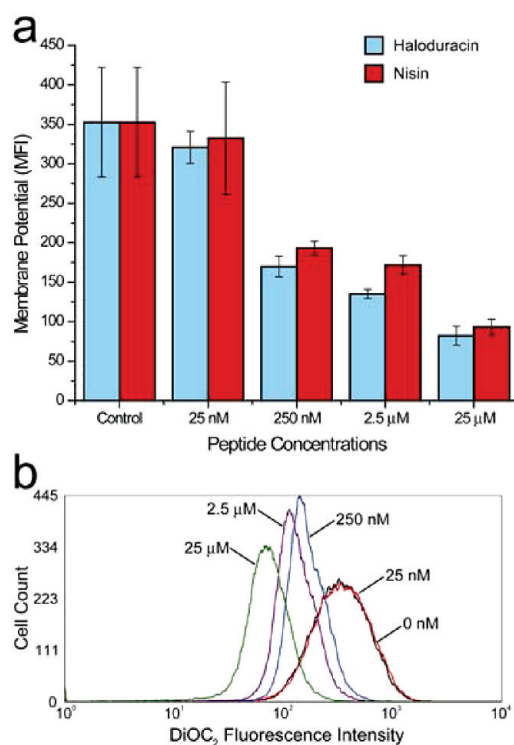
cally compromised at both lower and higher pH values. Moreover, at pH 7 and 8, essentially complete degradation was observed in 10 days (27).

Haloduracin was discovered during a search for alkaliphilic lantibiotic-producing bacteria. Its producer, *Bacillus halodurans* C-125, flourishes in basic environments with a pH greater than 9.5. For this reason, we speculated that haloduracin may have improved chemical stability compared to nisin at neutral pH. We monitored the stability of Hal $\alpha$ , Hal $\beta$ , and nisin in a solution of low buffer concentration at pH 7.5 using HPLC and MALDI-TOF MS analysis (Figure 3, panel a). Hal $\alpha$  displayed remarkable stability with >90% of intact peptide remaining after 36 days. The globular nature of this peptide, in addition to its low number of residues capable of undergoing oxidation, may contribute to its overall stability. Hal $\beta$  showed moderate stability as compared with Hal $\alpha$ , and the degradation of this linear peptide occurred gradually over the course of this assay. MS analysis of the additional peaks that were observed by HPLC shows that the products are mostly the result of oxidation ( $M + 16$ ); breakdown of the oligopeptide chain was not observed. Nisin displayed the poorest stability of the three with many oxidation products observed at early time points followed by significant degradation of the peptide chain. The increased susceptibility toward oxidation of both Hal $\beta$  and nisin may be due to their high number of thioether bridges. Agar dif-

fusion assays were utilized to evaluate the antimicrobial activities of haloduracin and nisin over time, using the same samples that were analyzed by HPLC. As shown in Figure 3, panel b, oxidation and degradation of nisin appears to have a much stronger deleterious effect on its bioactivity than oxidation of Hal $\beta$  has on the antimicrobial activity of haloduracin. The observed activity is not due to solo activity of Hal $\alpha$ , because Hal $\alpha$  has much weaker activity in the absence of Hal $\beta$ . It appears, therefore, that the structural requirements for Hal $\beta$  activity are not critically dependent on unoxidized thioether cross-links.

#### Hal $\alpha$ and Hal $\beta$ Act Sequentially To Affect Target

**Cells.** To address the potential roles of the individual peptides of haloduracin, we examined the order of binding events required to observe cell growth inhibitory activity. When target cells were incubated with Hal $\alpha$ , followed by stringent washing to remove unbound peptide and subsequent incubation with Hal $\beta$ , growth inhibition was observed (Figure 4, panel a). When the order of addition was reversed and cells were first exposed to Hal $\beta$ , washed, and then treated with Hal $\alpha$ , growth inhibition was not observed (Figure 4, panel b). When Hal $\alpha$  and Hal $\beta$  peptides were added at the same time, growth inhibition was observed (data not shown). The described outcome of sequential addition is similar to that observed for the two-peptide lantibiotic lactacin 3147 (21) and suggests a scenario in which Hal $\alpha$  binds



**Figure 5. Haloduracin and nisin display comparable membrane depolarization activities.** a) Mean fluorescence intensity (MFI) for haloduracin and nisin over a range of concentrations. b) Representative histogram of cell count versus DiOC<sub>2</sub> fluorescence intensity for haloduracin. Populations shown are for 0 nM (control, black), 25 nM (red), 250 nM (blue), 2.5 μM (violet), and 25 μM haloduracin (green).

a target on the outer surface of the bacterial cell and the resulting complex is required for Halβ to exert its synergistic effect.

**Membrane Depolarization by Haloduracin.** Given the known pore-forming activity of several lantibiotics, we examined the effect of haloduracin on membrane potential via flow cytometry with DiOC<sub>2</sub>, a membrane-potential-sensitive dye. Under physiological conditions, bacteria with intact cytoplasmic membranes maintain a membrane potential on the order of 100–200 mV (28). As shown in Figure 5, haloduracin rapidly (within 10 min) dissipated the membrane potential of *B. subtilis* cells in a concentration-dependent manner and at levels comparable to or lower than nisin. The extensive dissipation of membrane potential likely reflects damage of the cell membrane.

### Pore Formation by Haloduracin and Efflux of Potassium Ions.

The effects of haloduracin on membrane potential prompted the investigation of potassium efflux using a cell impermeable, potassium-sensitive dye (PBF). Treatment of a *M. luteus* cell suspension with Halα and Halβ triggered rapid efflux of intracellular potassium (Figure 6, panel a). Nearly identical results were observed for other Gram-positive strains such as *Bacillus subtilis* ATCC 6633 (data not shown). Potassium ion release was detected after a short lag time followed by a rapid signal increase that reached a plateau within 4–5 min. The observed potassium release was dose-dependent, and efflux was not detected in untreated samples. When cells were treated with Halα or Halβ alone at low micromolar concentrations, no leakage of potassium was observed. These findings, in addition to the results obtained from the sequential binding study described above, prompted us to further investigate the individual roles of the haloduracin peptides in pore formation. When Halα and Halβ were added together or when cells were preincubated with Halβ for 5 min followed by the addition of Halα, a short lag was observed for 20–30 s followed by rapid K<sup>+</sup> efflux. However, when cells were preincubated with Halα followed by the addition of Halβ, rapid onset of efflux was observed and the lag was essentially absent. The combined results of potassium efflux and sequential binding assays suggest that the biological role of Halα involves binding to its target, resulting in a complex that serves as a docking site for Halβ to bind and form pores. A similar mechanism has been proposed for lactacin 3147 (9), and it may be that this is a general mechanism for two-peptide lantibiotics for which the α-peptide has structural homology with mersacidin.

**Halα Mutants.** Antimicrobial activity in the nanomolar range often involves binding to a specific target molecule. On the basis of the comparable activity of haloduracin and nisin (a known lipid II-binding lantibiotic) against a range of bacteria, as well as the structural similarity of Halα with mersacidin, haloduracin most likely targets lipid II. The Halα peptide possesses a conserved CTLXEC thioether bridge motif (B-ring), which is believed to be important for lipid II binding (6, 29). This sequence is found in several class II lantibiotics including the single-peptide lantibiotics mersacidin and actagardine, as well as the Ltnα peptide from the two-peptide lantibiotic lactacin 3147 (30–32). Residing within this ring is a highly conserved glutamate residue, which has

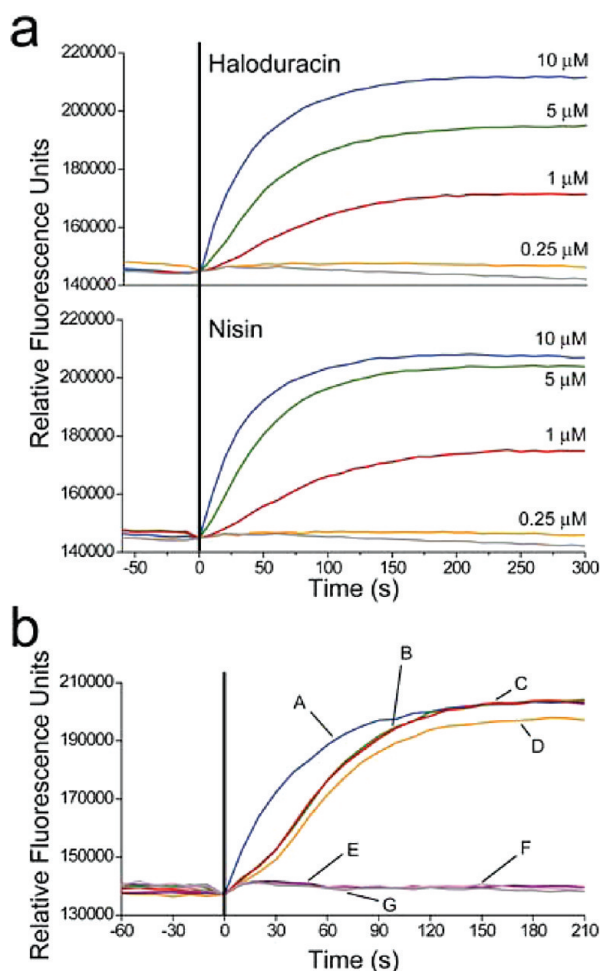
been shown for mersacidin to be essential for binding to lipid II (33).

Haloduracin biosynthesis was recently reconstituted *in vitro* (6), providing a method for the production of haloduracin mutants (29). The effects of individually disrupting the B- and C-thioether rings of Hal $\alpha$  were probed by the mutation of Cys23 and Cys27 to alanines, respectively. Additionally, the conserved glutamate residing in the conserved CLTXEC ring (B-ring) was mutated to the isosteric glutamine (Figure 1). It has been shown previously that these mutations do not affect the formation of the other ring structures (29).

All Hal $\alpha$  mutants retained the ability to inhibit *L. lactis* HP cell growth in liquid medium when incubated in the presence of wild-type Hal $\beta$ . Quantitative IC<sub>50</sub> values highlight the importance of the thioether rings of Hal $\alpha$  in the antimicrobial activity of haloduracin with IC<sub>50</sub> values of 335  $\pm$  29 and 1180  $\pm$  48 nM for wild-type Hal $\beta$  combined with Hal $\alpha$  C23A and C27A, respectively. The highly conserved glutamate residue residing in the B-ring of Hal $\alpha$  also was important but not required for activity and displayed an IC<sub>50</sub> of 964  $\pm$  76 nM for Hal $\alpha$  E22Q in combination with wild-type Hal $\beta$ .

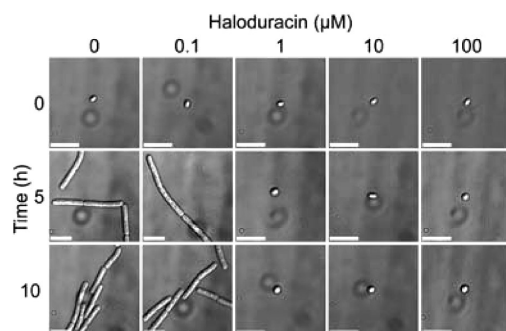
Maintaining biological activity at all despite the absence of conserved thioether rings present in Hal $\alpha$  was unanticipated as these motifs are generally considered to be the basis of lantibiotic bioactivity and disruption of a ring has often resulted in abolished activity (1, 34–39). The relatively modest reduction of bioactivity of the highly conserved B-ring mutant (10-fold) was therefore most unexpected. The retention of activity for the C27A and E22Q mutants was also unexpected as these mutants had no activity in agar diffusion assays (29). Similar increased activity of two-peptide lantibiotics in liquid media compared to solid agar media has been observed previously (40). Additional studies are necessary to investigate whether the reduced potency of Hal $\alpha$  mutants in combination with wild-type Hal $\beta$  is reflective of the ability of Hal $\alpha$  mutants to bind lipid II, to effectively recruit Hal $\beta$  for synergistic activity, or both.

**Inhibition of Spore Outgrowth.** Nisin inhibits the outgrowth of spores from several pathogenic Gram-positive bacteria including *Bacillus* and *Clostridium* (15, 41). To determine if haloduracin is capable of preventing spore outgrowth, *Bacillus anthracis* (Sterne 7702) was selected as a model. Spores were germinated in Brain Heart Infusion (BHI) medium in the presence of a range of haloduracin concentrations (Figure 7). For cultures containing



**Figure 6.** Potassium ion release from *Micrococcus luteus* cells induced by haloduracin and nisin. (a) Responses to 10 (blue), 5 (green), 1 (red), 0.25 (orange), and 0  $\mu$ M (gray) haloduracin or nisin. (b) The effects on potassium release by preincubating either Hal $\alpha$  or Hal $\beta$  prior to addition of its counterpeptide: (A, blue) 5  $\mu$ M Hal $\alpha$  preincubation followed by 5  $\mu$ M Hal $\beta$  addition; (B, green) 5  $\mu$ M Hal $\beta$  preincubation followed by 5  $\mu$ M Hal $\alpha$  addition; (C, red) simultaneous addition of 5  $\mu$ M Hal $\alpha$  and 5  $\mu$ M Hal $\beta$ ; (D, orange) addition of 5  $\mu$ M nisin; (E, violet) addition of 5  $\mu$ M Hal $\alpha$ ; (F, pink) addition of 5  $\mu$ M Hal $\beta$ ; and (G, gray) a control without haloduracin. Vertical line (black) indicates time of antibiotic addition.

low amounts of haloduracin (0.1  $\mu$ M), differential interference contrast (DIC) microscopy revealed chains of vegetative bacilli after 5 and 10 h. However, no bacilli were present within cultures supplemented with 1, 10, or 100  $\mu$ M haloduracin. This dose-dependent response shows that haloduracin was equally effective as nisin in



**Figure 7.** Inhibition of *Bacillus anthracis* spore outgrowth by haloduracin. Scale bars equal 5.0  $\mu\text{m}$  for all images.

the inhibition of *B. anthracis* spore outgrowth (17). Hal $\alpha$  and Hal $\beta$  also inhibited spore outgrowth when incubated independently, but only at 100  $\mu\text{M}$  concentrations, whereas vegetative bacilli were observed in cultures containing 10  $\mu\text{M}$  or less of either peptide

## METHODS

**Materials, Cultures, and Conditions.** All chemicals and HPLC grade solvents were purchased from Sigma-Aldrich, unless otherwise stated. Tris and MOPS buffers were obtained from Fisher and  $\alpha$ -cyano-4-hydroxy-cinnamic acid from Fluka. All media including Luria Bertoni (LB), Brain Heart Infusion (BHI), Todd Hewitt (TH), Mueller Hinton Broth (MHB), and M17 supplemented with 0.5% glucose (w/v) (GM17) were purchased from BD Biosciences. Factor Xa was purchased from New England Biolabs. Supplementary Table S1 describes the bacteria strains, source, and culture conditions for all bacteria used for this study. All Hal $\alpha$  mutants were prepared as described previously (6, 29).

**Specific Activity Determination.** Microtiter plates were used to determine the  $\text{IC}_{50}$  values (the concentration at which 50% growth inhibition is observed) of indicator strains. For assays with haloduracin, a 1:1 ratio of Hal $\alpha$ :Hal $\beta$  was used. Serial dilutions of Hal $\alpha$  and Hal $\beta$  peptides were prepared in sterile deionized water (SDW). Ninety-six-well and forty-eight-well microtiter plates (Corning Costar) were utilized for anaerobic and aerobic strains, respectively (see Materials, Cultures, and Conditions above). For 96-well plates, the total volume of culture in each well was 200  $\mu\text{L}$ ; the experimental wells contained 50  $\mu\text{L}$  of diluted Hal $\alpha$  and Hal $\beta$  peptides at defined concentrations and 150  $\mu\text{L}$  of a 1-in-10 dilution (approximately  $1 \times 10^8$  CFU  $\text{mL}^{-1}$ ) of a culture of indicator strain diluted in fresh growth medium. In addition, each plate contained several blank (150  $\mu\text{L}$  fresh growth medium and 50  $\mu\text{L}$  SDW) and control wells (150  $\mu\text{L}$  of untreated 1-in-10 diluted culture and 50  $\mu\text{L}$  SDW). For 48-well plates, the total volume in each well was 300  $\mu\text{L}$ ; the experimental wells contained 75  $\mu\text{L}$  of diluted Hal $\alpha$  and Hal $\beta$  peptides at defined concentrations and 225  $\mu\text{L}$  of a 1-in-10 dilution (approximately  $1 \times 10^8$  CFU  $\text{mL}^{-1}$ ) of a culture of indicator strain diluted in fresh growth medium. In addition, each plate contained several blank (225  $\mu\text{L}$  fresh growth medium and 75  $\mu\text{L}$  SDW) and control wells (225  $\mu\text{L}$  of untreated 1-in-10 diluted culture and 75  $\mu\text{L}$  SDW). The optical density at 600 nm ( $\text{OD}_{600}$ )

(Supplementary Figure S5). Hence, the spore outgrowth inhibition depicted in Figure 7 is the result of a synergistic mechanism of both peptides.

**Conclusion.** This study illustrates the potent antimicrobial activity displayed in a synergistic manner by the two peptides of haloduracin. The activity of this two-peptide lantibiotic rivals that of nisin, which has been used worldwide as an effective preservative against food-borne pathogens. Haloduracin displayed stability higher than that of nisin at pH 7, which bodes well for potential use. Despite significant differences in the structures of both the  $\alpha$ - and  $\beta$ -peptides of lacticin 3147 and haloduracin (Figure 1), these two compounds appear to operate by a similar mechanism that involves recognition by the  $\alpha$ -peptides of a target at the cell surface, likely lipid II, and utilization of the  $\beta$ -peptides for membrane permeabilization. As such, these compounds combine the two activities of nisin into separate peptides (42).

was recorded at hourly intervals from 0 to 5 h with an additional measurement at 18 h using a BioTek Synergy 2 plate reader. Plates were incubated according to optimal growth conditions (see Materials, Cultures, and Conditions above). The triplicate readings were averaged, and blanks (growth medium and SDW only) were subtracted from these readings. Growth curves were developed using control (culture and SDW only) readings to ensure sufficient OD changes for accurate inhibition assessment. Curve fits for  $\text{IC}_{50}$  determination were produced by fitting the data with Origin8 software using the dose-response curve with the equation  $y = A_1 + (A_2 - A_1)/(1 + 10^{(p \log x_0 - x)/p})$ , with  $p$  = variable Hill slope. A 50% growth inhibition was determined as half the final  $\text{OD}_{600} \pm 0.05$ . Nisin  $\text{IC}_{50}$  values were determined using identical procedures as described above.

Specific activities for individual peptides were determined essentially as described above, except that peptides were evaluated independently. Specific activity was assessed against the indicator strain *L. lactis* HP, and nisin standards were used as controls.

**Membrane Potential Assays.** Membrane potential was measured indirectly using the membrane potential-sensitive dye, 3,3'-diethyloxycarbocyanine iodide (DiOC $_2$ , Molecular Probes/Invitrogen). *B. subtilis* ATCC 6633 cultures (in triplicate) were grown to a density of  $4 \times 10^6$  cells  $\text{mL}^{-1}$ , and aliquots were transferred to tubes containing DiOC $_2$  (final concentration of 300 nM). Cells were incubated with the dye for 20 min at 30  $^\circ\text{C}$  under aeration conditions prior to the addition of either haloduracin or nisin at 25  $\mu\text{M}$ , 2.5  $\mu\text{M}$ , 250 nM, 25 nM, or 0 nM. Following addition of peptides, cultures were incubated at 30  $^\circ\text{C}$  with aeration for an additional 10 min prior to analysis. The membrane potential was assessed by measuring the *B. subtilis*-associated DiOC $_2$  fluorescence by flow cytometry (BD Biosciences LSR II flow cytometer) with excitation at 488 nm with an argon laser and measurement of emission through a band-pass filter at 530/30 nm. A minimum of 50,000 events were de-



ected for each sample, and the data were analyzed using the FCS Express 3.00.0311 V Lite Stand-alone software. The data were plotted as the geometric mean of the fluorescence intensity (MF).

**Potassium Ion Release Assay.** Potassium efflux was measured using the  $K^+$ -sensitive fluorescent dye 1,3-benzenediacarboxylic acid, 4,4'-(1,4,10,13-tetraoxa-7,16-diazacyclo-octadecane-7,16-diylbis(5-methoxy-6,2-benzofurandiyl))bis (PBFI, Molecular Probes/Invitrogen). A culture of *M. luteus* was grown to an  $OD_{600}$  of 1.0 and washed five times with assay buffer (5 mM HEPES, 5 mM glucose, pH 7.2). Washed cells were resuspended in one-fifth the volume of assay buffer containing PBFI (final concentration of 10  $\mu$ M) and aliquots were added to UV-transparent microtiter plates (Nunc). Sample fluorescence (excitation = 346 nm, emission = 505 nm) background data was collected (BioTek Synergy 2 plate reader, excitation filter 360/40, emission filter 508/20) until baseline signal was achieved, followed by the addition of haloduracin (Hal $\alpha$  and Hal $\beta$  in a 1:1 ratio) or nisin at 10  $\mu$ M, 5  $\mu$ M, 1  $\mu$ M, 250 nM, or 0 nM. For preincubation studies of the haloduracin peptides, baseline signal was achieved, followed by the addition of either Hal $\alpha$  or Hal $\beta$  to wells at the concentrations indicated and incubation for 5 min prior to the addition of Hal $\beta$  or Hal $\alpha$ , respectively.

**Inhibition of *Bacillus anthracis* Spore Outgrowth.** *B. anthracis* spores at a concentration of  $4.0 \times 10^6$  spores  $mL^{-1}$  were incubated in Brain Heart Infusion (BHI) medium supplemented with haloduracin (0.1, 1, 10, or 100  $\mu$ M at a Hal $\alpha$ :Hal $\beta$  ratio of 1:1) or 0.1 M MOPS (pH 6.8) as a control. For assessment of Hal $\alpha$  and Hal $\beta$  used independently, spores were incubated in BHI medium supplemented with the peptide at 0.1, 1, 10, or 100  $\mu$ M or with 0.1 M MOPS (pH 6.8) as a control. As a positive control, spores were treated with nisin under the same conditions and concentrations as used for Hal $\alpha$  and Hal $\beta$ . Cultures were incubated at 37  $^{\circ}C$  under aeration conditions and ambient  $CO_2$  (0.03%  $CO_2$ ). At 0, 5, and 10 h, samples were removed from the *B. anthracis* cultures and fixed by incubation in 3% formaldehyde (v/v) for 30 min at 37  $^{\circ}C$ . Samples were mounted on glass microscope slides in 20% glycerol (v/v). Differential interference contrast (DIC) microscopy images were collected with an Applied Precision assembled DeltaVision epifluorescence microscope equipped with an Olympus Plan Apo x100 oil objective with a numerical aperture of 1.42 and a working distance of 0.15 mm. Images were processed with SoftWoRx Explorer Suite.

**Acknowledgment:** We thank I. Gut for assistance with spore outgrowth and microscopy studies. This work was supported by the Howard Hughes Medical Institute.

**Supporting Information Available:** This material is available free of charge via the Internet at <http://pubs.acs.org>.

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