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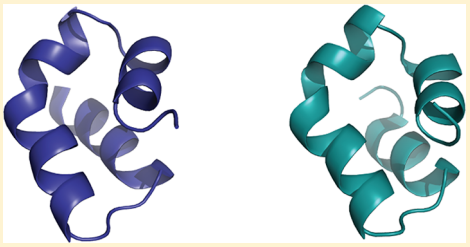
Solution Structures of the Linear Leaderless Bacteriocins Enterocin 7A and 7B Resemble Carnocyclin A, a Circular Antimicrobial Peptide

Christopher T. Lohans,[‡,†] Kaitlyn M. Towle,[‡,†] Mark Miskolzie,[†] Ryan T. McKay,[†] Marco J. van Belkum,[†] Lynn M. McMullen,[§] and John C. Vederas*[,†]

[†]Department of Chemistry, and [§]Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, Alberta, Canada T6G 2G2

Supporting Information

ABSTRACT: Leaderless bacteriocins are a class of ribosomally synthesized antimicrobial peptides that are produced by certain Gram-positive bacteria without an N-terminal leader section. These bacteriocins are of great interest due to their potent inhibition of many Gram-positive organisms, including food-borne pathogens such as *Listeria* and *Clostridium* spp. We now report the NMR solution structures of enterocins 7A and 7B, leaderless bacteriocins recently isolated from *Enterococcus faecalis* 710C. These are the first three-dimensional structures to be reported for bacteriocins of this class. Unlike most other linear Gram-positive bacteriocins, enterocins 7A and 7B are highly structured in aqueous conditions. Both peptides are primarily α-helical, adopting a similar overall fold. The structures can be divided into three separate α-helical regions: the N- and C-termini are both α-helical, separated by a central kinked α-helix. The overall structures bear an unexpected resemblance to carnocyclin A, a 60-residue peptide that is cyclized via an amide bond between the C- and N-termini and has a saposin fold. Because of synergism observed for other two-peptide leaderless bacteriocins, it was of interest to probe possible binding interactions between enterocins 7A and 7B. However, despite synergistic activity observed between these peptides, no significant binding interaction was observed based on NMR and isothermal calorimetry.



Ribosomally synthesized antimicrobial peptides produced by bacteria, also known as bacteriocins, are of great recent interest due to their potential uses for food preservation.[1] Gram-positive bacteria produce a diverse array of bacteriocins with many having a variety of post-translational modifications.[1,2] Most bacteriocins are initially produced with an N-terminal extension, referred to as a leader peptide.[3] During the maturation of these bacteriocins, the leader peptide is proteolytically removed, often by a dedicated protease.[3] Leader peptides play numerous roles in the production and export of bacteriocins, including the protection of the producer organism, targeting the immature bacteriocin to enzymes responsible for post-translational modifications and directing the bacteriocin to transporter proteins involved in export.[3,4] Among the post-translational modifications controlled by leader peptides are the formation of lanthionine and methyllanthionine bridges in the lantibiotics,[5] the linkage of cysteine sulfur to alpha-carbon bridges in the sactibiotics,[6] and, possibly, the cyclization of the N- to C-terminus of the circular bacteriocins.[7]

The leaderless bacteriocins are distinguished by the absence of this common N-terminal extension seen in the structural genes of most antimicrobial peptides from bacteria.[8] Accordingly, the leaderless bacteriocins do not undergo any further post-translational modifications, and export is likely mediated by a dedicated ABC transporter.[8,9] However, many leaderless bacteriocins bear an N-terminal formylmethionine residue, a feature not commonly found in mature bacterial peptides.[10,11] Furthermore, the gene clusters responsible for the production of leaderless bacteriocins differ from those involved in the production of other bacterial antimicrobial peptides.[12] One notable feature is the occasional occurrence of two clustered structural genes encoding similar leaderless bacteriocins, which could be a result of gene duplication.[12] One such gene cluster is responsible for the production of enterocin L50A and L50B, the first two-peptide leaderless bacteriocin to be reported.[12] Enterocins L50A and L50B are highly homologous and display 72% amino acid identity.[12] More recently, another two-peptide leaderless bacteriocin was isolated from a culture of *Enterococcus faecalis* 710C and characterized.[11] The individual peptides, enterocins 7A and 7B, showed high levels of homology to enterocin L50A and L50B (98% and 95% amino acid identity, respectively). Approximately 20 distinct leaderless bacteriocins have been reported to date.[8]

Leaderless bacteriocins are of particular interest due to their relatively broad spectra of activity, with inhibition of important food pathogens such as *Clostridium* spp. and *Listeria monocytogenes*.[11] These bacteriocins are also active against vancomycin-resistant enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA),[11] which occur frequently in hospital-acquired infections. However, despite the potential industrial and therapeutic utility of the leaderless bacteriocins, little is known about their three-dimensional

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structures in solution. Circular dichroism (CD) and fluorescence experiments have been reported for preliminary evaluation of the structures of aureocin A53 and lacticin Q_2 from *S. aureus* A53 and *Lactococcus lactis* QU5, respectively.[10,13] Structural characterization of the leaderless bacteriocins is an essential prerequisite for an understanding of their mode of action. It may also provide valuable clues that account for the broad spectra of activity exhibited by these bacteriocins. Hence, we examined the solution structures of enterocin 7A and 7B using NMR studies and now report that they have an unexpected resemblance to large circular bacteriocins such as carnocyclin A.

MATERIALS AND METHODS

Isolation of Enterocins 7A and 7B. The approach used for the purification of enterocins 7A and 7B was based on literature precedent, with some modifications.[11] An overnight culture of *E. faecalis* 710C was grown in BD Difco All-Purpose Tween (APT) broth at 37 °C without shaking. This culture was used to inoculate (5% v/v) 1 L of APT, which was incubated at 37 °C, without shaking, for 22 h. The culture was then centrifuged (10000*g*, 10 min, 4 °C) to pellet the bacteria. The supernatant was loaded onto a SP Sepharose Fast Flow column (20 mL resin, 1 mL/min; Sigma-Aldrich) preequilibrated with 20 mM sodium phosphate (pH 6.9). The column was washed with 100 mL of 20 mM sodium phosphate (pH 6.9) and then with 100 mL of 20 mM sodium phosphate (pH 6.9) with 0.2 M NaCl. Enterocins 7A and 7B were eluted with 100 mL of 20 mM sodium phosphate (pH 6.9) with 1.0 M NaCl.

The elution fraction was desalted using a Bond Elut C18 10 g, 60 mL cartridge (Agilent) preconditioned with 50 mL of methanol and 100 mL of deionized water. After the elution fraction was loaded onto the cartridge, it was washed with 50 mL of each of the following: 30% ethanol, 30% acetonitrile, and 40% isopropyl alcohol (IPA). Enterocins 7A and 7B were eluted from the cartridge using 50 mL of 80% IPA acidified with 0.1% trifluoroacetic acid (TFA).

The 80% IPA 0.1% TFA elution fraction was concentrated *in vacuo* to approximately 10 mL. Enterocins 7A and 7B were purified by HPLC using a Vydac C_{18} Peptide & Protein column (5 μm, 4.6 mm × 250 mm). Chromatography was monitored using a UV/vis detector set at 220 nm. The solvents used were water with 0.1% TFA (solvent A) and acetonitrile with 0.1% TFA (solvent B). The solvent gradient (using a flow rate of 1 mL/min) was initially held at 30% solvent B for 5 min, before being ramped up to 71% B over the course of 26 min. Enterocins 7A and 7B eluted at 27 and 25 min, respectively (Figure S9, Supporting Information). The organic solvent was removed *in vacuo*, and the remainder was frozen and lyophilized. Approximately 2 mg of each peptide was obtained per liter of media.

Circular Dichroism. CD spectra were acquired using an OLIS DSM 17 CD spectrophotometer (Olis). Sample concentrations of 0.8 mg/mL were prepared in unbuffered H_2O, in 20 mM sodium phosphate (pH 7.0), and in 50% trifluoroethanol (TFE). Samples were analyzed in a 0.2 mm quartz cuvette.

NMR Spectroscopy. All NMR experiments were performed at 27 °C on a Varian (now Agilent Inc.) VNMRS 700 MHz spectrometer with VNMRJ 3.2 host control and equipped with a triple resonance HCN cryogenically cooled probe and Z-axis pulsed-field gradients. Solutions of each enterocin were prepared to a final concentration of 0.8 mM in 300 μL of a 9:1 mixture of H_2O and D_2O. The pH of the samples was approximately 6. For the sample containing both enterocin 7A and 7B, the concentration of each peptide was approximately 0.8 mM. D_2O magnetic susceptibility matched 5 mm Shigemi NMR tubes were used for experiments performed on enterocins 7A and 7B individually. Standard 5 mm NMR tubes were used for experiments performed on the mixture of enterocin 7A and 7B. An internal reference standard of 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) was included at a final concentration of 0.01% w/v.

One dimensional ^{1}H NMR, two-dimensional homonuclear ^{1}H−^{1}H-TOCSY, -gCOSY, and -NOESY spectroscopy along with natural abundance 2D-^{1}H,^{15}N-HSQC and ^{1}H,^{13}C-HSQC data sets were acquired for both enterocins 7A and 7B individually (experimental details are in the Supporting Information). A two-dimensional ^{15}N-HSQC data set was acquired for the mixture of enterocins 7A and 7B. Spectra were processed using NMRPipe[14] and analyzed using NMRView.[15] Chemical shifts were assigned manually using techniques described in the literature.[16,17] Chemical shifts assignments for enterocin 7A and 7B can be found in the Supporting Information.

Structure Calculations. The structures of enterocins 7A and 7B were calculated using CYANA 2.1.[18] NOE crosspeaks were almost entirely automatically assigned by CYANA, with minimal manually assigned crosspeaks. For enterocin 7A, 1481 crosspeak NOEs (166 long-range, 212 medium range, 373 short-range) were used in the final structure calculation. Chemical shift assignments for enterocin 7A have been deposited in the BMRB (accession number 19094), and coordinates for the structure have been deposited in the PDB (accession number 2m5z). For enterocin 7B, 1005 crosspeak NOEs (107 long-range, 173 medium range, 355 short-range) were used in the final structure calculation. Chemical shift assignments for enterocin 7B have been deposited in the BMRB (accession number 19101), and coordinates for the structure have been deposited in the PDB (accession number 2m60). Backbone overlays of the 20 calculated structures of enterocins 7A and 7B can be found in Figure S3.

Isothermal Calorimetry. Calorimetry experiments were performed using a VP-ITC isothermal titration calorimeter (GE Healthcare). Enterocin 7A and 7B were prepared to concentrations of 0.5 mM and 0.02 mM, respectively, in 20 mM sodium phosphate (pH 6.9). The enterocin 7B solution was placed into the calorimeter cell, while the enterocin 7A solution was drawn into the calorimeter syringe. The calorimeter was then allowed to equilibrate at 37 °C until steady baseline was achieved. The enterocin 7A solution was injected (15 × 3 μL, 300 s intervals) into the cell, and the resulting changes in temperature were monitored (Figure S8).

RESULTS

Enterocins 7A and 7B (Ent7A and Ent7B) are a two-peptide leaderless bacteriocin of 44 and 43 amino acids, respectively, sharing 74% amino acid identity (Figure S1). We isolated these peptides from a culture of *E. faecalis* 710C through a modification of the previously reported purification.[11] The solvent dependence of their structures was initially analyzed through CD characterization (Figure 1). Both peptides showed a high degree of helical secondary structure in unbuffered water (Ent7A 29% helicity, Ent7B 41% helicity), phosphate-buffered water (Ent7A 39%, Ent7B 37%), and 50% TFE (Ent7A 66%, Ent7B 46%). Although this may be typical for the structure-

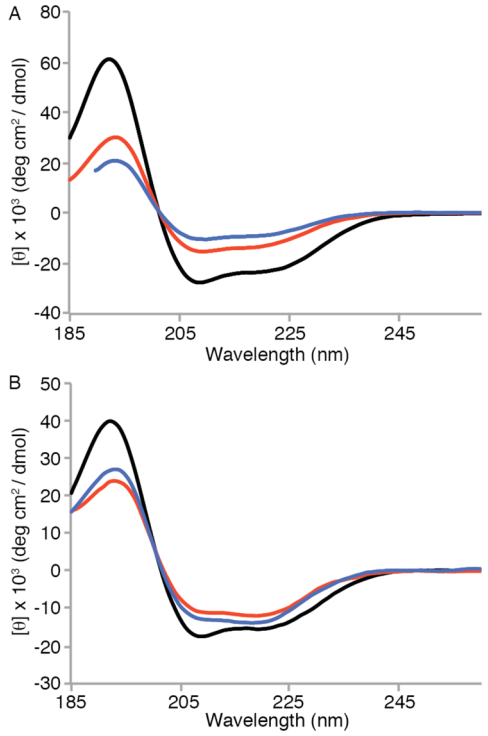

Figure 1. CD spectra for (A) enterocin 7A and (B) enterocin 7B. 50% TFE, black; 20 mM sodium phosphate (pH 7.0), red; unbuffered water, blue.

inducing cosolvent TFE,[19] the extent of structuring found in aqueous conditions is unusual. Because of the high degree of secondary structure in aqueous conditions, we chose these conditions for the determination of the NMR solution structures of these peptides.

Although attempts were made to prepare isotopically labeled enterocins 7A and 7B from *E. faecalis* 710C grown in ^{13}C,^{15}N-enriched media, their level of production in this labeled media was prohibitively low. In addition, the high quality and lack of overlap of the NMR spectra of unlabeled samples allowed the solution structures to be approached by a predominantly homonuclear approach. 1D proton NMR spectra of both peptides displayed that the amide proton chemical shifts were well dispersed. Similarly, natural abundance ^{15}N-HSQC experiments revealed a large chemical shift dispersion in the amide region for both ^{15}N and ^{1}H resonances (Figure S2), a further indication of the highly structured nature of these peptides. These data suggested that it was feasible to determine the structures of enterocins 7A and 7B through homonuclear means, and so COSY, TOCSY, and NOESY data sets were acquired for these peptides.

Chemical shifts were manually assigned based on the standard identification of spin systems in the TOCSY data sets and ordering of these spin systems based on interresidue NHNH(i, i +1), αNH(i, i +1), and βNH(i, i +1) NOE crosspeaks from the NOESY data sets. A peaklist encompassing all of the NOE crosspeaks was prepared for each peptide (the majority unassigned), and these data in combination with the chemical shift assignments were supplied to CYANA 2.1.[18] The resulting solution structures of enterocin 7A and enterocin 7B are shown in Figure 2 (the backbone variation can be found in Figure S3, Ramachandran plots in Figure S4). The statistics describing the structure calculations (Table 1) indicate that both peptides are highly structured, as indicated by the low root mean squared deviation (rmsd) values. Furthermore, the relatively high proportion of long-range NOEs is consistent with the compact nature of these peptides.

Structurally, both enterocins 7A and 7B are quite similar, with both peptides adopting the same overall fold (Figure 3,

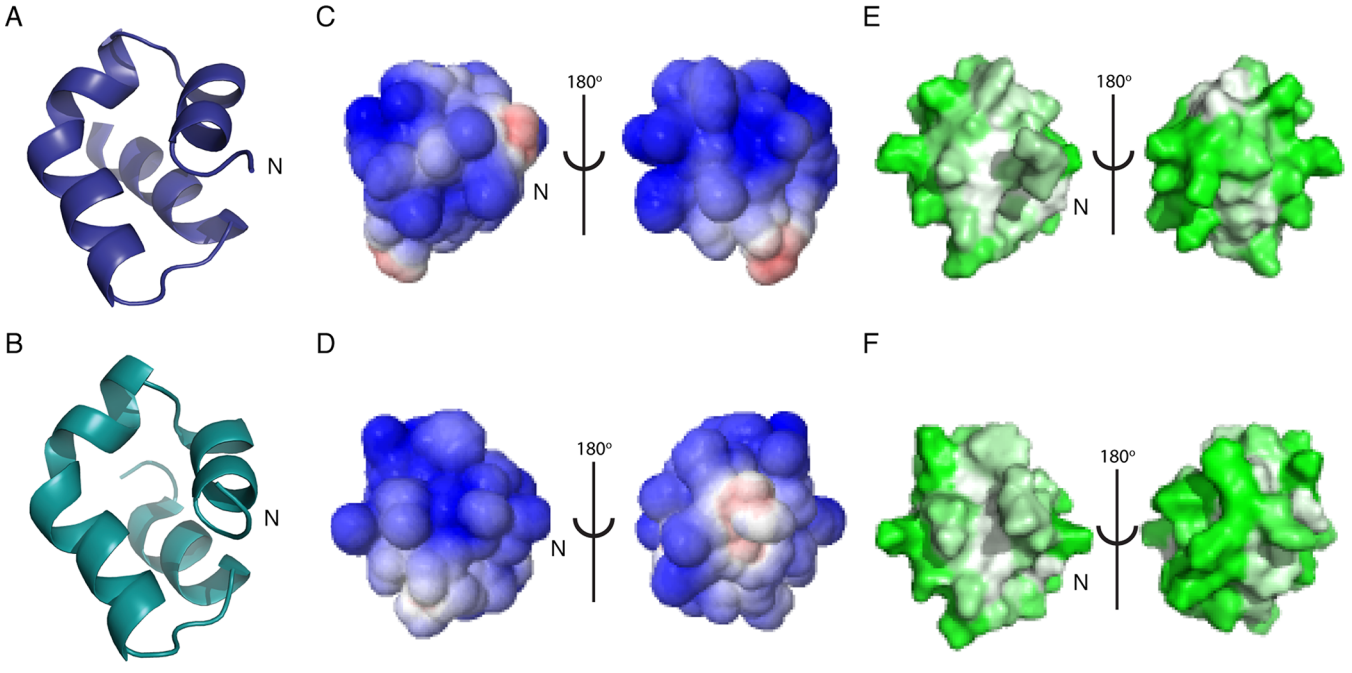

Figure 2. Solution structures of (A) enterocin 7A and (B) enterocin 7B. Electrostatic potential surface maps of (C) enterocin 7A and (D) enterocin 7B. Cationic regions are indicated in blue, while anionic regions are in red. Electrostatic potential surface maps were calculated using the APBS implementation of the PDB2PQR (version 1.8) online pipeline.[20] Hydrophobic surface maps of (E) enterocin 7A and (F) enterocin 7B. Hydrophobic residues are indicated in white, and hydrophilic residues are in green. The position of the N-terminus is indicated by the letter N.

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Table 1. Structure Calculation Statistics for Enterocins 7A and 7B

	enterocin 7A	enterocin 7B
total NOE peak assignments	1481	1005
short ($\mid i-j \mid \leq 1$)	373	355
medium ($1 < \mid i-j \mid < 5$)	212	173
long ($\mid i-j \mid \geq 5$)	166	107
average target function value	0.40	0.64
RMSD for full peptide		
backbone atoms (Å)	0.48 ± 0.15	0.74 ± 0.20
heavy atoms (Å)	0.92 ± 0.11	1.39 ± 0.31

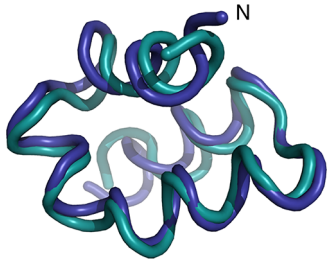

Figure 3. Overlay of the backbones of enterocins 7A (dark blue) and 7B (light blue). The N-termini are indicated with the letter N.

rmsd 1.463 over 507 atoms). As predicted from the CD data, both peptides are almost completely α-helical. The positions of the α-helices are supported by the presence of interresidue NOEs consistent with this secondary structure (Figure S5 and S6). These are further supported through chemical shift index analysis, as implemented in NMRView.[15] The structures feature an N-terminal α-helix extending between residues 4 and 10. This α-helix is followed by a longer α-helical region extending from residue 14 to 29, with a kink around residue 19. Finally, another α-helix extends from residue 33 to the C-terminus. The three helical regions are amphipathic, burying the hydrophobic residues in the core of the peptide (Figure S7). Numerous long-range NOEs were observed between hydrophobic amino acids from different α-helices. Furthermore, some of the aromatic residues were buried in the peptide interior. The positioning of some hydrophobic residues relative to the aromatic side-chains gave rise to some atypical chemical shifts (i.e., the γ-methyl group of Ile-40 from Ent7A has a chemical shift of −0.13 ppm). As such, the structures are compact, with the N-terminal and C-terminal α-helices in contact with each other via hydrophobic interactions. Similar as was predicted for leaderless bacteriocin aureocin A53 based on fluorescence results,[10] the three tryptophan residues in enterocins 7A and 7B are positioned at the surface.

As may be expected based on the primary sequences of enterocins 7A and 7B, electrostatic potential maps indicate that the surfaces of these peptides are highly cationic (Figure 2C,D).[20] The cationic residues are fairly spread out throughout the peptide, and the majority of the surface is positively charged. Although the positions of the cationic residues are almost fully conserved between enterocins 7A and 7B, the anionic residues are not. There are also some smaller relatively uncharged patches found on the surface. Hydrophobic surface maps of enterocins 7A and 7B demonstrate the amphipathic character of these peptides (Figure 2E,F), revealing distinct patches of hydrophobic and hydrophilic amino acids. The N-terminal moieties of these peptides demonstrate a hydrophilic patch surrounded by more hydrophobic residues. Conversely, the C-terminal portions show a defined hydrophobic patch surrounded by hydrophilic residues.

Because of the synergistic activity reported for some two-peptide leaderless bacteriocins, we performed activity tests to determine if enterocins 7A and 7B were also synergistic. Synergistic activity was observed against a selection of indicator organisms (*Lactococcus lactis* subsp. *cremoris* HP, *Brochothrix campestris* ATCC 43754, *Carnobacterium maltaromaticum* UAL26, *E. faecium* BFE900, *Lactobacillus sakei* DSM 20017; data not shown). On the basis of this synergism, it was of interest to determine if there was any structural interaction between enterocins 7A and 7B. A mixed solution of enterocins 7A and 7B was prepared, and a natural abundance ^{15}N-HSQC experiment was run on the mixture. The overlap of this spectrum with the ^{15}N-HSQC spectra acquired individually for enterocins 7A and 7B is shown in Figure 4. The mixing of the

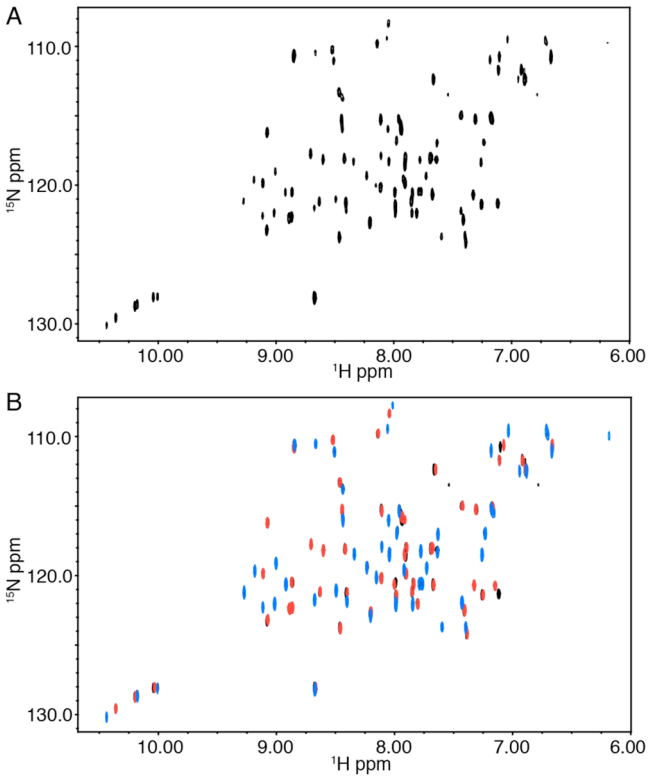

Figure 4. (A) ^{15}N-HSQC spectrum for a mixture of enterocins 7A and 7B. (B) The ^{15}N-HSQC spectrum of the mixture (black) overlaid with the individual ^{15}N-HSQC spectra of enterocin 7A (red) and enterocin 7B (blue).

two peptides together resulted in only minimal changes in the chemical shifts of two amide protons in enterocin 7A. This suggests that there are no significant binding interactions between the two pure peptides, at least in aqueous solution. As a further investigation into the possibility of binding between enterocin 7A and 7B, isothermal calorimetry (ITC) experiments were conducted. However, no evolution of heat was observed upon the mixing of the two peptides (Figure S8), further indicating that there is no binding interaction in aqueous solution.

DISCUSSION

In terms of structure, enterocins 7A and 7B (and presumably other homologous leaderless bacteriocins) behave quite

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Ent7A  MG---AIAKLVAKFGWPIVKKYYK---QIMQFIGEG----WAINKIIDWIKKHI 44
Ent7B  MG---AIAKLVAKFGWPFIKKFYK---QIMQFIGQG----WTIDQIEKWLKRH- 43
LnqQ   MAGFLKVVQLLAKYGSKAVQWAWANKGKILDWLNAGQAIDWVVSKIKQILGIK- 53
A53    MS-WLNFLKYIAKYGKKAVSAAWKYKGKVLEWLNVGPTLEWVWQKLKKIAGL-- 51
       *.    . : :**:*   :.  :    ::::::. *    *. .:: .
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Figure 5. Sequence alignment of enterocin 7A (Ent7A), enterocin 7B (Ent7B), lacticin Q (LnqQ), and aureocin A53 (A53), using Clustal Omega.[32] Conserved residues are indicated with an asterisk, residues with strongly similar properties with a colon, and residues with weakly similar properties with a period. The horizontal lines above the sequences indicate the α-helical regions of enterocin 7A and 7B.

differently than other Gram-positive bacteriocins, most of which exhibit random coil conformations in water. As was initially suggested based on CD results for aureocin A53,[10] enterocins 7A and 7B are both highly structured in aqueous conditions. CD analyses of lacticin Q similarly suggested structure in buffer.[13] In contrast, pediocin-like (type IIa) bacteriocins of similar size, such as leucocin A[21,22] and carnobacteriocin B2,[23] exist as a random coil in aqueous conditions. The same has been found for two-component bacteriocins whose precursors have N-terminal leaders, such as plantaricin E/F and plantaricin J/K.[19] Such linear peptides require the use of structure-inducing solvents (such as TFE) or micelles to adopt defined conformations. Bacteriocins that have additional covalent linkages in their sequences can assume more defined conformations. Examples include the lantibiotics that possess lanthionine and methyllanthionine bridges,[24] the cyclic bacteriocins that have N- to C-termini amide linkages,[25] and the sactipeptides[2] (also called sactibiotics)[26] that have unusual sulfur-to-alpha-carbon bridges.[27,28] The fact that the linear leaderless bacteriocins enterocins 7A and 7B maintain a defined structure in water in the absence of covalent modifications distinguishes them from most other known Gram-positive bacteriocins.

Most of the work toward understanding the mode of action of leaderless bacteriocins has focused on lacticin Q[13,29] and aureocin A53.[30] The antimicrobial activity of both of these bacteriocins was attributed to membrane-permeation, followed by the leakage of essential molecules and dissipation of membrane potential. Lacticin Q was found to permeabilize liposomes at much lower concentrations than did nisin A or pediocin PA-1.[13] Lantibiotics (such as nisin A) and type IIa bacteriocins with a YGNGV motif[31] (such as pediocin PA-1) require docking molecules (lipid II and mannose phosphotransferase, respectively) not present in these liposomes, explaining their relatively diminished potency in these systems.[13] The observation that lacticin Q shows potent membrane-permeabilizing activity toward these liposomes implies that a specific receptor molecule may not be required for activity. Lacticin Q was suggested to form a "huge toroidal pore" in target membranes resulting in the leakage of molecules as large as proteins, something not previously reported for antimicrobial peptides.[29] However, it was proposed that the activity of aureocin A53 results from generalized membrane permeabilization as opposed to the formation of discrete pores.[30] This difference in mode of action is unexpected, considering the high sequence homology between aureocin A53 and lacticin Q (47% amino acid identity).

Although enterocins 7A and 7B are approximately 7−10 amino acids shorter than aureocin A53 and lacticin Q, there are regions of sequence homology (via Clustal Omega)[32] between these bacteriocins (Figure 5). This homology is notable in the N-terminal α-helical region, and even more pronounced in the C-terminal portion of the kinked central α-helix. Furthermore, as was suggested for aureocin A53[10] and lacticin Q,[13] all of the helical regions of enterocins 7A and 7B are amphipathic (Figure S7). The highly cationic nature of these peptides may be expected to attract them to the anionic phospholipids found in bacterial membranes. This likely corresponds to the especially cationic surface of enterocins 7A and 7B (Figure 2). However, there was evidence that aureocin A53 binds more strongly to neutrally charged membranes than to negatively charged membranes.[30] This was attributed to the predicted hydrophobic surface regions and fluorescence measurements that suggested that several tryptophan residues were present on the peptide surface.[30] As both enterocins 7A and 7B also demonstrate hydrophobic surface regions (Figure 2), it is possible that a combination of ionic and hydrophobic interactions contributes to the binding of these peptides to the membranes of susceptible bacteria. Following the initial binding interaction of enterocins 7A and 7B to the membrane, it is likely that the α-helices unpack, thereby exposing the hydrophobic core and allowing the peptide to insert into the membrane.[33] However, the next step toward membrane permeabilization is not yet clear. Whether enterocins 7A and 7B form huge toroidal pores like lacticin Q or cause a generalized membrane permeabilization without pore formation like aureocin A53 cannot be predicted from similarities in sequence homology alone. Mode of action studies using the methodology applied to lacticin Q and aureocin A53 would provide an indication as to how enterocin 7A and 7B exert their antimicrobial effects.

It is also unclear if the two individual peptides, enterocin 7A and 7B, possess slightly different roles in membrane permeabilization. Many two-peptide bacteriocins whose precursors have leaders are inactive or have reduced activity individually.[34] However, enterocins 7A and 7B each display strong activity by themselves without their partner. Therefore, membrane permeabilization is unlikely to be dependent on the presence of both peptides. However, if enterocins 7A and 7B do indeed form complex pores structures, it is conceivable that these pores could consist of both peptides, due to their structural similarity. Enterocins L50A and L50B were previously reported to demonstrate significant synergistic activity.[12] Despite the very high sequence homology between enterocins L50A and 7A, and between enterocins L50B and 7B, a previous report found no synergistic activity between enterocins 7A and 7B against *L. sakei*.[11] Our results indicate synergistic activity against several indicator organisms, including modest synergism against *L. sakei*. Comparison of NMR spectra acquired for the peptides individually with a spectrum wherein they were mixed did not reveal any significant changes in backbone chemical shifts of either peptide. Similarly, ITC did not reveal any significant binding between enterocins 7A and 7B. As these peptides are individually active, it may be that there is indeed no specific binding interaction between the two peptides. However, it is also possible that binding interactions between these peptides only develop following insertion into a membrane environment.

Unexpectedly, certain structural features of enterocins 7A and 7B were remarkably homologous to the three-dimensional structure of carnocyclin A, a 60-residue circular bacteriocin with a saposin fold.[25] Specifically, the C-terminal portion of the central kinked α-helix and the C-terminal α-helix of enterocins 7A and 7B aligned with α-helices α1 and α2 of carnocyclin A (with an rmsd of 1.210 over 169 atoms for enterocin 7B; Figure 6A). Furthermore, the amphipathicity of these particular

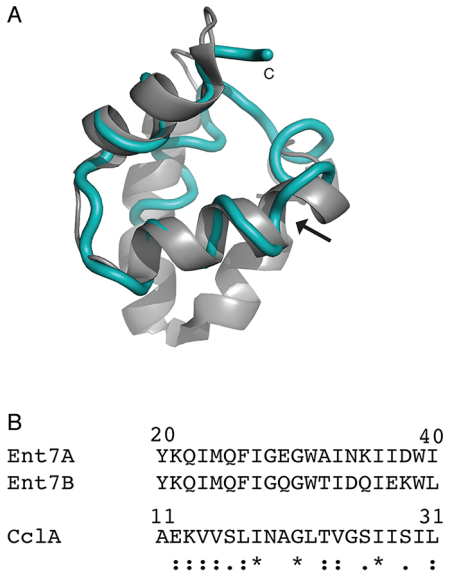

Figure 6. (A) Overlay of the structures of enterocin 7B (blue) and carnocyclin A (gray). The arrow indicates the point at which the N- and C-termini of carnocyclin A are linked, on the α-helix in the background. (B) Sequence alignment of a portion of enterocins 7A and 7B (Ent7A and Ent7B, respectively) with a portion of carnocyclin A (CclA), using Clustal Omega.[32] Conserved residues are indicated with an asterisk, residues with strongly similar properties with a colon, and residues with weakly similar properties with a period.

enterocin α-helices matches those in carnocyclin A. Alignment of these moieties of these bacteriocins revealed sequence homology that was missed by aligning their full amino acid sequences (Figure 6B). Also, in carnocyclin A and enterocins 7A and 7B, there is a cationic surface positioned in a similar position relative to the homologous α-helices. Carnocyclin A, like lacticin Q, can interact directly with the membrane without the need for a surface receptor.[35] However, carnocyclin A is believed to exert at least part of its antimicrobial effects through the formation of anion-selective channels.[35] The structure of enterocin AS-48, a 70-residue circular bacteriocin, has been elucidated through both NMR spectroscopy[36] and X-ray crystallography.[37] Enterocin AS-48 is structurally similar to carnocyclin A, despite the presence of an additional α-helical region. Furthermore, the α1 and α2 regions of enterocin AS-48 are oriented similarly to the C-terminal α-helices of enterocins 7A and 7B. Like carnocyclin A, enterocin AS-48 acts by permeabilizing the target cell membrane; however this is accomplished through the formation of nonselective pores.[38] The homologous regions between enterocins 7A and 7B and circular bacteriocins carnocyclin A and enterocin AS-48 may represent a common motif involved in the attraction of these peptides to the membrane followed by insertion therein. The differing modes of action upon insertion into the membrane are potentially a function of the other portions of these peptides. That a similar motif can arise through such different means is noteworthy. Carnocyclin A and enterocin AS-48 require an N- to C-terminus cyclization to maintain the orientation of the component α-helices. A similar motif is achieved by these leaderless bacteriocins due to the tight packing of the hydrophobic core.

The structures of enterocins 7A and 7B are expected to be similar to those of aureocin A53, lacticin Q, and several other leaderless bacteriocins. Sequence alignment suggests that the amino acids in the helical regions of enterocins 7A and 7B are homologous to regions of aureocin A53 and lacticin Q (Figure 5) but potentially have longer linker regions between them. However, there are other, shorter leaderless bacteriocins such as aureocin A70[39] and enterocin Q[40] for which the enterocin 7A and 7B structures may not be representative. It has been noted that leaderless Gram-positive bacteriocins possess some similarities to phenol-soluble modulins, a group of hemolytic peptides produced by many staphylococci.[12,41] Apart from some sequence homology, both groups comprise amphipathic α-helical peptides of a similar size that are ribosomally synthesized without N-terminal leader sequences. It may be the case that the structures of the phenol-soluble modulins prove to be similar to those of enterocins 7A and 7B. Overall, the structural motif shared by the leaderless and circular bacteriocins may be responsible for their initial membrane binding and insertion, but subsequent mechanisms of membrane permeabilization due to the other parts of these peptides appear divergent.

■ ASSOCIATED CONTENT

Supporting Information

Sequence alignments, chemical shift assignments, NMR spectra, Ramachandran plots, peptide solution structures, ITC results. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: 780-492-5475; e-mail: john.vederas@ualberta.ca.

Author Contributions

‡These authors contributed equally.

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Notes

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■ ABBREVIATIONS USED

APT, All-Purpose Tween; CD, circular dichroism; gCOSY, gradient correlation spectroscopy; HPLC, high pressure liquid chromatography; HSQC, heteronuclear single quantum coherence; IPA, isopropyl alcohol; ITC, isothermal calorimetry; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser effect spectroscopy; rmsd, root-mean-square deviation; TFA, trifluoroacetic acid; TFE, trifluoroethanol; TOCSY, total correlation spectroscopy

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