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Identification of an N-Terminal Formylated, Two-Peptide Bacteriocin from *Enterococcus faecalis* 710C

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ABSTRACT: *Enterococcus faecalis* 710C, isolated from beef product, has a broad antimicrobial activity spectrum against foodborne pathogens. Two bacteriocins, enterocin 7A (Ent7A) and enterocin 7B (Ent7B), were purified from the culture supernatant of *E. faecalis* 710C and characterized using matrix-assisted laser desorption ionization-time-of-flight mass spectrometry and electrospray infusion tandem mass spectrometry analyses. These data and subsequent genetic analysis showed that Ent7A and Ent7B are produced without N-terminal leader sequences and have amino acid sequences that are identical to those of enterocins MR10A and MR10B, respectively. However, the observed masses for Ent7A and Ent7B are 5200.80 and 5206.65 Da (monoisotopic mass), respectively, which are higher than the theoretical molecular masses of MR10A and MR10B, respectively. This study provides evidence that both Ent7A and Ent7B are formylated on the N-terminal methionine residue. Purified Ent7A and Ent7B are active against spoilage microorganisms and foodborne pathogens, including *Clostridium sporogenes*, *Listeria monocytogenes*, and *Staphylococcus aureus* as well as *Brevundimonas diminuta*, which has been associated with infections among immune-suppressed cancer patients.

KEYWORDS: *Enterococcus faecalis*, leaderless bacteriocin, mass spectrometry, formylation, antimicrobial activity

INTRODUCTION

Application of naturally occurring microorganisms that produce antimicrobial substances for the prevention of bacterial foodborne illness has been a rapidly emerging area of research and development.[1−3] Of special interest are lactic acid bacteria (LAB) that grow naturally on food and the bacteriocins they secrete.[4−7] Bacteriocins are ribosomally synthesized antimicrobial peptides from bacteria. *Enterococcus* is a genus of LAB that has a large range of environmental niches, including food,[8] birds,[9] and plants,[10] and is a member of autochthonous bacteria associated with the human and animal intestinal tracts. Many enterococci are bacteriocin producers, and some of these bacteriocins, often called enterocins, exhibit strong antimicrobial activity against a remarkably broad spectrum of foodborne pathogens, including *Listeria* spp., *Clostridium* spp., and *Staphylococcus aureus*.[4] Many bacteriocins produced by Gram-positive bacteria contain an N-terminal leader peptide that directs the secretion of the bacteriocin and that is cleaved off during the secretion process.[4,11−13] However, some enterocins do not have a leader peptide, and the secretion is likely dependent on a dedicated ABC transporter.[14] These leaderless enterocins are produced either as individual peptides or as two-peptide bacteriocins.[4]

Enterococcus faecalis 710C was isolated from ground beef on a KF (Kenner Fecal) *Streptococcus* agar plate. This organism has a broad spectrum of antimicrobial activity, including common Gram-positive foodborne pathogens such as *Clostridium* spp., *Listeria monocytogenes*, and methicillin-resistant *S. aureus* (MRSA) and a Gram-negative organism, *Brevundimonas diminuta*, which has been suggested to cause infections in immune-suppressed individuals, such as cancer patients.[15] In the present study, we report that *E. faecalis* 710C makes two leaderless bacteriocins, enterocin 7A (Ent7A) and enterocin 7B (Ent7B), that exhibit a broad spectrum of activity. Purification of Ent7A and Ent7B, MS/MS sequence analysis, and sequencing of their structural genes demonstrated that they have identical amino acid sequences as enterocins MR10A and MR10B.[9] It was reported previously that the theoretical molecular weights of MR10A and MR10B differed from the weights observed by matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) experiments and that this difference could be attributed to possible oxidation in the methionine residues or other alterations of the peptides during purification.[9] Our mass spectral results demonstrated that the discrepancy between the theoretical and measured molecular weight is due to the presence of N-terminal formylmethionine in the primary sequences of both Ent7A and Ent7B. Bacteriocins with formylated N-termini have been described previously.[16−20] However, in most of these studies, statements referring to formylation of bacteriocins were not supported by mass spectral data. Our work describes methodology to recognize N-formylation of bacteriocins through high-resolution mass spectrometric analysis. In addition, we also investigated the presence of virulence factors in *E. faecalis*

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Table 1. Antimicrobial Spectrum of *E. faecalis* 710C against Indicator Organisms

indicator strain	result[a]
Brochothrix campestris ATCC[b] 43754	+
Brevundimonas diminuta UFM[c]1	+
Campylobacter jejuni ATCC 700819	−
Carnobacterium divergens UAL9	+
Carnobacterium maltaromaticum UAL8A, UAL8B, UAL8C2, UAL26, UAL307, JG126	+
Clostridium botulinum spores and viable cells[d]	+
Clostridium butyricum ATCC 8260, viable cells	+
Clostridium difficile 3195, 76; viable cells	+
Clostridium perfringens CL5626, R783; viable cells	+
Clostridium sporogenes 25779, 7955; viable cells	+
Escherichia coli AW 1.7	−
Escherichia coli GGG[e]10	−
Enterococcus faecium BFE900 and VRE strains	+
vancomycin-resistant *Enterococcus faecium*[f] CL3745, E2155, E2217, E2352, M1008, S769, R493, R704, R846	+
Lactobacillus sakei DSM 20017, 706	+
Leuconostoc gelidum UAL 187	+
Listeria innocua ATCC 33090	+
Listeria monocytogenes ATCC 15313, CDC7762, FS-15	+
Pediococcus acidilactici PAC 1.0	+
Salmonella enterica serovar Typhimurium 18	−
Staphylococcus aureus ATCC 23235	+
methicillin-resistant *Staphylococcus aureus* R468, R507, R667, R719, R776, R870, R948, R1230, R1262, R1578[f]	+

[a] +, inhibition; −, no inhibition. [b] American Type Culture Collection. [c] University of Alberta, Food Microbiology Laboratory Culture Collection. [d] Proteolytic strains of *C. botulinum* used in this study were *C. botulinum* 368B, 2B, A6, and A62, and non-proteolytic strains were DB2, 13983IIB, and 17B. [e] Agriculture and Agri-Food Canada, Lacombe Research Station. [f] Provincial Laboratory for Public Health, Edmonton, AB.

710C to evaluate whether this LAB strain has the potential to be used safely in food biopreservation.

MATERIALS AND METHODS

Bacterial Strains and Culture. *E. faecalis* 710C (obtained from the University of Alberta collection of lactic acid bacteria) was grown at 37 °C in All-Purpose Tween (APT) broth (APT; Difco, BD Diagnostics, Sparks, MD). *Lactobacillus* spp. were grown at 25 °C on APT agar or in APT broth. Viable cells of *Clostridium* spp. were grown anaerobically at 39 °C in Reinforced Clostridial Medium (RCM; Oxoid, Hampshire, U.K.). *Campylobacter jejuni* ATCC 700819 and *B. diminuta* UFM1 were grown on Campylobacter Blood-Free Selective Agar (mCCDA) agar plates (Oxoid). For use in antimicrobial activity assays, *Campylobacter* spp. and *B. diminuta* were grown on tryptic soy agar (Difco) supplemented with 0.5% yeast extract (Difco) and were incubated at 39 °C in an anaerobic jar flushed with a mixture of 4.98% CO_2 with a balance of nitrogen to create microaerophilic conditions. All other indicator strains (Table 1) were grown in tryptic soy broth (TSB; Difco) (with 0.5% yeast extract) or on TSB agar plates with 0.5% added yeast extract. All strains were maintained as stocks in TSB or APT supplemented with glycerol (40%) and stored at −80 °C.

The cultivation of *C. botulinum* spores and viable cells was performed as follows: proteolytic strains of *C. botulinum* used in this study were *C. botulinum* 368B, 13983IIB, A6, and A62, and nonproteolytic strains were DB2, 2B, and 17B (University of Alberta Food Microbiology laboratory collection). Spore production for strain DB2 was done in sporulation medium (SM) containing 50 g L^{-1} tryptone (Difco) L^{-1} and 10 g peptone (Difco) L^{-1}, whereas RCM (Difco) was used for strain A6. Spores of all other strains were produced in trypticase−peptone−glucose−yeast extract broth (TPGY) containing, per liter, 50 g of tryptone, 5 g of peptone, 20 g of yeast extract (Difco), 4 g of glucose (Fisher Scientific Canada, Ottawa, ON, Canada), and 1 g of sodium thioglycollate (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada). Before use, all media were incubated in an anaerobic chamber for at least 24 h in an atmosphere of 5% CO_2, 10% H_2, and balance N_2 (Praxair Canada, Edmonton, AB, Canada) to ensure anaerobic conditions.

For spore production, 100 μL of spore suspensions was inoculated into 5 mL of appropriate medium, heat-shocked in a water bath for 15 min at either 55 °C (nonproteolytic strains) or 75 °C (proteolytic strains), and incubated at 37 °C under anaerobic conditions for 48 h. The 5 mL culture was then used to inoculate a 250 mL volume of the appropriate medium, and the culture was incubated at 37 °C under anaerobic conditions for 14 days with periodic monitoring by phase contrast microscopy. Spores were harvested by centrifuging at 16270*g* for 20 min, followed by five washes (100 mL) and a final resuspension (10 mL) in sterile 0.9% NaCl, and then a 1 h heat treatment at 65 °C to destroy residual toxin and vegetative cells. *C. botulinum* strains were stored as spore stocks in 0.9% (w/v) NaCl at 4 °C, and were enumerated using RCM medium containing 2.5% (w/v) agar.

Activity Assays. Bacteriocin activity was monitored by spot-on-lawn assays.[21] The supernatant of a 24 h culture of *E. faecalis* 710C was filtered through 0.22 μm Millipore Express PLUS polyethersulfone membrane (Millipore Corp., Billerica, MA), spotted on solid (1%) agar, and allowed to air-dry. Soft (0.5%) agar was inoculated with the indicator organism (1.2% inoculum) and poured onto the solid agar. Plates were incubated at appropriate temperatures for growth of the respective indicator strains. After 24−48 h of incubation, plates were examined for zones of clearing.

Isolation and Purification of Bacteriocins. *E. faecalis* 710C was grown in 1 L of APT broth (5% inoculum) at 37 °C for 22 h. The culture was centrifuged at 10000*g* for 20 min at 4 °C. The supernatant was filtered (0.22 μm filter, Millipore) and loaded onto a cation-exchange column at 8 mL/min (HiPrep 16/10 SP FF; GE Healthcare Life Sciences) that was preconditioned with 50 mM sodium acetate buffer, pH 4.6. Detection of peptides was monitored by a UV detector at 280 nm. The column was washed with 5 column volumes of sodium acetate buffer after supernatant was loaded onto the column. Bacteriocins were eluted in a gradient of 2 M NaCl in the same buffer. Fractions were tested for activity against *L. sakei* DSM20017. Active fractions were desalted using C18 cartridges (Sep-Pak C18, Waters; prewashed with 2-propanol containing 0.1% trifluoroacetic acid (TFA), conditioned with 0.1% TFA) and eluted in 40−70% 2-propanol with 0.1% TFA. The 2-propanol was removed by RotaVAP (Büchi Corp., New Castle, DE) at 35 °C. Active peptides were separated by reverse-phase HPLC (5 μm, 150 × 4.6 mm Eclipse XDB-C18 (Agilent Technologies Canada Inc., Mississauga, ON, Canada), preconditioned with 40% acetonitrile with 0.1% TFA) in a linear gradient of 40−70% acetonitrile containing 0.1% TFA, at a flow rate of 1 mL/min. Detection of peptides was monitored by a UV detector at 280 nm. Antimicrobial activity in flow-through and fractions from each purification step were tested using spot-on-lawn assays. Protein concentration measured at 280 nm by a NanoDrop spectrophotometer.

Mass Spectrometry Analysis. Active peptides were analyzed by MALDI-TOF MS for molecular weight detection. α-Cyano-4-hydroxycinnamic acid (HCCA) or 2,5-dihydroxybenzoic acid (DHB) was used as matrix. Spectra were obtained on an AB Sciex Voyager Elite MALDI-TOF MS system (AB Sciex, Foster City, CA) operating in

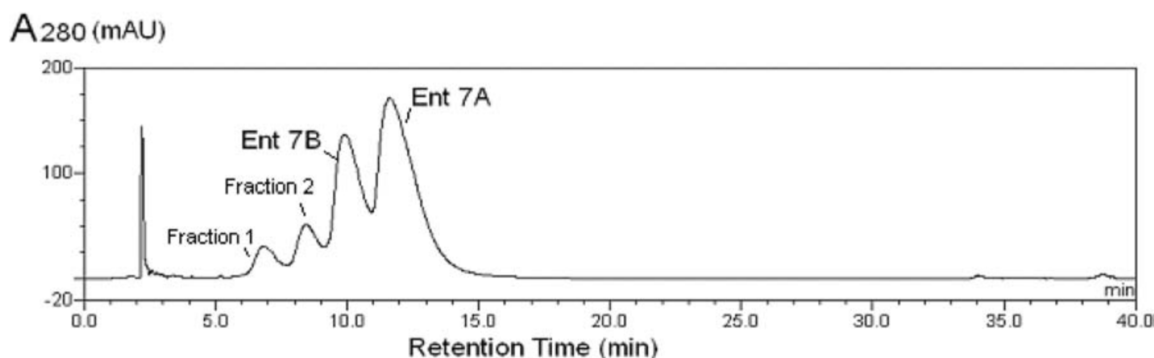

Figure 1. Separation of Ent7A and Ent7B by RP-HPLC. Elution of peptides was monitored by a UV detector at 280 nm.

negative ion mode. Peptide sequence information was obtained on a Q-TOF Premier mass spectrometer (Micromass, UK/Waters, Milford, MA) operating in positive-ion nanoelectrospray (nano-ESI) mode. Active fractions from RP-HPLC were further purified by C4 ZipTip (MilliPore) to remove TFA. Eluents from C4 ZipTip were infused to the Q-TOF Premier mass spectrometer by nano-ESI to produce MS/MS spectra, which were compared with the theoretical fragment ions of enterocin MR10A and MR10B.

The exact mass of the intact peptide was measured on a Bruker Apex-Qe 9.4T FTICR mass spectrometer (Bruker Daltonics, Billerica, MA) by infusion electrospray ionization while co-infusing the sample with bovine insulin to provide an internal calibration reference. The peptides were dissolved at a concentration of approximately 0.5 μM in 1:1 acetonitrile/water with 0.2% formic acid added.

Activity Assays of Purified Ent7A and Ent7B. Purified Ent7A and Ent7B (dissolved in water) were tested against the representative foodborne pathogens *C. jejuni* ATCC 700819, *B. diminuta* UFM1 (isolated from a pig processing plant), viable cells of *Clostridium sporogenes* ATCC 25779, *L. monocytogenes* FS-15, and *S. aureus* ATCC 23235 using spot-on-lawn assays.

The antimicrobial activity of purified Ent7A was tested against both spores and viable cells of *C. botulinum*. Briefly, 5 μL of purified enterocin 7A (1.8 mg mL^{-1}) was spotted on APT agar (1.5% w/v), dried for 10 min, and overlaid with 5 mL of semisolid (0.75% w/v) RCM (Difco) seeded with either 3×10^3 CFU mL^{-1} heat-shocked clostridial spores or with 50 μL of an overnight culture of vegetative cells. For the spore inhibition test, proteolytic and nonproteolytic strains were heat-shocked for 15 min at 75 and 55 °C, respectively. Plates were incubated at 37 °C in an anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, MI) under an atmosphere of 5% CO_2, 10% H_2, and balance N_2 for 24 h. Plates were then examined for clear zones of inhibition.

Gene Amplification and Sequencing. Total DNA of *E. faecalis* 710C was isolated using DNeasy Blood & Tissue Kit (Qiagen Inc., Mississauga, ON, Canada) and used as template for PCR. To amplify nucleotide positions 1−287 of MR10A-MR10B genes, forward primer (5′-ATGGGAGCAATCGCAAAATTAG-3′) and reverse primer (5′-TTAATGTCTTTTTAGCCATTTTCAATTTG-3′) (GenBank: DQ366596.1) were used. The genes were amplified with 30 cycles of 94 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 30 s. PCR product was purified by using a QIAquick PCR purification kit (Qiagen). The PCR product was sequenced using an ABI Big Dye version 3.1 Terminator sequencing kit (Applied Biosystems).

Identification of Virulence Factors and Enterolysin A Gene. Screening of the genes associated with virulence factors and the enterolysin A gene in *E. faecalis* 710C was performed by PCR. The enterolysin gene was amplified by PCR using the primers EntLysAfw (5′-TGGGATCCTGTAGGTGGAGG-3′) and EntLysArev (5′- CC-ATGAACCTCCGAGGACCC-3′), which amplifies a 355 bp product. The PCR reaction contained 100 ng of template DNA, 25 pmol of each primer, 200 μmol of each dNTP, 1× Taq polymerase reaction buffer, and 1 U of Taq polymerase (GE Healthcare Bio-Sciences Corp, Piscataway, NJ) in a 50 μL volume. The enterolysin A gene was amplified in 32 cycles, where each cycle included a denaturing step at 94 °C for 1 min, annealing at 61 °C for 1 min, and extension at 72 °C for 40 s. The presence of gelatinase was confirmed by screening on Todd Hewitt Agar (Becton Dickinson, Heidelberg, Germany), as well as by PCR.[22] The presence of a β-hemolysin (cytolysin) was determined by detection of hemolysis on horse blood agar containing 5% horse blood (Becton Dickinson) and by PCR.[22] The presence of other virulence factors, such as adhesion to collagen (Ace), aggregation substance (AS, *asa*1 gene), and resistance to vancomycin (*van*B gene), was determined by PCR.[22] The *Enterococcus* endocarditis antigen genes of *Enterococcus faecium* and *E. faecalis* (efa_{fm} and efa_{fc}, respectively) were PCR amplified according to a method described by Eaton and Gasson.[8]

RESULTS AND DISCUSSION

Antimicrobial Spectrum of *E. faecalis* 710C. The antimicrobial activity of *E. faecalis* 710C was tested against a wide variety of indicator strains (Table 1). Strain 710C has strong antimicrobial activity against Gram-positive foodborne pathogens such as *Listeria* spp., *Clostridium* spp., and MRSA. In addition, *E. faecalis* 710C is active against Gram-negative *B. diminuta*. Generally, a bacteriocin from LAB is active against Gram-positive organisms; however, a few characterized bacteriocins have been found to be active against Gram-negative bacteria. These bacteriocins include E 50-52 from *Enterococcus faecium* NRRL B-30746, which has activity against *C. jejuni* and *Salmonella* spp.[23] *E. faecalis* 710C has no activity against *C. jejuni*, *Escherichia coli*, or *Salmonella* spp. (Table 1).

Purification of Ent7A and Ent7B. Enterocins 7A and 7B were partially purified from *E. faecalis* 710C medium supernatant through cation-exchange chromatography. The active peptide fraction was desalted on C18 reverse-phase cartridges. Subsequent separation by RP-HPLC using a linear gradient of 40−70% acetonitrile containing 0.1% TFA yielded pure Ent7B and Ent7A, which eluted with retention times of 9.9 and 11.6 min, respectively (Figure 1). Typically, 1 L of culture yielded about 10 mg of Ent7A and 8 mg of Ent7B.

MALDI-TOF spectra of Ent7A and Ent7B showed a single peak, at m/z 5199.5 and 5205.3 Da, respectively, further indicating that the enterocins had been purified to homogeneity. The MALDI-TOF MS spectra were acquired in negative mode; hence, the molecular weights observed are for the deprotonated forms ($[M - H]^-$) of Ent7A and Ent7B. More accurate

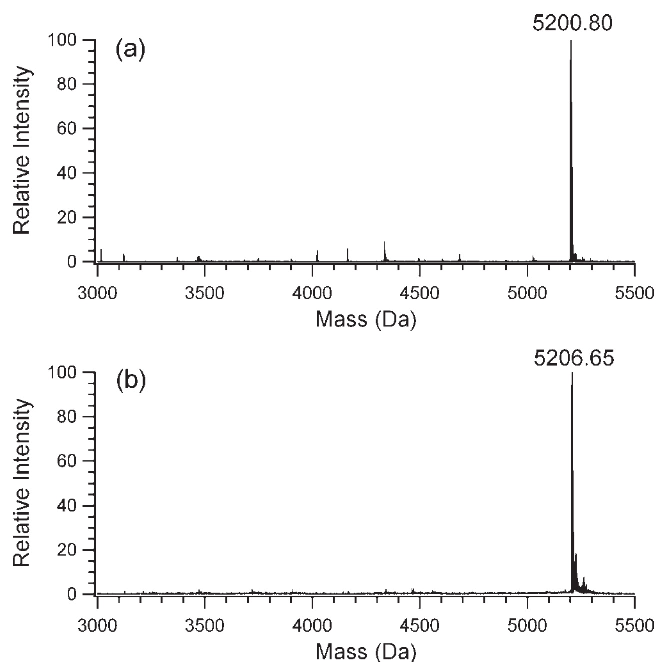

Figure 2. Nano-ESI MS spectra (deconvoluted to zero charge) of HPLC-purified peptides with monoisotopic mass labeled (a) Ent7A and (b) Ent7B.

molecular weight determinations of Ent7A and Ent7B, as well as HPLC fractions 1 and 2 (Figure 1), were done on a Q-TOF Premier mass spectrometer, from which the peaks 5200.80 Da (Ent7A, monoisotopic mass) (Figure 2a), 5206.65 Da (Ent7B, monoisotopic mass) (Figure 2b), 5222.60 Da (Fraction 1, monoisotopic mass), and 5216.60 Da (fraction 2, monoisotopic mass) were seen. It was noted that molecular masses of Ent7A and Ent7B were very similar to reported masses of enterocins MR10A (5201.58 Da) and MR10B (5207.5 Da) from *E. faecalis* MRR 10-3.[9] To investigate whether 710C contains genes that might be similar to those encoding for MR10A and MR10B, PCR was performed on genomic DNA of 710C using primers based on the 5′ part of the gene for MR10A and the 3′ part of the gene for MR10B. The PCR product was sequenced and the DNA sequence matched perfectly with DNA encoding MR10A and MR10B.[9]

MR10A and MR10B belong to a group of enterocins that are produced without a leader peptide, and these bacteriocins all contain a methionine as an N-terminal residue (Figure 3). In eubacteria and in the organelles of eukaryotes, *N*-formylmethionine (fMet) is the first amino acid in a nascent peptide chain, because fMet is coded by the start codon (AUG). The fMet residue is usually cleaved when the second residue is a nonbulky amino acid.[26] Before fMet can be removed by what is called N-terminal Met Excision (NME), the formyl group of fMet is removed by peptide deformylase (PDF), followed by the cleavage of methionine by Met aminopeptidase (MAP).[26] It should be noted that removal of the N-formyl group is not always followed by NME.[26] Interestingly, several leaderless bacteriocins contain a nonbulky amino acid such as Ala and Gly as the second residue in the peptide chain (Figure 3). It would therefore be expected that the methionine at the N-terminus is cleaved off. However, for removal of the first methionine residue, the N-terminus of the peptide needs to be exposed to PDF and MAP. When the N-terminus is buried inside the peptide, inserted into the membrane, or sterically hindered, PDF/MAP cannot excise fMet.[26] Because these bacteriocins are hydrophobic at the N-terminus and are subject to secretion, it is likely that the N-termini of Ent7A and Ent7B are protected from PDF/MAP activity when these peptides are inserted in the membrane and are secreted by the membrane-bound ABC transporter. A similar situation is described for the leader peptidase Lep,[27] an inner membrane protein of *E. coli*, which retains its fMet residue after insertion in the membrane. The N-terminus of Lep is facing the periplasm and is shielded from PDF and MAP activity.[27]

Amino Acid Sequence and Identification of N-Formylation. Martín-Platero et al. noted mass differences of 26.2 and 26.5 Da between the theoretical and measured masses of MR10A and MR10B, respectively.[9] These authors suggested that the mass discrepancy was due to the oxidation of methionine or other alterations during the purification process; however, an oxidation would result in only a 16 Da increase in mass. Therefore, tandem mass spectrometry (MS/MS) analyses of the 5200.80 and 5206.65 Da peaks were done to determine the sequences and chemical modifications of Ent7A and Ent7B. Comparison of MS/MS fragment peaks of Ent7A with theoretical fragments from MR10A[9] showed that the observed y-ion series matched the theoretically predicted y-ion series from the y_2 to y_{43} ions (MR10A has 44 amino acids in total). This confirmed that Ent7A has the same sequence as MR10A except for the N-terminal Met, which was observed to be 28 Da higher than predicted. The amino acid sequences of Ent7A and Ent7B are listed in Figure 3, except for Leu and Ile, which were deduced from the genes coding for Ent7A and Ent7B.

For N-terminal Met, the observed mass difference could in principle represent a modification by either dimethylation (addition of $2 \times CH_2$) or formylation (addition of CO) (DeltaMass, http://www.abrf.org/index.cfm/dm.home). Hence, the Q-TOF instrument was precalibrated for an accuracy of 10−20 ppm for masses of 100−500 Da. The b-ion series of Ent7A was compared with theoretically dimethylated or formylated MR10A fragment ions (Table 2). In peptide MS/MS sequencing the b-ion and y-ion series are formed through cleavage of the amide bond on the peptide backbone with the structure of the resulting b-ion typically being a cyclic oxazolone, which requires the presence of a carbonyl at the $n - 1$ position for a given b_n ion. Therefore, a b_1 ion (i.e., for the N-terminal residue) is not normally observed for most peptides because its formation would require the presence of a carbonyl group from the nonexistent $n - 1$ amino acid. However, if an acetyl or formyl group was present on the amino group at the N-terminus, a b_1 ion could be seen, because the carbonyl necessary to form the cyclic oxazolone structure of a b-ion is available. In contrast, dimethylation on Met could not lead to formation of this type of structure. Although the formylated b_1 ion peak of Ent7A is weak, its presence can be confirmed and also distinguished from an *N,N*-dimethyl modification by the difference in exact mass for two CH_2 groups versus a CO group. The experimental b_1−b_4 ions (the first four amino acids from the N-terminus) of Ent7A closely match those of the theoretically N-formylated MR10A (Table 2).

The b-ion series of MR10A with formylation on N-terminal Met was then compared with the MS/MS spectrum, and all b_1−b_{43} peaks were found except the b_{14} peak. Information from both b-ions and y-ions further confirmed that Ent7A has the same sequence as MR10A with formylation of the amino group of Met on the N-terminus. The same analyses were performed on

Figure 3. Amino acid sequences of leaderless bacteriocins: (a) amino acid sequence alignments of Ent7A (identical to MR10A[9]) with EntL50A[17] (identical to 62-6A[18]); (b) amino acid sequence alignments of Ent7B (identical to MR10B[9]) with EntL50B[17] (identical to 62-6B [18]); different residues are highlighted; (c) amino acid sequences of enterocin EJ97,[24] enterocin Q,[25] and lacticin Q.[19].

Table 2. Experimental Molecular Weights of b_1 to b_4 Ion Series of Ent7A Determined by Nano-ESI MS/MS and theoretical Molecular Weights of the Formylated or Dimethylated MR10A

		ion series (Da)			
	sample	b_1	b_2	b_3	b_4
experimental	Ent7A	160.043 ± 0.003	217.062 ± 0.004	288.098 ± 0.006	401.178 ± 0.008
theoretical	Met1 formylated MR10A	160.043	217.065	288.102	401.186
theoretical	Met1 dimethylated MR10A	160.079	217.101	288.138	401.222

Ent7B. Results revealed that Ent7B has the same sequence as MR10B with a corresponding formylated Met on the N-terminus.

Accurate mass measurements of the intact peptides, Ent7A and Ent7B, were conducted on a Bruker 9.4T Apex-Qe FTICR instrument, which had been internally calibrated by bovine insulin to an accuracy of 1 ppm. The theoretical m/z value of a sextuply charged Ent7A peptide ($[M + 6H]^{6+}$) is 867.8241 Da if N-terminally formylated and 867.8301 if dimethylated. We found a m/z of 867.8233 Da, a difference of 0.9 and 7.9 ppm for the formylated versus dimethylated species, respectively. Similarly, the theoretical m/z value of a septuply charged Ent7B peptide ($[M + 7H]^{7+}$) is 744.8399 Da if N-terminally formylated and 744.8451 if dimethylated. We found a m/z of 744.8393 Da, a difference of 0.8 and 7.8 ppm for the formylated versus dimethylated species, respectively. Accurate mass measurements on the intact peptides clearly favor the formylated species. The resulting empirical formulae calculated for Ent7A ($C_{252}H_{390}N_{60}O_{55}S_2$) and Ent7B ($C_{251}H_{380}N_{62}O_{55}S_2$) match those of formylated MR10A and MR10B, respectively.

The presence of *N*-formylmethionine was postulated to explain why N-terminal sequencing by Edman degradation was blocked for enterocins L50A and L50B and enterocins 62-6A and 62-6B.[17,18] EntL50A and EntL50B are identical to 62-6A and 62-6B, respectively, and they show a high degree of similarity with MR10A/Ent7A and MR10B/Ent7B, respectively (Figure 3). Nano-LC-MS/MS analysis indeed seemed to confirm that the N-terminal methionines of EntL50A and EntL50B were formylated.[20] Lacticin Q is another leaderless enterocin that has been reported to have an N-formylated methionine based on a 159 Da decrease after cyanogen bromide treatment.[19] On the basis of these data, it seems that most, if not all, leaderless enterocins contain an N-terminal fMet residue and that deformylation of fMet and MAP activity are likely prevented in the same manner.

The first 10 residues of MR10A and MR10B were successfully sequenced by Edman degradation.[9] However, it should be noted that *N*-formyl groups are easily removed by mild aqueous acid treatment in contrast to most other *N*-acyl groups.[28,29] Because such conditions are frequently used during HPLC purification of peptides (e.g., TFA/water/CH_3CN), partial deformylation to liberate the N-terminus as a free amino group can occur after collection of peptide-containing fractions if they are permitted to stand in the solvent even for modest periods of time. Therefore, it is possible that enterocins that are produced with an N-terminal fMet residue can be successfully sequenced by Edman degradation.

Oxidation of N-Terminal Formylated Met. The molecular weights of 5222.60 Da (HPLC fraction 1, monoisotopic mass) and 5216.60 Da (fraction 2, monoisotopic mass) differ from those of Ent7B (5206.65 Da, monoisotopic mass) and Ent7A (5200.80 Da, monoisotopic mass) by 16 Da, respectively. MS/MS analysis was performed on peaks of 5222.60 and 5216.60 Da to determine whether they were chemically modified Ent7A and Ent7B. The y-ion series of the 5222.60 Da peak match those of Ent7B from y_1-y_{39} ($y_{40}-y_{43}$ not observed), indicating that (1) the 5222.60 Da mass has the same amino acid sequence as Ent7B and (2) chemical modification did not occur on the C-terminus of Ent7B. Each of the b_1-b_{38} ions of the 5222.60 Da peak is 15.99 Da (atomic weight of oxygen) more than the corresponding b-ion series of Ent7B ($b_{39}-b_{43}$ not observed), indicating that fraction 1 contains the mono-oxidized form of Ent7B and that the site of oxidation was the N-terminal methionine (which was also formylated). The same analysis was performed on the 5216.60 Da peak and showed that fraction 2 contained Ent7A with an oxidized N-terminal formylated Met.

During the aerobic growth of *E. faecalis* 710C, Met was prone to oxidation by active oxygen species (AOS) into methionine sulfoxide.[30] This oxidation process is reversible because

Table 3. Presence of the Virulence Factors and Vancomycin Resistance Gene in *E. faecalis* 710C

virulence factor	result[a]
ace[b]	+
aggregation substance (*asa*1)	+
enterolysin A	+
gelatinase	+
cytolysin	+
β-hemolysis	+
$EfaA_{fc}$[c]	+
$EfaA_{fm}$[c]	−
VanB	−

[a] + indicates presence of gene or positive result on plate assays; −, negative result [b] ace, collagen adhesion. [c] $EfaA_{fc/m}$,endocarditis antigen from *E. faecalis* or *E. faecium*, respectively.

methionine sulfoxide reductase (Msr), an enzyme present in all living organisms,[31,32] can reduce methionine sulfoxide to methionine.[30] Oxidation of Met can also occur following exposure of Ent7A and Ent7B to environmental oxidizing agents.[33]

Addition of oxygen to Ent7A/Ent7B decreases their hydrophobicity. Therefore, oxidized Ent7A/Ent7B eluted with a lower concentration of acetonitrile than Ent7A/Ent7B during the RP-HPLC purification procedure. In our study, both HPLC fraction 1 (oxidized Ent7B) and fraction 2 (oxidized Ent7A) were active against indicator *L. sakei* DSM 20017. However, the activity of oxidized Ent7A/7B is only 25% of the nonoxidized Ent7A/7B.

An oxidized and a nonoxidized form of EntL50A were also detected by Izquierdo et al.,[20] whereby oxidation occurred at Met[24].

Activity of Purified Ent7A and Ent7B. Both peptides Ent7A and Ent7B were tested individually against a number of indicator strains. The minimal inhibitory concentrations (MIC) of Ent7A and Ent7B against *L. sakei* DSM20017 are 4 and 6 μg/mL, respectively. Both Ent7A and Ent7B were active not only against Gram-positive bacteria (*C. sporogenes* 25779, *L. monocytogenes* FS-15, and *S. aureus* 23235) but also against the Gram-negative bacterium, *B. diminuta* UFM1. No synergistic effect between Ent7A and Ent7B was observed when *L. sakei* DSM 20017 was used as the indicator organism.

Most bacteriocins from Gram-positive bacteria are effective against Gram-positive bacteria, and only a few have been shown to be effective against Gram-negative organisms. One explanation is that Gram-negative bacteria have an outer membrane, which prevents the entry of most bacteriocins from Gram-positive bacteria. In this experiment, both Ent7A and Ent7B were active against Gram-negative *B. diminuta* cells. Neither Ent7A nor Ent7B, alone or combined, was effective against the Gram-negative pathogen *Salmonella typhimurium*.

Potential Use of Ent7A and Ent7B for Food Safety. Enterocins 7A and 7B are broad-spectrum antimicrobial bacteriocins produced by enterococci. They are active against various common foodborne pathogens including *Listeria* spp., *Clostridium* spp., and *S. aureus*. Therefore, Ent7A and Ent7B can be considered as candidates for food preservation. Recently, virulence factors from enterococci have been identified as a concern when enterococcal cultures are added directly to food products.[34,35] Some of these virulence factors, including gelatinase, adhesion to collagen, aggregation substance, and endocarditis antigen, were determined to be produced also in this study for *E. faecalis* 710C. PCR-based methods have been applied to screen the presence of virulence traits and bacteriocin genes in enterococci.[36] In this study, the presence of genes associated with well-known virulence factors and vancomycin resistance in *E. faecalis* 710C was examined by PCR-based or plate assays (Table 3).[22] *E. faecalis* 710C was positive for the presence of the adhesion to collagen (*ace*), aggregation substance (*asa*1), enterolysin, gelatinase, cytolysin, and endocarditis antigen and was also β-hemolytic on sheep blood agar. The presence of virulence factors makes this strain unsuitable for use in foods. According to the qualified presumption of safety (QPS) concept of the European Food Safety Authority (EFSA), bacteria considered for use in food with antibiotic resistances or virulence factors would not be considered to obtain QPS status. The presence of an *asa1* gene furthermore indicates the presence of a sex pheromone plasmid. This gene is usually located on such pheromone-responsive plasmids, which facilitate aggregation of strains to other enterococcal strains, resulting in a highly efficient plasmid transfer. This implies the strain has a high capacity also for horizontal gene transfer, which is a further detrimental aspect from a safety point of view. However, as our experiment demonstrated, Ent7A and Ent7B could be isolated, and the purified forms of Ent7A and Ent7B, instead of the live enterococcal culture, could be applied to control the growth of pathogens in ready-to-eat foods.

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