Three Novel Lantibiotics, Ticins A1, A3, and A4, Have Extremely Stable Properties and Are Promising Food Biopreservatives

Bingyue Xin, Jinshui Zheng, Ziya Xu, Congzhi Li, Lifang Ruan, Donghai Peng, Ming Sun

State Key Laboratory of Agricultural Microbiology, College of Life Science and Technology, Huazhong Agricultural University, Wuhan, China

Lantibiotics are antimicrobial peptides with potential applications as the next generation of antimicrobials in the food industry and/or the pharmaceutical industry. Nisin has successfully been used as a food preservative for over 40 years, but its major drawback is its limited stability under neutral and alkaline pH conditions. To identify alternatives with better biochemical properties, we screened more than 100 strains of the *Bacillus cereus* group. Three novel lantibiotics, ticins A1 (4,062.98 Da), A3 (4,048.96 Da), and A4 (4,063.02 Da), which were highly thermostable (121°C for 30 min) and extremely pH tolerant (pH 2.0 to 9.0), were identified in *Bacillus thuringiensis* BMB3201. They all showed potent antimicrobial activities against all tested Gram-positive bacteria and greater activities than those of nisin A against *Bacillus cereus* and *Listeria monocytogenes*, two important foodborne pathogens. These three novel lantibiotics, with their extremely stable properties and potent antimicrobial activities, have the potential for use as biopreservatives.

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by various bacteria that are active against other bacteria either of the same species (narrow spectrum) or across genera (broad spectrum) (1). They are classified into two groups: posttranslationally modified bacteriocins and unmodified or cyclic bacteriocins (2). Lantibiotics (lanthipeptides) are the best-characterized modified bacteriocins, and their modifications include the formation of *meso*-lanthionine and 3-methylanthionine residues, as well as dehydrated amino acids (Dha and Dhb) (3). Lanthionine (Lan) consists of two alanine residues cross-linked via a thioether linkage; 3-methylanthionine (MeLan) contains one additional methyl group. Lanthipeptides are classified into four classes depending on their biosynthetic enzymes. For class I lanthipeptides, dehydration is carried out by a LanB dehydratase, and cyclization is catalyzed by a LanC cyclase. Class II lanthipeptides are modified by LanM lanthionine synthetases, which have an N-terminal dehydration domain and a C-terminal cyclization domain. For class III and IV lanthipeptides, the dehydration and cyclization reactions are catalyzed by multifunctional enzymes (RamC/LabKC or LanL).

In the past decade, research on bacteriocins or lantibiotics has focused primarily on lactic acid bacteria (LAB), primarily because of their potential applications as preservatives in the food industry and/or as viable alternatives to antibiotics in medicine (2, 4). These bacteriocins also have several desirable properties that make them suitable for food preservation, as follows: (i) they are generally recognized as safe (GRAS), (ii) they are nontoxic to eukaryotic cells, (iii) they can be inactivated by digestive proteases, (iv) they are pH and heat tolerant, (v) they have a relatively broad antimicrobial spectrum against many Gram-positive foodborne pathogenic and spoilage bacteria, (vi) they show a bactericidal mode of action and no cross-resistance with antibiotics, and (vii) they can be conveniently modified by genetic manipulation (5, 6). Nisin is the most extensively studied lantibiotic, and it has been approved for use as a food preservative to combat foodborne pathogens for more than 40 years (7). However, the drawback of nisin is its limited stability at neutral and alkaline pHs, so identifying alter-

natives that have better biochemical properties for application as food additives is necessary (8).

The *Bacillus cereus* group comprises the species *B. anthracis*, *B. cereus*, *B. thuringiensis*, *B. cytotoxicus*, *B. mycoides*, *B. pseudomycoides*, and *B. weihenstephanensis*. These can be isolated from diverse environments, such as soil, water, and animal hosts (9, 10). Our previous work proved that the *B. cereus* group is an excellent reservoir of novel lanthipeptides (11). All three lantibiotics reported to date within this group exhibit broad-spectrum antimicrobial activity against some clinically important Gram-positive pathogens, including multidrug-resistant strains (11–13). In this study, we applied a targeted screening method to identify and characterize three novel class II lantibiotics, ticins A1, A3, and A4, in *B. thuringiensis* BMB3201. Their extreme stability at high temperatures and in strong bases and potent antimicrobial activity, especially against the foodborne pathogens *B. cereus* and *Listeria monocytogenes*, make them appropriate for further investigation for applications in the food industry.

MATERIALS AND METHODS

Strain screening. More than 100 strains (mainly *B. cereus* and *B. thuringiensis*) isolated from the soil of different provinces in China were screened for antimicrobial activity. For each strain, the antimicrobial activity of the cell-free supernatant (from the exponential growth phase and the stationary growth phase) was tested against two important foodborne

Received 3 June 2015 Accepted 16 July 2015

Accepted manuscript posted online 31 July 2015

Citation Xin B, Zheng J, Xu Z, Li C, Ruan L, Peng D, Sun M. 2015. Three novel lantibiotics, ticins A1, A3, and A4, have extremely stable properties and are promising food biopreservatives. *Appl Environ Microbiol* 81:6964–6972. doi:10.1128/AEM.01851-15.

Editor: M. A. Elliot

Address correspondence to Ming Sun, m98sun@mail.hzau.edu.cn.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AEM.01851-15>.

Copyright © 2015, American Society for Microbiology. All Rights Reserved. doi:10.1128/AEM.01851-15

TABLE 1 Antimicrobial activities of *Bacillus thuringiensis* BMB3201 culture supernatant and purified ticin A4 and relative antimicrobial activities of ticin A4 and nisin A

Indicator strain (reference) ^a	Broth medium ^b	Culture supernatant ^c	MIC (μM) ^d	
			Ticin A4	Nisin A
Gram-negative bacteria				
<i>Sphingobacterium</i> strain Pri1 (11)	NB	—	—	—
<i>Pseudomonas aeruginosa</i> ATCC 27853	NB	—	—	—
<i>Pseudomonas putida</i> Pri3 (11)	NB	—	—	—
<i>Pseudomonas psychrophila</i> Pri5 (11)	NB	—	—	—
<i>Escherichia coli</i> BL21 (32)	LB	—	—	—
<i>Escherichia coli</i> DH5α (32)	LB	—	—	—
<i>Erwinia herbicola</i> LS005 (33)	LB	—	—	—
<i>Enterobacter</i> strain Bom2 (11)	NB	—	—	—
Gram-positive bacteria				
<i>Bacillus amyloliquefaciens</i> X1	LB	+	0.98	3.91
<i>Bacillus cereus</i> ATCC 14579	LB	+	3.91	7.82
<i>Bacillus cereus</i> ATCC 49064	LB	+	0.49	0.98
<i>Bacillus cereus</i> VD 048	LB	+	0.49	1.95
<i>Bacillus firmus</i> DS-1	LB	+	1.95	3.91
<i>Bacillus subtilis</i> 168 (34)	LB	+	7.81	0.98
<i>Bacillus subtilis</i> Bsn5 (35)	LB	+	1.95	1.95
<i>Bacillus thuringiensis</i> BMB171 (36)	LB	+	0.975	1.95
<i>Bacillus thuringiensis</i> YBT1518 (37)	LB	+	0.49	1.95
<i>Bacillus pumilus</i> SCG I (11)	LB	+	3.91	1.95
<i>Listeria monocytogenes</i> LM201 (15)	TSBYE	+	1.95	3.91
<i>Listeria monocytogenes</i> LM605	TSBYE	+	0.98	3.91
<i>Paenibacillus</i> strain X2 (11)	LB	+	1.95	7.82
<i>Staphylococcus aureus</i> CMCC 26003	NB	+	15.63	3.91
<i>Staphylococcus aureus</i> ATCC 43300	NB	+	31.25	3.91
<i>Staphylococcus sciuri</i> Bom1 (11)	NB	+	3.91	7.82
<i>Microbacterium</i> strain Pri2 (11)	NB	+	3.91	0.98
<i>Enterococcus faecalis</i> ATCC 29212	NB	+	0.49	0.49

^a See references for the sources of the marked strains. Unmarked strains were isolated by our group. ATCC, American Type Culture Collection; CMCC, China Medical Culture Collection.

^b NB, nutrient broth; LB, Luria broth; TSBYE, tryptic soy broth supplemented with yeast extract.

^c A minus sign indicates that the culture supernatant of *B. thuringiensis* BMB3201 has no antimicrobial activity to the indicator strain. A plus sign indicates that the culture supernatant of *B. thuringiensis* BMB3201 has antimicrobial activity to the indicator strain.

^d The highest concentrations of nisin A and ticin A4 were 125 μM. For each indicator strain, the antimicrobial activities of ticins A1, A3, and A4 were essentially comparable, so we have listed the MIC of only ticin A4. A minus sign indicates the absence of activity against indicator strains, even at the highest concentrations of the indicated peptides.

pathogens, *B. cereus* ATCC 49064 and *L. monocytogenes* LM201 (14, 15). Then we tested the stability of the cell-free supernatant of antimicrobial-producing strains at a high temperature (100°C for 30 min) and under strong-acid (pH 2.0 for 24 h) and strong-base (pH 9.0 for 24 h) conditions. The BMB3201 strain produced potent and stable antimicrobial agents and therefore was selected for further study.

Cultures and media. *B. thuringiensis* strain BMB3201 was propagated in Luria-Bertani (LB) agar plates at 28°C. The other strains used as indicator strains and the media used are listed in Table 1.

Genome sequencing and sequence analysis. The whole genome of *B. thuringiensis* BMB3201 was sequenced using paired-end sequencing technology on the Illumina HiSeq2500 platform and assembled by Abyss. The bacteriocin biosynthetic gene clusters were predicted by BAGEL 3.0 (16).

DNA manipulation, RNA manipulation, cDNA synthesis, and transcriptional analysis. The total DNA of *B. thuringiensis* was obtained as described previously (17). Total RNA was extracted from *B. thuringiensis* BMB3201 after incubation in LB medium for 10 h (28°C and 220 rpm), using the RNeasy minikit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The cDNA was synthesized using the RNA PCR kit (AMV; TaKaRa, Dalian, China) version 3.0. The transcription of some genes in two lantibiotic biosynthetic gene clusters was analyzed using PCR

amplification with the primers II-MF, II-MR, II-AF, II-AR, I-BF, I-BR, I-CF, and I-CR (Table 2).

Purification of antimicrobial agents. *B. thuringiensis* BMB3201 was grown in LB broth at 28°C into the early stationary phase. Cells were removed by centrifugation (12,000 rpm, 10 min, and 4°C), and the cell-

TABLE 2 Primers used in this study

Primer	Sequence	Size of PCR product (bp)
16S-F	5'-GCTGGCACCTTGACGGTACC-3'	108
16S-R	5'-CGCTTTACGCCAATAATTCGG-3'	
II-MF	5'-AACGTGCGAAAGTGGGAAA-3'	125
II-MR	5'-CGATTTCCAAACCAAGTTCG-3'	
II-AF	5'-AGGTGGTAGTTTGGGAGTCA-3'	100
II-AR	5'-CGTAGTCAATACCGCACCAC-3'	
I-BF	5'-GCTCAGCGGTAGGAATAGCT-3'	155
I-BR	5'-TTCTATCGGTGTGGTGAAC-3'	
I-CF	5'-TGAGTGGTAGGTATCTCGGAG-3'	138
I-CR	5'-GCATGTAACCAGCATAGCCA-3'	

FIG 1 Identification of two putative lanthipeptide synthetic gene clusters in *B. thuringiensis* BMB3201. (A) Two putative lanthipeptide synthetic gene clusters and amino acid sequence alignment of the precursor peptides for these two gene clusters. (B) Kinetics of antimicrobial substances produced during the growth of *B. thuringiensis* BMB3201. The optical density of BMB3201 culture was measured at 600 nm (diamonds). The antimicrobial substance concentration was expressed as the inhibition zone diameter (squares). The indicator strain is *B. thuringiensis* BMB171. (C) Transcription of genes coding for lanthipeptide and ticin production in the exponential growth phase of *B. thuringiensis* BMB3201. The 16S rRNA gene was used as a positive control.

free supernatant (2 liters) was shaken with 200 g of Amberlite XAD-7HP (Sigma, St. Louis, MO) for 12 h at 4°C. The resin was collected by filtration and sequentially washed with 2 liters of distilled water and 1 liter of 30% (vol/vol) ethanol. The active substances were eluted with 500 ml of 80% (vol/vol) ethanol, pH 2.0. Then the eluate was collected and condensed in a rotary evaporator at 42°C under vacuum, and the concentrate was lyophilized. The freeze-dried fraction was dissolved in distilled water, followed by centrifugation. The resulting supernatant was referred to as the antimicrobial crude extract (CE).

High-performance liquid chromatography (HPLC) analysis was performed on the Waters 1525 Breeze system. The mobile phase consisted of acetonitrile and HPLC-grade water containing 0.1% trifluoroacetic acid (TFA). The CE (50 µl) of *B. thuringiensis* BMB3201 was loaded into an Agilent HC-C18 (2) column (5 µm; 4.6 by 250 mm; Agilent) and separated by a linear gradient of 10% to 50% acetonitrile from 0 to 60 min at a flow rate of 1.0 ml/min. The eluate was monitored at a wavelength of 220 nm, and fractions were manually collected for a bioassay of antibacterial activity against *B. cereus* ATCC 49064. The active fractions were collected and lyophilized. They were then dissolved in double-distilled water (ddH₂O), and the concentration of bacteriocin preparation was adjusted to 0.5 mg/ml.

Nisin A was purified from commercial nisin (Sigma-Aldrich, Shanghai, China) using the procedure described above. The purities of nisin A and ticins A1, A3, and A4 were determined by HPLC using the above-described procedure, and the values were all greater than 95% based on the ratio of absorption peak area at 220 nm.

Assay of antimicrobial activity. Antimicrobial activity was detected by the agar well diffusion method (11). The MICs of the bacteriocins for the various indicator strains were measured by serial 2-fold HPLC-purified bacteriocin solutions.

LC-MS/MS analysis. Liquid chromatograph-mass spectrometry (LC-MS) and LC-tandem MS (MS/MS) were both performed by the Agilent 6540 Ultra High Definition (UHD) Accurate-Mass quadrupole time of flight (Q-TOF) LC-MS system. The analytical column was an Agilent HC-C18 (2) column (5 µm; 4.6 by 250 mm; Agilent). The MS operating conditions were as follows: the capillary voltage was 3,500 V, the temperature was 350°C, the nebulizer pressure was 35 lb/in², and the flow rate of the drying gas was 9 liters/min. The scanning range of the Q-TOF was *m/z* 100 to 3,000. Detailed sequence information for three antimicrobial peptides was obtained using the targeted MS/MS mode. The target ion was isolated and fragmented by adding a voltage of 35 V.

Lantibiotics	Calculated <i>m/z</i>	Measured <i>m/z</i>	Difference	Dehydration/All T or S
Ticin A1 (TinA1)	4206.77 Da	4062.98 Da	143.79 Da	8/12
Ticin A2 (TinA2)	4308.86 Da	-	-	-
Ticin A3 (TinA3)	4192.74 Da	4048.96 Da	143.78 Da	8/12
Ticin A4 (TinA4)	4206.81 Da	4063.02 Da	143.79 Da	8/12

FIG 2 Verification of the molecular mass of three antimicrobial substances by LC/MS. The table indicates the possible number of dehydrated serine residues or threonine residues in these three lantibiotics. Three fractions correspond to three peaks (A, B, and C), with retention times of 49.8 min, 50.8 min, and 51.9 min, that were active against *B. thuringiensis* BMB171.

Thermal, pH, and enzymatic stabilities of ticin A4. To test the thermal stability of ticin A4, the bacteriocin preparations ($\sim 30 \mu\text{M}$) were incubated at 28°C for 2 weeks, 80°C and 100°C for 1 h, and 121°C for 30 min. In addition, nisin A ($\sim 30 \mu\text{M}$) and ticin A4 ($\sim 30 \mu\text{M}$) were also exposed to 100°C for 2 h and 4 h. The residual bacteriocin activities against *B. subtilis* 168 were determined as described above. To study the sensitivity of ticin A4 to pH, the bacteriocin preparations ($\sim 30 \mu\text{M}$) were adjusted to pH 2.0 to 11.0, using 5 M NaOH or 1 M HCl solution, followed by incubation at 37°C for 24 h. Nisin A ($\sim 30 \mu\text{M}$) was used for comparison. The residual antimicrobial activity was assessed after neutralizing the sample to pH 5.0. The effects of various degradative enzymes (1 mg/ml) on ticin A4 were determined. The tested enzymes were α -chymotrypsin (1,200 U/mg), α -amylase (50 U/mg), pepsin (≥ 250 U/mg), bromelain (3 to 7 U/mg), papain (1.5 to 10 U/mg), protease (type XIII; ≥ 0.6 U/mg), and trypsin ($\geq 10,000$ U/mg). The solutions of ticin A4 (100 mg/ml) and enzymes were mixed at a 1:1 ratio (vol/vol) and incubated at 37°C for 1 h, and the remaining activity posttreatment was determined with the agar well diffusion method described above.

Bactericidal activity of ticin A4. The effect of ticin A4 on a sensitive indicator bacterial strain was assessed by incubating exponential-phase *B. subtilis* 168 with purified ticin A4. A 30-ml culture of the indicator grown in LB broth was incubated at 37°C . The culture was divided into three aliquots when the optical density at 600 nm (OD_{600}) reached 0.3. One sample was used as a control, and different amounts of ticin A4 were added to the other two samples. The flasks were then incubated at 37°C for 240 min, and samples were removed at different times to measure the number of viable cells on LB agar plates and the OD_{600} . Three independent experiments were performed.

Nucleotide sequence accession numbers. The whole-genome shotgun sequencing results for *B. thuringiensis* BMB3201 have been deposited in GenBank under accession no. [LDEU00000000](#). The version described in this paper is version [LDEU01000000](#). The nucleotide sequence of the ticin gene cluster has been deposited in GenBank under accession no. [KR869822](#).

RESULTS

Screening and identification of *B. cereus* group strains that produce antimicrobials with base- and heat-resistant features. The *B. cereus* group contains diverse novel lanthipeptides, and recently reported lantibiotics in this group have been shown to exhibit high efficacy against certain Gram-positive pathogens (11, 13). Therefore, we screened more than 100 strains (mainly *B. cereus* and *B. thuringiensis*) for antimicrobial activity against two important foodborne pathogens, *B. cereus* ATCC 49064 and *L. monocytogenes* LM201 (14, 15), and tested the stability of the cell-free supernatant of antimicrobial-producing strains under high-temperature, strong-acid, and strong-base conditions. More than 50% of the strains exhibited antimicrobial activity against one of the indicator organisms. Of these, one *B. thuringiensis* strain, BMB3201, produced an antimicrobial substance in the exponential phase (Fig. 1B), and the antimicrobial substance was effective against all of the tested Gram-positive bacteria (Table 1). The antimicrobials were stable over a wide pH range (pH 2.0 to 9.0) and at a high temperature (100°C , 30 min). In addition, the antimicrobials were

Ticin A1

FIG 3 Electrospray ionization (ESI)-MS/MS spectra and proposed primary structure of ticin A1. Fragment ions are indicated. The putative thioether bridging of ticin A1 is shown. The underlined threonine residues are not dehydrated. Dehydrated serine residues or threonine residues are displayed in black. Three of six additional residues were also dehydrated, but we could not determine which ones from the current data; these six amino acids are displayed in gray.

able to be digested by different proteolytic enzymes, so we deduced that the antimicrobials were bacteriocins.

Genome sequencing and identification of two novel lanthipeptide gene clusters. To identify the gene (cluster) responsible for the synthesis of the antimicrobial agents, we sequenced the genome of the strain BMB3201 and analyzed all of the putative bacteriocin biosynthetic gene clusters. Only two putative lanthipeptide gene clusters could potentially synthesize the bacteriocins (Fig. 1A). One lanthipeptide gene cluster consisted of five genes, including one structural gene (*lanA*), two genes (*lanB* and *lanC*) coding for enzymes involved in the posttranslational modification of the precursor peptide, one gene coding for glycosyltransferase, and one gene (*lanT*) coding for an ABC transporter-related protein that may contribute to the secretion of the lanthipeptides (Fig. 1A). This gene cluster was also present in the genome of *B. cereus* F65185, and it was regarded as a type I-5 lanthipeptide gene cluster in *B. cereus* group strains (11). The second lanthipeptide gene cluster consists of 11 genes, including 4 highly homologous structural genes (*tinA1*, *tinA2*, *tinA3*, and *tinA4*), 1 gene (*tinM*) encoding a modification enzyme, 3 genes (*tinEFG*) encoding an ABC

transporter that could be involved in immunity, 1 gene (*tinT1*) encoding a transporter with an associated protease domain, 1 gene (*tinT2*) encoding an HlyD family secretion protein, and 1 regulatory gene (*tinR*) (Fig. 1A). The BLASTP analysis of the TinA protein revealed that this gene cluster was not reported in previous studies. Four precursor peptides (TinA1, TinA2, TinA3, and TinA4) are nearly identical (identities above 88%), with differences at positions 2, 3, 4, 10, 11, 14, 17, and 40 of the core peptide (Fig. 1A), and they show no similarity to any other known lantibiotic precursor peptide sequences.

To reveal the genetic determinants of the antimicrobials, we tested the transcription of the genes in these two gene clusters in the exponential growth phase of strain BMB3201. Both the *tinA1* and *tinM* genes in the ticin gene cluster were transcribed in the early stationary phase of growth (Fig. 1C). For the class I lanthipeptide gene cluster, no transcripts of the *lanB* gene were detected. This result suggested that the type I lanthipeptide gene cluster is not responsible for the production of antimicrobial substances.

Purification and characterization of antibacterial substances produced by *B. thuringiensis* BMB3201. Purification of antimi-

FIG 4 Agar well diffusion inhibition assay with various indicator strains to determine the relative antimicrobial activities of ticin A4 and nisin A. The concentrations of ticin A4 or nisin A in eight wells were identical; the values were 125 μ M, 62.5 μ M, 31.25 μ M, 15.63 μ M, 7.81 μ M, 3.91 μ M, 1.95 μ M, and 0.98 μ M, respectively.

crobal compounds from the 18-h cultures of *B. thuringiensis* BMB3201 was achieved by concentration on Amberlite XAD-7 HP resin and then separation of the antimicrobial crude extract (CE) by reverse-phase HPLC (RP-HPLC). As shown in Fig. 2, only three fractions corresponding to three peaks with retention times of 49.8 min, 50.8 min, and 51.9 min were active against *B. thuringiensis* BMB171. The chemical nature of these three antimicrobial substances was analyzed by LC-MS. The measured molecular masses of fractions A (m/z 4,063.02 Da), B (m/z 4,048.96 Da), and C (m/z 4,062.98 Da) were determined from their monoisotopic signals. The calculated molecular masses of the predicted mature peptide of TinA1, TinA3, and TinA4 (called ticins A1, A3, and A4, respectively) were all 143.79 Da higher than the calculated mass, indicating that 8 out of 12 serine residues or threonine residues were dehydrated and that spontaneous hydrolysis of the Dhb residue occurred at the N terminus of ticins A1, A3, and A4, resulting in a 2-oxobutyl residue instead of a Dhb (18).

We then used LC-MS/MS to analyze the detailed sequence information for these three antimicrobial substances. The marked fragments of fractions A, B, and C correspond to the fragments of ticin A1, ticin A3, and ticin A4 (Fig. 2 and 3; see also Fig. S1 in the

supplemental material). In the MS/MS spectrum of ticins A1 and A3, the molecular mass difference of fragment ions b16 and b17 was 101.04 Da, indicating that Thr17 was not dehydrated. The data on fragment ions also indicated that Ser1, Thr5, Thr13, Thr15, and Thr20 were dehydrated. Three of six residues (Ser32, Ser36, Ser41, Thr30, Thr31, and Thr38) were also dehydrated, but we could not determine exactly which ones from the current data. In addition, no cleavage was observed from Thr20 to Cys25 and Thr30 to Cys42, indicating the presence of an intramolecular thioether bridge (Lan and/or MeLan). Based on the above-described data, we surmised that there were possible Lan and MeLan bridges in these three lantibiotics (Fig. 3; see also Fig. S1).

Antimicrobial activities of lantibiotics. The HPLC-purified ticin A4 had inhibitory activity against all of the tested Gram-positive bacteria, including *B. amyloliquefaciens*, *B. cereus*, *B. firmus*, *B. thuringiensis*, *B. subtilis*, *B. pumilus*, *Enterococcus faecalis*, *L. monocytogenes*, *Microbacterium*, *Paenibacillus*, *Staphylococcus aureus*, and *Staphylococcus sciuri*, but not against any of the tested Gram-negative bacteria (Table 1). For each indicator strain, the antimicrobial activities of these three lantibiotics were essentially comparable. In addition, we compared the antimicrobial activities

FIG 5 Thermal, pH, and enzymatic stabilities of ticin A4. *B. subtilis* 168 was used as the indicator strain in these four experiments. (A) Stability of ticin A4 in response to heat. (B) Relative stabilities of ticin A4 and nisin A at 100°C for 2 h and 4 h. Means and standard deviations are presented. (C) Relative stabilities of ticin A4 and nisin A under various pH conditions. Means and standard deviations are presented. (D) Enzymatic stability of ticin A4.

of ticin A4 and nisin A (Table 1 and Fig. 4). Surprisingly, ticin A4 showed 2-fold to 8-fold higher activity against *B. cereus* ATCC 14579, ATCC 49064, and *B. thuringiensis* BMB171 than did nisin A. Ticin A4 demonstrated 2-fold to 4-fold higher activity against *L. monocytogenes* LM201 and LM605 than did nisin A. Ticin A4 showed activity similar to that of nisin A against *E. faecalis* ATCC 29212 and *B. subtilis* 168 and 2-fold lower activity against *B. pumilus* SCG I. Its antimicrobial activity against *S. aureus* ATCC 43300 and *S. aureus* CMCC 26003 was 4-fold to 8-fold lower than that of nisin A. These results illustrated that ticin A4 had more effective antimicrobial activity against the strains that belong to the same group (*B. cereus* and *B. thuringiensis*) and the foodborne pathogen *L. monocytogenes* than did nisin A.

Stability of ticin A4 to pH, enzymes, and thermal treatment. The HPLC-purified ticin A4 was tested for sensitivity to pH, degradative enzymes, and temperature. As shown in Fig. 5C, 100% stability was retained in a wide pH range, from 2.0 to 9.0. The residual antibacterial activities were greatly decreased when the pH was greater than 10.0. In parallel experiments, nisin A was less stable than ticin A4 under neutral and alkaline conditions.

Ticin A4 remained active after storage at 28°C for 2 weeks and after incubation at 80°C and 100°C for 1 h (Fig. 5A). Ticin A4 retained most of its antimicrobial activity when autoclaved at 121°C for 30 min. We also treated nisin A and ticin A4 solutions at 100°C for 2 h and 4 h, and their thermal stabilities were compara-

ble (Fig. 5B). Ticin A4 lost its activity to various degrees when digested with various proteolytic enzymes but not α -amylase (Fig. 5D). In addition, the thermal, pH, and enzymatic stabilities of ticin A1 and A3 were also tested. The stability of these two lantibiotics was basically identical to that of ticin A4 (data not shown).

Bactericidal activity of ticin A4. To study the mode of action of ticin A4, the indicator strain *B. subtilis* 168 was treated with different concentrations of ticin A4, and both the OD₆₀₀ of the culture and the number of viable cells were monitored at various time points. The addition of two different concentrations (1× MIC and 2× MIC) of ticin A4 caused the OD₆₀₀s of the culture to decrease to various degrees (Fig. 6A). The number of viable cells also showed a rapid decrease after treatment with different concentrations of ticin A4 (Fig. 6B). These results indicated that ticin A4 acts as a bactericidal compound.

DISCUSSION

Lantibiotics are the best-characterized modified bacteriocins and can inhibit several clinically important Gram-positive pathogens, including several multidrug-resistant ones (19–23). The lantibiotic nisin has been used as an effective food preservative against foodborne pathogens for over 40 years (7). Owing to its limited stability at neutral and alkaline pH, many researchers have focused on finding alternatives with better biochemical properties

FIG 6 Effect of ticin A4 on the optical density (A) and the number of viable cells (B) in *B. subtilis* 168 culture. Ticin A4 was added at 60 min (indicated with an arrow). Means and standard deviations are presented.

(8, 24). In this study, we screened a large number of the *B. cereus* group strains, which are regarded as a reservoir of novel lanthipeptides, and identified three heat- and alkali-resistant lantibiotics. This study not only identified three promising food biopreservatives but also provided a new approach to identify special properties of bacteriocins.

In addition, the aim of this study was to identify heat- and alkali-resistant bacteriocins in strains of the *B. cereus* group. Other types of bacteriocins with special properties may also be found in this group. Thuricin CD, a two-peptide bacteriocin consisting of Trn- α and Trn- β , is produced by *B. thuringiensis* DPC 6431 and shows a narrow spectrum of antibacterial activity against a wide range of clinically significant *Clostridium difficile* strains. This bacteriocin has been suggested as a potential treatment for *C. difficile*-associated disease (CDAD) (25). Genome mining for radical SAM protein determinants has predicted some sactibiotic-like gene clusters in the genome of some *B. cereus* and *B. thuringiensis* strains (26). In further research, we will focus on these narrow-spectrum bacteriocins by screening strains of the *B. cereus* group.

Owing to the growing consumer demand for safe food, lactic acid bacterial bacteriocins have attracted significant attention because of their great potential as nontoxic additives for preserving food and for preventing food spoilage by several foodborne pathogenic bacteria (5). Until now, only nisin and pediocin PA-1/AcH have been used as commercial biopreservatives. The bacteriocins applied as biopreservatives are classified as generally recognized as safe (GRAS). Ticins A1, A3, and A4 were produced by *B. thuringiensis*, which is the most successful insect pathogen used for insect control and is safe for humans (27, 28). Moreover, these three lantibiotics (>1 mg/ml) had no hemolytic activity. However, more detailed safety testing to assess the feasibility of widespread use of these three lantibiotics needs to be performed before they can be used as biopreservatives.

The antimicrobial activity tests illustrated that these three lantibiotics have stronger antimicrobial activity against *B. cereus* and *L. monocytogenes* strains than does nisin A. *B. cereus* is a human pathogen that causes diarrheal or emetic-type illnesses and is considered the third most important cause of collective food poison-

ing incidents in Europe (29, 30). *L. monocytogenes* is a facultative intracellular foodborne pathogen that can cause listeriosis in animals and humans, especially immunocompromised individuals (31). These characteristics of ticin A4 indicate the potential for its use against *B. cereus* and *L. monocytogenes*-associated foodborne diseases.

Differences in the production of ticins A1, A2 (not detected), A3, and A4 are probably related to differences in the transcription of their structural genes. In fact, each structural gene (*tinA1*, *tinA2*, *tinA3*, and *tinA4*) had its own promoter and terminator (data not shown). In addition, the *lanB* gene in the type I lanthipeptide gene cluster also had its own promoter (data not shown), and it might be transcribed only under certain conditions. We will perform further experiments to confirm these hypotheses.

In previous studies intended to identify antimicrobial peptides with certain properties, researchers mainly focused on bacteria surviving in particular ecological environments. For example, geobacillin I was identified in the thermophilic bacterium *Geobacillus thermodenitrificans* NG80-2 and showed greater stability than nisin A (8). In our research, we screened a large number of *B. cereus* group strains, which are regarded as reservoirs of novel lanthipeptides, and identified three heat- and alkali-resistant lantibiotics. Members of the *B. cereus* group can be found in diverse environments under no specific extreme stress. Our observations indicate that novel antimicrobial peptides with particular features can be found not only in specific organisms that exist in particular environments but also in those that contain diverse novel bacteriocins.

ACKNOWLEDGMENTS

We thank Michael Gänzle, University of Alberta, for discussions, suggestions about writing the manuscript, and critical reading of the manuscript. We thank Liu Mei, associate professor of Huazhong Agricultural University, for kindly providing us with the two strains *Listeria monocytogenes* LM201 and LM605.

This work was supported by grants from the National High Technology Research and Development Program (863) of China (2011AA10A203), the China 948 Program of the Ministry of Agriculture

(2011-G25), the National Basic Research Program (973) of China (2009CB118902), and the National Natural Science Foundation of China (31170047 and 31171901).

REFERENCES

- Cotter PD, Hill C, Ross RP. 2005. Bacteriocins: developing innate immunity for food. *Nat Rev Microbiol* 3:777–788. <http://dx.doi.org/10.1038/nrmicro1273>.
- Cotter PD, Ross RP, Hill C. 2013. Bacteriocins—a viable alternative to antibiotics? *Nat Rev Microbiol* 11:95–105. <http://dx.doi.org/10.1038/nrmicro2937>.
- Zhang Q, Yu Y, Velasquez JE, van der Donk WA. 2012. Evolution of lanthipeptide synthetases. *Proc Natl Acad Sci U S A* 109:18361–18366. <http://dx.doi.org/10.1073/pnas.1210393109>.
- Zhu X, Zhao Y, Sun Y, Gu Q. 2014. Purification and characterisation of plantaricin ZJ008, a novel bacteriocin against *Staphylococcus* spp. from *Lactobacillus plantarum* ZJ008. *Food Chem* 165:216–223. <http://dx.doi.org/10.1016/j.foodchem.2014.05.034>.
- Gálvez A, Abriouel H, Lopez RL, Ben Omar N. 2007. Bacteriocin-based strategies for food biopreservation. *Int J Food Microbiol* 120:51–70. <http://dx.doi.org/10.1016/j.ijfoodmicro.2007.06.001>.
- Snyder AB, Worobo RW. 2014. Chemical and genetic characterization of bacteriocins: antimicrobial peptides for food safety. *J Sci Food Agric* 94:28–44. <http://dx.doi.org/10.1002/jsfa.6293>.
- Delves-Broughton J, Blackburn P, Evans RJ, Hugenholtz J. 1996. Applications of the bacteriocin, nisin. *Antonie Van Leeuwenhoek* 69:193–202. <http://dx.doi.org/10.1007/BF00399424>.
- Garg N, Tang W, Goto Y, Nair SK, van der Donk WA. 2012. Lantibiotics from *Geobacillus thermodenitrificans*. *Proc Natl Acad Sci U S A* 109:5241–5246. <http://dx.doi.org/10.1073/pnas.1116815109>.
- Guinebreière MH, Auger S, Galleron N, Contzen M, De Sarrau B, De Buyser ML, Lamberet G, Fagerlund A, Granum PE, Lereclus D, De Vos P, Nguyen-The C, Sorokin A. 2013. *Bacillus cytotoxicus* sp. nov. is a novel thermotolerant species of the *Bacillus cereus* group occasionally associated with food poisoning. *Int J Syst Evol Microbiol* 63:31–40. <http://dx.doi.org/10.1099/ijs.0.030627-0>.
- Vilas-Bôas GT, Peruca AP, Arantes OM. 2007. Biology and taxonomy of *Bacillus cereus*, *Bacillus anthracis*, and *Bacillus thuringiensis*. *Can J Microbiol* 53:673–687. <http://dx.doi.org/10.1139/W07-029>.
- Xin B, Zheng J, Xu Z, Song X, Ruan L, Peng D, Sun M. 2015. The *Bacillus cereus* group is an excellent reservoir of novel lanthipeptides. *Appl Environ Microbiol* 81:1765–1774. <http://dx.doi.org/10.1128/AEM.03758-14>.
- Wang J, Ma H, Ge X, Zhang J, Teng K, Sun Z, Zhong J. 2014. Bovicin HJ50-like lantibiotics, a novel subgroup of lantibiotics featured by an indispensable disulfide bridge. *PLoS One* 9:e97121. <http://dx.doi.org/10.1371/journal.pone.0097121>.
- Wang J, Zhang L, Teng K, Sun S, Sun Z, Zhong J. 2014. Cerecidins, novel lantibiotics from *Bacillus cereus* with potent antimicrobial activity. *Appl Environ Microbiol* 80:2633–2643. <http://dx.doi.org/10.1128/AEM.03751-13>.
- Khalil AA, Deraz SF, Elrahman SA, El-Fawal G. 2015. Enhancement of mechanical properties, microstructure, and antimicrobial activities of zein films cross-linked using succinic anhydride, eugenol, and citric acid. *Prep Biochem Biotechnol* 45:551–567. <http://dx.doi.org/10.1080/10826068.2014.940967>.
- Wu Y, Zheng J, Wang Y, Li S, Jin H, Li Z, Bi D, Sun M, Liu M. 2015. Draft genome sequence of *Listeria monocytogenes* LM201, isolated from foodstuff. *Genome Announc* 3(1):e01417–14.
- van Heel AJ, de Jong A, Montalbán-Lopez M, Kok J, Kuipers OP. 2013. BAGEL3: automated identification of genes encoding bacteriocins and (non-) bactericidal posttranslationally modified peptides. *Nucleic Acids Res* 41:W448–W453. <http://dx.doi.org/10.1093/nar/gkt391>.
- Arantes O, Lereclus D. 1991. Construction of cloning vectors for *Bacillus thuringiensis*. *Gene* 108:115–119. [http://dx.doi.org/10.1016/0378-1119\(91\)90495-W](http://dx.doi.org/10.1016/0378-1119(91)90495-W).
- Martin NI, Sprules T, Carpenter MR, Cotter PD, Hill C, Ross RP, Vederas JC. 2004. Structural characterization of lacticin 3147, a two-peptide lantibiotic with synergistic activity. *Biochemistry* 43:3049–3056. <http://dx.doi.org/10.1021/bi0362065>.
- Severina E, Severin A, Tomasz A. 1998. Antibacterial efficacy of nisin against multidrug-resistant Gram-positive pathogens. *J Antimicrob Chemother* 41:341–347. <http://dx.doi.org/10.1093/jac/41.3.341>.
- Galvin M, Hill C, Ross RP. 1999. Lacticin 3147 displays activity in buffer against gram-positive bacterial pathogens which appear insensitive in standard plate assays. *Lett Appl Microbiol* 28:355–358. <http://dx.doi.org/10.1046/j.1365-2672.1999.00550.x>.
- Kruszewska D, Sahl HG, Bierbaum G, Pag U, Hynes SO, Ljungh A. 2004. Mersacidin eradicates methicillin-resistant *Staphylococcus aureus* (MRSA) in a mouse rhinitis model. *J Antimicrob Chemother* 54:648–653. <http://dx.doi.org/10.1093/jac/dkh387>.
- Dischinger J, Basi Chipalu S, Bierbaum G. 2014. Lantibiotics: promising candidates for future applications in health care. *Int J Med Microbiol* 304:51–62. <http://dx.doi.org/10.1016/j.ijmm.2013.09.003>.
- Sandiford SK. 2014. Advances in the arsenal of tools available enabling the discovery of novel lantibiotics with therapeutic potential. *Expert Opin Drug Discov* 9:283–297. <http://dx.doi.org/10.1517/17460441.2014.877882>.
- Field D, Connor PM, Cotter PD, Hill C, Ross RP. 2008. The generation of nisin variants with enhanced activity against specific gram-positive pathogens. *Mol Microbiol* 69:218–230. <http://dx.doi.org/10.1111/j.1365-2958.2008.06279.x>.
- Rea MC, Sit CS, Clayton E, O'Connor PM, Whittall RM, Zheng J, Vederas JC, Ross RP, Hill C. 2010. Thuricin CD, a posttranslationally modified bacteriocin with a narrow spectrum of activity against *Clostridium difficile*. *Proc Natl Acad Sci U S A* 107:9352–9357. <http://dx.doi.org/10.1073/pnas.0913554107>.
- Murphy K, O'Sullivan O, Rea MC, Cotter PD, Ross RP, Hill C. 2011. Genome mining for radical SAM protein determinants reveals multiple sactibiotic-like gene clusters. *PLoS One* 6:e20852. <http://dx.doi.org/10.1371/journal.pone.0020852>.
- Ibrahim MA, Griko N, Junker M, Bulla LA. 2010. *Bacillus thuringiensis*: a genomics and proteomics perspective. *Bioeng Bugs* 1:31–50. <http://dx.doi.org/10.4161/bbug.1.1.10519>.
- Bravo A, Likitvivatanavong S, Gill SS, Soberon M. 2011. *Bacillus thuringiensis*: a story of a successful bioinsecticide. *Insect Biochem Mol Biol* 41:423–431. <http://dx.doi.org/10.1016/j.ibmb.2011.02.006>.
- Ankolekar C, Labbe RG. 2010. Physical characteristics of spores of food-associated isolates of the *Bacillus cereus* group. *Appl Environ Microbiol* 76:982–984. <http://dx.doi.org/10.1128/AEM.02116-09>.
- Ramarao N, Sanchis V. 2013. The pore-forming haemolysins of *Bacillus cereus*: a review. *Toxins* 5:1119–1139. <http://dx.doi.org/10.3390/toxins5061119>.
- Cossart P, Toledo-Arana A. 2008. *Listeria monocytogenes*, a unique model in infection biology: an overview. *Microbes Infect* 10:1041–1050. <http://dx.doi.org/10.1016/j.micinf.2008.07.043>.
- Sambrook J, Russell DW. 2006. The condensed protocols from molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Silo-Suh LA, Lethbridge BJ, Raffel SJ, He H, Clardy J, Handelsman J. 1994. Biological activities of two fungistatic antibiotics produced by *Bacillus cereus* UW85. *Appl Environ Microbiol* 60:2023–2030.
- Cao M, Helmann JD. 2002. Regulation of the *Bacillus subtilis* bcrC bacterin resistance gene by two extracytoplasmic function sigma factors. *J Bacteriol* 184:6123–6129. <http://dx.doi.org/10.1128/JB.184.22.6123-6129.2002>.
- Deng Y, Li CZ, Zhu YG, Wang PX, Qi QD, Fu JJ, Peng DH, Ruan LF, Sun M. 2014. ApnI, a transmembrane protein responsible for subtilomycin immunity, unveils a novel model for lantibiotic immunity. *Appl Environ Microbiol* 80:6303–6315. <http://dx.doi.org/10.1128/AEM.02280-14>.
- Peng D, Luo Y, Guo S, Zeng H, Ju S, Yu Z, Sun M. 2009. Elaboration of an electroporation protocol for large plasmids and wild-type strains of *Bacillus thuringiensis*. *J Appl Microbiol* 106:1849–1858. <http://dx.doi.org/10.1111/j.1365-2672.2009.04151.x>.
- Wang P, Zhang C, Zhu Y, Deng Y, Guo S, Peng D, Ruan L, Sun M. 2013. The resolution and regeneration of a cointegrate plasmid reveals a model for plasmid evolution mediated by conjugation and *oriT* site-specific recombination. *Environ Microbiol* 15:3305–3318. <http://dx.doi.org/10.1111/1462-2920.12177>.