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The *Bacillus cereus* Group Is an Excellent Reservoir of Novel Lanthipeptides

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Lantibiotics are ribosomally synthesized peptides that contain multiple posttranslational modifications. Research on lantibiotics has increased recently, mainly due to their broad-spectrum antimicrobial activity, especially against some clinical Gram-positive pathogens. Many reports about various bacteriocins in the *Bacillus cereus* group have been published, but few were about lantibiotics. In this study, we identified 101 putative lanthipeptide gene clusters from 77 out of 223 strains of this group, and these gene clusters were further classified into 20 types according to their gene organization and the homologies of their functional genes. Among them, 18 types were novel and have not yet been experimentally verified. Two novel lantibiotics (thuricin 4A-4 and its derivative, thuricin 4A-4D) were identified in the type I-1 lanthipeptide gene cluster and showed activity against all tested Gram-positive bacteria. The mode of action of thuricin 4A-4 was studied, and we found that it acted as a bactericidal compound. The transcriptional analysis of four structural genes (*thiA1*, *thiA2*, *thiA3*, and *thiA4*) in the thuricin 4A gene cluster showed that only one structural gene, *thiA4*, showed efficient transcription in the exponential growth phase; the other three structural genes did not. In addition, the putative transmembrane protein ThiI was responsible for thuricin 4A-4 immunity. Genome analysis and functional verification illustrated that *B. cereus* group strains were a prolific source of novel lantibiotics.

Bacteriocins are ribosomally synthesized peptides produced by bacteria and exhibit antimicrobial activity against other bacteria (either in the same species or across genera) (1); they include posttranslationally modified bacteriocins and unmodified bacteriocins (2). Lanthipeptides (lantibiotics) are peptides that undergo multiple posttranslational modifications, and these are the best-characterized modified bacteriocins (3). Modifications include the formation of *meso*-lanthionine and 3-methyllanthionine as well as dehydrated amino acids (Dha and Dhb). Lanthipeptides are classified into four different classes on the basis of their biosynthetic enzymes (4). In class I lanthipeptides, the dehydration is carried out by a dehydratase (LanB) and cyclization is catalyzed by a cyclase (LanC). Class II lanthipeptides are modified by LanM proteins, which perform both dehydration and cyclization. In class III and IV lanthipeptides, the dehydration and cyclization reactions are catalyzed by multifunctional enzymes (RamC/LabKC or LanL).

The *Bacillus cereus* group currently contains eight species: *B. anthracis*, *B. cereus*, *B. thuringiensis*, *B. cytotoxicus*, *B. weihenstephanensis*, *B. mycoides*, *B. pseudomycoides*, and *B. toyonensis* (5, 6). To date, bacteriocins of the *B. cereus* group have been identified or characterized mainly in strains of *B. cereus* and *B. thuringiensis*. More than 20 bacteriocins have been reported in these two species (7, 8). They are active against closely related bacilli, such as the foodborne pathogen *B. cereus*, and are also potentially useful for the control of some pathogenic bacteria. For example, thuricin CD, a two-peptide bacteriocin consisting of Trn-α and Trn-β, shows antibacterial activity against *Clostridium difficile* and *Listeria monocytogenes*, and it is suggested as a potential treatment against *C. difficile*-associated disease (CDAD) (9).

Many lantibiotics exhibit broad-spectrum antimicrobial activity against clinical Gram-positive pathogens, including multidrug-resistant strains, and have clinical applications in the treatment of multidrug-resistant pathogens (10–14). So far, most reports of bacteriocins from the *B. cereus* group are about unmodified bacteriocins, while few are on class I bacteriocins or lantibiotics (8). Recent studies suggested that the *B. cereus* group may contain some unidentified lanthipeptide gene clusters (15, 16). Therefore, the purpose of this study was to systematically investigate the distribution and diversity of lanthipeptide gene clusters in this group. Putative biosynthetic gene clusters were predicted by BAGEL3 (17) from 223 genomes of *B. cereus* group strains. A novel class I lanthipeptide-encoding gene cluster, thuricin 4A, which has multiple structural genes and exists in 10 *B. cereus* group strains, was selected for validation of the antibacterial activity of *in silico*-predicted lanthipeptides.

MATERIALS AND METHODS

Gene cluster identification and classification. The genome sequences of 223 *B. cereus* group strains (see Table S1 in the supplemental material) were retrieved from GenBank (ftp://ftp.ncbi.nlm.nih.gov/genomes). Prediction of lanthipeptide biosynthetic gene clusters was carried out with BAGEL3 (17). The predicted results for each strain were used for further analysis. First, all genes involved in lanthipeptide biosynthesis were searched against the NCBI database to predict their function. Second, several gene clusters which had the same gene organization and identity of the same functional genes of greater than 85% were classified as a type.

DNA manipulation, RNA extraction, cDNA synthesis, and RT-qPCR. The bacterial strains, plasmids, and primers used in this study are listed in Table S3 in the supplemental material. Molecular biology cloning techniques and DNA detection assays were performed as described by

Received 14 November 2014 Accepted 19 December 2014

Accepted manuscript posted online 29 December 2014

Citation 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. doi:10.1128/AEM.03758-14.

Editor: R. M. Kelly

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.03758-14.

doi:10.1128/AEM.03758-14

Sambrook and Russell (18). Total DNA of *B. thuringiensis* was obtained as described by Arantes and Lereclus (19).

B. thuringiensis T01001 was obtained from the Collection of *Bacillus thuringiensis* and *Bacillus sphaericus*, Institut Pasteur, Paris. It was inoculated from glycerol stocks into 5 ml Luria-Bertani (LB) broth and grown for 12 to 16 h at 28°C. Twenty microliters of activated culture was subcultured in 20 ml LB broth and grown for 6 h, 16 h, 26 h, and 36 h. Cells were collected by centrifugation (12,000 rpm, 10 min, 4°C). RNA was extracted from cells using the RNeasy minikit (Qiagen, Germany) according to the manufacturer's protocol. The first-strand cDNA was synthesized according to the manufacturer's instructions for the RNA PCR kit (AMV ver. 3.0; TaKaRa, Dalian, China).

Real-time quantitative PCR (RT-qPCR) was performed using the StepOne thermal cycler (Applied Biosystems, Foster City, CA). The $2^{-\Delta\Delta CT}$ method (20) was used to analyze the real-time quantitative PCR data. The primers for the endogenous control 16S rRNA gene and four specific structural genes (*thiA1*, *thiA2*, *thiA3*, and *thiA4*) are listed in Table S3 in the supplemental material. The amplification efficiencies of each genes were obtained by the equation $E = 10^{(-1/\text{slope})}$. The standard reactions were carried out in volumes of 20 μl containing 10 μl SYBR green PCR master mix (Applied Biosystems, Madrid, Spain), 1.5 μl forward primer (5 μM), 1.5 μl reverse primer (5 μM), 2 μl DNA template (10 ng/μl), and 5 μl double-distilled water (ddH_2O). Cycling conditions were 95°C for 10 min and 40 cycles of 95°C for 15 s, 50°C for 30 s, and 72°C for 15 s. Fluorescence data were collected at the end of each cycle. Each sample was run in triplicate with two biological replicates.

Cloning and overexpression of the thuricin 4A biosynthetic gene cluster. The DNA fragment containing the thuricin 4A gene cluster was amplified from *B. thuringiensis* T01001 with the primers F1 and R1 (see Fig. 3A and Table S3 in the supplemental material). The PCR was run in a 580BR3396 thermal cycler (Bio-Rad) with the following thermal cycle: denaturation at 94°C for 10 min, followed by 30 cycles of 94°C for 30 s and annealing and extension at 60°C for 9 min, with a final extension at 72°C for 10 min. The DNA fragment and plasmid pEMB0630 were both digested with NotI and ligated with T4 DNA ligase. The recombinant plasmid pBMB1661 was electroporated into *B. thuringiensis* BMB171 as described by Peng et al. (21). The transformant *B. thuringiensis* BMB1661, which had antimicrobial activity against *B. thuringiensis* BMB171, was screened by PCR amplification with the primer pair F1/R1. The transformant was then sequenced with Megabace 1000 automated sequencers.

Cloning and overexpression of *thiI*. The DNA fragment which contained the *thiA4* gene and its promoter sequence was amplified using splicing by overlap extension PCR (SOE-PCR) with the primers A-F, A-R, I-F, and I-R (see Fig. 6A and Table S3 in the supplemental material) from *B. thuringiensis* T01001. After digestion with EcoRI and HindIII, the DNA fragment was ligated into pHT304 (19) and then transformed into BMB171 by electroporation (the recombinant plasmid was named pHT304-I). pHT304 was also transformed into BMB171 as the negative control.

Bacteriocin activity assay and verification of *thiI* immune function. For the various indicator strains and their culture conditions, see Table 3. The bacteriocin activity assay was performed using a previously described method with some modifications (22). Twenty milliliters of agar was seeded with 10^6 CFU of indicator strain in a petri dish, and holes (diameter, 6 mm) were bored in the agar. Then, 50 μl of 2-fold dilutions of the bacteriocin preparation was added to the wells. In the bacteriocin tolerance assay, *B. thuringiensis* BMB171, BMB171(pHT304), and BMB171(pHT304-I) were used as indicator strains. The plates were kept at 4°C for 2 h and subsequently incubated at a temperature suitable for the growth of the test strains.

The MICs of the bacteriocin for the various indicator strains were determined with high-pressure liquid chromatography (HPLC)-purified bacteriocin solutions using the well diffusion assays described above.

Purification of antibacterial peptides. *B. thuringiensis* T01001 was inoculated from glycerol stocks into 5 ml Luria-Bertani (LB) broth and grown overnight. The activated culture was subcultured in 500 ml LB medium at 28°C with agitation at 220 rpm for 16 h. Cells were then removed by centrifugation (12,000 rpm, 10 min, 4°C), and the cell-free supernatant fluid was shaken with 100 g of Amberlite XAD-7HP (Sigma, St. Louis, MO, USA) for 12 h at 4°C. The resin was sequentially washed with 1 liter of distilled water, 750 ml of 30% (vol/vol) ethanol, and 500 ml of 80% (vol/vol) ethanol. The active substances were eluted with 250 ml of 80% (vol/vol) ethanol, pH 2.0, and then the eluate was collected and lyophilized into powder. The generated powder was dissolved in distilled water followed by centrifugation. The resulting supernatant is referred to as antimicrobial crude extract (CE) (23). *B. thuringiensis* BMB171 and BMB1661 (with addition of kanamycin [50 μg/ml]) were cultured for 12 h, and CEs were prepared by the procedure as described above.

HPLC analysis was carried out on the Waters 1525 Breeze system. The mobile phase consisted of acetonitrile and HPLC-grade water containing 0.1% trifluoroacetic acid (TFA). Fifty microliters of the CE of *B. thuringiensis* T01001 was loaded into an Agilent HC-C_{18} (2) column (particle size, 5 μm; 4.6 mm [inside diameter] by 250 mm) and separated by a linear gradient of 30% to 80% acetonitrile from 0 to 10 min at a flow rate of 1.0 ml/min. The eluate was monitored at a wavelength of 254 nm, and fractions were manually collected for the bioassay of antibacterial activity against *B. thuringiensis* BMB171. The active fractions were collected and lyophilized into powder. They were then dissolved in ddH_2O, and the concentration of bacteriocin preparation was quantified to 1 μg/μl. In addition, the CEs of *B. thuringiensis* BMB171 and *B. thuringiensis* BMB1661 were separated under the same HPLC conditions as described above.

LC-MS/MS analysis. Liquid chromatography-mass spectrometry (LC-MS) and LC-tandem MS (LC-MS/MS) were both performed with 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-C_{18} (2) column (particle size, 5 μm; 4.6 mm [inside diameter] by 250 mm). The MS operating conditions were as follows: capillary voltage, 3,500 V; nebulizer pressure, 35 lb/in^2 gauge; flow rate of drying gas, 9 liters/min; and temperature, 350°C. The scanning range of Q-TOF was *m/z* 100 to 3,000. Data were acquired at the rate of 1 spectrum/s. Detailed sequence information for these two peptide antimicrobial substances was further investigated with the targeted MS/MS mode. The target ion was isolated and fragmented by adding a voltage of 35 V.

Effects of temperature and pH on antimicrobial activities of the lantibiotics. To test the thermal stabilities of thuricin 4A-4 and thuricin 4A-4D, the bacteriocin preparations (~10 μM) were exposed to 28°C, 60°C, 80°C, 100°C, and 121°C for 30 min. The residual bacteriocin activities against *B. thuringiensis* BMB171 were determined as described above. To establish the sensitivities of these two peptides to pH, the bacteriocin preparations (~10 μM) were adjusted to pH 2.0 to 9.0, using 1 M NaOH or 1 M HCl solution, followed by incubation at 28°C for 2 h. The residual antimicrobial activity was assessed after neutralizing the sample to pH 6.5.

Thuricin 4A-4 mode of action. The effect of thuricin 4A-4 on the sensitive indicator strain *B. thuringiensis* BMB171 was assessed by incubating the exponential-phase *B. thuringiensis* BMB171 with the purified thurincin 4A-4. A 40-ml culture of the indicator strain (*B. thuringiensis* BMB171) grown in LB broth was incubated at 28°C. The culture was divided in four aliquots when the optical density at 600 nm (OD_{600}) reached 0.5. One was used as a control, while different amounts of thuricin 4A-4 were added to the other three. The flasks were then incubated at 28°C for 120 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.

RESULTS

The *B. cereus* group contains 20 types of lanthipeptide gene clusters. A total of 101 putative lanthipeptide biosynthetic gene clusters were identified in 78 of the 223 strains of the *B. cereus* group with available genome sequences (Table 1; see Table S1 in the supple-

TABLE 1 Distribution of the lanthipeptide gene cluster in the *B. cereus* group strains

Species (no. of strains)	No. of lanthipeptide gene clusters[a]		
	Class I	Class II	Total
B. anthracis (29)	0	0	0
B. cereus (147)	25	53	78
B. thuringiensis (37)	7	11	18
B. cytotoxicus (1)	0	0	0
B. mycoides (3)	1	2	3
B. pseudomycoides (1)	0	1	1
B. weihenstephanensis (4)	1	0	1
B. toyonensis (1)	0	0	0
Total (223)	34	67	101

[a] Class III or class IV lanthipeptide gene clusters were not identified in any of the genomes.

mental material). Most of the gene clusters were identified from strains of *B. cereus* and *B. thuringiensis*. On the basis of their gene organizations and the homologies of functional genes (*lanB* or *lanM* genes), these gene clusters were classified into 20 types, including 6 types of class I (Fig. 1) and 14 types of class II (Fig. 2). No putative lanthipeptides belonging to class III or IV were predicted, except for two putative genes encoding LanL-like lanthionine synthetases involved in modification of type IV lanthipeptides from some *B. cereus* strains (see Table S1 in the supplemental material). However, adjacent to these genes, putative structural genes or other genes involved in lanthipeptide secretion and immunity could not be found.

None of the 6 types of class I lanthipeptide biosynthetic gene clusters have yet been biochemically characterized. All the putative precursor peptides (LanA peptides) of these gene clusters had the conserved motif F-(N/D)-L-(N/D/E) and contained cysteine, threonine, and serine residues (see Table S2 in the supplemental material). The putative precursor peptides in the type I-3, I-5, and I-6 gene clusters had no similarity to any known class I lantibiotics precursor peptides (see Table S2). Two types of gene clusters, I-1 and I-4, contained two or more similar structural genes (see Table S2). In type I-2, different gene clusters in different strains contained different copies of the structural gene (*lanA*) (Fig. 1; see Table S2). This situation has rarely been reported in class I lantibiotic biosynthetic gene clusters.

In the 14 types of class II lanthipeptide gene clusters, each of the

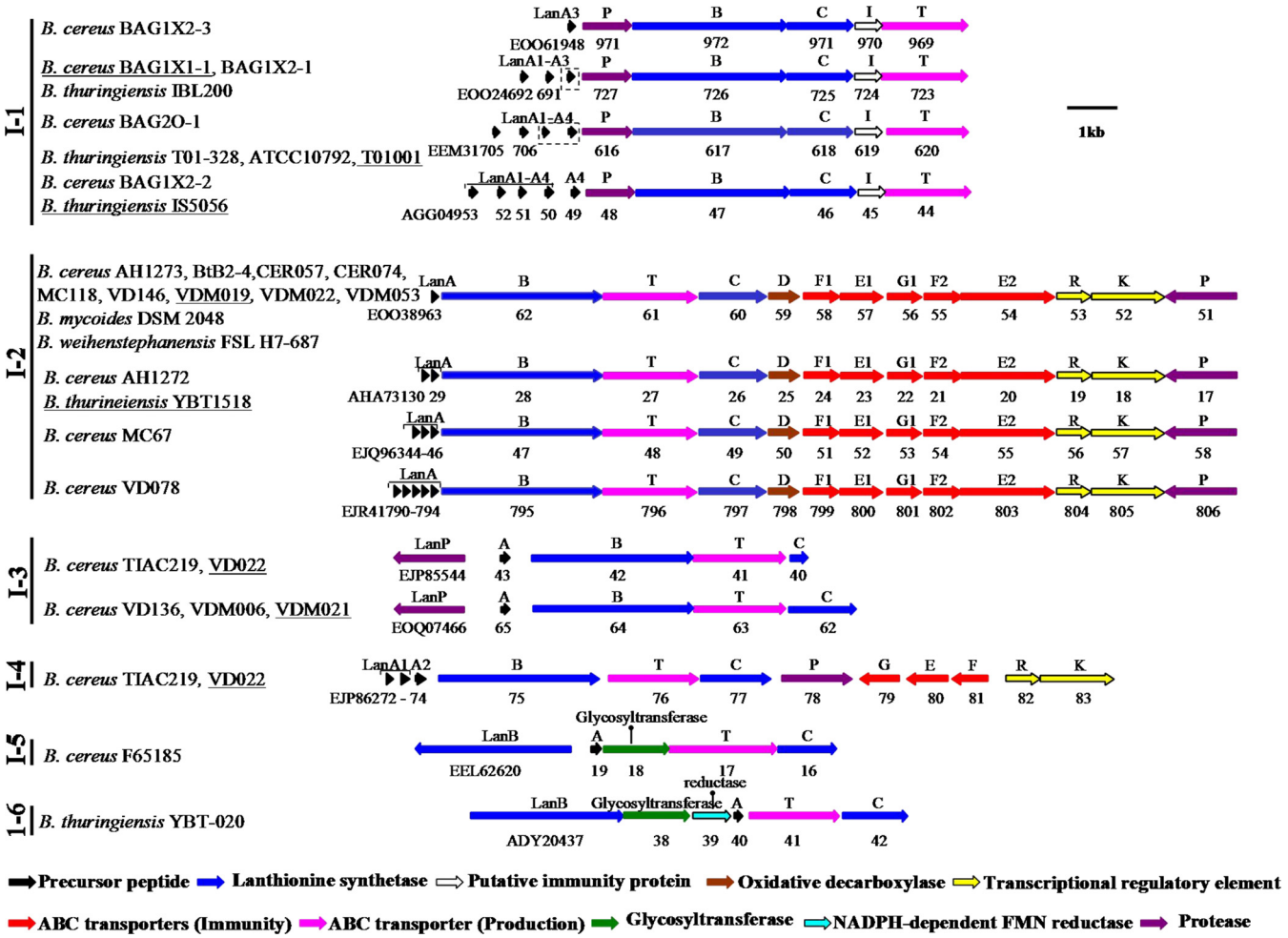

FIG 1 Line diagram of 6 predicted types of class I lanthipeptide gene clusters in the *B. cereus* group strains. The genes in the dashed boxes were not annotated. The numbers refer to NCBI annotation numbers of the underlined strains.

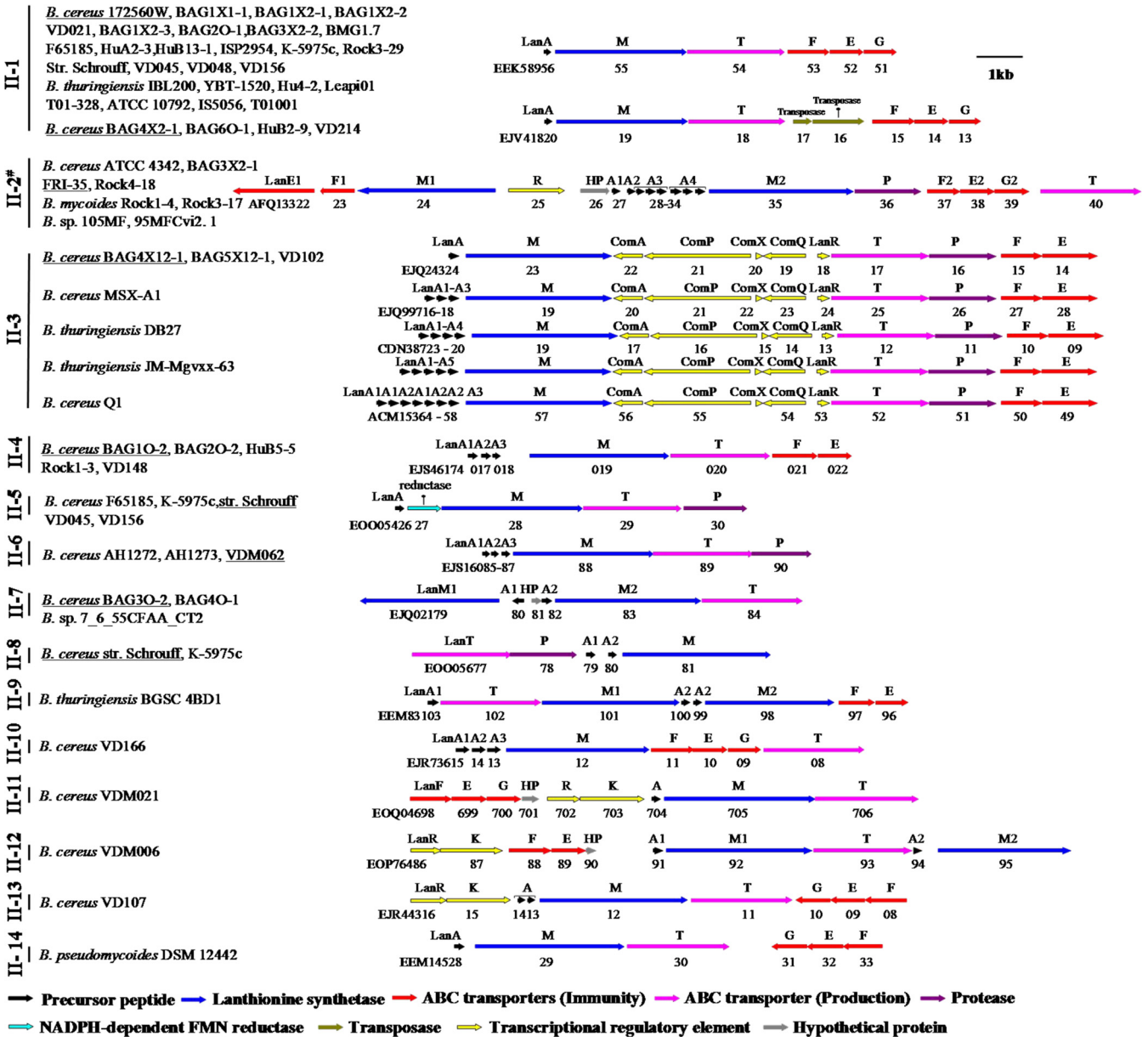

FIG 2 Line diagram of 14 predicted types of class II (B) lanthipeptide gene clusters in the *B. cereus* group strains. The numbers refer to NCBI annotation numbers of the underlined strains. #, the gene cluster in different strains might have different numbers of structural genes, which could not be determined by the available sequence.

putative structural peptides (LanA peptides) was characterized by an N-terminal leader sequence with a conserved G (G/A/S) cleavage site (see Table S2 in the supplemental material). Four of them, II-2, II-7, II-9, and II-12, might produce two-peptide lanthipeptides. The type II-1 and type II-3 gene clusters had significant similarity with known lantibiotic (cerecidin and thuricin) gene clusters (15, 16), but the other 10 types of gene clusters were not biochemically characterized (see Table S2). The putative precursor peptides of II-5 and II-12 showed no similarity to any other known lantibiotic precursor peptides (see Table S2).

Characterization of the thuricin 4A biosynthetic gene cluster. To confirm the synthetic function of these predicted gene clusters, we selected the first type of class I lanthipeptide gene cluster for verification. This type of gene cluster contains multiple structural genes and exists in 10 *B. cereus* group strains. In these 10 strains, the sequence diversity of the structural genes is low (<1%), and the major differences among different gene clusters are the numbers of the structural genes (Fig. 1).

In *B. thuringiensis* T01001, the sequence of the thuricin 4A gene cluster (GenBank accession number KP133062) was verified by PCR amplification and Sanger sequencing (Fig. 3A). It consisted of nine genes, four tandem structural genes (*thiA1*, *thiA2*, *thiA3*, and *thiA4*), two genes (*thiB* and *thiC*) coding for enzymes involved in the posttranslational modification of the precursor peptides, one gene (*thiP*) coding for a putative extracellular serine protease which may remove the leader peptide of the precursor

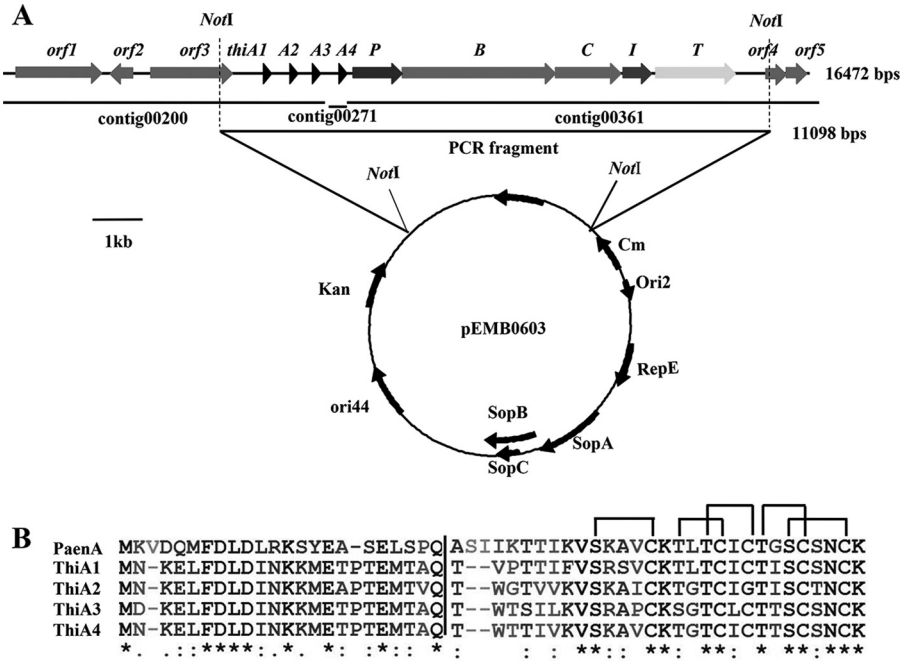

FIG 3 Characterization and cloning of the thuricin 4A biosynthetic gene cluster. (A) Construction of thuricin 4A gene cluster expression vector pBMB1661. (B) Amino acid sequence alignment of four precursor peptides with PaenA. Conserved residues are in boldface, and the cleavage site of the processing protease is represented by a vertical line. Thioether bridging of paenibacillin is shown.

peptides, and one gene (*thiT*) coding for an ABC transporter-related protein that may contribute to secretion of the lanthipeptides (Table 2). *thiI*, a gene encoding a putative membrane protein with five putative transmembrane domains, may be responsible for lantibiotic immunity. The protein sequences of the four precursor peptides (ThiA1, ThiA2, ThiA3, and ThiA4) had identities of 58%, 54%, 48%, and 58% to PaenA (the precursor peptide of paenibacillin), respectively (Table 2). As indicated in Fig. 3B, ThiA1, ThiA2, ThiA3, ThiA4, and PaenA had a high level of amino acid sequence similarity in the C termini but were more diverse in the N termini and the leader peptides.

Transcriptional analysis and cloning of the thuricin 4A biosynthetic gene cluster. To verify this gene cluster, we first tested the transcription of four structural genes by qPCR. Relative quantification was carried out by the $2^{-\Delta\Delta CT}$ method (20). The amplification efficiencies of the endogenous gene and four structural genes ranged from 92% to 108%, with an r^2 value of ≥0.992 (data not shown). It was found that all four structural genes were tran-

TABLE 2 Open reading frames analysis of the thuricin 4A gene cluster in *B. thuringiensis* serovar thuringiensis T01001 using BLASTp

Predicted open reading frame	No. of amino acids	Protein homology (accession no./no. of amino acids)[a]	Identity (%) in aligned region	Expectation value
thiA1	51	PaenA, paenibacillin precursor peptide, *P. polymyxa* OSY-DF (AFS60100; 53)	29/50 (58)	6.00E−17
thiA2	51	PaenA, paenibacillin precursor peptide, *P. polymyxa* OSY-DF (AFS60100; 53)	27/50 (54)	8.00E−15
thiA3	51	PaenA, paenibacillin precursor peptide, *P. polymyxa* OSY-DF (AFS60100; 53)	24/50 (48)	2.00E−13
thiA4	51	PaenA, paenibacillin precursor peptide, *P. polymyxa* OSY-DF (AFS60100; 53)	29/50 (58)	2.00E−16
thiP	324	SubP, subtilisin-like protease, *B. subtilis* MMA7 (AGL93172; 324)	118/314 (38)	3.00E−69
thiB	1,023	SubB, lanthionine synthetase B-like protein, *B. subtilis* MMA7 (AGL93173; 1,036)	379/1,038 (37)	0
thiC	436	PaenC, lantibiotic cyclase, *P. polymyxa* OSY-DF (AFS60103; 423)	135/436 (31)	1.00E−69
thiI	183	Transmembrane protein responsible for subtilomycin immunity, *B. subtilis* BSn5 (ADV95128; 196)	53/180 (29)	4.00E−12
thiT	536	Antibiotic ABC transporter ATP-binding protein, *S. haemolyticus*, (WP_011276513; 575)	245/538 (46)	2.00E−173

[a] Results are from a BLASTp search of the GenBank protein database in October 2014.

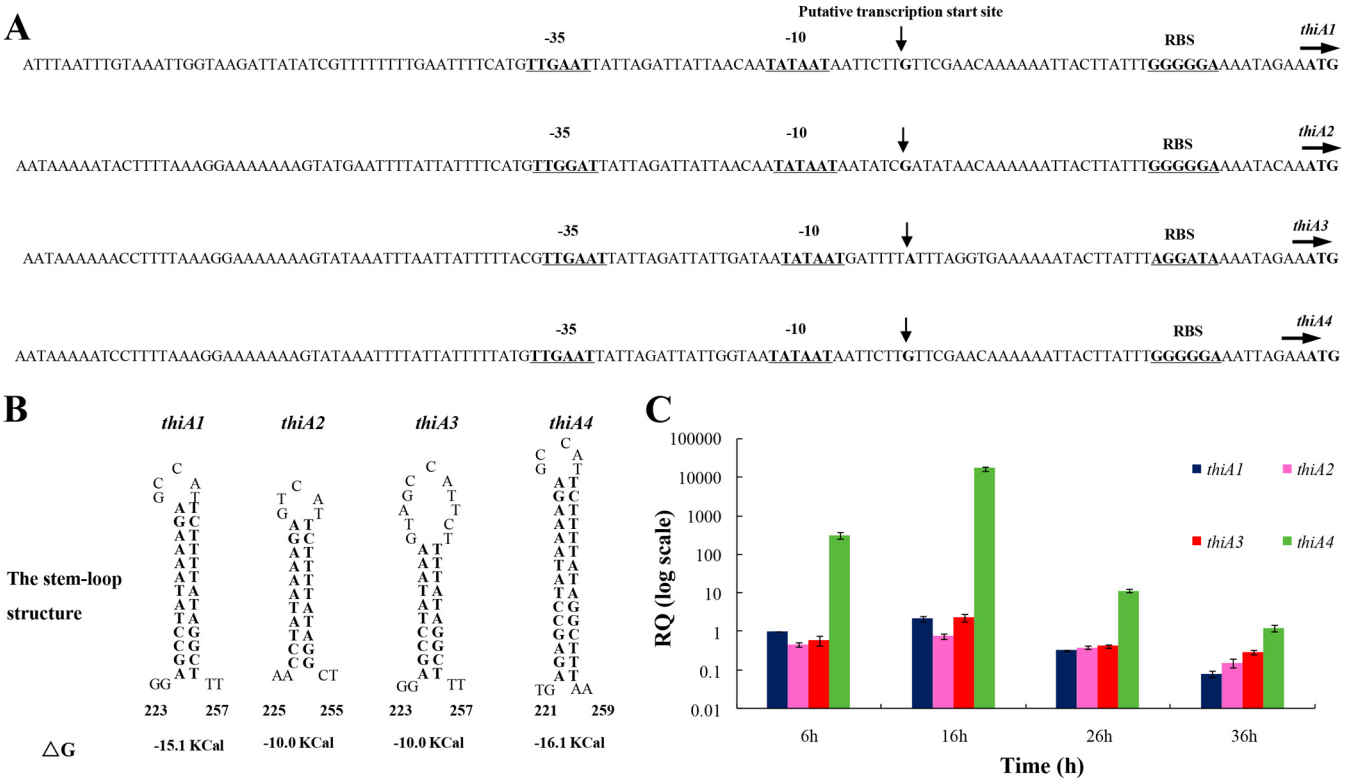

FIG 4 Analysis of transcription of four structural genes (*thiA1*, *thiA2*, *thiA3*, and *thiA4*). (A) The promoters of the four putative structural genes. Their −35 and −10 regions, putative transcription start sites, ribosomal binding sites (RBS), and the start codons for the *thiA1*, *thiA2*, *thiA3*, and *thiA4* genes are indicated. (B) Nucleotide sequence of the stem-loop structures located in the four structural genes. Nucleotides are numbered from the putative transcription start site of each structural gene. (C) RT-qPCR for the relative quantification (RQ) of *thiA1*, *thiA2*, *thiA3*, and *thiA4* mRNAs in *B. thuringiensis* serovar thuringiensis T01001 at different time points during bacterial growth. Error bars represent standard deviations.

scribed in the exponential growth phase. Although they had similar promoters and terminators (Fig. 4A and B), the cDNA quantity of *thiA4* was much higher than those of the other three structure genes (Fig. 4C).

Second, a DNA fragment (~11 kbp) containing the entire putative gene cluster was cloned into the vector pEMB0603 and transferred into a surrogate host, *B. thuringiensis* BMB171 (Fig. 3A). A transformant, designated *B. thuringiensis* BMB1661, exhibited antibacterial activity against the parent strain *B. thuringiensis* BMB171, demonstrating that the putative thuricin 4A biosynthetic gene cluster codes for the synthesis of an active antibacterial peptide(s).

***B. thuringiensis* strains T01001 and BMB1661 both produce two novel lantibiotics, thuricin 4A-4 and its derivative thuricin 4A-4D.** The kinetics of the antimicrobial substance production assay showed that T01001 produced an antimicrobial compound in the exponential phase (Fig. 5A). Antimicrobial peptides in the culture supernatants of T01001 and BMB1661 were concentrated on Amberlite XAD-7 HP resin and separated by reverse-phase HPLC (RP-HPLC). The CEs of T01001 and BMB1661 both showed high antimicrobial activity ($>1.6 \times 10^5$ arbitrary units [AU]/ml) compared to that of BMB171. Separation of active peptides from T01001 and BMB1661 by RP-HPLC generated two fractions with activity against BMB171 (Fig. 5B), while the corresponding preparation from BMB171 did not yield fractions with antimicrobial activity.

Subsequently, these two target fractions were analyzed by LC-MS. The measured molecular masses of fractions A-A′ (*m/z* 2,786.3) and B-B′ (*m/z* 2,886.3) were determined from their monoisotopic signals (Fig. 5C). The calculated molecular mass of the predicted mature peptide of ThiA4, termed thuricin 4A-4, was 2,965.4 Da. The observed molecular masses of fractions A and A′ were ~179 Da less than the calculated mass, indicating that 3 serine residues and 7 threonine residues in thuricin 4A-4 were dehydrated. Spontaneous hydrolysis of Dhb at the N terminus of thuricin 4A-4, analogous to LtnA2 (24), was predicted to yield 2-oxobutyryl residue (+1). It can thus be concluded that fractions A and A′ contain thuricin 4A-4. However, the *m/z* of fractions B and B′ (*m/z* 2,886.3) did not correspond to any of the predicted products of the structural genes of the gene cluster, ThiA1, ThiA2, ThiA3, or ThiA4.

Fractions A and B were further analyzed using LC-MS/MS. As illustrated in Fig. S1A in the supplemental material, all the marked fragment ions of fraction A corresponded to fragments of thuricin 4A-4, confirming the identity of fraction A as thuricin 4A-4. Furthermore, judging from the fracture site, the pattern of Lan or MeLan cross-linking of thuricin 4A-4 (Fig. 3B and 5D) was identical to that of paenibacillin (23, 25). Surprisingly, the parent ion (*m/z* 930.1051) and a series of fragment ions (gray) of thuricin 4A-4 also appeared in the MS/MS spectrum of fraction B (see Fig. S1 in the supplemental material). The molecular masses of fragment ions y22, y23, y24, y25, y26, b20, and b27 of fraction B were about 100 Da (monoisotopic) higher than those of the corresponding fragment ions of thuricin 4A-4. However, fragment ions corresponding to y14 and y15 with 100 Da added could not be identified in the MS/MS spectrum of fraction B. These results suggested that fraction B was derived from

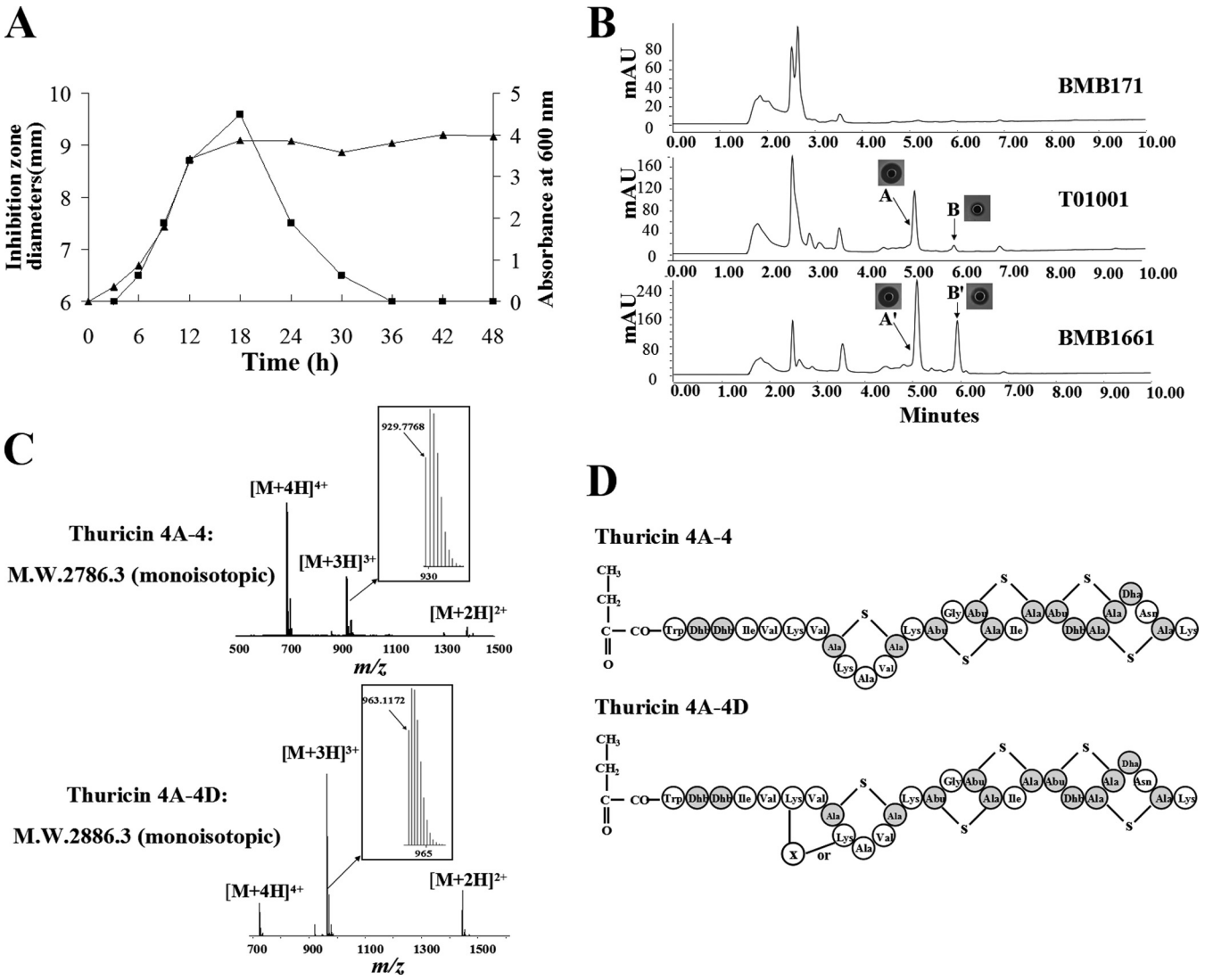

FIG 5 Purification and identification of two lantibiotics, thuricin 4A-4 and thuricin 4A-4D. (A) Kinetics of antimicrobial substances produced during the growth of *B. thuringiensis* T010001. The optical density of the T01001 culture was measured at 600 nm (▲). The antimicrobial substance concentration was expressed as inhibition zone diameter (■). (B) Reverse-phase HPLC profiles of crude extracts of *B. thuringiensis* BMB171, T01001, and BMB1661. (C) MS profiles of the HPLC-purified antimicrobial peptides thuricin 4A-4 and thuricin 4A-4D. (D) Schematic representation of thuricin 4A-4 and thuricin 4A-4D structures. For thuricin 4A-4D, the added group (approximately 100 Da) is represented as X.

thuricin 4A-4 by addition of an unknown compound to the side chain of an amino acid between Val6 and Cys13 of thuricin 4A-4. In that range of amino acids, the most reactive side chains of amino acids were Lys7 and Lys10. Therefore, we suggest that the side chain of Lys7 or Lys10 reacted to an 118-Da substance to produce the derivative of thuricin 4A-4, thuricin 4A-4D (Fig. 5D; see Fig. S1 in the supplemental material).

Antimicrobial activities and stabilities of thuricin 4A-4 and thuricin 4A-4D. The HPLC-purified thuricin 4A-4 and thuricin 4A-4D showed broad-spectrum antimicrobial activity and were active against all of the tested Gram-positive bacteria, including *B. cereus*, *B. firmus*, *B. thuringiensis*, *B. subtilis*, *B. pumilus*, *Enterococcus faecalis*, *Microbacterium*, *Paenibacillus*, *Staphylococcus aureus*, and *Staphylococcus sciuri* (Table 3). Both compounds were inactive against all of the Gram-negative bacterial strains tested. Comparing the MIC values of these two antimicrobial peptides, the antibacterial activity of thuricin 4A-4 was higher than that of thuricin 4A-4D for each indicator bacterial strain (Table 3).

The residual antibacterial activities of thuricin 4A-4 and thuricin 4A-4D remained unchanged when the temperature was below 80°C (see Table S4 in the supplemental material). After exposure to temperatures exceeding 90°C, the antibacterial activities of thuricin 4A-4 and thuricin 4A-4D decreased, and they were eliminated at 121°C. Thuricin 4A-4 and thuricin 4A-4D retained most of their antimicrobial activities when exposed to different pH values from 2 to 9 (see Table S4 in the supplemental material).

The putative membrane protein ThiI confers thuricin 4A-4 immunity. The *thiI* gene codes for a polypeptide of 183 residues that has low similarity (29%) with known lantibiotic immunity protein ApnI (26) (Table 2). To test whether it contributes thuricin 4A-4 immunity, we heterologously expressed *thiI* with the *thiA4* promoter in *B. thuringiensis* BMB171 (Fig. 6A). The recombinant strain, *B.*

TABLE 3 Antimicrobial spectra of thuricin 4A-4 and thuricin 4A-4D

Indicator strain (reference)[a]	Medium[b]-incubation temp (°C)	MIC (μM)[c]	
		Thuricin 4A-4	Thuricin 4A-4D
Gram-negative bacteria			
Sphingobacterium strain Pri1	NB-28	NA	NA
Pseudomonas putida Pri3	NB-28	NA	NA
Pseudomonas psychrophila Pri5	NB-28	NA	NA
Escherichia coli BL21 (18)	LB-37	NA	NA
Escherichia coli DH5α (18)	LB-37	NA	NA
Erwinia herbicola LS005 (35)	LB-28	NA	NA
Enterobacter strain Bom2	NB-37	NA	NA
Gram-positive bacteria			
Bacillus cereus UW85 (35)	LB-28	7.8	21.6
Bacillus firmus BCRC11944	LB-28	0.326	1.35
Bacillus subtilis 168 (36)	LB-28	5.2	10.8
Bacillus subtilis Bsn5 (37)	LB-28	5.2	10.8
Bacillus thuringiensis BMB171 (38)	LB-28	1.3	5.2
Bacillus thuringiensis YBT1518 (39)	LB-28	1.3	2.6
Bacillus pumilus SCG I	LB-28	0.65	86.6
Paenibacillus X3	LB-28	2.6	10.8
Staphylococcus aureus X4	NB-37	1.3	2.6
Staphylococcus sciuri Bom1	NB-37	1.95	43.2
Microbacterium strain Pri2	NB-37	5.2	5.2
Enterococcus faecalis Bom3	NB-37	2.6	5.2

[a] Strain BCRC 11944 was obtained from Bioresource Collection and Research Center (BCRC); other strains for which references are not provided were isolated by our group.
[b] NB, nutrient broth; LB, Luria broth.
[c] The highest concentrations of thuricin 4A-4 and thuricin 4A-4D were 332.8 μM and 345.6 μM, respectively. NA, no activity against the indicator strain, even with the highest concentration of the indicated peptide.

thuringiensis BMB171 (pHT304-I), was four times more resistant to thuricin 4A-4 than the control strain *B. thuringiensis* BMB171 (pHT304) (Fig. 6B and C). This means that ThiI is a novel immunity protein conferring immunity to producer strains.

Thuricin 4A-4 mode of action. To study the putative mode of action of thuricin 4A-4, an exponentially grown culture of the indicator strain *B. thuringiensis* BMB171 was treated with different concentrations of thuricin 4A-4 and both the OD_{600} of the culture and the number of viable cells were monitored at different times. The addition of thuricin 4A-4 caused OD_{600} values of the culture to rapidly change (Fig. 7A). A series of thuricin 4A-4 concentrations (1× MIC, 2× MIC, and 4× MIC) were tested, and various degrees of decrease in the OD_{600} were observed. The number of viable cells also showed various degrees of decrease after the treatment with different concentrations of thuricin 4A-4 (Fig. 7B). These results indicated that thuricin 4A-4 acts as a bactericidal compound.

DISCUSSION

Lantibiotics inhibit many clinical pathogens, including some multidrug-resistant ones (10–14). Therefore, the identification of novel lantibiotics may provide new tools for treatment of multidrug-resistant pathogens. In this study, we analyzed the genome sequences of the *B. cereus* group strains and identified a large number of putative lanthipeptide gene clusters, most of which have not yet been experimentally verified. The functional verification of one gene cluster also supported the synthetic function of these predicted gene clusters. The comprehensive bioinformatic assessment of putative lanthipeptide gene clusters in a single species supports conclusions about the distribution and diversity of lanthipeptides in this species, and in addition, we identified a prolific source of novel lantibiotics, the *B. cereus* group.

In our genome analysis, we provided some excellent candidate strains for detailed validation experiments and quick discovery of many more new lantibiotics. Although there are numerous lanthipeptides awaiting discovery and biochemical characterization, some of the more promising antimicrobial substances should be given more attention in future research. One of the most important of these is the precursor peptide of lanthipeptide, which has no similarity to any known lantibiotics and/or the lanthipeptide gene cluster that contains some rare modifying enzymes, such as in the I-5 and I-6 gene clusters of the *B. cereus* group. This might result in some new antimicrobial activity (9). Next is the precursor peptide of lanthipeptide, which has similarity to well-applied lantibiotics. Some studies have confirmed that products of the site-directed mutagenesis of bacteriocin peptides or their analogs might have new features, such as different three-dimensional structures, inhibitory spectra, and thermal stabilities (27, 28). For example, geobacillin I is a nisin analog but showed increased stability compared to that of nisin A (29).

Of the 20 types of lanthipeptides, 12 types were identified in different strains. The BLASTP analysis of the lanthipeptide synthetases in the *B. cereus* group showed that any two proteins with similar functions (LanB or LanM proteins) within the same type have a high level of amino acid sequence identity, generally more than 90%, while the identity of genes from other types or other species is low, usually below 40% (see Table S5 in the supplemental material). This suggested that these 20 types of lanthipeptide gene clusters independently evolved or at least had no recent horizontal gene transfer, and most of them are likely transmitted by intraspecies horizontal gene transfer.

The function of the predicted novel lantibiotic biosynthetic gene cluster thuricin 4A in *B. thuringiensis* serovar thuringiensis T01001 was verified by purification of thuricin 4A and heterologous expression of the cluster. It is worth mentioning that the broad inhibitory spectrum of thuricin 4A-4 may allow development of applications. In reported lantibiotic gene clusters, the number of structural genes rarely exceeds two (4, 16). Interestingly, the number of structural gene in the thuricin 4A gene cluster was four. Moreover, the levels of expression of the four structural genes differed substantially although the genes have similar promoters and terminators. Purification of antimicrobial peptides confirmed that thuricin4 A-4 is the only product and additionally identified a derivative, thuricin 4A-4D, which may result from succinylation at a lysine residue. The lantibiotic subtilin (*m/z* 3,319.6) also occurred as succinylated subtilin (*m/z* 3,419.6) (30). N-terminally succinylated subtilin retains less than 10% antibacterial activity, and succinylation thus may present a novel mechanism of self-protection for the producer strain (31). MS/MS analysis showed that the molecular weight difference between thuricin 4A-4 and thuricin 4A-4D and the reduced antibacterial activity of thuricin 4A-4D are consistent with succinylation at Lys7 or Lys10 of thuricin 4A-4. However, the confirmation of succinylation requires further experimentation.

A previous report proposed that the majority of bacteria and archaea produce at least one bacteriocin (32). The prevailing view is that

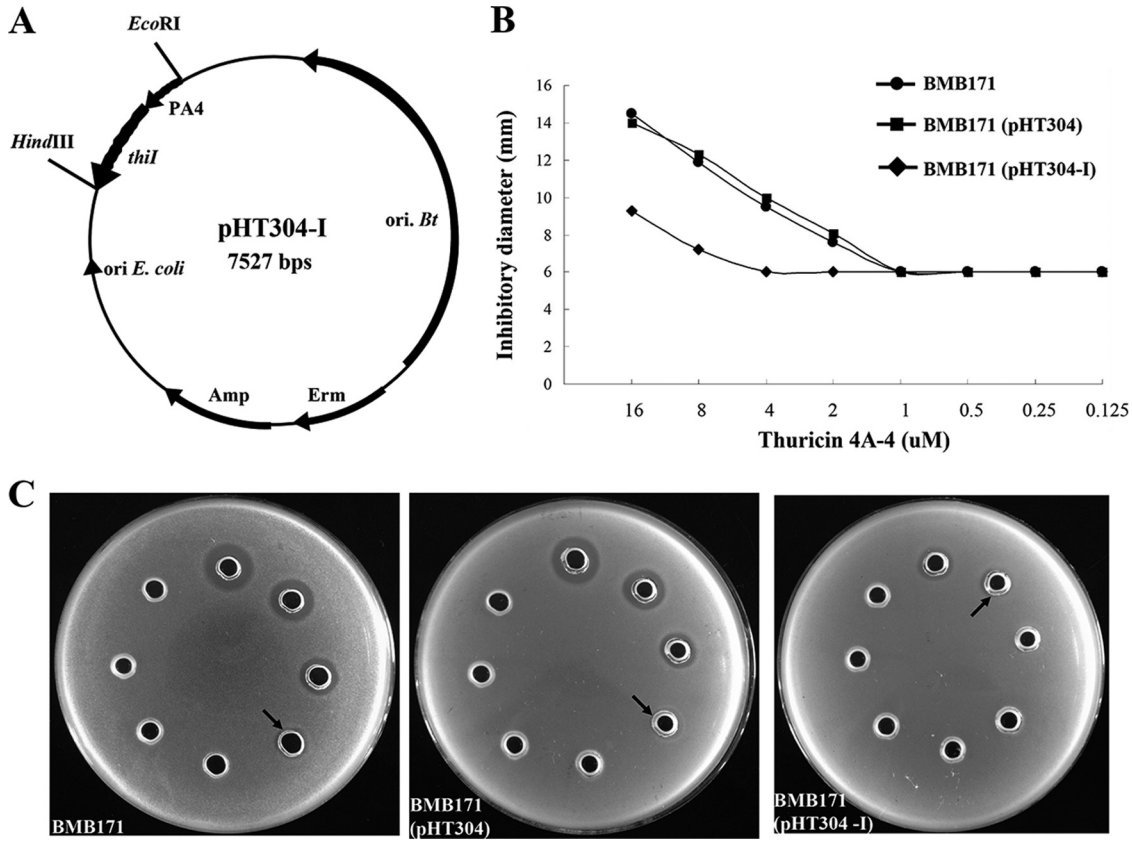

FIG 6 Validation of *thiI* gene immune function. (A) Construction of expression vector pHT304-I. (B) Thuricin 4A-4 immunity of BMB171 and its mutants. (C) MICs of thuricin 4A-4 to BMB171 and its mutants. The arrows indicate the MIC of thuricin 4A-4 to the indicator bacteria.

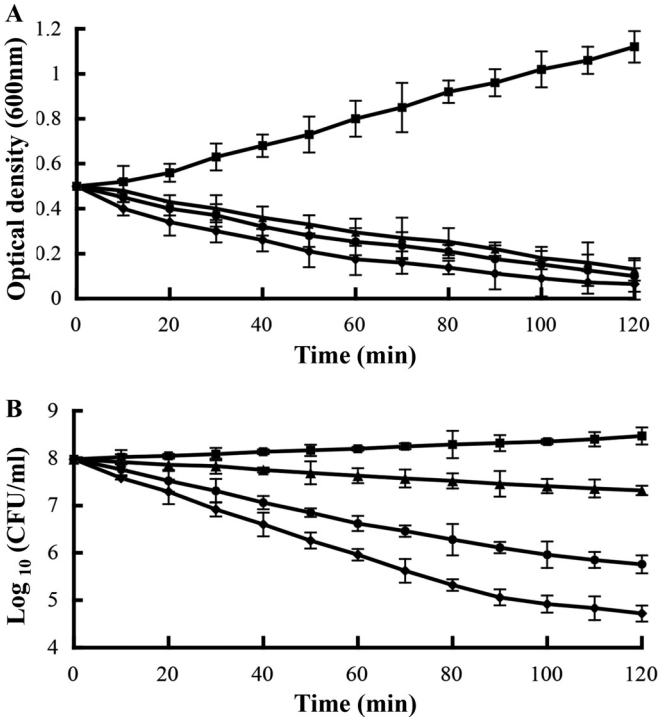

FIG 7 Effect of thuricin 4A-4 on the optical density (A) and the number of viable cells (B) of a *B. thuringiensis* BMB171 culture. Thuricin 4A-4 concentrations: 0 (■), 1× MIC (▲), 2× MIC (●), and 4× MIC (◆). Means and standard deviations are presented.

the production of antimicrobial compounds might facilitate the competition of a producer and be a competitive advantage in certain environment (33, 34). The bacteria of the *B. cereus* group can survive in various ecological environments, and this ability is generally considered to be associated with their robust endospores, while the production of antimicrobial substances confers a double advantage for their survival and growth in different habitats (7). Two reported lantibiotics (cerecidins and thuricin) and thuricin 4A-4 had a broad spectrum of antibacterial activity (15, 16). This suggests that the lantibiotics may play an important role in survivability and adaptability to the environment.

In total, our research revealed that the *B. cereus* group has a variety of novel lanthipeptides, and it will greatly enrich the understanding of the distribution and diversity of the lanthipeptide gene cluster in the *B. cereus* group.

ACKNOWLEDGMENTS

We thank Michael Gänzle, University of Alberta, for his discussion on the writing and critical reading of the manuscript. We thank Edward C. Mignot, Shandong University, for linguistic advice. We are grateful to all institutions and individuals who provided strains and vectors.

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 (G25), the National Basic Research Program (973) of China (2009CB118902), the National Natural Science Foundation of China (31170047 and 31171901), International Scientific Cooperation of Hubei Province (2011BFA019), and the Fundamental Research Fund for Central University (2011PY056).

REFERENCES

1. **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.
2. **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.
3. **Willey JM, van der Donk WA.** 2007. Lantibiotics: peptides of diverse structure and function. Annu Rev Microbiol **61:**477–501. http://dx.doi.org/10.1146/annurev.micro.61.080706.093501.
4. **Knerr PJ, van der Donk WA.** 2012. Discovery, biosynthesis, and engineering of lantipeptides. Annu Rev Biochem **81:**479–505. http://dx.doi.org/10.1146/annurev-biochem-060110-113521.
5. **Guinebretiere 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.
6. **Guinebretiere MH, Velge P, Couvert O, Carlin F, Debuyser ML, Nguyen-The C.** 2010. Ability of *Bacillus cereus* group strains to cause food poisoning varies according to phylogenetic affiliation (groups I to VII) rather than species affiliation. J Clin Microbiol **48:**3388–3391. http://dx.doi.org/10.1128/JCM.00921-10.
7. **Abriouel H, Franz CMAP, Omar NB, Gálvez A.** 2011. Diversity and applications of *Bacillus* bacteriocins. FEMS Microbiol Rev **35:**201–232. http://dx.doi.org/10.1111/j.1574-6976.2010.00244.x.
8. **de la Fuente-Salcido NM, Casados-Vázquez LE, Barboza-Corona JE.** 2013. Bacteriocins of *Bacillus thuringiensis* can expand the potential of this bacterium to other areas rather than limit its use only as microbial insecticide. Can J Microbiol **59:**515–522. http://dx.doi.org/10.1139/cjm-2013-0284.
9. **Rea MC, Sit CS, Clayton E, O'Connor PM, Whittal 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.
10. **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.
11. **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.
12. **Kruszewska D.** 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.
13. **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.
14. **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.
15. **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.
16. **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.
17. **Van Heel AJ, de Jong A, Montalbán-López M, Kok J, Kuipers OP.** 2013. BAGEL3: automated identification of genes encoding bacteriocins and (non-)bactericidal posttranslationally modified peptides. Nucleic Acids Res **15:**448–453.
18. **Sambrook J, Russell DW.** 2006. The condensed protocols from molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
19. **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.
20. **Livak KJ, Schmittgen TD.** 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. Methods **25:**402–408. http://dx.doi.org/10.1006/meth.2001.1262.
21. **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.
22. **Cintas LM, Rodriguez JM, Fernandez MF, Sletten K, Nes IF, Hernandez PE, Holo H.** 1995. Isolation and characterization of pediocin L50, a new bacteriocin from *Pediococcus acidilactici* with a broad inhibitory spectrum. Appl Environ Microbiol **61:**2643–2648.
23. **He Z, Kisla D, Zhang L, Yuan C, Green-Church KB, Yousef AE.** 2007. Isolation and identification of a *Paenibacillus polymyxa* strain that coproduces a novel lantibiotic and polymyxin. Appl Environ Microbiol **73:**168–178. http://dx.doi.org/10.1128/AEM.02023-06.
24. **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.
25. **He Z, Yuan C, Zhang L, Yousef AE.** 2008. N-terminal acetylation in paenibacillin, a novel lantibiotic. FEBS Lett **582:**2787–2792. http://dx.doi.org/10.1016/j.febslet.2008.07.008.
26. **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.
27. **Caetano T, Krawczyk JM, Mosker E, Sussmuth RD, Mendo S.** 2011. Heterologous expression, biosynthesis, and mutagenesis of type II lantibiotics from *Bacillus licheniformis* in *Escherichia coli*. Chem Biol **18:**90–100. http://dx.doi.org/10.1016/j.chembiol.2010.11.010.
28. **Wang G, Manns DC, Churey JJ, Worobo RW.** 2014. Development of a homologous expression system for and systematic site-directed mutagenesis analysis of thurincin H, a bacteriocin produced by *Bacillus thuringiensis* SF361. Appl Environ Microbiol **80:**3576–3584. http://dx.doi.org/10.1128/AEM.00433-14.
29. **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.
30. **Chan WC, Bycroft BW, Leyland ML, Lian LY, Roberts GC.** 1993. A novel post-translational modification of the peptide antibiotic subtilin: isolation and characterization of a natural variant from *Bacillus subtilis* A.T.C.C. 6633. Biochem J **291:**23–27.
31. **Heinzmann S, Entian KD, Stein T.** 2006. Engineering *Bacillus subtilis* ATCC 6633 for improved production of the lantibiotic subtilin. Appl Microbiol Biotechnol **69:**532–536. http://dx.doi.org/10.1007/s00253-005-0023-9.
32. **Klaenhammer TR.** 1988. Bacteriocins of lactic acid bacteria. Biochimie **70:**337–349. http://dx.doi.org/10.1016/0300-9084(88)90206-4.
33. **Riley MA, Wertz JE.** 2002. Bacteriocin diversity: ecological and evolutionary perspectives. Biochimie **84:**357–364. http://dx.doi.org/10.1016/S0300-9084(02)01421-9.
34. **Majeed H, Gillor O, Kerr B, Riley MA.** 2011. Competitive interactions in *Escherichia coli* populations: the role of bacteriocins. ISME J **5:**71–81. http://dx.doi.org/10.1038/ismej.2010.90.
35. **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.
36. **Cao M, Helmann JD.** 2002. Regulation of the *Bacillus subtilis bcrC* bacitracin 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.
37. **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.
38. **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.
39. **Wang PX, Zhang CY, Zhu YG, Deng Y, Guo SX, Peng DH, Ruan LF, 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.