RESEARCH LETTER

Biosynthesis and transcriptional analysis of thurincin H, a tandem repeated bacteriocin genetic locus, produced by *Bacillus thuringiensis* SF361

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Abstract

Thurincin H, a bacteriocin produced by *Bacillus thuringiensis* SF361 isolated from honey, strongly inhibited the growth of *Bacillus cereus* F4552. The bacteriocin was purified by 65% ammonium sulfate precipitation of the culture supernatant, followed by octyl-sepharose CL-4B and reverse-phase HPLC. The molecular mass of the bacteriocin was determined to be 3139.51 Da and the 14 amino acids of the bacteriocin at the N-terminus were identified. The complete amino acid sequence of mature thurincin H was deduced from three structural genes, *thnA1*, *thnA2*, and *thnA3* found in tandem repeats on the chromosome, all of which encode for the same bacteriocin, thurincin H. The genetic determinants for thurincin H biosynthesis consist of 10 ORFs, including three thurincin H structural genes. Northern hybridization elucidated that the transcription of all three bacteriocin structural genes was regulated by a putative promoter located upstream of *thnA1*.

Introduction

Bacteriocins are relatively small (3–10 kDa), cationic antibacterial peptides produced by Gram-positive and -negative bacteria (Klaenhammer, 1993). They can exert their antagonistic activity by disrupting membrane ionic potential through pore formation on the target cell membrane (Héchard & Sahl, 2002). Bacteriocins have been studied as potential alternatives to food preservatives that include sodium benzoate, potassium sorbate and sulfur dioxide that are currently used in the food industry (Ross *et al.*, 2002). Bacteriocins from food-grade microorganisms such as lactic acid bacteria are generally regarded as safe due to historical use in foods and the sensitivity of the bacteriocins to intestinal proteolytic enzymes when applied in the food, livestock and agricultural industry. Nisin is a bacteriocin produced by *Lactococcus lactis* ssp. *lactis* that has been applied to processed cheeses and other dairy products for $>$ 40 years in $>$ 50 countries of the world (Ross *et al.*, 2002; Delves-Broughton, 2005).

The *Bacillus* genus has been known to produce various types of bacteriocins (Stein, 2005). Subtilin, produced by *Bacillus subtilis*, is a lantibiotic, containing post-translationally modified unusual amino acids, such as lanthionine and methyllanthionine, and contain disulfide linkages between cysteine residues in the structure. The genes associated with subtilin production, immunity, modification, transport and regulatory system have been well investigated (Kleerebezem, 2004). Another lantibiotic, subtilosin A, produced by *B. subtilis* has been well studied and the genetic determinants for production and immunity (*sbo-alb*) have been identified (Babasaki *et al.*, 1985; Zheng *et al.*, 1999). *Bacillus cereus* is phylogenetically very similar to *Bacillus thuringiensis* and produces several bacteriocins exerting antibacterial activity against closely related *Bacillus* species (Naclerio *et al.*, 1993; Oscáriz *et al.*, 1999, 2006; Sebei *et al.*, 2007). Moreover, *B. thuringiensis* strains have been reported to produce a variety of bacteriocins (Paik *et al.*, 1997; Cherif *et al.*, 2001, 2003; Ahern *et al.*, 2003; Kamoun *et al.*, 2005; Gray *et al.*, 2006b; Chehimi *et al.*, 2007) that show high levels of antibacterial activity against closely related *Bacillus* species.

Previously, we isolated bacteria from different varieties of honey to determine the incidence of antimicrobial production and it was observed that as many as 92.5% of bacteria

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from specific honey varieties produced antimicrobial compounds (Lee *et al.*, 2008a). In this study, a bacterial strain, *B. thuringiensis* SF361, isolated from sunflower honey was selected to characterize the nature of the antimicrobial activity exhibited by the producer strain, which showed a high level of antagonistic activity against Gram-positive food-borne pathogens. To understand the primary structure and the genetic determinants of the bacteriocin, the structural and accessory genes were cloned and transcriptional analysis was performed to elucidate how the structural genes are transcribed.

Materials and methods

Screening and identification of the producer strain

An isolate (no. 361) from sunflower honey (South Dakota), screened by Lee *et al.* (2008a), displaying a high level of antibacterial activity against *B. cereus* F4552, was selected for further characterization of the antibacterial compound. Gram staining and 16S rRNA gene sequencing by PCR with set 1 primers (Table 1) was conducted to identify the producer strain as described by Lee *et al.* (2009a). To amplify *gyrB* gene as described by Manzano *et al.* (2003), set 2 primers (Table 1) were designed to perform PCR under the following conditions; one cycle of 3 min at 94 °C, followed by 30 cycles of 30 s denaturation at 94 °C, 30 s annealing at 57 °C, and 30 s extension at 72 °C and the last cycle of 8 min at 72 °C.

The PCR products were eluted using Qiagen Gel extraction kit (Qiagen, Valencia, CA) and the eluted products were sequenced by the Cornell University Life Science Core Laboratories Center (Ithaca, NY). The sequencing results were analyzed using NCBI BLAST homology search (BLASTN) to identify the producer strain.

Table 1. Primers used in this study

Primer sets		Sequence (5′–3′)
1	16S-FOR	AGAGTTTGATCCTGGCTCAG
	16S-REV	AAGGAGGTGATCCAGCCGCA
2	BCFW1	GTTTCTGGTGGTTTACATGG
	BCRW1	CAACGTATGATTTAATTCCACC
3	AB17R	GCCGATGGGTAAAGAAGCCCTATA
	AB6F	CTCTTTCTGATAAAGTTATGGACATAATAATA
4	AB7R	TTCACCATATGTTTTAACATCCTC
	AB13R	TATTATTATGTCCATAACTTTATCAGAAAGAG
5	AB12F	GTATCCAAAGTTTACAATATGAACATTG
	AB19R	AATTGAGATGAAACAAGGCATCCA
6	AB18F	GATACACATGCATTGGTAAAGGGC
	AB20R	ACCAACAGTTACATTTTTAGTTCCTGG
7	AB19F	TGGATGCCTTGTTTCATCTCAATT
	AB21R	CCACTGTTCTAAACCATATTGCTG
8	AB20F	CCAGGAACTAAAAATGTAACTGTTGGT
	AB22R	GGAACATAGGGAATAACAATCTTCCA
9	AB1F	GATTGGACTTGTTGGAGTTGCTTA
	AB4R	TTAGCTTGCAGTACTAGCCCCTGT

Antibacterial spectrum of bacteriocin producer

Bacillus thuringiensis SF361 was replica plated on tryptic soy agar (BD, Sparks, MD) plates and incubated at 30 °C for 24 h. The antibacterial spectrum of *B. thuringiensis* SF361 was determined against 41 indicator strains including food spoilage and pathogenic bacteria (Table 2) by deferred inhibition assay as described by Lee *et al.* (2009a). The antibacterial activity was measured by arbitrary determination of units ranging from (no activity) to +++++ (highest activity) by the diameter of clear zone formed around the producer colonies.

Purification of bacteriocin and activity assay

The producer strain was inoculated into 1.5 L of tryptic soy broth (BD) and grown at 30 °C with agitation (250 r.p.m.) for 15 h. The culture supernatant was recovered by centrifugation at 15 000 ***g*** for 20 min at 4 °C and precipitated with ammonium sulfate to 65% saturation. The precipitate was collected by centrifugation at 16 000 ***g*** for 10 min at 4 °C and dissolved with sterile deionized water. The concentrated bacteriocin solution was loaded onto octyl-sepharose CL-4B (GE Healthcare, Piscataway, NJ) equilibrated with 1.7 M ammonium sulfate. The hydrophobic column was initially eluted with 200 mL of 1.7 M ammonium sulfate, and then with a decreasing concentration gradient of 1.7 M ammonium sulfate and deionized water (250 mL). After the first gradient elution, the column was eluted with 200 mL of deionized water and an increasing gradient elution (0–70%) with ethanol (250 mL). The final elution was performed with 100 mL of 70% ethanol. The pooled active fractions from the column were injected onto RP-HPLC system (Agilent 1100 series, Agilent Technologies, Wilmington, DE) using a C18 RP column (Discovery Bio Wide pore C18, 4.6 150 mm, Supelco, Bellefonte, PA). The system was run with a linear gradient of HPLC-grade deionized water and acetonitrile containing 0.05% trifluoroacetic acid (TFA; Fisher Scientific, Hampton, NH) as the mobile phase. The signal intensity was monitored at 214 nm. The active peak separated by RP-HPLC was fractionated and lyophilized to perform chemical characterization studies.

The antibacterial activity of the fractions were determined by the spot-on-lawn test (Lee *et al.*, 2008b) using *B. cereus* F4552 as an indicator strain. The arbitrary activity units were calculated by the reciprocal of the highest

Table 2. Antibacterial spectrum of *Bacillus thuringiensis* SF361

Indicator strains	Incubation		Producer strain
	Media	Temperature (°C)	*Bacillus thuringiensis* SF361*
Bacillus subtilis ATCC 6633	TSB	37	++
Bacillus subtilis ATCC 6537	TSB	37	+
Bacillus subtilis CU1065(WT)	TSB	37	+++
Bacillus subtilis LRB90	TSB	37	+
Bacillus subtilis LRB91	TSB	37	+
Bacillus cereus F4552	TSB	37	+++++
Bacillus cereus ATCC 11778	TSB	37	
Bacillus cereus F4810	TSB	37	+++++
Bacillus cereus Northland	TSB	37	+++++
Bacillus cereus Northview P2E018	TSB	37	+++++
Geobacillus stearothermophilus ATCC 12980	TSB	50	+++++
Bacillus thuringiensis EG10368	TSB	37	+++++
Bacillus megaterium LRB89	TSB	37	+++++
Listeria monocytogenes F2 586 1053	TSB	37	++++
Listeria monocytogenes 2289	TSB	37	++++
Listeria innocua ATCC 2283	TSB	37	+++++
Listeria ivanovii ATCC 19119	TSB	37	+++++
Micrococcus luteus	TSB	37	+
Paenibacillus kobensis M	TSB	37	
Staphylococcus aureus ATCC 9144	TSB	37	+
Staphylococcus aureus ATCC 8095	TSB	37	+
Staphylococcus aureus ATCC 25923	TSB	37	
Lactobacillus acidophilus L-39	APT	30	
Lactobacillus plantarum B246	APT	30	
Lactobacillus plantarum EH22G	APT	30	
Lactobacillus helveticus L-31	APT	30	
Lactobacillus delbrueckii 4797	APT	30	
Lactobacillus buchneri ATCC 4005	APT	30	
Lactobacillus fermentum ATCC14931	APT	30	
Lactobacillus casei 30SC	APT	30	
Lactobacillus brevis B155	APT	30	
Leuconostoc mesenteroides C-33	APT	30	
Leuconostoc mesenteroides 548D	APT	30	
Enterococcus faecalis 8043	APT	30	
Carnobacterium piscicola CU216	APT	30	+++++
Pediococcus cerevisiae E66	APT	30	
Escherichia coli BF2	TSB	37	
Escherichia coli O157:H7 ATCC 43889	TSB	37	
Salmonella Typhimurium ATCC 14028	TSB	37	
Salmonella Typhimurium Montevideo	TSB	37	
Salmonella Enteritidis	TSB	37	

*Antibacterial activity of the producer strain was graded by (no activity) to ++++ (highest activity).

dilution showing inhibition per milliliter of the fractions tested.

Determination of N-terminal sequence and NanoESI-qTOF MS

The purified active bacteriocin from RP-HPLC was subjected to N-terminal sequencing by Edman degradation, performed by the Synthesis and Sequencing Facility at Johns Hopkins University School of Medicine (Baltimore, MD).

Electrospray ionization (ESI)-MS was performed to determine the accurate molecular mass of the HPLC-purified bacteriocin at the Proteomics and Mass Spectrometry Facility, Donald Danforth Plant Science Center (St. Louis, MO). The bacteriocin was analyzed on an Applied Biosystems QSTAR XL hybrid quadrupole time-of-flight (TOF) MS system equipped with a nanoelectrospray source.

DNA manipulation and sequence analysis

Basic DNA manipulation techniques were performed as described by Sambrook & Russell (2001). From the results of NCBI BLAST homology search (BLASTP) with the N-terminal sequence, two hypothetical proteins identified by the unfinished whole-genome shotgun sequencing of *B. cereus* AH1134 (GenBank accession no.: NZ_ABDA02000035) and their genetic flanking regions were selected to design six sets of primers (sets 3–8) to clone the bacteriocin biosynthesis genes of *B. thuringiensis* SF361 (Table 1). PCR was performed with a program of the following conditions: initial one cycle of 94 °C for 3 min, followed by 30 cycles of a set of reactions composed of 30 s at 94 °C, 30 s at 60 °C, and 2 min at 72 °C, and one last cycle of 72 °C for 8 min. The PCR products were eluted and sequenced as described in a previous section. Genomic analyses were performed with LASERGENE software (Dnastar Inc., Madison, WI) and the NCBI BLAST homology search (BLASTP).

RNA manipulation and Northern hybridization

Total RNA was extracted from exponentially growing *B. thuringiensis* SF361 in Luria–Bertani medium (BD) after 5 h of incubation (30 °C, 250 r.p.m.) with RNeasy Protect Bacteria Mini Kit (Qiagen). Ten micrograms of total RNA and 5 μg of RNA marker (0.1–1.0 kb; Sigma, St. Louis, MO) as a size reference were run in formaldehyde agarose gel [1.5% (w/v)] and transferred to Hybond-N+ positively charged nylon transfer membrane (GE Healthcare) as described by Sambrook & Russell (2001). Northern hybridization was performed with Amersham Gene Image AlkPhos Direct Labelling and Detection System (GE Healthcare). The partial structural gene of thurincin H was amplified with the chromosomal DNA as a template using a set of primers (set 9; Table 1) designed based on the sequence of a structural gene, *thnA2*, and its termination codon (TAA). The PCR product with a size of 96 bp was labeled by the labeling system, and then used as a probe. After signal generation, the signal on the membrane was exposed and developed on a Kodak BioMax Light Film (Kodak, Rochester, NY).

Nucleotide sequence accession number

The annotated DNA sequences in this paper were deposited to GenBank, whose accession numbers are FJ160904 and FJ977580.

Results

Identification of the bacteriocin producer

The producer was a Gram-positive, spore-forming bacterium that was identified as *B. thuringiensis* by 16S rRNA gene sequencing. To complement 16S rRNA gene sequencing, the partial sequence of *gyrB* gene encoding subunit B protein of DNA gyrase (topoisomerase type II) was amplified by PCR using a specific-primer set. The sequence of *gyrB* of the producer showed the highest identity (99%) to *gyrB* sequences of several *B. thuringiensis* strains. Based on these results, the producer was designated as *B. thuringiensis* SF361.

Antibacterial spectrum of the bacteriocin producer

The inhibitory spectrum of *B. thuringiensis* SF361 was determined against a broad range of Gram-positive and -negative bacteria. All of *Bacillus* spp. except *B. cereus* ATCC 11778 were sensitive to the antagonistic activity of the producer (Table 2). The producer also exhibited a high level of antibacterial activity against *Listeria* spp. Among the lactic acid bacteria, the growth of only one bacterium (*Carnobacterium piscicola* CU216) was inhibited by the antibacterial activity. *Bacillus thuringiensis* SF361 did not exhibit antimicrobial activity against any of the Gram-negative bacteria tested.

Purification, N-terminal sequencing, and MS of thurincin H

Ammonium sulfate precipitation of the culture supernatant resulted in the highest yield (65%) in terms of the antibacterial activity. The active compound was eluted with deionized water from octyl-sepharose CL-4B (Fig. 1a). All of the active fractions were pooled for further purification by RP-HPLC. The HPLC chromatogram showed that one main peak containing the antibacterial activity was eluted with 65% acetonitrile containing 0.05% TFA (Fig. 1b). The purified bacteriocin was designated as thurincin H.

N-terminal sequencing identified 14 amino acid residues with four unidentified residues, DWTXWSXLVXAAXSVELL (X, unidentified amino acid). The molecular mass of thurincin H was calculated to be 3139.51 Da by nanoESI-qTOF MS (Fig. 1c).

Analyses of the gene cluster of thurincin H

The PCR product from set 4 primers was sequenced to determine the nucleotide sequence of the coding region of thurincin H. Three ORFs (*thnA1*, *A2*, and *A3*) were arranged in tandem on the sequence and all three genes encode for the identical bacteriocin, thurincin H (Fig. 2a). Upstream of *thnA1*, a putative promoter with 35 and 10 sequences was predicted and three probable ribosome-binding sites were located 8 bp upstream of each putative start codon (ATG) of all three structural genes. However, no other start codons were found upstream of the annotated start codons

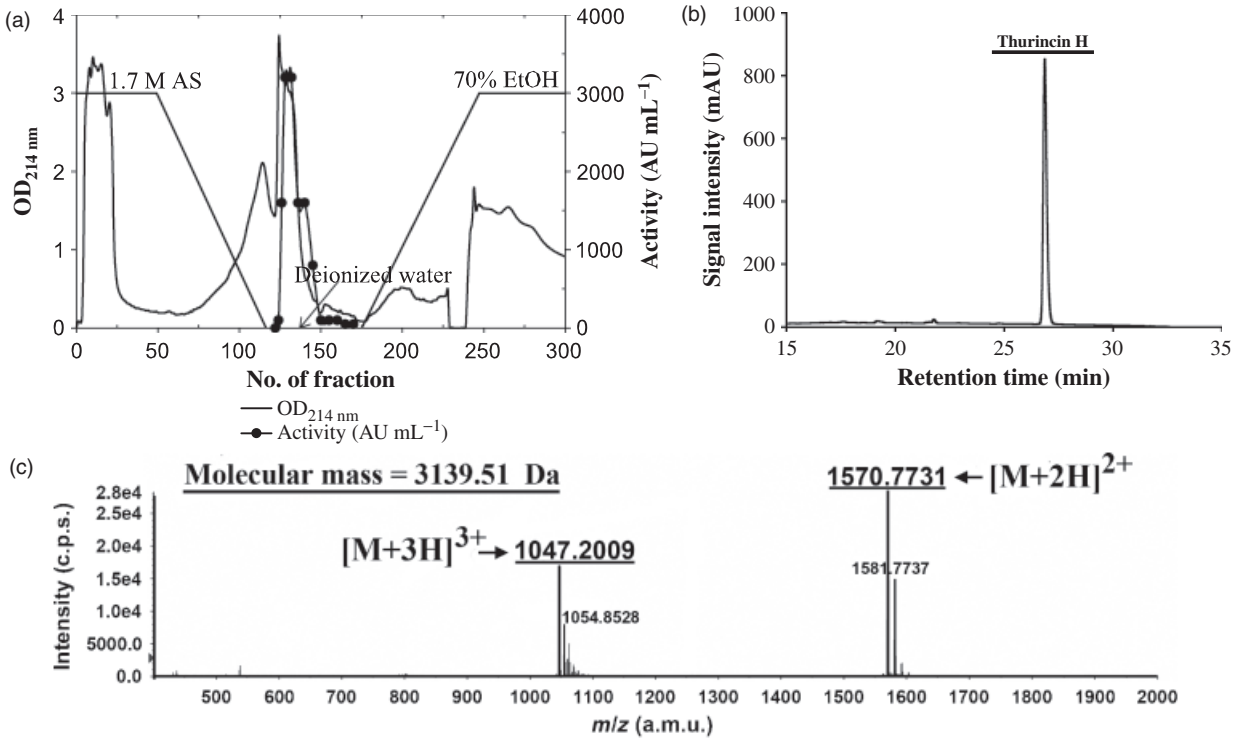

Fig. 1. (a) Chromatogram of bacteriocin produced by *Bacillus thuringiensis* SF361 on octyl-sepharose CL-4B. AS, ammonium sulfate. (b) Chromatogram of octyl-sepharose CL-4B active fractions on RP-HPLC with a C18 column. (c) NanoESI-qTOF mass spectrum of HPLC-purified thurincin H.

for each gene. A potential *rho*-independent termination site (nucleotide 938–983) was identified downstream of *thnA3*. Three ORFs of thurincin H were translated into 40-amino acid-long prepeptide bacteriocins, METPVVQPRDWTCWS CLVCAACSVELLNLVTAATGASTAS. They consist of an identical nine amino acid leader peptide and an identical mature bacteriocin comprised of 31 amino acids. The calculated molecular mass and pI of the mature thurincin H based on the primary structure was 3147.61 Da and 3.7, respectively. The difference of the actual and the calculated molecular mass of thurincin H was 8.1 Da.

Six PCR products obtained by primer sets 3–8 yielded a genetic organization with a size of approximately 8.7 kb. In total, seven ORFs flanking three thurincin H structural genes were annotated (Fig. 2b). ThnP [465 amino acids (aa)] and ThnB (459 aa) showed homology to the epidermin leader peptide-processing serine protease EpiP and AlbA of *B. subtilis* ssp. *subtilis* strain 168, an Fe–S oxidoreductase, radical SAM superfamily, respectively. *thnR* encodes for a putative transcriptional regulator, GntR family (127 aa) and two ABC transporters, ThnD (288 aa), an ATP-binding protein and ThnE (215 aa), a permease protein, are encoded by two ORFs downstream of *thnR*. ThnT (569 aa) is predicted to be an ABC-secretion protein. However, *thnI*, encoding a hypothetical protein composed of 95 amino acids, showed no similarity to any known bacteriocin production genes.

Transcriptional analysis of thurincin H genes

Total RNA of *B. thuringiensis* SF361 was analyzed by Northern hybridization to determine whether the thurincin H genes were transcribed independently or as a single transcriptional unit. Using a PCR fragment with a partial sequence of all three structural genes, a single transcript of thurincin H genes with an approximate size of 700 nucleotides was identified (Fig. 3).

Discussion

We attempted to clone the structural and accessory genes of thurincin H produced by *B. thuringiensis* SF361, a bacterial isolate from US domestic honey. By comparison of the 18 amino acids at the N-terminus of thurincin H with all known antimicrobial peptide sequences using BLASTP, it was revealed that two identical proteins of *B. cereus* AH1134 with unknown functions showed 77.8% identities to thurincin H. Both of the hypothetical proteins (locus tag: BCAH1134_C0273 and C0274) were annotated by the unfinished shotgun genome sequencing of *B. cereus* AH1134. The two proteins were composed of 40 amino

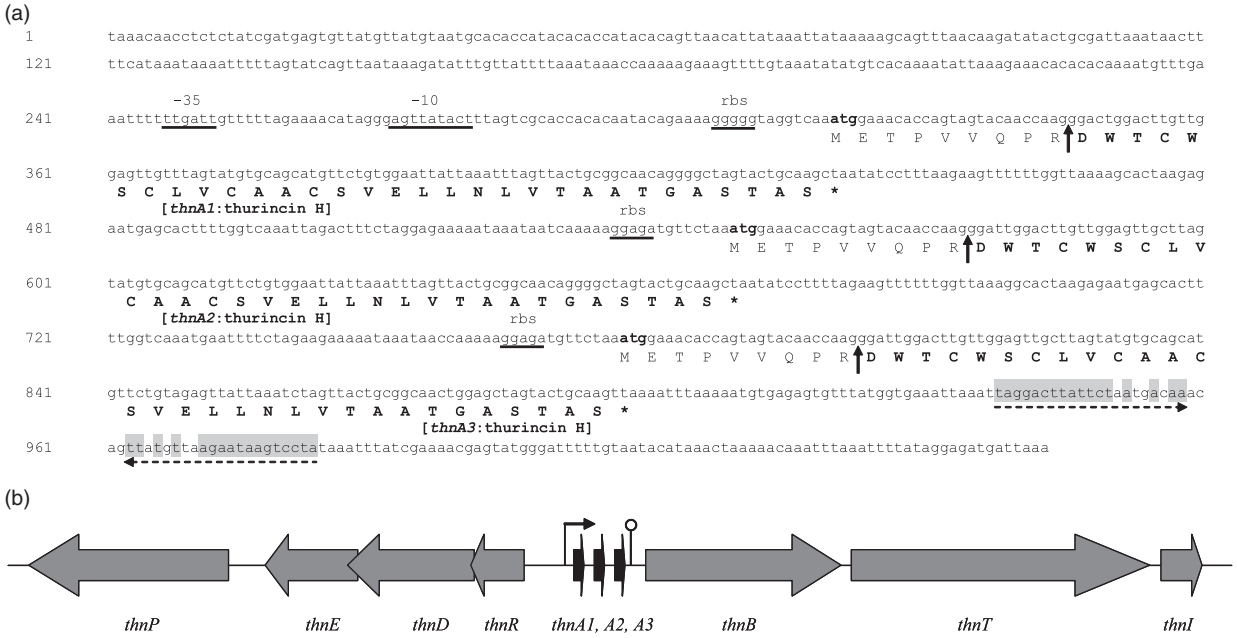

Fig. 2. (a) Nucleotide sequence and deduced amino acid sequence encoding thurincin H (*thnA1*, *thnA2*, and *thnA3*). Start codons (atg) of three genes are specified in bold. Putative ribosomal binding sites (rbs) and 35 and 10 sequences of predicted promoter are underlined. Upward arrows indicate the peptide cleavage processing sites, resulting in mature thurincin H; the corresponding encoded amino acids are in bold. Two facing dotted arrows and the shaded nucleotides represent a putative transcriptional termination site with a stem–loop structure. (b) Genetic organization of thurincin H structural genes (black arrows) with the anking genes for the bacteriocin production (gray arrows). Bent arrow and lollipop represent the promoter and transcription termination site of the thurincin H structural genes, respectively.

acids and were identical to each other in their primary structure and were subsequently found to be identical to the deduced amino acid sequence of thurincin H by PCR. However, one additional ORF encoding the same protein by the annotation of the genome sequence of *B. cereus* AH1134 was identified. It was assumed that this ORF may have been overlooked during annotation.

Five sets of primers (sets 3, 5, 6, 7 and 8), designed based on the up- and downstream regions of the ORFs encoding the two proteins of *B. cereus* AH1134 were used for amplification of the flanking regions of thurincin H genes. The additional PCR showed that the annotated seven ORFs (locus tag: BCAH1134_C0269, C0270, C0271, C0272, C0275, C0276 and C0277) flanking two genes (locus tag: BCAH1134_C0273 and C0274) of *B. cereus* AH1134 were identical to the flanking genes, *thnP*, *E*, *D*, *R*, *B*, *T*, and *I* of thurincin H structural genes with the same transcriptional directions (Fig. 2b). ThnP and B are suggested to be related to the processing of thuricin H, resulting in the mature bacteriocin. Thn D and E are postulated to be associated with bacteriocin resistance, as determined by BLASP. ThnT is predicted to be involved as a transporter for bacteriocin production. Interestingly, a transcription regulator, ThnR, which may be related to two-component regulatory systems for bacteriocin synthesis was found without the typical paired histidine kinase in the genetic organization. It is assumed that ThnI may be an immunity protein of thurincin H in terms of its short length (95 aa) compared with subtilosin A immunity protein (AlbB; 53 aa) (Zheng *et al.*, 2000) even though there was no functional or homologous evidence. Functional analyses will be conducted for the accurate annotation of each ORF.

The reported N-terminal sequences of four similar bacteriocins to thurincin H from three *B. thuringiensis* and one *B. cereus* strains showed high identities to the N-terminal sequence of thurincin H: thuricin S (DWTXWSXL), thuricin 17 (DWTCWSCLVVAACSVELL), bacthuricin F4 (DWTXWSXL) and cerein MRX1 (DWTCWSCLVCAACSVELL) (Kamoun *et al.*, 2005; Gray *et al.*, 2006a, b; Chehimi *et al.*, 2007; Sebei *et al.*, 2007). The determined molecular masses of thuricin S (3137.61 Da), thuricin 17 (3162 Da), bacthuricin F4 (3160.05 Da), and cerein MRX1 (3137.93 Da) were slightly different from thurincin H (3139.51 Da). Recently, a bacthuricin F4 production-associated gene was identified to be plasmid encoded (Kamoun *et al.*, 2009). To determine the thurincin H operon location, Southern hybridization was performed using a PCR product of the genetic locus, which included the three structural genes of thurincin H as a probe to determine the bacteriocin operon location. As confirmation for the chromosomal location,

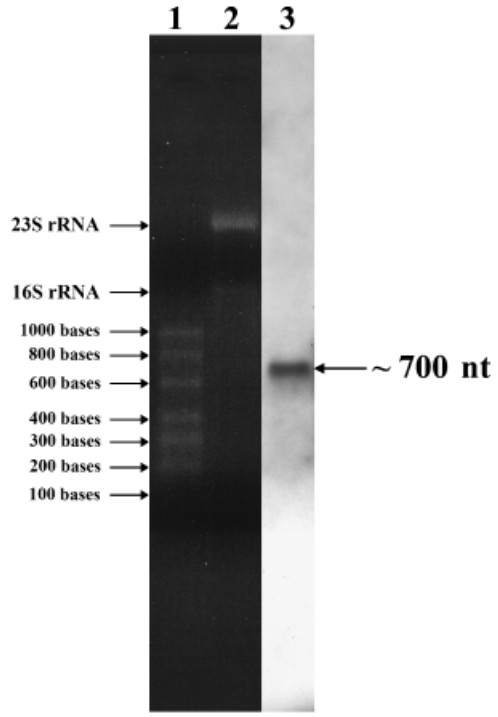

Fig. 3. Northern hybridization of total RNA extracted from *Bacillus thuringiensis* SF361. Transcript of thurincin H genes with an approximate size of 700 nt is indicated with an arrow. Lane 1, RNA marker (0.1–1.0 kb); lane 2, total RNA of *B. thuringiensis* SF361 (10 μg); lane 3, a signal representing the thurincin H transcript detected using chemiluminescence detection.

PCR-amplified 16S rRNA gene was used as a probe. Southern hybridization with chromosomal and plasmid DNA of *B. thuringiensis* SF361 revealed the bacteriocin gene to be chromosomally encoded (data not shown), which is different compared with the bacthuricin F4. This suggests that the producer strain of thurincin H is different from that of bacthuricin F4, located on a plasmid DNA (Kamoun *et al.*, 2009). As a further confirmation of the hybridization results, it was elucidated that the phage minor structural protein gene (GenBank accession no.: FJ499396) reported by Kamoun *et al.* (2009) was not amplified by PCR with the chromosomal or plasmid DNA from *B. thuringiensis* SF361.

It was revealed that three thuricin 17 bacteriocin genes had identical genetic structures to three thurincin H genes (Lee *et al.*, 2009b). However, it was elucidated that there is one additional amino acid, methionine, in leader peptide of thurincin H (METPVVQPR), compared with the leader peptide of thuricin 17 (ETPVVQPR). A homologous gene located downstream of thuricin 17 (*albA*) was also identified downstream of the thurincin H structural genes (*thnB*) with the same transcriptional orientation (Fig. 2b). However, two genes (*secE* and *nusG* encoding preprotein translocase SecE subunit and transcriptional antitermination factor, respectively) upstream of the thuricin 17 genes (Lee *et al.*, 2009b) were not matched to the two consecutive genes upstream of the thurincin H genes. On the contrary, *thnR* encoding a transcription regulator flanked by a gene encoding for the ATPase component of the ABC-type multidrug transport system (ThnD) was annotated upstream of the thurincin H genes (Fig. 2b). Both genes were elucidated to be encoded on the noncoding strand of the thurincin H genes. It is interesting that the identical bacteriocin structural genes were located downstream of two totally different genes with the opposite transcriptional direction from the same species of bacilli. It can be assumed that this may occur during horizontal gene transfer or by certain types of recombination events.

The thurincin H leader peptide is significantly shorter (9 aa residues) than typical bacteriocin leader peptides except those of three circular bacteriocins, MFL (circularin A), MDILLE (uberolysin), and MKKAVIVE (subtilosin A) (Zheng *et al.*, 1999; Kemperman *et al.*, 2003; Wirawan *et al.*, 2007), resulting in a prepeptide that barely spans the cytoplasmic membrane for export. A unique type of export and processing system might be expected for thurincin H and the three circular bacteriocins based on the leader peptide sequence lengths.

It was found that only eight out of 120 nucleotides are different for all three thurincin H genes (Fig. 2a). Two intergenic sequences (intergenic 1: 109 nt between *thnA1* and *thnA2*; intergenic 2: 108 nt between *thnA2* and *thnA3*) showed very high similarity (93% identity), indicating that the *thnA* genes may be the result of tandem duplication mutations of a single gene. However, neither of the intergenic sequences showed any significant similarity to the noncoding region downstream of *thnA3* (167 nucleotides). Moreover, the two intergenic regions did not contain a notable transcriptional terminator with a stem–loop structure, suggesting that the three genes might be controlled under the same promoter upstream of *thnA1*. From the Northern hybridization, only one signal representing mRNA with an approximate size of 700 nt was detected (Fig. 3). The size was similar to that of the predicted mRNA size (700 nt; Fig. 2a, nucleotides 281–291 to 983) of all three genes transcribed under the identical putative promoter upstream of *thnA1*. Therefore, the analysis confirmed that no putative promoter regions are predicted within both intergenic sequences and elucidated that the transcription of all three genes is controlled by a single promoter. To our knowledge, this is the first report showing multiple copies of identical bacteriocin structural genes in tandem that are regulated by the same promoter.

The genetic redundancy of multiple copies of genes has been discovered to express elevated level of resistance to antibiotics. Multiple tandem structural genes encoding the same bacteriocin under the control of the same promoter

may be associated with the survival of the producer strain in a niche environment where a variety of microorganisms compete for the limited nutrients. Production of three times the amount of bacteriocin under the same control at the transcriptional level during a given time would allow for rapid increases in bacteriocin levels, thus making it possible to compete more efficiently against other bacteria.

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