PURIFICATION, CHARACTERIZATION, SITE-DIRECTED MUTAGENESIS AND MODE OF ACTION STUDIES OF THE *BACILLUS* BACTERIOCIN THURINCIN H

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PURIFICATION, CHARACTERIZATION, SITE-DIRECTED MUTAGENESIS AND MODE OF ACTION STUDIES OF THE *BACILLUS* BACTERIOCIN THURINCIN H

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Bacteriocins are ribosomally synthesized antimicrobial peptides or proteins produced by bacteria, which usually inhibit the growth of closely related species. Bacteriocins produced by *Bacillus* species have attracted emerging interests by both food and pharmaceutical industries due to their broader antimicrobial spectrum, compared with the widely used bacteriocins produced by lactic acid bacteria. Thurincin H is an anti-listerial bacteriocin produced by *Bacillus thuringiensis* SF361, a strain originally isolated from US domestic honey. Purified thurincin H exhibited a broad antimicrobial activity against various foodborne pathogens and spoilage microorganisms across several genera, including *Bacillus*, *Carnobacterium*, *Geobacillus*, *Enterococcus*, *Listeria*, and *Staphylococcus*.

A rapid and simple method was developed to produce and purify large amounts of thurincin H. The purified thurincin H was characterized regarding its thermal and acid stability, and its inhibitory effect against *B. cereus* spores. Systematic conservative and non-conservative site directed mutagenesis were performed to identify the critical amino acids in the native thurincin H production pathway, using an optimized thurincin H heterologous expression system newly developed in this study. The thurincin H gene cluster was confirmed by heterologously expressing a

bioinformatically identified gene cluster in a sensitive *B. thuringiensis* strain. The sensitive strain acquired complete immunity and produced thurincin H at a higher level compared with the natural producer. The bactericidal thurincin H caused cell morphology changes in a concentration dependent manner, but did not induce cell membrane permeability, which indicated a novel mode of action that is different from the generalized pore-forming mechanism for most bacteriocins.

BIOGRAPHICAL SKETCH

Gaoyan Wang was born to Ruiling Gao and Guanglun Wang in Jiaozhou, China and grew up in the same city until she graduated from high school. She obtained her B.S. degree in Food Science at the Ocean University of China in her beautiful hometown of Qingdao, which is located along the seaside. In 2007, she went to Clemson University in the United States to study antimicrobial peptides as an alternative treatment for acne. Her continued interest in food science and microbiology motivated her to pursue a Ph.D. degree in the Department of Food Science at Cornell University under Dr. Randy W. Worobo, working on antimicrobial peptides and microbial food safety. After completing her Ph.D., she is planning to start her career in the food industry.

This dissertation is lovingly dedicated to my family, my mother Ruiling Gao, father Guanglun Wang, grandparents and brother Wenhui Wang.

Their support, encouragement, and constant love

have sustained me throughout my life.

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CHAPTER 1

JUSTIFICATION AND INTRODUCTION

Antimicrobial peptides or proteins are widely produced and used as a defensive strategy, by a wide range of organisms that include microorganisms, plants, insects, and animals (Bowdish *et al.*, 2005; Ganz, 2003). Bacteriocins are the antimicrobial peptides or proteins produced by bacteria, which are genetically encoded and ribosomally synthesized (Cotter *et al.*, 2005). Bacteriocins usually exhibit a relatively narrow inhibition spectrum of activity against closely related species (Cotter *et al.*, 2005). It has been suggested that a significant percentage of bacteria can produce at least one bacteriocin (Klaenhammer, 1988; Riley, 1998). This competitive advantage may assist the bacteriocin-producers in preventing the invasion of other strains or species into an occupied niche by limiting nutrient competition of neighboring cells (Majeed *et al.*, 2011).

Since lactic acid bacteria (LAB) are generally recognized as safe (GRAS status) according to the FDA, LAB bacteriocins have been widely applied in food systems to ensure food safety, enhance food quality, and minimize economic loss by inhibiting the growth of foodborne pathogens and spoilage bacteria. Nisin is widely used in the food industry and has been extensively studied, not only from the chemical and genetic basis, but also from its application in various foods (Chen & Hoover, 2003). Using food grade bacteriocins as natural food preservatives could also provide an option for the food processors to face the “natural” food trend challenges that are being demanded by consumers. Bacteriocins are considered as “natural” food

preservative systems since they are not regarded as chemical preservatives. In addition to food systems, the interest of bacteriocin application has been expanded to veterinary and human medicines in recent decades (Hillman, 2002; Ryan *et al.*, 1999). As the most studied group of bacteriocins, LAB bacteriocins can be divided into three main classes based on their structural and chemical characteristics: the class I lantibiotics containing lanthionine; the class II non-lanthionine-containing bacteriocins; and the class III heat-labile large proteins (Cotter *et al.*, 2005). LAB bacteriocins generally kill sensitive strains by forming pores in their cell membrane, causing cell membrane potential and proton motive force disruption (Moll *et al.*, 1996).

Compared to LAB bacteriocins, *Bacillus* bacteriocins are attracting increasing research interests due to their high diversity and much broader inhibition spectra. Research on *Bacillus* bacteriocins to date indicates their promising application potentials in agricultural, environmental and pharmaceutical industries, as well as in the food industry as a means to control various spoilage and pathogenic microorganisms (Abriouel *et al.*, 2011; Lee & Kim, 2011). The limited in-depth research on the bacteriocin structural and chemical characteristics has made it difficult to clearly and accurately classify those highly diverse *Bacillus* bacteriocins, with the only systematic classification proposed in 2010 (Abriouel *et al.*, 2011). As summarized in the classification, *Bacillus* spp. also produce a large group of lantibiotics, which have similar structures and biochemical characteristics to LAB lantibiotics (Abriouel *et al.*, 2011).

Thurincin H is a 31 amino acid hydrophobic bacteriocin produced by *B. thuringiensis* SF361, a strain originally isolated from US domestic honey (Lee *et al.*,

2009). It forms a hairpin structure with a helical backbone maintained by four pairs of unique sulfur to α-carbon thioether bridges, catalyzed by a putative radical S-adenosylmethionine superfamily enzyme (Sit *et al.*, 2011). Those thioether linkages are quite different from all studied LAB bacteriocins (Cotter *et al.*, 2005) or *Bacillus* lantibiotics (Abriouel *et al.*, 2011), and only exist in three other *Bacillus* bacteriocins (Fluhe *et al.*, 2012).

Studies on the biochemical and genetic characteristics, structures and mechanisms of action for this new type of *Bacillus* bacteriocins are still limited. Further investigation of these bacteriocins is needed to provide insights into their unique nature or their relatedness to already-known bacteriocins. Thurincin H for example, requires a large scale bacteriocin and purification system to provide sufficient purified bacteriocin to perform subsequent biochemical characterizations, toxicity evaluation and structure analysis. Completely new heterologous expression systems and site directed mutagenesis are needed to facilitate the exploration of variants with new characters, structures and functions. Commonly used *Escherichia coli* (Ingham *et al.*, 2005; Lohans & Vederas, 2012) or lactic acid bacteria (Rodriguez *et al.*, 2003) expression systems are not suitable since the unique posttranslational modification involved in the thurincin H biosynthesis are not compatible. Completely novel mechanism of action for thurincin H might exist since it has a unique structure. Its mode of action could be not predicted by only comparing the structures of thurincin H with other extensively characterized bacteriocins.

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CHAPTER 2

LARGE SCALE PURIFICATION, CHARACTERIZATION, AND SPORE OUTGROWTH INHIBITORY EFFECT OF THURINCIN H, A BACTERIOCIN PRODUCED BY *BACILLUS THURINGIENSIS* SF361

ABSTRACT

Large scale purification of the highly hydrophobic bacteriocin thurincin H was accomplished via a novel, rapid, and simple two-step method: ammonia sulfate precipitation and C18 solid phase extraction. The inhibition spectrum and stability of thurincin H, as well as its antagonistic activity against *Bacillus cereus* F4552 spores were further characterized. In the purification method, secreted proteins contained in the supernatant of a 40-hour culture of *B. thuringiensis* SF361 was precipitated by 68% ammonia sulfate and purified by reverse phase chromatography with a yield of 18.53 mg/L. Silver stained SDS-PAGE, high-performance liquid chromatography (HPLC), and liquid chromatography–mass spectrometry (LC-MS) confirmed the high purity of the prepared sample. Thurincin H exhibited a broad antimicrobial activity against 22 tested bacterial strains among 6 different genera including *Bacillus*, *Carnobacterium*, *Geobacillus*, *Enterococcus*, *Listeria*, and *Staphylococcus*. There was no detectable activity against any of the selected yeast or fungi. The bacteriocin activity was stable for 30 min at 50°C, and decreased to undetectable levels within 10 minutes at temperatures above 80°C. Thurincin H was stable from pH 2-7 for at least 24

hours, although gradual loss of activity occurred in alkaline solutions within 24 hours at room temperature. Thurincin H is germicidal against *B. cereus* spores in brain heart infusion broth, but not in Tris-NaCl buffer. The efficient purification method enables the large scale production of pure thurincin H, which is an adequate preparation for further downstream biochemical studies. The broad inhibitory spectrum of this bacteriocin may be of interest as a potential natural biopreservative in the food industry, particularly in acidic and acidified food.

INTRODUCTION

Foodborne disease and spoilage caused by microorganisms have long been a challenge for public health concerns and the food processing industry. It is estimated that 9.4 million illnesses, 55,961 hospitalizations, and 1,351 deaths were caused by 31 different foodborne pathogens each year in the United States (Scallan *et al*., 2011). Among the bacteria, yeasts, and molds responsible for general food spoilage and their resulting economic losses, spore forming bacteria are of particular concerns for the food industry due to their high heat resistance, and higher tolerance to drying, freezing, and chemical disinfectants (Nicholson *et al*., 2000).

Bacteriocins are ribosomally synthesized peptides or proteins produced by bacteria that exhibit antimicrobial activity against other bacteria mostly within the same species (narrow spectrum) or sometimes across different genera (broad spectrum) (Cotter *et al*., 2005). Bacteriocins produced by Gram-positive bacteria, most commonly lactic acid bacteria, have been widely applied in the food industry because of their effectiveness against various foodborne pathogens and spoilage microorganisms (Settanni & Corsetti , 2008). *Bacillus* spp. have gained recent

research interest since they are considered rich producers of different types of antibiotics, antimicrobial proteins or peptides, and antifungal substances (Stein, 2005; Abriouel *et al*., 2011). *Bacillus* spp. strains generally exhibit a broad inhibition spectrum and occasionally inhibit yeasts and molds, even bacterial spores (Abriouel *et al*., 2011). For example, bacteriocin AS-48 was reported to inhibit the outgrowth of *B. cereus* spores (Abriouel *et al*., 2002).

Thurincin H is a bacteriocin produced by *Bacillus thuringiensis* SF361, a strain isolated from US domestic honey (Lee *et al*., 2009). The producer strain inhibits the growth of several Gram-positive foodborne pathogens and food spoilage microorganisms, such as *L. monocytogenes* and *B. cereus*, based on overlay assays (Lee *et al*., 2009). The mature thurincin H is composed of 31 amino acids with a molecular mass of 3139.51Da (Lee *et al*., 2009). According to a recent three dimensional NMR spectroscopy study, the helical backbone of mature thurincin H folds to form a hairpin structure with helical backbones stabilized by four sulfur to α-carbon bridges (Sit *et al*., 2011).

The objective of this study was to develop an efficient and reproducible method to produce and purify large amounts of highly pure thurincin H. Additionally, to further evaluate its potential as a natural preservative, the inhibition spectrum, stability under various conditions, as well as its antagonistic activity against *Bacillus* spores were determined.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The thurincin H producing strain *B. thuringiensis* SF361, and the indicator strain *B. cereus* F4552, were cultivated in

trypticase soy broth (TSB) or on trypticase soy agar (TSA) (BD, Sparks, MD) at 37°C. Yeast and fungi were cultivated in potato dextrose agar (PDA) (Hardy diagnostics, Santa Maria, CA) or potato dextrose broth (PDB) (BD, Sparks, MD) adjusted pH to 3.5 with a 10% sterile tartaric acid solution added after autoclaving. Phosphate buffered saline (PBS) at pH 7 was formulated with 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO, and 0.24 g KH_2PO_4 per liter. In spore germination studies, spores were incubated in brain heart infusion (BHI) broth (BD, Sparks, MD) at 30°C for 1 hour with 250 rpm shaking. All chemicals and reagents were either autoclaved at 121°C for 15 minutes or filtrated by polyethersulfone membrane (0.22 µm), prior to use.

***B. cereus* F4552 spore preparation and quantification.** One milliliter of a 5 hour *B. cereus* F4552 culture in TSB was evenly spread on SPO 8 solid agar (Pol *et al*., 2001) and incubated at 20°C for 7 days. The resulting spores were transferred from the plate surface and resuspended in sterile Milli-Q water (Millipore Corporation, Billerica, MA). The suspension was washed three times in ice cold sterile Milli-Q water, heat treated at 80°C for 10 min, and stored at -20°C until needed. To determine the concentration, spores were serially diluted and plated on TSA plates. Colonies were counted after incubating for 16 hours at 37°C and the concentration of spores were calculated. Two independently prepared spore crops were used throughout this study.

Quantification of bacteriocin activity. The bacteriocin activity was determined by a previously described microtiter plate assay method (Daba *et al*., 1991), modified for the current study. In brief, using untreated, clear, flat bottom 96 micro well plates (Thermo Scientific, Nunc, Denmark), 50 µl of bacteriocin diluted

two-fold in the appropriate buffer was mixed with 150 µl of 1.33% (v/v) *B. cereus* F4552 overnight culture in TSB in each well and incubated at 37°C for 8 hours. The absorbance at 600 nm (A_{600}) of each well was measured using a Synergy HT Multi-Mode Microplate Reader (BioTek, Winooski, VT). One arbitrary unit (AU) was defined as the amount of bacteriocin in the 50 µl sample that caused a 50% growth inhibition when compared with the control groups.

Total protein concentration measurement. The concentration of total protein was measured by the Pierce BCA protein assay kit according to the manufacturer's protocol (Thermo scientific, Rockford, IL).

Thurincin H production and C18 purification. *B. thuringiensis* SF361 was streaked on a TSA plate and incubated at 37°C for 14 hours. A single colony was inoculated into 5 ml of TSB and incubated at 37°C for 12 hours with shaking at 225 rpm. A 1.5 ml aliquot of this incubation was added into 150 ml and incubated at 37°C for 40 hours with shaking at 225 rpm. Pooled supernatants from multiple incubations (750 ml in total) were collected after centrifugation (13,000 x g, 4°C, 40 min). The crude protein fraction was precipitated using ammonium sulfate at a final concentration of 68% saturation. Pelleted precipitates were resuspended in 150 ml of PBS and purified using C18 Sep Pak Plus tC18 Environmental Cartridges (Waters, Milford , MA). To accomplish this, an SPE cartridge was first equilibrated with 20 ml methanol followed by 20 ml PBS buffer. After the supernatant samples were loaded on to the cartridge, 20 ml each of increasing concentrations of acetonitrile (30%, 35 %, 45%, 50%, 100%) were consecutively applied to the column. The supernatant, crude protein extract, and each eluted fraction from the cartridge were separately collected

and analyzed by high-performance liquid chromatography (HPLC) to evaluate purity as described below. Those fractions (35%-50%) eluted from the SPE cartridge were pooled, vacuum-centrifuged to remove the acetonitrile mobile phase, and resuspended in 60 ml PBS buffer. Three independent preparations were conducted and purification results were summarized in Table 2.1.

High Performance Liquid Chromatography (HPLC). The purity of thurincin H preparations was monitored via HPLC using a Jupiter 300 C5 column (250 mm x 4.6 mm; 5 µm particle size; 300 Å pore size; Phenomenex, Torrance, CA) connected to an Agilent series 1100 HPLC system with in-line degasser, quaternary pump, and diode array detector set to monitor at 216 nm (Manns *et al*., 2012). A 50 µl sample was loaded onto the column, and active fractions were resolved using a starting mobile phase of 5% acetonitrile in water supplemented with 0.1% trifluoroacetic acid (TFA) and linearly increased to 100% acetonitrile (0.1% TFA) over a 30 min period at a 1 ml/min flow rate.

Tricine SDS-PAGE and silver staining. Thurincin H preparations were visually monitored on a three-layer tricine gel system consisting of a loading layer (4% acrylamide, 30% solution, 29:1 ratio; Bio-Rad, Hercules, CA), a stacking layer (10% acrylamide), and a resolving layer (15% acrylamide supplemented with 9% glycerol) as previously described (Manns *et al*., 2012). An appropriate amount of each sample and 2 µl of Precision Plus Protein Dual Xtra Standards Marker (Bio-Rad) ranging from 2 kDa to 250 kDa were boiled for 5 min in PAGE buffer (Schägger H, 2006), rapidly chilled on ice, and loaded onto the gel built on a mini-Protean III gel platform (Bio-Rad, Hercules, CA). After 120 min at 110 V, gels were thoroughly washed using

Milli-Q water and fixed in 5% glutaraldehyde for 1 hour with gentle shaking. Gels were rinsed multiple times and silver-stained using the recommended standard protocol (Bio-Rad, Hercules, CA).

Liquid Chromatography–Mass Spectrometry (LC-MS). LC-MS was performed at the Proteomics and Mass Spectrometry Facility at Cornell University Institute of Biotechnology to determine the accurate molecular weight mass (Ithaca, NY).

Minimum Inhibitory Concentration (MIC) of thurincin H against different bacterial strains. The MICs of thurincin H against 27 different bacteria were determined by the microtiter plate assay method described earlier. The lowest concentration (μg/ml) of purified thurincin H that allowed 50% growth of each strain was determined as the MIC (Eijsink *et al*., 1998). At least three independent assays were performed for each strain.

Inhibition against yeast and fungi. Inhibition to different yeasts and fungi strains were evaluated via a spot on lawn assay (Fujita *et al*., 2007). Fifty microliters of 48 hour cultures of yeast or fungi incubated in PDB were inoculated into 8 ml soft PDA (0.75% agar) and overlaid on a PDA base plate. Ten microliters of thurincin H (500 μg/ml) were spotted on top and incubated at 30°C. The presence/absence of clear inhibition zones were periodically checked within 24-48 hours. At least three assays were conducted for each strain.

Acidic and basic stability. Pure thurincin H resuspended in sterile water was mixed with sterile pH-adjusted TSB (varying integrally from 1 to 12, 8.2, 8.4, 8.6, and 8.8) at a 1:10 ratio. The initial bacteriocin activity of diluted thurincin H in TSB (at pH

7) was 320 AU/ml. After a 24-hour incubation time at 22°C, each mixture was neutralized by HCl or NaOH. Residual activity was measured by microtiter plate method. Six independent experiments were performed.

Thermal stability. Thurincin H (640 AU/ml) in PBS buffer was heated in a water bath at varying temperatures (50°C, 60°C, 70°C, 80°C, 90°C) for a set time period and immediately cooled on ice after treatment. Residual bacteriocin activity was measured by microtiter plate method. Six independent experiments were performed.

Inhibitory effect of thurincin H against *B. cereus* spores. Fifty microliters of thurincin H (500 µg/ml) diluted 1:2 in Tris-NaCl buffer (10 mM Tris, 10 mM NaCl, pH 7.4) was added to the wells of an untreated, clear, flat bottom 96 micro well plate (Thermo Scientific, Nunc, Denmark) and mixed with 150 µl of *B. cereus* F4552 spores resuspended in BHI. The final concentrations of spores in the mixture were 10^4, 10^5, 10^6, and10^7 CFU/ml. Tris-NaCl buffer served as a negative control. The minimum concentration that caused 50% inhibition of spore outgrowth was determined. Two independent experiments in triplicate were performed.

Effect of thurincin H on spore hydration. Absorbance of spore suspension at 600 nm (A_{600}) decreased as the spore rehydration in the germinating process caused alteration in its light-scattering behavior (Moir & Smith, 1990; Hornstra *et al*., 2005). Using a 96 well microtiter plate, a 150 µl aliquot of *B. cereus* spores resuspended in BHI or Tris-NaCl was combined with 50 µl of purified thurincin H in Tris-NaCl buffer to a final concentration of 100 µg/ml, 10 µg/ml, or 0 µg/ml (negative control). The final concentration of spores in presence/absence of thurincin H was approximately

10^7 CFU/ml. A_{600} was immediately read for 1 hour at a 2 min intervals. Before each reading, plates were automatically shaken rapidly for 30 s. The result was presented as the percentage of A_{600} at each time point normalized to the initial point. Two independent experiments were performed in duplicate.

Inhibitory effect upon germination. Spores were resuspended in BHI or Tris-NaCl buffer with or without thurincin H at a concentration of approximately 10^7CFU/ml and incubated in 30°C for 60 min. For the experimental group, the final concentration thurincin H was 100 µg/ml. After 60 min, all samples were immediately serially diluted with 0.1% peptone water and plated on TSA plate. Two independently prepared spores crops were assayed in triplicate.

RESULTS AND DISCUSSION

Bacteriocin production and purification. Based on a preliminary time-course study between incubation time and bacteriocin activity of cell-free supernatant, initial bacteriocin activity was detected after 8 hours, reached a stable activity maximum between 36 hours and 46 hours, and remained stable for at least 90 hours throughout an incubation time course at 37°C (data not shown). This bacteriocin activity curve is consistent with previous reports indicating the production of bacteriocins are triggered under conditions of high stress, such as overpopulation and nutrient limitation during early stationary phase (Riley & Gordon, 1999; Singh & Banerjee, 2008). For the three independent thurincin H purification trials, the supernatant was harvested after 40 hours of incubation at 37°C.

Based on the HPLC result of cell free supernatant in Figure 2.1 (A), most of the compounds residing in the supernatant were eluted prior to thurincin H (22.1 min).

No significant peaks were detected after 22.1 min, indicating the strong hydrophobicity of thurincin H. Following the ammonia sulfate precipitation step, the concentration and purity of thurincin H was significantly increased and was the dominant peak in the elution profile (Figure 2.1, B). The strongly hydrophobic nature of thurincin H was exploited to optimize its large scale purification using high capacity C18 cartridges. One hundred and fifty milliliters of crude protein extract resuspended in PBS buffer was loaded onto a C18 cartridge and eluted with increasing concentrations of acetonitrile. A single HPLC resolved peak resulted from the 35%, 45%, and 50% acetonitrile eluates as shown in Figure 2.1 (C). Each step of the purification process was visually assessed via silver-stained SDS-PAGE as the effects of each stage is shown, culminating in the presence of one single band for the final purified thurincin H (Figure 2.1, D). With a molecular mass below 4 kDa, 5% (v/v) glutaraldehyde demonstrated superior fixing results compared with acidified methanol (40% methanol and 10% acidic acid mixture). For further confirmation of purity and identity, the LC-MS data showed an intact molecular mass of 3139.52 Da, which is consistent with the previously reported molecular mass of thurincin H (data not shown) (Lee *et al*., 2009).

The hydrophobic nature of thurincin H was critical in devising a scaled-up purification process. The amino acid sequence showed that thurincin H is overall negatively charged, while the 3D structure elucidated by NMR demonstrated that the uncharged residues form a hydrophobic region on one side of the hairpin loop structure (Sit *et al*., 2011). This unique feature formed theoretical basis for the purification method as thurincin H binds to the C18 column more tightly than most of

the substances in the supernatant shown in Figure 2.1 (A).

Thurincin H was previously purified in small quantities using hydrophobic octyl-sepharose CL-4B cartridge (GE Healthcare, Piscataway, NJ). Subsequent to a crude ammonia sulfate precipitation and application to the cartridge, thurincin H was eluted by a continuously decreasing gradient of ammonia sulfate followed by an increasing gradient of ethanol in water (Lee *et al*., 2009). This laborious purification method is not feasible to purify large quantities of pure thurincin H, since the thurincin H peak overlap with neighboring contaminating peaks leading to a decreased yield as shown by the chromatography results. Furthermore, hundreds of samples had to be collected in order to simply identify the target thurincin H. This new C18 purification cartridge and the columns in HPLC both used carbon chain-based hydrophobic stationary phase as well as a similar composition for the mobile phase/eluent. A range of 35-50% acetonitrile was sufficient to elute the pure thurincin H from the cartridge, greatly increasing the reproducibility of the method compared with using a gradient-based mobile clean-up and elution scheme. This new purification method is simple and fast, giving an 81% bacteriocin activity recovery rate (Table 2.1) in only two steps. This is far superior than most purification methods, since usually more than two purification steps were applied during the entire purification process, resulting is cumulative losses at each step (Pingitore *et al*., 2007).

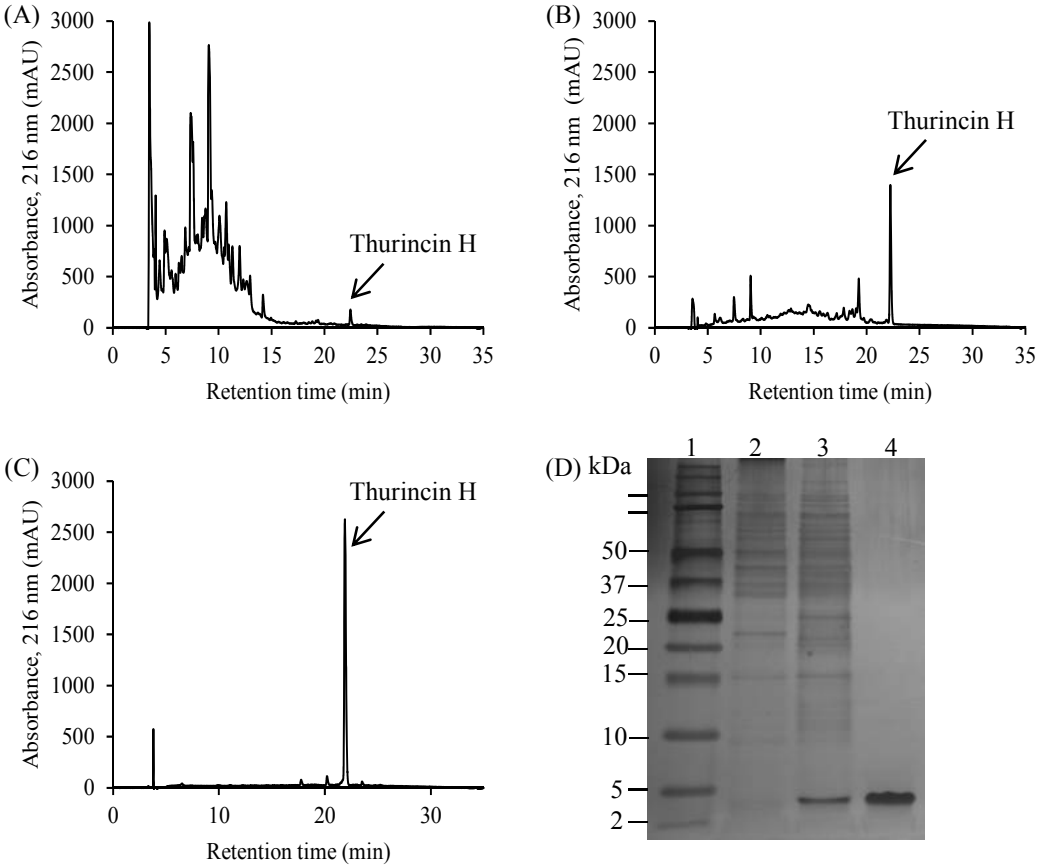

Figure 2.1 Purification of thurincin H. (A) HPLC of 40 hour supernatant. (B) HPLC of semi-purified thurincin H after ammonia sulfate precipitation. (C) Thurincin H after C18 purification. (D) SDS-PAGE and silver staining. Lane (1), Bio-Rad Precision Plus Protein™ Dual Xtra Standards; Lane (2), supernatant; Lane (3), semi-purified thurincin H after ammonia sulfate precipitation; lane (4), thurincin H after C18 purification.

Table 2.1 Purification summary of thurincin H[#]

Purification Step	Volume	Protein Concentration	Total Protein	Bacteriocin Activity	Total Activity	Specific Activity	Yield	Purification factor
Unit	(ml)	(mg/ml)	(mg)	(AU/ml)	(AU)	(AU/mg)	%	(fold)
Supernatant	750	5.506*	4129.500±115.564	267±80	200250±69282	48±18	100%†	1[†]
Ammonia sulfate precipitation	150	0.523	78.450±3.813	1280±554	192000±55426	2447±637	96%±34%	50±18
C18 purification（single peak on HPLC）	60	0.232	13.920±6.622	2702±1001	162120±39105	11647±2561	81%±9%	240±163

[#]Values shown in the table are means and standard deviations of three independent purification experiments. [†] The total activity of the supernatant was arbitrarily made as 100% yield and 1 fold of purification, serving as the starting point for subsequent purification results to compare. *Concentration of total concentration of protein was measured using BCA protein assay with results accurate to μg/ml.

Antimicrobial spectrum and MIC. A larger scale purification of thurincin H permitted an accurate examination of its inhibitory spectrum, allowing for a better comparison with the previously described spectrum (Lee *et al*., 2009). Instead of showing a typically narrow spectrum like lactic acid bacteriocins (Drider *et al*, 2006), thurincin H exhibited a wide antimicrobial spectrum against one or more species across several genera including *Bacillus, Listeria, Carnobacterium, Enterococcus, Staphylococcus* and *Geobacillus*. Among all the 22 sensitive strains, 14 of them are spore formers in or close to the *Bacillus* genus. The MICs were strain dependent, ranging from 0.28 nM to 21.9 nM for Gram-positive strains. None of the three Gram-negative bacteria were inhibited by thurincin H (Table 2.2).

Thurincin H is ineffective against any of the 9 selected yeast strains (*Candida albicans* 3153A, *Dekkera anomala*, *Geotrichum candidum* 755, *Pichia stipitis* CBS 6054, *Rhodotorula mucilaginosa*, *Saccharomyces bisporus*, *Saccharomyces cerevisiae*, *Zygosaccharomyces baillii*, and *Zygosaccharomyces bisporus*) or the 10 mold strains (*Aspergillus flavus*, *Aspergillus niger* 2270, *Byssochlamys fulva* G -1, *Byssochlamys fulva* H25, *Neosartorya fischeri, Penicillium expansum* 7861, *Penicillium vermiculation, Rhizopus oligosporus, Rhizopus oryzae,* and *Talaromyces flavus*). Several *Bacillus* strains were reported to inhibit growth of yeast and mold, but such studies only tested either the crude supernatant or a partially purified solution of bacteriocin (Abriouel *et al*., 2011). Those substances that inhibit the yeast or mold cannot be traced specifically to the bacteriocin in questions, and any inhibitory effects may be due other substances in solution. For example, the thurincin H producer *B. thuringiensis* SF361 also produces a 13.484 kD antifungal protein, YvgO, which

inhibits a wide variety of filamentous fungi ranging across several genera, including *Aspergillus*, *Penicillium*, and *Byssochlamys* (Manns *et al*., 2012). Furthermore, a highly purified bacteriocin is required to verify the activity of the specific bacteriocin under review, as more than one antimicrobial compounds are often produced by the same strain. For instance, *Bacillus subtilis* JM4 was reported to produce two antimicrobial peptides that differed by only one amino acid, subpeptin JM4-A and subpeptin JM4-B (Wu *et al*., 2005).

Table 2.2 MIC of thurincin H against different bacteria

Strains	Medium	Incubation (Hour)	Temp (℃)	MIC	
				(μg/ml)	(nM)
Bacillus cereus F4552	TSB	12	37	1.7	0.54
Bacillus cereus F4810	TSB	12	37	1.7	0.54
Bacillus cereus Northland	TSB	12	37	4.70	1.50
Bacillus cereus Northview P2E018	TSB	12	37	7.05	2.25
Bacillus licheniformis	TSB	12	37	68.75	21.90
Bacillus megaterium LRB89	TSB	17	37	0.44	0.14
Bacillus subtilis ATCC 6537	TSB	14	37	4.60	1.47
Bacillus subtilis CU1065(WT)	TSB	17	37	36.94	11.77
Bacillus subtilis LRB90	TSB	14	37	9.23	2.94
Bacillus subtilis LRB91	TSB	14	37	2.31	0.73
Bacillus subtilis ATCC 6633	TSB	14	37	1.15	0.37
Bacillus thuringiensis SF361	TSB	12	37	18.44	5.87
Bacillus thuringiensis EG10368	TSB	12	37	0.85	0.27
Carnobacterium piscicola CU216	APT	12	30	0.88	0.28
Enterobacter agglomerans J-1	TSB	12	37	-‡	-
Enterococcus mundtii EM	TSB	12	37	68.75	21.90
Geobacillus stearothermophilus ATCC 12980	TSB	24	50	0.88	0.28
Listeria innocua ATCC 2283	TSB	17	37	0.88	0.28
Listeria ivanovii ATCC 19119	TSB	12	37	0.88	0.28
Listeria monocytogenes F2 586 1053	TSB	17	37	1.76	0.56
Listeria monocytogenes 2289	TSB	17	37	1.76	0.56
Paenibacillus larvae subsp *larvae* ATCC 25747	TSB	12	37	-	-
Pseudomona syringae pv. *papulans* 51	TSB	12	37	-	-
Staphylococcus aureus ATCC 9144	TSB	14	37	36.94	11.77
Staphylococcus aureus ATCC 8095	TSB	14	37	18.44	5.87
Streptococcus faecalis ATCC 8043	TSB	12	37	-	-
Vibrio parahaemolyticus G1-166 (03:k6)	TSB	12	37	-	-

‡ The growth of the strain was not inhibited by the applied thurincin H in the experiment.
§There was no discernible deviation in the assay of the MICs for all samples measured.

Stability. Similar to many acid-stable yet alkaline-labile class I and class II lactic acid bacteriocins (Chen & Hoover, 2003), thurincin H is stable from pH 2-7 up to at least 24 hours. Bacteriocin activity was partially lost in pH 8-9 solutions, and completely lost at pH 9-11 within 24 hours at room temperature, as shown in Figure 2.2 (A).

When thurincin H was exposed to elevated temperatures in PBS buffer, the bacteriocin activity remained for 30 min at 50°C but half of the activity dropped at 50°C by 60 min, at 60°C by 10 min, and at 70°C in less than 10 min. Thurincin H lost most of its activity within 3 min temperatures above 80°C, as in Figure 2.2 (B). Compared with thurincin H, some lactic acid bacteria could remain stable after extreme heat treatments (eg. plantaricin LP84, 20 min at 121°C) (Suma *et al*., 1998).

Samples treated at 100°C for 10 min and at pH 10 for 12 hours (and subsequently neutralized) were analyzed via HPLC and LC-MS. The peak representing the intact thurincin H at 22.1 min completely disappeared and several novel, yet earlier eluting peaks dominated the HPLC spectra (data not shown). Furthermore, an intact molecular mass for thurincin H was not detected in the heat or alkaline processed samples via LC-MS. These results indicated that the loss of activity was not simply due to a conformational change, but chemical degradation. The peptide backbone of thurincin H after thermal and basic condition treatment might be degraded.

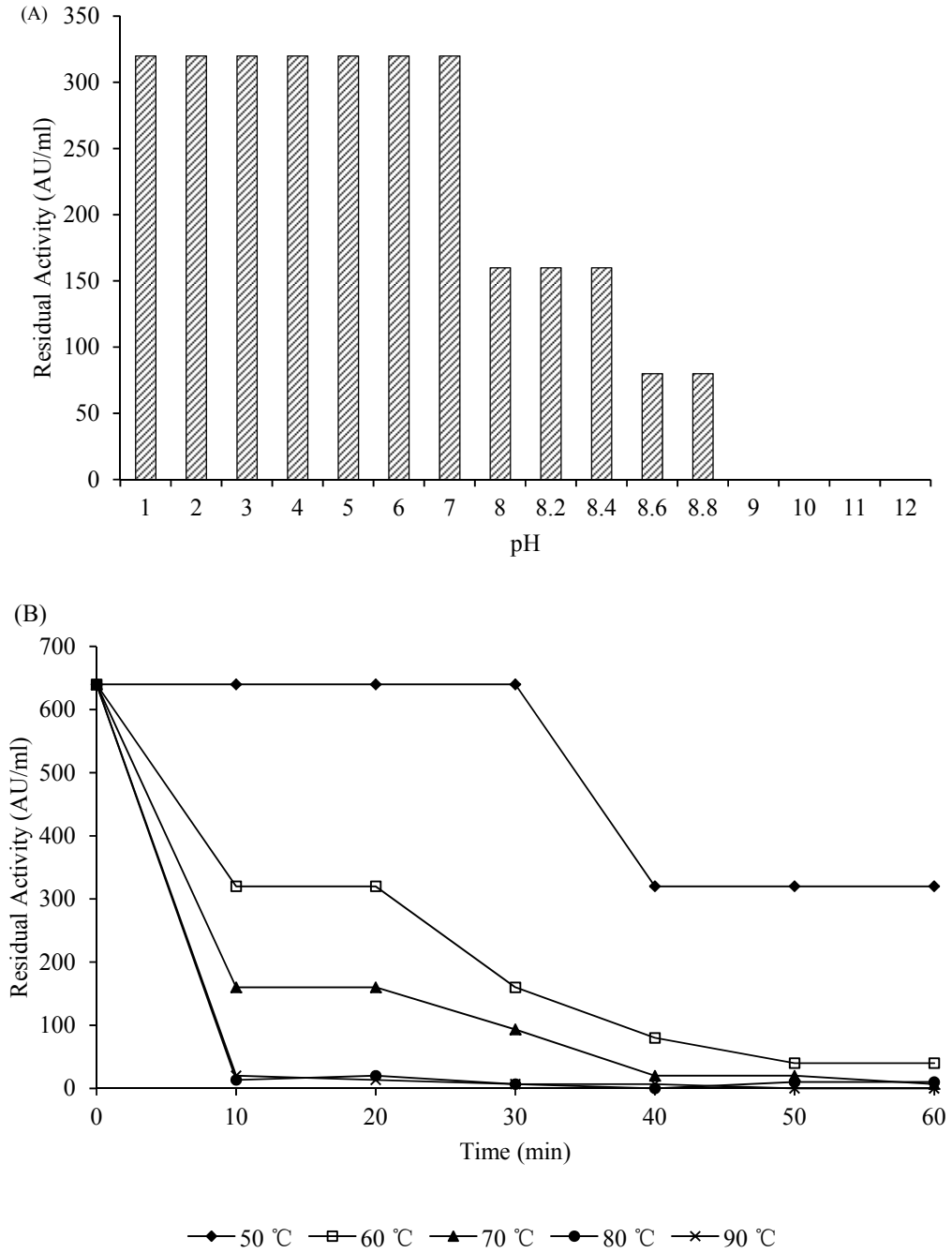

Figure 2.2 Stability of thurincin H. (A) Thurincin H was stable under acidic conditions for 24 hours, but gradually lost activity in alkaline conditions. (B) Thurincin H was heat labile. The data in shown in the figure are means of multiple different independent experiments, and there was no discernible deviation in the microtiter bacteriocin activity assays for all the samples measured.

Effect of thurincin H on *Bacillus* spore outgrowth. The MICs of thurincin H against *B. cereus* F4552 spores were 3.8 μg/ml for 10^4 CFU/ml, and 7.6 μg/ml for 10^5-10^7 CFU/ml at 30°C in BHI for 12 hours. At the same incubation medium and temperature, no optical density increase of *B. cereus* was observed at the concentration of equal to or higher than 2 times of MIC for up to one week. After incubating thurincin H with spores for one week at 30°C, the suspension was centrifuged and plated out on BHI agar plate. No colonies were found on the plates, which indicated that no viable spores or germinated spores or vegetative cells exist in the mixture (data not shown).

To determine if thurincin H could induce or block spore germination initiation, the A_{600} of spores incubated in the presence of thurincin H was measured (Stewart *et al*., 1981). As shown in Figure 2.3 (A), when spores were resuspended in Tris-NaCl buffers (restricting germination), the A_{600} remained stable with or without the presence of thurincin H. When spores were resuspended in a nutrient-rich BHI broth (encouraging germination), the A_{600} dropped at the same level in the presence or absence of thurincin H. These results indicate that thurincin H could neither induce spore germination initiation in nutrient deficient conditions nor block spore germination initiation in nutrient sufficient conditions.

To determine if thurincin H kills intact spores before spore germination initiation, thurincin H was mixed with *B. cereus* spores in Tris-NaCl buffers or BHI nutrient broth. The results showed that the spores were killed only when germination is induced by matrix nutrients. Spores in Tris-NaCl buffer in presence of thurincin H, did not kill spores compared with control, as seen in Figure 2.3 (B). This indicates that

germination is a prerequisite for the bacterial sporicidal action of thurincin H.

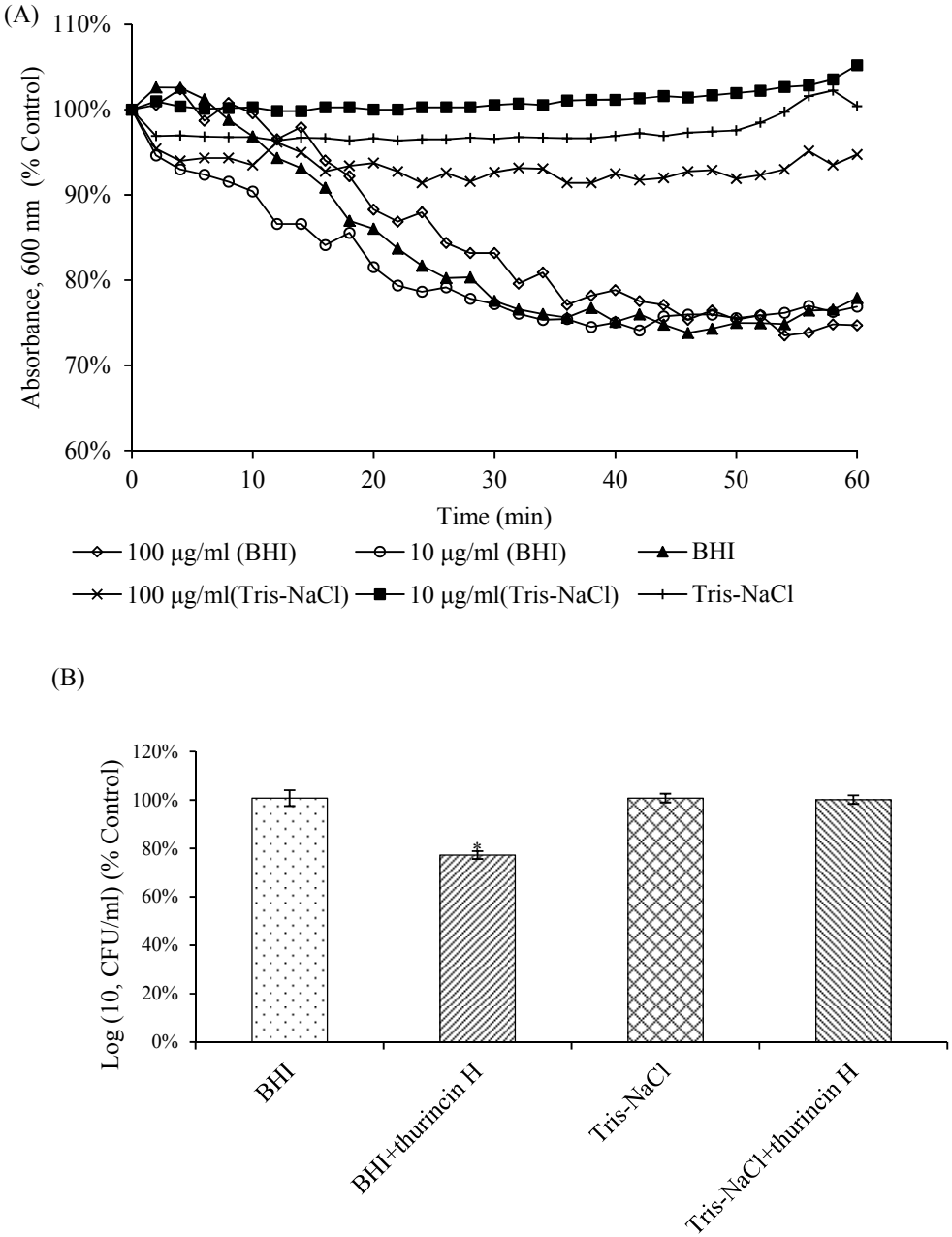

Figure 2.3 Inhibitory effect of thurincin H against *Bacillus* spores. (A) Thurincin H does not prevent *Bacillus* spore germination initiation in BHI or induce *Bacillus* spore germination initiation in Tris-NaCl buffer. (B) Germination is required for the action of thurincin H. Error bars describe the standard deviation for three independent experiments. * shows significant lower value, $p<0.05$.

CONCLUSIONS

A new large scale production and purification system exploiting the strongly hydrophobic feature of thurincin H was developed. Highly pure thurincin H showed a wide inhibition spectrum against primarily Gram-positive bacteria, but not against tested yeasts and molds. Nascent studies involving potential biological modes of action reveal that germination initiation is a prerequisite for the sporicidal action of thurincin H.

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CHAPTER 3

DEVELOPMENT OF A HETEROLOGOUS EXPRESSION SYSTEM FOR THE SYSTEMATIC SITE-DIRECTED MUTAGENESIS OF THURINCIN H, A BACTERIOCIN PRODUCED BY *BACILLUS THURINGIENSIS* SF361

ABSTRACT

Thurincin H is an antimicrobial peptide produced by *Bacillus thuringiensis* SF361. With a helical back bone, the 31 amino acids of thurincin H form a hairpin structure maintained by four pairs of very unique sulfur to α-carbon thioether bonds. The production of thurincin H depends on a putative gene cluster containing 10 open reading frames. The gene cluster includes three tandem structural genes (*thnA1, thnA2* and *thnA3*) encoding the three identical 40 amino acid thurincin H prepeptides, and seven other genes putatively responsible for prepeptide processing, regulation, modification, exportation, and self-immunity. A heterologous thurincin H expression system was developed by transforming a thurincin H deficient host with the novel expression vector pGW133. The host, designated *B. thuringiensis* SF361thnH$^-$, was constructed by deletion of the three tandem structural genes from the chromosome of the native thurincin H producer. The thurincin H expression vector pGW133 was constructed by cloning the thurincin H native promoter, *thnA1,* and a Cry protein terminator into the *E. coli-B. thuringiensis* shuttle vector pHT315. Thirty three different pGW133 variants, each containing a different point mutation in the *thnA1* gene, were generated and separately transformed into *B. thuringiensis*

SF361thnH$^-$. Those site-directed mutants contain either a single radical or conservative amino acid substitution on the thioether linkage-forming positions, or a radical substitution on other non-alanine amino acids. Bacteriocin activities of *B. thuringiensis* SF361thnH$^-$ carrying different pGW133 variants against three different indicator strains were subsequently compared.

INTRODUCTION

Bacteriocins are ribosomally synthesized, antimicrobial peptides or proteins produced by bacteria, usually with a narrow inhibitory spectrum, although notable exceptions exist (Cotter *et al.*, 2013). Bacteriocins produced by lactic acid bacteria (LAB) have been extensively studied and used as natural food preservatives. Due to the fact that lactic acid bacteria are generally recognized as safe (GRAS status), they have been investigated as potential agents for preventing spoilage and enhancing the safety of foods. The LAB bacteriocins can be divided into three main classes: the class I lantibiotics, containing a lanthionine thioether bond linking the sulfur atom of cysteines with the β-carbon of other amino acids; the class II non-lanthionine-containing bacteriocins; and the class III heat-labile large proteins (Cotter *et al.*, 2005). In recent years, bacteriocins produced by *Bacillus* spp. have gained increasing research interest since many of them exhibit a broader antimicrobial spectrum compared with most lactic acid bacteriocins, anticipating potential applications in the food, agricultural, and pharmaceutical industries in controlling various spoilage and pathogenic microorganisms (Abriouel *et al.*, 2011; Lee & Kim, 2011). One *Bacillus* bacteriocin, thurincin H, is an antimicrobial peptide

produced by *Bacillus thuringiensis* SF361, a strain originally isolated from US domestic sunflower honey. It exhibits inhibitory activity against a wide range of Gram-positive bacteria including different foodborne pathogens and spoilage bacteria, such as *Listeria monocytogenes*, *Bacillus cereus*, and *Micrococcus* spp.(Lee *et al.*, 2009). Thurincin H contains four pairs of unique sulfur to α-carbon thioether bridges (Figure 3.1) which is quite different from those extensively studied group of class I lantibiotics, since the structure of lantibiotics was maintained by sulfur to β-carbon bridges (Willey & van der Donk, 2007).

As elucidated by bioinformatics studies, the *thnP-thnI* gene cluster is responsible for the production of mature active thurincin H. It is composed of three tandem thurincin H prepeptide genes (*thnA1*, *thnA2* and *thnA3*), as well as the *thnP, thnB, thnD*, *thnE, thnT*, *thnR, thnI* genes, putatively required for thurincin H prepeptide processing, regulation, modification, exportation, and immunity. It was proposed that *thnA1*, *thnA2* and *thnA3* genes are first translated into three identical 40 amino-acid thurincin H prepeptides and subsequently modified by ThnB, a member of the radical S-adenosylmethionine (SAM) superfamily of enzymes, to form the thioether bonds. The leader peptide is subsequently cleaved and mature thurincin H is exported to the extracellular environment (Lee *et al.*, 2009). The mature 31 amino acids of thurincin H feature a helical backbone that is folded over to a hairpin structure maintained by linking the sulfur atoms of the four cysteines (Cys4, Cys7, Cys10 and Cys13) to the α-carbons of one asparagine (Asn19), two threonines (Thr22 and Thr25) and one serine (Ser28) (Sit *et al.*, 2011b).

In this study, a heterologous thurincin H expression system was constructed by introducing the newly constructed expression vector pGW133 to *B. thuringiensis* SF361thnH$^-$, in which the three tandem structural genes (*thnA1*, *thnA2* and *thnA3*) were in-frame deleted from the chromosome of the native thurincin H producer. The expression vector was developed based on the *E. coli-B. thuringiensis* shuttle vector pHT315 (Arantes & Lereclus, 1991). A commonly used expression system such as *Escherichia coli* (Ingham *et al.*, 2005; Lohans & Vederas, 2012) or lactic acid bacteria (Rodriguez *et al.*, 2003) was not used in the expression of thurincin H since the genes in the *thnP-thnI* gene cluster are indispensable for the biosynthesis and maturation of the thurincin H. These genes might not exist or could not be expressed in those commonly used hosts.

Using the newly developed expression system, systematic site-directed mutagenesis was performed on the mature thurincin H to explore the altered bacteriocin activity of 33 thurincin H variants, aiming to determine the critical amino acids that are critical for its inhibitory activity. Specifically, radical and conservative single site directed mutagenesis were performed to mutate the sulfur to α-carbon bond forming amino acids. Additionally, radical single site directed mutagenesis was performed to mutate all the non- sulfur to α-carbon bond forming amino acids.

MATERIALS AND METHODS

Bacterial strains, culture conditions, plasmids and primers. Both *Bacillus* and *E. coli* strains used in this study were cultivated at 37°C in trypticase soy broth (TSB) or on trypticase soy agar (TSA) (BD, Sparks, MD). *B. cereus* F4552 was used as the

indicator strain for testing the antimicrobial activity of the constructed heterologous expression system. *E. coli* DH5α was used for cloning recombinant plasmids. *E. coli* K12 ER2925 (NEB, MA) was used to produce demethylated plasmids as a preparation for *B. thuringiensis* transformation (Macaluso & Mettus, 1991). Erythromycin (25 μg/ml) or ampicillin (100 μg/ml) was supplemented in TSA plates used to select transformants of *B. thuringiensis* or *E. coli*, respectively. *B. cereus* F4552, *B. thuringiensis* EG10368, and *Listeria monocytogenes* 2289 were used as indicator strains in evaluating the bacteriocin production activity of each constructed mutant. All chemicals and reagents were either autoclaved at 121°C for 15 min or filtered through a polyethersulfone (PES) membrane (0.22 μm; Celltreat, China). All plasmids and primers used in expression vector construction are listed in Table 3.1. Primers used in site directed mutagenesis are listed in Table 3.2. All primers used in this study were synthesized by Integrated DNA Technologies (Coralville, Iowa). All DNA sequencings were performed at the Biotechnology Resource Center at Cornell University (Ithaca, NY) by an Applied Biosystems Automated 3730xl DNA Analyzer using Big Dye Terminator chemistry and AmpliTaq-FS DNA Polymerase.

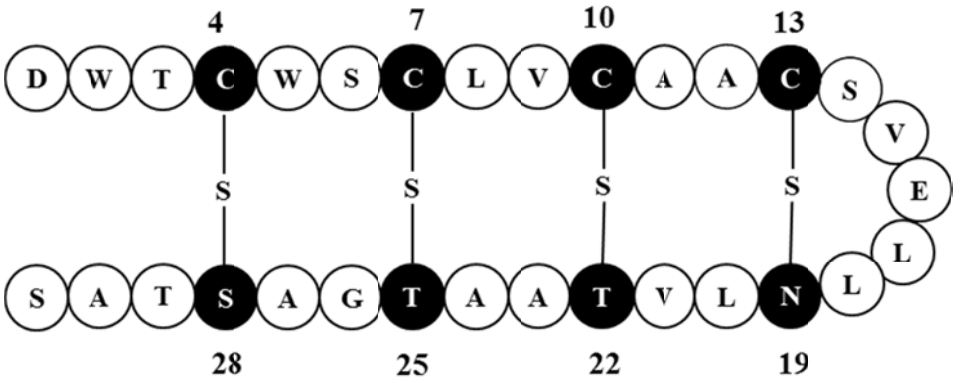


Figure 3.1 Structure of thurincin H. Four sulfur to α-carbon thioether linkages maintain the hairpin structure.

Table 3.1 Strains and plasmids used in this study

Strains	Description	Reference
E. coli DH5α	F^- φ80*lac*ZbM15 b(*lac*ZYA-*arg*F) U169 *rec*A1*end*A1 *hsd*R17 (rk^-, mk^+) *gal*$^-$ *pho*A *sup*E44 λ^- *thi*$^-$1*gyr*A96 *rel*A1	NEB, MA
E. coli K12 ER2925	*Produce demethylated plasmids, ara-14 leuB6 fhuA31 lacY1 tsx78 glnV44 galK2 galT22 mcrA dcm-6 hisG4 rfbD1 R(zgb210::Tn10)TetS endA1 rpsL136 dam13::Tn9 xylA-5 mtl-1 thi-1 mcrB1 hsdR2*	NEB, MA
B. thuringiensis SF361	Thurincin H producer strain, carrying the putative thurincin H producing gene cluster	Lab stock
B. thuringiensis SF361thnH$^-$	Thurincin H producer strain with in-frame deletion of the *thnA1*, *thnA2* and *thnA3*	This study
B. cereus F4552	Indicator strain, sensitive to thurincin H	(Lee *et al.*, 2009)
B. thuringiensis EG10368	Indicator strain, sensitive to thurincin H	(Lee *et al.*, 2009)
Listeria monocytogenes 2289	Indicator strain, sensitive to thurincin H	(Lee *et al.*, 2009)

Table 3.2 Plasmids and primers used in developing expression system

Plasmids	Description	References
pMAD	Vector for efficient allelic replacement, thermosensitive, carrying *bga*B, Ery^R Amp^R	(Arnaud *et al.*, 2004)
pMADΔ*thnA1A2A3*	pMAD carrying the upstream and downstream DNA sequences of *thnA1*, *thnA2* and *thnA3*, used for homologous recombination	This study
pHT315	*E. coli-B. thu*ringiensis shuttle vector, 6.5 KB, Ery^R Amp^R	(Arantes & Lereclus, 1991)
pGW131	*thnA1, thnA2 and thn A3* genes with native promoter (P_{nat}) and native terminator (T_{nat}), cloned in pHT315	This study
pGW132	*thnA1* gene with native promoter (P_{nat}) and native terminator(T_{nat}), cloned in pHT315	This study
pGW133	*thnA1* gene with native promoter (P_{nat}) and Cry protein terminator(T_{cry}), cloned in pHT315	This study
Primers	**Sequence**	**Restriction enzyme**
DEL1	gatgga*ggatcc*aataccaattttgtttttagactttctttcatgcttatccatat	*Bam*HI
DEL2	gatgga*aagctt*ttgtactactggtgtttccatttgacc	*Hin*dIII
DEL3	gatgga*aagctt*ggagctagtactgcaagttaaaatttaaaaatgtgagag	*Hin*dIII
DEL4	gaagga*ccatgg*tgcatatcaatttgcacaaatgttttcgactt	*Nco*I
DTC1	cattgttaaatatggattccttaatttgctcctttatctgttg	NA
DTC2	cgctctcgtttgggtttttatgaactacc	NA
TH01	gatgga*ctgcag*gtaaatatatgtcacaaaatattaaagaaacacacacaaaatgtttg	*Pst*I
TH02	gatggt*gaattc*gtttttagtttatgtattacaaaaatcccatactcgttttcg	*Eco*RI
TH03	atggaaacaccagtagtacaaccaagg	NA
TH04	ttagcttgcagtactagcccctgt	NA
TH05	gatgga*ctgcag*gtaaatatatgtcacaaaatattaaagaaacacacacaaaatgtttgaaa tttttgattgtttttagaaaacatagggagttatactttagtcgcaccacacaatacagaaaag ggggtaggtcaaatggaaacaccagtagtacaaccaagg	*Pst*I
TH06	gatggt*gaattc*gtttttagtttatgtattacaaaaatcccatactcgttttcgataaatttatagg acttattcttaacataactgtttgtcattagaataagtcctaatttaatttcaccataaacactctc acatttttaaattttagcttgcagtactagcccctgt	*Eco*RI
TH07	gatggt*gaattc*tagtaaaacggacatcactccgtttcaatggaggtgatgtccgttttattta atttcaccataaacactctcacatttttaaatttta	*Eco*RI
DTC3	ctgcaaggcgattaagttgggtaac	NA
DTC4	cggataacaatttcacacaggaaacagcta	NA

Bacteriocin activity assays. Bacteriocin activity on TSA plates was detected using a deferred antagonism assay as previously described (Birri *et al.*, 2010) with modifications. *B. thuringiensis* SF361 (on TSA) and *B. thuringiensis* SF361thnH$^-$ carrying different plasmids (on TSA with 25μg/ml Erythromycin) were incubated for 12 h at 37°C. Single colonies were subsequently spotted on TSA and incubated for at 37°C for 15 h. Fifty microliters of overnight indicator strain culture was inoculated into 8 ml soft TSA (50°C, 0.75% agar) and overlaid on top of the plates. After an incubation at 25°C for 12 h, the diameter of the inhibition zones around the colonies were measured.

Bacteriocin activity in liquid medium was detected by a previously described microtiter plate assay (Daba *et al.*, 1991) modified for the current study. In brief, using untreated, clear, flat bottom 96 micro well plates (Thermo Scientific, Nunc, Denmark), 50 μl of bacteriocin diluted two-fold in the appropriate buffer was mixed with 150 μl of 1.33% (v/v) *B. cereus* F4552 overnight culture in TSB and incubated at 37°C for 8 h. The absorbance at 600 nm (A_{600}) of each well was measured using a Synergy HT Multi-Mode Microplate Reader (BioTek, Winooski, VT). One arbitrary unit (AU) was defined as the amount of bacteriocin in the 50 μl sample that caused a 50% growth inhibition compared with control group.

General DNA manipulation. General molecular cloning methods used in this study were performed as previously described by Sambrook and Russell (Sambrook & Russell, 2001), unless otherwise indicated. PrimeSTAR® Max DNA Polymerase (Takara, R045) was used for all PCR reactions, including colony PCR, using a total 50 μl mixture

volume. The PCR protocol includes a template denaturation step at 98°C (1 min) followed by 30 cycles of denaturing at 98°C (10 s), annealing at 55°C (10 s), and polymerization at 72°C (30-60 s), with one final extension at 72°C (7 min). Generally, to construct recombinant plasmids, PCR products were purified, double digested with high fidelity restriction enzymes (NEB, MA) for 2 h at 37°C and ligated to the double digested plasmids overnight at 16°C by T4 ligase. Recombinant plasmids were transformed using commercial competent *E. coli* DH5α by the heat shock method according to the standard protocol (NEB, MA). Plasmids were purified from *E. coli* DH5α using QIAprep Spin Miniprep Kit (QIAGEN) and passed through *E. coli* K12 ER2925 to produce a demethylated plasmid in preparation for electroporation into *B. thuringiensis* strains (Macaluso & Mettus, 1991). Recombinant plasmids were transformed to *B. thuringiensis* strains as previously described (Lereclus *et al.*, 1989). Sequences of all recombinant plasmids were confirmed by DNA sequencing.

Non-thurincin H producing host construction. The non-thurincin H producing host (*B. thuringiensis* SF361thnH$^-$) was constructed by an in-frame deletion of *thnA1*,*thnA2* and *thnA3* from the wild type producer *B. thuringiensis* SF361 using a homologous recombination method through the theromosentive suicide plasmid pMAD, as described by Arnaud (Arnaud *et al.*, 2004). pMAD is a plasmid which carries a *bgaB* gene encoding a thermostable galactosidase for blue and white screening (Arnaud *et al.*, 2004). A 984 bp *Bam*HI/*Hin*dIII DNA fragment (with primer DEL1 and DEL2) and an 895 bp *Hin*dIII/ *Nco*I DNA fragment (with primer DEL3 and DEL4) corresponding to the

regions upstream and downstream of *thnA1*, *thnA2 and thnA3* were amplified by PCR and cloned into pMAD. In ligating the fragments into pMAD, the upstream, downstream, and digested pMAD were mixted in the same reaction at a ratio of 3:3:1. The recombinant plasmid pMADΔ*thnA1A2A3* transformed *E. coli* DH5α, was passed through *E. coli* K12 ER2925, and subsequently transformed *B. thuringiensis* SF361 as previously described (Lereclus *et al.*, 1989). Tranformants were inoculated in 5 ml TSB, incubated for 3 h at 42°C followed by 3 h at 30°C, and plated on TSA containing X-Gal (50 μg/ml). White colonies were selected for further confirmation. Using primers designed accroding to the chromosomal region flanking the *thnA1,thnA2* and *thnA3* genes (DTC1 and DTC2), DNA fragments amplified by colony PCR from the wild type producer and the selected white colonies were compared for size differences. The expected sequence in the deletion mutant was also confirmed by DNA sequencing.

To determine if all other genes neccessary for thurincin H production still function properly after the deletion, a complementation experiment was performed. Specifically, a fragment containing the native promoter (P_{nat}), three tandem structural genes (*thnA1, thnA2* and *thnA3*), and native terminator (T_{nat}) was amplified from *B. thuringiensis* SF361 using primers TH01 and TH02, and cloned into pHT315, resulting in the plasmid pGW131. The pGW131 was transformed into *B. thuringinesis* SF361thnH$^-$ and colonies displaying a white morphology with the expected DNA sequence and was plated on TSA (Erythromycin 25μg/ml). The bacteriocin production of the deletion mutant and transformants were detected using a deferred antagonism assay as described above (Birri

et al., 2010) and compared to the wild type producer.

Construction of heterologous expression vectors. In preparation for site directed mutagenesis, two plasmids each carrying a single copy of the structural gene *thnA1* under the native promoter were constructed. First, *thnA1* was amplified using primers TH03 and TH04, and purified using QIAquick PCR Purification Kit (Qiagen, USA). Using the purified *thnA1* gene as a template, an insertion fragment containing the native promoter (P_{nat}), *thnA1* gene, and the native terminator (T_{nat}) was amplified with primers TH05 and TH06, cloned into pHT315, resulting in the pGW132 plasmid. Second, using the purified pGW132 inserts as the template, another fragment containing the native promoter (P_{nat}), *thnA1*, and Cry terminator (T_{cry}) (Wong & Chang, 1986) was amplified with primers TH01 and TH07, and cloned into pHT315, resulting in the plasmid pGW133. In plasmid pGW133, the reverse primer contained the sequence of the Cry terminator loop. These two constructed plasmids (pGW132 and pGW133) were used separately to transform *B. thuringiensis* SF361thnH$^-$. The antimicrobial activity of both transformants was detected by the modified deferred antagonism assay as described above.

Thurincin H production in broth by *B. thuringiensis* SF361thnH$^-$pGW133 was compared to the wild type producer. Fresh overnight colonies of *B. thuringiensis* SF361 and *B. thuringiensis* SF361thnH$^-$ pGW133 from TSA (Erythromycin 25µg/ml) were inoculated into 5 ml of TSB and incubated at 37°C for 12 hours with shaking at 225 rpm. A 1.5 ml aliquot of this incubation was added into 150 ml TSB and incubated at 37°C for up to 90 h with shaking at 225 rpm. Samples were taken every 3 h, centrifuged (8000 x *g*,

4°C, 5 min), filtered (PES membrane, 0.22μM) and stored at -20°C. The antimicrobial activity of each sample was quantified by the microtiter plate method as described above. Five independent experiments were performed.

Site directed mutagenesis. A total of thirty three different pGW133 plasmids, each with a different mutated *thnA1* gene, was generated by using a QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Mutagenesis reactions were performed in a BioRad T100 thermal cycler by using *PfuUltra* HF DNA polymerase in the kit according to the manufacturer's guidelines with modifications. Each reaction mixture contained 2.5 μl 10x reaction buffer, 18.5 ng plasmid template, 62.5 ng of each oligonucleotide primer, 0.5 μl dNTP mix, 0.75 μl QuickSlotion reagent, 0.5μl QickChange Lightning Enzyme, and was brought up to 25 μl with sterile Milli-Q water (Millipore Corporation, Billerica, MA). The mutagenesis reaction was performed with a program of the following conditions: initial one cycle of 95°Cfor 2 min, followed by 18 cycles of a set of reactions composed of 20 s at 95°C 10 s at 60°C, and 4 min at 68°C, with one final cycle of 68°C for 5 min. The resulting products were digested for 5 min at 37°C using the endonuclease *Dpn*I to eliminate the methylated and hemi-methylated DNA containing the non-mutated sequence originating from the template plasmids. The nicked vector containing the expected mutation was transformed to *E. coli* XL10-Gold ultracompetent cells and the nick was sealed by *E. coli* DNA repair systems. Plasmids were purified, passed through *E. coli* K12 ER2925, and transformed *B. thuringiensis*

SF361thnH$^-$. The DNA sequences of all mutated plasmids were verified by DNA sequencing.

Antimicrobial activity assay on plates. Inhibitory activities of the 33 obtained site directed mutants were compared to *B. thuringiensis* SF361thnH$^-$pGW133 by the deferred antagonism assay using *B. cereus* F4552, *B. thuringiensis* EG10368 and *L. monocytogenes* 2289 as indicator strains, as described above. Four independent sets of experiments were performed on each strain.

RESULTS

Thurincin H deficient expression host engineered from wild type producer. The structural genes *thnA1, thnA2* and *thnA3* were deleted in-frame based on the double crossover homologous recombination method using the thermosenstive plasmid pMAD (Figure 3.2, A). The deletion of the *thnA1*, *thnA2* and *thnA3* on the white colonies was first confirmed by PCR, where the amplicon was run on agarose gels and revealed that the product from each deletion mutant was smaller than the wild type (Figure 3.2, B). Subsequent confirmation was made by DNA sequencing (data not shown).

Deletion mutants with the expected DNA sequence lost the ability to inhibit the sensitive indicator strain *B. cereus* F4552 (Figure 3.2, C, colony 2). As further verification, pGW131 containing the native promoter (P_{nat}), *thnA1*, *thnA2* and *thnA3*, and the thurincin H native terminator (T_{nat}) was constructed and was used to transform the deletion mutants, in order to test if it could complement the production of thurincin H prepeptide. The results showed that *B. thuringiensis* SF361 thnH$^-$ carrying pGW131 (Figure 3.2, C, colony

3) exhibited a similar sized zone of inhibition compared to the wild type (Figure 3.2, C, colony 1), indicating that the structural gene in the pGW131 successfully complemented the mature thurincin H production and secretion pathway in the constructed mutant.

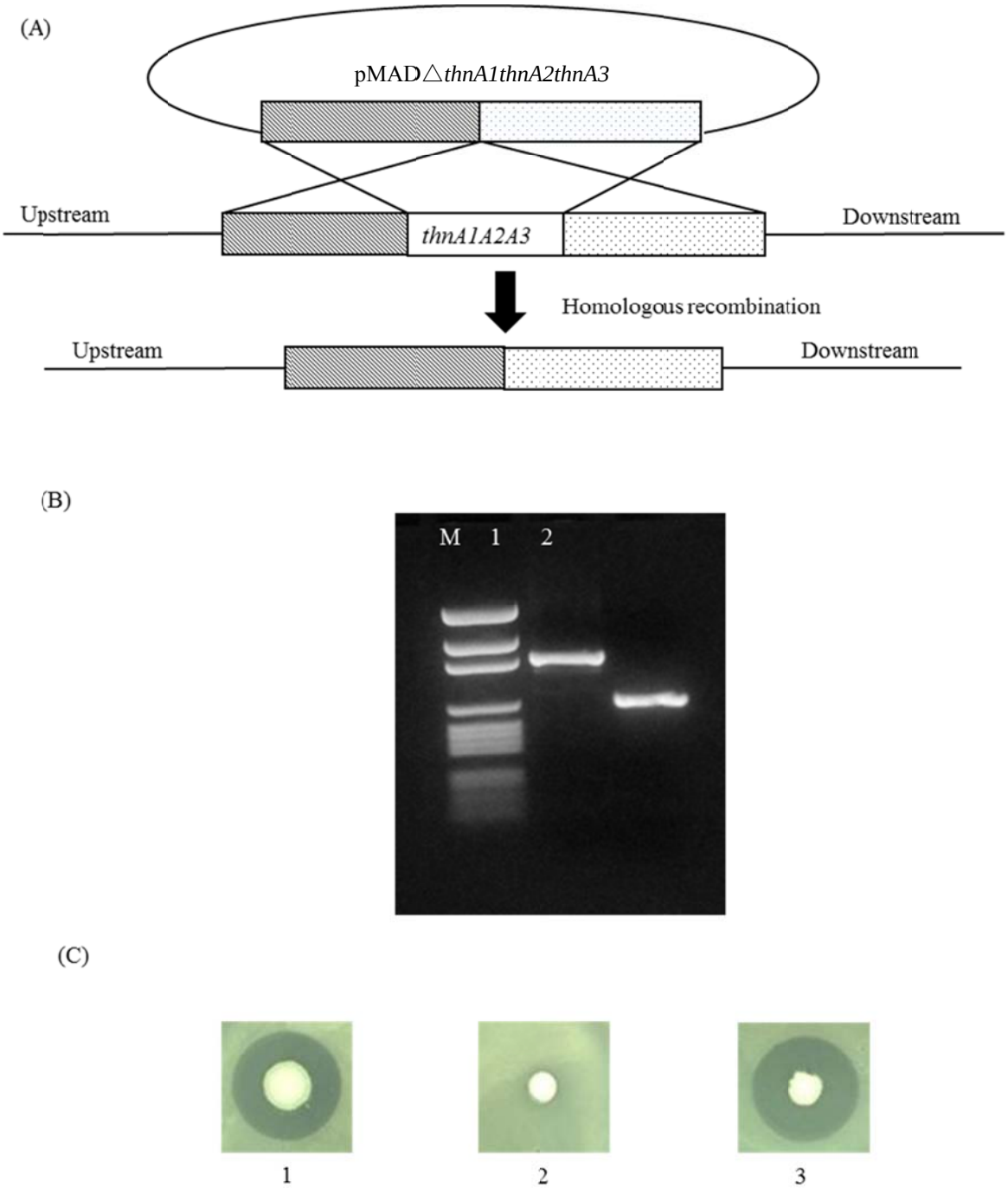


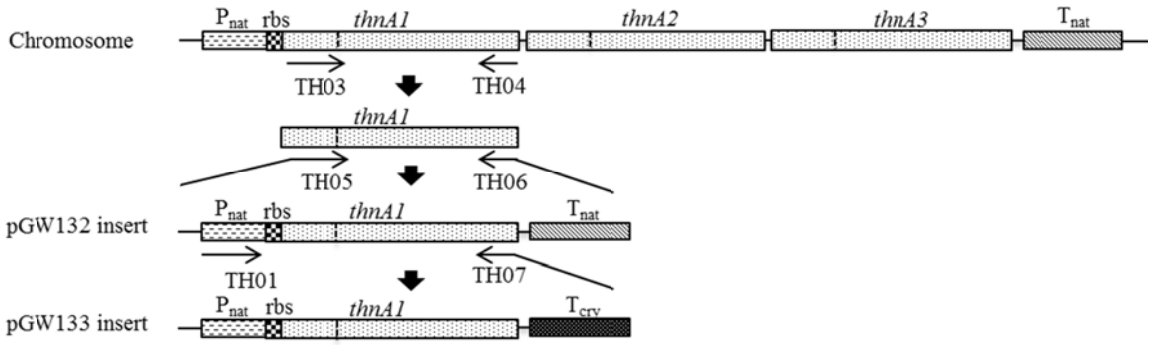
Figure 3.2 In-frame deletion of structural genes (*thnA1*, *thnA2* and *thnA3*) by homologous recombination. (A) Scheme of homologous recombination using pMAD; (B) Confirmation of deletion by PCR. Lane 1, BenchTop pGEM® DNA Markers (Promega); Lane 1, WT; Lane 2, deletion mutants; (C) Bioactivity assay of WT (left), deletion mutants (middle), and *B. thuringiensis* SF361thnH⁻pGW131(right).

Expression vector construction and optimization. The plasmid construct, pGW132, containing the native promoter (P_{nat}), *thnA1,* and native terminator (T_{nat}) was preliminarily constructed to express thurincin H in preparation for site direct mutagenesis (Figure 3.3, A). *B. thuringiensis* SF361thnH$^-$ pGW132 (Figure 3.3, B, colony 3) exhibited a significantly smaller inhibition zone compared with the wild type (Figure 3.3, B, colony 1).

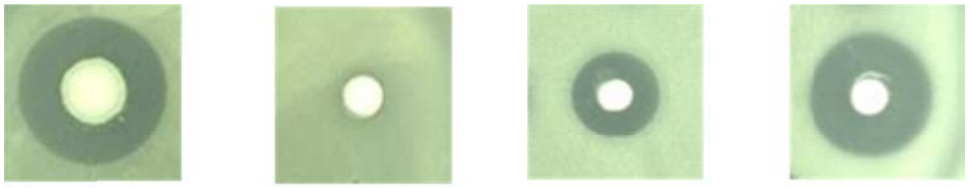
To improve the heterologous expression level of thurincin H, the native promoter (T_{nat}) was replaced with a CryIAa protein terminator (T_{cry}) originally found in *B. thuringiensis* subsp. *kurstaki* HD1, leading to the construction of pGW133 (Figure 3.3, A). The results indicated that bacteriocin activity of *B. thuringiensis* SF361thnH$^-$ pGW133 was significantly increased compared with pGW132, reaching a similar expression level as the wild type (Figure 3.3, colony 4).

To evaluate the bacteriocin production of *B. thuringiensis* SF361thnH$^-$ pGW133 in liquid broth, it was incubated in TSB without antibiotics and compared with the bacteriocin production level of the wide type producer under the same conditions. The production level of *B. thuringiensis* SF361thnH$^-$ pGW133 reached similar levels as the wild type producer (Figure 3.3, C). No selective antibiotics were added to the TSB medium during the 90 h production since pHT315 has been reported to be stably maintained in a *B. thuringiensis* host without selective pressure (Arantes & Lereclus, 1991; Okay *et al.*, 2008).

(A)


Chromosome
Pnat
rbs
thnA1
thnA2
thnA3
Tnat
TH03
TH04
thnA1
TH05
TH06
pGW132 insert
Pnat
rbs
thnA1
Tnat
TH01
TH07
pGW133 insert
Pnat
rbs
thnA1
Tcry

(B)



1 2 3 4

(C)

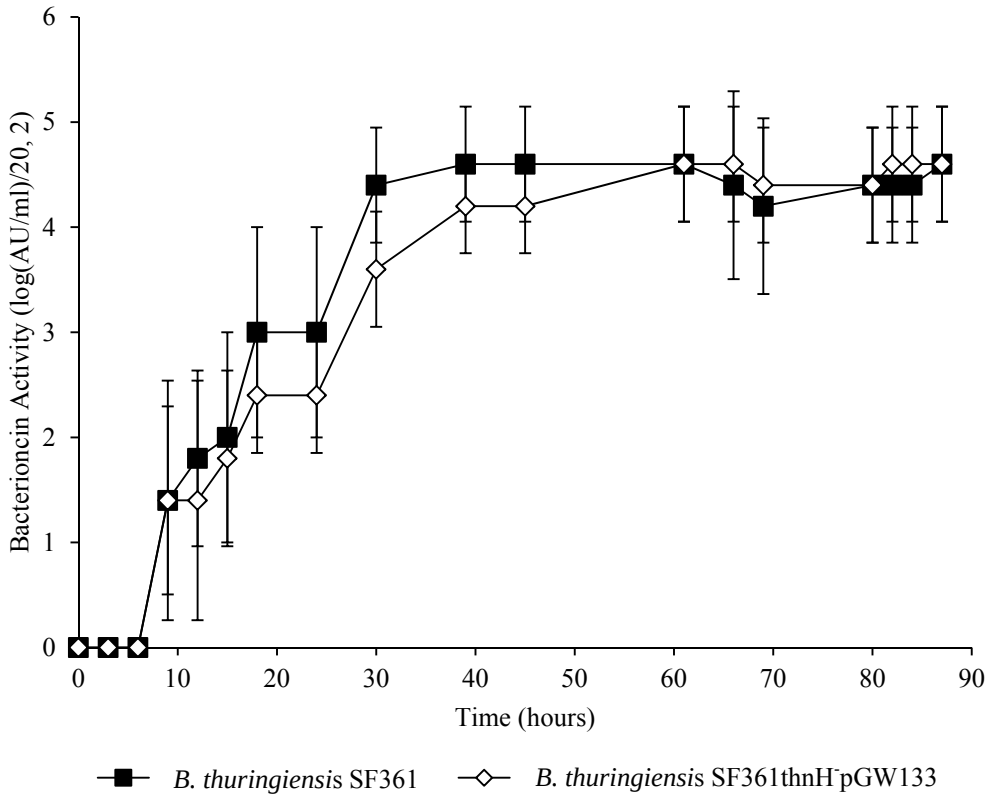


Figure 3.3 Construction of different expression vectors and measurement of their bacteriocin production levels. (A) Scheme of plasmids pGW132 and pGW133 construction; (B) Bacteriocin assay of pGW132 and pGW133 on solid TSA media; 1, WT; 2, *B. thuringiensis* thnH$^-$; 3, *B. thuringiensis* thnH$^-$pGW132; 4, *B. thuringiensis* SF361thnH$^-$pGW133; (C) Bacteriocin activity of *B. thuringiensis* thnH$^-$pGW133 in TSB.

Site directed mutagenesis. Mature thurincin H is composed of six alanines at non-sulfur to α-carbon bond forming positions (A11, A12, A23, A24, A27, and A30) in addition to 25 other amino acids throughout the rest of the peptide (Figure 3.1). The 33 single amino acid site-directed mutations on the pGW133 plasmids were categorized into three groups: 1) 8 radical substitutions at thioether bond forming positions; 2) 8 conservative substitutions at thioether bond forming positions; 3) 17 radical substitutions at non-thioether bond forming positions (Table 3.3). Those mutants were systematically generated to elucidate the tolerance of the biosynthesis, regulation, and transportation pathway toward each amino acid substitution in leaderless thurincin H. The results of the bacteriocin activity change were measured by deferred antagonism assay and are summarized in Table 3.3.

In groups 1 and 2, the inhibitory activity was completely lost as a result of any of the eight single cysteine-alanine/cysteine-serine mutations. However, in the thioether acceptor sites of group 1, bacteriocin activity disappeared in N19A and T22A, was partially retained in T25A and S28A. Remarkably, in group 2, relatively high partial bacteriocin activities were retained in all four thioether acceptor sites, N19Q, T22S, T25S, and S28T. This result indicated that all four cysteines (C4, C7, C10, C13), the donors of the thioether bonds, are critical in maintaining the bacteriocin activity. However, the thurincin H pathway does have limited tolerance towards amino acid substitution in the thioether acceptor positions (N19, T22, T25, and S28).

In group 3, bacteriocin activity was completely abolished when W (2, 5), L (8, 17,

20), and T29 were substituted by alanine. Partial activity was lost when E16, G26, D1, V (9, 15, 21), L18 and T3 were mutated. Very high activity was retained when S (6, 14, 31) were substituted with alanine. Tryptophan could not be substituted at either position probably because the featured indole functional group plays an essential role. As for leucine and threonine, their substitutability depended on their positions. Remarkably, all three serine-alanine mutations maintained high bacteriocin activity, regardless of their positions in the peptide.

Table 3.3 Primers used in site directed mutagenesis

Mutation	Primer[a]	Sequence
Radical substitutions (thioether linkages forming positions)		
C4A	AM 4	agtagtacaaccaagggactggactgcatggagttgtttagtatgtg
C7A	AM 7	agggactggacttgttggagtgcattagtatgtgcagcatgttct
C10A	AM 10	ggactggacttgttggagttgtttagtagcagcagcatgttctgtg
C13A	AM 13	cttgttggagttgtttagtatgtgcagcagcatctgtggaattattaaatttagttact
N19A	AM 19	gtttagtatgtgcagcatgttctgtggaattattagcattagttactgcggcaacag
T22A	AM 22	gttctgtggaattattaaatttagttgcagcggcaacaggggc
T25A	AM 25	aaatttagttactgcggcagcaggggctagtactg
S28A	AM 28	atttagttactgcggcaacaggggctgcaactgcaagctaaaatttaaaaatg
Conservative substitutions (thioether linkages forming positions)		
C4S	CM4	acaaccaagggactggactagttggagttgtttag
C7S	CM7	actggacttgttggagtagtttagtatgtgcagcatg
C10S	CM10	tggacttgttggagttgtttagtaagtgcagcatgttctg
C13S	CM13	ggagttgtttagtatgtgcagcaagttctgtggaattattaaattta
N19Q	CM19	agtatgtgcagcatgttctgtggaattattacaattagttactgcggcaac
T22S	CM22	tctgtggaattattaaatttagttagtgcggcaacagggg
T25S	CM25	tattaaatttagttactgcggcaagtggggctagtactgcaag
S28T	CM28	ttactgcggcaacaggggctacaactgcaagctaaaattta
Radical substitutions (none thioether linkages forming positions)		
D1A	AM 1	accagtagtacaaccaagggcatggacttgttggagttgtt
W2A	AM 2	caccagtagtacaaccaagggacgcaacttgttggagttgtttagtatg
T3A	AM 3	tagtacaaccaagggactgggcatgttggagttgtttagtatg
W5A	AM 5	tacaaccaagggactggacttgtgcaagttgtttagtatgtgcagc
S6A	AM 6	ccaagggactggacttgttgggcatgtttagtatgtgcagcatgt
L8A	AM 8	ctggacttgttggagttgtgcagtatgtgcagcatgttctg
V9A	AM 9	actggacttgttggagttgtttagcatgtgcagcatgt
S14A	AM 14	gagttgtttagtatgtgcagcatgtgcagtggaattattaaatttagttactg
V15A	AM 15	ttgtttagtatgtgcagcatgttctgcagaattattaaatttagttactgcgg
E16A	AM 16	gtatgtgcagcatgttctgtggcattattaaatttagttactgcg
L17A	AM 17	tatgtgcagcatgttctgtggaagcattaaatttagttactgcggcaac
L18A	AM 18	tgcagcatgttctgtggaattagcaaatttagttactgcggcaacag
L20A	AM 20	tgcagcatgttctgtggaattattaaatgcagttactgcggcaacag
V21A	AM 21	cagcatgttctgtggaattattaaatttagcaactgcggcaacaggg
G26A	AM 26	aaatttagttactgcggcaacagcagctagtactg
T29A	AM 29	ctgcggcaacaggggctagtgcagcaagctaaaatttaaa
S31A	AM 31	cggcaacaggggctagtactgcagcataaaatttaaaaatgtgagagtgt

[a] Only one of the two primers (one pair) used in each site directed mutagenesis reaction was listed in this table. The other primer of the pair is complementary to the sequence listed above. Codons for the mutated amino acids were underlined.

Table 3.4 Inhibitory activity of site directed mutants.

	Mutation	Sizes of inhibition zones (mm)[#]		
		B. cereus **F4552**	***B. thuringiensis*** **EG10368**	***L. monocytogenes*** **2289**
GROUP 1	C4A	-	-	-
Radical	C7A	-	-	-
Substitution	C10A	-	-	-
(thioether linkages forming position)	C13A	-	-	-
	N19A	-	-	-
	T22A	-	-	-
	T25A	13.9±0.4	12.0±0.0	-
	S28A	13.7±0.4	11.8±0.8	9.8±0.5
	C4S	-	-	-
	C7S	-	-	-
GROUP 2	C10S	-	-	-
Conservative	C13S	-	-	-
Substitution	N19Q	8.6±1.0	9.0±0.0	-
(thioether linkages	T22S	11.7±0.7	9.6±0.6	-
forming position)	T25S	10.3±0.4	8.8±0.8	-
	S28T	13.5±0.6	11.0±0.9	-
	D1A	8.8±0.7	6.8±0.5	-
	W2A	-	-	-
	T3A	6.2±0.9	7.5±0.5	-
	W5A	-	-	-
	S6A	14.4±0.7	12.8±0.8	10.1±0.6
GROUP 3	L8A	-	-	-
Radical	V9A	9.9±0.7	8.3±0.5	-
Substitution	S14A	14.6±0.4	12.0±1.1	10.5±0.0
(None thioether	V15A	9.9±0.7	10.5±0.9	
Linkages forming	E16A	10.1±1.4	8.8±0.3	-
position)	L17A	-	-	-
	L18A	10.9±0.8	10.1±1.2	-
	L20A	-	-	-
	V21A	7.1±0.8	8.3±0.8	-
	G26A	9.0±0.0	8.1±0.3	-
	T29A	-	-	-
	S31A	15.2±0.9	13.7±0.8	9.9±0.6
No Substitution	+ Control	16.3±1.5	14.1±1.2	10.3±0.8

[*]Size of inhibition zones were measured with the accuracy of 1 mm (± 0.5 mm). Means and standard deviations of four independent experiments were presented in the table.

DISCUSSION

In-frame deletion and complementation. A deletion mutant of the wild type producer was chosen as the expression host in our study since there are generally always potential technical limitations to the express proteins or peptides in other hosts. For example, the structural gene of the protein might contain rarely used codons in the desired host (Gustafsson *et al.*, 2004) or the proteins expressed in the desired host might be degraded by native proteolytic enzymes (Jensen *et al.*, 2000; Murashima *et al.*, 2002; Narayanan & Chou, 2009). In this particular case of the extensive modified thurincin H, the sulfur to α-carbon bonds were very unique and are only reported to exist in three other bacterial peptides to date (Kawulka *et al.*, 2004; Liu *et al.*, 2010; Sit *et al.*, 2011a). These bonds are not reported in proteins expressed by commonly used expression hosts such as *E. coli* (Richard *et al.*, 2004) or lactic acid bacteria (Rodriguez *et al.*, 2003), rendering the deletion mutant a logical host for expression of sited directed mutants.

During construction of *B. thuringiensis* SF361thnH$^-$ by homologous recombination, five of the 65 white colonies from the X-gal plates were radomly selected for DNA sequencing. All five potential mutants possessed the expected sequence without incorporating any changes in the regions flanking the gene cluster, indicating a high heterlogous recombination accuracy. In addition, this in-frame deletion method did not introduce any antibiotic resistance markers into the chromosome.

The pGW131 complementation experiment indicated that the thurincin H prepeptide translated from the plasmids was correctly modified by the chromosome and

encoded the radical SAM enzyme to form the unique sulfur to α-carbon thioether bonds. In addition, cleavage of the leader prepeptide and transportation of the peptide to the extracellular environment were not affected. The deletion mutant still does not show increased sensitivity to the active thurincin H, likely due to the protection of the intact putative immunity protein on the chromosome.

Construction of the heterologous expression vector. To construct a plasmid for site directed mutagenesis, the expression vector had to carry only one structural gene for the mutagenesis reaction, and express sufficient mature thurincin H to facilitate downstream studies. As stated in the results, pGW131 successfully complemented thurincin H production in the deletion mutant, but it is not a sufficient vector for site directed mutagenesis since the three tandem copies of the structural gene makes it difficult for the primers to specifically bind to an accurate position. Thereafter, according to the requirements, pGW132 was constructed. However, the bacteriocin activity of pGW132 was lower than the wild type, even though multiple copies (15 copies/cell) of this plasmid exist in each cell (Arantes & Lereclus, 1991).

To sufficiently improve the production of the thurincin H, our method was to improve the mRNA stability by changing the thurincin H native terminator in pGW132 to a more stable Cry protein terminator, resulting in pGW133. This strategy was inspired from the remarkably high production of Cry proteins in *B. thuringiensis* species. One reason for high Cry protein production is due to the longer mRNA half-life caused by its stable terminator (Agaisse & Lereclus, 1995). It was reported that the 3' terminal

fragment of the *cryIAa* gene from *B. thuringiensis* subsp. *kurstaki* HD1 forms a strong Rho-independent terminator (ΔG=-13.4 kcal /mol), which was predicted to reduce 3'-5' exoribonuclease sensitivity (Wong & Chang, 1986). Compared with the Cry terminator, the native thurincin terminator is less GC rich and forms a less strong Rho-independent stem loop (ΔG=-9.16kcal/mol). In previous research, the fusion of this *Cry* terminator to the *penP* gene in *E. coli* and *B. subtilis* enhanced the mRNA stability and consequently protein expression (Wong & Chang, 1986). To our knowledge, this is the first time mRNA stability enhancement was used as a strategy to improve the bacteriocin production level. As such, it could be considered as an alternative in future research for the heterologous expression of bacteriocins.

Comparing the production of the three plasmids constructed in our research, pGW131 contains high copies (15 copies) of the structural genes (Arantes & Lereclus, 1991), but its production level is similar to wild type. This might be caused by limited regulation and posttranslational modification systems. Thurincin H was found to inhibit the growth of its producer *B. thuringiensis* SF361 at a relatively high concentration (unpublished data). Self-toxicity was found to be a limiting factor for the natural producer to express higher levels of bacteriocin, despite the fact that the immunity protein usually protects the producer up to a certain level (Heinzmann *et al.*, 2006; Kim *et al.*, 1998). It is also possible that only a certain level of promoter activation could be initiated, even when extra promoter binding sites existed in the cell. This could be due to sigma factor limitation, or RNA polymerase limitation, or other unknown reasons. In this case, less

thurincin H prepeptide mRNA would be transcribed in *B. thuringiensis* SF361thnH$^-$ pGW132 than in the wild type. This might also explain why three repeated prepeptide structural genes exist after the native promoter in the wild type producer, since a higher copy numbers increases the bacteriocin prepeptides production, giving the producer an evolutionary advantage.

Technically, in constructing pGW132 and pGW133, two rounds of PCR were used to amplify the inserts since the forward and/or reverse primers were longer than commonly used primers. Long primers with secondary hairpin structures (TH05&TH06, TH01&07) can easily to lead to nonspecific binding, causing multiple PCR products. When genomic DNA of *B. thuringiensis* was used as template, multiple bands and smears were amplified due to non-specific binding (data not shown). To eliminate non-specific binding, *thnA1* encoding the thurincin H prepeptide was first amplified by PCR and purified as a template.

In some heterologous expression systems for less-extensively modified bacteriocins, only the mature bacteriocin encoding sequence was incorporated in the expression vector (Ingham *et al.*, 2005; Richard *et al.*, 2004). However, in our constructed system, the leader peptide was included in all the expression vectors, since generally the leader peptide of the bacteriocin can be involved in keeping the bacteriocin prepeptide in an inactive state inside the cell, facilitating transportation of the bacteriocin across the membrane, and can play an essential role in the formation of lanthionine/ methyllanthionine in lantibiotics (Riley & Gillor, 2007). Similarly, it was suggested that

in the maturation of subtilosin A, formation of the three pairs of sulfur to α-carbon thioether bonds is the first modification on the prepeptide and is dependent on the leader peptide (Fluhe *et al.*, 2012). To maintain this critical functionality, the prepeptide sequence was required.

Most bacteriocins are consistently produced on appropriate solid media, but some bacteriocins can only be produced in liquid media under certain conditions, either by high inoculation rates or artificial induction (Gobbetti, 2013; Maldonado-Barragan *et al.*, 2009; Quadri *et al.*, 1997). Production of thurincin H from *B. thuringiensis* SF361thnH$^-$ pGW133 in TSB could reach the similar level of production as the WT without the presence of antibiotics in TSB. The high yield of thurincin H in liquid is of essential importance because this system could be easily used as a heterologous expression system for site directed mutagenesis experiments to produce thurincin H variants. Those variants could be purified from the liquid for further research.

Site directed mutagenesis. Genetic engineering has been used as a tool to construct bacteriocin mutants with new features. For example, replacing the threonine at residue 6 with isoleucine in subtilosin A not only enhanced its bactericidal activity, but also rendered the mutant hemolytic (Huang *et al.*, 2009). Deferred antagonism assays were conducted to evaluate the bacteriocin activity of *B. thuringiensis* SF361thnH$^-$ pGW133 transformed by mutant variants of the thurincin H structural gene. The method could only detect the active bacteriocin that had been secreted into the extracellular environment, and therefore reflects the tolerance of the entire biosynthesis, regulation,

and transportation pathway against a different amino acid point mutation. In the mutants that lost activity, it is possible that any step in the biosynthesis was affected, such as the mutation completely abolishing bacteriocin production, or the recognition of the radical SAM enzyme which modifies thurincin H derivatives (Fluhe *et al.*, 2012), or the transportation, etc. However, the bacteriocin activity loss caused by thioether bond amino acid substitution, as in group 1 and 2, were highly likely to be a result of interference of the thioether bond formation. It was reported that the three pairs of sulfur to α-carbon thioether bonds in subtilosin A were sensitive to single amino acid substitution and none of the thioether bonds were formed if any of the six amino acids (C4, C7, C13, F22, T28, and F31) in the sulfur to α-carbon bond forming positions was substituted with alanine *in vivo*. However, their study only detected the thioether bond formation using high performance liquid chromatography and high resolution mass spectrometry, and did not directly report the bacteriocin activity of their mutants (Fluhe *et al.*, 2012). In the thurincin H variants, partial activity was retained with some of the mutants (T25A, S28A, N19Q, T22S, T25S, S28T) containing substitutions in the four thioether bonds. It is possible that partial thioether bonds were formed, or the complete four pairs of thioether bonds were formed, but bacteriocin activity was lost due to the amino acid substitutions.

CONCLUSIONS

A heterologous expression system was developed to sufficiently express adequate levels thurincin H from one structural gene. This structural gene was under the native promoter and included a modified Cry protein terminator which significantly improved

the expression level through enhancing mRNA stability. Our research analyzed a complete, systematic site directed mutagenesis on thurincin H, which is representative of bacteriocins produced by *Bacillus* spp. with unique sulfur to α-carbon thioether bonds. These thurincin H variants could be sufficiently purified, and new features of the variants could be explored in future research, such as 3D structural changes, inhibitory spectrum changes, and thermal stability changes.

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CHAPTER 4

HETEROLOGOUS EXPRESSION OF RECOMBINANT AND NATIVE THURINCIN H IN AN ENGINEERED NATURAL PRODUCER

ABSTRACT

The *Bacillus* bacteriocin, thurincin H, exhibits a wide inhibitory spectrum of activity against various foodborne pathogens such as *Listeria monocytogenes*, and dairy spoilage bacteria, especially different *Bacillus* species. Previously, we constructed three plasmids to express native thurincin H heterologously in an engineered natural producer, *B. thuringiensis* SF361thnH$^-$. This host is deficient in thurincin H production since the structural genes *thnA1*, *thnA2* and *thnA3* were in-frame deleted from the chromosome of the natural producer *B. thuringiensis* SF361. The expression vectors were constructed by cloning the native thurincin H promoter, three (or one) copies of structural genes, and the native (or Cry protein) terminator into the *E. coli-B. thuringiensis* shuttle vector pHT315. In this study, three corresponding expression vectors (pGW134, pGW135, and pGW136) were constructed to express recombinant thurincin H-His6 in the same host, in which a six-histidine tag was fused to the C terminus of each structural gene. The resulting low bacteriocin production indicated that the His tag might negatively interfere with subsequent post-translational modification or exportation after the thurincin H-His6 prepeptide was translated. In order to overexpress native thurincin H, two

additional plasmids (pGW137 and pGW138) were constructed, consisting of the sporulation-dependent Cry protein dual promoter BtI and BtII, the *thnA1* structural gene, and the thurincin H native or Cry protein terminator. However, the production was low on LB plates and was abolished on sporulation plates. It is possible that the resulting thurincin H prepeptide was not correctly modified or exported to the extracellular environment due to the undesired biochemical and physiological changes during the sporulation phase.

SHORT COMMUNICATIONS

Thurincin H is an antimicrobial peptide composed of 31 amino acids produced by *Bacillus thuringiensis* SF361, which was originally isolated from US honey (Lee et al., 2009). It exhibits inhibitory activity against different foodborne pathogens and spoilage bacteria, such as *Listeria monocytogenes*, *Micrococcus* and *Bacillus* species, which are common pathogens in various dairy products (Konosonoka *et al.*, 2012; Lee *et al.*, 2009). Thurincin H contains four pairs of unique sulfur to α-carbon thioether bonds catalyzed by the putative radical S-adenosylmethionine superfamily enzyme (ThnB) present to maintain the hairpin structure with a helical back bone (Sit et al., 2011). As elucidated by bioinformatics studies, the 8.14-kb *thnP-thnI* gene cluster is responsible for the thurincin H production and exportation pathway. This gene cluster consists of three tandem thurincin H prepeptide genes (*thnA1*, *thnA2* and *thnA3*), as well as the *thnP*, *thnB*, *thnD*, *thnE*, *thnT*, *thnR*, *thnI* genes, related to thurincin H prepeptide processing, regulation, modification, export, and self-immunity (Lee et al., 2009).

Heterologous expression of bacteriocins or recombinant bacteriocins with a purification tag has been a research interest to increase the yield or to simplify the purification process. For extensively modified bacteriocins, like lantibiotics, usually similar species were used as the expression hosts in which the natural systems are still available to modify certain unique bonds (Rodriguez et al., 2003). In this study, we constructed five plasmids based on the *E. coli-B. thuringiensis* shuttle vector pHT315, aiming to express sufficient C terminal His-tagged thurincin H, or overexpress native thurincin H using a previously constructed *B. thuringiensis* SF361thnH$^-$ as an expression host.

Both *E. coli* and *Bacillus* strains were cultivated in trypticase soy broth (TSB) or on trypticase soy agar (TSA) (Difco, BD, MD) at 37°C. General molecular cloning methods used in this study were performed as previously described by Sambrook and Russell (Sambrook & Russell, 2001). Generally, to construct a recombinant plasmid based on pHT315, an insertion fragment was amplified using one step or two-step PCR by PrimeSTAR® Max DNA Polymerase (Takara, R045; Dalian, China) in a total volume of 50 µl. The PCR conditions include a template denaturation step at 98°C (1 min) followed by 30 cycles of denaturing at 98°C (10 sec), annealing at 55°C (10 sec), and polymerization at 72°C (60 sec), with one final hold at 72°C (7 min). Transformants of *E. coli* and *Bacillus* were selected on TSA plates supplemented with 100 µg/ml ampicillin and 25 µg /ml erythromycin, respectively. Recombinant plasmids were purified from *E. coli* using QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany) and passed through

E. coli K12 ER2925 to produce demethylated plasmids as a preparation for *B. thuringiensis* transformation (Macaluso & Mettus, 1991). Recombinant plasmids were transformed to *B. thuringiensis* strains as previously described by Lereclus et al (Lereclus et al., 1989). Bacterial strains and primers used in this study were listed in Table 4.1.

Table 4.1 Bacterial strains and plasmids used in this study.

Strains	Properties	References
E. coli DH5α	Cloning host, produced methylated plasmid	NEB, MA
E. coli K12 ER2925	*Dam dcm,* produce demethylated plasmid	NEB, MA
B. thuringiensis SF361	Thurincin H producer strain, carrying the putative thurincin H producing gene cluster	(Lee et al., 2009)
B. thuringiensis SF361thnH$^-$	Thurincin H producer strain with in-frame deletion of the *thnA1*, *thnA2* and *thnA3* genes	See chapter 3
B. cereus F4552	Indicator strain, sensitive to thurincin H	(Lee et al., 2009)
Plasmid	**Properties**	**References**
pHT315	*E. coli*-B. thuringiensis shuttle vector, 6.5 KB, Ery^R Amp^R	(Arantes and Lereclus, 1991)
pGW131	*thnA1* gene with native promoter (P_{nat}) and native terminator(T_{nat}), cloned in pHT315	See chapter 3
pGW134	*thnA1his6, thnA2 his6 and thnA3 his6* genes with native promoter (P_{nat}) and native terminator (T_{nat}), cloned in pHT315	This study
pGW135	*thnA1his6* gene with native promoter (P_{nat}) and native terminator(T_{nat}), cloned in pHT315	This study
pGW136	*thnA1his6* gene with native promoter (P_{nat}) and Cry protein terminator(T_{cry}), cloned in pHT315	This study
pGW137	*thnA1* gene with BtI&II dual promoter ($P_{BtI\&II}$) and Cry protein terminator(T_{nat}), cloned in pHT315	This study
pGW138	*thnA1* gene with BtI&II dual promoter ($P_{BtI\&II}$) and Cry protein terminator(T_{cry}), cloned in pHT315	This study
Primers	**Sequences**	**References**
TH21	gatgat*ctgcag*aatgcacaccatacacaccataca cagtta	This study
TH22	ttagtggtggtggtggtggtggcttgcagtactagcccctgt	This study
TH23	cag*aagctt*aatttataggacttattcttaacataactgtttgtcattaga ataagtcctaatttaattagtggtggtggtggtggtg	This study

Table 4.1 (Continued)

TH24	gatgga*ctgcag*gtaaatatatgtcacaaaatattaaag aaacacacacaa aatgtttg	This study
TH25	gcttgcagtactagcccctgt	This study
TH27	gatgga*gaattc*tagtaaaacggacatcactccgtttcaatggaggtg atgtccgttttatttaatttcaccataaacactctcacatttttaaattttagt ggtggtggtggtggtggcttgcagtactagcccctgt	This study
DTC3	ctgcaaggcgattaagttgggtaac	See chapter 3
DTC4	cggataacaatttcacacaggaaacagcta	See chapter 3
TH28	gatgga*ctgcag*tagttgcactttgtgcatttttcataagatgagtcat atgttttaaattgtagtaatgaaaaacagtattatatcataatgaattggt atcttaataaaagagatggaggtaacttatggaaacaccagtagtaca accaagg	This study
TH29	gatggt*gaattc*gtttttagtttatgtattacaaaaatcccatactcgtttt c	This study
TH30	gatggt*gaattc*tagtaaaacggacatcactccgtttcaatggaggtg atgtccgttttatttaatttcaccataaacactctcacatttttaaatttta	This study

A fragment containing the native promoter, *thnA1his6*, *thnA2his6*, *thnA3his6*, and the native terminator (noted as P_{nat}*thnA1his6thnA2his6thnA3his6*$T_{nat,}$ same as following) was synthesized (Figure 4.1, A) by GenScript (Piscataway, NJ) and cloned into pHT315, resulting in the plasmid pGW134. Gene synthesis was used to produce the fragment, since 18 bp additions of DNA sequences encoding the six-histidine tag after each of the three tandem structural genes by PCR is very difficult. Using primers TH21and TH22, the fragment containing P_{nat}*thnA1*$_{his6}$ was amplified from *B. thuringiensis* SF361 genomic DNA, purified, and used as an amplification template. Using this template, a second fragment P_{nat}*thnA1his6* T_{nat} was amplified with primers TH21 and TH23 (Figure 4.1, B), and cloned into pHT315, resulting in the plasmid pGW135. Using primers TH24 and TH25, a fragment containing P_{nat}*thnA1* was amplified from previously constructed

pGW131, purified, and used as an amplification template. Using this template, a fragment P_{nat}*thnA1his6* T_{cry} was amplified with primers TH24 and TH27 (Figure 4.1, C), and cloned into pHT315, resulting in the plasmid pGW136. The bacteriocin production of *B. thuringiensis* SF361thnH$^-$ carrying those three plasmids was determined by a modified deferred antagonism assay (Birri *et al.*, 2010) against *B. cereus* F4452. However, inhibition zones formed by those constructs were significantly lower than the wild type producer (Figure 4.2). In earlier work, two plasmids similar to pGW134 and pGW136 but lacking the His tag were constructed. These constructs displayed an inhibitory activity as high as the wild type producer (See chapter 3). These results indicated that the extracellular thurincin H-His6 was either produced at a lower level or has a lower antimicrobial activity due to the His tag. Attempts were also made to produce and purify thurincin-His6 in TSB broth. A 1% inoculation of *B. thuringiensis* SF361thnH$^-$pGW135 ovenight culture into 150 ml TSB broth was incubated at 37°C for 16 hours with shaking at 225 rpm. The supernatant was collected by centrifugation (10000 x g, 20 min, 4°C). Crude protein extract was precipitated by 40%-65% saturated ammonia sulfate solution, resuspended in 15 ml LEW buffer (50 mM NaH_2PO_4, 0.3 M NaCl, pH 7.5), and purified by High Specific PrepEase Histidine-tagged Protein Purification Kit (USB, Cleveland, Ohio) according to the standard protocol. Based on an imidazole gradient concentration test, an optimal elution buffer (50 mM NaH_2PO4, 0.3 M NaCl, 0.25 M imidazole, pH 8.0) was chosen to elute thurincin H-His6 from the nikel column. The 0.25 M imidizole buffer did not inhibit *B. cereus* F4552 (data not shown). A low bacteriocin activity against *B.*

cereus F4552 was detected in both the supernatant and 0.25 M imidizole eluate, using a spot on lawn method (Fujita et al., 2007). Directly loading supernatant to the nickel column led to very low binding efficacy.

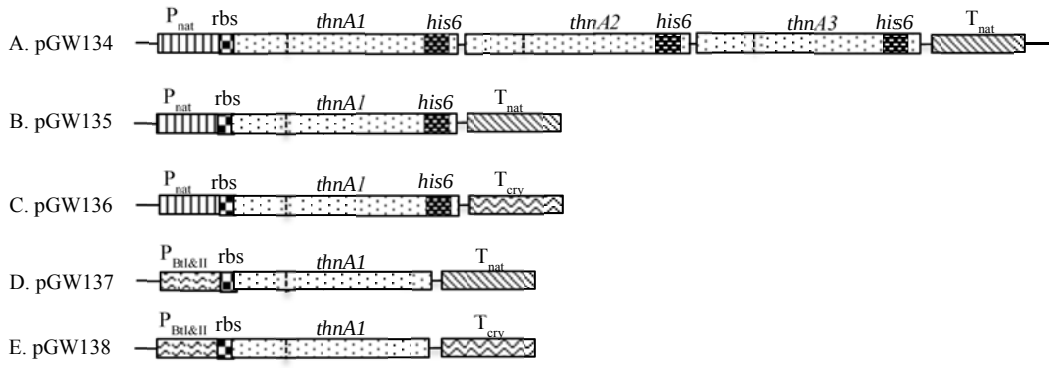

Figure 4.1 Insertion fragments scheme in different expression vectors.

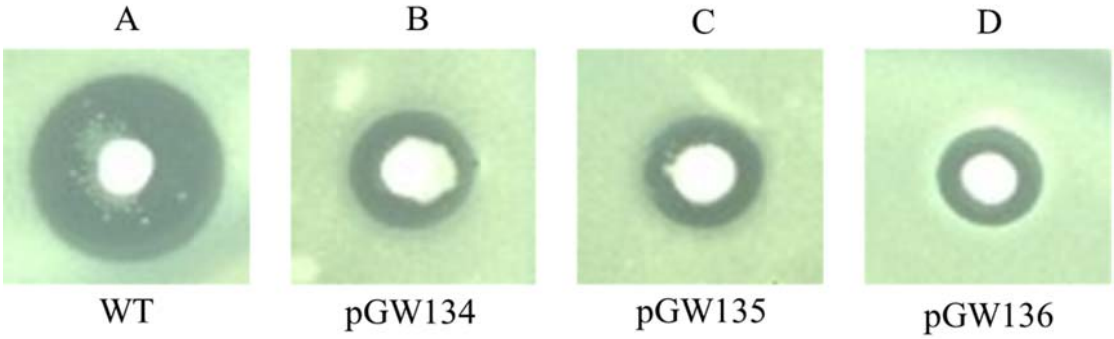

Figure 4.2 Inhibitory activities of *B. thuringiensis* SF361thnH$^-$ carrying different thurincin H-His6 expression vectors. (A) Wild type *B. thuringiensis* SF361; (B) *B. thuringiensis* SF361thnH$^-$pGW134; (C) *B. thuringiensis* SF361thnH$^-$pGW135; (D) *B. thuringiensis* SF361thnH$^-$pGW136.

To determine if the His tag on thurinicn H interfered with the natural production of mature thurincin H, pGW135 was subsequently transformed to wild type *B. thuringiensis* SF361. The bacteriocin activity of *B. thuringiensis* SF361pGW135 showed a lower thuricin H activity when compared to the wild type producer (Figure 4.3), even

though pGW135 contains 15 copies of His-tagged thurincin H precursor structural genes (Lereclus et al., 1989). This indicated that the thurincin H-His6 precursor peptides have been translated from plasmid pGW135 and negatively interfered with one or more steps of regulation, modification, or exportation along the native thurincin H production pathway. While many bacteriocins fused with a His tag have been reported to retain high activity, such as bacteriocin 51 (Yamashita et al., 2011) and enterocin P (Herranz & Driessen, 2005), a His tag could also impair the expression or activity of other enzymes (Freydank *et al.*, 2008; Sabaty *et al.*, 2013), or sometimes result in a change in the crystal structure (Loschi et al., 2004). In the case of thurincin H, the low production of thurincin H-His6 and native thurincin H production could be caused by several reasons. The addition of 6 hydrophilic histidines to the small 31 amino acid hydrophobic thurincin H (Sit et al., 2011) might alter the hydrophobicity of thurincin H-His6 prepeptide drastically from the native thurincin H. The formation of the unique sulfur to α-carbon thioether bonds catalyzed by S-adenosylmethionine enzyme might also be affected, since the hydrophilic tag might interfere with the distinguished hydrophobic patch surrounding the whole hairpin structure. In some bacteriocin expression systems such as piscicolin 126 (Gibbs et al., 2004), His tags were fused to the N terminus and cleaved off prior to being used for subsequent research. However, in this system, the His-tag could not be added to the N-terminus of the thurincin H prepeptide since the 9 amino acid leader peptide will eventually be cleaved off the thurincin H prepeptides.

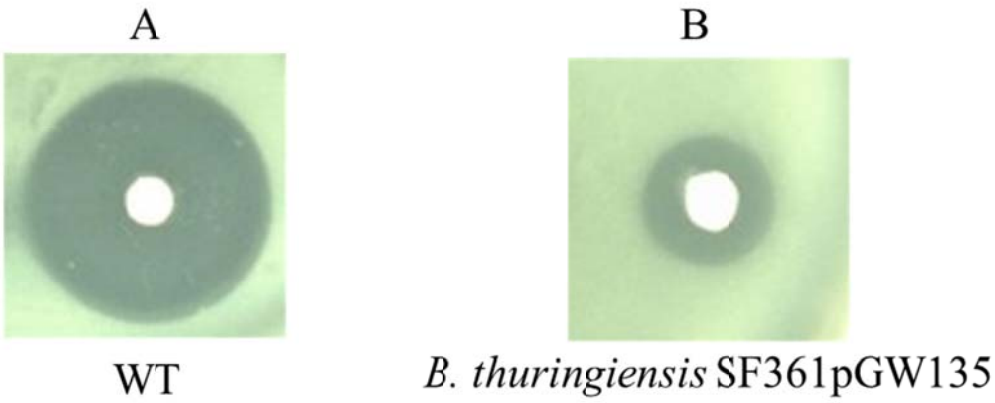

Figure 4.3 Inhibitory activities of *B. thuringiensis* SF361and *B. thuringiensis* SF361 carrying thurincin H-His6 expression vector. (A) Wild type *B. thuringiensis* SF361; (B) *B. thuringiensis* SF361pGW135.

Two additional plasmids carrying *thnA1* under the dual Cry protein overlapping dual BtI and BtII promoters were constructed in an attempt to overexpress native thurincin H. A template fragment was amplified from pGW131 using DTC3 and DTC4 as primers. Using this fragment as a template, a first fragment containing the BtI and BtII dual promoters ($P_{BtI\&II}$), *thnA1*, and the native terminator (T_{nat}) was amplified with primers TH28 and TH29 (Figure 4.1, D), and cloned into pHT315, resulting in plasmid pGW137. A second fragment containing the BtI and BtII promoters ($P_{BtI\&II}$), *thnA1*, and Cry terminator (T_{cry}) was amplified with primers TH28 and TH30 (Figure 4.1, E), and cloned into pHT315, resulting in pGW138. The two constructed plasmids were separately transformed to *B. thuringiensis* SF361thnH$^-$. The antimicrobial activity of the resulting transformants was detected by the modified deferred antagonism assay as described above, except that in order to test the effect of different media on the expression, the transformants were spotted on LB and SPO 8 plates and incubated for 72 hours at room temperature (22°C) before being overlaid with *B. cereus* F4552. On LB plates, *B.*

thuringiensis SF361thnH$^-$ carrying pGW137 and pGW138 exhibited smaller inhibition zones compared to that of the wild type producer (Figure 4.4, colonies A, B, C). On SPO 8 plates, *B. thuringiensis* SF361thnH$^-$ carrying pGW137 and pGW138 did not exhibit any inhibition zones. However, the wild type producer still exhibited an inhibition zone on this sporulation media (Figure 4.4. colonies D, E, F). The rationale of *B. thuringiensis* species producing high yields of Cry proteins was adapted in the last two plasmid constructs (pGW137 and pGW138). The high expression of Cry proteins in *B. thuringiensis* is partially the result of strong overlapping promoters (BtI and BtII), and stable mRNA from the strong terminator (Agaisse & Lereclus, 1995). Several proteins such as chitinase (Hu et al., 2009) have been heterologously expressed in *B. thuringiensis* strains using the overlapping promoter and terminator based on pHT315. The same strategy was attempted in our research to produce thurincin H at higher levels. However, the results indicate that a decrease in thurincin H production was observed under these experimental conditions. The reason might be that the production of the active thurincin H not only depends on the amount of thurincin H precursor peptide, but also heavily relies on the cooperation of the other components related to the regulation, modification, and export. In addition, the overlapping promoter was turned on once sporulation starts, but changes in cellular morphology, biochemistry, and physiology are also induced in the cell (Errington, 1993). These changes might negatively affect other steps in the thurincin H modification and export pathways. To our knowledge, this is the first time the Cry protein promoter and terminator was adopted to express a bacteriocin in *B. thuringiensis*.

Our research indicates that in bacteriocins requiring extensive posttranslational modification, posttranslational modification, regulation and exportation need to be taken into consideration, not only production and expression level of the structural genes.

Technically, two step PCR was used to construct the plasmids in order to reduce nonspecific binding since some of the primers used in this study were longer than usual (up to 156 bp). The *B. thuringiensis* SF361 genomic DNA contains three tandem structural genes which additionally enhance non-specific binding and non-specific amplification of fragments. We first amplified a short core fragment to serve as a template DNA in PCR, and ensured the sequence accuracy in the final products.

Our research underscores the risks of using a His-tag system as a means for purification in a heterologously expressed bacteriocins, especially those extensively modified bacteriocins, such as thurincin H, since the His tag may negatively interfere with subsequent modification or exportation steps. A Cry protein promoter and terminator should also be carefully considered since changes occurring during sporulation might exert a negative effect on the function of other components in the pathway. In future research, a xylose inducible expression system will be constructed to control the expression time and level of the structural genes.

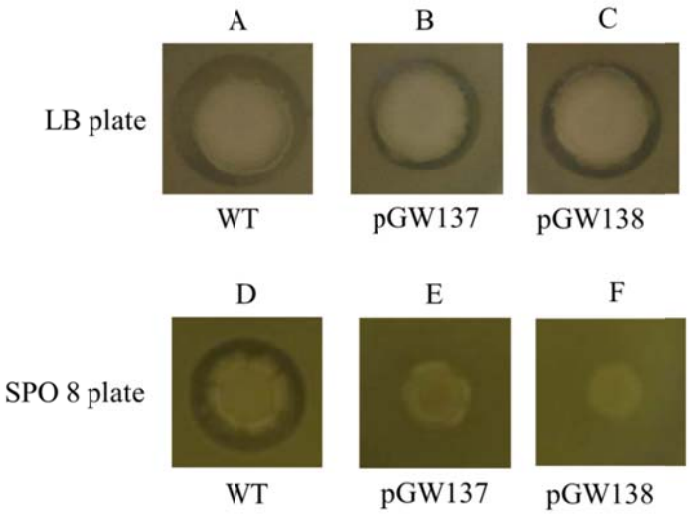

Figure 4.4 Inhibitory activities of *B. thuringiensis* SF361thnH$^-$ carrying different thurincin H-His6 expression vectors with Cry protein promoters and native (or Cry) terminators. (A) *B. thuringiensis* SF361 on LB agar; (B) *B. thuringiensis* SF361thnH$^-$ pGW137 on LB agar; (C) *B. thuringiensis* SF361thnH$^-$pGW138 on LB agar; (D) *B. thuringiensis* SF361 on SPO8 agar; (E) *B. thuringiensis* SF361thnH$^-$pGW137 on SPO8 agar; (F) *B. thuringiensis* SF361thnH$^-$pGW138 on SPO8 agar.

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CHAPTER 5

NATURALLY SENSITIVE *BACILLUS THURINGIENSIS* EG10368 PRODUCES THURINCIN H AND ACQUIRED IMMUNITY AFTER HETEROLOGOUS EXPRESSION OF THE ONE-STEP AMPLIFIED THURINCIN H GENE CLUSTER

ABSTRACT

Heterologous expression of bacteriocin genetic determinants (or operons) has long been a research interest for the functional analysis of genes involved in bacteriocin expression, regulation, modification, and immunity. Previously, in order to identify new bacteriocin operons, genomic libraries of the bacteriocin producer strains were usually required. This method is tedious and time consuming. For the first time, we directly amplified an 8.14-kb bioinformatically identified thurincin H gene cluster using a one-step PCR with 100% accuracy. This amplified gene cluster was cloned into pHT315, resulting in plasmid pGW139, and subsequently transformed to *Bacillus thuringiensis* EG10368, a strain naturally sensitive to thurincin H. Heterologous expression of the gene cluster makes the sensitive *B. thuringiensis* EG10368 produce thurincin H at a higher level compared to the wild type producer, *B. thuringiensis* SF361. Moreover, *B. thuringiensis* EG10368pGW139 becomes completely immune to thurincin H. The results indicate that one-step PCR method is a promising tool to accurately amplify long bacteriocin gene clusters used in further functional analysis and can be an effective way to produce bacteriocins at

a higher level without the need for cloning large chromosomal fragments.

SHORT COMMUNICATION

Thurincin H is bacteriocin produced by *B. thuringiensis* SF361. It exhibits a broad inhibitory spectrum, especially against various spore forming *Bacillus* species such as *Bacillus cereus,* a toxin producing pathogen often associated with dairy products (Lee *et al.*, 2009; Schoeni & Wong, 2005). The secondary structure of the 31 amino acid leaderless mature thurincin H peptide forms a hairpin structure with a helical backbone maintained by four pairs of very unique sulfur to α-carbon thioether bridges (Sit et al., 2011). This unique thioether bond is quite different from the more common sulfur to β-carbon thioether bonds existing in extensively studied lantibiotics produced by lactic acid bacteria (Cotter et al., 2005).

As elucidated by bioinformatics studies, the 8.14-kb *thnP-thnI* gene cluster is responsible for the production and exportation of mature, active thurincin H. It consists of three tandem bacteriocin-like precursor genes (*thnA1*, *thnA2* and *thnA3),* as well as *thnP, thnB, thnD*, *thnE, thnT* ,*thnR, thnI*, genes which have the putative functions related to thurincin H prepeptide processing, regulation, modification, exportation, and self-immunity (Lee et al., 2009). The objective of this study was to accurately amplify the thurincin H gene cluster using a rapid one-step PCR method and test if the gene cluster can be heterologously expressed at high levels in the sensitive strain *B. thuringiensis* EG10368.

Bacillus and *E. coli* strains used in this study were cultivated in trypticase soy broth (BD, Sparks, MD) or on trypticase soy agar (BD, Sparks, MD) at 37°C. The *E. coli*-*B. thuringiensis* shuttle vector pHT315 (Arantes & Lereclus, 1991) was used as a thurincin H gene cluster cloning and expression vector. *E. coli* DH5α was used as a cloning host for the pGW139 recombinant plasmid. *E. coli* K12 ER2925 (NEB, MA) was used to produce demethylated plasmids, used to increase the efficiency of transformation to *B. thuringiensis* (Macaluso & Mettus, 1991). Erythromycin (25 μg/ml) or ampicillin (100 μg/ml) was used to select *B. thuringiensis* or *E. coli* transformants, respectively. All strains, plasmids, and primers used in this study are listed in Table 5.1.

Table 5.1 Strains, plasmids, and primers used in this study

Strains	**Properties**	**Reference**
E. coli DH5α	Host for cloning thurincin H gene cluster to plasmids pHT315	NEB, MA
E. coli K12 ER2925	*dam dcm* strain, produce demethylated plasmids	NEB, MA
B. thuringiensis SF361	Thurincin H producer strain	(Lee et al., 2009)
B. thuringiensis EG10368	Sensitive to thurincin H	(Lee et al., 2009)
B. thuringiensis EG10368pGW139	*B. thuringiensis* EG10368 carrying recombinant plasmid pGW139	This study
B. cereus F4552	Sensitive to thurincin H	(Lee et al., 2009)
B. thuringiensis SF361thnH^{-}	Native thurincin H producer with a deletion of *thnA1*, *thnA2* and *thnA3* genes	(Lee et al., 2009)
Plasmids	**Properties**	**Reference**
pHT315	pHT315 *E. coli-B. thuringiensis* shuttle vector, 6.5 KB, Ery^{R} Amp^{R}	(Arantes & Lereclus, 1991)
pGW139	pHT315 carrying the 8.14-kb thurincin H gene cluster, Ery^{R} Amp^{R}	This study
Primers	**Sequences**	**Reference**
TH81	gatgga*ctgcag*ttattgggaaatcgctttatagacatcaagcaaaccatta	This study
TH82	gatggt*gaattc*ctatatttctgaagtatacaatgtgaccattccatg tgttctattt	This study
SequencingPrimer_1	ctgcaaggcgattaagttgggtaac	See chapter 3
SequencingPrimer_2	cggataacaatttcacacaggaaacagcta	See chapter 3
SequencingPrimer_3	ttattgggaaatcgctttatagacatcaagcaaacc	This study
SequencingPrimer_4	ttagtaacatcaagagcatcattaccagccgcagcaa	This study
SequencingPrimer_5	gatccaggtgaaatgttatcttttaaatcaggatg	This study
SequencingPrimer_6	agcatgaactgtagtaataggtatgaaagtt	This study
SequencingPrimer_7	aacaggaagtagtacaccaaataaaatacttaga	This study
SequencingPrimer_8	taacatggatgttccccccaatatatgaaagcataatct	This study
SequencingPrimer_9	atctgcatgatgggatagggctaatgcaactgcaaa	This study
SequencingPrimer_10	catctgcatgaatacctatactacgagccatttga	This study
SequencingPrimer_11	attataaattataaaaagcagtttaacaagatatactgcgattaaa	This study
SequencingPrimer_12	cttttggtcaaattagactttctaggagaaaaataaataatca	This study
SequencingPrimer_13	ttttataggagatgattaaagtgaatggctatttattttgga	This study
SequencingPrimer_14	aaacagatgtaatgtaagatgtcgtcattgctatgga	This study
SequencingPrimer_15	cataatgaaattagccgaaaatgatgttcgtttacg	This study
SequencingPrimer_16	atttttttaacttacaagcacctcaaatggact	This study
SequencingPrimer_17	attatcgcaccgataccatatattattggctatat	This study
SequencingPrimer_18	gcagaaatgtatgaaaccttaaatggaatagaggatataaaat	This study
SequencingPrimer_19	tgtttcaggaggtcaagcacaatttatagccttttta	This study
SequencingPrimer_20	aagtgaaaatagaacacatggaatggtcacattgtatacttcagaaatatag	This study

To amplify the 8.14-kb thurincin H gene cluster, 100 ng of purified *B. thuringiensis* SF361 genomic DNA was used as the PCR reaction (50 µl) template. PrimeSTAR® Max DNA Polymerase (Takara, R045A; Dalian, China) was used as the master mix. TH81 and TH82 were used as primers, along with the restriction enzymes *Pst*I and *Eco*RI. The DNA polymerase from Takara claimed to be able to amplify up to 10 kb from *E. coli* genomic DNA with an extension time of 5 kb/sec. The manufacture's standard PCR protocol failed to amplify the gene cluster from *B. thuringiensis* SF361 genomic DNA (data not shown). To adapt the protocol to serve the current system's needs, the extension time in each of the 30 amplification cycles was afterwards modified to be 4 mins (about 2 kb/min), with an additional 7 minutes extension time at the end. The optimized PCR method includes a template denaturation step at 98°C (1 min), followed by 30 cycles of denaturing at 98°C (10 sec), annealing at 55°C (10 sec), and polymerization at 72°C (4 mins), with one final hold at 72°C (7 min). PCR products showed a single band on a 1.5% agarose gel (Figure 5.1). With the modified conditions, a PCR product approximately 8 kb in length was amplified as expected.

Molecular cloning experiments were performed as described by Sambrook and Russell (Sambrook & Russell, 2001). Generally, the purified PCR product was double *Pst*I-HF/ *Eco*RI-HF digested (NEB, MA), purified, and ligated overnight at 16°C to the double *Pst*I-HF/ *Eco*RI-HF digested pHT315 at a ratio of 1:1 by T4 ligase (NEB, MA). The ligation mixture was first transformed to *E. coli* DH5α. The recombinant plasmid pGW139 was purified from the transformants, and DNA sequencing of the thurincin H

gene cluster inserts in pGW139 was performed at the Biotechnology Resource Center at Cornell University (Ithaca, NY). DNA sequencing results showed 100% accuracy when compared with reported sequences (GenBank: FJ977580.1). Primers used in sequencing the gene cluster are listed in Table 5.1. Purified pGW139 was subsequently passed through *E. coli* K12 ER2925 and transformed to *B. thuringiensis* EG10368 strains as previously described (Lereclus et al., 1989).

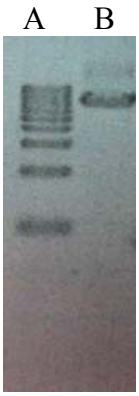

Figure 5.1 Amplification of thurincin H gene cluster by PCR. (A) 1 Kb plus DNA ladder (Invitrogen, Carlsbad, California). (B) 8.14-kb thurincin H gene cluster amplified by one-step PCR.

To test the inhibitory activity of *B. thuringiensis* EG10368 and *B. thuringiensis* EG10368pGW139, a deferred antagonism assay was performed as previously described using *B. thuringiensis* SF361 as a positive control and *B. thuringiensis* EG10368 as an indicator strain (Birri et al., 2010) . Inhibition zones formed around the wild-type producer *B. thuringiensis* SF361 (Figure 5.2, A) but not around *B. thuringiensis* EG10368 (Figure 5.2, B), indicating that *B. thuringiensis* EG10368 itself does not produce self-active antimicrobial compounds. The obvious inhibition zone formed around *B.*

thuringiensis EG10368pGW139 was larger than the wild type (Figure 5.2, C), indicating that mature thurincin H was produced from the gene cluster carried in pGW139 at a higher level than the wild type producer. Similar results were obtained using *B. cereus* F4552 as an indicator strain (data not shown). To test if immunity was acquired by *B. thuringiensis* EG10368pGW139, *B. thuringiensis* EG10368 was overlaid on top of those three colonies described above. Remarkably, no inhibition zones were formed around any of those colonies (Figure 5.2, D, E, F). This result indicates that the originally sensitive *B. thuringiensis* EG10368 acquired complete immunity after pGW139 was transformed into it. Since those two strains are in the same *Bacillus* species and share highly similar genetic machinery, it is very probable that all the genes in the 8.14-kb gene cluster were able to be expressed and function in sensitive strain *B. thuringiensis* EG10368. However, the concrete functions of each gene in the cluster need to be further confirmed.

pGW139 was constructed based on the high copy number plasmid pHT315 (15 copies per cell) (Arantes & Lereclus, 1991), theoretically resulting in 15 fold expression of the gene cluster compared with the wild type producer *B. thuringiensis* SF361. *B. thuringiensis* EG10368pGW139 expressed a higher level of thurincin H compared with the wild type producer (Figure 5.2, C). Similarly, we also found that transforming pGW139 into *B. thuringiensis* SF361thnH$^-$ (a WT producer with *thnA1*, *thnA2* and *thnA3* deleted in-frame) led to a higher inhibitory activity compared with the wild type producer (data not shown). Those two results are likely caused by the high copy numbers of genes related to thurincin H biosynthesis, regulation, modification, exportation, and immunity,

since it was reported that separately expressing additional copies of bacteriocin precursor genes, regulatory components, or immunity proteins led to increased extracellular bacteriocin production. An example of this has been reported for nisin Z (Cheigh et al., 2005). Nisin Z production was increased by introducing multiple copies of the structural genes (*nisZ*), a two-component regulatory system (*nisRK*), or the immunity protein (*nisFEG*), into the wild type producer *Lactococcus lactis* subsp. *lactis* A164 (Cheigh et al., 2005). Furthermore, additional copies of subtilin immunity genes *spaIFEG* were also integrated into the genome of the producer strain *Bacillus subtilis* ATCC 6633 and improved the production of the lantibiotic subtilin, since the tolerance level of *B. subtilis* ATCC 6633 toward subtilin was enhanced (Heinzmann et al., 2006).

Previously, in screening new bacteriocins or bacteriocin gene clusters, genomic libraries were routinely constructed to screen for the bacteriocin producing DNA fragment, requiring a large amount of laborious of work (Roh et al., 2010). With the increasing numbers of complete bacterial genomes available, genome mining using different web servers such as BAGEL (de Jong et al., 2006) is becoming an emerging strategy for identifying new putative bacteriocins and bacteriocin gene clusters (Wang et al., 2011). To our knowledge, no research has been reported to directly amplify those whole putative bacteriocin gene clusters for functional confirmation. Our one-step PCR method perfectly complemented the needs to analyze the functions of those bioinformatically identified putative bacteriocin genes or gene clusters. The combination of genome data mining and our method, provide a new strategy of screening new

bacteriocins genes (clusters) and their functionality.

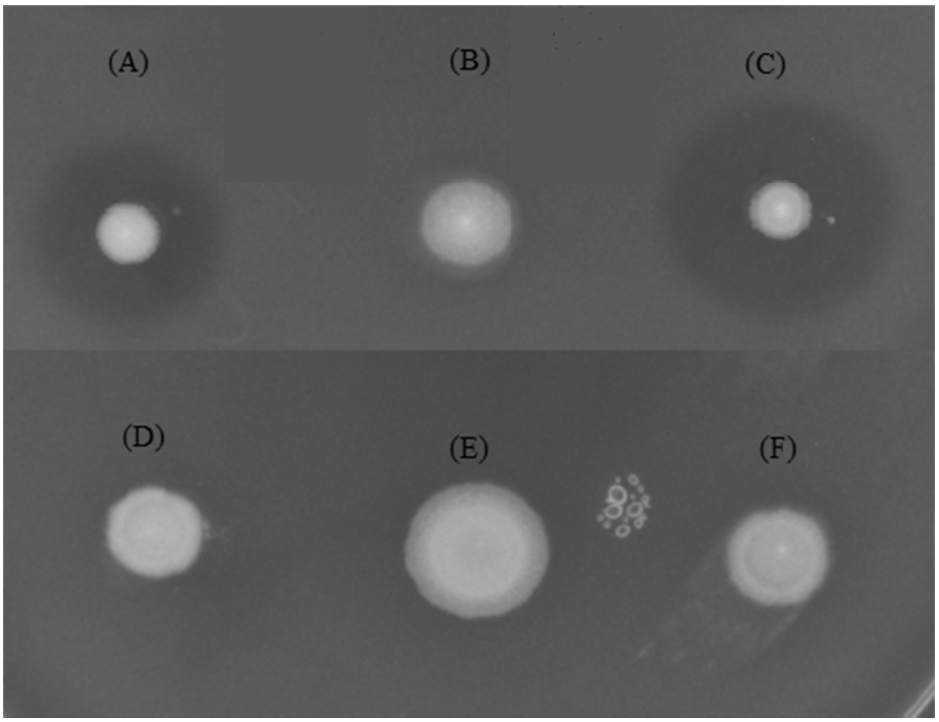

Figure 5.2 inhibitory activities of *B. thuringiensis* EG10368pGW139 and its acquired immunity. (A) colony, *B. thuringiensis* SF361; overlay, *B. thuringiensis* EG10368. (B) colony, *B. thuringiensis* EG10368; overlay, *B. thuringiensis* EG10368. (C) colony, *B. thuringiensis* EG10368pGW139; overlay, *B. thuringiensis* EG10368. (**D**) colony, *B. thuringiensis* SF361; overlay, *B. thuringiensis* EG10368pGW139. (E) colony, *B. thuringiensis* EG10368; overlay, *B. thuringiensis* EG10368pGW139. (**F**) colony, *B. thuringiensis* EG10368pGW139; overlay, *B. thuringiensis* EG10368pGW139.

In conclusion, a simple one step PCR method was developed to accurately amplify the 8.14-kb thurincin H gene cluster. Heterologous expression of the thurincin H gene cluster using the high copy plasmid pHT315 in the naturally sensitive strain *B. thuringiensis* EG10368 led to a higher thurincin H production in the extracellular environment when compared with the wild type producer. The sensitive strain also acquired complete immunity. Our method provides a new strategy to study the functions of putative bacteriocin gene clusters and to heterologously express higher levels of bacteriocins. Any genes within the thurincin H gene cluster could be directly amplified using the above method and separately studied in future research.

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CHAPTER 6

THURINCIN H CAUSES VIABILITY DECREASE AND MORPHOLOGICAL CHANGES IN *BACILLUS CEREUS* F4552

ABSTRACT

Thurincin H is an anti-listerial bacteriocin produced by *Bacillus thuringiensis* SF361. This bacteriocin is a hydrophobic anionic peptide folded to form a hairpin structure by four pairs of sulfur to α-carbon thioether bonds. It exhibits inhibitory activity against a wide range of Gram-positive foodborne pathogens and spoilage bacteria that include *Listeria monocytogenes*, *B. cereus*, *B. subtilis*, and some lactic acid bacteria. Incubation of *B. cereus* F4552 with thurincin H for 1 hour resulted in a significant decrease in cell viability. However, thurincin H did not cause decrease in optical density or changes in the cell membrane permeability. Under scanning electron microscopy, *B. cereus* F4552 treated with thurincin H (32 MIC) showed regular rod-shaped cells, while cells treated with thurincin H (256 MIC) showed loss of cell integrity and rigidity. These results suggest that thurincin H might not act on the bacterial cell membrane to cause intracellular contents efflux like most extensively studied lantibiotics, but rather kills sensitive bacteria through a novel mechanism.

INTRODUCTION

Antimicrobial peptides have been widely found in most living organisms:

prokaryotes, plants, and animals including vertebrates and invertebrates (Garcia-Olmedo *et al.*, 1998; Lehrer & Ganz, 1999; Tossi *et al.*, 2000). These antimicrobial peptides and proteins have diverse chemical structures and play essential roles in the innate immunity and early defense systems to protect their hosts (Zasloff, 2002). Bacteriocins are antimicrobial peptides produced by bacteria. They are ribosomally synthesized peptides and exhibit antimicrobial activity against other bacteria mostly within the same species, or sometimes across different genera (Cotter *et al.*, 2005). Bacteriocins produced by Gram-positive bacteria, most commonly lactic acid bacteria, have been extensively studied and are currently used by the food industry because of their GRAS status (generally recognized as safe) and effectiveness against various foodborne pathogens and spoilage microorganisms (Settanni & Corsetti, 2008).

Bacteriocins produced by lactic acid bacteria can be divided into three main classes: the class I lantibiotics containing lanthionine; the class II non-lanthionine-containing bacteriocins; and the class III heat-labile, large proteins (Cotter *et al.*, 2005). Class II bacteriocin can be further divided into three subcategories: class IIa containing pediocin-like bacteriocins, class IIb containing two-peptide bacteriocins, and class IIc containing other bacteriocins (Drider *et al.*, 2006). Recently, *Bacillus* spp. have gained recent research interest since they produce a diverse array of bacteriocins (Abriouel *et al.*, 2011; Stein, 2005), usually with a broader inhibition spectra compared to bacteriocins produced by lactic acid bacteria (Abriouel *et al.*, 2011).

One *Bacillus* bacteriocin, thurincin H, is an antimicrobial peptide produced by *B. thuringiensis* SF361, a strain originally isolated from US domestic sunflower honey. It exhibits inhibitory activity against a wide range of Gram-positive bacteria including different foodborne pathogens and spoilage bacteria, such as *Listeria monocytogenes*, *Bacillus cereus*, and *Micrococcus* spp. (Lee *et al.*, 2009). Thurincin H contains four pairs of unique sulfur to α-carbon thioether bridges which are different from the extensively studied class I lantibiotics, since the structure of lantibiotics is maintained by sulfur to β-carbon bridges.

The objective of this study was to preliminarily characterize the mode of action of thurincin H.

MATERIALS AND METHODS

Chemicals, bacterial strains and culture conditions. The indicator strain *B. cereus* F4552 was cultivated in trypticase soy broth (TSB; BD, Sparks, MD) or on trypticase soy agar (TSA; BD, Sparks, MD) at 37°C. Phosphate buffered saline (PBS) at pH 7 was formulated with 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4 per liter. Nisin from *Lactococcus lactis* (2.5%, balanced with sodium chloride and denatured milk solids) was purchased from Sigma-Aldrich (St. Louis, MO). All chemicals and reagents were autoclaved at 121°C for 15 minutes prior to use.

Thurincin H purification and purity confirmation. Thurincin H was produced by *B. thuringiensis* SF361. The thurincin H sample used in this study was purified using

ammonia sulfate precipitation and C18 solid phase extraction. The purity of thurincin H was confirmed by HPLC and LC-MS as previously described (See chapter 2).

Growth of *B. cereus* with the presence of thurincin H. The relation between different levels of thurincin H and its effect on *B. cereus* growth was assayed by incubating the indicator strain with thurincin H in 96 well plates. In brief, 50 µl of two-fold diluted thurincin H in PBS buffer was prepared in untreated clear flat bottom 96 microtiter plates (Thermo Scientific, Nunc, Denmark), mixed with 150 µl of 1.33% (v/v) *B. cereus* F4552 overnight culture in TSB in each well and incubated at 37°C for 15 hours. The final inoculation of *B. cereus* was 1% (v/v). The absorbance at 600 nm (A_{600}) for each well was measured by the Synergy HT Multi-Mode Microplate Reader (BioTek, Winooski, VT) for up to 15 hours at 1 hour intervals. Seven independent experiments were performed.

Bactericidal effect of thurincin H on *B. cereus* viability. The bactericidal effect of thurincin H was assessed by incubating exponential phase *B. cereu*s F4552 with purified thurincin H in PBS buffer. An overnight culture of *B. cereus* was diluted 100 fold in 5 ml TSB and incubated at 37°C for 4 hours. Cultures were centrifuged (5000 x *g*, 4°C, 5 min) and resuspended in an equal volume of thurincin H (16 MIC). Samples were taken for viability counts every 10 minutes for up to1 hour. Nisin (16 MIC) was used as a positive control and PBS buffer was used as negative control. Four independent experiments were performed.

Effect of thurincin H on *B. cereus* optical density. The effect of thurincin H on the optical density of *B. cereus* F4552 was assessed by incubating exponential phase *B. cereus* F4552 with the purified thurincin H. An overnight culture of *B. cereus* was diluted 100 fold in 5 ml TSB and incubated at 37°C for 4 hours. Cultures were centrifuged (5000 x *g*, 4°C, 5 min) and resuspended in an equal volume of PBS buffer containing thurincin H at a concentration of 256 MIC and 32 MIC. Two hundred microliters of the culture suspension was added to each well of 96 well microtiter plates and incubated at 37°C for up to 1 hour. The absorbance (A_{600}) was read every 6 minutes by the Synergy HT Multi-Mode Microplate Reader. Nisin (8 MIC and 2 MIC) were used as positive controls and PBS buffer was used as a negative control. Five independent experiments were performed.

Membrane permeability. To determine if thuricin H alters the membrane permeability of sensitive cells, LIVE/DEAD *Bac*Light Bacterial Viability Kit (Molecular probe, L7012, Eugene, Oregon) was used according to the manufacturers recommendations (Swe *et al.*, 2009). An overnight culture of *B. cereus* was diluted 100 fold in 5 ml TSB and incubated at 37°C for 4 hours. Bacterial cells were collected by centrifugation and resuspended in an equal volume of PBS buffer containing thurincin H (256 MIC). Cells suspensions were incubated at 37°C with gentle shaking. One hundred microliters of the cell suspension sample was taken out every 15 min for up to 60 minutes. Samples were washed twice by 0.85% NaCl, resuspended in 200 µl of 0.85% NaCl and placed on ice. After the treatment, 100 µl of cell suspension was mixed with 0.15 µl SYTO9 green-fluorescent nucleic acid stain and propidium iodide red-fluorescent nucleic

acid stain (ratio 1:1). After incubating the plate at room temperature for 15 minutes, green (excitation wavelength: 485 nm/ 20 nm; emission wavelength: 528 nm/20 nm) and red (excitation wavelength: 485 nm/ 20 nm; emission wavelength: 645 nm/40 nm) fluorescent signal was measured by a Synergy HT Multi-Mode Microplate Reader. All the samples were immediately plated onto TSA to determine the viability count after the fluorescent signal measurement. Nisin (2 MIC) was used as a positive control and PBS buffer was used as negative control. Three independent experiments were performed.

Scanning electron microscopy (SEM). An overnight culture of *B. cereus* was diluted 100 fold in 25 ml TSB and incubated at 37°C for 5 hours. Cultures were centrifuged (5000 x *g*, 4°C, 3 min) and resuspended in an equal volume of PBS buffer containing thurincin H (32 MIC and 256 MIC), nisin (2 MIC) or PBS buffer (control). Cell suspensions were incubated at 37°C for 30 minutes with gentle shaking. Suspensions were centrifuged and resuspended in two volumes of PBS buffer to stop the bacteriocin treatment. Each sample was diluted and plated for viability counting in duplicate. At the same time, the control, nisin-treated, and thurincin H treated *B. cereus* cells (approximately 10^6 CFU) were deposited onto a 0.22 μm filter membrane. Cells were fixed using 2.5% (w/v) glutaraldehyde in 0.05 M sodium cacodylate buffer for 1 h. Fixed cells were rinsed three times for 5 minutes with cacodylate buffer. A secondary fixation was conducted using 2% (w/v) osmium tetroxide for 30 min, followed by rinsing with cacodylate buffer three times as described above. To maintain cell morphology in its natural form after bacteriocin treatment, the cells were dehydrated using gradient ethanol

solutions of 25% (v/v), 50%, 70%, 95%, and 100%, for 5 min each. *B. cereus* cells in absolute ethanol were critical point-dried with carbon dioxide (Bal-Tec Critical Point Dryer CPD 030). Filter membranes with dried cells were mounted to SEM stubs with carbon tapes placed on the top. The stubs with samples were coated with evaporated carbon. Images were acquired with a Zeiss LEO 1550 field emission scanning electron microscope at a voltage of 1.5 kV. The working distance was 2-3 mm. Aperture size was 30 µm. The accompanying software SmartSEM (Carl Zeiss Microscopy, LLC, Germany) was used to scan samples and acquire images. Two images with high and low magnifications for each treated sample were presented.

RESULTS

Effect of thurincin H on *B. cereus* growth. The effect of thurincin H against *B. cereus* F4552 in liquid was evaluated in TSB broth in microtiter plate. The results indicate that the optical density increase of *B. cereus* could be partially or completely inhibited by adding thurincin H at different concentrations into TSB broth (Figure 6.1). This is the rationale of the bacteriocin activity quantification method, and the method to determine bacteriocin MIC in microtiter plates (Daba *et al.*, 1991; Faye *et al.*, 2002). The time-course results indicated that at 10-13 hours incubation time, at a certain threshold concentration of thurincin H, the standard deviations of A_{600} were significantly high. These results suggested that 10-13 hours should be avoided as incubation times for bacteriocin activity quantification or MIC determination, since it caused inconsistencies

between different independent experiments. In the study of thurincin H, 8 hours was used as the incubation time.

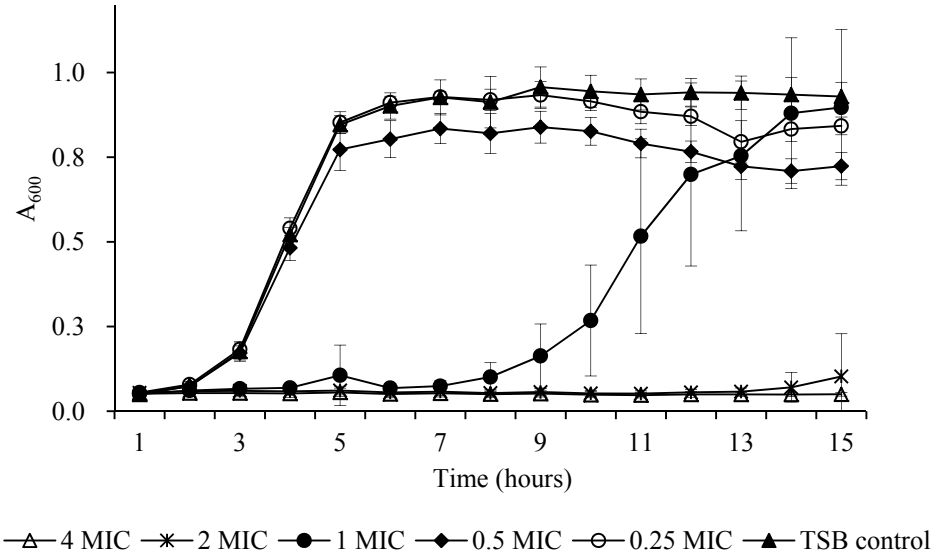


Figure 6.1 A_{600} change of indicator strain *B. cereus* F4552 incubated with thurincin H at different concentrations. Means and standard deviations are shown in the figure.

Effect of thurincin H on *B. cereus* viability. Both thurincin H (16 MIC) and nisin (16 MIC) resulted in decreased cell viability under the experimental conditions. However, thurincin H showed a mild gradual reduction, while nisin caused a rapid drastic reduction within 10 minutes (Figure 6.2). The actual concentration in terms of MIC are the same for the two bacteriocins, but nisin showed a more dramatic effect and 2 higher log reductions than thurincin H at the end of 60 minutes.

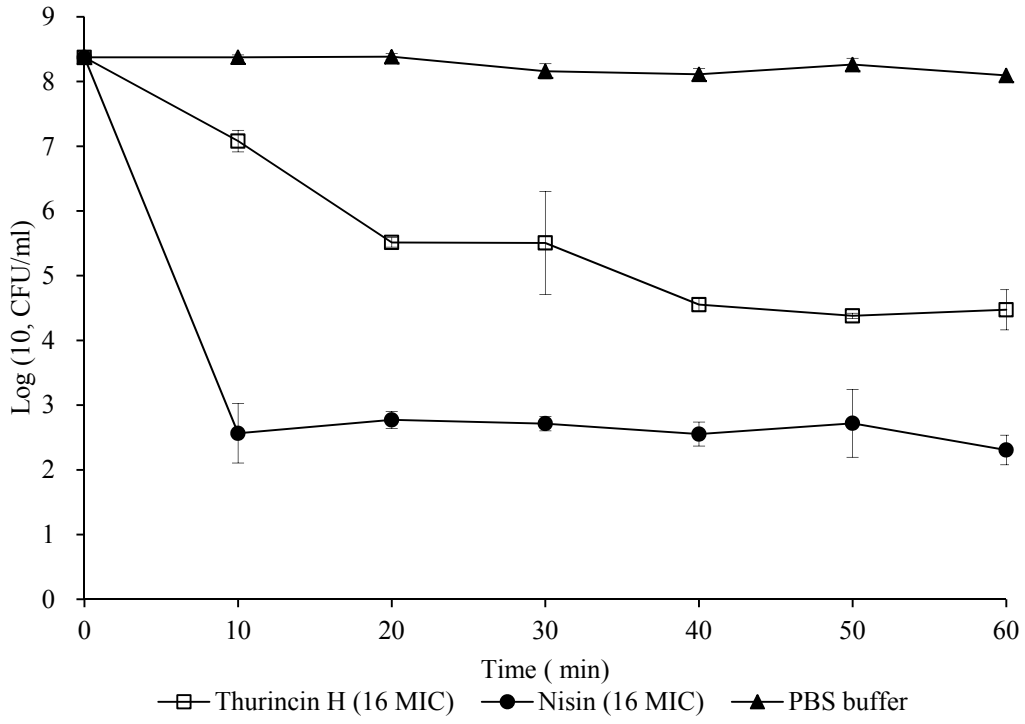


Figure 6.2 Bactericidal effect of thurincin H (16 MIC) and nisin (16 MIC) against *B. cereus* F4552. Means and standard deviations of each time point are included.

Effect of thurincin H on *B. cereus* optical density (OD). Thurincin H (256 MIC) did not cause any optical density reduction compared with the buffer control (Figure 6.3). On the other hand, nisin at 2 MIC and 8 MIC, caused significant decreases in the optical density (Figure 6.3). In preliminary experiments, a series of thurincin H concentrations, including 2 MIC, 4 MIC, 16 MIC, 64 MIC were tested, and none of them resulted in a decrease in optical density (data not shown).

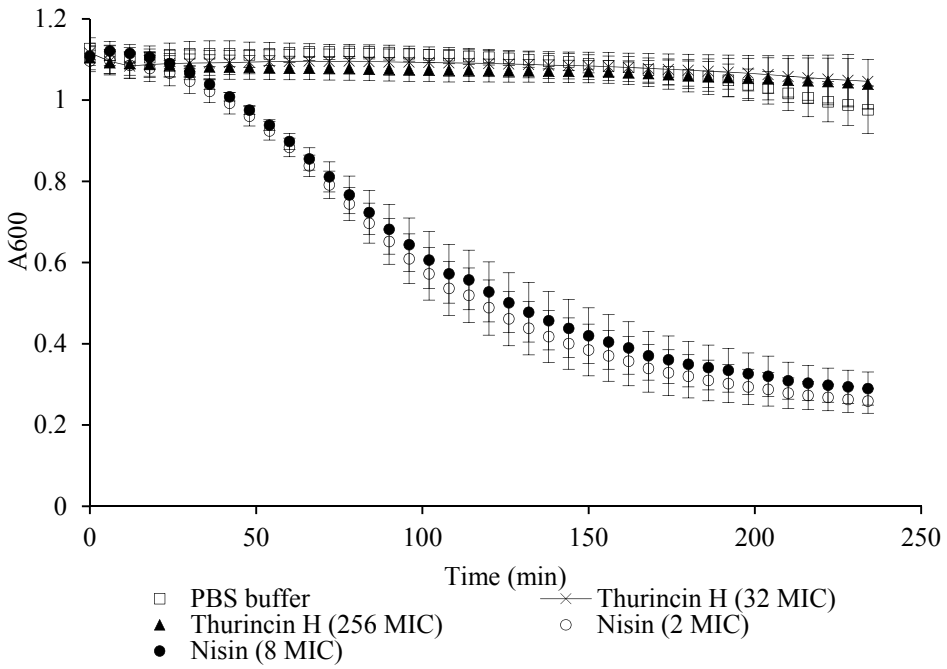
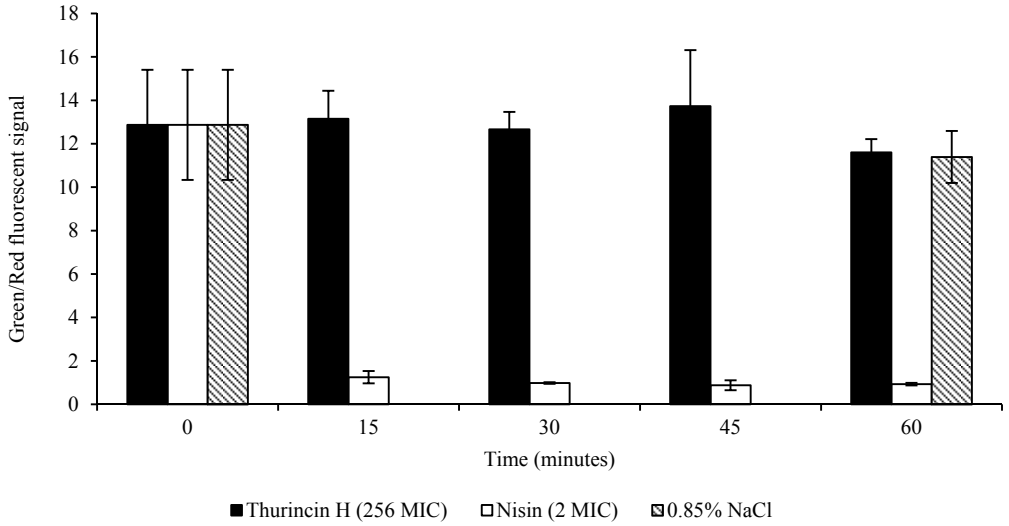


Figure 6.3 *B. cereus* incubated with thurincin H (32 MIC & 256 MIC) and absorbance monitored at A_{600}.

Membrane permeability studies. Mixtures of SYTO9 green-fluorescent nucleic acid stain and the red-fluorescent nucleic acid stain propidium iodide were used in this study. When both dyes are mixed with a bacteria population at optimal ratio, bacteria with intact Cell membranes of viable cells result in a green fluorescence, while propidium iodide only penetrates bacteria with damaged membranes, and result in a red fluorescence (Swe *et al.*, 2009). The results from these studies indicate that thurincin H at a relatively high concentration (256 MIC) did not alter the cell membrane integrity (Figure 6.4, A) while at the same time resulted in 99.99% reduction in the viability count (Figure 6.4, B). On the other hand, nisin (2 MIC) caused significant cell membrane damage (Figure 6.4, A) with a similar cell viability reduction as thurincin H (Figure 6.4, B).

(A)



(B)

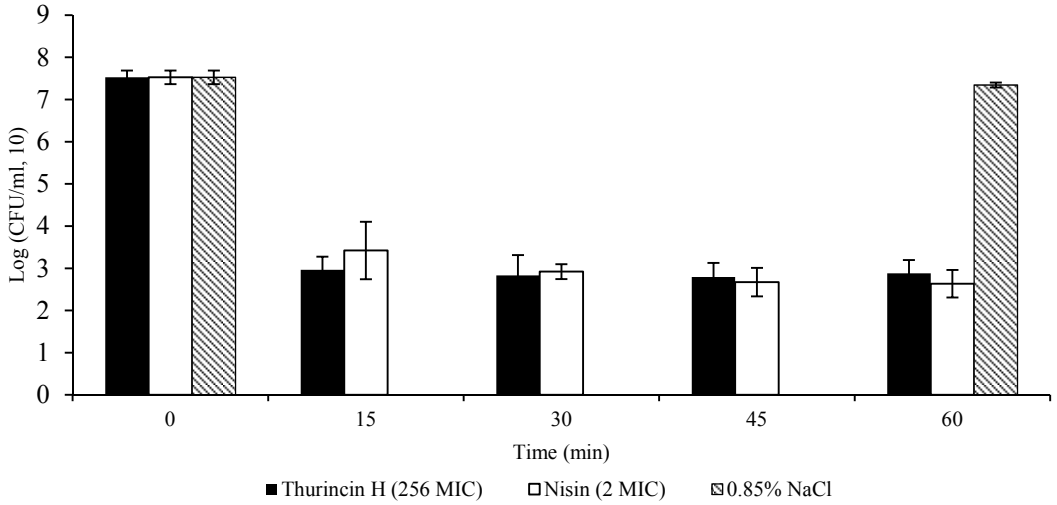


Figure 6.4 Cell membrane permeability and cell viability change of *B. cereus* F4552 after thurincin H and nisin treatment. (A) Green/red fluorescent ratio changed caused by thurincin H and nisin (2 MIC); (B) *B. cereus* cell viability reduction caused by thurincin H (256 MIC) and nisin (2 MIC).

Morphological changes of *B. cereus* with thurincin H exposure. Viability cell assays indicated that thurincin H (32 MIC) resulted in a 2 log reduction of *B. cereus*, while thurincin H (256 MIC) and nisin (2 MIC) caused a 4 log reduction (data not shown). However, thurincin H (32 MIC) treated *B. cereus* showed a similar morphology to the control, with a smoothly surface and regular rod shape (Figure 6.5, CD & AB). Thurincin H (256 MIC) treated *B. cereus* showed a collapsed and flat rod shape (Figure 6.5, EF). Nisin treated cells showed coarse, collapsed surface with perforations (Figure 6.5, GH). Morphology of all the cells under SEM were consistent and only one field of view was presented.

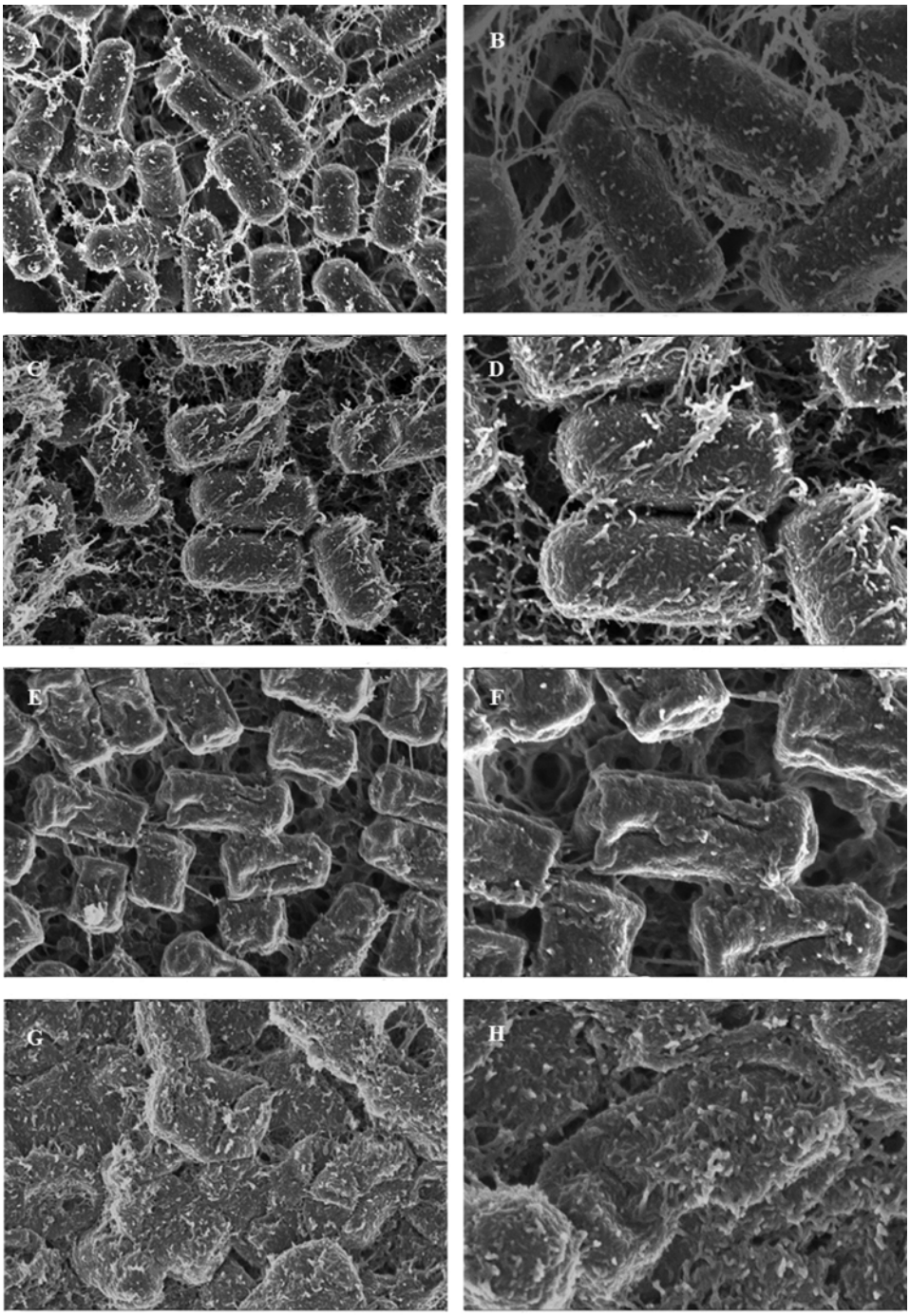


Figure 6.5 SEM of *B. cereus* F4552 cells after thurincin H and control treatments. (A) PBS, low magnification; (B) PBS, high magnification; (C) Thurincin H (32 MIC), low magnification; (D) Thurincin H (256 MIC), high magnification; (E) Thurincin H (256 MIC), low magnification; (F) Thurincin H (256 MIC), high magnification; (G) Nisin (2 MIC), low magnification; (H) Nisin (2 MIC), high magnification.

DISCUSSION

Although hundreds of bacteriocins have been discovered and characterized to date, the most extensively studied group of bacteriocins are produced by lactic acid bacteria, because of their GRAS status and immediate application by food industry as natural preservatives. The mode of action of lantibiotics produced by lactic acid bacteria is usually generalized as forming pores in the membrane of sensitive bacteria, with nisin as a prototype (Moll *et al.*, 1996). Pore formation in cell membrane results in the dissipation of membrane potential and the efflux of small metabolites from sensitive cells, and leads to cell death (Cotter *et al.*, 2005). The cell membrane damage effect of nisin showed in this study (Figure 6.4) is consistent with previously reported pore forming effects (Wiedemann *et al.*, 2004). At high concentrations, nisin can also cause lysis of the cell wall (Wiedemann *et al.*, 2004), as shown in the Figure 6.3, the optical density decreased significantly within 1 hour of treatment exposure.

Bacillus bacteriocins are another diverse category of bacteriocins. Most of the reported *Bacillus* bacteriocins have been found to be active on the cell membrane and result in efflux of the intracellular contents, similar to that of the lantibiotics produced by lactic acid bacteria (Abriouel *et al.*, 2011). One exception is mersacidin, a tetracyclic peptide bacteriocin produced by *B. subtilis* strain HILY-85,54728. This peptide is active against methicillin- and vancomycin-resistant *S. aureus* strains by targeting the cell wall

precursor lipid II and thereby inhibiting cell wall synthesis. It does not modify the bacterial cell membrane permeability like most other lantibiotics (Brotz *et al.*, 1997).

Unlike the mode of action of nisin and most bacteriocins with a pore forming mechanism, the results of thurincin H mode of action experiments presented here suggested that thurincin H did not cause cell membrane damage according to the cell membrane permeability studies (Figure 6.4). It also did not cause cell lysis either, since decrease in the optical density of the *B. cereus* indicator strain was not observed (Figure 6.3). It is possible that the mechanism of action of thurincin H is targeting the cell wall, since thurincin H (256 MIC) caused cell wall collapse as observed by SEM at varying concentrations of thurincin H. Thurincin H (32 MIC) may not be sufficient concentration to cause obvious cell morphology changes under SEM, even though it results in the loss of viability of the *B. cereus* sensitive cells.

CONCLUSIONS

The mode of action of thurincin H was preliminary characterized in this study. The bactericidal thurincin H resulted in decreased cell viability, but did not cause cell membrane permeability or cell wall lysis. Remarkably, *B. cereus* only showed loss of cell integrity when treated with thurincin H at a high concentration. Our studies suggest that thurincin H inactivates sensitive indicator strains in a different mechanism compared to nisin and most lantibiotics.

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CHAPTER 7

CONCLUSIONS AND PROSPECTUS

CONCLUSIONS

The hydrophobic bacteriocin, thurincin H, is encoded by three tandem structural gene repeats, *thnA1*, *thnA2*, and *thnA3,* that have a single inducible promoter. Mature thurincin H, which exhibits a helical backbone, is folded over to form a hairpin structure by four unique sulfur to α-carbon thioether bridges (Sit *et al.*, 2011). This type of thioether bridges is reported in only four *Bacillus* bacteriocins (Fluhe *et al.*, 2012), and are quite different from the extensively studied group of lantibiotics that contain sulfur to β-carbon thioether bridges (Twomey *et al.*, 2002). This uniqueness makes it difficult to be heterologously expressed or genetically modified in most commonly used systems.

Thurincin H was produced and purified from a 40 hour incubated supernatant of *B. thuringiensis* SF361 via a novel, rapid, and simple two-step method: ammonia sulfate precipitation and C18 solid phase extraction, with a yield of 18.53 mg/L. The purified thurincin H was stable for 30 min at 50°C, and decreased to undetectable levels within 10 minutes at temperatures above 80°C. Thurincin H was also stable from pH 2-7 for at least 24 hours, although gradual loss of activity occurred under alkaline conditions within 24 hours. It was found that thurincin H does not prevent germination initiation of *B. cereus* F4552, and killed the spores only in the presence of nutrients.

To construct a heterologous expression host for thurincin H, *thnA1, thnA2* and *thnA3* were deleted in-frame from the chromosome of the wild type producer by

homologous recombination. The deletion mutants maintained the functions to posttranslationally modify thurincin H prepeptides, cleave the leader peptides and export the bacteriocin to extracellular environment. Using this deletion mutant as a host, several expression vectors were constructed to express native or His-tagged thurincin H. Those vectors contained different combinations of native promoter or Cry protein dual promoters, one copy or three copies of structural genes, and the native or Cry protein terminators, based on an *E. coli-B. thuringiensis* shuttle vector pHT315. Two of those vectors expressed in thurincin H deletion mutants reached similar production levels as the wide type producer.

The plasmid containing the native promoter, one copy of structural gene and the Cry protein terminator was selected as the expression vector for subsequent site directed mutagenesis. Mutated thurincin H variants were produced by site directed mutagenesis, including twenty five single radical amino acid substitutions throughout the thurincin H peptide, as well as four additional conservative amino acid substitutions at cysteine sites (C4, C7, C10, C13) and the four thioether acceptor sites (N19, T22,T25, S28). Critical amino acids which maintained the inhibitory effect of thurincin H were identified, without which, the antimicrobial activity was partially or completely compromised.

The putative thurincin H producing gene cluster was accurately amplified by one step PCR and heterologously expressed in the sensitive strain *B. thuringiensis* EG10368. The sensitive strain acquired complete immunity to thurincin H and produced thurincin H at a level higher than the wild type producer *B. thuringiensis* SF361. To our knowledge,

this is the first time one step PCR was employed to directly amplify such a long bacteriocin gene cluster for functional studies.

In the mode of action study, thurincin H exhibited a bactericidal effect against sensitive strain *B. cereus* F4552. However, it did not alter cell membrane permeability, even at a concentration that most *B. cereus* cells had been killed. This indicated that thurincin H acts on the indicator strains via a novel mode of action, since causing damage to cell membrane by forming pores was the generalized killing mechanism for most of bacteriocins. Studies on the morphological changes of *B. cereus* cells treated with thurincin H indicated that at a low concentration, cells were killed but regular rod shape were observed under scanning electron microscopy, while at a higher concentrations of thurincin H treatment, collapsed flat rods lacking of cell rigidity were observed.

PROSPECTUS

The author developed methods to purify thurincin H on a large scale basis, which is an important prerequisite to subsequent characterizations. The genetic advances made in this study also opened doors for further in depth investigation for this bacteriocin. The following studies are suggested for future work.

Thurincin H further characterization and application. Bacteriocins produced by lactic acid bacteriocins attracted most extensive research interests in recent decades and were widely applied in food industry due to its GRAS status (Berlec & Strukelj, 2009). Even though *Bacillus* bacteriocins have a great potential regarding the inhibition spectrum, safety concerns are an important issue in its application. In order to receive

approval from FDA to use *B. thuringiensis* SF361 as protective strain, or using its fermentation extract as a food additive, *B. thuringiensis* SF361 will have to be evaluated as GRAS by support of further research. An alternative way is to conduct extensive research on toxicity of purified thurincin H in animal and human models to evaluate the possibility of using pure thurincin H as a food additive.

New characteristics of mutated thurincin H variants. It was reported that proteins with point mutations could acquire completely novel features (Huang *et al.*, 2009). The inhibitory activities of thurincin H deletion mutant carrying mutated thurincin H expression plasmid variants were compared in this study. Those expressing thurincin H variants could be further purified according the method established in Chapter 2, and further analyzed. Expected features, such as enhanced stability, could also be explored by substituting those critical amino acids related to stability with a series of different alternative amino acids to screen for expected mutants (von der Osten *et al.*, 1993).

Functional analysis of genes in the thurincin H cluster. The immunity protein(s) were identified to be encoded by genes within the cluster. However, the exact gene(s) responsible for immunity needs to be further revealed by expressing specific gene(s) in sensitive strains. The functions of all the genes (*thnP, thnB, thnD*, *thnE, thnT*, *thnR,* and *thnI*) in the gene cluster are still putatively identified by bioinformatics studies and need to be confirmed by complementation and separate functional analysis studies.

Further characterization of thurincin H mode of action. Permeability of the *B. cereus* cell membrane was not altered based on results obtained, and are suggested to be

further confirmed by potassium (Riazi *et al.*, 2012) or ATP efflux assays (Li *et al.*, 2005). Experiments characterizing the disruption of membrane potential (van Kuijk *et al.*, 2012) or proton motive force (Pham *et al.*, 2004) are also suggested to monitor the mode of action in real time. Both low and high concentrations of thurincin H treatments are recommended, which will complement the observations under the scanning electron microscopy as described in Chapter 6.

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