Short communication: Naturally sensitive *Bacillus thuringiensis* EG10368 produces thurincin H and acquires immunity after heterologous expression of the one-step-amplified thurincin H gene cluster

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ABSTRACT

Heterologous expression of bacteriocin genetic determinants (or operons) has long been a research interest for the functional analysis of genes involved in bacteriocin biosynthesis, regulation, modification, and immunity. Previously, construction of genomic libraries of the bacteriocin producer strains was usually required to identify new bacteriocin operons, a method that 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 method with 100% accuracy. This amplified gene cluster was cloned into plasmid 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 with the wild-type producer, *B. thuringiensis* SF361. Moreover, *B. thuringiensis* EG10368pGW139 acquired complete immunity to thurincin H. The results indicated that one-step PCR is a promising tool to accurately amplify long bacteriocin gene clusters used in bacteriocin functional analysis studies and it is an effective way to produce bacteriocins at a higher level, without the need to clone large chromosomal fragments.

Key words: bacteriocin, gene cluster amplification, heterologous expression, immunity

Short Communication

Bacteriocins produced by *Bacillus* species have potential to be used as natural preservatives in a variety of dairy products, such as milk and cheese (Abriouel et al., 2011). Thurincin H is a bacteriocin produced by *Bacillus thuringiensis* SF361. It exhibits a broad inhibitory spectrum, especially against *Listeria monocytogenes*, *Micrococcus*, and a variety of spore-forming

Bacillus species, which are common foodborne pathogens or spoilage microorganisms in dairy products (Schoeni and Wong, 2005; Lee et al., 2009). It also inhibits several *Staphylococcus aureus* strains that are resistant to multiple antibiotics (G. Wang, unpublished data). The secondary structure of the 31-AA leaderless mature thurincin H peptide forms a hairpin structure, with a helical backbone maintained by 4 pairs of unique sulfur to α -carbon thioether bridges (Sit et al., 2011). This 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 (GenBank accession number: FJ977580.1). It consists of 3 tandem bacteriocin-like precursor genes (*thnA1*, *thnA2*, and *thnA3*), as well as *thnP*, *thnB*, *thnD*, *thnE*, *thnT*, *thnR*, and *thnI* genes, which have 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 could be heterologously expressed at high levels in the sensitive strain *B. thuringiensis* EG10368.

Bacillus and *Escherichia coli* strains used in this study were cultivated in trypticase soy broth (Becton Dickinson Co., Sparks, MD) or on trypticase soy agar (Becton Dickinson Co.) at 37°C. The *E. coli*-*B. thuringiensis* shuttle vector pHT315 (Arantes and Lereclus, 1991) was used as a cloning and expression vector for the thurincin H gene cluster. No promoters or terminators for expressing the genes in the cluster exist in the vector itself. *Escherichia coli* DH5 α was used as a cloning host for the pGW139 recombinant plasmid, and *E. coli* K12 ER2925 (New England Biolabs, Ipswich, MA) was used to produce demethylated plasmids, which are used to increase the efficiency of transformation to *B. thuringiensis* (Macaluso and Mettus, 1991). Erythromycin (25 μ g/mL) and ampicillin (100 μ g/mL) was used to select

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Table 1. Strains, plasmids, and primers used in this study

Item	Property	Reference
Strain		
<i>Escherichia coli</i> DH5 α	Host for cloning thurincin H gene cluster to plasmids pHT315	New England Biolabs (Ipswich, MA)
<i>E. coli</i> K12 ER2925	<i>Dam dcm</i> strain, produce demethylated plasmids	New England Biolabs
<i>Bacillus thuringiensis</i> SF361	Thurincin H producer strain	Lee et al., 2009
<i>B. thuringiensis</i> EG10368	Sensitive to thurincin H	Lee et al., 2009
<i>B. thuringiensis</i> EG10368pGW139	<i>B. thuringiensis</i> EG10368 carrying recombinant plasmid pGW139	This study
<i>Bacillus cereus</i> F4552	Sensitive to thurincin H	Lee et al., 2009
<i>B. thuringiensis</i> SF361thnH	Native thurincin H producer with a deletion of <i>thnA1</i> , <i>thnA2</i> , and <i>thnA3</i> genes	Lee et al., 2009
Plasmids		
pHT315	pHT315 <i>E. coli</i> - <i>B. thuringiensis</i> shuttle vector, 6.5 kb, Ery ^R Amp ^R	Arantes and Lereclus, 1991
pGW139	pHT315 carrying the 8.14-kb thurincin H gene cluster	This study
Primers		
	Sequence	
TH81F	gatggactgcagttattgggaatcgctttatagacatcaagcaaaccatta	This study
TH82R	gatgggtgaattcctatatatttctgaagtatacaatgtgaccattccatg tgttctattt	This study
SequencingPrimer_1	ctgcaaggcgattaagtgtgggtaac	G. Wang, unpublished data
SequencingPrimer_2	cggataacaatttcacacaggaacagcta	G. Wang, unpublished data
SequencingPrimer_3	ttattgggaatcgctttatagacatcaagcaaacc	This study
SequencingPrimer_4	ttagtaacatcaagagcatcattaccagccgagcaa	This study
SequencingPrimer_5	gatccaggtgaaatgttatcttttaaatcaggatg	This study
SequencingPrimer_6	agcatgaactgtagtaataggtatgaaagtt	This study
SequencingPrimer_7	aacaggaagtagtacaccaataaaaacttaga	This study
SequencingPrimer_8	taacatggatgttcccccaatatatgaaagcataatct	This study
SequencingPrimer_9	atctgcatgatgggatagggctaatacgaactgcaaa	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	attttttaacttacaagcacctcaaatggact	This study
SequencingPrimer_17	attatcgccaccgataccatatattattggctatat	This study
SequencingPrimer_18	gcagaaatgtatgaaccttaaatggaatagaggatataaaat	This study
SequencingPrimer_19	tgtttcaggaggtcaagcacaatttatagccttttta	This study
SequencingPrimer_20	aagtgaaaatagaacacatggaatgggtcacattgtatacttcagaaatatag	This study

Figure 1. Amplification of the thurincin H gene cluster by one-step PCR; (A) 1-kb Molecular Ruler (Bio-Rad, Hercules, CA); (B) 8.14-kb thurincin H gene cluster amplified by one-step PCR (negative control with no template was routinely used in PCR reactions, but not shown in the figure).

B. thuringiensis and *E. coli* transformants, respectively. All strains, plasmids, and primers used in this study are listed in Table 1.

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 μ L) template. PrimeSTAR Max DNA Polymerase (R045A; Takara, Dalian, China) was used as the master mix, and TH81F and TH82R 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/s. The manufacturer'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 subsequently modified to be 4 min (about 2 kb/min), with an additional 7-min extension time at the end, because the extension time (1 kb/5 s) might not be sufficient for extension in each cycle. The optimized PCR method included 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 extension at 72°C (4 min), with one final extension at 72°C (7 min). The PCR products showed a single band on a 1.5% agarose gel (Figure 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 (2001). Generally, the purified PCR product was double digested with *Pst*I-HF/*Eco*RI-HF (New England Biolabs), purified, and ligated overnight at 16°C to the double digested (*Pst*I-HF/*Eco*RI-HF) pHT315 at a ratio of 1:1 by T4 ligase (New England Biolabs). The ligation mixture was first transformed to *E. coli* DH5 α . 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). The DNA sequencing results showed 100% accuracy compared with reported sequences (GenBank: FJ977580.1). Primers used in sequencing the gene cluster are listed in Table 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).

To test the inhibitory activities 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 2A) but not around *B. thuringiensis* EG10368 (Figure 2B), indicating that *B. thuringiensis* EG10368 itself did not produce self-active antimicrobial compounds. The obvious inhibition zone formed around *B. thuringiensis* EG10368pGW139 was larger than that of the wild type (Figure 2C), indicating that mature thurincin H was produced at a higher level from the gene cluster carried in pGW139 than from the wild-type producer. Similar results were obtained using *Bacillus cereus* F4552 as an indicator strain (data not shown). To test whether *B. thuringiensis* EG10368pGW139 was sensitive or resis-

Figure 2. Inhibitory activities of *Bacillus 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 (---). Images in this figure represent multiple independent experiments with reproducible results. Antibacterial activity of the producer strain is indicated from – (no activity) to +++++ (highest activity).

tant to thurincin H, *B. thuringiensis* EG10368 pGW139 was overlaid on top of those 3 colonies described above. Remarkably, no inhibition zones were formed around any of those colonies (Figure 2, panels D, E, F), indicating that the originally sensitive *B. thuringiensis* EG10368 acquired complete immunity after pGW139 was transformed into it. Because those 2 strains are in the same *Bacillus* species and share highly similar genetic machinery, it is probable that all the genes in the 8.14-kb gene cluster were able to be expressed and were functional in sensitive strain *B. thuringiensis* EG10368. However, the concrete functions of each gene in the cluster need to be further confirmed.

Plasmid pGW139 was constructed based on the high-copy-number plasmid pHT315 (15 copies per cell) (Arantes and Lereclus, 1991), theoretically resulting in high copies of the gene cluster being expressed. *Bacillus thuringiensis* EG10368pGW139 expressed a higher level of thurincin H compared with the wild-type producer (Figure 2C). Similarly, we found that transforming pGW139 into *B. thuringiensis* SF361thnH[–] (a wild-type producer with *thnA1*, *thnA2*, and *thnA3*

deleted in-frame) led to a higher inhibitory activity compared with the wild-type producer (data not shown). These 2 findings likely result from the high copy numbers of genes related to thurincin H biosynthesis, regulation, modification, exportation, and immunity, because it has been reported (Cheigh et al., 2005) that separately expressing additional copies of bacteriocin precursor genes, regulatory components, or immunity proteins led to increased extracellular bacteriocin production. An example of this was reported for nisin Z (Cheigh et al., 2005). Nisin Z production was increased by introducing multiple copies of the structural genes (*nisZ*), the 2-component regulatory system (*nisRK*), or the immunity protein (*nisFEG*), into the wild-type producer *Lactococcus lactis* ssp. *lactis* A164 (Cheigh et al., 2005). Furthermore, additional copies of subtilin immunity genes *spaIFEG* were integrated into the genome of the producer strain *Bacillus subtilis* ATCC 6633 and improved the production of the lantibiotic subtilin, because 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 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 an emerging strategy for identifying putative bacteriocins and bacteriocin gene clusters (Wang et al., 2011). To our knowledge, no research has been reported to directly amplify 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 for screening new bacteriocins genes (clusters) and their functionality.

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-number plasmid pHT315 in the naturally sensitive strain *B. thuringiensis* EG10368 led to higher production of thurincin H in the extracellular environment 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. Besides gene function analysis, this research provided a fundamental genetic manipulation method for expressing a higher level of bacteriocins and subsequently reducing production cost, which will promote its downstream application in various dairy products.

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