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ARTICLE

Comparison of NheA toxin production and doubling time between *Bacillus cereus* and *Bacillus thuringiensis*

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Abstract In this study, we compared the toxin gene expression, NheA toxin production, doubling time, and viable cell number for several strains of the food poisoning bacteria *Bacillus cereus* and the microbial pesticide *Bacillus thuringiensis*. The two *B. cereus* and six *B. thuringiensis* strains evaluated were confirmed to possess and transcribe the *nheABC*, *hblCDA*, and *cytK* genes using polymerase chain reaction (PCR) and reverse-transcription PCR. NheA toxin production was compared based on the absorbance at 414 nm using a Tecra BDE-VIA kit. The NheA-specific production (absorbance/viable cell number) values indicated that the two *B. thuringiensis* var. *kurstaki* isolates from microbial pesticide produced the highest amount of toxin (0.66–0.95) than other *B. thuringiensis* (0.14–0.45) and the *B. cereus* strains (0.19–0.31). However, the *B. thuringiensis* strains had longer doubling time (20–26 min) than the *B. cereus* strains (18–19 min). Interestingly, two *B. thuringiensis* var. *kurstaki* isolates produced the highest amount of NheA toxin, and their doubling times (20–22 min) were close to those of the *B. cereus* strains tested.

Keywords *B. cereus* · *B. thuringiensis* · Doubling time · Microbial pesticide · NheA toxin

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Introduction

Bacillus cereus and *Bacillus thuringiensis*, gram-positive, motile rod-shaped, and spore-forming bacteria, are classified into the same *Bacillus cereus* group due to their high genetic and biochemical similarities. Although the two have many features in common, *B. cereus* is known to cause food poisoning, whereas *B. thuringiensis* has been used in formulations for microbial pesticides.

B. cereus can cause food poisoning in two different ways: emetic or diarrheal. The emetic syndrome is caused by the intake of an emetic toxin called cereulide, which is stable against heat, acidic pH, and digestive enzymes [1]. Vomiting usually occurs with this syndrome [1–3]. In contrast, diarrheal syndrome is caused by the intake of bacteria or spores that germinate inside the human intestine, creating enterotoxins [4, 5]. Hbl, Nhe, and CytK are major enterotoxins that cause the diarrheal syndrome. The Hbl toxin consists of three protein subunits, Hbl L2, L1, and binding protein B [6, 7], which are encoded by the *hblC*, *hblD*, and *hblA* genes, respectively [8, 9]. Likewise, the Nhe toxin is made of three proteins, NheA, B, and C, which are the products of the *nheA*, *nheB*, and *nheC* genes, respectively [10]. CytK is a single protein [11] encoded by the *cytK* gene.

During sporulation, *B. thuringiensis* produces various insecticidal proteins [12], encoded by different kinds of *cry* genes [13]. To date, more than 500 types of *cry* genes have been discovered and among them, the *cry1* subfamily is known as a major group [14]. Since these crystal proteins show highly effective pesticidal properties on insects including libythea celtis and diptera [15, 16], *B. thuringiensis* has been widely adopted as an eco-friendly pesticide material [17]. Due to recent increasing demand,

the market share for microbial pesticides in the crop protection market has increased consistently every year, reaching $300 million in 2013. This figure is expected to increase to $450 million by 2023 [18].

Many studies have been conducted to evaluate the safety of *B. thuringiensis*. The results revealed no association between excessive exposure of humans, mice, rats, rabbits, and sheep to *B. thuringiensis* and the outbreak of food poisoning [19–28]. Another study reported that although some *B. thuringiensis* strains were found in the feces of workers using pesticides, there was no direct correlation between the use of pesticides and food poisoning [29, 30]. Microbial pesticides made of *B. thuringiensis* have been assessed to be safe. However, some studies have shown that *B. thuringiensis* strains contain some enterotoxigenic genes [30–33] and do produce enterotoxins [34]. In addition, there have been a few reports on the outbreak of food poisoning caused by *B. thuringiensis*, not *B. cereus,* although it was unclear whether the strains that caused food poisoning were derived from microbial pesticides [35, 36].

Several studies have been conducted on the presence of enterotoxigenic genes in *B. cereus* and *B. thuringiensis* and on enterotoxin production by the bacteria [37–39]. However, little is known about the amount of toxin produced or the rate of bacterial growth with regard to doubling time. Comparing doubling time and toxin production in pesticide isolates would be helpful to understand the safety of using these bacteria commercially. In this study, we used polymerase chain reaction (PCR) and RT-PCR (reverse-transcription PCR) to investigate the presence and expression of *hblCDA*, *nheABC*, *cytK,* and *cry* genes in *B. cereus* and *B. thuringiensis* strains. We also measured and compared the amount of NheA toxin produced, along with the number of viable cells and their doubling time.

Materials and methods

B. cereus and *B. thuringiensis* strains

The two *B. cereus* and six *B. thuringiensis* strains used in this study are listed in Table 1. Two *B. thuringiensis* var. *kurstaki* and two *B. thuringiensis* var. *aizawai* were isolated from commercial microbial pesticides. Each sample was diluted with sterile phosphate buffer and incubated in mannitol egg yolk polymyxin agar selective medium for *B. cereus*. After 24 h of incubation, pink colonies were collected and confirmed with API 50CH and API 20E systems (bioMerieux, Inc., Marcy I'Etoile, France). PCR was used to classify the colonies into three different groups: emetic toxin-producing *B. cereus* (positive for *groEL* and *ces* genes), enterotoxin-producing *B. cereus* (positive for *groEL* gene), or crystal toxin-producing *B. thuringiensis* (positive for *groEL* and *cry* genes).

Table 1 Bacterial strains used in this study

Bacterium	Strain
Bacillus cereus	ATCC[a] 14579
	ATCC 11778
	KCTC[b] 1510
	KCTC 1513
Bacillus thuringiensis	Pesticide isolate 1 (var. kurstaki)
	Pesticide isolate 2 (var. kurstaki)
	Pesticide isolate 3 (var. aizawai)
	Pesticide isolate 4 (var. aizawai)

[a] American type culture collection

[b] Korean collection for type cultures

Nucleic acids extraction, primers, and PCR conditions

For the incubation of bacterial strains, Luria–Bertani (LB) and Brain–Heart-Infusion (BHI) media (Difco, Detriot, MI, USA) were used. All bacteria were plated on LB agar and incubated overnight at 37 °C. A single colony from each strain was inoculated in 5 mL of LB broth and incubated aerobically at 37 °C for 8 h. For the extraction of chromosomal DNA, the Power Prep™ DNA Extraction kit (KogeneBiotech, Seoul, Korea) was used. Total RNA was extracted using the Minibest Universal RNA Extraction kit (Takara, Otsu, Shiga, Japan), and the Quantitect Reverse-Transcription kit (Qiagen, Hilden, Germany) was used for cDNA synthesis. Conventional PCR and RT-PCR were performed using the Step One Plus real-time system (AB, Foster, CA USA). The primers used in this study were synthesized by Cosmogenetech (Seoul, Korea) and are listed in Table 2.

The PCR mixture is comprised of 1 μL template DNA, 2.5 μL of 10 × Taq buffer, 0.5 μL of dNTP stock solution (containing 10 mM of dNTP), 0.125 μL Taq polymerase (Solgent, Daejeon, Korea), and 1 μL of primer stock solution (10 pmol/μL) in a total volume of 25 μL. The following PCR procedure was used: 95 °C for 5 min, 40 cycles consisting of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, with a final extension cycle at 72 °C for 5 min. PCR products were analyzed in 1.0% agarose gel in a Tris–acetate EDTA (TAE) buffer supplemented with ethidium bromide (0.5 μg/mL TAE), with 100 bp plus (TIANGEN, Bejing, China) used as a molecular mass marker. The RT-PCR mixture is comprised of 1 μL of cDNA, 2.5 μL of 10 × Taq buffer, 0.5 μL of dNTP stock solution (containing 10 mM of dNTP), 0.125 μL Taq polymerase, and 1 μL of primer stock solution (10 pmol/

Table 2 Oligonucleotide sequences of primers used in this study

Gene primer		Sequences (5′ → 3′)	Amplicon size (bp)	References
nheA	NA-F1	ATT ACA GGG TTA TTG GTT ACA GCA GT	475	[49]
	NA-R1	AAT CTT GCT CCA TAC TCT CTT GGA TGC T		
nheB	NB-F1	GTG CAG CAG CTG TAG GCG GT	328	
	NB-R1	ATG TTT TTC CAG CTA TCT TTC GCA AT		
nheC	NC-F1	GCG GAT ATT GTA AAG AAT CAA AAT GAG GT	557	
	NC-R1	TTT CCA GCT ATC TTT CGC TGT ATG TAA AT		
hblA	FhblA	GCA AAA TCT ATG AAT GCC TA	884	[11]
	RhblA	GCA TCT GTT CGT AAT GTT TT		
hblC	FhblC	CCT ATC AAT ACT CTC GCA A	695	
	RhblC	TTT CCT TTG TTA TAC GCT GC		
hblD	HDF	ACC GGT AAC ACT ATT CAT GC	829	
	HDR	GAG TCC ATA TGC TTA GAT GC		
cytK	FCytK	CGA CGT CAC AAG TTG TAA CA	565	[11]
	R2CytK	CGT GTG TAA ATA CCC CAG TT		
groEL	Rba1F	TGC AAC TCT ATT ACG ACA AGC T	238	[40]
	Rba1R	TTA CCA ACG CGC TCC ATT GCT T		[50]
ces	RCesF1	GGT GAC ACA TTA TCA TAT AAG GTG	120	[50]
	RCesR2	ATT CAA CAT AAT ATT ATA CGC CGT		
cry1	c1Aa	ATT CGC TAG GAA CCA AGC	398	[41]
	c1Ad	AAT CCG GTC CCC ATA CAC		

μL) in a total volume of 25 μL. During RT-PCR, one sample with total RNA was used as the negative control and the overall procedure proceeded with the same.

Measurement of NheA toxin

A commercial kit, the Tecra BDE-VIA (3 M, St. Paul, MN, USA), was used for the detection of enterotoxin. The Tecra kit has been reported to detect NheA toxin in the Nhe complex. For the immunoassay, overnight cultures originating from a single colony were diluted 1:100 in fresh BHI broth and incubated at 37 °C with moderate shaking (150 rpm). The supernatant was isolated by centrifugation and subjected to enterotoxin immunoassays in accordance with the manufacturers' instructions. Positive or negative readings of the results were carried out by referring to the color card supplied with the kit. To compare the relative toxin productivity, the amount of NheA toxin produced was measured at a wavelength of 414 nm using a 680 XR microplate reader (Bio-Rad, Hercules, CA, USA). Although the manufacturer did not provide a standard curve, the effective range of the measured absorbance values was 0.2–3.0. According to the manufacturer's instruction, the larger the absorbance within the effective range, the higher was the amount of Nhe toxin. This experiment was repeated twice, and the average values are shown in Table 5.

Measurement of doubling time and viable cell number

Each *B. cereus* and *B. thuringiensis* strain was plated on LB agar and incubated overnight at 37 °C. A single colony of each strain was inoculated in 5 mL of BHI broth and incubated aerobically at 37 °C with shaking at 80 rpm for 12–18 h. Afterward, 100 μL of the overnight culture was re-inoculated in 9.9 mL of fresh BHI broth and incubated at 37 °C with moderate shaking (150 rpm). Viable cells were counted every 20 min, and colony forming units (CFUs) were calculated by multiplying the average number of colonies by the dilution factor. Doubling time during the log phase was determined from the linear part of a semi-logarithmic plot of the number of CFUs per milliliter over time.

Results

Expression of various toxin genes in *B. cereus* and *B. thuringiensis* strains

To examine whether the strains used in this study produced emetic toxin, we confirmed the presence of the *ces* gene, an emetic toxin synthesis gene, using PCR. All eight strains showed negative results, confirming that they

Table 3 PCR detection of various toxin genes and *groEL* gene

Strain	Nhe complex			Hbl complex			cytK	GroEL	ces	Cry1
	nheA	*nheB*	*nheC*	*hblA*	*hblC*	*hblD*	*cytk*	*groEL*	*ces*	*cry1*
Bacillus cereus ATCC 14579	+	+	+	+	+	+	+	+	−	−
Bacillus cereus ATCC 11778	+	+	+	+	+	+	+	+	−	−
Bacillus thuringiensis KCTC 1510	+	+	+	+	+	+	+	+	−	+
Bacillus thuringiensis KCTC 1513	+	+	+	+	+	+	+	+	−	+
Bacillus thuringiensis pesticide isolate 1 (var. kurstaki)	+	+	+	+	+	+	+	+	−	+
Bacillus thuringiensis pesticide isolate 2 (var. kurstaki)	+	+	+	+	+	+	+	+	−	+
Bacillus thuringiensis pesticide isolate 3 (var. aizawai)	+	+	+	+	+	+	+	+	−	+
Bacillus thuringiensis pesticide isolate 4 (var. aizawai)	+	+	+	+	+	+	+	+	−	+

+: PCR product of the expected size was observed

−: No PCR product was observed

Table 4 Expression of enterotoxin genes based on mRNA analysis

Strain	Nhe complex			Hbl complex			cytK
	nheA	*nheB*	*nheC*	*hblA*	*hblC*	*hblD*	*cytk*
Bacillus cereus ATCC 14579	+	+	+	+	+	+	+
Bacillus cereus ATCC 11778	+	+	+	+	+	+	+
Bacillus thuringiensis KCTC 1510	+	+	+	+	+	+	+
Bacillus thuringiensis KCTC 1513	+	+	+	+	+	+	+
Bacillus thuringiensis pesticide isolate 1 (var. kurstaki)	+	+	+	+	+	+	+
Bacillus thuringiensis pesticide isolate 2 (var. kurstaki)	+	+	+	+	+	+	+
Bacillus thuringiensis pesticide isolate 3 (var. aizawai)	+	+	+	+	+	+	+
Bacillus thuringiensis pesticide isolate 4 (var. aizawai)	+	+	+	+	+	+	+

+: Reverse-transcription PCR product of the expected size was observed

−: No RT-PCR product was observed

were not emetic toxin-producing strains (Table 3). The *groEL* gene-specific amplicon found only in the *Bacillus cereus* group was detected in all eight strains [40]. Next, we confirmed the presence of the *cry* gene by using PCR to distinguish between *B. cereus* and *B. thuringiensis* [41]. As shown in Table 3, the DNA fragment specific to the *cry* gene was amplified only in the six *B. thuringiensis* strains, including the reference strains and microbial pesticide isolates. In addition, we investigated the presence of the enterotoxigenic genes *nheABC*, *hblACD*, and *cytK*, for which all eight strains showed positive results (Table 3). The expression of each gene was then determined using RT-PCR, and as shown in Table 4, all eight strains exhibited positive results for the *nheABC*, *hblACD*, and *cytK* genes. Thus, we confirmed that the *B. cereus* and *B. thuringiensis* strains used in this study had several enterotoxigenic genes, which were all expressed at the mRNA level.

Comparison of NheA toxin production and doubling time

The Tecra BDE-VIA kit used to identify Nhe toxin production by the *B. cereus* and *B. thuringiensis* strains showed that all eight strains produced NheA toxin (Table 5). All of the measured absorbance values were within the effective range, but since there was no standard curve, quantitative evaluation was not possible. However, the absorbance values tended to be higher in *B. thuringiensis* (0.67–2.24) than in *B. cereus* (1.04–1.20). The *B. thuringiensis* strains (0.91–2.24) isolated from commercial microbial pesticides had higher absorbance than the *B. thuringiensis* reference strains (0.67–0.84). Among the *B. thuringiensis* variants isolated from microbial pesticide, *kurstaki* (1.87–2.24) showed higher absorbance than *aizawai* (0.91–1.21). On the other hand, the viable cell number at the time the absorbance of the NheA

Table 5 Growth and NheA toxin production by *B. cereus* and *B. thuringiensis* strains

Strain	Toxin production (ABS)[a]	Cell number ($\times 10^8$ CFU)	NheA-specific production (ABS/10^8 CFU)	Doubling time (min)
Bacillus cereus ATCC 14579	1.20	6.33	0.19	18
Bacillus cereus ATCC 11778	1.04	3.38	0.31	19
Bacillus thuringiensis KCTC 1510	0.67	2.43	0.28	22
Bacillus thuringiensis KCTC 1513	0.84	5.88	0.14	25
Bacillus thuringiensis pesticide isolate 1 (var. kurstaki)	2.24	2.35	0.95	20
Bacillus thuringiensis pesticide isolate 2 (var. kurstaki)	1.87	2.85	0.66	22
Bacillus thuringiensis pesticide isolate 3 (var. aizawai)	0.91	2.50	0.36	25
Bacillus thuringiensis pesticide isolate 4 (var. aizawai)	1.21	2.66	0.45	26

Cells were grown in Brain–Heart-Infusion broth and incubated at 37 °C with moderate shaking. Results of NheA toxin production, viable cell count, and doubling time are means of duplicates

Absorbance results >0.2 are considered to be positive based on ELISA. O/S, off scale, absorbance >3.0

[a] The amount of NheA toxin production was detected using the Tecra BDE-VIA kit and was estimated from absorbance at 414 nm

toxin measured was lower in the microbial pesticide *B. thuringiensis* (2.35–2.85 $\times$ 10^8 CFU/mL) than in *B. cereus* (3.38–6.33 $\times$ 10^8 CFU/mL). Taking into account both the toxin absorbance and viable cell number, the NheA-specific production (absorbance/10^8 CFU) indicated that the microbial pesticide *B. thuringiensis* var. *kurstaki* (0.66–0.95) produced the highest amount of toxin than other *B. thuringiensis* (014–0.45) and *B. cereus* (0.19–0.31) strains tested. However, the doubling time was 18–19 min for *B. cereus* and 20–26 min for *B. thuringiensis*; thus, *B. cereus* had a faster growth rate than *B. thuringiensis*. Among the microbial pesticide isolates, the doubling time of *B. thuringiensis* var. *kurstaki* (20–22 min) was faster than that of *B. thuringiensis* var. *aizawai* (25–26 min).

Discussion

Biochemical and genetic similarities between *B. cereus* and *B. thuringiensis* make it difficult to correctly distinguish them. The examination of crystal toxins under a microscope is commonly used to distinguish the two, but the process is laborious and time-consuming [42]. In this study, we conducted PCR assays with primers specific to the *cry1* gene to distinguish the two strains [41], resulting in the detection of *cry1* gene-specific amplicon in only six *B. thuringiensis* strains. Some of the major enterotoxins related to the diarrheal syndrome induced by *B. cereus* include Hbl, Nhe, and CytK. These enterotoxins are known to be destabilized or destroyed as they pass through digestive organs such as the stomach and intestines, but the diarrheal syndrome caused by *B. cereus* persists after the enterotoxins have passed through the digestive system. This suggests that the diarrheal syndrome is caused by an enterotoxin or enterotoxins produced by the *B. cereus* that proliferate in the intestine. At present, the safety of *B. thuringiensis* as a microbial pesticide has been verified in many animal experiments [43]. However, it is still unclear why excessive intake of *B. thuringiensis* microbial pesticides does not cause food poisoning, even though like *B. cereus*, they have several diarrhea-causing enterotoxin genes and produce enterotoxins [43]. Our PCR and RT-PCR results correspond well with previous studies reports indicating that similar to *B. cereus*, *B. thuringiensis* also has several enterotoxigenic genes, some of which are expressed [30–33]. The amount of NheA toxin produced by each strain evaluated was measured based on the absorbance after immunoassays, and all eight strains showed positive results. This is consistent with the results of previous studies showing that most *B. cereus* isolated from nature and from various samples produce the Nhe toxin [44, 45].

However, research comparing the amount of toxin produced by each *B. cereus* and *B. thuringiensis* strain and the difference in doubling time over the same period has yet to be conducted. Although the results of this study were not quantitative, the microbial pesticide *B. thuringiensis* produced more toxin than the food poisoning bacteria *B. cereus*. Moreover, among the microbial pesticides evaluated, *B. thuringiensis* var. *kurstaki* exhibited higher toxin production than *B. thuringiensis* var. *aizawai*. However, most enterotoxins are destroyed as they pass through the stomach; therefore, the number of spores and vegetative cells that survive in the intestines, their growth rate, and their ability to produce toxins seem to affect the pathogenicity. The infective dose and the incubation period necessary for the diarrheal syndrome caused by *B. cereus* to occur are 10^5–10^7 CFU and 8–16 h, respectively [4, 46]. Therefore, it is estimated that the greater the toxin production ability and the faster the growth rate of *B. cereus* or

B. thuringiensis vegetative cells proliferating in the intestine, the greater the pathogenicity.

The doubling time for the microbial pesticide *B. thuringiensis* is 20–26 min (mean 23.3 min), while that of *B. cereus* is 18–19 min (mean 18.5 min), with an average difference of 4.8 min. Thus, the growth of *B. thuringiensis* was about 26% slower than that of *B. cereus*. The slower growth of *B. thuringiensis* may result from its large sized plasmid, which would correspond to the finding that *E. coli* carrying a plasmid has a slower growth rate than one without a plasmid due to the metabolic burden [47]. The *cry* genes in *B. thuringiensis* are usually present in plasmids, and when these plasmids are removed, the plasmid-lost *B. thuringiensis* cannot be distinguished from *B. cereus* [48]. Although both *B. cereus* and *B. thuringiensis* produce food poisoning-causing enterotoxins, the slower growth rate of the latter may partly explain why it is safe to include *B. thuringiensis* in microbial pesticides. The difference in doubling time between the two strains in the intestine may affect the time it takes to reach the infective dose. However, the growth rate for the microbial pesticide *B. thuringiensis* was slower than that for *B. cereus*, and *B. thuringiensis* var. *kurstaki* (mean 21 min) grew faster and produced more Nhe toxin than *B. thuringiensis* var. *aizawai* (mean 25.6 min). Therefore, when a large amount of the former is used as a microbial pesticide in crops, more attention should be paid to the safety of the crops and the resulting food. Further studies on the enterotoxin production and growth rate of clinical isolates of *B. cereus* and *B. thuringiensis* are needed.

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