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A fundamental study on bio-control of environmental mosquito problems: Genetic and biological characterization of potentially novel insecticide bacteria

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We have previously isolated two *Bacillus cereus* strains, Ae10 and Cx5, that displayed stable retention in mosquito larvae guts, indicating a high potential as novel host cells for application in mosquito control. However, *B. cereus* has also been recognized as an enterotoxin-producing strain. In order to elucidate the presence of enterotoxin genes in strains Ae10 and Cx5, primers specific to the structural genes of *B. cereus* enterotoxins, multicomponents haemolysin BL (HBL) and non-haemolytic enterotoxin (Nhe), and single components, BceT and EntFM, were designed and used for gene amplification. The PCR results indicated the presence of seven enterotoxin genes, all except *bceT*, in each bacterium tested. Multicomponent genes were confirmed to be present in a single gene cluster. Southern hybridization with the genomic DNA of strain Cx5 indicated that only single copies of *nheA* and *entFM* genes were present on the chromosome. An immunoassay against the *nheA* gene product displayed positive results in all strains. The bacteria harbored haemolytic activity, and also displayed positive results in Vero cell cytotoxicity tests. Oral feeding to mice did not lead to abnormal symptoms, and negative results were obtained in a rabbit skin irritation assay.

Key words: *Bacillus cereus*, *Bacillus thuringiensis*, enterotoxin, biopesticide

Introduction

In many tropical areas, mosquito-borne diseases such as dengue, malaria, filariasis, and Japanese encephalitis, remain a serious problem. Various measures have been taken in order to control the mosquito population in these areas. Chemical pesticides such as dichlorodiphenyltrichloroethane (DDT), gammaxane, malathion, and chlordane have been applied to some extent. However, not only has mosquito resistance against these chemicals been reported, but the pesticides themselves present threats to both human health and the ecosystem (4, 17). Therefore as an alternative, biopesticides such as *Bacillus thuringiensis* serovar *israelensis* (*Bti*) and *Bacillus sphaericus*, are being used in worldwide field tests designed to control the populations of *Aedes, Culex*, and *Anopheles* larvae.

Bti produces a mosquitocidal crystal protein (δ-endotoxin) consisting of five protoxins encoded by *cry4A*, *cry4B*, *cry10A*, *cry11A*, and *cyt1A* during sporulation. The toxins are toxic to mosquito larvae especially *Aedes* and blackflies (20). On the other hand, highly toxic *B. sphaericus* strains 2297, 2362, and 1593 contain binary toxin genes (51- and 42-kDa protein genes) that exhibit toxicity against mosquito larvae, particularly *Culex* (3). However, utilization of these bacteria has been limited by several disadvantages. The mosquito larvicidal crystals of the bacteria are not stable for long periods in the environment and the crystals and spore-crystal complexes rapidly sediment from the water surface, which is the predominant larval feeding zone. The direct use of *Bti* cells

also has its drawbacks, as the cells do not exhibit stable habitation in the environment. While the toxicity of *B. sphaericus* has been found to be more persistent than that of *Bti*, it has narrow host range specificity and mosquito larvae resistance against its toxins has already been reported (16). Therefore, the isolation and/or development of a bacterial strain with (i) larvicidal activity having a broad host range specificity, (ii) stable habitation, and (iii) non-hazardous properties, is desired.

In order to develop a novel biopesticide for mosquito control, we screened for microorganisms that physically associate with mosquito larvae and display stable habitation in the environment. We have previously reported the presence of the Gram-negative *Enterobacter amnigenus* as one of the predominant strains in the guts of *Anopheles dirus* larvae (12). We also identified *B. cereus* strains Ae10 and Cx5 from the guts of *Aedes aegypti*, and *Culex quinquefasciatus* larvae, respectively. Particularly, the stable presence of *B. cereus* strains Ae10 and Cx5 was observed in the guts of *C. quinquefasciatus* larvae even after a seven-day feeding period (14). The results raised the possibilities that *B. cereus* strains may be applicable as new mosquito larvicide host cells with long persistence. However, *B. cereus* has been recognized as the cause of food poisoning due to its production of enterotoxin. On the other hand, *Bti*, a very closely related strain genetically, has been accepted as a biological pesticide worldwide (10). Before use as a host strain for mosquito control, the investigation of enterotoxin genes in *B. cereus* strains Ae10 and Cx5 strains and their comparison to those of *Bti* are of high priority.

Recently, the enterotoxin genes from *B. cereus* have been classified into two groups. Multicomponent enterotoxins, namely haemolysin BL (HBL) and non-haemolytic enterotoxin (Nhe), and the single component enterotoxins, BceT and EntFM (1, 2, 7, 9, 19). HBL is encoded by the *hblC*, *hblD* and *hblA* genes, while Nhe is comprised of the protein products of the *nheA*, *nheB* and *nheC* genes. In each case, the three genes have been found to comprise a single gene cluster. All three genes of the HBL are required for maximal enterotoxin activity.

In this study, we designed primers specific to the structural genes of all eight enterotoxin genes in order to investigate their presence in *Bacillus cereus* Ae10 and Cx5 and in *Bacillus thuringiensis* var. israelensis 4Q2-72. The PCR results revealed that all genes except *bceT* existed in each strain. We further characterized the genes as well as the biological activities of the bacteria to obtain more data concerning enterotoxins in *B. cereus* and *B. thuringiensis* var. *israelensis*.

Materials and methods

Bacterial strains

B. cereus strains Ae10 and Cx5 were isolated from the guts of *Aedes aegypti* and *Culex quinquefasciatus* larvae collected from the natural habitat in Thailand, respectively, as the dominant population strains. The bacteria were deposited in the Bangkok MIRCEN Culture Collection (code number TISTR 1395 and 1396). The *Bacillus thuringiensis* var. *israelensis* (*Bti*) strain 4Q2-72, which produces mosquito larvicidal crystal protein, was kindly provided from Prof. D.H. Dean, Ohio State University. *B. sphaericus* 2297 harboring the mosquito larvicidal binary toxin genes was a gift from Prof. A.A. Yousten, Department of Biology, Virginia Polytechnic and State University. *B. subtilis* ANA-1 (*arg-15 hsdR hsdM ΔaprA3* Amy^- Npr^-) was a derivative strain of *B. subtilis* NA-1 (13). *B. cereus* NVH 1230-88, isolated after a food borne outbreak in Norway in 1988, was kindly provided from Prof. P.E. Granum, Dept. of Pharmacology, Microbiology and Food Hygiene, Norwegian School of Veterinary Science, Oslo, Norway.

PCR reactions

In order to investigate the presence of enterotoxin genes, specific PCR primers corresponding to eight enterotoxin genes, *hblC*, *hblD*, *hblA*, *nheA*, *nheB*, *nheC*, *bceT* and *entFM* genes, were designed and shown in Table 1. The PCR conditions were optimized and were as follows; 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 51°C for 30 sec, and 72°C for 1 min. One to three micrograms of genomic DNA from each bacterium, 100 μM dNTP, and 0.5 U of DNA polymerase KOD dash (Toyobo, Osaka, Japan) were used in a 50 μl reaction mixture.

DNA sequencing

DNA sequencing was performed with a Perkin-Elmer ABI Prism 310 automatic sequencer (Perkin Elmer Applied Biosystems, Foster City, CA, USA). The ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit was used according to the manufacturer's instructions. Triplicate reactions were performed for one sample and both strands of the genes were sequenced. The nucleotide sequences of the PCR amplified genes were obtained. All deduced amino acid sequences were compared with the previous sequence of *B. cereus* FM1 (2). The sequences of *B. cereus* Ae10 and Cx5 were submitted to GenBank with the accession numbers AF192766 and AF192767, respectively.

DNA hybridization

One microgram of the bacterial genomic DNA was digested with *Eco*RV and applied to 1% agarose gel electrophoresis. The DNA was blotted to the Gene Screen Plus membrane (NEN Research Products, Boston, MA, USA). The PCR amplified *entFM* and *nheA* genes from *B. cereus* Cx5 were labeled with Digoxigenin-11-dUTP (Boehringer Mannheim, Mannheim, Germany) and used as the probes. The hybridized reactions were performed at 42°C for 16 h and the membranes were washed twice with 2x SSC-0.1% SDS for 15 min at room temperature followed by washing two times with 0.1x SSC-0.1% SDS at 68°C for 15 min. The hybridized fragments were identified by color detection

Table 1. Primers for amplifying full-length enterotoxin genes of *Bacillus cereus*.

Gene	Primer sequences	Amplified size (kbp)
entFM	5'– ATGAAAAAAGTAATTGCAGG –3' 5'– TTAGTATGCTTTTGTGTAACC –3'	1.2
bceT	5'– ATGAAAGAGTTAGTTTCAACAGCG –3' 5'– TTATCCGCCGAAGTAAAAGAAGAC –3'	1.0
hblC	5'– ATGAAAACTAAAATAATGACAGG –3' 5'– TTATAGATTTCCAAAAAACGC –3'	1.3
hblD	5'– ATGAAAAAATTTCCATTCAAAG –3' 5'– TTAGCGGCTTTTAAATTCATCTGG –3'	1.1
hblA	5'– ATGATAAAAAAAATCCCTTAC –3' 5'– CTATTTTTGTGGAGTAACAG –3'	1.1
nheA	5'– GTGAAAAAGACTTTAATTACAGG –3' 5'– TTAATGTACTTCAACGTTTGTAACG –3'	1.1
nheB	5'– ATGACAAAAAAAACCTTATAAAG –3' 5'– TTATGCTTTTTTCGTATCTAC –3'	1.2
nheC	5'– ATGCAGAAACGATTTTATAAAAAATGTC –3' 5'– TTACTTCGCCACACCTTCATG –3'	1.0

using BCIP-NBT as substrates.

Immunological assay

The *Bacillus* diarrhoeal enterotoxin visual immunoassay, BDE kit (Tecra Diagnostics, Biotech Australia, Roseville, Australia) was used for immunological detection. The antibody of the kit recognizes the *nheA* gene product. The experiments were carried out according to the manufacturer's instructions.

Vero cells cytotoxicity

The assays were performed by using African green monkey kidney (Vero) cells with the procedure of Gentry and Dalrymple (5). The Vero cells were provided from the Department of Virology, AFRIMS, Bangkok, Thailand. One hundred microliters of Vero cells (10^5 cells/ml) in MEM medium (No. 11700-077, Life Technologies, Rockville, MD, USA) were added to 96-well microtiter plates. The supernatant of bacterial cultures were filtrated through 0.22 μm φ membrane (Millipore, Bedford, MA, USA), then added to the Vero cells culture plates. After 48 h of the incubation at 37°C in 5% CO_2 incubator, detached cells, medium and toxin were removed and attached cells were stained by 0.13% crystal violet in 5% ethanol-2% formalin-PBS for 20 min. Excess staining was washed off with water and the plates were air-dried. The dried plates were pursued intensively for the cytotoxic activity under an inverted microscope. The activity was quantitated by successive elution of the stain in the well using 200 μl of 50% ethanol and measured the absorbance at 595 nm. The toxicity was determined as the dilution that corresponded to the optical density by using untreated cells as the control. Experiments were repeated two times.

Haemolytic activity assay

Haemolytic activity was detected by the formation of a clear zone around the bacterial colonies on blood agar plates after incubation of the bacteria at 30°C for 16 h. Human blood agar plates contained 1% Tryptone, 0.5% Yeast extract, 0.5% NaCl, 1.5% agar and 10% human red blood cells. Sheep blood agar plates contained 2% Nutrient base of heart extract, 2% peptone, 0.5% NaCl, 1.5% agar and 5% sheep red blood cells.

Rabbit skin irritation

Cotton cloth absorbed with the bacterial cultures were attached on normal and scratched skin at the top of the hind legs of three New Zealand white rabbits. Observations for

skin irritation, hypersensitivity, rash and pus were performed at 24, 48 and 72 h. Experiments were performed at Toxicology Unit, National Institute of Health, Nonthaburi, Thailand.

Mouse oral feeding assay

The bacteria (8×10^7 cells/ml) were orally fed to 20 g body weight of ICR mice. Symptoms of diarrhea, inactiveness and abnormal eating behavior were observed for 14 days. Experiments were performed at Toxicology Unit, National Institute of Health, Nonthaburi, Thailand.

Results and Discussion

We investigated the presence of enterotoxin genes in *B. cereus* Ae10, Cx5 and *Bti* 4Q2-72 by PCR using the designed primers and genomic DNA as a template. As a result, we observed specific amplification of the structural genes for all enterotoxin genes in each strain, with the exception of *bceT* (Table 2). As expected, we did not observe amplification when genomic DNA of *B. subtilis* ANA-1 and *B. sphaericus* 2297 were used as template. As in the cases of *B. cereus* Ae10, Cx5 and *Bti* 4Q2-72, the presence of multiple enterotoxin genes in single *Bacillus* strains has been previously reported (8, 11, 18). Concerning the *bceT* gene, we carried out PCR under various conditions, but this did not lead to gene amplification in any of the strains. The examined strains may not harbor the *bceT* gene, or the gene may have an unexpectedly high divergence in its nucleotide sequence. The same tendency has been observed in a previous report by Mantynen and colleagues (15). They explored the *bceT* gene in 108 strains of *B. cereus* by PCR and found that only the *bceT* gene model strain, B-4ac, displayed a specific amplification.

In order to investigate whether the tripartate enterotoxin genes, HBL and Nhe, were clustered together on the chromosome, PCR was performed using the 5' primer of the first gene and the 3' primer of the third gene in each cluster. The results showed a single PCR fragment with a length corresponding to the sum of the three genes arranged in a single cluster (data not shown). The genes were organized in an order of *hblCDA* and *nheABC*. Furthermore, Total Southern blot hybridization of *entFM* and *nheA* genes of *B. cereus* Cx5 displayed only one region on the chromosomal DNA that hybridized with the probe (data not shown). The results indicated the presence of a single copy of each gene on the chromosome of *B. cereus* Cx5. This is a fortunate characteristic when one is aiming to carry out disruption of these genes.

The *entFM* genes, single enterotoxin genes, amplified by PCR from *B. cereus* Ae10, Cx5 and *Bti* 4Q2-72, were cloned and sequenced. The *Bti* 4Q2-72 EntFM contained a longer stretch of consecutive Asn residues than the reference strain, *B. cereus* FM1. This difference in sequence may have a correlation with the different enterotoxic activity observed between *B. cereus* FM1 and *B. thuringiensis*. Interestingly, we found that the *B. cereus* Cx5 EntFM showed more Gln-Thr repeats near the N-terminus than the proteins from other strains (Fig. 1).

We next examined the biological activities of the *B. cereus* strains Ae10 and Cx5. General toxicity of the cells was investigated by mouse oral feeding assay and rabbit skin irritation assays. Both strains displayed negative results. The negative results in rabbit skin irritation sug-

Table 2. The investigation of the enterotoxin genes by PCR.

	Tripartite genes						Single genes	
	Haemolysin BL			Non-haemolysin				
	hblC	*hblD*	*hblA*	*nheA*	*nheB*	*nheC*	*entFM*	*bceT*
Strain	1.3kb	1.1kb	1.1kb	1.1kb	1.2kb	1.0kb	1.2kb	1.0kb
B. cereus Ae10	+	+	+	+	+	+	+	–
B. cereus Cx5	+	+	+	+	+	+	+	–
B. cereus NVH1230-88	+	+	+	+	+	+	+	–
B. thuringiensis var. *israelensis* 4Q2-72	+	+	+	+	+	+	+	–
B. subtilis ANA-1	–	–	–	–	–	–	–	–
B. sphaericus 2297	–	–	–	–	–	–	–	–

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Bc.Cx5          32  ALKEINGQTQTQTQTQTTVTETKTVETTSELKYTVTADVLNVRSG
Bc.Ae10         32  ALKEING----QTQTQTTVTETKTVETKSDLKYTVTADVLNVRSG
Bti.4Q2-72      32  ALKEING----QTQTQTTVTETKTVETTSELKYTVTADVLNVRSG
Bc.FM1          32  ALKEING----QTQTQTTVTETKTVETKSDLKYTVTADVLNVRSG

Bc.Cx5         287  TNN----NNVTNNVQQPGKDVQKPTTGGDTSSIAGFARSLNGSPY
Bc.Ae10        282  TNN----------VQQPVKTYKTNNRWN-TSSIAGFARSLNGSPY
Bti.4Q2-72     282  TNNNNNNNVTNNVQQRSKDVQNPTTGGDTSSIAGFARSLNGSPY
Bc.FM1         282  TNN--------NNVQQPGKDVQKPTTGGDTSSIAGFARSLNGSPY
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Fig. 1. Multiple sequence alignment of *entFM* single gene enterotoxins from various strains of *Bacillus cereus*(*Bc*) and *Bacillus thuringiensis* var. *israelensis*(*Bti*). Bold letters show characteristic amino acids.

Table 3. Biological activities of various *Bacillus* species.

Strain	Cytotoxicity (Vero cells)	Haemolytic activity		Mouse oral feeding assay	Rabbit skin irritation	BDE[c]
		Human[a]	Sheep[b]			
Bc. Ae10	+	+	+	–	–	+
Bc. Cx5	+	+	+	–	–	+
Bc. NVH1230-88	+	+	+	nd	nd	+
Bti. 4Q2-72	+	+	+	–	nd	+
*B. subtilis*ANA-1	–	–	–	nd	nd	–
B. sphaericus 2297	–	+	+	–	nd	–

nd: not determined.

[ab]Haemolytic activity toward human[a] and sheep[b] red blood cells.

[c]*Bacillus* diarrhoeal enterotoxin visual immunoassay was used.

gest that the bacteria do not show extraintestinal pathogenesis. A further detailed examination was carried out by measuring cytotoxicity against Vero cells (African Green monkey kidney cells) and haemolytic activities towards human and sheep red blood cells. Direct detection of the NheA enterotoxin was performed with the *Bacillus* diarrhoeal enterotoxin visual immunoassay, BDE kit. The *B. cereus* strains Ae10 and Cx5, along with *Bti.* 4Q2-72, all showed positive results in Vero cells cytotoxicity, human and sheep red blood cell haemolytic activities and the BDE immunoassay (Table 3). *B. subtilis* ANA-1 displayed negative results in these assays. *B. sphaericus* displayed negative results in the cytotoxicity against Vero cells and BDE assay, but proved positive in haemolytic activity. In particular, the BDE immunoassay directly displayed that the NheA enterotoxin was produced in *B. cereus* strains Ae10 and Cx5. These results somewhat contradict with the fact that mouse oral feeding did not lead to any symptoms of diarrhea. Granum and colleagues have pointed out that *B. cereus* food poisoning is caused by ingestion of cells or spores, and that the food poisoning is due to enterotoxins produced in the human small intestine, rather than by preformed enterotoxins prior to ingestion (6). Thus, the negative results in mouse oral feeding assay may suggest the lack of colonizability of the bacteria in the mouse intestine. Therefore, it is still of interest to study the relationship between the presence and/or expression of enterotoxin genes in *B. cereus* strains and their actual pathogenic properties. We are now proceeding to disrupt the enterotoxin genes in these *B. cereus* strains by replacing them with a combination of mosquito larvicidal toxin genes. Genetic engineering should lead to strains with the desira-

ble mosquito larvicidal activity and non-hazardous properties, along with their original features, their broad host range specificity and stable habitation in the environment.

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