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Cloning of Novel Enterotoxin Genes from *Bacillus cereus* and *Bacillus thuringiensis*

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A novel enterotoxin gene was cloned from *Bacillus cereus* FM1, and its nucleotide sequence was determined. Previously, a 45-kDa protein causing characteristic enterotoxin symptoms in higher animals had been isolated (K. Shinagawa, p. 181–193, *in* A. E. Pohland et al., ed., *Microbial Toxins in Foods and Feeds*, 1990) from the same *B. cereus* strain, but no report of cloning of the enterotoxin gene has been published. In the present study, a specific antibody to the purified enterotoxin was produced and used to screen the genomic library of *B. cereus* FM1 made with the lambda gt11 vector. An immunologically positive clone was found to contain the full protein-coding region and some 5′ and 3′ flanking regions. The deduced amino acid sequence of the cloned gene indicated that the protein is rich in beta structures and contains some unusual sequences, such as consecutive Asn residues. In order to clone enterotoxin genes from *Bacillus thuringiensis*, two PCR primers were synthesized based on the nucleotide sequence of the *B. cereus* gene. These primers were designed to amplify the full protein-coding region. PCR conducted with DNA preparations from the *B. thuringiensis* subsp. *sotto* and *B. thuringiensis* subsp. *israelensis* strains successfully amplified a segment of DNA with a size almost identical to that of the protein-coding region of the *B. cereus* enterotoxin. Nucleotide sequences of the amplified DNA segments showed that these *B. thuringiensis* strains contain an enterotoxin gene very similar to that of *B. cereus*. Further PCR screening of additional *B. thuringiensis* strains with four primer pairs in one reaction revealed that some additional *B. thuringiensis* strains contain enterotoxin-like genes.

Bacillus cereus is a rod-shaped, spore-forming, gram-positive bacterium commonly found in soil. This bacterium is often isolated from raw milk and dairy products as a contaminant. *B. cereus* like other bacilli produces a large amount of proteolytic enzymes which digest casein to cause abnormal flavor or odor in milk. Besides degrading dairy products, there is a human health problem associated with *B. cereus*, because some strains are known to produce an extracellular enterotoxin. Shinagawa et al. (15) reported that *B. cereus* produces an enterotoxin which causes diarrhea in higher animals. Although a 45-kDa protein that shows typical enterotoxin characteristics was purified from *B. cereus* (14), the gene coding for the toxin has not been cloned.

Bacillus thuringiensis, a species closely related to *B. cereus* (6), is well known for its pathogenicity to a variety of insects. While *B. cereus* is considered a bacterium potentially harmful to humans, *B. thuringiensis* is regarded as a beneficial organism because of its insecticidal activity. In fact, *B. thuringiensis* is distinguishable from *B. cereus* for its production of crystalline inclusion bodies made of insecticidal proteins. Differences between two bacillus species may not go beyond the production of insecticidal proteins. Several *B. thuringiensis* strains appear to produce an enterotoxin similar to that of *B. cereus* (4). The report by Carlson and Kolstø (4) raised a concern that *B. thuringiensis* may cause diarrhea in humans and other higher animals. However, *B. thuringiensis* has been used for decades in numerous commercial insecticides which have been applied on a large scale to food crops. Unlike for *B. cereus*, there is no report that substantiates the human health problem caused by *B. thuringiensis* (10). In order to understand differences in human pathogenicity between *B. cereus* and *B. thuringiensis*, it seems appropriate to compare enterotoxins from these bacteria.

In this report, we have cloned and sequenced the enterotoxin genes from *B. cereus* FM1, *B. thuringiensis* subsp. *sotto*, and *B. thuringiensis* subsp. *israelensis*. In addition, we have demonstrated that some *B. thuringiensis* strains of other subspecies contain a DNA sequence that is homologous to the enterotoxin gene.

MATERIALS AND METHODS

Bacterial strains. *B. cereus* FM1 was kindly provided by K. Shinagawa, Department of Veterinary Medicine, Iwate University, Iwate, Japan. *B. thuringiensis* subsp. *sotto* and *B. thuringiensis* subsp. *israelensis* have been described previously (8). Other *B. thuringiensis* strains which were used to screen for the enterotoxin gene were obtained from the U.S. Department of Agriculture, Peoria, Ill. *Escherichia coli* Y1088 (Stratagene) was used for cloning the total genomic DNA isolated from *B. cereus* FM1. *E. coli* Y1090 (Stratagene) was used for protein expression.

Cloning and sequencing of the *B. cereus* enterotoxin gene. A genomic library of *B. cereus* FM1 was constructed with a total DNA preparation from *B. cereus*. The DNA preparation was digested with *Eco*RI, ligated into lambda gt11 (Stratagene), and packaged with GigapakII Gold (Stratagene) according to the manufacturer's instructions. Ampicillin-resistant plaques were screened by enzyme immunoassay with a polyclonal antibody made in guinea pigs to the purified *B. cereus* FM1 enterotoxin. The bacteriophage containing the enterotoxin gene was designated lambda-gF1. The gene in lambda-gF1 was subcloned into *Eco*RI-digested pBluescript II SK(−) (Stratagene) to create pBF1, and successive unidirectional deletions were made with exonuclease III (7).

Plasmids from *E. coli* were purified by the method of Birnboim and Doly (2). DNA sequences were obtained by the dideoxy chain termination method (13) with [α-^{32}P]dATP (Amersham) and a Sequenase version II kit from U.S. Biochemicals. Restriction enzymes and DNA-modifying enzymes were purchased from Takara. All enzymes were used according to the instructions provided by the manufacturers.

Protein analysis and antiserum production. An enterotoxin preparation from *B. cereus* FM1 was made as described by Shinagawa (14). The preparation was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 12.5% gel. An antiserum to *B. cereus* enterotoxin was made in

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TABLE 1. PCR primers used to screen *B. thuringiensis* strains for the enterotoxin gene

Primer	Sequence	Nucleotides of *entS*	Product size (bp)
TY123	5′-GGTTTAGCAGCAGCTTCTGTAGCTGGCG	19–46	261
TY124	5′-CTTGTCCAACTACTTGTAGCACTTGGCC	262–289	
TY123	5′-GGTTTAGCAGCAGCTTCTGTAGCTGGCG	19–46	581
TY125	5′-GTTTCGTTAGATACAGCAGAACCACC	574–599	
TY123	5′-GGTTTAGCAGCAGCTTCTGTAGCTGGCG	19–46	857
TY126	5′-GTAACGTTATTGTTATTGTTATTGTTAACG	846–875	
TY123	5′-GGTTTAGCAGCAGCTTCTGTAGCTGGCG	19–46	1,222
TY127	5′-CAGAACTAATACGTACACCAGTTGCATCTG	1211–1240	

guinea pigs. The antigen used to produce the antiserum was further purified by SDS-PAGE followed by electroelution with a Bio-Rad 422 electroeluter.

To detect the enterotoxin expressed by lambda-gF1 in *E. coli* Y1089, proteins produced by the bacteriophage were extracted according to the instructions from Stratagene and blotted onto nitrocellulose membranes with a Bio-Rad Trans-Blot apparatus. After the blotting, the membranes were incubated with the polyclonal enterotoxin antibody for 16 h at 4°C followed by an additional incubation with a protein A-horseradish peroxidase conjugate (Bio-Rad) for 30 min at room temperature. Localization of primary antibody was visualized with 4-chloro-1-naphthol as described in the instructions from Bio-Rad.

Cloning of *B. thuringiensis* enterotoxin genes. *B. thuringiensis* enterotoxin genes were cloned from *B. thuringiensis* subsp. *sotto* and *B. thuringiensis* subsp. *israelensis* by PCR with total DNA preparations as templates and the primers ENT-A (5′-ATG AAA AAA GTA ATT TGC AGG) and ENT-B (5′-TTA GTA TGC TTT TGT GTA ACC). Total DNA was purified from *B. thuringiensis* as described by Kalman et al. (9). The sequences used in these primers correspond to nucleotides 1 to 21 (for ENT-A) and 1249 to 1269 (for ENT-B) of the enterotoxin protein-coding region of *B. cereus* FM1. The amplified enterotoxin genes were blunt ended with T4 DNA polymerase and T4 polynucleotide kinase and ligated into *Sma*I-digested pBluescript II SK(−). The genes thus cloned were sequenced as described above.

PCR screening of *B. thuringiensis* enterotoxin genes. A PCR technique reported by Kalman et al. (9) was employed to detect the enterotoxin gene in different *B. thuringiensis* strains. As shown in Table 1, the sequences used in the specific primers were selected from various regions of the enterotoxin gene cloned from *B. thuringiensis* subsp. *sotto* (*entS*). These multiple primers were designed to be used in the same reaction mixture.

RESULTS AND DISCUSSION

SDS-PAGE showed that a crude enterotoxin preparation from *B. cereus* FM1 contained several protein bands (Fig. 1A), one of which was positively identified as the enterotoxin by immunoblotting (Fig. 1B). The size of the immunologically positive protein was 45 kDa, as reported by Shinagawa (14) for the enterotoxin. Since only one protein band reacted positively with the antibody, the blotting confirmed that the antibody was highly specific to the enterotoxin.

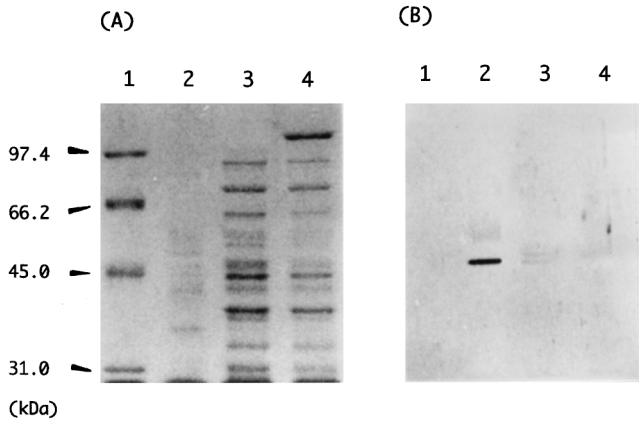

FIG. 1. SDS-PAGE and immunoblot showing detection of the enterotoxin in a crude *B. cereus* preparation, lambda gt11, and lambda gt11 containing the enterotoxin gene (lambda-gF1). (A) SDS-PAGE gel (10% polyacrylamide) stained with Coomassie brilliant blue. (B) Immunoblotting analysis. Lanes: 1, molecular mass markers; 2, crude enterotoxin; 3, lambda-gF1; 4, lambda gt11.

In order to clone the enterotoxin gene from *B. cereus* FM1, several thousand bacteriophage plaques were screened with the enterotoxin antibody. The screening revealed one positive clone, called lambda-gF1. SDS-PAGE showed two immunologically positive protein bands in lambda-gF1 at around 45 kDa (Fig. 1B). Analysis of lambda DNA indicated that a 2.6-kb *Eco*RI *B. cereus* DNA fragment was cloned. The 2.6-kb insert in lambda-gF1 was subcloned into pBluescript II SK(−), and its nucleotide sequence was determined (Fig. 2). The sequence determination revealed an open reading frame (ORF) encoding a 45-kDa protein with 422 amino acid residues. In the immediate vicinity of this ORF, a potential ribosome-binding site was found (11). The 5′ region flanking this ORF contains a stretch of sequence similar to that of the *B. thuringiensis cry* gene promoter, which was reported by Wong et al. (17) and Brown and Whiteley (3). The sequence analysis clearly indicated that a complete enterotoxin gene was cloned from *B. cereus* FM1. The cloned gene was designated *entFM.*

The predicted amino acid sequence of the *B. cereus* enterotoxin was analyzed to understand this protein. The N-terminal portion, which consists of 19 amino acid residues, appeared to be a signal peptide sequence which is relatively hydrophobic. This finding can explain the previous report (14) that *B. cereus* secretes the toxin into the culture medium. Since SDS-PAGE of lambda-gF1 showed two immunologically positive protein bands, one at 45 kDa and the other slightly larger, it is likely that some enterotoxin molecules produced by *E. coli* still contain the signal peptide. Sequence comparisons revealed that there were no statistically significant similarities to other known enterotoxin sequences, such as those reported by Stieglitz et al. (16), Ogawa et al. (12), and Betley and Mekalanos (1). The protein has a repeating Glu-Thr sequence around residue 40. Additionally, this protein has a unique region around residue 280 consisting of consecutive Asn residues. The secondary structure of this protein was predicted by computer with an algorithm developed by Chou and Fasman (5). The prediction indicated that the protein is rich in beta structures.

To determine the location of the *entFM* gene in *B. cereus*, whether it is on a plasmid or the chromosome, total DNA from *B. cereus* FM1 was separated into plasmid and chromosome fractions by $CsCl_2$ density gradient centrifugation and blotted onto a sheet of nylon membrane (Hybond N^+). DNA on the membrane was allowed to hybridize with a radioactive probe specific to the *entFM* gene. The result indicated that the enterotoxin gene is on the chromosome (data not shown).

In order to clone and sequence putative enterotoxin genes which may be present in *B. thuringiensis*, the oligonucleotide primers ENT-A and ENT-B were synthesized. An enterotoxin gene was then amplified by PCR with these primers from DNA

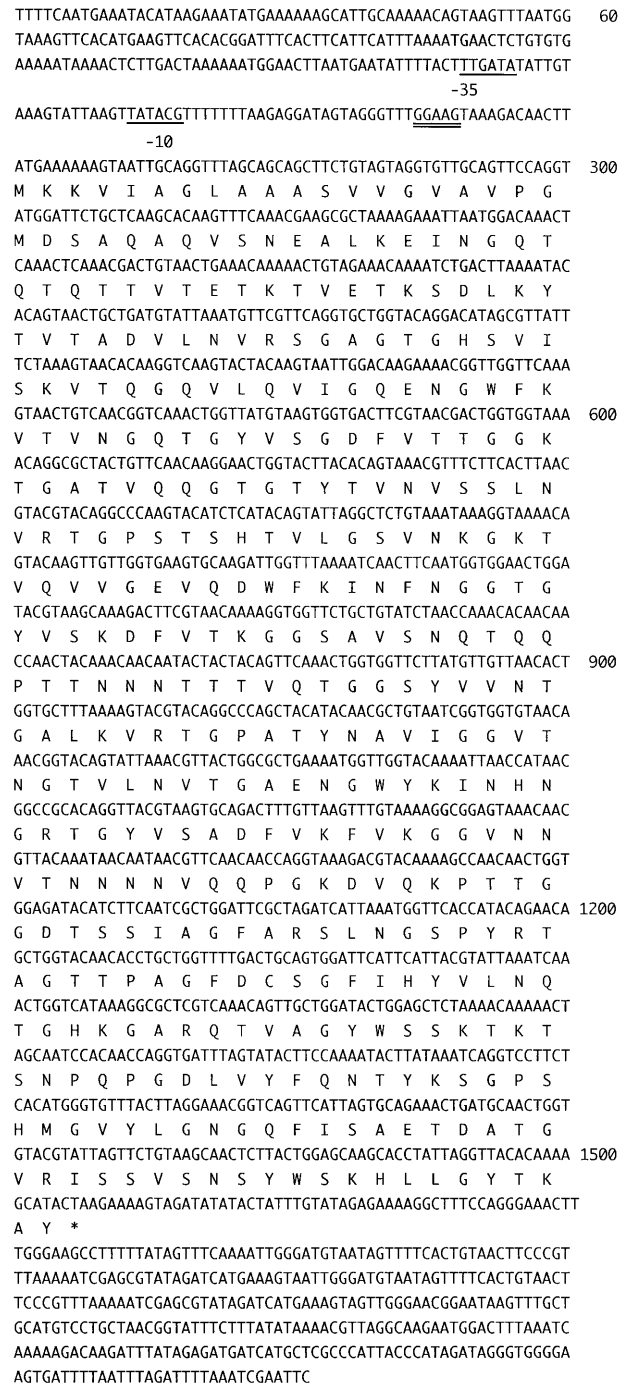

FIG. 2. Nucleotide sequence and predicted amino acid sequence of the enterotoxin gene (*entFM*) from *B. cereus* FM1. The ribosomal-binding site is double underlined, and −35 and −10 consensus sequences are underlined. Asterisk, termination triplet.

templates prepared from *B. thuringiensis* subsp. *sotto* and *B. thuringiensis* subsp. *israelensis*. From both DNA templates, PCR amplified DNA molecules of the right size for the enterotoxin. The amplified genes were then cloned and sequenced. The gene cloned from *B. thuringiensis* subsp. *sotto* was designated *entS*, and the gene from *B. thuringiensis* subsp. *israelensis* was designated *entI*. The sequence analysis of *entS* showed a continuous ORF encoding a 45-kDa protein of 430 amino acid residues. The deduced amino acid sequence of the EntS protein was found to be 99% identical to the sequence of the EntI protein and 97% identical to the sequence of the EntFM protein (Fig. 3).

A number of *B. thuringiensis* strains were screened by PCR to determine if the enterotoxin gene is commonly present in *B. thuringiensis. B. thuringiensis* subsp. *sotto* enterotoxin-specific primers were designed to produce a characteristic pattern of four amplified DNA fragments of 261, 581, 857, and 1,222 bp (Table 1). When a complete set of these DNA fragments is seen, the presence of the enterotoxin gene in a particular *B. thuringiensis* strain is highly likely. A lack of any particular fragment or fragments indicates sequence heterogeneity between the *entS* gene and the other gene detected in *B. thuringiensis*. The results of this PCR screening are summarized in Table 2. *B. thuringiensis* subsp. *morrisoni* HD12 and *B. thuringiensis* subsp. *tolworthi* HD125 showed only the 261-bp PCR product, suggesting that these strains contain no enterotoxin gene or one that is quite different from the *entS* gene. The PCR screening did not produce the 857-bp fragment with *B. thuringiensis* subsp. *galleriae* HD29. This indicates that the enterotoxin gene in this strain is somewhat different from the *entS* gene. The remaining *B. thuringiensis* strains appeared to contain an enterotoxin gene that was similar to the *entS* gene, if not identical. This PCR experiment was repeated under different conditions, as PCR sometimes fails to amplify the target DNA sequence. Since we obtained highly consistent results, it is likely that those *B. thuringiensis* strains which did not show the positive PCR results do not contain the enterotoxin gene.

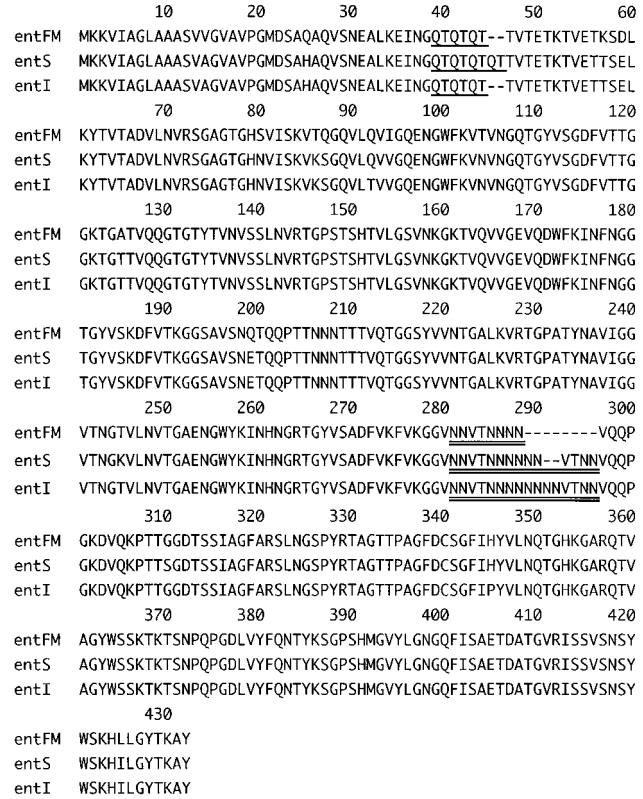

FIG. 3. Comparison of amino acid sequences predicted from nucleotide sequences of *entFM*, *entS*, and *entI* genes. Repeated sequences are underlined, and Asn-rich regions are double underlined.

TABLE 2. Results of PCR screening of the enterotoxin genes in *B. cereus* and *B. thuringiensis* strains

Strain or subspecies	Presence of amplified DNA fragment (bp):			
	243	555	827	1,192
B. cereus FM-1	+	+	+	+
B. thuringiensis subsp.:				
sotto	+	+	+	+
israelensis ONR-60A	+	+	+	+
kurstaki HD-1	+	+	+	+
morrisoni HD-12	+	−	−	−
galleriae HD-29	+	+	−	+
aizawai HD-112	+	+	+	+
tolworthi HD-125	+	−	−	−
kenyae HD-136	+	+	+	+
darmstadiensis HD-147	+	+	+	+
entomocidus HD-198	+	+	+	+

Our results confirm a common distribution of the enterotoxin gene between *B. thuringiensis* and *B. cereus* strains. Interestingly, we discovered that some *B. thuringiensis* strains did not contain the enterotoxin gene as it is known in *B. cereus*. Carlson and Kolstø (4) pointed out a possibility that *B. thuringiensis* is a *B. cereus* variant that has acquired *cry*-containing plasmids. From this point of view, *B. thuringiensis* subsp. *morrisoni* HD12 and *B. thuringiensis* subsp. *tolworthi* HD125 may be more advanced in the evolutionary process. *B. cereus*, being the more primitive organism, could acquire a *cry* gene to become an *ent*-positive *B. thuringiensis* strain and then evolve to an *ent*-negative *B. thuringiensis* strain like *B. thuringiensis* subsp. *morrisoni* and *B. thuringiensis* subsp. *tolworthi*. Or, these *ent*-negative *B. thuringiensis* strains have an ancestor different from that of *ent*-positive *B. thuringiensis* strains. The report of McClintock et al. (10) stating that there are no published human health problems associated with *B. thuringiensis* leads to further speculation that (i) minor differences in amino acid sequences between *B. cereus* and *B. thuringiensis* enterotoxins may reduce the pathogenicity of *B. thuringiensis* and/or (ii) *B. thuringiensis* does not multiply in the human intestine. The results of this study of cloned enterotoxin genes from *B. thuringiensis* will allow us to eliminate a human health concern by removing enterotoxin genes by genetic engineering from the commercial *B. thuringiensis* strains.

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