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Riboprint and Virulence Gene Patterns for *Bacillus cereus* and Related Species

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A total of 72 *Bacillus cereus* strains and 5 *Bacillus thuringiensis* strains were analyzed for their EcoRI ribogroup by ribotyping and for the presence or absence of seven virulence-associated genes. From these 77 strains, 42 distinctive ribogroup were identified using EcoRI, but the two species could not be discriminated by their EcoRI ribogroup. The 77 strains were also examined by PCR for the presence of seven virulence-associated genes, *cerAB*, *pi-plc*, *entFM*, *bceT*, *hblA*, *hblC*, and *hblD*. All five *Bacillus thuringiensis* strains were positive for these genes. Although differences in the patterns of virulence genes were observed among the different *B. cereus* strains, within any given ribogroup the patterns of the seven virulence genes was the same. Pulsed-field gel electrophoresis (PFGE) analysis in combination with available chromosomal maps for a selected group of *B. cereus* strains revealed significant differences in their chromosome size and the placement of virulence genes. Evidence for significant rearrangements within the *B. cereus* chromosome suggests the mechanism through which the pattern of virulence-associated genes varies. The results suggest linkage between ribogroups and virulence gene patterns as well as no apparent containment of the latter within any particular species boundary.

Keywords: *Bacillus cereus*, *Bacillus thuringiensis*, ribotype, ribogroup, virulence genes

The *Bacillus cereus* group, which consists of *B. cereus* and *B. thuringiensis*, is of great interest both economically and medically. *B. thuringiensis* is characterized by its ability to produce the most widely used biological insecticides worldwide [34, 41], whereas *B. cereus* has been implicated as a food poisoning agent. Since these two species share a great number of phenotypic and biochemical characteristics, differentiation of the species is, by no means, easy. *B. cereus* and *B. thuringiensis* have been reported to exhibit >99% similarity in their 16s rRNA base sequences, which suggests they are the same species [4]. The similarity between these two species has been intensively investigated at the genetic level, but their taxonomic relationship is not yet clear [14, 22, 29, 39, 46]. *B. thuringiensis* and *B. cereus* differ only by the presence or absence of δ-endotoxin (toxin crystal), respectively, which has insecticidal activity. Recently, an examination of the genome structure, multilocus enzyme electrophoresis, and sequence analysis suggest that *B. cereus*, *B. thuringiensis*, and *B. anthracis* are very closely related and the latter two proposed to be descendents of *B. cereus* [23].

B. cereus has been implicated in many food poisoning outbreaks as well as a number of opportunistic infections. The two predominant illnesses caused by *B. cereus* are diarrheal and emetic types of food poisoning. The diarrheal type was first recognized after a hospital outbreak in Oslo, Norway, in 1948, and the emetic type described about 20 years after several outbreaks in London, involving rice [21, 32]. Neither is usually life threatening, but it is of concern to the food industry because *B. cereus* spores are heat resistant and therefore have a high probability of contaminating dried products such as milk powder [43].

There is confusion regarding how many different virulence factors are produced by *B. cereus* and which proteins are responsible for food poisoning [38]. Hemolysin BL is composed of three antigenically distinct proteins, B (37.5 kDa), L_1 (38.2 kDa), and L_2 (43.5 kDa). It is responsible for the diarrheal food poisoning syndrome and necrotizing infections such as endophthalmitis [6]. The genes encoding B, L_2, and L_1 are *hblA*, *hblC*, and *hblD*, respectively, and they are arranged in tandem in the genome [42]. The non-hemolytic enterotoxin (NHE) complex, consisting of proteins with molecular masses of 39, 45, and 105 kDa, are cytotoxic in low concentrations [37]. Agata *et al.* [1]

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cloned and sequenced a single protein, BceT, of calculated molecular mass 41 kDa, which was reported to be a diarrheal enterotoxin. However, some *B. cereus* strains involved in a food poisoning outbreak do not have the gene encoding the BceT protein [21]. Cereolysin AB is produced from tandemly arranged genes for two distinct protein products, phospholipase C (*cerA*) and sphingomyelinase (*cer B*) [18]. Phospholipase C confers a lecithinase-positive phenotype to its host, and sphingomyelinase causes lysis of sphingomyelin-rich erythrocytes [18, 25]. Phosphatidylinositol-specific phospholipase C (PI-PLC) cleaves the phosphatidylinositol-glycan-ethanolamine anchor and it has been cloned from *B. thuringiensis* [35] and *B. cereus* [33]. Shinagawa *et al.* [44] purified a 45 kDa protein, EntFM, showing typical enterotoxin characteristic, that was subsequently cloned and sequenced [3]. A cytotoxic protein (CytK) of 34 kDa had been isolated from *B. cereus* strains that were involved in several food poisoning outbreaks and reported to cause necrotic enteritis [36].

Ribosomal RNA is considered to be a good biological clock for discriminating between different genera, species, and, in certain cases, subspecies [5]. Although 16S rRNA sequences have been used as the basis for taxonomic classification of organisms for the last two decades, the sequences do not vary sufficiently for subspecies typing. However, the flanking sequences, which have a less direct biological role, vary with high frequency. Thus, the diversity of flanking sequences is used for subtyping. The differences in these flanking regions can be scanned by looking for restriction fragment length polymorphism (RFLP). A pattern can be generated by probing the multiple copies of the rRNA operon, which are flanked by polymorphic restriction sites [7].

In this study, two closely related species, *B. cereus* and *B. thuringiensis*, were analyzed by ribotyping and virulence-associated gene screening. The correlation between the presence of virulence-associated genes and ribotype of the strains was determined.

MATERIALS AND METHODS

Bacterial Strains

The bacterial isolates used in this study are listed in Table 1. A total of 31 whey isolates were identified as *B. cereus* according to

Table 1. Characteristics of 77 strains of *B. cereus* and *B. thuringiensis*.

Strains[a]	Source[b]	Origin	cer TAQ-1[c]	Pi-plc	entFM	bceT	hblA	hblD	hblC	Ribogroup
W1-1-3	W.N.	Whey	0.11	+	+	–	–	–	–	116-100-S-1
ATCC10987	ATCC	Unknown	0.08	+	+	–	–	–	–	116-100-S-2
W24-1-1	W.N.	Whey	0.14	+	+	–	–	–	–	116-100-S-2
W39-1-2	W.N.	Whey	0.04	+	+	+	+	+	+	116-100-S-3
W41-1-1	W.N.	Whey	4.88	+	+	+	+	+	+	116-100-S-5
W44-1-1	W.N.	Whey	0.30	+	–	–	–	–	–	116-100-S-7
W45-2-1	W.N.	Whey	0.34	+	+	–	–	–	–	116-100-S-8
W30-1-3	W.N.	Whey	0.11	+	+	–	–	–	–	116-102-S-1
W32-2-3	W.N.	Whey	0.01	+	+	–	–	–	–	116-102-S-2
W35-1-2	W.N.	Whey	0.08	+	+	–	–[d]	+	–[d]	116-102-S-3
W47-1-1	W.N.	Whey	0.72	+	+	–	–	–	–	116-102-S-5
W50-1-1	W.N.	Whey	0.79	+	+	–	–	–	–	116-102-S-5
W50-2-1	W.N.	Whey	0.83	+	+	–	–	–	–	116-102-S-5
W48-2-1	W.N.	Whey	0.81	+	+	–	+	+	+	116-102-S-6
W51-1-3	W.N.	Whey	0.76	+	+	–	+	+	+	116-102-S-6
W49-1-1	W.N.	Whey	0.33	+	+	–	–	–	–	116-102-S-7
W39-1-3	W.N.	Whey	0.09	+	+	+	+	+	+	116-103-S-2
W46-1-2	W.N.	Whey	0.72	+	+	–	+	+	+	116-103-S-3
W52-2-2	W.N.	Whey	0.18	+	+	–	+	+	+	116-103-S-7
7-P	USDA	Unknown	0.05	+	+	+	+	+	+	116-108-S-6
7-V	USDA	Unkown	3.93	+	+	+	+	+	+	116-108-S-6
F4433/73	USDA	Meat loaf	0.48	+	+	–	+	+	+	116-108-S-7
F837/76	Wong	Prostate infection	0.29	+	+	–	+	+	+	116-117-S-8
W38-1-1	W.N.	Whey	0.23	+	+	–	–	–	–	116-56-S-1
F58	W.N.	Unknown	0.83	+	+	–	–	–	–	116-56-S-1
F58-2	W.N.	Unknown	0.80	+	+	–	–	–	–	116-56-S-1
ATCC14579	ATCC	Unknown	3.55	+	+	+	+	+	+	116-56-S-2
17-B	USDA	Unknown	3.45	+	+	+	+	+	+	116-56-S-2
17-P	USDA	Unknown	3.21	+	+	+	+	+	+	116-56-S-2

Table 1. Continued.

Strains[a]	Source[b]	Origin	cer TAQ-1[c]	Pi-plc	entFM	bceT	hblA	hblD	hblC	Ribogroup
W27-1-1	W.N.	Whey	4.59	+	+	+	+	+	+	116-56-S-2
W37-1-1	W.N.	Whey	5.21	+	+	+	+	+	+	116-56-S-2
W42-1-1	W.N.	Whey	4.69	+	+	+	+	+	+	116-56-S-2
W5-2-1	W.N.	Whey	3.24	+	+	+	+	+	+	116-56-S-2
B4ac-1	USDA	Pea soup	4.02	+	+	+	+	+	+	116-56-S-2
B4ac-2	USDA	Pea soup	4.95	+	+	+	+	+	+	116-56-S-2
B6ac	USDA	Unknown	4.47	+	+	+	+	+	+	116-56-S-2
H129	W.N.	Unknown	5.29	+	+	+	+	+	+	116-56-S-2
H224	W.N.	Unknown	5.60	+	+	+	+	+	+	116-56-S-2
R-96	USDA	Unknown	3.47	+	+	+	+	+	+	116-56-S-2
Soc 67	Wong	Periodontic infection	3.40	+	+	+	+	+	+	116-56-S-2
S232	W.N.	Unknown	0.21	–	+	–	–	–	–	116-56-S-5
AM232	W.N.	Unknown	5.57	+	+	+	+	+	+	116-56-S-6
SSA-70	USDA	Unknown	0.13	+	+	+	+	+	+	116-58-S-7
T-2	USDA	Unknown	0.1	+	+	+	+	+	+	116-58-S-7
F4370/75	USDA	Barbecue chicken	0.48	–	–	–	–[d]	+	–[d]	116-62-S-3
NCTC11145	USDA	Meat loaf	0.37	+	+	–	+	+	+	116-62-S-5
ATCC10876	ATCC	Unknown	3.70	+	+	+	+	+	+	116-62-S-7
45814/70	USDA	Unknown	3.85	+	+	+	+	+	+	116-62-S-7
F4810/72	USDA	Rice	3.97	+	+	+	+	+	+	116-62-S-7
MGBC142	Wong	Endophthalmitis patient	3.25	+	+	+	+	+	+	116-62-S-7
MGBC145	Wong	Endophthalmitis patient	3.24	+	+	+	+	+	+	116-62-S-7
NCTC11143	USDA	Cooked rice	4.37	+	+	+	+	+	+	116-62-S-7
JAP-IV	USDA	Unknown	0.16	+	+	–	–	–	–	116-62-S-8
F4552/75	USDA	Vomitus	0.18	+	+	–	–	–	–	116-64-S-3
3-V	USDA	Unknown	0.45	+	+	–	–	–	–	116-64-S-4
5056	USDA	Meat loaf	3.49	+	+	+	+	+	+	116-67-S-2
H-13	USDA	Unknown	4.33	+	+	+	+	+	+	116-67-S-3
W6-1-1	W.N.	Whey	0.16	+	+	–	–	–	–	116-72-S-5
ATCC11778	ATCC	Unknown	3.75	+	+	+	–[d]	–[d]	–[d]	116-84-S-1
2-O	USDA	Unknown	3.14	+	+	+	+	+	+	116-84-S-1
2-P	USDA	Unknown	4.33	+	+	+	+	+	+	116-84-S-1
2-V	USDA	Unknown	2.64	+	+	+	+	+	+	116-84-S-1
W53-1-3	W.N.	Whey	4.89	+	+	+	+	+	+	116-84-S-1
MAC-1	USDA	Macaroni and Cheese	3.62	+	+	+	+	+	+	116-84-S-1
T-1	USDA	Unknown	4.05	+	+	+	+	+	+	116-84-S-1
RAL-14	USDA	Unknown	0.15	–	–	–	+	+	–[d]	116-84-S-4
W15-1-2	W.N.	Whey	3.72	+	+	+	+	+	+	116-84-S-6
W2-1-1	W.N.	Whey	0.01	+	+	+	–[d]	+	–[d]	116-99-S-1
W7-1-1	W.N.	Whey	3.53	+	+	+	+	+	+	116-99-S-3
W20-2-3	W.N.	Whey	0.23	+	+	+	+	+	+	116-99-S-4
W43-1-1	W.N.	Whey	0.23	+	–	–	–	–	–	116-99-S-5
W43-1-2	W.N.	Whey	0.27	+	+	–	–	–	–	116-99-S-6
B. thur. 824	Qualicon	Unknown	0.19	+	+	+	+	+	+	116-106-S-3
B. thur. 639	Qualicon	Unknown	0.22	+	+	+	+	+	+	116-108-S-1
B. thur. 713	Qualicon	Unknown	0.23	+	+	+	+	+	+	116-108-S-1
B. thur. 833	Qualicon	Unknown	0.18	+	+	+	+	+	+	116-108-S-1
B. thur. 826	Qualicon	Unknown	3.82	+	+	+	+	+	+	116-62-S-7

[a]All strains are *Bacillus cereus*, except for five *Bacillus thuringiensis* strains described as *B. thur.*

[b]USDA: USDA, Philadelphia, PA; Qualicon: Qualicon, Inc., Wilmington, DE; W.N.: Wyeth Nutritionals Inc., Milton, VT; ATCC: American Type Culture Collection, 10801 University Blvd, Manassas, VA; Wong: Amy C. Lee Wong, Department of Food Microbiology and Toxicology, University of Wisconsin, Madison, WI.

[c]The values were taken from the work by Kim *et al.* [30].

[d]Negative on PCR test but positive on Southern hybridization.

biochemical tests and microbiological tests described in the FDA *Bacteriological Analytical Manual* (BAM). *B. thuringiensis* was differentiated from *B. cereus* by δ-endotoxin production.

Ribotyping

Ribotyping was performed by the Laboratory for Molecular Typing (Cornell University, Ithaca, NY, U.S.A.) using an automated ribotyping system (RiboPrinter, Microbial Characterization System, Qualicon, Wilmington, DE, U.S.A.). EcoRI was used for genomic DNA digestion. Ribotypes of 13 *B. cereus* and 19 *B. thuringiensis* strains were provided by Qualicon. They are *B. cereus* QLN-20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, and 41, and *B. thuringiensis* QLN-3, 4, 5, 6, 7, 8, 9, 11, 12, 13, 14, 15, 16, 36, 38, 39, 43, 44, and 45.

Virulence Gene Typing

Seven sets of primers were selected based on known *B. cereus* virulence-associated genes and cytolytic toxin genes (Table 2). GenBank sequences for the *B. cereus* hemolysin BL binding component, B protein (Accession No. L20441), L_2 and L_1 proteins (Accession No. U63928), *cerAB* gene (Accession No. M24149), and *bceT* gene (Accession No. D17312) were used to design primers. The primer set entFM2-F and entFM2-R was designed based upon the sequence reported by Asano *et al.* [3]. The names and sequences of primer sets PC-105/106 and Certaq-5'/3' were taken from the work by Damgaard *et al.* [16] and Kim *et al.* [30].

B. cereus were grown in 5 ml of Brain Heart Infusion broth (BHI) (Difco, St. Louis, MO, U.S.A.) overnight at 37°C at 250 rpm. A 500-μl portion of the culture was centrifuged and the pellet was resuspended in 95 μl of 1×PCR buffer (Perkin-Elmer). Lysozyme (Sigma) was added to the final concentration of 2 mg/ml, and the mixture was then incubated for 15 min at 37°C. Proteinase K (Boehringer Mannheim) was added to the final concentration of 200 μg/ml. After a 1-h incubation at 60°C, the mixture was boiled for 8 min, and 1 μl of the mixture was used for PCR.

All PCR reactions were carried out in a 50-μl volume consisting of 1×PCR buffer, 1.5 mM $MgCl_2$, 100 μM each of dATP, dGTP, dCTP, and dTTP (New England Biolabs, Beverly MA, U.S.A.), 2.5 U of AmpliTaq DNA polymerase (Perkin-Elmer, Foster City, CA, U.S.A.), and 500 nM of each primer. The cycling conditions were as follows: one cycle of 94°C for 5 min, 40 cycles of 94°C for 30 sec, each annealing temperature for 30 sec, and 72°C for one min, and one hold for 10 min at 72°C. The annealing temperatures for each primer were 59°C for Certaq-5'/3', 53°C for PC105/106, 55°C for bceT-2F/2R, 50°C for entFM2-F/R, 55°c for HblA-F/R, 57°C for L1/BC-F/R, and 50°C for L2/BC-F/R. The PCR products were verified by agarose gel electrophoresis and ethidium bromide staining. All PCR reactions were performed in a Perkin-Elmer model 2400 thermal cycler.

Pulsed-Field Gel Electrophoresis (PFGE) Analysis

Bacteria were grown in 5 ml of BHI broth overnight at 37°C to an optical density of 0.6 at 600 nm. The cells were harvested and the plugs for PFGE were prepared as described by Kolsto and colleagues [12, 31]. Genomic DNA was digested with NotI enzyme (New England Biolabs) for PFGE analysis. The Gene Path Strain Typing System from Bio-Rad Laboratories (Richmond, CA, U.S.A.) was used. The DNA in one-fourth of the plug was electrophoresed through 1% agarose gels (SeaKem GTG, FMC Corp., Rockland, ME, U.S.A.) in 0.5×TBE (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA). Electrophoresis was performed with a pulse time of 34.9 sec for 19.7 h, pulse time of 66.0 sec for 19.5 h, and pulse time of 120 sec for 19.5 h. *Saccharomyces cerevisiae* chromosomes (size range 260 to 1,900 kb; Bio-Rad Laboratories) and *lambda* concatemers (size range 50 to 1,000 kb; Bio-Rad Laboratories) were used as size markers.

Southern Hybridization

DNA fragments separated on a 1.0% agarose gel were transferred overnight in 20× SSC (1× SSC is 0.3 M NaCl with 0.03 M sodium citrate) onto Hybond-N^+ (Amersham). Genomic DNA was hybridized with gene probes produced from *B. cereus* 17-B. The probes were labeled by PCR with digoxigenin 11 dUTP (Boehringer Mannheim). Hybridization was performed at 60°C overnight in 5× SSC; 0.02%

Table 2. PCR primers used in this study

Primer name	Primer sequence 5'→3'	Location of primer 5'→3'
Certaq-5'[a]	TTA TGC TGC TGA CTA TGA AAA TCC TTA	467–493
Certaq-3'[a]	ATA CAG CTG CTC TTA CGA ACC AAT C	927–903
PC 105[b]	CGC TAT CAA ATG GAC CAT GG	260–279
PC 106[b]	GGA CTA TTC CAT GCT GTA CC	828–809
bceT-2F	TTA CAT TAC CAG GAC GTG CTT	1,354–1,374
bceT-2R	TGT TTG TGA TTG TAA TTC AGG	1,781–1,761
entFM2-F	ACA GGC CCA AGT ACA TCT CAT A	667–688
entFM2-R	CGT TGT TTA CTC CGC CTT TTA C	1,081–1,060
HblA-F	GAT TAC AGA TTT GCG AGG TGA	826–849
HblA-R	CGA GCG CCT TTA TGA CCA G	1,280–1,262
L1/BC-F	AAG AAA CGA CCG CTC AAG AAC AAA	2,654–2,667
L1/BC-R	ACA ATA ACT ACC GCT CCT CCA ATA	3,341–3,318
L2/BC-F	TTC CTA TCA ATA CTC TCG CAA CA	1,241–1,263
L2/BC-R	TGG CTT TCA TCA GGT CAT ACT C	2,125–2,104

[a]Names and sequence are taken from the work of Kim *et al.* [30].

[b]Names and sequence are taken from the work of Damgaard *et al.* [16].

SDS; 0.1% sodium lauryl sarcosine; 1% Blocking Reagent (Boehringer Mannheim). Following hybridization, filters were washed twice at room temperature for 10 min each in 5× SSC with 0.1% SDS. Nonradioactive detection was conducted according to the protocol provided with the Genius 3 Nucleic Acid Detection Kit (Boehringer Mannheim).

RESULTS

Virulence Gene Distribution Among *B. cereus*

The PCR conditions were established for the seven virulence genes, and 72 *B. cereus* and 5 *B. thuringiensis* strains were tested (Table 1). A total of 46 (64%) of the *B. cereus* strains were positive by PCR for the *hblA*, *hblC,* and *hblD* genes of hemolysin BL. The primer sets that were designed to amplify a portion of each gene gave 455, 884, and 688 bp fragments for the *hblA*, *C*, and *D* genes, respectively. Three genes encoding hemolysin BL (*hblC*, *hblD*, and *hblA*) are arranged in tandem in the genome [42]. It was interesting to note that three strains (W2-1-1, W35-1-2, and F4370/75) were positive for *hblD*, but negative for *hblA* and *hblC*, and one strain (RAL-14) was positive for both *hblA* and *hblD*, but negative for *hblC*. These four strains were further analyzed by Southern hybridization to determine if any portion of the *hblA* and/or *hblC* was present. EcoRI- and HindIII-digested DNA fragments were hybridized with a dig-labeled PCR probe from *hblA* and *hblC*. All the four strains were positive by Southern hybridization for those two genes. However, the sizes of the hybridized fragments in these four strains were different than strain 17-B (Fig. 1), indicating sequence divergence in the flanking restriction enzyme recognition. Sequence variation within the genes might account for the negative PCR result observed for the four strains.

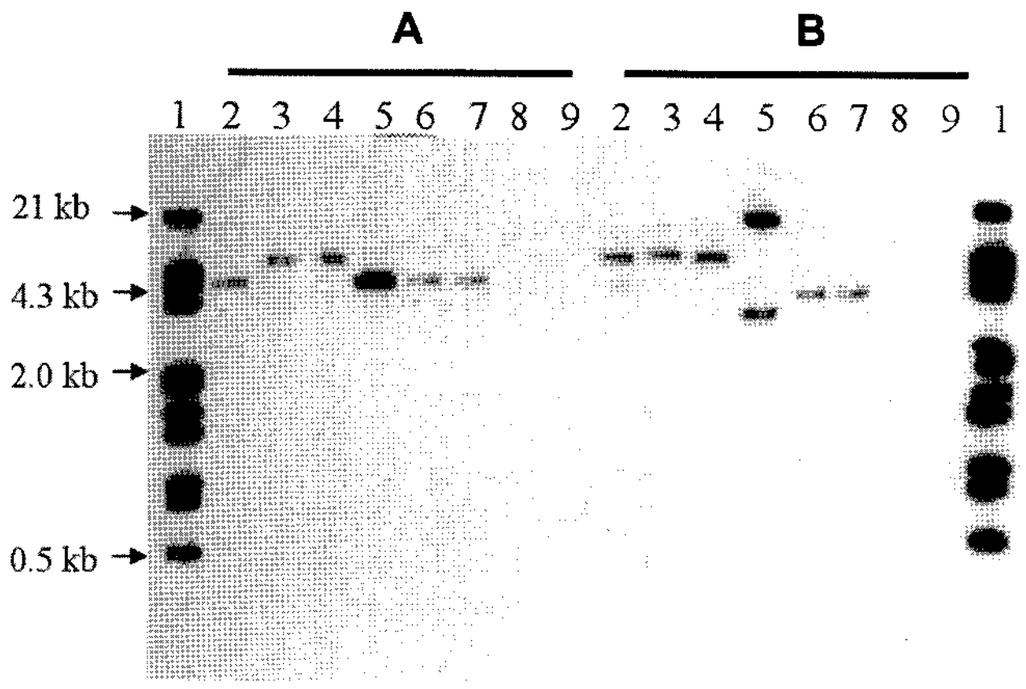

Fig. 1. Southern hybridization of *hblC*⁺ *B. cereus* strains. The *hblC* gene was probed with a digoxigenin-labeled 884-bp *hblC* fragment amplified from *B. cereus* 17-B using primer set L2/BC-F/R.
The DNA was digested with EcoRI (**A**) and HindIII (**B**). Lanes: 1, digoxigenin-labeled lambda DNA, BamHI/EcoRI digested; 2, *B. cereus* 17-B; 3, *B. cereus* F837/76; 4, *B. cereus* F4370/75; 5, *B. cereus* RAL-14; 6, *B. cereus* W35-1-2; 7, *B. cereus* W2-1-1; 8, *B. cereus* F58; 9, *B. cereus* W47-1-1. Lanes 2 and 3 are positive controls, and lanes 8 and 9 are negative controls.

A total of 41 (57%) of the *B. cereus* strains tested were positive by PCR for the *bceT* gene, and a 428-bp fragment was observed with the bceT-2F/bceT-2R primer set. In addition, 69 (96%) and 68 (94%) of the *B. cereus* strains were positive for the genes encoding the phosphatidylinositol-specific phospholipase C (PI-PLC) and Ent FM, respectively. A 569 bp and a 415 bp fragment were observed as expected for PI-PLC and *entFM*, respectively. Seventy-two (100%) of the *B. cereus* strains were positive for *cer*AB with fragment size of 461 bp. The five *B. thuringiensis* strains were tested and shown to be positive by PCR for the seven genes encoding *cer*AB, three components of hemolysin BL, *bceT*, PI-PLC, and *entFM*.

Comparison of the Ribotype Patterns of *B. cereus* and *B. thuringiensis*

A total of 72 *B. cereus* and 5 *B. thuringiensis* strains were ribotyped, and from these a total of 42 distinct ribotypes were identified (Table 1). The 32 ribotypes provided by Qualicon as well as the 42 ribotypes identified in this study were analyzed to determine if there are any distinctive ribotype patterns between these two species. However, there were no species-specific patterns that could be used to separate one species from another. When the ribotype patterns were subject to group analysis based on similarity, many *B. cereus* strains were dispersed to *B. thuringiensis* ribogroups. All the 7 *B. cereus* strains typed as 84-S-1 showed >92% similarity with *B. thuringiensis* strain QLN-44, and all the 7 *B. cereus* strains typed as 62-S-7 showed >86% similarity with that of *B. thuringiensis* strain QLN-11 (Fig. 2). Moreover, all the 14 *B. cereus* strains typed as 56-S-2 were shown to have >87% similarity with the ribotype of *B. thuringiensis* strain QLN-44 (Fig. 2). In addition, the ribopattern of *B. thuringiensis* 826 was similar to type 62-S-7, as were seven *B. cereus* strains (ATCC 10876, F4810/72, MGBC 142, MGBC 145, NCTC 11143, W15-1-2, and 45814/70). Among the strains in this type, 62-S-7, *B. cereus* strains MGBC 142 and MGBC 145 were isolated from an endophthalmitis patient, and *B. cereus* F4810/72 was reported to be an emetic toxin producer [2].

Strains within the same ribogroup had the same virulence gene profile (Table 1). Strains of ribotypes 108-S-1, 108-S-6, 56-S-2, 62-S-7, and 84-S-1 were all positive by PCR for the six virulence genes tested, whereas strains of ribotypes 100-S-2, 102-S-5, and 56-S-1 were all negative for the *bceT* and *hbl* genes. The strains of ribotype 102-S-6 were also all negative for the *bceT* gene.

Mapping of Virulence Genes

The NotI DNA fragments of the *B. cereus* strains were hybridized to the dig-labeled probes for virulence genes,

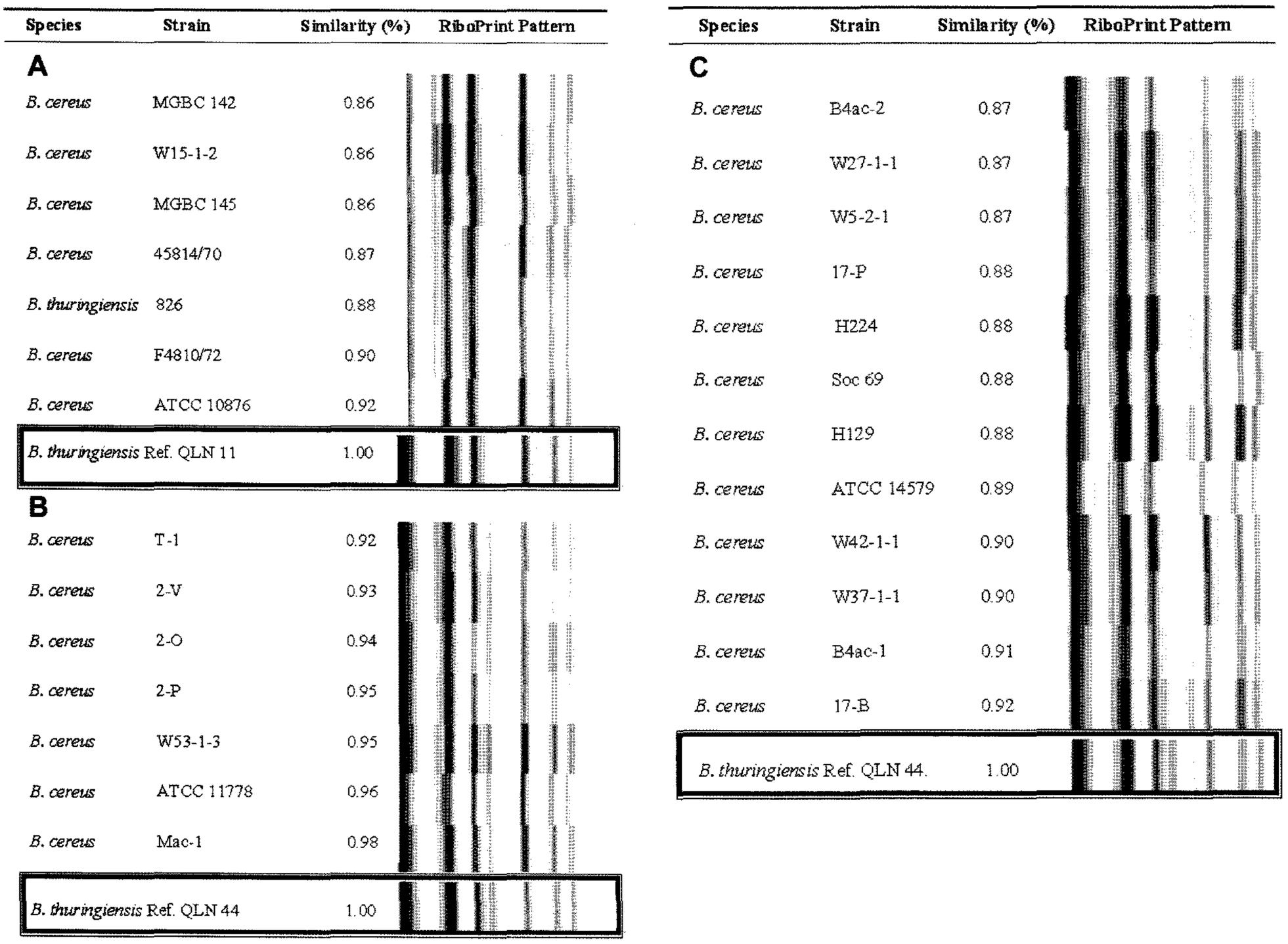

Fig. 2. Comparison of *B. cereus* ribopatterns with *B. thuringiensis* ribopatterns.
Seven *B. cereus* ribopatterns of type 62-S-7 (**A**), 7 *B. cereus* ribopatterns of type 84-S-1 (**B**), and 12 *B. cereus* ribopatterns of type 56-S-2 (**C**) were compared with the patterns from *B. thuringiensis* QLN 11 and *B. thuringiensis* QLN 44.

cerAB, *hblA*, *hblC*, *hblD*, and *bceT*. The probe containing *cerAB* always hybridized to one of the largest NotI fragments of *B. cereus* DNA. The probes containing the *hblA* gene hybridized to an approximately 700-kb NotI fragment in all strains of ribotypes 56-S-2, 62-S-7, and 84-S-1, and hybridized to a larger fragment (in all strains of ribotype 108-S-1 and 108-S-6) or smaller fragment size in other strains (in strains W52-2-2 and W39-1-2). The probe containing the *bceT* gene hybridized to 670–780 kb NotI fragments in all six strains of ribotype 62-S-7, and to 1,160 kb and 1,133 kb fragments in *B. thuringiensis* strain 639 and 824, respectively. It is notable that most strains of three ribogroups, 56-S-2, 62-S-7, and 84-S-1, with high similarity (>85%) in their ribopatterns, had the *hblA* containing the NotI-fragment of a similar size (about 700 kb). In other words, the region surrounding *hblA* is relatively consistent in these groups.

Most probes for the seven virulence genes hybridized to one of the fragments derived from NotI-digested DNA. However, no hybridization with the *bceT* probe was observed in any NotI fragment of some strains. For example, strains ATCC 11778 and ATCC 14579 were positive by PCR for the *bceT* gene, but the probe did not hybridize to any NotI fragment. Instead, the probe hybridized to unmigrated DNA. The *bceT* gene might be located in a large plasmid, which was unable to enter the gel in these strains. Five *B. cereus* strains, ATCC 10876, ATCC 10987, ATCC 11778, ATCC 14579, and F837/76, in which physical maps are available [9–11, 13], were subject to Southern hybridization with a *hblA* probe (Fig. 3). The *hblA* was absent in strain ATCC 10987, in agreement with the PCR results. The *hblA* probe hybridized to a 670 and a 700 kb NotI fragment in strains ATCC 10876 and ATCC 14579, respectively. These *hblA*-containing fragments appear to be located in a less conserved region of chromosome in both strains (Fig. 4). Interestingly, the *hblA* probe hybridized to extrachromosomal DNA in *B. cereus* F837/76.

DISCUSSION

The close relationship between *B. cereus* and *B. thuringiensis* was investigated by ribotyping and virulence gene typing. *B. cereus* and *B. thuringiensis* have been reported to

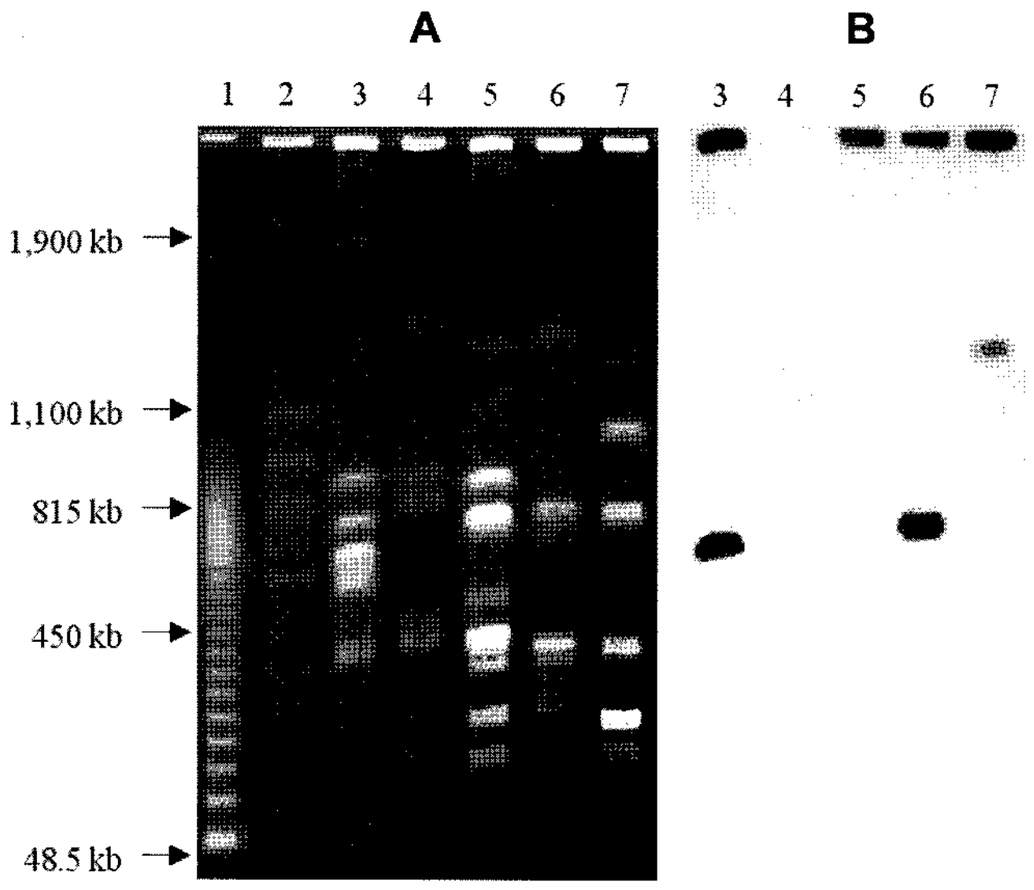

Fig. 3. Genomic mapping and localization of *hblA* NotI fragments of genomic DNA from *B. cereus* strains, separated by PFGE at 120-s for 19.5 h (**A**), blotted onto a nylon membrane, and hybridized with *hblA* probe (**B**).
Lanes: 1, lambda concatemers; 2, *S. cerevisiae* chromosomes; 3, *B. cereus* ATCC 10876; 4, *B. cereus* ATCC 10987; 5, *B. cereus* ATCC 11778; 6, *B. cereus* ATCC 14579; 7, *B. cereus* F837/76. The *hblA* probe hybridized to the 670 and 700 kb NotI fragment in strains ATCC 10876 and ATCC 14579, respectively. The *hblA* probe hybridized to a plasmid-derived band (1,250 kb) in *B. cereus* F837/76.

exhibit >99% similarity in their 16s rRNA base sequences, which suggests they are the same species [4]. *B. thuringiensis* is considered to be a harmless pesticide and does not cause illness in humans. However, *B. thuringiensis* has been reported to produce enterotoxins [15, 17, 40] and to cause illness in mammals [27]. *B. thuringiensis* may be involved in cases of human disease because its proper identification on the basis of δ-endotoxin production requires specialized techniques that may not be employed routinely [15].

The plasmid coding for the δ-endotoxin (*cryIA*) of *B. thuringiensis* can be transferred to *B. cereus*, and these transformed strains produce toxin crystals [20]. Acrystalliferous *B. thuringiensis* strains are indistinguishable from *B. cereus*, whereas some *B. cereus* strains (ATCC 21281 and ATCC 21282) have been found to contain the *cryIA* gene, which is a unique feature of *B. thuringiensis* [8, 45]. Those *B. cereus* strains have been reported to be toxic to larvae of black fly and mosquitoes [24], but typical crystal inclusions were not observed in these two strains [8]. It has been suggested that an additional factor(s) may be required for crystal formation [19]. These strains might have been misidentified as *B. cereus* because of the lack of crystalline inclusion bodies.

The plasmids carrying the *cry* genes are transmissible between species [19, 20, 28]. By achieving a stable residence in the cell, the transferred plasmid could cause the cell to adapt a new habitat. Through this evolutionary process, *B. cereus* may assume the characteristics of *B. thuringiensis*.

A total of 72 available ribogroups were analyzed, but no distinctive differences were observed between these two species. The three ribogroups that accounted for the majority of the *B. cereus* isolates, 84-S-1, 62-S-7, and 56-S-2, showed >92%, >86%, and >87% similarity to the ribopatterns of *B. thuringiensis* QLN 44, QLN 11, and QLN 44, respectively (Fig. 2). Some strains of *B. cereus* and *B. thuringiensis* belong to the same ribogroup (*i.e.*, 62-S-7). Our ribotyping data clearly demonstrate the close

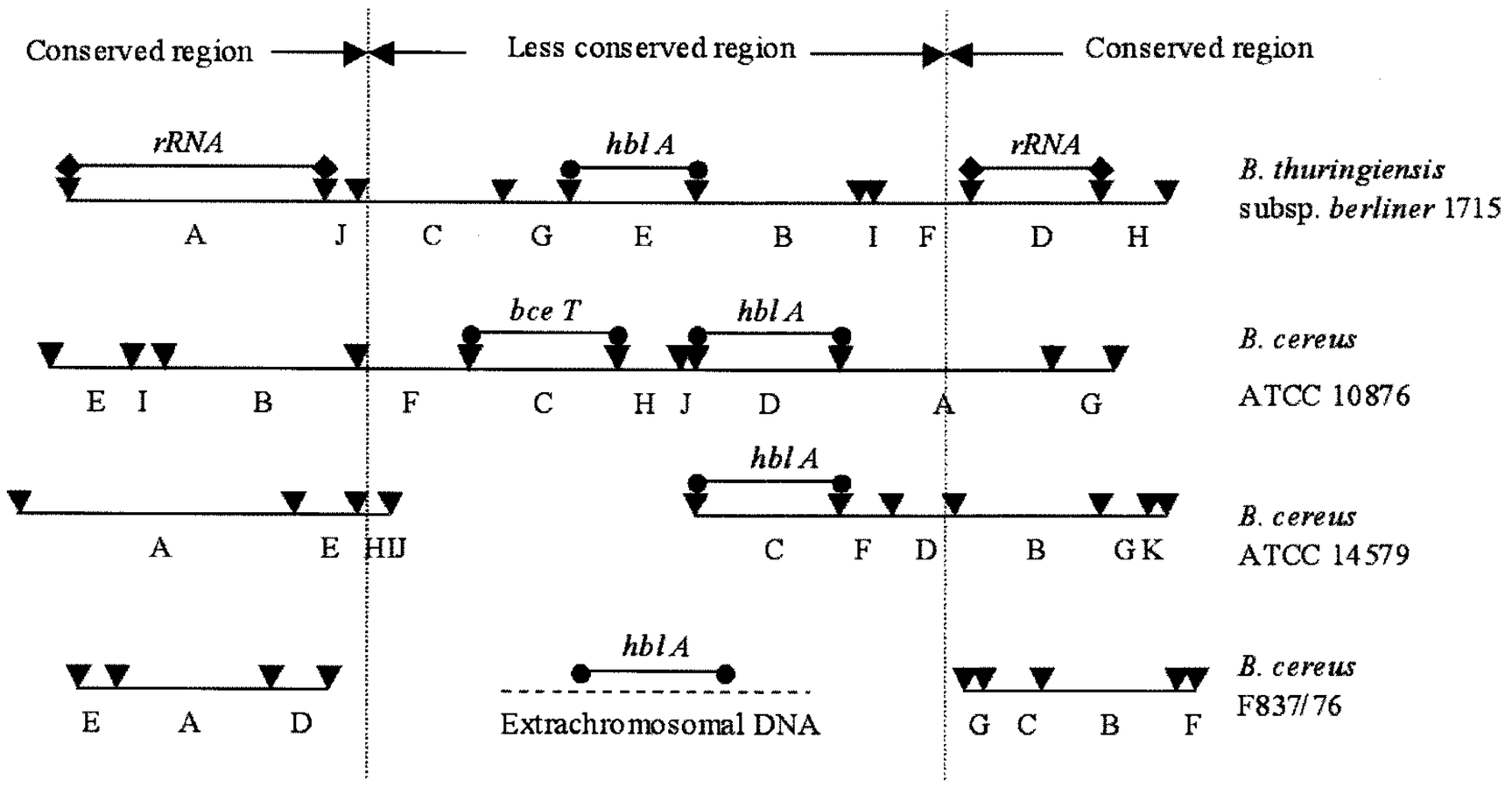

Fig 4. Location of *hblA* genes in NotI maps of three *B. cereus* strains and one *B. thuringiensis* strain.
The NotI chromosome maps were constructed by Carlson *et al.* [8–10, 12]. *hblA* is located in the D fragment (670 kb) of *B. cereus* ATCC 10876 and the C fragment (700 kb) of *B. cereus* ATCC 14579. *hblA* is located in extrachromosomal DNA in *B. cereus* F837/76.

relationship between *B. cereus* and *B. thuringiensis*, with as high degree of similarity in their ribopatterns between these two species as those observed within one species. With these results, it is assumed that they are too close to be differentiated as two different species based on the current information from 16S rRNA sequencing, or ribotyping.

In this study, seven virulence-associated genes were examined both in *B. cereus* and *B. thuringiensis* strains (Table 1); virulence genes are located in various parts of the *B. cereus* genome. The *B. cereus* genome has a more conserved region on the basis of a consistent arrangement of genes in virtually all strains, whereas in the other regions, deletions, additions, and inversions are apparent [13]. The conserved part of chromosome in *B. cereus* contains *dnaA*, *gyrA/B*, and rRNA genes [11]. The less conserved region of the chromosome contains the genes that are often plasmid-encoded such as β-*lacI*, β-*lacII*, and *inA*. Genetic exchange may occur in this unstable part of chromosome [8, 11], and it may cause the variation in chromosome size of *B. cereus*. Genome sequencing of *B. cereus* ATCC 14579 revealed a discrete region with different G+C content [26]. The presence of putative prophages at the border of the discrete region also supports the idea of phage-mediated recombination between closely related organisms.

The *cerAB* gene is always found in one of the largest NotI fragments located in the conserved part of the chromosome, based upon Southern hybridization. Three nucleotides within *cerAB* were reported to be species specific between *B. cereus* and *B. thuringiensis*, and a fluorogenic probe-based PCR assay was developed based on the three base differences [30]. In this study, only 63% of 72 *B. cereus* tested gave a positive signal (a ΔRQ value higher than 0.4) with the cerTAQ-1 probe. It is interesting to note that strains within the same ribogroup were either all positive or all negative with the probe (except one strain, 38-1-1, in ribogroup 56-S-1 with a small difference in ΔRQ value), implying that the sequence variation in the *cerAB* gene is consistent within a given ribogroup. It can be assumed that the sequence polymorphism in *cerAB* follows overall chromosomal divergence within the species. However, the segregation of *cerAB* sequences has not reached to the point in which these two species can be differentiated.

The *hblA* gene was localized in three strains of *B. cereus* for which chromosomal maps [9–11, 13] are available. The *hblA* is located in the less conserved part of the chromosome of *B. cereus* strains ATCC 10876 and ATCC 14579 (Fig. 4). *hblA* has been reported to be located in the less conserved part of the chromosome in *B. thuringiensis* subsp. *berliner* 1715 [11]. Interestingly, the *hblA* probe hybridized to an extrachromosomal band in *B. cereus* F837/76. *B. cereus* F837/76 has a small chromosome (2.4 Mb), which shows high homology with the conserved part of the large *B. cereus* chromosome (strain ATCC 10987) by cross-hybridization [13]. The *hblA* gene is located in either a less conserved part of some *B. cereus* chromosome or in the plasmid of some other *B. cereus* strains. The absence of the *hblA* gene in 31% of the *B. cereus* examined in this study could be explained by the location of the *hblA* gene. The *hblA* gene located in the less conserved part of the chromosome may be subject to genomic exchanges, which can cause the loss of the gene. Although *hblA* is located in a less conserved part of chromosome in the *B. cereus* strains tested, a good correspondence between ribotype and the presence of *hblA* was found in all other ribogroups (Table 1).

Three enterotoxins are generally accepted as being involved in diarrhea-type food poisoning; hemolysin BL (HBL), non-hemolytic enterotoxin (NHE), and BceT. A total of 42 distinctive ribotypes were identified in this study, and the presence or absence of two enterotoxins, *hblA* and *bceT*, in each ribogroup was determined (Table 1). These data can be used as a reference to determine the presence of two enterotoxins in unknown isolates that are assigned to one of these previously identified ribogroup. The genes encoding three components of the NHE complex (NHE-39, NHE-45, and NHE-105) were not screened in this study.

Riboprint patterns may be diagnostic for the food poisoning potential of a given *Bacillus* sp. if sufficient evidence can be gathered to suggest a correlation to the virulence gene portfolio or, more appropriately, its capacity to cause illness. Unfortunately, the capacity to cause illness is complicated and not fully understood for *B. cereus*, and the discrimination between it and the commercially important *B. thuringiensis* is convoluted [37].

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