Reliable Identification of *Bacillus cereus* Group Species Using Low Mass Biomarkers by MALDI-TOF MS

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Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)-based pathogen identification relies on the ribosomal protein spectra provided in the proprietary database. Although these mass spectra can discern various pathogens at species level, the spectra-based method still has limitations in identifying closely-related microbial species. In this study, to overcome the limits of the current MALDI-TOF MS identification method using ribosomal protein spectra, we applied MALDI-TOF MS of low-mass profiling to the identification of two genetically related *Bacillus* species, the food-borne pathogen *Bacillus cereus*, and the insect pathogen *Bacillus thuringiensis*. The mass spectra of small molecules from 17 type strains of two bacilli were compared to the morphological, biochemical, and genetic identification methods of pathogens. The specific mass peaks in the low-mass range (m/z 500–3,000) successfully identified various closely-related strains belonging to these two reference species. The intensity profiles of the MALDI-TOF mass spectra clearly revealed the differences between the two genetically-related species at strain level. We suggest that small molecules with low molecular weight, 714.2 and 906.5 m/z can be potential mass biomarkers used for reliable identification of *B. cereus* and *B. thuringiensis*.

Keywords: MALDI-TOF MS, food-borne pathogen, discrimination, *Bacillus cereus*, *Bacillus thuringiensis*

Introduction

Matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS) has emerged as a new technology for species identification [1–7]. The MALDI-TOF MS-based identification method can be used to analyze the protein profiles of intact bacterial cells. The method is a rapid and powerful tool because of its speed, reproducibility, and sensitivity of analysis [8, 9]. The speed and simplicity of sample preparation and result acquisition associated with minimal consumable cost make this method suitable for routine and high-throughput use for microorganism identification [1, 10–13]. The identification of the target microorganism using MALDI-TOF MS is based on the comparison of the protein mass spectra of the microorganism obtained from MALDI-TOF MS to the

spectrum in a reference database. Bacterial MALDI spectra databases usually contain mass peaks of ribosomal proteins accounting for >20% of the total cellular proteins [14, 15]. However, one drawback in using the MALDI-TOF MS-based identification method is that some species do not have sufficient differences in their ribosomal protein sequences. For instance, *Shigella* spp. and *Escherichia coli* cannot be distinguished using MALDI-TOF MS [16]. Similarly, the members of the *Bacillus* spp., such as *B. cereus*, *B. anthracis*, *B. thuringiensis*, *B. mycoides*, *B. megaterium*, and *B. subtilis*, cannot be distinguished by proteins having less than 50 kDa of molecular weight when microfluidic capillary electrophoresis is applied [17].

The *Bacillus cereus* group [18] of rod-shaped, Gram-positive bacteria comprises six closely-related *Bacillus* species: *B. cereus*, *B. anthracis*, *B. thuringiensis*, *B. mycoides*,

B. pseudomycoides, and *B. weihenstephanensis* [19, 20]. The *B. cereus* species is an important pathogen in humans and animals [21–24]. Of all the *Bacillus* spp., the most common pathogen in humans is *B. cereus*, an opportunistic pathogen involved in various illnesses such as food poisoning [25, 26]. Another pathogen is *B. thuringiensis*, which produces δ -endotoxin crystals, commonly referred to as Bt toxin. This pathogen was used in biopesticides because of its lethality to insect larvae [27]. The *B. cereus* group species can be distinguished from other members of the aerobic endospore-forming bacteria because of its inability to produce acid from mannitol and lecithinase production [18]. Members of the *B. cereus* group exhibit different phenotypic characteristics used for their identification. For example, *B. anthracis* is non-motile, non-hemolytic, and non-penicillin resistant, which distinguishes it from *B. cereus*, *B. thuringiensis*, and *B. mycoides* [28]. Both *B. cereus* and *B. thuringiensis* are motile; however, *B. thuringiensis* can be distinguished from *B. cereus* based on its ability to produce the Bt toxin [29–31]. However, it is known that phenotypic tests and sequence analysis of the 16S rRNA genes could fail to differentiate between members of the *B. cereus* group [32]. Species-specific identification of *B. cereus* group species using polymerase chain reaction (PCR) is problematic because of the close homology between particular species of this group [33], for which reason some authors have suggested as genetically being the same species [30, 34].

Current laboratory methods used for the identification of the *B. cereus* group are mainly based on the morphological, physiological, and biochemical characteristics of the species. Conventional identification methods are time-consuming, taking up to 48 h [12, 35, 36] and are unreliable because of poor discrimination between closely-related species, such as *B. cereus*, *B. thuringiensis*, and *B. mycoides*. In addition, supplementary tests are often recommended for an accurate identification of species that are misidentified, i.e., in cases where the species identified is discordant with the reference information or are unidentified [37]. Therefore, rapid and reliable, closely-related *Bacillus* identification methods are required for food safety control by routine analysis.

In this study, we developed a pathogen identification method by seeking biomarker candidates to distinguish between *B. cereus* and *B. thuringiensis* while overcoming the limits of current MALDI-TOF MS-based identification techniques. We attempted to find biomarkers with differences in intensity profiles of specific low-mass peaks that vary by species using MALDI-TOF MS analysis.

Materials and Methods

Bacterial Strains

We analyzed 22 strains belonging to the *B. cereus* group and related species obtained from the Korean Collection for Type Culture (KCTC) (Table 1). Freeze-dried cultures in Pyrex ampules were resuspended in 0.5 ml sterile water. A 100- μ l volume of each suspension was smeared onto mannitol-egg yolk-polymyxin (MYP, USA) agar plates and incubated at 30°C for 24 h.

Biochemical Analysis

Biochemical identification of the bacterial strains was carried out using VITEK 2 Compact (bioMérieux SA, Marcy l'Etoile, France). A colony showing pink color on MYP agar was cultured on nutrient agar to make bacterial suspensions, which were prepared using 3 ml of sterile saline and adjusted to a McFarland standard (2.0) using DensiChek (bioMérieux). The *Bacillus* identification card (BCL) (bioMérieux) was filled automatically in the VITEK vacuum chamber, sealed, and incubated at 35.5°C, and read automatically every 15 min for 16 h. Data were analyzed automatically using the VITEK2 database version 7.01. Identification results were reported as described previously [37]. Briefly, the species was reported as either (i) correctly identified to a single species or more than one species in a “slashline,” in which case supplementary testing was required to distinguish between similar species; (ii) misidentified (where the species identified using BCL was discordant with the results of 16S rRNA sequencing); or (iii) unidentified.

PCR Amplification and Sequencing of 16S rRNA Genes

Bacterial genomic DNA was extracted using a Labopass Tissue Mini Prep Kit (Cosmo Genetech Co. Ltd., Korea), according to the manufacturer's instructions. The 16S rRNA sequences were PCR amplified with the primers 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3') [38, 39] using the following conditions: initial denaturation at 94°C for 3 min; followed by 35 cycles of denaturation at 94°C for 30 sec; annealing at 58°C for 30 sec; and extension at 72°C for 30 sec. PCR products were purified and sequenced at Macrogen, Korea, using the primers 785F (5'-GGATTAGATACCCTGGTA-3') and 907R (5'-CCGTCAATTCMTTTRAGTTT-3'). The resulting sequences, approximately 1.5 kb in size, were aligned and submitted for a similarity search in the National Center for Biotechnology Information (NCBI) database using the BLAST 2 Sequence [40]. Query sequences with $\geq 99\%$ sequence similarity were used for the identification of the PCR-amplified 16S rRNA sequences. Partial 16S rRNA sequences were compared with known sequences in the NCBI Genbank database. The sequences were as follows: *B. cereus* (NR114582.1, NR115526.1, NR074540.1, NR112630.1, NR113266.1, NR115714.1), *B. thuringiensis* (NR043403.1, NR114581.1), *B. anthracis* (NR041248.1), *B. weihenstephanensis* (NR024697.1), *B. mycoides* (NR115993.1, NR036880.1, NR113990.1), *B. pseudomycoides* (NR113991.1).

Table 1. Assignment of *Bacillus* species using biochemical, transmission electron microscope analysis, and 16S rRNA gene sequencing.

No.	Strain ID	Biochemical analysis	16S rRNA gene sequencing	Presence of crystal	Assignment of species	
1	<i>B. cereus</i>	KCTC 1014	<i>B. cereus</i> / <i>B. thuringiensis</i> / <i>B. mycoides</i>	<i>B. thuringiensis</i>	+	<i>B. thuringiensis</i>
2	<i>B. cereus</i>	KCTC 1661	<i>B. cereus</i> / <i>B. thuringiensis</i> / <i>B. mycoides</i>	<i>B. thuringiensis</i>	-	<i>B. cereus</i>
3	<i>B. cereus</i>	KCTC 3624	<i>B. cereus</i> / <i>B. thuringiensis</i> / <i>B. mycoides</i>	<i>B. thuringiensis</i>	-	<i>B. cereus</i>
4	<i>B. cereus</i>	KCTC 3124	<i>B. cereus</i> / <i>B. thuringiensis</i> / <i>B. mycoides</i>	<i>B. thuringiensis</i>	-	<i>B. cereus</i>
5	<i>B. cereus</i>	KCTC 1013	<i>B. cereus</i> / <i>B. thuringiensis</i> / <i>B. mycoides</i>	<i>B. thuringiensis</i>	-	<i>B. cereus</i>
6	<i>B. thuringiensis</i>	KCTC 1508	<i>B. cereus</i> / <i>B. thuringiensis</i> / <i>B. mycoides</i>	<i>B. cereus</i>	-	<i>B. cereus</i>
7	<i>B. thuringiensis</i>	KCTC 3452	<i>B. cereus</i> / <i>B. thuringiensis</i> / <i>B. mycoides</i>	<i>B. thuringiensis</i>	+	<i>B. thuringiensis</i>
8	<i>B. thuringiensis</i>	KCTC 1524	<i>Brevibacillus laterosporis</i>	<i>B. cereus</i>	-	<i>B. cereus</i>
9	<i>B. thuringiensis</i>	KCTC 1525	<i>B. cereus</i> / <i>B. thuringiensis</i> / <i>B. mycoides</i>	<i>B. thuringiensis</i>	-	<i>B. cereus</i>
10	<i>B. thuringiensis</i>	KCTC 1511	<i>B. cereus</i> / <i>B. thuringiensis</i> / <i>B. mycoides</i>	<i>B. thuringiensis</i>	+	<i>B. thuringiensis</i>
11	<i>B. thuringiensis</i>	KCTC 1507	<i>B. cereus</i> / <i>B. thuringiensis</i> / <i>B. mycoides</i>	<i>B. thuringiensis</i>	+	<i>B. thuringiensis</i>
12	<i>B. mycoides</i>	KCTC 3453	<i>Brevibacillus laterosporis</i>	<i>B. cereus</i>	-	<i>B. cereus</i>
13	<i>B. megaterium</i>	KCTC 3708	<i>Brevibacillus laterosporis</i>	<i>B. cereus</i>	-	<i>B. cereus</i>
14	<i>B. megaterium</i>	KCTC 1098	<i>B. cereus</i> / <i>B. thuringiensis</i> / <i>B. mycoides</i>	<i>B. cereus</i>	-	<i>B. cereus</i>
15	<i>B. megaterium</i>	KCTC 1316	<i>B. cereus</i> / <i>B. thuringiensis</i> / <i>B. mycoides</i>	<i>B. cereus</i>	+	<i>B. thuringiensis</i>
16	<i>B. megaterium</i>	KCTC 2194	<i>B. megaterium</i>	<i>B. megaterium</i>		<i>B. megaterium</i>
17	<i>B. megaterium</i>	KCTC 3712	<i>B. smithii</i>	<i>B. megaterium</i>		<i>B. megaterium</i>
18	<i>B. megaterium</i>	KCTC 1366	<i>Brevibacillus laterosporis</i>	<i>B. cereus</i>	-	<i>B. cereus</i>
19	<i>B. megaterium</i>	KCTC 2178	<i>B. megaterium</i>	<i>B. megaterium</i>		<i>B. megaterium</i>
20	<i>B. megaterium</i>	KCTC 3007	<i>B. cereus</i> / <i>B. thuringiensis</i> / <i>B. mycoides</i>	<i>B. thuringiensis</i>	-	<i>B. cereus</i>
21	<i>B. subtilis</i>	KCTC 13241	Unidentified	<i>B. megaterium</i>		<i>B. megaterium</i>
22	<i>B. subtilis</i>	KCTC 3135	Unidentified	<i>B. megaterium</i>		<i>B. megaterium</i>

Observation of Parasporal Crystals Using Transmission Electron Microscope (TEM)

Bacillus cereus group and related species were grown on MYP medium for 1 day at 30°C. Bacterial cells were prepared for TEM analysis, as described previously [41]. Briefly, bacterial cells were resuspended in Karnovsky's fixative prepared in 0.1 M sodium cacodylate buffer, 10% paraformaldehyde, and 8% glutaraldehyde and stored at 4°C overnight. Later, the pellets were washed three times with 0.05 M sodium cacodylate buffer and post-fixed with 1% osmium tetroxide in 0.05 M cacodylate buffer for 2 h at 4°C. Subsequently, the samples were washed with cold distilled water twice and stained in 0.5% uranyl acetate overnight. Samples were then subjected to a serial dehydration process using three 10-min incubations in ethanol at 30%, 50%, 70%, 80%, 90%, and 100% each. The dehydrated cells were embedded in Spurr's resin mixture and cured at 70°C for 48 h. Ultra-thin sections were prepared using an ultramicrotome (EM UC7, Germany). Samples were observed using the energy-filtering TEM (LIBRA 120 equipped with Olympus SIS-iTEM version 5.0 [build 1243], Carl-Zeiss, Germany).

MALDI-TOF MS Analysis

Bacterial colonies grown on culture medium were directly placed on the MALDI target plate as a thin layer and allowed to air dry. Two microliters of 70% formic acid (Merck, Germany) were applied to the sample layer [13, 42], allowed to dry, and overlaid with 2 µl of matrix solution consisting of 10 mg/ml α-cyano-4-hydroxy-cinnamic acid (Fluka, Switzerland) saturated in 2.5% trifluoroacetic acid (Sigma-Aldrich, USA) and 50% acetonitrile (Honeywell Burdick & Jackson, Fisher Scientific). The matrix and analyte were allowed to co-crystallize, and the target plate was inserted into the MALDI-TOF MS instrument. Mass spectra were automatically acquired with MALDI-TOF MS operating in a linear positive mode by accumulating 1,000 laser shots in the *m/z* ranges 2,000–20,000 and 500–3,000 using Thinkerbell LT (ASTA Inc., Korea). External calibration of mass spectra was performed using recombinant *E. coli* extracts (ASTA Inc.) in a protein mass range of 3,000–20,000 Da and Peptide Calibration Standard II (Bruker Daltonics, Germany) in a small molecule mass range of 500–3,000 Da.

Results and Discussion

Assignment of Reference *Bacillus* Species

The use of reference strains with a well-defined taxonomic status is a prerequisite for reliable identification of unidentified strains [12]. Moreover, errors in conventional identification methods may lead to discordant results of MALDI-TOF MS identification [3]. Due to the high genetic and phenotypic similarity between *B. thuringiensis* and *B. cereus* [31, 43], they might represent the same species [30], thus could not be distinguished based on sequencing alone [44]. For these reasons, we paid special attention to the verification of 22 strains to ensure specific biomarkers for reliable identification of *B. cereus* and *B. thuringiensis*. Twenty-two strains of *Bacillus* species obtained from stock collection were investigated in this study, including *B. cereus* ($n = 5$), *B. thuringiensis* ($n = 6$), *B. mycoides* ($n = 1$), *B. megaterium* ($n = 8$), and *B. subtilis* ($n = 2$) (Table 1). We examined the strains based on biochemical identification, 16S rRNA gene sequencing, and parasporal crystal observation. As shown in Table 1, the strains belonging to five *Bacillus* species were identified into one of three *Bacillus* species: *B. cereus*; *B. thuringiensis*; and *B. megaterium*.

Biochemical analysis using BCL card identified 20 of the 22 strains but two strains of *B. subtilis* remained unidentified by the analysis. Of these identified 20 strains, 12 strains were matched with the strain ID information. However, eight strains showed ambiguous identification different from the original nomenclature. Biochemical identification of the bacterial strains was confirmed via PCR amplification and partial sequencing of the 16S rRNA genes using the primer pairs 27F/1492R and 785F/907R. The matched 12 strains with original nomenclature were also confirmed as *B. cereus*, *B. thuringiensis* or *B. megaterium* by 16S rRNA gene sequencing. Two unidentified strains (KCTC 13241 and 3135) in the biochemical analysis were identified as *B. megaterium* by 16S rRNA gene sequencing. Among the

mismatched eight strains with strain ID information, three strains identified as *B. cereus*/*B. thuringiensis*/*B. mycoides* in the biochemical analysis were confirmed with similar results by 16S rRNA gene sequencing but five strains were identified as a *B. cereus* or *B. megaterium*. To distinguish between *B. cereus* and *B. thuringiensis*, 17 strains identified as *B. cereus* or *B. thuringiensis* were observed for the presence or absence of cuboid or diamond-shaped parasporal crystals using TEM [43, 45]. The parasporal crystals were observed only in 5 out of the 17 strains, and the KCTC 1014, 3452, 1511, 1507, and 1316 strains were finally identified as *B. thuringiensis* (Fig. 1).

Differentiation between *B. cereus* and *B. thuringiensis* Strains

Protein mass patterns of *B. cereus* and *B. thuringiensis* were theoretically distinguished using an algorithm that applied the intensity factor to the relative intensity of protein mass peaks [13]. However, these two species could not be distinguished because both were in the highly reliable ranking when evaluating unused reference strains in the MALDI-TOF MS database [13]. This may not represent each species because of the high sequence diversity between *B. cereus* and *B. thuringiensis* strains [46]. Therefore, we compared the heat maps showing the relative peak intensities to determine if there are common protein markers that distinguish the two species when using several well-validated standard strains. MALDI-TOF MS spectra were derived from three *Bacillus* species, *B. cereus* ($n = 12$), *B. thuringiensis* ($n = 5$), and *B. megaterium* ($n = 5$), grown on blood agar plates (BAP), MYP, and trypticase soy agar (TSA) media. Seventy peaks were extracted from the MALDI-TOF MS spectra and ordered according to the relative intensity for 22 strains of the three *Bacillus* species. m/z Bins at 1,200 ppm intervals were made at 3,000–20,000. Different colors indicated different peak intensities; red indicated high intensity, green indicated low intensity,

Fig. 1. Transmission electron microscopy images of parasporal crystals of (a) KCTC 1014, (b) KCTC 3452, (c) KCTC 1511, (d) KCTC 1507, and (e) KCTC 1316.

Fig. 2. Heat map of protein mass peaks derived from 22 bacterial strains assigned into three *Bacillus* species, *B. cereus* ($n = 12$), *B. thuringiensis* ($n = 5$), and *B. megaterium* ($n = 5$).

Red color indicates high peak intensity, green indicates low peak intensity, and black indicates absence of peak.

and black indicated absence of peaks. The heat map in Fig. 2 demonstrated a high degree of spectral reproducibility and indicated a relatively high degree of similarity between spectra from strains belonging to the same species members and, conversely, distinct spectra from strains belonging to different species. *B. megaterium* ($n = 5$) was characterized as having significantly different protein mass peaks, m/z of 3373, 3864, 4303, and 5194, compared with *B. cereus* ($n = 12$) and *B. thuringiensis* ($n = 5$). *B. cereus* and *B. thuringiensis* shared two dominant mass peaks: m/z 3356 (double-charged ion), and 6712. The specific peak at m/z 6712 was the spore marker protein of *B. cereus* and *B. thuringiensis* [47–49]. However, no protein

mass peaks were found to allow for the reliable differentiation between the two species. MALDI-TOF protein mass spectra of the two species were not different even when the culture conditions were changed (data not shown). As previously reported, we reaffirmed that the discovery of species-specific protein markers using MALDI-TOF MS protein profiling was hampered by the high genetic similarity existing within *B. cereus* and *B. thuringiensis*.

Discovery of Species-Specific Biomarkers Using Low-Mass Profiling by MALDI-TOF

Wilson *et al.* reported that *B. cereus* excretes two catecholates

Fig. 3. MALDI-TOF MS spectra obtained from the representative strains of *B. cereus* (KCTC 3124) (A) and *B. thuringiensis* (KCTC1507) (B) in the low-mass range (500–3,000 m/z).

BAP: blood agar plates; MYP: mannitol–egg yolk–polymyxin agar; TSA: trypticase soy agar

siderophores, petrobactin (m/z 719.3 $[M + H]^+$, which contains 3,4-dihydroxybenzoyl moieties) and bacillibactin (m/z 883.2 $[M + H]^+$, which contains 2,3-dihydroxybenzoyl moieties), whereas *B. thuringiensis* produces only bacillibactin [50]. Therefore, we attempted to identify small molecules that could potentially distinguish between the strains of *B. cereus* and *B. thuringiensis* studied here using rapid and accurate MALDI-TOF MS. The MALDI-TOF MS spectra obtained from the representative strains of *B. cereus* (KCTC 3124) and *B. thuringiensis* (KCTC 1507) in the range of 500–3,000 m/z are shown in Fig. 3. These strains were cultured on BAP, MYP, and TSA, respectively. The spectra of *B. cereus* and *B. thuringiensis* shared mass peaks at m/z 714.2 and 906.5; however, the intensity of the peaks greatly differed between *B. cereus* and *B. thuringiensis*, regardless of the culture conditions. The dominant mass peaks were 714.2 m/z for *B. cereus* and 906.5 m/z for *B. thuringiensis*. The heat map in Fig. 4 shows the spectral- and species-specific reproducibility for differences of intensity profiles from 17 verified strains: *B. cereus* ($n = 12$) and *B. thuringiensis* ($n = 5$). At this time, the tolerance of mass peak was 500 ppm. The list of the peak of low molecules detected by MALDI-

TOF was shown in the light blue section of Fig. 4. Thirty-nine mass peaks were listed that were detected more than 50% in each species, and the intensity profile for each peak was visually represented by a heat map. Ten *B. cereus* and three *B. thuringiensis* strains showed high peak intensities at m/z 714 and 906, respectively (Fig. 4). However, two *B. cereus* (KCTC 1611 and 3007) and two *B. thuringiensis* (KCTC 3452 and 1316) strains showed contradictory results. While KCTC 1661 and 3007 strains were classified as *B. cereus* strains because of the absence of parasporal crystals, the intensity profiles of these strains were similar to those of *B. thuringiensis* strains. *B. thuringiensis* harbors the *Cry* genes that encode insecticidal crystal toxins, and *B. thuringiensis* strains that lack *Cry* genes are indistinguishable from *B. cereus* [43, 51]. Thus, these strains may be *B. thuringiensis* strains that could not be distinguished from *B. cereus* because they lack the *Cry* genes and do not produce crystals. Indeed, KCTC 1661 and 3007 strains did not contain *Cry1Ab/Ac* and *Cry2A*, which are encoded by the abundant *Cry1* and *Cry2* genes in most *B. thuringiensis* isolates [52] when tested using the ImmuneStrips (Agdia Incorporated, USA) (data not shown). KCTC 3452 and

Fig. 4. Heat map of the small molecule mass peaks derived from *B. cereus* ($n = 12$) and *B. thuringiensis* ($n = 5$) in the low-mass range (500–3,000 m/z).

KCTC 1316 were assigned to *B. thuringiensis* because of the presence of crystals. These two strains also had the *Cry* genes; *Cry1Ab/Ac* in KCTC 3452, and *Cry1Ab/Ac* and *Cry2A* in KCTC 1316 were detected by the ImmuneStrips (Agdia Incorporated, USA) (data not shown). However, the low-mass patterns of these strains were similar to those of *B. cereus*. *Cry* genes encoded by the plasmid show a high potential for horizontal gene transfer between *B. thuringiensis* and *B. cereus* [25, 53, 54]. It is possible that KCTC 3452 and KCTC 1316 underwent horizontal transfer of *Cry* genes, which made it difficult to distinguish between the two species, *B. cereus* and *B. thuringiensis*.

When the four strains were reclassified according to the low-mass patterns analyzed by MALDI-TOF, the intensity rate of two mass peaks, m/z 714.2 and 906.5, was significantly

exchanged between the two species (Table 2). For *B. cereus*, the intensity rate of m/z 714.2 increased from 85.4 to 97.3 and that of m/z 906.5 decreased from 37.6 to 2.7, whereas for *B. thuringiensis*, the intensity rate of m/z 714.2 decreased from 45.0 to 13.5 and that of m/z 906.5 increased from 63.3 to 100.0. These changes indicated that these two peaks have increased potential as biomarkers for the distinction of the two species. Also, low-mass patterns from MALDI-TOF MS could distinguish between *B. cereus* and *B. thuringiensis*, even if the morphological phenotype was misleading.

Evaluation of Biomarker Candidates to Distinguish *B. cereus* and *B. thuringiensis*

The biomarker candidates 714.2 and 906.5 m/z were evaluated for reliable discrimination of the *B. cereus* and *B. thuringiensis* strains isolated from actual contaminated food. The twenty-six isolates were identified as twenty *B. cereus* and six *B. thuringiensis* by the presence of parasporal crystals. Of the 26 isolates, 23 strains were consistent with the results of identification by MALDI-TOF MS biomarkers, as shown in Table 3. Three strains (R33, R42, and R50) were identified as *B. cereus* because no crystals were identified, but the MALDI-TOF MS biomarkers were identified as *B. thuringiensis*. This may be the *B. thuringiensis* deficient in the *Cry* gene as described above, which could not be distinguished in the conventional method. Therefore, the MALDI-TOF MS biomarkers presented in this study showed that it is well applied to actual samples in distinguishing

Table 2. Peak intensity rates for two mass peaks, m/z 714.2 and 906.5.

Species	Mass peak (m/z)	Reclassification criteria	
		Presence of crystals by TEM	Low-mass patterns by MALDI
<i>B. cereus</i>	714.2	85.4	97.3
(BC, $n = 12$)	906.5	37.6	2.7
<i>B. thuringiensis</i>	714.2	45.0	13.5
(BT, $n = 5$)	906.5	63.3	100.0

TEM, transmission electron microscope; MALDI, matrix-assisted laser desorption/ionization.

Table 3. Evaluation of the biomarker candidates, m/z 714.2 and 906.5, to distinguish *B. cereus* and *B. thuringiensis*.

No	Isolated strains from food	TEM		MALDI-TOF		
		Presence of crystals	Identification	Relative peak intensity rates		Identification
				714.2 ± 0.5	906.5 ± 0.5	
1	R1	-	<i>B. cereus</i>	86	14	<i>B. cereus</i>
2	R2	-	<i>B. cereus</i>	100	0	<i>B. cereus</i>
3	R3	-	<i>B. cereus</i>	98	2	<i>B. cereus</i>
4	R23	-	<i>B. cereus</i>	100	0	<i>B. cereus</i>
5	R24	-	<i>B. cereus</i>	95	5	<i>B. cereus</i>
6	R33	-	<i>B. cereus</i>	23	77	<i>B. thuringiensis</i>
7	R34	+	<i>B. thuringiensis</i>	29	71	<i>B. thuringiensis</i>
8	R42	-	<i>B. cereus</i>	14	86	<i>B. thuringiensis</i>
9	R43	+	<i>B. thuringiensis</i>	30	70	<i>B. thuringiensis</i>
10	R44	+	<i>B. thuringiensis</i>	14	86	<i>B. thuringiensis</i>
11	R45	+	<i>B. thuringiensis</i>	18	82	<i>B. thuringiensis</i>
12	R46	-	<i>B. cereus</i>	90	11	<i>B. cereus</i>
13	R47	+	<i>B. thuringiensis</i>	0	100	<i>B. thuringiensis</i>
14	R48	-	<i>B. cereus</i>	92	8	<i>B. cereus</i>
15	R49	+	<i>B. thuringiensis</i>	0	100	<i>B. thuringiensis</i>
16	R50	-	<i>B. cereus</i>	0	100	<i>B. thuringiensis</i>
17	R256	-	<i>B. cereus</i>	91	9	<i>B. cereus</i>
18	R257	-	<i>B. cereus</i>	100	0	<i>B. cereus</i>
19	R258	-	<i>B. cereus</i>	100	0	<i>B. cereus</i>
20	R259	-	<i>B. cereus</i>	100	0	<i>B. cereus</i>
21	R260	-	<i>B. cereus</i>	100	0	<i>B. cereus</i>
22	R261	-	<i>B. cereus</i>	100	0	<i>B. cereus</i>
23	R262	-	<i>B. cereus</i>	100	0	<i>B. cereus</i>
24	R263	-	<i>B. cereus</i>	100	0	<i>B. cereus</i>
25	R264	-	<i>B. cereus</i>	100	0	<i>B. cereus</i>
26	R265	-	<i>B. cereus</i>	100	0	<i>B. cereus</i>

B. cereus from *B. thuringiensis*. These findings will help in the development of efficient diagnostic tools based on the MALDI-TOF MS database for the rapid identification of biomarkers to differentiate between *B. cereus* and *B. thuringiensis* strains.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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