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Classification of *Bacillus cereus* and *Bacillus thuringiensis* by Metabolomic Profile Differentiation Using Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry

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Abstract

Bacillus species are aerobic spore-forming bacteria that are known pose a significant risk to human health. Even though numerous methods for classifying *Bacillus cereus* and *Bacillus thuringiensis* have been reported, it remains very difficult to accurately and rapidly distinguish these *Bacillus* species since they are genetically very similar. In this study, we described to identify biomarkers for classifying *B. cereus* and *B. thuringiensis* by metabolomic profiling using ultra-performance liquid chromatography–tandem mass spectrometry. Established method were provided for classifying *B. thuringiensis* and *B. cereus* led to the identification of 92 and 38 potential candidate biomarkers in cellular and media fractions, respectively. Among them, one particular ion (1193.676 m/z) had accurately classified for three food samples with the suspected contamination of *B. cereus* or *B. thuringiensis*. Our study proposed a new biomarker to rapidly distinguish *B. cereus* group members and might be very useful in the food, cosmetic, and agricultural industries.

Keywords

Biomarker, Metabolite, Ultra-performance liquid chromatography-tandem mass spectrometry, *Bacillus cereus*

Introduction

Bacillus species must be classified for public human health and various methods for classifying *Bacillus* species were recently reported to assist in the identification of biological agents and environmental factors affecting food safety [1, 2]. *Bacillus* species, which is one of the largest and most ubiquitous genera of bacteria, has gained notoriety among taxonomists because of the extreme phenotypic diversity and heterogeneity of member species [3]. Presently, the *Bacillus* genus includes 268 species and seven subspecies that are conventionally classified into three groups on the basis of the morphologies of the spores and sporangia [3]. Of these bacteria, member species of the *Bacillus cereus* group are primarily found in the environment and laboratory [4]. Although these bacteria are considered to be contaminants and can infect humans, few pose serious threats to public health.

The members of the *B. cereus* group, which include *Bacillus anthracis*, *B. cereus*, *Bacillus mycoides*, and *Bacillus thuringiensis*, are characterized by their inability to ferment mannitol and produce lecithinase [5]. These species are abundantly found in soil and many types of foods. In particular, *B. cereus* has been reported

to cause food poisoning because of the production of complex enterotoxins [6, 7], and *B. thuringiensis* is frequently used as a biological pesticide because it produces intracellular protein crystals that are toxic to a wide variety of larvae [6]. Moreover, it is difficult to classify the phenotypes of members of the *B. cereus* group because of the high similarities of 16S rRNA/DNA and ribosomal proteins between *B. cereus* and *B. thuringiensis* [3, 8, 9].

Members of the *B. cereus* group are conventionally classified based on biochemical test results and 16S rRNA gene sequencing [6, 8-10]. In fact, these methods have been successfully applied to distinguish members of the *B. cereus* group from other aerobic endospore-forming bacteria. However, since it is difficult to differentiate *B. cereus* from *B. thuringiensis*, more recent approaches have employed matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry (MS) [11-14] or liquid chromatography with tandem mass spectrometry (LC-MS/MS) [11, 15]. MALDI-TOF MS is based on the comparison of the protein or DNA spectrum of the studied specimen to a database of reference spectra which cannot distinguish between closely related microbial species. In addition, LC-MS/MS-based proteomic, genomic, and lipidomic methods have been used to identify biomarkers for bacterial classification. However, none of previously reported methods are capable of differentiating *B. cereus* from *B. thuringiensis*.

Here, we reported potential biomarkers to differentiate the metabolomic profiles of *B. cereus* and *B. thuringiensis* which were identified using ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS). To test the proposed method, *B. megaterium* was additionally analyzed as this species was genetically assigned as a different species based on the phenotypic and phylogenetic similarities with pathogenic *B. cereus*. After purification of metabolites from the cellular and media fractions, differentially expressed metabolites were subjected to UPLC-MS/MS to identify potential biomarker candidates. Then, ChemSpider software (http://www.chemspider.com/) was used to search for biomarkers against the Kyoto Encyclopedia of Genes and Genomes (KEGG) and LIPID MAPS databases. This approach is first successful to classify *B. cereus* and *B. thuringiensis* and proposed for a novel biomarker that should prove useful in product quality in the food, cosmetic, and agricultural industries.

Materials and Methods

Chemicals and solvents

All chemicals (Sigma-Aldrich Corporation, St. Louis, MO, USA) and solvents (Honeywell - Burdick & Jackson, Muskegon, MI, USA) were high-performance liquid chromatography grade.

Table 1: The *Bacillus* species and strains used as test sets, evaluation sets, and unknown sets in this study.

Sample No.	Species	Strain ID	Test set/Evaluation set/Unknown set	Display Color in Figure
1	*Bacillus cereus*	KCTC 3124	Test set	Blue
2	*Bacillus cereus*	KCTC 1508	Test set	Blue
3	*Bacillus cereus*	KCTC 1524	Test set	Blue
4	*Bacillus cereus*	KCTC 1525	Test set	Blue
5	*Bacillus cereus*	KCTC 3453	Test set	Blue
6	*Bacillus cereus*	KCTC 1366	Test set	Blue
7	*Bacillus thuringiensis*	KCTC 3452	Test set	Purple
8	*Bacillus thuringiensis*	KCTC 1511	Test set	Purple
9	*Bacillus thuringiensis*	KCTC 1507	Test set	Purple
10	*Bacillus thuringiensis*	KCTC 1316	Test set	Purple
11	*Bacillus megaterium*	KCTC 3712	Test set	Orange
12	*Bacillus megaterium*	KCTC 2178	Test set	Orange
13	*Bacillus megaterium*	KCTC 13241	Test set	Orange
14	*Bacillus megaterium*	KCTC 3135	Test set	Orange
15	*Bacillus cereus*	KCTC 1661	Evaluation set	
16	*Bacillus cereus*	KCTC 3624	Evaluation set	
17	*Bacillus thuringiensis*	KCTC 1517	Evaluation set	
18	*Bacillus cereus* group* isolated from Foods		Unknown	
19	*Bacillus cereus* group* isolated from Foods		Unknown	
20	*Bacillus cereus* group* isolated from Foods		Unknown	

* Ha et al. [17]

Bacterial strains and culture

Verified and reclassified information about the 17 *Bacillus* strains (Korean Collection for Type Cultures [KCTC], Jeongeup, South Korea) used in this study is shown in table 1. Freeze-dried cultures of the bacteria were aseptically suspended in 0.5 mL of water in a PYREX® ampule (Corning Inc., Corning, NY, USA). For each sample, a 100-µL aliquot of the suspension was smeared onto a mannitol-egg yolk-polymyxin agar plate (Difco Laboratories, Inc., Detroit, MI, USA), which was then incubated at 30 ˚C for 24 h.

Sample preparation

To separate the cellular and media fractions, the cultured bacterial samples were centrifuged at 900 × g. Then, the cells were washed three times with phosphate-buffered saline and centrifuged again. Following treatment with 1 mL of methanol, the metabolites from cells (0.3 g) were extracted by 20 strokes in a Dounce tissue homogenizer. Then, the samples were centrifuged again at 900 × g and the supernatant was transferred into a new tube. After drying in a SpeedVac™ Vacuum Concentrator, the cellular metabolite was stored at -80 ˚C until UHPLC-MS/MS analysis.

For media preparation, the extraction of metabolites were performed under acidic (pH 2), neutral (pH 7), or basic (pH 10) conditions. After adjusting the pH to 7.0 with 1 M NaOH, the media were combined with 5 mL of ethyl acetate and shaken for 10 min at 120 rpm to extract neutral metabolites. Then, the supernatant containing the neutral metabolites was collected and the media layer was adjusted to pH 2 with 1 M HCl to extract acidic metabolites. After each sample was combined with 5 mL of ethyl acetate and shaken for 10 min at 120 rpm at room temperature, the supernatant containing the acidic metabolites was collected and the media layer was adjusted to pH 12 with 2 M NaOH. Finally, 5 mL of ethyl acetate was added and the basic metabolites were extracted for 10 min at 120 rpm. After centrifugation (3000 rpm, 5 min, room temperature), the supernatant containing the basic metabolites were collected. The fractions of acidic, neutral, and basic metabolites were pooled, dried under a mild flow of nitrogen gas, and stored at -80 ˚C until analysis by UHPLC-MS/MS.

UPLC-MS/MS

All UPLC-MS/MS analyses were performed using an ACQUITY UPLC I-Class system (Waters Corporation, Milford, MA, USA) equipped with a Synap G2 mass spectrometer (Waters Corporation) and an electrospray ion source. The extracted samples were separated using a 10 cm analytical column (Zorbax 300SB C18, 0.075 × 100 mm; Agilent Technologies, Santa Clara, CA, USA) with an acetonitrile gradient of 3% - 100% in 0.1% formic acid over a 20 min period. The flow rate for UPLC was 0.4 mL/min. The effluent from UPLC was directly electro sprayed into the port of the mass spectrometer. The mass spectrometer was operated in the auto MS/MS acquisition mode and automatically switched between full-scan MS and MS/MS acquisition. After acquiring MS spectra over a mass range of m/z 100 - 1200 Da, the three most intense peptide ions were sequentially isolated and fragmented by quadrupole TOF collision-induced dissociation. MS/MS spectra were acquired over a mass range of m/z 100 - 1200 Da. The voltage between the ion spray tip and spray shield was 3.0 kV. Drying nitrogen gas heated to 250 ˚C was applied at a flow rate of 10 L/min. The collision energy was increased from 16 to 45 V for parent and daughter ion scanning.

Statistical analysis and identification

The UPLC-MS/MS data were imported into Progenesis 5.0 QI small molecule discovery analysis software (Nonlinear Dynamics, Newcastle upon Tyne, UK) to search for differentially expressed metabolites. Default parameters were employed for automatic sensitivity, peak picking, and retention time limits. After ion generation, *Bacillus* species were compared through principal component analysis (PCA) to assess their relatedness. Molecules with a two-fold difference in expression levels by PCA were considered as potential candidate biomarkers. Potential candidate biomarkers generated by PCA were identified by ChemSpider searches against the KEGG and LIPID MAPS online databases. The following parameters were applied: precursor tolerance of 5 ppm; optional search parameters for the theoretical fragment, fragment tolerance of 5 ppm; and elemental composition filtering (H0-150, C0-100, N0-10, and O0-30). Compounds with scores >30 were considered acceptable.

Results and Discussion

Methods to differentiate *B. megaterium* from *B. thuringiensis* and *B. cereus*

Rapid, cost-effective and accurate methods to classify among *Bacillus* species are urgently needed to protect human health against infection with bacteria that are widely used in agriculture and industry. Among these commercially exploited bacteria, *B. cereus* is specially a known pathogen of human disease that needs to be discriminated from *B. thuringiensis* which was frequently used in biopesticide. However, it is difficult to accurately differentiate these species with current methods because of the high degree of genetic homogeneity between them. In short, the aim of this study was to test the applicability of MS-based metabolomic profiling for classification of *B. cereus* and *B. thuringiensis*.

To evaluate the proposed method, an attempt was first made to classify *B. cereus*, *B. thuringiensis*, and *B. megaterium*. Generally, *B. megaterium* can be genetically distinguished from *B. cereus* and *B. thuringiensis*. All candidate biomarkers in the cellular and media fractions were examined using six *B. cereus* strains, four *B. thuringiensis* strains, and four *B. megaterium* strains. A schematic illustrating the sample preparation method is shown in figure 1 and the analyzed strains are listed in table 1. After sample preparation, fractions from the cellular and media were analyzed by UPLC-MS/MS. The resultant ion data were extracted by peak picking using Progenesis 5.0 QI software. Then, the biomarker candidates were tested for the ability to differentiate *B. megaterium* from *B. cereus* and *B. thuringiensis*.

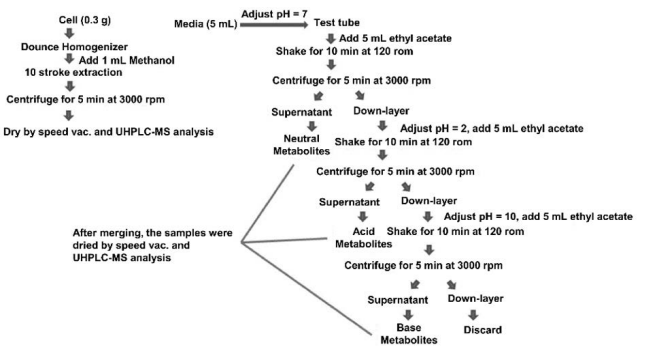

Figure 1: Scheme of sample preparation.

A total of 9,548 and 7,754 ions, respectively, were detected in the cellular and media fractions (Figure 2A and 2B, respectively). Of the generated ions, those with a maximum fold change >2 and probability (p) value of <0.05 by analysis of variance (ANOVA) were included for PCA to identify potential biomarkers. The results of both the cellular and media fractions are shown in figures 2C and 2F, respectively. PCA of the cellular fraction (Figure 2C) showed a close relationship between *B. cereus* and *B. thuringiensis*, while *B. megaterium* was relatively distant. A total of 4,120 ions were eventually considered as candidate biomarkers (Supplementary Table 1). In addition, 2,462 ions had p-values <0.05 (ANOVA) and q-values <0.05 (the expected proportion of false positives) with resolving power (probability of finding a real difference) of >0.9995 (Supplementary Table 1), which is statistically very significant. The standardized normalized abundances of the top 10 ranked candidates are shown in figures 2D (downregulated ions) and 2E (upregulated ions). As is shown in figures 2D and 2E, both the up- and downregulated ions of *B. megaterium* could be accurately differentiated from those of *B. thuringiensis* and *B. cereus*.

While PCA of the media accurately differentiated all three *Bacillus* species (Figure 2F), only 172 ions were considered as candidate biomarkers (Supplementary Table 2). However, the q-values were very low despite the significant p-values (ANOVA) and the high resolving power of >0.9995, with the exception of one ion at 514.1291 m/z. This result may be explained by the low concentration of metabolites in the media fraction due to dilution with the volume of the media.

Taken together, these results suggest that the proposed method can easily and rapidly differentiate *B. megaterium* from *B. cereus* and *B. thuringiensis* in accordance with the parameters described in previous reports [6, 16].

Potential biomarkers to classify *B. cereus* and *B. thuringiensis*

After evaluating the proposed method, an attempt was made to classify *B. cereus* and *B. thuringiensis* since these species are generally classified in the same group because the species are genetically very close. In total, 9,627 ions were detected in the cellular fraction (Figure 3A) and 8,076 in the media fraction (Figure 3B). Among these, ions with a maximum fold change of >2 and p value <0.05 (ANOVA) were further examined by PCA to identify potential candidate biomarkers. The results are shown in figures 3C and 3D, and the candidate biomarkers identified in the cellular and media fractions are

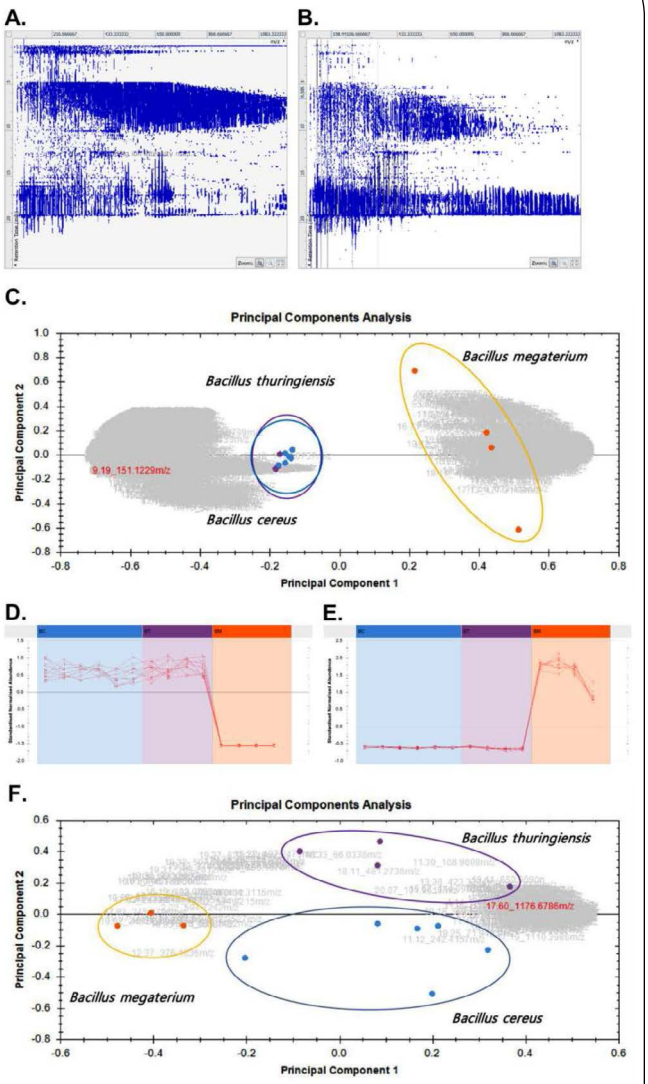

Figure 2: Classification of *B. cereus*, *B. thuringiensis*, and *B. megaterium* in the cells and the media.
The ions were generated through peak picking with Progenesis 5.0 QI software in the cellular **(A)** and media **(B)** fractions. The cutoff values for candidate biomarkers were a maximum fold change of >2 and a p value of < 0.05 (ANOVA). PCA was used to evaluate the candidate biomarkers in the cellular **(C)** and media **(F)** fractions. Notably, in the cellular fraction, the standardized normalized abundance of the up- **(D)** and down- **(E)** regulated top 10 ranking of these candidates were useful to differentiate *B. megaterium* from *B. cereus* and *B. thuringiensis*.

listed in supplementary tables 3 and 4, respectively. PCA accurately differentiated 92 and 38 candidate biomarkers in the cellular and media fractions of *B. cereus* from *B. thuringiensis*, respectively. Although some ions had a resolving power of >0.9995 and p - value <0.05 (ANOVA), none had significant q-values. The lowest q-values of the cellular and media fractions were 0.85 and 0.3, respectively. These results may lead to false positives during distinction between *B. cereus* and *B. thuringiensis*, which may make it difficult for distinguishing between the two bacteria, similar to that observed in previous reports [6, 8, 17-19].

Evaluation of biomarkers to distinguish between *B. cereus* and *B. thuringiensis*

Given the low p-valve by ANOVA and the high resolving

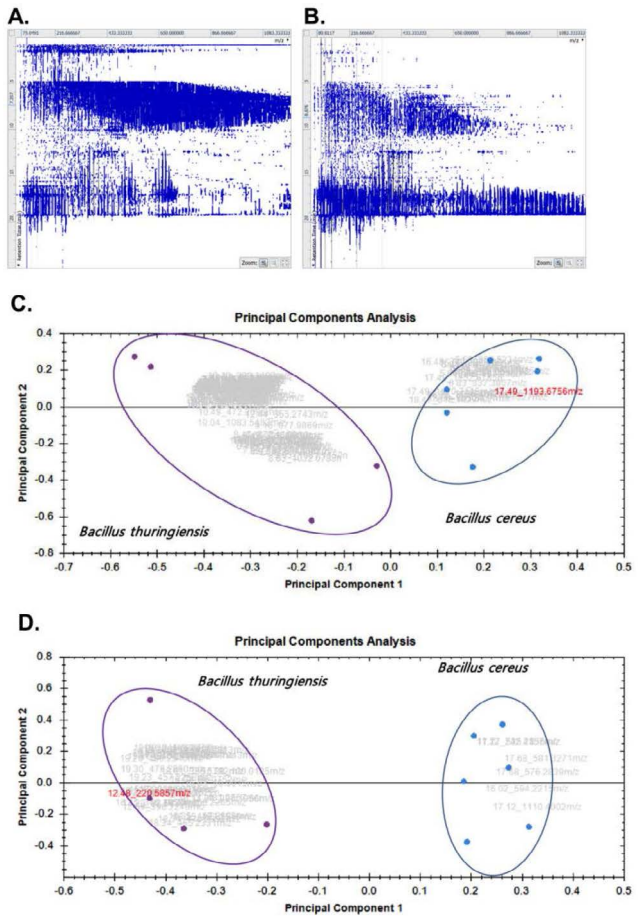

Figure 3: Classification of *B. cereus* and *B. thuringiensis* in the cellular and media fractions. The ions were generated through peak picking with Progenesis 5.0 QI software in the cellular **(A)** and media **(B)** fractions. The cutoff values for candidate biomarkers were a maximum fold change of >2 and p - value of < 0.05 (ANOVA). PCA was used to evaluate the candidate biomarkers in the cellular **(C)** and media **(D)** fractions.

power of both the cellular and media fractions to differentiate *B. cereus* from *B. thuringiensis*, although the q-values were relatively high, these ions were further examined for the ability to classify *B. cereus* and *B. thuringiensis*. For this purpose, potential candidate biomarkers were screened in three strains of known *Bacillus* species (two *B. cereus* and one *B. thuringiensis* strains) and the data obtained from the extracted ions were used to construct a chromatograph. As shown in figure 4, only one of the candidate biomarkers (1193.676 m/z) was sufficient to differentiate *B. cereus* from *B. thuringiensis*. The ion at 1193.676 m/z, which appeared at 17.50 min, was more intense in *B. thuringiensis* (Figure 4), and subsequently was used to accurately classify 13 strains of *B. cereus* and *B. thuringiensis*.

A search of all candidate biomarkers with ChemSpider against the KEGG and LIPID MAPS online databases revealed 10 and 4 compounds in the cellular and media fractions, respectively. However, the ion identified at 1193.676 m/z was not retrieved from the database search, thus further analysis is warranted.

Finally, three food samples with suspected contamination of *B. cereus* or *B. thuringiensis* were analyzed to test the ability of the proposed method to accurately classify the *Bacillus* species based on this ion. The bacteria in the samples were classified as *B. cereus*, since the ion at 1193.676 m/z was not detected. Taken together, these results show that *B. cereus* and *B. thuringiensis* can be accurately classified according to the ion at 1193.676 m/z. However, further investigations with more *Bacillus* species are needed to verify these results.

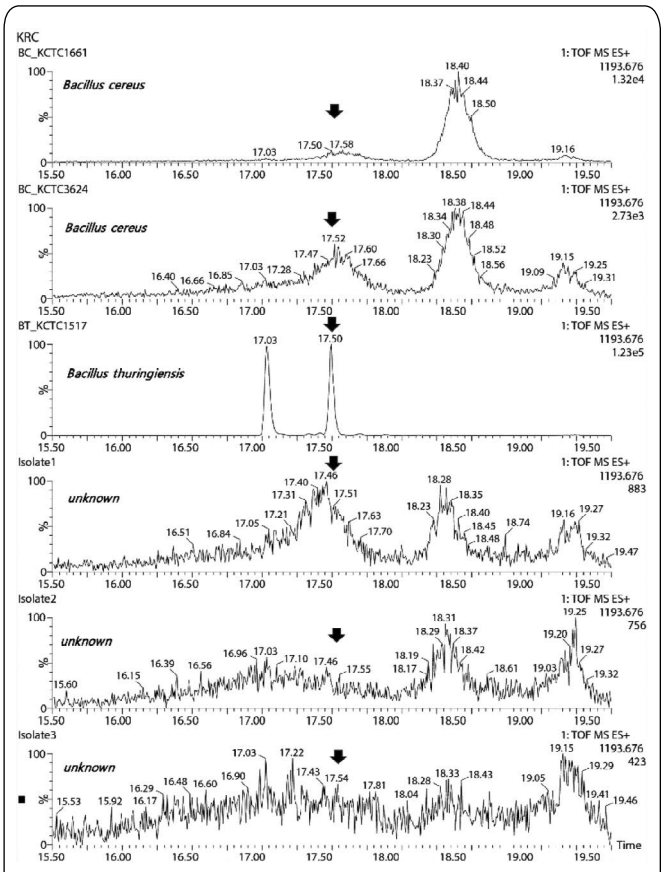

Figure 4: Evaluation and application of the biomarker at 1193.676 m/z to classify *B. cereus* and *B. thuringiensis*. The ion at 1193.676 m/z was used to classify *B. cereus* and *B. thuringiensis* in actual cultured food samples.

Conclusion

As consumer awareness of health and hygiene has improved recently, foodborne pathogen testing for food safety is increasing and accurate identification of foodborne bacteria is necessary. *Bacillus cereus*, one of the food poisoning bacteria, can't be distinguishable from *Bacillus thuringiensis* used as a biopesticide by conventional biochemical identification in routine microbiological tests. Because of their high genetic homology, the two bacteria were difficult to distinguish in the MALDI-TOF mass spectrometry-based identification method, which mainly analyzes ribosomal proteins. In this study, we examined the applicability of MS-based metabolomic profiling for classification of *B. cereus* and *B. thuringiensis*. Additionally, UPLC-MS/MS analysis condition applied short running time to detect more ions. The proposed method has been evaluated as to whether *B. megaterium* is correctly distinguished from *B. cereus* and *B. thuringiensis*. Statistically significant the p-values, q-values, and resolving

power results clearly indicated that MS-based metabolomic profiling method can be applied very effectively to classify *Bacillus* species, consistent with previous reports [6, 16]. Thus, we performed experiments to discover potential biomarkers that could classify *B. cereus* and *B. thuringiensis* in this way. Since, *B. cereus* and *B. thuringiensis* are genetically very close to date, there is no report to distinguish them using mass spectrometry. Most of the previous studies were attempts to genetically classify other species using MALDI-TOF [11-14] or HPLC-MS/MS [11, 15]. We have detected 92 and 38 ions as potential biomarker candidates for classifying *B. cereus* and *B. thuringiensis* in the cellular and media fractions, respectively. Among the biomarker candidates, 1193.676 m/z ion (at 17.50 min) distinguished these species, which were lower in *B. cereus* and higher in *B. thuringiensis*.

Using this specific ion, we applied to discriminate *B. cereus* group strains isolated from actual food samples in unknown set. Three *B. cereus* group strains were identified as *B. cereus*, since the biomarker at 1193.676 m/z was not detected. These three unknown samples were confirmed as *B. cereus* by other analyses (data not shown).

This study is successful approach to propose a biomarker to correctly classify *B. cereus* and *B. thuringiensis* using UPLC-MS/MS and would be useful in the food, cosmetic, and agricultural industries. However, further validation is required using more test samples to ensure the reliability of this method.

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Conflict of Interest

The authors declare no conflict of interest.

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