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Development of a Rapid and Accurate Identification Method for *Citrobacter* Species Isolated from Pork Products Using a Matrix-Assisted Laser-Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS)

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Previous detection methods for *Citrobacter* are considered time consuming and laborious. In this study, we have developed a rapid and accurate detection method for *Citrobacter* species in pork products, using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS). A total of 35 *Citrobacter* strains were isolated from 30 pork products and identified by both MALDI-TOF MS and 16S rRNA gene sequencing approaches. All isolates were identified to the species level by the MALDI-TOF MS, while 16S rRNA gene sequencing results could not discriminate them clearly. These results confirmed that MALDI-TOF MS is a more accurate and rapid detection method for the identification of *Citrobacter* species.

Keywords: *Citrobacter* species, MALDI-TOF MS, pork products, identification

Citrobacter species (spp.) are facultative anaerobes of the *Enterobacteriaceae* family and are considered putrefactive bacteria and opportunistic pathogens [1]. *Citrobacter* are classified into 11 species by DNA hybridization assay: *C. amalonaticus*, *C. braakii*, *C. farmeri*, *C. freundii*, *C. gillenii*, *C. koseri*, *C. murliniae*, *C. rodentium*, *C. sedlakii*, *C. werkmanii*, and *C. youngae* [3, 4]. The *Citrobacter* genus is generally present in human and animal gastrointestinal tracts [8] and commonly found in water, soil, and food products [14]. Previous studies demonstrated that biogenic amines are associated with the putrescence activity of bacteria in meat [16, 17]. To the best of our knowledge, *Citrobacter* spp. cannot be identified distinctly, since rapid and accurate identification methods are not available yet. In order to prevent potential threats of foodborne illness caused by *Citrobacter*, a new monitoring system for putrefactive bacteria is required. For the last decade, matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) has been widely utilized for the reliable identification of various microorganisms [2, 13]. Recently, Kolínská *et al.* [11] identified *Citrobacter* spp. using the MALDI-TOF MS on clinical samples, but there is no report on *Citrobacter* spp. from food samples to date. In this study, we have successfully developed a rapid and reliable assay for the identification of three *Citrobacter* spp. (*C. braakii*, *C. freundii*, and *C. werkmanii)* on pork products, using the MALDI-TOF MS.

A total of 30 samples were obtained from raw pork meat (pork fat, belly, rib, hind legs, and sirloin), semi-processed pork meat (cured, smoked, emulsified, and mixed meat),

and pork meat products (roasted ham, vacuum-packaged sausages, and bacon) in four meat processing plants located in the Gyeonggi province, Korea from March to June of 2013. Homogenized samples were serially diluted and spread onto xylose lysine desoxycholate (XLD) selective media (Difco-BBL, Detroit, MI, USA). After incubation, colonies with different morphological characteristics (color, shape, or size) were selected to identify specific *Citrobacter* species, based on the previous studies, and inoculated into broth for enumeration [10, 12].

Genomic DNA was extracted from each grown culture using a G-spin Genomic DNA extraction kit (Intron Biotechnology Inc., Seongnam, Korea) according to the manufacturer's instructions. The DNA concentration was measured using a MaestroNano Spectrophotometer (Maestrogen, Las Vegas, NV, USA). The genomic DNA isolated from 35 strains were utilized to amplify the 16S rRNA gene portion for sequencing, using 16S universal primers that were adopted from a previous report (27F: AGAGTTTGATCCTGGCTCAG; and 492R: GGCTACCTT GTTACGACTT) (Bionics Co., Ltd., Seoul, Korea) [9]. Approximately 1,465 bp of partial 16S rRNA gene sequences from each isolate were analyzed using a basic local alignment search tool (BLAST) algorithm for homologous gene identification.

To perform the MALDI-TOF MS assay, 35 isolates from pork products were grown on tryptic soy media (Difco-BBL) at 37°C overnight. Bacterial cells from an individual colony were deposited directly on a target polished steel microscout target plate (MSP 96; Bruker Daltonik GmbH, Bremen, Germany) overlaid with 0.5 µl of 99% formic acid and 1 µl of α-cyano-4-hydroxycinnamic acid matrix solution in acetonitrile:water:trifluoroacetic acid (TFA) (50:47.5:2.5 (v/v)). After crystallization at room temperature, measurements were performed on an autoflex speed TOF/TOF mass spectrometer (Bruker Daltonik GmbH). Ionization was performed with laser irradiation using flex analysis software 3.4. Raw spectral data were imported into the latest Biotyper software 3.1 (Bruker Daltonik GmbH), which contains spectra of approximately 4,613 species. Integrated pattern-matching algorithms were recorded as log scores with maximum values of 3.0. Scores higher than

Table 1. Comparison of 16S rRNA gene sequencing and MALDI-TOF MS system for identifying 35 isolates of *Citrobacter* species.

Identification of *Citrobacter* isolates by 16S rRNA gene sequencing			Identification of *Citrobacter* isolates by MALDI-TOF MS system		
			MALDI-TOF MS results	No. of log score value[c]	
No. of *Citrobacter* isolates	Description	Accession No.[b]		Score value > 2.0	1.8 < Score value < 2.0
3	C[a]. *freundii*	KM222619.1	*C. freundii*	3	
	C. werkmanii	KJ865550.1			
12	*C. freundii*	KM222633.1	*C. braakii*	11	1 (1.939[d])
	C. werkmanii	KJ830707.1			
	C. braakii	NR_117750.1			
7	*C. freundii*	KM222633.1	*C. werkmanii*	7	
	C. werkmanii	KJ865550.1			
1	*C. werkmanii*	KJ865550.1	*C. freundii*	1	
	C. freundii	KJ726569.1			
	C. braakii	NR_117750.1			
6	*C. freundii*	KF245926.1	*C. braakii*	6	
	C. braakii	KC139411.1			
5	*C. freundii*	JN831090.1	*C. freundii*	3	2 (1.974[e] and 1.857[f])
1	*C. braakii*	AB741663.1	*C. freundii*	1	
	C. freundii	GU586147.1			

[a]C: *Citrobacter*.

[b]Accession numbers have been deposited in the NCBI database.

[c]Number of isolates identification (log) score values of >2.0 indicate species-level, and between 1.7 and 2.0 indicate genus level identification.

[d]Species log score value of isolate No. 4 was considered for identification at the genus level (1.939).

[e]Species log score value of isolate No. 29 was considered for identification at the genus level (1.974).

[f]Species log score value of isolate No. 37 was considered for identification at the genus level (1.857).

1.7 were required for identification at the genus level and scores over 2.0 were required for species-level identification. Scores below 1.7 were not assigned to a species or genus [18].

A total of 176 *Enterobacteriaceae* were isolated from XLD selective media (Difco-BBL) and 16S rRNA gene sequencing was performed to identify *Citrobacter* spp. (Table 1). From the 16S rRNA gene sequencing results, 35 isolates were concurrently identified as *C. braakii*, C. *freundii*, and C. *werkmanii*. Only five isolates were identified up to the species level as *C. freundii*, whereas the other 30 isolates were only identified up to the genus level based on the BLAST database. Since genus *Citrobacter* has high sequence homology among species, it is hard to discriminate them to an accurate species level through a 16S rRNA sequencing approach.

The MALDI-TOF MS assay was performed to identify specific *Citrobacter* spp. and compare these data with the 16S rRNA gene sequencing results. The database contained 21 different *Citrobacter* profiles with 10 different species: *C. amalinaticus* (2 profiles); *C. braakii* (2); *C. famerii* (1); *C. freundii* (4); *C. gilenii* (1); *C. koseri* (6); *C. murliniae* (1); *C. rodentium* (1); *C. sedlakii* (2); and *youngae* (1) (Bruker Corporation, MALDI-TOF/TOF systems overview). However,

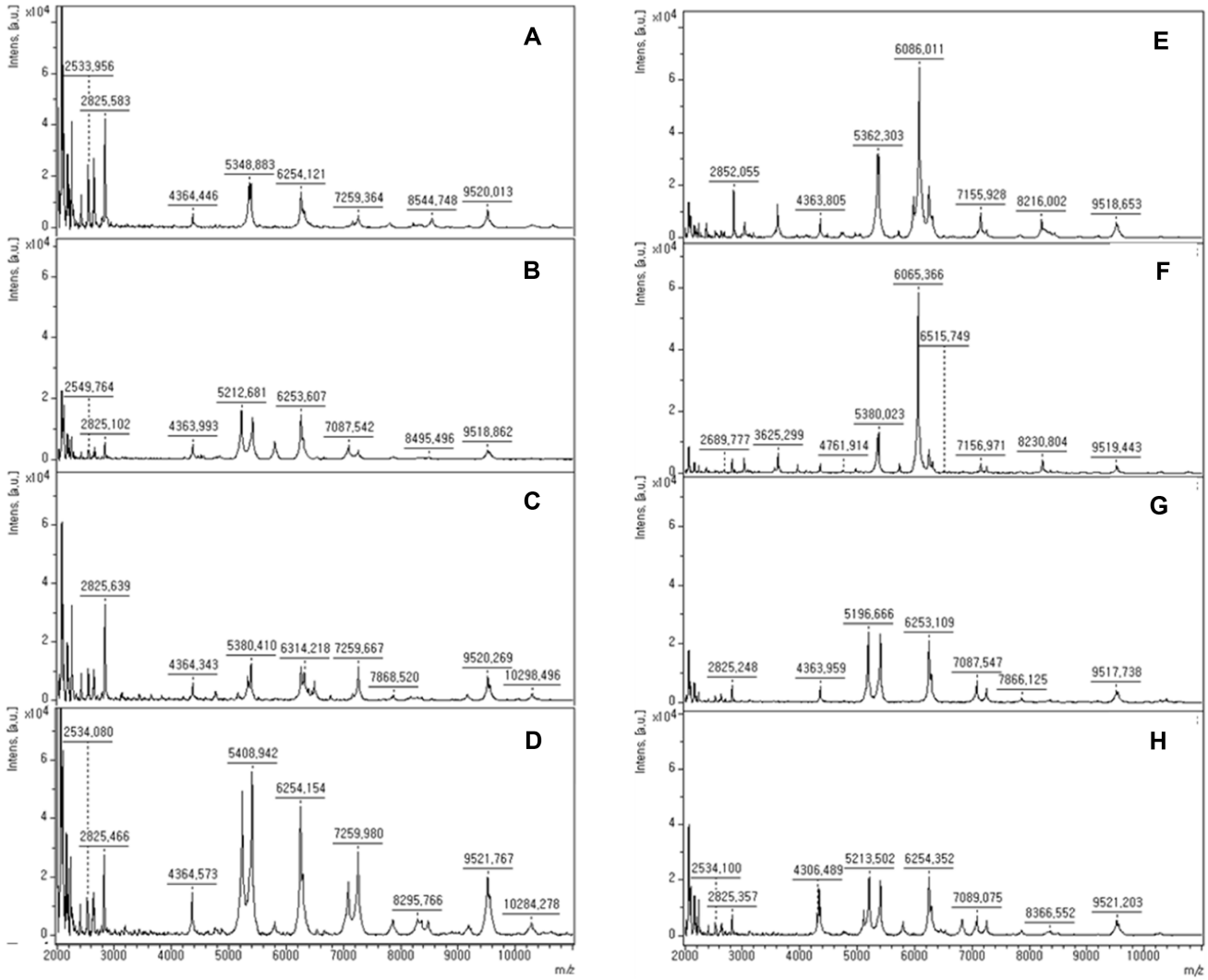

Fig. 1. MALDI-TOF MS of eight reference strains.

Absolute intensities (Da) of ions and mass values (*m/z*) are shown on the y axis, and the x axis, respectively. The *m/z* value stands for the mass-to-charge ratio. (**A**) *C. amalonaticus* NCCP 15699; (**B**) *C. braakii* KCTC 2006; (**C**) C. *farmeri* NCCP 15641; (**D**) *C. freundii* KCTC 2359; (**E**) *C. rodentium* KACC 15163; (**F**) *C. sedlakii* KACC 15164; (**G**) *C. werkmanii* KACC 15165; and (**H**) *C. youngae* KACC 16595.

a profile for *C. werkmanii* was not included in the latest database. To compare results from 16S rRNA gene sequencing and MALDI-TOF MS data, we added a *C. werkmanii* profile into the database. With the database from Bruker 3.1 and the spectrum that we created, reference strains from Korean Collection for Type Culture (KCTC), Korean Agricultural Culture Collection (KACC), and National Culture Collection for Pathogens (NCCP) strains were used and identified to the species level (Fig. 1).

To confirm the developed assay, all strains were independently cultivated three times and subjected to three analogous MALDI-TOF MS analyses. As expected, all isolates were successfully classified to the three different species *via* the MALDI-TOF MS. The most predominant species was identified as *C. braakii* (18 isolates), followed by *C. werkmanii* (7 isolates), based on mass spectra (Table 1). Interestingly, one of the *Citrobacter* isolates showed a different result compared with the sequencing results. In the sequencing result, one isolate was identified as both *C. freundii* and *C. werkmanii*, whereas MALDI-TOF MS identified it as *C. freundii*. The *C. freundii*-specific mass values were only identified as *m/z* ions 2,618 ± 0.45, 3,545 ± 1.31, 4,761 ± 2.62, 5,169 ± 0.72, 5,238 ± 1.31, 5,428 ± 0.30, 5,444 ± 2.19, 6,396 ± 1.80, 7,290 ± 3.40, 7,893 ± 0.59, 7,949 ± 3.21, 8,297 ± 0.36, 8,337 ± 3.93, 8,386 ± 1.84, 8,400 ± 2.86, 8,903 ± 0.32, 8,932 ± 0.01, 8,947 ± 0.26, 9,230 ± 0.73, and 10,323 ± 4.25. The strain isolated from meat products was identified as *C. freundii* (*m/z* ions 8,337, 8,903, and 8,932), whereas the *C. werkmanii*-specific mass values were not detected (data not shown).

Based on these species-specific mass values, the strain isolated from meat products was identified as *C. freundii*. Unlike *C. freundii*, other *Citrobacter* spp. isolates exhibited discrepancies from the 16S rRNA gene sequencing results. Reference strains of *C. freundii*, *C. braakii*, *C. youngae*, and *C. werkmanii* were difficult to distinguish by 16S rRNA gene sequencing because of their high level of homologous sequences. Therefore, species-specific mass values measured by the MALDI-TOF MS are crucial for the discrimination and identification among similar *Citrobacter* spp. (Fig. 2). Each *Citrobacter* species has its unique species-specific mass values. Specifically, an identification method that uses specific mass spectra was confirmed through several studies utilizing MALDI-TOF MS [5]. Whole-cell mass spectra demonstrated that bacterial isolates can be identified to the genus, species, and subspecies levels [6, 15]. Differences in the mass spectra of unknown strain samples could be used for the interrelationship of food origin with the types of bacterial isolates identified in MALDI-TOF MS as being predominant [9].

In this study, we have established a rapid and accurate method for the identification of 35 *Citrobacter* strains isolated from pork meat products that had been difficult to discriminate using routine identification methods such as 16S rRNA gene sequencing. Many conventional identification methods are time-consuming and labor-intensive in terms

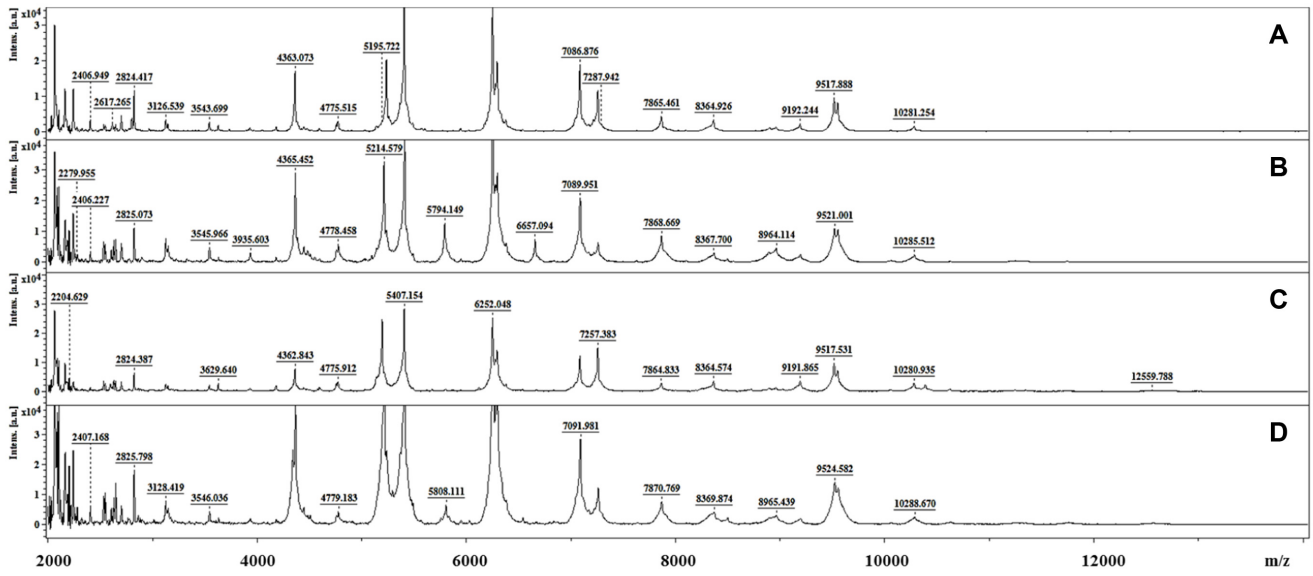

Fig. 2. Comparison of mass spectra of four reference strains.
(**A**) *C. freundii* KCTC 2359; (**B**) *C. braakii* KCTC 2006; (**C**) *C. werkmanii* KACC 15165; and (**D**) *C. youngae* KACC 16595.

of sample preparation and rapid data acquisition. However, the MALDI-TOF MS assay can be utilized as a rapid bacterial identification method with considerable potential for cost-effectiveness and accuracy [5, 7, 9] when a reference database is available, including species-specific mass values of reference strains. Our results may improve on the current limitation of MALDI-TOF MS in identifying bacteria that were not previously included in the MALDI-TOF database by adding approximately 4,613 species spectra detected by specific mass values. Furthermore, our results will provide a critical standard method and approach for detecting other potential foodborne pathogens in foods.

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