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Application News

No. B114

MALDI-TOF Mass Spectrometry

MALDI-MS Proteotyping of *Cutibacterium Acnes*

Introduction

C. acnes is a major member of human skin commensal type of bacteria. The members of *C. acnes* are classified into three types, I, II, and III, based on traditional phenotypic characteristics such as serotypes, sugar content of the cell-wall, fatty acid profile, and morphology. Recently, these were assigned to novel subspecies as follows: type I that included *C. acnes* subsp. *acnes*, type II that included *C. acnes* subsp. *defendens*, and type III that included *C. acnes* subsp. *elongatum*. In addition, Type I is subdivided into subtypes IA_1, IA_2, IB, and IC by DNA-based approach, multilocus sequence typing (MLST). MALDI-MS fingerprinting technique, which uses unidentified two to five peaks has been applied to rapid identification of subtypes for *C. acnes* strains; however, IA_1 and IA_2 have not been differentiated yet. In addition, reported *m/z* values of the biomarker peaks are different among researchers.

However, detected peaks of proteins can be assigned using theoretical masses calculated from amino-acid sequences translated from DNA sequences. Using the "MALDI-MS proteotyping" method, microorganisms are identified or classified based on identified biomarker peaks. In this application news, 24 strains of *C. acnes* are classified at the subtype level by MALDI-MS proteotyping using eMSTAT Solution™ and Strain Solution™ [(1)].

K. Teramoto

Experiment

Sample strains

A total of 24 stains of *C. acnes* (Table 1) were purchased from Japan Collection of Microorganisms (RIKEN Bio Resource Center, Japan).

Table 1 Sample Strains

Sample name	Strains	Subtype*1
C. acnes subsp. *acnes* (type I)	JCM 6425^T	IA_1
	JCM 6495	IA_1
	JCM 18916	IA_1
	JCM 18922	IA_1
	JCM 18924	IA_1
	JCM 18907	IA_2
	JCM 18908	IA_2
	JCM 18910	IA_2
	JCM 18912	IA_2
	JCM 18917	IB
	JCM 18918	IB
	JCM 18923	IB
	JCM 18927	IB
C. acnes subsp. *defendens* (type II)	JCM 6473^T	II
	JCM 18911	II
	JCM 18913	II
	JCM 18914	II
	JCM 18915	II
	JCM 18920	II
	JCM 18921	II
	JCM 18926	II
C. acnes subsp. *elongatum* (type III)	JCM 18909	III
	JCM 18919^T	III
	JCM 18925	III

*1 Dekio, I.et al., Journal of medical microbiology **2012**, 61, 622-630.

Database construction of ribosomal proteins

Ribosomal proteins (SPs) are known as promising biomarker proteins, which are detected in bacteria by MALDI-MS. The theoretical masses of RPs were calculated for the following genome sequenced strains used in this study: *C. acnes* subsp. *acnes* JCM 6425^T, JCM 18916, and JCM 18918; *C. acnes* subsp. *defendens* JCM 6473^T and JCM 18920; and *C. acnes* subsp. *elongatum* JCM18909. Loss of N-terminal methionine is a possible post-translational modification.

MALDI-TOFMS

MALDI-TOFMS:
AXIMA Performance™, positive-ion linear mode. MALDI mass spectra for each sample were acquired in the range of *m/z* 2000–30000.

Matrix solution:
Sinapinic acid at a concentration of 10 mg/mL in 50 % acetonitrile with 1 % trifluoroacetic acid.
Methylenediphosphonic acid was used for additives of matrix solution.

Sample preparation:
Cell-lysates were obtained by grinding with zirconia beads from bacterial cells, and the beads and cell debris were removed by centrifugation. Protein fractions were obtained by ultra-filtration of the cell lysate. Sample/matrix mixture was prepared by mixing of 10 µL of matrix solution and 1 µL of protein fraction. One microliter of sample/matrix mixture was spotted onto the MALDI target plate and dried.

Peak matching of the biomarker proteins was judged from tolerance within 200 ppm. Peak matching and statistical analysis were performed using eMSTAT Solution. Cluster analysis was conducted using Strain Solution. A dendrogram was constructed using the unweighted pair group method with arithmetic mean (UPGMA) obtained from the Euclidian distance.

Result

Fig. 1 shows MALDI mass spectrum of *C. acnes* subsp. *acnes* JCM 6425^T, and asterisks (*) indicate RPs. The number of detected RPs was 31. Peak matching of MALDI mass spectra of *C. acnes* sample strains and biomarker RPs was carried out using Strain Solution. The assessment results for each biomarker protein are summarized in the binary biomarker matching table, in which score used was either 1 or 0. A dendrogram was constructed by cluster analysis using the biomarker matching table.

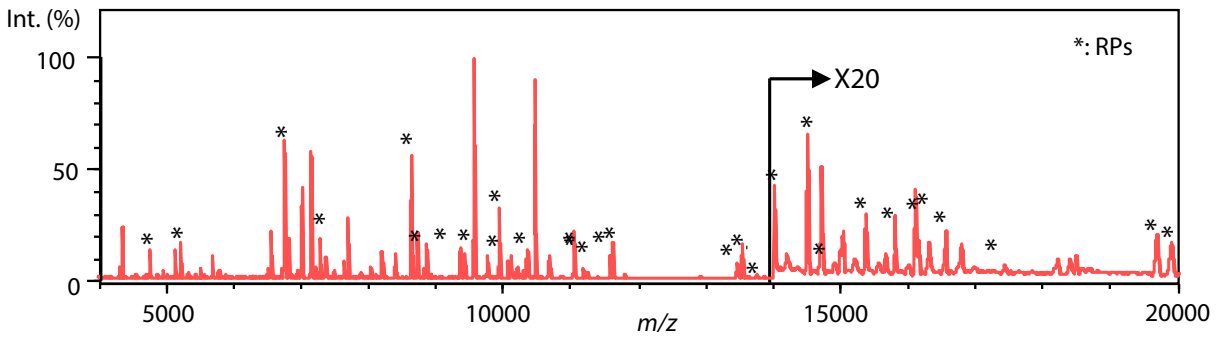

Fig. 1 MALDI mass spectrum of *C. acnes* subsp. *acnes* JCM 6425^T.

As biomarker proteins, a total of 10 kinds of proteins, which were not overlapping with the other peaks and could be detected with good reproducibility, were selected from the three types of strains. Following 6 kinds of biomarker proteins were detected at the same *m/z* values for each sample strain: L9, L29, L30, S8, S15, and S19. These 6 biomarker proteins can be used to identify *C. acnes* species. However, the other 4 kinds of biomarker proteins can be used to discriminate *C. acnes* strains at subspecies and/or subtypes because these detected masses were different among *C. acnes* strains reflecting slight mutation of amino-acid residues.

These 10 kinds of RPs were registered in a Strain Solution DB, and peak matching was carried out against 24 strains of *C. acnes*. As a result, subspecies of *C. acnes* strains were classified correctly, but subtype IA_1 and IA_2 for *C. acnes subsp. acnes* could not be differentiated. Biomarker proteins to distinguish subtype IA_1 and IA_2 were searched using eMSTAT Solution. As a result, it was suggested that a peak detected at *m/z* 7035 was a promising biomarker peak to differentiate subtype IA_1 and IA_2 because it was specific for IA_1 strains (Table 2).

Table 2 Peak Matrix

	m/z	IA1(15)	IA2(12)	IB(12)	II(24)	III(9)
☑	7004.92	0	11	12	0	0
☑	7019.87	13	6	7	24	9
☑	7034.53	15	0	0	0	0
☑	7046.91	0	0	0	2	0
☑	7049.97	0	3	0	7	0
☑	7062.00	0	0	0	0	9

The peak matrix created by the eMSTAT Solution shows the number of times the peak was detected in each sample group (repeated measurements). Pink color indicates that the peak were detected in all mass spectra of each sample group. For example, antitoxin at *m/z* 7035 was detected in all 15 repeated measurements in IA_1, but not in any of the other samples.

Although this peak (*m/z* 7305) has been used by the other researchers to differentiate subtypes of *C. acnes* strains using the MALDI-MS fingerprinting approach, it has not been characterized [2], [3]. In addition, the subtypes characterized by the biomarker peak detected at *m/z* 7035 varied among researchers. For example, Nagy *et al.* [2] reported that a biomarker peak detected at *m/z* 7035 was common among subtype IA_1 and IA_2, but Dekio *et al.* [3] reported that it was characteristic for subtype IA_1. Our results were in agreement with the results reported by Dekio *et al*. Because the biomarker protein that was detected at *m/z* 7035 was easily purified by a combination of ultra filtration and step-wise fractionation, peptide mass fingerprinting analysis of the biomarker protein of *C. acnes* subsp. acnes JCM 6425^T was performed from fragments derived from trypsin digestion. As a result, the biomarker protein was identified as an antitoxin. Calculated masses and detected masses of antitoxin for each subtypes were matched within a difference of±200 ppm. In addition, biomarker peaks detected around *m/z* 7200, which had been used in previous studies by others, were identified as CsbD-like protein [1]. Calculated masses and detected masses of CsbD-like protein for each subtypes were also matched within a difference of ±200 ppm (Fig. 2). Amino-acid sequences and calculated masses for each subtype are summarized (Table 3).

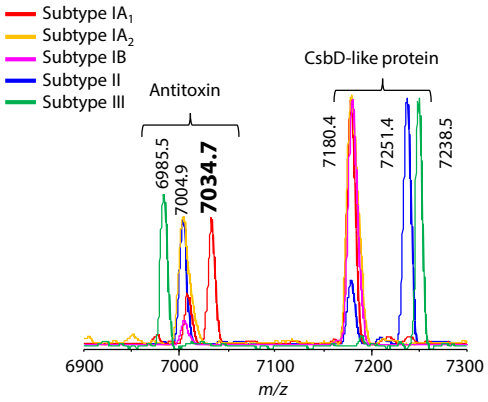

Fig. 2 MALDI mass spectra of *C. acnes* in the range of *m/z* 6900–7300.

Table 3 Amino-acid sequences of antitoxin and CsbD-like protein with calculated masses.

Antitoxin (66 aa)
Calculated mass: *m/z* 7034.6 (IA_1)
MGLFDKAKDAISDRQDDIKNQASQHSDQVEQGIDKAGNTVDDKTGGKFSDQIDKGQDALKDKLGDL
Calculated mass: *m/z* 7004.5 (IA_2, IB, IC, and III)
MGLFDKAKDAISDRQDDIKNQASQHSDQVEQGIDKAGNAVDDKTGGKFSDQIDKGQDALKDKLGDL
Calculated mass: *m/z* 6985.5 (II)
MGLFDKAKDAISDHQDDIKNQASQHSDQVEQGIDKAGNAVDDKTGGKFSDQIDKGQDALKDKLGDL
CsbD-like protein (71 aa)
Calculated mass: *m/z* 7179.9 (IA_1, IA_2, IB, IC and III)
MGLSDKINSKSDEAVGAAKEKIGGLTDDSDLKSAGADQKASGKVAQKVEDVKDKANDLKHNVQAAADKLKG
Calculated mass: *m/z* 7238.0 (III)
MGLSDKINSKSDEAVGAAKEKIGGLTDDSDLKSEGADQKASGKVAQKVEDVKDKANDLKHNVQAAADKLKG
Calculated mass: *m/z* 7251.0 (II, ST 42 and 46)
MGLSDKINSKSDEAVGAAKEKIGGLTDDSDLKSEGANQKASGKVAQKVEDVKDKANDLKHNIQAAADKLKG
Calculated mass: *m/z* 7265.0 (II, ST 43)
MGLSDKINSKSDEAVGAAKEKIGGLTDDSDLKSEGANQKASGKVAQKVEDVKDKANDLKHNVQAVADKLKG

Table 4 Biomarker matching table in a binary format of the sample strains.

Strains		Biomarker proteins																
subtype	JCM	L9	L29	L30	S8	S15	S19	Antitoxin	L6		L13			L15		L23		
		16119	8755	6787	14526	10081	10380	7035	19679	19707	16154	16168	16182	15358	15385	11181	11200	
IA_1	6425^T	1	1	1	1	1	1	1	0	1	0	1	0	0	1	0	1	
	6495	1	1	1	1	1	1	1	0	1	0	1	0	0	1	0	1	
	18916	1	1	1	1	1	1	1	0	1	0	1	0	0	1	0	1	
	18922	1	1	1	1	1	1	1	0	1	0	1	0	0	1	0	1	
	18924	1	1	1	1	1	1	1	0	1	0	1	0	0	1	0	1	
IA_2	18907	1	1	1	1	1	1	0	0	1	0	1	0	0	1	0	1	
	18908	1	1	1	1	1	1	0	0	1	0	1	0	0	1	0	1	
	18910	1	1	1	1	1	1	0	0	1	0	1	0	0	1	0	1	
	18912	1	1	1	1	1	1	0	0	1	0	1	0	0	1	0	1	
IB	18917	1	1	1	1	1	1	0	0	1	1	0	0	0	1	0	1	
	18918	1	1	1	1	1	1	0	0	1	1	0	0	0	1	0	1	
	18923	1	1	1	1	1	1	0	0	1	1	0	0	0	1	0	1	
	18927	1	1	1	1	1	1	0	0	1	1	0	0	0	1	0	1	
II	6473^T	1	1	1	1	1	1	0	1	0	0	0	1	1	0	0	1	
	18911	1	1	1	1	1	1	0	1	0	0	0	1	1	0	0	1	
	18914	1	1	1	1	1	1	0	1	0	0	0	1	1	0	0	1	
	18915	1	1	1	1	1	1	0	1	0	0	0	1	1	0	0	1	
	18920	1	1	1	1	1	1	0	1	0	0	0	1	1	0	0	1	
	18921	1	1	1	1	1	1	0	1	0	0	0	1	1	0	0	1	
	18913	1	1	1	1	1	1	0	1	0	0	1	0	1	0	0	1	
	18926	1	1	1	1	1	1	0	1	0	0	1	0	1	0	0	1	
III	18909	1	1	1	1	1	1	0	1	0	1	0	0	0	1	1	0	
	18919^T	1	1	1	1	1	1	0	1	0	1	0	0	0	1	1	0	
	18925	1	1	1	1	1	1	0	1	0	1	0	0	0	1	1	0	

A dendrogram was generated using Strain Solution by UPGMA cluster based on the biomarker matching table (Table 4). Fig. 3 shows the resulting dendrogram of *C. acnes* strains together with their phylotypes. A total of 24 *C. acnes* strains used in this study were split into three major clusters that correlated with traditional phylogenetic classification defined as types I, II, and III by two RSPs selected from L23, L15, and L6. In addition, members of type I were classified into phylotypes as IA_1, IA_2, and IB by L13 and antitoxin. As a result, we successfully classified 24 strains of *C. acnes* phylotypes based on 10 RSPs and antitoxin for IA_1.

This is the first report that has successfully classified the phylotypes of *C. acnes* strains as IA_1, IA_2, IB, II, and III based on MALDI-MS proteotyping, using assigned biomarker proteins such as RSPs and antitoxin. Although MLST is widely recognized as the gold-standard technique allowing the differentiation of phylotypes of *C. acnes*, our proposed method is still a viably important workflow, which enabled us to distinguish the phylotypes of *C. acnes* independent of the DNA-based technique.

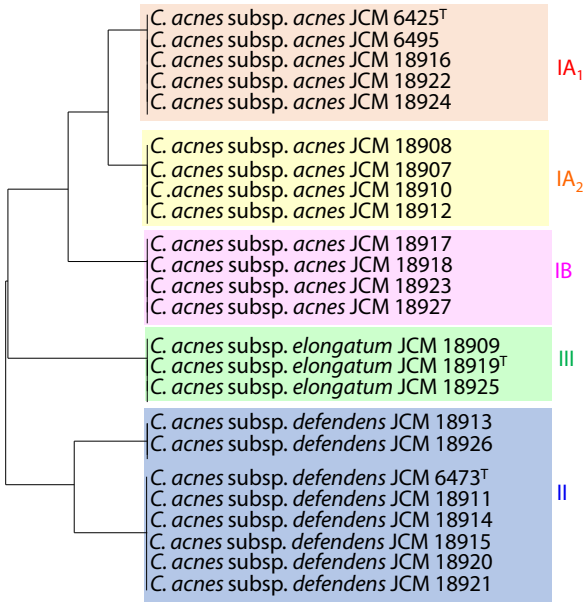

Fig. 3 Dendrogram of *C. acnes* strains based on variation of 10 ribosomal subunit proteins and antitoxin.

Summary

C. acnes strains, which are generally analyzed by the DNA-based approach, were successfully classified at the subtype levels by MALDI-MS proteotyping. Here, we used two software; Strain Solution is useful for peak assignment of bacterial mass spectra and cluster analysis, and eMSTAT Solution is useful for statistical analysis of the mass spectra from bacteria and biomarker search for typing of bacteria.

<References>
(1) Teramoto, K. *et al., Proc. Jpn. Acad., Ser. B* 2019, **95**, 612-623.
(2) Nagy, E. *et al., Anaerobe* 2013, **20**, 20–26.
(3) Dekio, I. et al., Journal of medical microbiology 2012, **61**, 622-630.

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