

### 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<sub>1</sub>, IA<sub>2</sub>, 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<sub>1</sub> and IA<sub>2</sub> 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™<sup>(1)</sup>.

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#### ■ 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* <sup>1</sup> |
|--|------------------------|-----------------------|
| <i>C. acnes</i> subsp. <i>acnes</i> (type I)       | JCM 6425 <sup>T</sup>  | IA <sub>1</sub>       |
|  | JCM 6495               | IA <sub>1</sub>       |
|  | JCM 18916              | IA <sub>1</sub>       |
|  | JCM 18922              | IA <sub>1</sub>       |
|  | JCM 18924              | IA <sub>1</sub>       |
|  | JCM 18907              | IA <sub>2</sub>       |
|  | JCM 18908              | IA <sub>2</sub>       |
|  | JCM 18910              | IA <sub>2</sub>       |
|  | JCM 18912              | IA <sub>2</sub>       |
|  | JCM 18917              | IB                    |
|  | JCM 18918              | IB                    |
|  | JCM 18923              | IB                    |
|  | JCM 18927              | IB                    |
| <i>C. acnes</i> subsp. <i>defendens</i> (type II)  | JCM 6473 <sup>T</sup>  | II                    |
|  | JCM 18911              | II                    |
|  | JCM 18913              | II                    |
|  | JCM 18914              | II                    |
|  | JCM 18915              | II                    |
|  | JCM 18920              | II                    |
|  | JCM 18921              | II                    |
| JCM 18926  | II                     |                       |
| <i>C. acnes</i> subsp. <i>elongatum</i> (type III) | JCM 18909              | III                   |
|  | JCM 18919 <sup>T</sup> | 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<sup>T</sup>, JCM 18916, and JCM 18918; *C. acnes* subsp. *defendens* JCM 6473<sup>T</sup> 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  $\mu$ L of matrix solution and 1  $\mu$ L 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<sup>T</sup>, 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.

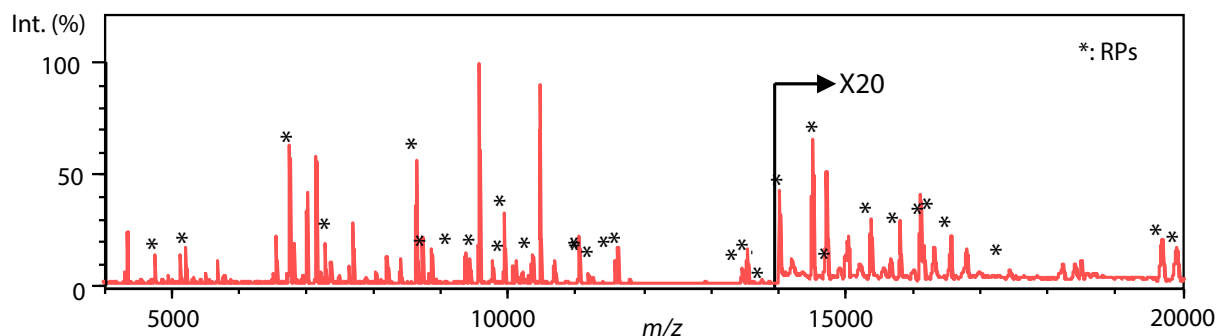


Fig. 1 MALDI mass spectrum of *C. acnes* subsp. *acnes* JCM 6425<sup>T</sup>.

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<sub>1</sub> and IA<sub>2</sub> for *C. acnes subsp. acnes* could not be differentiated. Biomarker proteins to distinguish subtype IA<sub>1</sub> and IA<sub>2</sub> 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<sub>1</sub> and IA<sub>2</sub> because it was specific for IA<sub>1</sub> strains (Table 2).

Table 2 Peak Matrix

| <input checked="" type="checkbox"/> | $m/z$   | IA1(15) | IA2(12) | IB(12) | II(24) | III(9) |
|-------------------------------------|---------|---------|---------|--------|--------|--------|
| <input checked="" type="checkbox"/> | 7004.92 | 0       | 11      | 12     | 0      | 0      |
| <input checked="" type="checkbox"/> | 7019.87 | 13      | 6       | 7      | 24     | 9      |
| <input checked="" type="checkbox"/> | 7034.53 | 15      | 0       | 0      | 0      | 0      |
| <input checked="" type="checkbox"/> | 7046.91 | 0       | 0       | 0      | 2      | 0      |
| <input checked="" type="checkbox"/> | 7049.97 | 0       | 3       | 0      | 7      | 0      |
| <input checked="" type="checkbox"/> | 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<sub>1</sub>, 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<sup>(2), (3)</sup>. In addition, the subtypes characterized by the biomarker peak detected at  $m/z$  7035 varied among researchers. For example, Nagy *et al.*<sup>(2)</sup> reported that a biomarker peak detected at  $m/z$  7035 was common among subtype IA<sub>1</sub> and IA<sub>2</sub>, but Dekio *et al.*<sup>(3)</sup> reported that it was characteristic for subtype IA<sub>1</sub>. 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<sup>T</sup> 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  $\pm 200$  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<sup>(1)</sup>. Calculated masses and detected masses of CsbD-like protein for each subtypes were also matched within a difference of  $\pm 200$  ppm (Fig. 2). Amino-acid sequences and calculated masses for each subtype are summarized (Table 3).

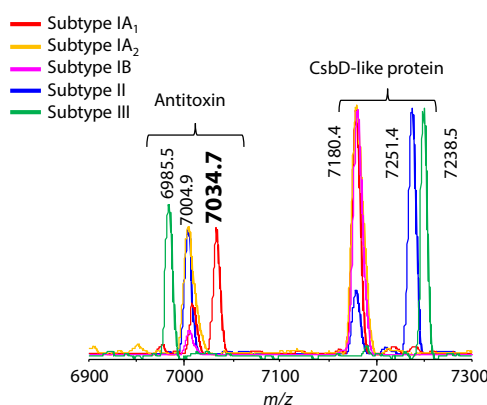


Fig. 2 MALDI mass spectra of *C. acnes* in the range of  $m/z$  6900–7300.

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

|   |
|---|
| Antitoxin (66 aa)   |
| Calculated mass: $m/z$ 7034.6 (IA <sub>1</sub> )  |
| MGLFDKAKDAISRQDDIKNQASQHSQVEQIGDKAGNI <del>V</del> DDKTGGKFSQIDKQDALKDKLGDL                       |
| Calculated mass: $m/z$ 7004.5 (IA <sub>2</sub> , IB, IC, and III)                                 |
| MGLFDKAKDAISRQDDIKNQASQHSQVEQIGDKAGN <del>V</del> DDKTGGKFSQIDKQDALKDKLGDL                        |
| Calculated mass: $m/z$ 6985.5 (II)  |
| MGLFDKAKDAISD <del>H</del> QDDIKNQASQHSQVEQIGDKAGN <del>V</del> DDKTGGKFSQIDKQDALKDKLGDL          |
| CsbD-like protein (71 aa)   |
| Calculated mass: $m/z$ 7179.9 (IA <sub>1</sub> , IA <sub>2</sub> , IB, IC and III)                |
| MGLSDKINSKSD <del>E</del> AVGAAKEKIGGLTDDSDLK <del>S</del> AGADQKASGKVAQKVEDVKDKANDLKHNVQAAADKLLG |
| Calculated mass: $m/z$ 7238.0 (III)   |
| MGLSDKINSKSD <del>E</del> AVGAAKEKIGGLTDDSDLK <del>S</del> EGADQKASGKVAQKVEDVKDKANDLKHNVQAAADKLLG |
| Calculated mass: $m/z$ 7251.0 (II, ST 42 and 46)  |
| MGLSDKINSKSD <del>E</del> AVGAAKEKIGGLTDDSDLK <del>S</del> EGANQKASGKVAQKVEDVKDKANDLKHNIQAAADKLLG |
| Calculated mass: $m/z$ 7265.0 (II, ST 43)   |
| MGLSDKINSKSD <del>E</del> AVGAAKEKIGGLTDDSDLK <del>S</del> EGANQKASGKVAQKVEDVKDKANDLKHNVQAVADKLLG |

**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<br>7035 | L6    |       | L13   |       | L15   |       | L23   |       |       |
|                 |                    | 16119              | 8755 | 6787 | 14526 | 10081 | 10380 |                   | 19679 | 19707 | 16154 | 16168 | 16182 | 15358 | 15385 | 11181 | 11200 |
| IA <sub>1</sub> | 6425 <sup>T</sup>  | 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 <sub>2</sub> | 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 <sup>T</sup>  | 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 <sup>T</sup> | 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     |

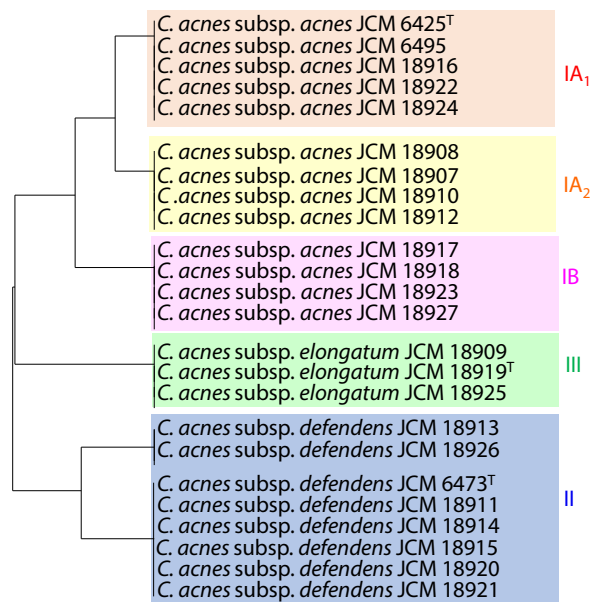
A dendrogram was generated using Strain Solution by UPGMA cluster based on the biomarker matching table (Table 4). Fig. 3 shows the resulting dendrogram of *C. acnes* strains together with their phylotypes. A total of 24 *C. acnes* strains used in this study were split into three major clusters that 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<sub>1</sub>, IA<sub>2</sub>, 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<sub>1</sub>.

This is the first report that has successfully classified the phylotypes of *C. acnes* strains as IA<sub>1</sub>, IA<sub>2</sub>, 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.

**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.

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

<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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