LAAN-C-XX-E019

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Application Note

No.34

Classification of Bacteria by MALDI-TOF MS Based on Ribosomal Protein Coding in *S10-spc-alpha* Operon at Strain Level

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LifeScience

1. Introduction

Identification of bacteria is associated with the environmental management and clinical microbiological testing in the food and pharmaceutical field, etc. The identification methods most commonly applied are morphological observation, physiological and biochemical characteristics testing, and DNA sequence analysis. Simple identification at the genus to species level is conducted by DNA sequence analysis, in which the 16S rRNA gene sequencing technique has become widely used in recent years; however, the following shortcomings of this approach underscore the need for a new microbial identification method.

1) Extraction of the DNA from the sample and determination of the DNA base sequence typically requires considerable time and effort.
2) These techniques require familiarity with gene handling, such as PCR and DNA sequencing.
3) Discrimination at the subspecies or strain level is generally difficult. Discrimination even at the species level can often be difficult depending on the species (Example: *Bacillus cereus* and *Bacillus thuringiensis*).

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MALDI-TOF MS (matrix-assisted laser desorption ionization time-of-flight mass spectrometry), a technique that has been used in recent years for the identification and classification of microorganisms (bacteria, yeast, mold), is beginning to attract more attention. Sample preparation for MALDI-TOF MS is simple, requiring only the mixing of a small amount of sample about the size of a single colony, with a very small amount of matrix solution (to assist in ionization). Microorganisms are identified by matching the mass spectrum[*3] of each species obtained in analysis with mass spectra previously recorded in the database (fingerprinting[*4]). As this technique also permits rapid analysis of multiple samples, MALDI-TOF MS is expected to overcome the weaknesses of conventional microbial identification.

Up to now, MALDI-TOF MS has primarily been used for identification of microorganisms at the species level. However, to elucidate the contamination pathways of microorganisms into food and create added value to fermented food, further development is required for applications such as the simple and rapid identification down to the subspecies – strain level for purposes that include typing. Here, we present an example and explanation of the *S10* GERMS method (*S10-spc-alpha* operon Gene Encoded Ribosomal protein Mass Spectrum), developed by the Laboratory of Environmental Microbiology, Faculty of Agriculture, Meijo University and the Japan's National Institute of Advanced Industrial Science and Technology. It is a highly precise microbial identification technique based on theoretical grounds, and is expected to satisfy these requirements.

*3 : The X-axis of the spectrum shows the mass (more precisely, the *m/z* value), and the Y-axis shows the signal intensity.
*4 : A method of identification in which mass spectral information obtained from known samples are recorded in a database as so-called fingerprints, and unknown samples are identified by matching their mass spectra with those recorded in the database.

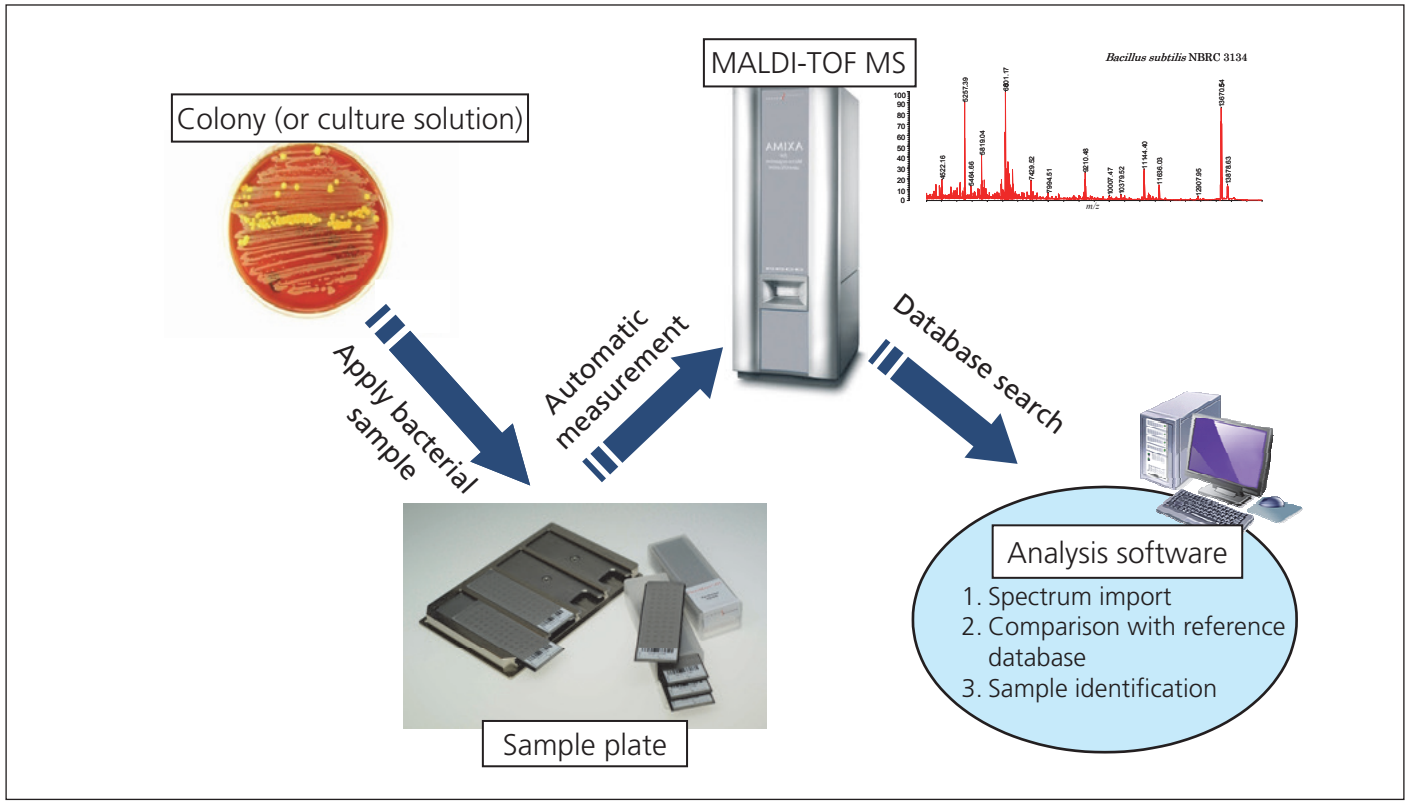

Fig. 1 General Scheme of Microbial Identification Based on MALDI-TOF MS

2. Microbial Identification Based on MALDI-TOF MS

The most common approach to microbial identification using MALDI-TOF MS relies on fingerprinting.
Fig. 1 shows the general scheme from sample preparation to microbial identification.

A major advantage of MALDI-TOF MS is that sample preparation is extremely simple. While preparation procedures may vary slightly, the flow is basically the same. The following is an example of the preparation process.

Step 1: Bacteria scraped from a single colony on agar medium are applied to a MALDI-TOF MS sample plate.

Step 2: Matrix solution consisting of sinapic acid[*5] or CHCA[*6] is added via micropipette to the bacteria that were applied to the sample plate. Bacteria and matrix solution are mixed using a micropipette, and the sample solution is dried before measurement.

MALDI-TOF MS measurement can then be conducted (Whole Cell MALDI-TOF MS: WC-MS) after completing this easy sample preparation process.
Typical examples (Escherichia coli and Bacillus subtilis) of the mass spectra obtained using samples prepared in this way are shown in Fig. 2. The amount of sample required for analysis is at the µg level, and given the number of bacteria in the example, a mass spectrum specific to each type of bacterium can be obtained with a sample that contains approximately 10^5 E. coli.

When a sample is prepared using this general method, approximately one hundred to one hundred-fifty peaks will be observed in the obtained mass spectrum, and each one of those peaks is derived from one of the proteins comprising that bacterium. Often, the amino acid sequence is different in proteins of the same type that are derived from different bacterial species. These differences in amino acid sequence result in differences in molecular weight for the protein, or in other words, with a different x-axis value (*m/z*) for the detected peak. Indeed, the mass spectral patterns differ considerably depending on the type of microorganism, as shown in Fig. 2, which clearly demonstrates the effectiveness of the fingerprinting method in identification of microorganisms.
An application system that combines the AXIMA series of Shimadzu MALDI-TOF MS instruments with software for microbial identification using a fingerprinting database of mass spectra for bacterial species is now being marketed in the United States and Japan as the Axima iD Plus Microbial Identification System.

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*5 : 4-Hydroxy-3,5-dimethoxycinnamic Acid
*6 : α-cyano-4-hydroxycinnamic acid

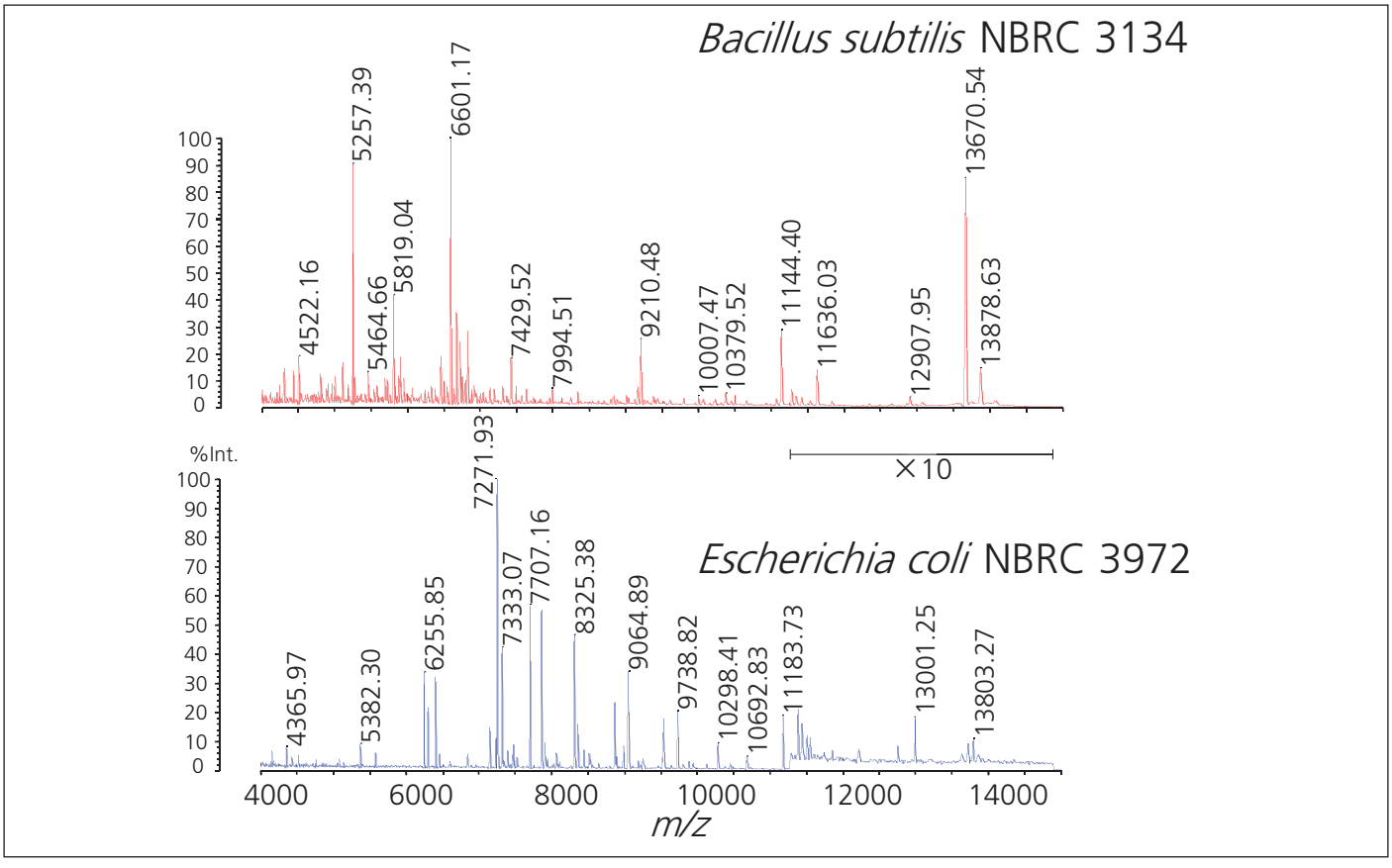

Fig. 2 Mass Spectra of Bacillus Subtilis and Escherichia Coli

3. Microbial Identification by MALDI-TOF MS Using Ribosomal Proteins as Biomarkers

Microbial identification by MALDI-TOF MS fingerprinting cannot be used to specify the protein from which each observed peak substance originates, but microbial identification using fingerprinting is not especially difficult.

When using this method,

1) There must be a theoretical basis for application to molecular phylogenetic analysis.
2) Because a microorganism's characteristic peaks appear based on differences in mass depending on the classification level, i.e., family, genus, species, and subspecies (Fig. 3), identification with good reproducibility at the species and subspecies level requires the selection of biomarker peaks with clear origins "that can be used for discrimination at the subspecies or strain level."

In addition, after having identified an observed peak component, one of the expectations of MALDI-TOF MS is that its application can be expanded to identify the subclass.

Upon identifying the protein from which a peak component observed in WC-MS is derived, efforts are now being focused on ribosomal proteins[*7]. Ribosomal proteins, from the standpoints of expression level (accounting for about 20 % of the total protein expression level in the logarithmic growth phase), isoelectric point (which include many that are basic and easy to ionize), and molecular weight range (molecular weights within the approximate range of 4000-15000 Da), are easy to observe as the principal component with MALDI-TOF MS, with more than 50 % of the peaks presumably derived from ribosomal proteins. By identifying the peaks derived from this ribosomal protein, molecular phylogenetic analysis based on theoretical rationale and the possibility of identification at the subspecies and strain level with good reproducibility can be expected with the following challenges:

1) There is little available ribosomal database information. Identification of peaks observed using WC-MS requires ribosomal protein gene sequence information, in other words, amino acid sequences. However, there are still not many bacterial strains for which genomic sequencing has been conducted.
2) There are ribosomal proteins that exhibit low peak detection sensitivity.

Below, we describe in detail the *S10*-GERMS method, newly developed to overcome the above problems,

*7 : Ribosomes are components in cells that synthesize proteins according to the genetic sequence. Ribosomal proteins are complexes of proteins that constitute the ribosome. They are composed of 54 protein subunits.

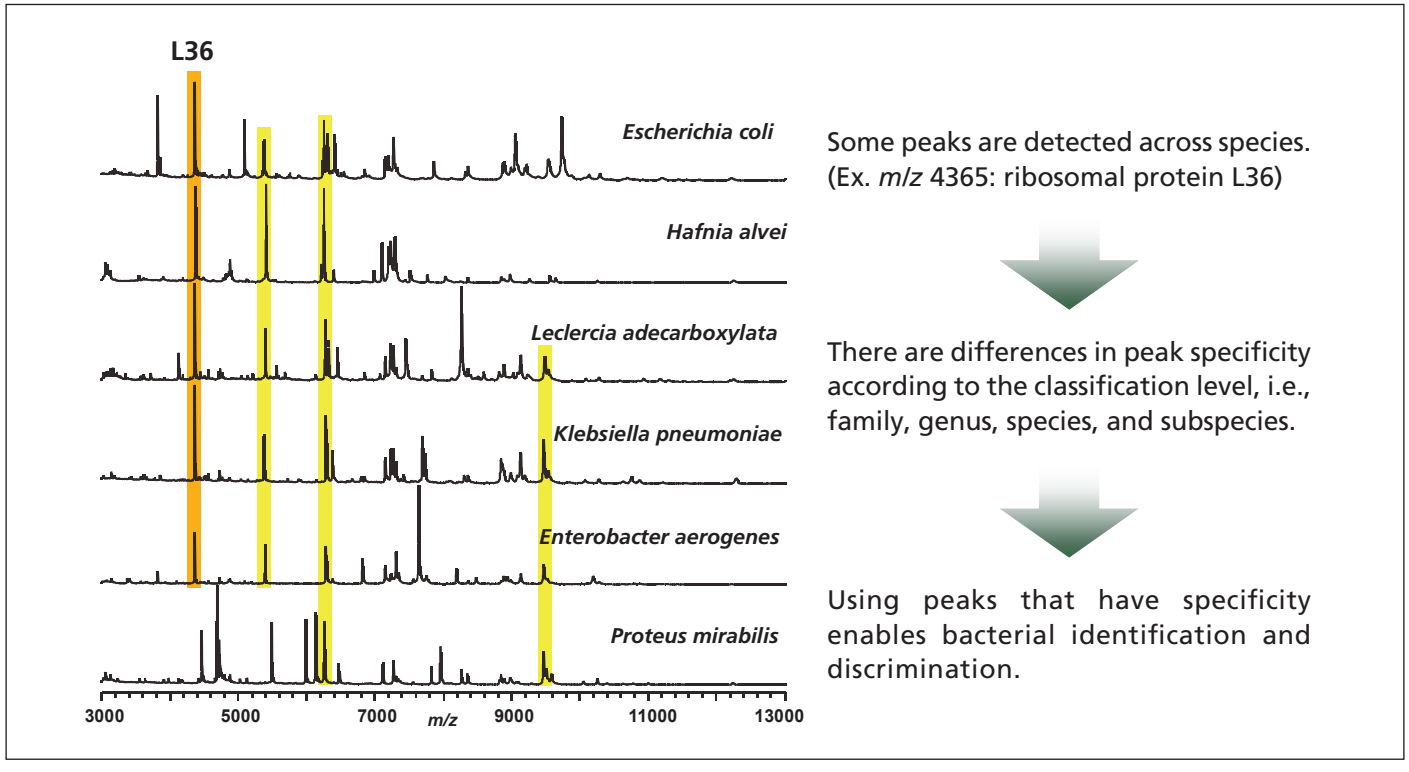

Fig. 3 Differences in Specificity Among Peaks

4. Microbial Identification by the *S10*-GERMS Method

In order to conduct analysis of the measured values based on theoretical rationale, we focused on the *S10-spc-alpha* operon[*8] which encodes bacterial ribosomal proteins. This operon, which exists commonly in bacteria, includes information on more than 25 ribosomal subunit proteins (Fig. 4).

If the DNA base sequence of this operon can be determined, the mass of the ribosomal protein coded therein can be determined, and that information can be used for identification of bacteria in conjunction with the peak obtained using WC-MS. This method, in which bacterial identification and discrimination is based on the information obtained by WC-MS together with the *S10-spc-alpha* operon genetic information, was established as the *S10*-GERMS method by Japan's National Institute of Advanced Industrial Science and Technology and Meijo University.*8

The workflow for constructing the ribosomal protein database to be used in the *S10*-GERMS method is shown below (Fig. 5).

Step 1: Measurement by MALDI-TOF MS (actual measured value) Acquire mass spectra of strains to be used for construction of the database by WC-MS. These are considered to be actual measurement values.

Step 2: Design of primers for sequencing the *S10-spc-alpha* operon Design primers based on the common nucleotide sequences of the genomic sequencing type strain *S10-spc-alpha* operon.

Step 3: Determination of *S10-spc-alpha* operon DNA base sequence and conversion to amino acid sequence Determine the DNA base sequences of the *S10-spc-alpha* operon strains for database construction, and then convert them to amino acid sequences.

Step 4: Construction of theoretical database of ribosomal proteins Calculate the theoretical mass values from the ribosomal protein amino acid sequences. Create a comprehensive database using a computer.

Step 5: Construction of accurate database using theoretical values, measured values and deciphered sequences Compare the theoretical values calculated in Step 4 with the actual measured values obtained using MALDI-TOF MS in Step 1, and exclude from the list of theoretical mass values ribosomal proteins that display low peak detection sensitivity. This step makes it possible to construct an accurate database.

Discrimination of bacterial samples becomes possible by matching the mass spectra of bacteria test samples with a database constructed in this manner. In the following section, we introduce an example in which discrimination of *Bacillus subtilis* (*B. subtilis*) was achieved at the subspecies-strain level using the *S10*-GERMS method.

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*8 : A region of the genome where multiple genes whose expression is controlled at the same time together

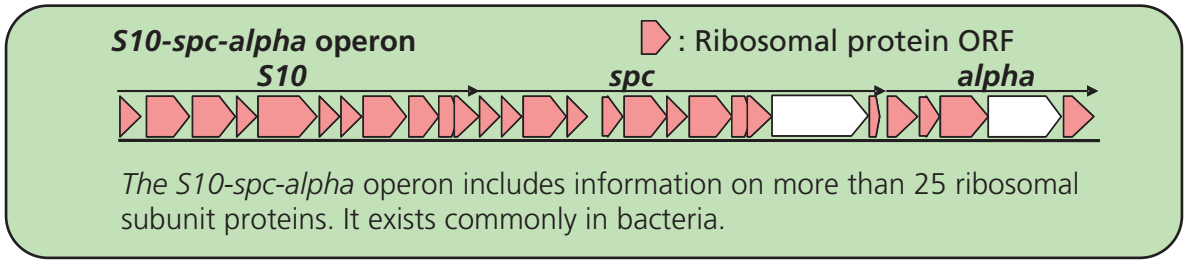
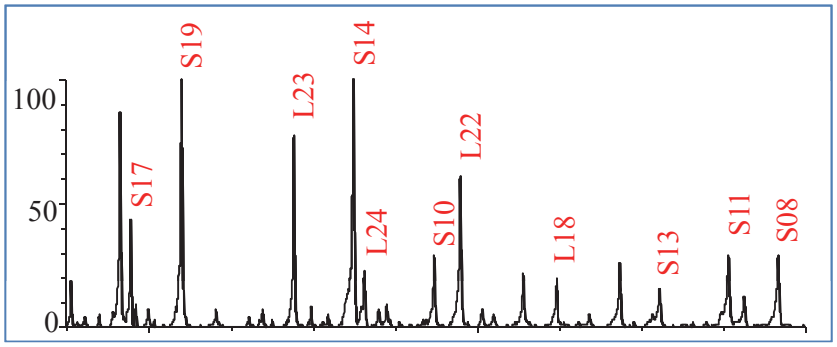


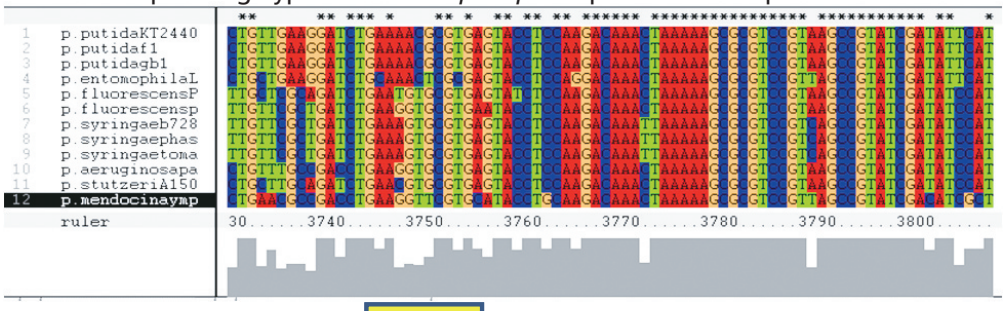
Fig. 4 What Is the S10-spc-alpha Operon?

Step 1: Measurement by MALDI-TOF MS (actual measured value)



Step 2: Design of primers for sequencing the *S10-spc-alpha* operon

Genome Sequencing Type Strain *S10-spc-alpha* Operon Base Sequence



Primer design

Primer	Sequence (5'-3')
-220f	TTCTTCAARGGCTACCGTCC
119f	CTTTTARCGGGCGTAKTSCG
615f	TTCACCGAAGAAGGTGTCTC
635r	GAGACACCTTCTTCGGTGAA
1505f	AACAAGAAGATGTAYCGCGC
1525r	GCGCGRTACATCTTCTTGTT
2310f	**AAGCAGCATTACCGTCTGGT**
2330r	ACCAGACGGTAATGCTGCTT
3265f	GAAGTAGCCGCTAAGTTGTC

<Point>
Primers are designed based on common sequences of the genomic sequencing type strain *S10-spc-alpha* operon.

Step 3: Determination of *S10-spc-alpha* operon DNA base sequence and conversion to amino acid sequence

S17 MAEAEKTVRT·····RAVEV
S19 MPRSLKKGPF ·····KKAKR
L23 MNQERVFKVL·····SSSAE
S14 MAKKSMKNRE·····VKASW
L24 MQKIRRDDEI·····KAVDA
S10 MQNQQIRIRL·····QISLG
L22 MEVAAKLSGA·····KVADK
L18 MTDKKVIRLR·····GGLEF
S13 MARIAGVNIP·····KPIRK
S11 MAKPAARPRK·····KKRRV
S08 MSMQDPLADM·····LCTVF

Fig. 5 Ribosomal Protein Database Construction — Workflow

Step 4: Construction of theoretical database of ribosomal proteins

		P. putida 14164	*P. f luorescens* 14160	*P. alcaligenes* 14159	*P. aeruginosa* 12689	*P. azotof ormans* 12693	*P. chlororaphis* 3904	*P. f ulva* 16637	*P. mendocina* 14162	*P. straminea* 16665	*P. stutzeri* 14165
S10	L22	**11912.0**	**11912.0**	11893.9	**11912.0**	**11912.0**	**11912.0**	**11912.0**	**11912.0**	**11912.0**	11897.9
	L23	*10900.7*	**10945.7**	10955.6	10950.7	**10945.7**	**10945.7**	*10900.7*	11015.7	11085.8	10920.6
	L29	**7173.3**	**7173.3**	7215.4	7202.4	**7173.3**	**7173.3**	**7173.3**	7205.4	7215.4	7274.4
	S10	**11753.6**	**11753.6**	*11783.6*	11767.6	**11753.6**	**11753.6**	**11753.6**	*11783.6*	11755.6	**11753.6**
	S17	**9902.5**	*9966.6*	9957.6	9955.6	*9966.6*	9984.6	**9902.5**	9974.6	10014.7	9973.6
	S19	**10218.1**	10246.1	10186.0	10227.1	10189.1	10204.0	**10218.1**	10176.0	10190.0	10163.0
spc	L14	**13410.9**	**13410.9**	*13396.8*	13412.9	**13410.9**	**13410.9**	**13410.9**	*13396.8*	**13410.9**	13436.9
	L18	12497.4	12556.4	12561.4	12531.4	**12512.4**	**12512.4**	12485.4	12413.3	12457.3	12477.3
	L24	**11330.2**	*11336.3*	11340.3	11471.5	*11336.3*	11345.3	**11330.2**	11344.3	11344.3	11413.4
	L30	6334.5	**6395.6**	6278.3	6347.4	**6395.6**	**6395.6**	6292.5	6363.5	6448.5	6463.6
	L36	**4435.4**	**4435.4**	4407.3	**4435.4**	**4435.4**	**4435.4**	**4435.4**	**4435.4**	**4435.4**	4421.4
	S08	13845.1	13962.3	13951.3	14040.4	13920.2	13973.3	13861.1	13928.2	13914.2	13869.1
	S14	11259.3	**11304.3**	11394.3	11435.3	**11304.3**	11274.2	11288.3	11359.2	11385.3	11326.2
alpha	S11	13529.5	13485.4	13517.5	**13513.5**	13499.4	**13513.5**	13543.5	13531.5	13493.4	13527.5
	S13	13126.3	13210.4	13177.4	13135.2	13164.5	13239.4	13140.3	13118.3	13058.3	13176.4

Step 5: Construction of accurate database using theoretical values, measured values and deciphered sequences

	P. putida NBRC 14164	*P. fulva* NBRC 16637	*P. fluorescens* NBRC 14160	*P. azotoformans* NBRC 12693	*P. chlororaphis* NBRC 3904	*P. aeruginosa* NBRC 12689	*P. mendocina* NBRC 14162	*P. straminea* NBRC 16665	*P. stutzeri* NBRC 14165	*P. alcaligenes* NBRC 14159
L22	11911.95	11911.95	11911.95	11911.95	11911.95	11911.95	11911.95	11911.95	11897.92	11893.91
L23	10900.65	10900.65	10945.74	10945.74	10945.74	10950.71	11015.74	11085.83	10920.64	10955.60
L29	7173.31	7173.31	7173.31	7173.31	7173.31	7202.35	7205.35	7215.39	7274.42	7215.44
S10	11753.58	11753.58	11753.58	11753.58	11753.58	11767.61	11783.61	11755.55	11753.58	11783.61
S17	9902.53	9902.53	9966.58	9966.58	9984.61	9955.60	9974.55	10014.66	9973.57	9957.61
S19	10218.07	10218.07	10246.12	10189.07	10204.04	10227.08	10175.98	10190.01	10162.99	10186.01
L18	12497.41	12485.36	12556.43	12512.38	12512.38	12531.43	12413.29	12457.30	12477.34	12561.35
L24	11330.24	11330.24	11336.25	11336.25	11345.26	11471.46	11344.27	11344.27	11413.37	11340.32
L30	6334.54	6292.46	6395.60	6395.60	6395.60	6347.44	6363.48	6448.54	6463.62	6278.33
L36	4435.39	4435.39	4435.39	4435.39	4435.39	4435.39	4435.39	4435.39	4421.36	4407.34
S08	13845.12	13861.12	13962.26	13920.23	13973.29	14040.42	13928.16	13914.18	13869.10	13951.25
S14	11259.27	11288.27	11304.32	11304.32	11274.24	11435.25	11359.15	11385.32	11326.23	11394.33
S11	13529.50	13529.50	13485.44	13485.44	13499.47	13499.47	13517.48	13479.39	13513.50	13503.46
S13	13126.31	13140.34	13210.43	13164.45	13239.43	13135.23	13118.33	13058.28	13176.42	13177.40

Fig. 5 (cont'd) Ribosomal Protein Database Construction — Workflow

5. Phylogenetic Analysis and Discrimination at Strain Level by *S10*-GERMS Method — Actual Example

B. subtilis is a type of resident bacteria widely present in the natural environment, including the air and soil. Because of its high heat resistance, it is widely known to be one of the microorganisms that cause bacterial contamination in food factories, etc. Fig. 6 shows examples of mass spectra of *Bacillus* bacteria type strains. Comparison of these with the data in a ribosomal protein database confirmed, with good repeatability, that the detected ribosomal protein is the same as that of Bacillus bacteria. Next, the homology of the 16S rRNA gene is 99.9 % (1473 of the 1475 bases match), with comparison of the mass spectra of *B. subtilis* subsp. *subtilis* NBRC 13719T, *B. subtilis* subsp. *Spizizenii* NBRC 101239T, and *B. subtilis* NBRC 104440 shown in Fig. 7. The differences among the masses of the ribosome subunit proteins L29, L22 and L18 peaks of the *B. subtilis* strains suggests that discrimination among these is possible at the strain level. Eight types of ribosomal proteins were singled out as biomarkers from among those encoded in the ribosomal protein *S10-spc-alpha* operon, and the results of cluster analysis[*9] for each strain of *B. subtilis* based on this information are shown in Fig. 8. The *S10*-GERMS method based on these 8 biomarkers was shown to be effective in discriminating among the variants of the B. subtilis strains at the subspecies and strain levels.

*9 : The classification technique in which similar items among a larger number of items are grouped together (clustered).

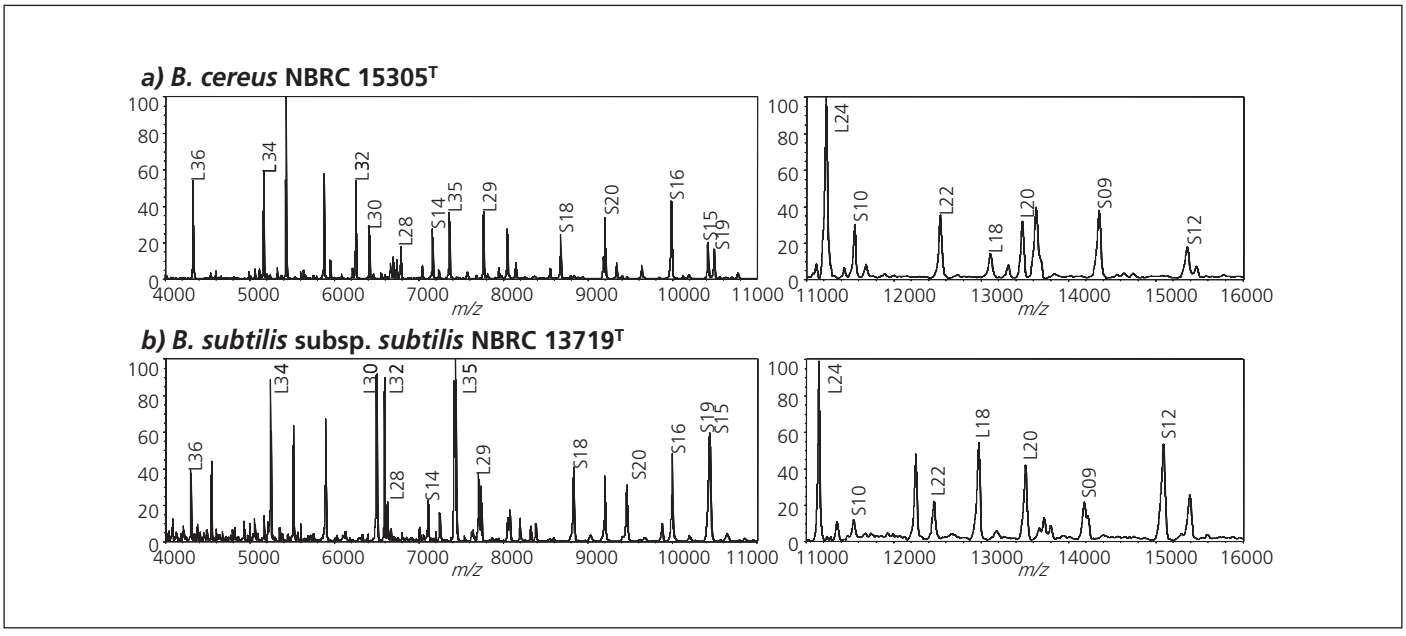
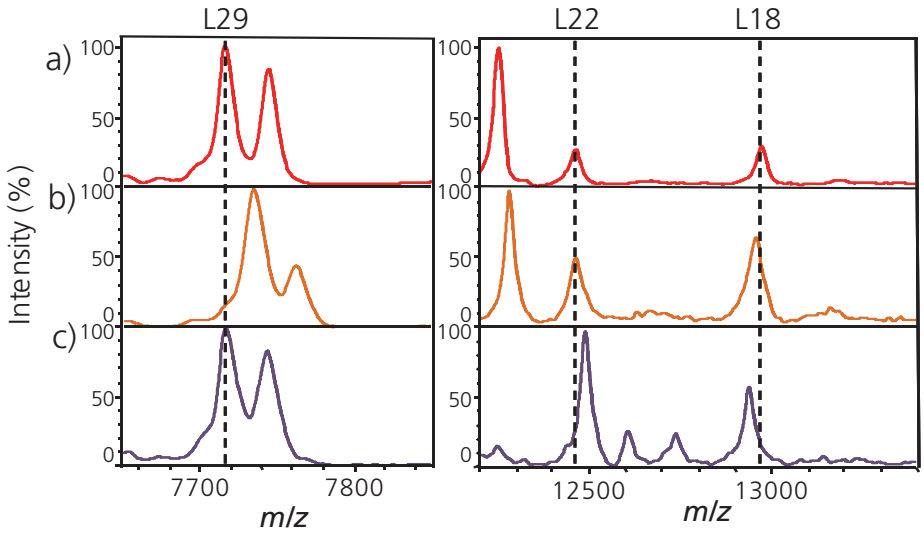


Fig. 6 MALDI Mass Spectra of *Bacillus* Type Strains



a) *B. subtilis* subsp. *subtilis* NBRC 13719^T
b) *B. subtilis* subsp. *spizizenii* NBRC 101239^T
c) *B. subtilis* NBRC 104440

Fig. 7 Comparison of Mass Spectra of *B. subtilis* Strains

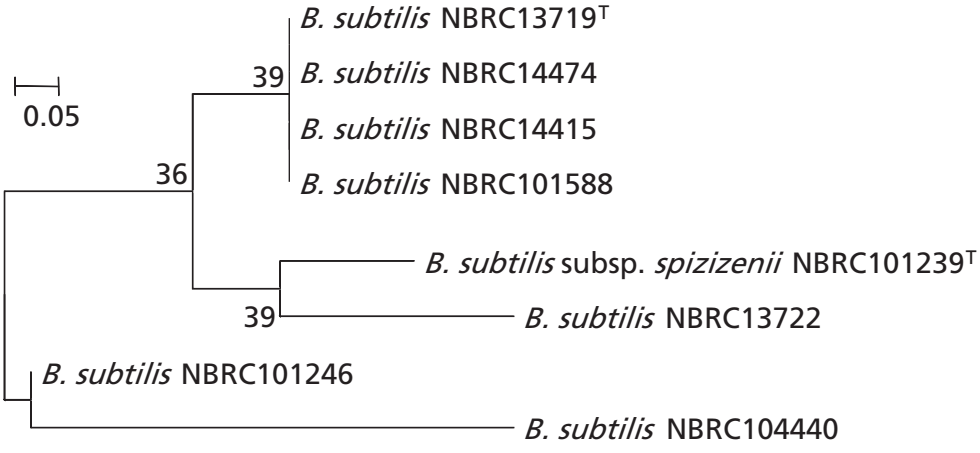


Fig. 8 Results of *B. subtilis* Cluster Analysis (by neighbor-joining method)

6. Conclusion

The advantages of conducting microbial identification by MALDI-TOF MS, namely fast and simple operation and low running costs, can be expected to shift attention to this alternative toward morphological, physiological and biochemical techniques. This in turn will advance progress in the field of clinical microbiology. On the other hand, further expectations of MALDI-TOF MS include the identification of microorganisms in addition to discrimination at the subspecies-strain level for the purpose of typing, etc. Discrimination of bacteria using MALDI-TOF MS has not reached the stature of conventional methods with respect to typing, but with the appearance of the *S10*-GERMS method based on theoretical rationale, it can be expected to occupy a position as one of the leading strain typing methods.

-Related Products-

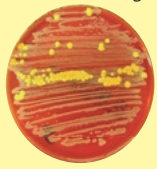
MALDI-TOF Mass Spectrometer Microorganism Identification and Classification System

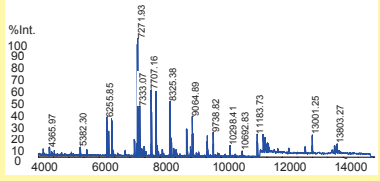
AXIMA Microorganism Identification System

Identify microorganisms quickly, easily, and reliably.

The AXIMA Microorganism Identification System identifies microorganisms (bacteria and fungi) by directly measuring them with a MALDI-TOF mass spectrometer to obtain their mass spectral data and then searching a database using the data.

- Requires only three steps—(1) sample preparation, (2) MALDI-TOF MS measurement, and (3) identification by database search. Each step is quick and easy.
- Completes analysis work within two minutes of the start of measurement.
- Enables the identification and classification of bacteria, mold, and yeast.
- Requires very few reagents and offers low running cost analysis.





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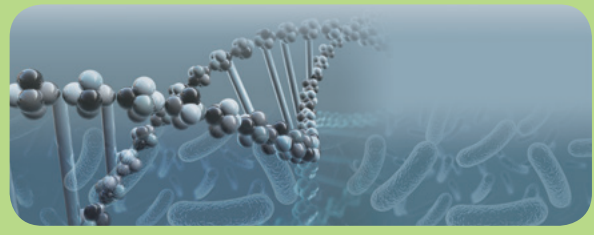
High-Precision Bacteria Characterization Software for AXIMA Microorganism Identification System

Strain Solution

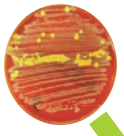
Differentiate bacteria even more accurately and reliably.

Strain Solution software uses a MALDI-TOF mass spectrometer, based on the *S10*-GERMS method, to discriminate bacteria at subspecies or strain level.
The bacteria can be characterized by preregistering markers that allow the user to differentiate the subspecies and strain of bacteria. Subsequently, these markers are matched to the MALDI-TOF MS measurement data to determine the target bacterial strain.

- Obtain more precise and reliable discrimination results than conventional microorganism identification methods using MALDI-TOF MS fingerprinting.
- Quickly characterize bacteria by genetic differences in the base sequence using MALDI-TOF MS.
- Create and customize bacterial strain databases.
- Perform molecular phylogenetic analysis using external software.



AXIMA Microorganism Identification System

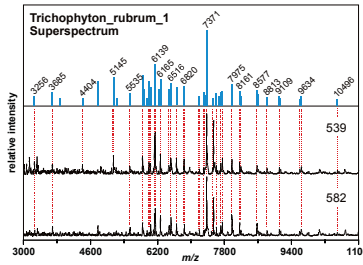


Colony
(or culture medium)

Sample plates

Database search

Export of peak list



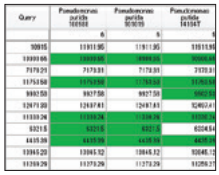
Comparison

AXIMA Microorganism Identification System Analysis Software
MS fingerprinting with a reference database (SuperSpectra)

- ✔ Bacterial strain identification
- ✔ Identification by fingerprinting method

MALDI-MS Fingerprinting Method

Strain Solution



Strain Solution
Database matching based on ribosomal proteins used as markers; cluster analysis using external software

- ✔ Rational basis for higher identification accuracy
- ✔ Molecular phylogenetic classification

***S10*-GERMSMethod**

Target sample bacterial strain

NOTE

- The software does not include marker peaks registered for samples other than the test sample. Bacterial strain marker peaks for the target bacterial species must be registered in advance.
- For Research Use Only. Not for use in diagnostic procedures.

This software was created from results obtained from an Aichi prefectural key Knowledge Hub research project on developing technologies for ensuring food safety and security (group leader: Professor Hiroto Tamura of the Department of Environmental Bioscience, Faculty of Agriculture, Meijo University), based on ideas from the Meijo University and the National Institute of Advanced Industrial Science and Technology (AIST).

[Acknowledgments]

The results in part of this Application Note are thanks to the support of the Aichi prefectural key Knowledge Hub research project on developing technologies for ensuring food safety and security.

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First Edition: April, 2014

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