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Review

Bacterial Pesticides: Mechanism of Action, Possibility of Food Contamination, and Residue Analysis Using MS

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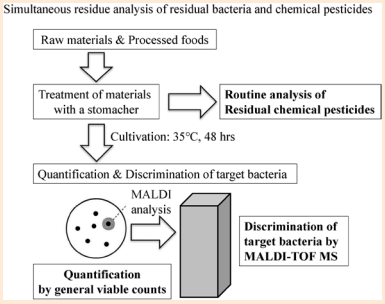
As Sustainable Development Goals (SDGs) and the realities of climate change become widely accepted around the world, the next-generation of integrated pest management will become even more important for establishing a sustainable food production system. To meet the current challenge of food security and climate change, biological control has been developed as one sustainable crop protection technology. However, most registered bacteria are ubiquitous soil-borne bacteria that are closely related to food poisoning and spoilage bacteria. Therefore, this review outlined (1) the mechanism of action of bacterial pesticides, (2) potential concerns about secondary contamination sources associated with past food contamination, and, as a prospective solution, focused on (3) principles and methods of bacterial identification, and (4) the possibility of identifying residual bacteria based on mass spectrometry.



Keywords: sustainable agriculture, biopesticide, *Bacillus*, residue analysis, MALDI-TOF MS, *S10*-GERMS.

Introduction

Of the current world population of approximately 8 billion, 800 million people are suffering from hunger. The chronic issue is how to produce food for the world's population, which is estimated to reach more than 9 billion by 2050. Meanwhile, forty percent of the global food crops are lost by pests and diseases leaving hundreds of millions of people without access to enough amount of food. Under these circumstances, The United Nations has adopted 2020 as the International Year of Plant Health to prevent the spread of plant pests and diseases and to raise global awareness.[1)]

In response to growing awareness of climate change, the Sustainable Development Goals (SDGs), which have been a major change since the Industrial Revolution, were agreed on by all member countries at the United Nations Summit in 2015. As the SDGs become widely accepted around the world, the establishment, promotion, and expansion of next-generation Integrated Pest Management will become even more important for achieving the establishment of a sustainable food production system.

This growing momentum has led to the launch of new agricultural policy initiatives aimed at reducing greenhouse gas emissions in the EU and the US in 2020, and in Japan in 2021. The EU's Farm to Fork strategy covers the entire agricultural and food chain from producer to consumer.[2)] The main goal of the Farm to Fork strategy is to support the conversion of 25% of all farmlands to organic agriculture by 2030. In the process, the overall use and potential risks of chemical pesticides will be reduced by 50% by 2030,[3)] which is closely linked to future crop protection strategies.

In the United States, the USDA Agriculture Innovation Agenda was published as a solution for farmers, consumers, and the environment in 2020.[4)] The goal set by the USDA agenda simultaneously achieves a 40% increase in agricultural production and a 50% reduction in ecological footprint by 2050, including carbon sequestration and greenhouse gases, water quality, and renewable energy.

In Japan, Measures of Achievement of Decarbonization and Resilience with Innovation (MeaDRI) was enacted in 2021.[5)] By 2050, MeaDRI's goal is to increase the proportion of organic farming by 25% and reduce the overall use of chemical pesticides by 50% at risk levels and the use of chemical fertilizers by 30%.

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This social environment places crop protection researchers increasingly responsible for sustainable food production that can meet the food production demands of the increasing global population with reducing environmental impacts more than ever before. Therefore, dramatic changes surrounding pesticide science and new initiatives in open science have encouraged the development of innovative crop protection technologies for reducing chemical pesticides, such as RNA pesticides, biopesticides, biostimulants, physical control, and pesticide delivery systems using nanoparticles which human health risk was assessed.[6,7] Biological control is defined as the suppression of populations of pests, weeds, and plant pathogens by living organisms which can reduce damage by invasive species and protect our environment by reducing the need for pesticides[8] and OECD, EU, and the USA also define the range of microbial pesticides as one of biopesticides, respectively.[9–11]

While biopesticides such as natural enemies and beneficial microbiomes have been used in classic plant protection technology, due to scientific advances, they are one of the technologies that are attracting more attention as a new direction for the future. Regarding chemical pesticides, not only their human safety but also the analysis technology for pesticide residues in crops and the environment has been established. Therefore, we can scientifically evaluate the probability and severity of adverse effects of biopesticides on human health and conduct risk management to reduce the adverse effects to a level that does not affect health caused by eating fresh and processed foods.

To accept the benefits of technological innovation in agrochemical science safely and securely, it is important to be aware of the potential risks of new technologies and to prepare solutions for them.

Most of the registered bacteria as biopesticides in IRAC and FRAC (Table 1) are ubiquitous soil-borne bacteria that are closely related to food poisoning and spoilage bacteria. Especially in food processing, the types of microorganisms originating from raw materials and the number of adhering bacteria are important indicators for quality control of potential hazards such as food poisoning and spoilage and may also have a significant impact on determining the shelf life of food products. In other words, if the types and number of bacteria adhering to food at the time of shipment from food industries can be determined, it will be able not to only provide safer and more secure food but can also reduce food loss and contribute to the Sustainable Development Goals (SDGs). Then, the thing is how we can establish the analysis method of residual bacteria.

This review outlined 1) the mechanisms of action of microorganisms, particularly bacterial pesticides, and 2) potential concerns about bacterial pesticides as a source of secondary contamination during food processing based on past cases of food contamination. As a prospective solution, this focused on 3) the principles of bacterial discrimination methods and 4) the possibility of identifying residual bacteria based on mass spectrom-

Table 1. Microbial pesticide listed in IRAC and FRAC.

Biological pesticides	Biological Group		Strain
Fungicide (FRAC 2022)	Bacteria	*Bacillus amyloliquefaciens*	QST713, FZB24, MBI600, D747, F727, AT-332
		Bacillus subtilis	AFS032321, Y1336, HAI-0404
		Gluconobacter cerinus	BC18B
		Pseudomonas chlororaphis	AFS009
		Streptomyces griseovirides	K61
		Streptomyces lydicus	WYEC108
	Fungi	*Trichoderma atroviride*	I-1237, LU132, SC1, SKT-1, 77B
		Trichoderma asperellum	T34, kd
		Trichoderma harzianum	T-22
		Trichoderma virens	G-41
		Clonostachys rosea	J1446, CR-7
		Coniothyrium minitans	CON/M/91-08
		Hanseniaspora uvarum	BC18Y
		Saccharomyces cerevisea	LAS02, DDSF623
		Talaromyces flavus	SAY-Y-94-01
Insecticide (IRAC2022)	Bacteria	*Bacillus thuringiensis*	aizawai, israelensis, kurstaki, tenebrionis
		Bacillus sphaericus	
	Fungi	*Burkholdera* spp	
		Wolbachis pipientis (Zap)	strain F52
		Beauveria bassiana	Apoka strain 97
		Metarhizium brunneun	
		Paecilomyces fumosoroseus	

etry.

The development of innovative crop protection technologies is an urgent issue, but new challenges also arise as a result. Countermeasures and/or solutions must be considered before the issues become apparent. This will become even more important in promoting and expanding new technology.

1. Mechanism of action of bacterial pesticides

Biopesticides have been used as a key technology for sustainable agriculture in EU countries as well as Japan to reduce environmental risk.[2,5] From concerns about climate change biopesticides have become more popular with the promotion of sustainable and environmentally friendly agriculture. Their active ingredient is an organism effective for controlling pests, such as antagonistic microorganisms, plant pathogenic microorganisms, insect pathogenic microorganisms, insect parasitic nematodes, and parasitic or predatory insects. Commercially available biopesticides are mainly formulated from living organisms. J. C. van Lenteren *et al.* summarized the insects and microorganisms known to date that are useful as biopesticides.[12]

Bacteria and filamentous fungi currently registered as active ingredients of microbial pesticides in the world are summarized in Table 1 based on the Insecticide Resistance Action Committee (IRAC) and Fungicide Resistance Action Committee (FRAC) by the International Resistance Action Committee, respectively.[13,14] In general, to promote pesticides safer and widely, people who engage should deepen their knowledge of how to use them, and for this purpose, it is necessary to scientifically elucidate the mechanisms of action of pesticides. Therefore, this review specifically focused on the mechanism of action of bacterial pesticides.

1.1. Bacterial insecticide

Both *Bacillus thuringiensis* and *B. sphaericus*, which exhibit insecticidal activity, are classified by IRAC as Microbial disruptors of insect midgut membranes. The mechanism of insecticidal action of entomopathogenic bacteria, *B. thuringiensis* has been well studied and the details have been becoming clearer.

In *B. thuringiensis* (Bt) during the spore-forming stage (the sporulation phase), Bt produces delta-endotoxins as pore-forming toxins, that possess toxic properties. These insecticidal crystalline proteins (Cry) are coded by a specific gene (Cry gene), which is a source of genes for the construction of transgenic plants resistant to insects.[15] Due to the diversity of the group of proteins, a database of delta-endotoxins has been constructed and is available through the *B. thuringiensis* delta-endotoxin nomenclature committee.[16]

These toxins are highly specific to their target insect of different orders: Lepidoptera, Diptera, Coleoptera, Hymenoptera, Hemiptera, Isoptera, Orthoptera, Siphonoptera, and Thisanoptera, and even mites and nematodes.[15,17]

As a representative, the insecticidal activity of *B. thuringiensis* in Lepidoptera is as follows; 1. ingestion of bacteria; 2. solubilization of the crystals; 3. activation protein; 4. binding of proteins to the receptors; 5. membrane pore formation and cell death.[17,18]

1.2. Bacterial fungicide

The fungicidal mechanisms of action of bacteria used as microbial pesticides in FRAC (Table 1) are as follows; competition, mycoparasitism, antibiosis, membrane disruption by fungicidal lipopeptides, lytic enzymes, and induced plant defense. Most of the registered bacteria in FRAC are soil-born bacteria that are recognized as plant growth-promoting rhizobacteria (PGPR) present in the rhizosphere, playing a crucial effect in plant growth promotion.

Therefore, the main target sites listed in FRAC were outlined based on the knowledge of the plant defense mechanism on the interaction between PGPR and plants. For more detail, readers are referred to the excellent reviews.[19–30]

1.2.1. Competition

This "Competition" is a classical idea. The active ingredient bacteria that are sprayed preventively on crops colonize and multiply on the crop surface (niches), aggressively monopolizing many of their habitats and nutritional sources. For this reason, even if spores of plant pathogens try to attach to their niches, the surface is already covered and protected by the active ingredient bacteria, and as a result, pathogens are unable to attach or colonize and the possibility of their infection is excluded from the crops. Those beneficial bacteria can act as a front-line defense against pathogen attacks.

1.2.2. Induced plant defense (*Fig. 1*)

Recent accumulated knowledge revealed that the defense mechanism of plants is composed of a primary defense system, named pattern-triggered immunity (PTI),[31] and a secondary defense system, named effector-triggered immunity (ETI).[32] In the PTI, the host plant receptors recognize pathogen associated molecular patterns (PAMPs), such as bacterial flagellin and fungal chitin, leading to triggering the primary immune defense system. The 22-amino acid epitope (flg22) of the N-terminal region of bacterial flagellin is conserved across a wide range of prokaryotic bacteria.[33] However, a plant's primary defense system is suppressed by pathogen effectors,[34] and the plant's secondary defense system, ETI is activated.[32,35] In the plant defense system, two resistance systems are induced by plant hormones. One is systemic acquired resistance (SAR) induced by salicylic acid (SA) whose level increases during pathogen infection.[36] The other is induced systemic resistance (ISR) which is jasmonic acid (JA)- and ethylene (ET)-dependent signal transduction pathway but salicylic acid-independent.[36] Since SAR is effective against a wide range of plant pathogens, it plays an important role as a defense mechanism to protect plants from pathogens. SAR induced by SA has an antagonistic relationship with the ABA-mediated environmental stress response,[38] and ABA suppresses upstream and downstream of SA in the SAR induction pathway.[39] Although in PGPR-activated host plants, the crosstalk between SA-dependent and JA/ET-dependent pathways has generally been considered to be antagonistic,[40] recent studies revealed that SA and JA/ET(Ethylene) signaling pathways are

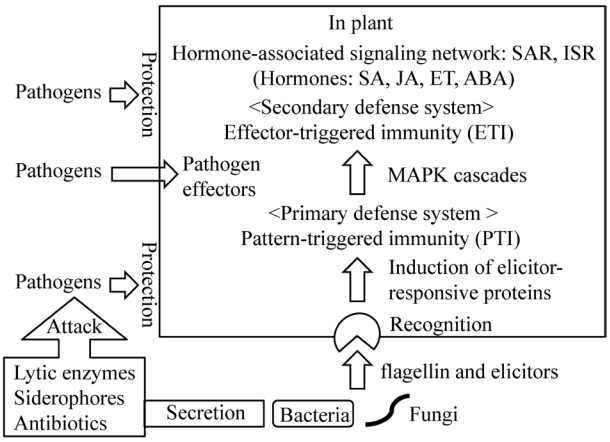

Fig. 1. Working model of induced plant defense mechanism by bacterial pesticides based on the knowledge from the relationship with rhizosphere bacteria. SA: salicylic acid; JA: jasmonic acid; ET: ethylene; ABA: abscisic acid.

activated by increasing the expression of SA and JA/ET marker genes *PR1* and *LOX2*, respectively leading to control plant diseases.[41] This suggests that PGPR simultaneously activates the SAR and ISR pathways in host plants.[42,43] In the signal transduction cascade, mitogen-activated protein kinases (MAPKs) also act as transduction of various extracellular stimuli into internal cellular responses in JA and ET pathways.[44]

Plant hormones, SA, JA, ET, and ABA induced in level through interactions with beneficial microorganisms play a vital role in making a defense system against pathogens by depending on each other leading to the protection of host plants from external enemies. Since this defense mechanism is a complicated network involving four hormones basic research on interactions between rhizosphere microorganisms and plants will pave the way for the widespread use of bacterial pesticides.

2. Potential concerns about bacterial pesticides as food contaminants

Due to the growing concerns about food safety and environmental risks associated with the use of chemical pesticides, the importance and market of biopesticides will be increased more and more.[6,7] Bacterial pesticides are assessed to pose a low risk to humans and the environment.[22,45] However, those bacteria originating from the soil environment are the same bacteria known to cause food spoilage and deterioration at the species level, implying that it is difficult to distinguish between the bacteria colonized on the crop and bacteria that cause rot and spoilage. In food processing, many treatments are carried out to reduce the number of bacteria attached to raw materials within a short period as follows; cleaning including pH changes, rapid temperature changes during heating and cooling, decreases in water activity due to drying and salting, and use of additives. However, it seems difficult to completely remove bacteria during food processing. Therefore, those bacteria become an economic hazard because they cause significant economic loss through product recalls and withdrawals of suspected food in the distribution chain as the use of ready-to-eat (RTE) foods has increased around the world. Food industries may not be completely risk-free from bacterial contamination.

Do the active ingredient bacteria in the microbial pesticides become a source of secondary contamination from raw materials during food processing? In particular, *Bacillus* spp. such as *B. amyloliquefaciens*, *B. subtilis*, and *B. cereus* group including *B. thuringiensis* form endospores. Those spores, which are stable to heat and pH, *etc.* have persisted on the crops and been detected in bread and other raw materials.[46]

While *B. subtilis* is beneficial in fermented soybean foods[47] and bacterial fungicide, those bacteria also become a source of secondary contamination originally from raw materials during food processing leading to rod and spoilage as well as *B. amyloliquefaciens*, which could be possibly misidentified as *B. subtilis* (Table 2).[48]

Another bacterial fungicide, *Pseudomonas chlororaphis* produces siderophores with low-molecular-mass molecules less than 1000 Da have high specificity and affinity for chelating effect on Fe^{3+}.[49] In general, Pseudomonads produce siderophores for their growth under iron-limiting conditions in particular to chelate Fe^{3+} and gain growth advantages.[50,51] The ability of siderophore secretion in *P. fluorescens* indicated an important role in the biofilm formation and spoilage potential.[52]

Table 2. Origin (%) of identified species, number of sample and of isolates for each food source. (F. Valerio, P. De Bellis, M. Di Biase, S. L. Lonigro, B. Giussani, A. Visconti, P. Lavermicocca and A. Sisto: Diversity of spore-forming bacteria and identification of *Bacillus amyloliquefaciens* as a species frequently associated with the ropy spoilage of bread. *Int. J. Food Microbiol.* **156**, 278–285, 2012)

	Durum wheat semolina	Other raw materials (grain, brewer's yeast, improvers)	Bread
Number of samples	69	12	12
Number of isolates	132	22	22
Species	Origin (%) of the identified species		
B. amyloliquefaciens	56.1	27.3	68.2
B. lichenifoemis	6.8	9.1	4.5
B. subtilis	2.3	4.6	9.1
B. simples	2.3	—	—
B. cereus group	18.9	9.1	18.2
B. pumilus	1.5	4.6	—
B. megaterium	—	13.6	—
B. mojavensis	0.7	4.6	—
B. oleronius	—	4.6	—
B. safensis	—	4.6	—
Others	11.4	13.7	—
H′	1.41	2.26	0.93
S	10	12	4
E	0.61	0.91	0.67

H′: Shannon diversity index; S: Richness; E: Evenness.

It's important to notice that the bacteria in microbial pesticides are the same ones at the species or genus level, that cause food spoilage and rot. This raises a new concern about food loss. To prevent this, it's vital to develop new analytical methods that can discriminate these bacteria at the strain level.

3. The principles of bacterial discrimination methods

As the increase of the potential risk of an economic hazard with the widespread of bacterial pesticides, therefore, rapid and cost-effective bacterial identification is going to become more important than ever in routine microbiology laboratories. In terms of SDGs, the establishment of a new analytical method will also bring the following benefits: decreasing food waste with maintaining resources of food supply and unnecessary recalls and decreasing the risk of starvation.

In the history of bacterial identification, over the last 20 years, 16S rRNA gene sequencing has been used widely for the identification of bacterial isolates. Due to the improvement in the accuracy of 16S rRNA gene sequencing techniques, the isolate, which shares less than 98.7–99% similarity based on 16S rRNA gene sequencing, is assigned as a novel species.[53] However, in some cases, the isolate with greater than 99% similarity of 16S rRNA gene sequencing exhibits less than the DNA–DNA hybridization value of 70%.[54,55] Therefore, the usage of the 16S rRNA gene sequencing technique is still limited from family to species and is not applicable to discrimination at the strain level. On the other hand, unique signatures for bacterial characterization were observed by mass spectrometry (MS) obtained from bacterial extracts in 1975[56] and low molecular biomarkers such as lipids were analyzed for bacterial profiling.[57] As a result of the development of a robust MS approach for the rapid and cost-effective identification of microorganisms, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis of whole cells by soft ionization is developed and successfully applied to identify not only clinically important microorganisms in diagnostic laboratories but also foodborne bacteria in food industries and institutes for health.[58–60] Furthermore, MALDI-TOF MS showed better potential to identify bacteria at the subspecies level than 16S rRNA gene sequencing whose similarity is 99–100% by using statistical coefficient and permitted the typing of microbial isolates at the strain or serovar level using discriminating peaks.[61,62] The technique most taken in the bacterial identification by MALDI-TOF MS is the fingerprint method which is the comparison of mass spectra of target isolates with those of known reference strains in a well-characterized commercially available database because the fingerprint is rather easy, rapid, high throughput and low-cost than conventional techniques.[63–68] However, one important demerit of the fingerprint method is the limitation in its discriminability at the strain or serovar level, even at the subspecies level, because the phylogenetically close bacteria by MALDI-TOF MS analysis give us very similar mass spectra.

Fortunately, the most informative masses with molecular weight range (m/z) from 4000 to 15000 observed by MALDI-TOF MS analysis are derived mostly from ribosomal and other housekeeping proteins.[69–71]

Given that their observed masses can be theoretically deduced from their corresponding amino acid sequences associated with the target genes, the masses as biomarkers give us a useful clue to develop the bioinformatics-based approach for reliable discrimination of closely related foodborne bacteria at the strain or serovar level.[71–75]

To address the solution to this challenge for the establishment and standardization of a simple and reliable discrimination method at the strain level, therefore, the *S10-spc-alpha* operon was selected for biomarker mines as the following reasons: this operon 1) encodes more than half of the bacterial ribosomal proteins, 2) is highly conserved among bacterial genomes, 3) its genome size is approximately 15–18 kb.[76,77] Moreover, the sequences of ribosomal proteins in this operon suggest that horizontal gene transfer may have played a significant role in the evolution of this operon.[76] The standardized MALDI-TOF MS method with a combination of genomics and proteomics was designated *S10*-GERMS (*S10-spc-alpha* operon Gene-Encoded Ribosomal protein Mass Spectrum) method.[78–80] The *S10*-GERMS method offers an accurate means to construct a database by comparing the experimentally observed mass-to-ion ratio (m/z) values of the selected biomarkers with their theoretically calculated m/z values and has been employed as a typing method for various taxa. Since the masses of identified peaks of ribosomal proteins are deduced based on their corresponding amino acid sequences associated with the target genes the bioinformatics-based approach has been developed for a highly reliable advanced discrimination method at strain level with a validation procedure.

Construction procedures of the working database for MALDI-TOF MS analysis are illustrated as follows (Fig. 2); briefly, first, MALDI-TOF MS analysis of the genome-sequenced strains is performed to have the observed m/z values. Second, the theoretical m/z values of ribosomal proteins in this operon are calculated by sequence data from NCBI databank for genome-sequenced strains or determination of the DNA sequence by using designed primers against the consensus DNA sequences, and then the candidate biomarkers are selected by comparison with the theoretical m/z values of each ribosomal protein *in silico*. Third, the reliable m/z values of candidate biomarkers are corrected by comparing the observed m/z values of the candidate biomarkers with their *in silico*-calculated m/z values (working database). Then, the proteotyping of an isolate is performed by the results of mass-matching profiles of the selected biomarkers using the working database. The application of the standardized *S10*-GERMS method for bacterial proteotyping is as follows; classification of genus *Pseudomonas*,[78,79] classification of genus *Bacillus*,[81] classification of genus *Sphingomonaceae*,[82] characterization of the *Lactobacillus casei* group,[83] characterization of enterohemorrhagic *Escherichia coli* at serovar level.[84,85]

Since the *S10*-GERMS method based on the combination of

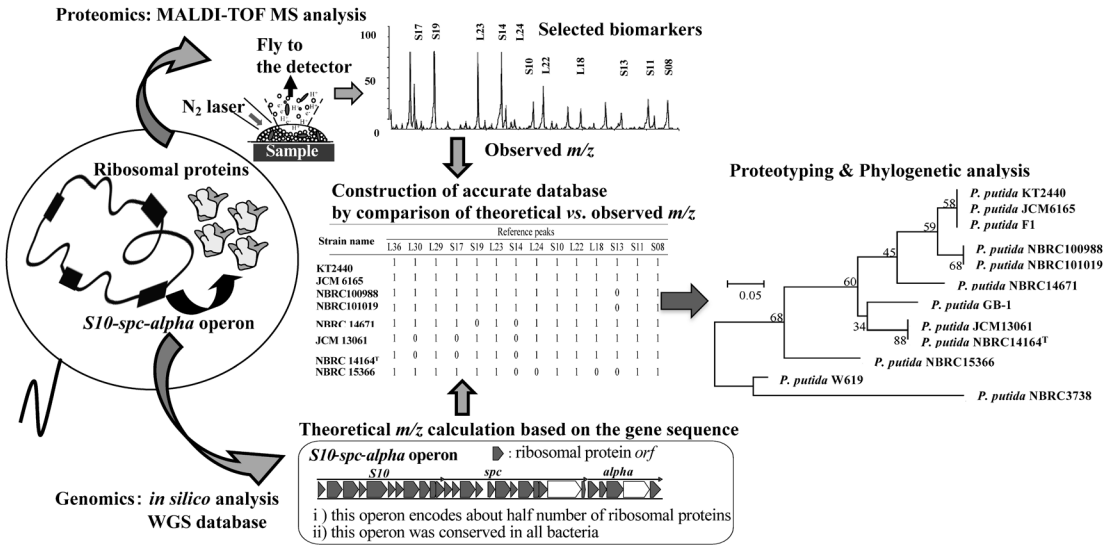

Fig. 2. Principle of the *S10*-GERMS method. Reused with permission from H. Tamura: "MALDI-TOF MS based on ribosomal protein coding in *S10-spc-alpha* operons for proteotyping" In MALDI-TOF and TANDEM MS for Clinical Microbiology, ed. by H. N. Shah and S. E. Gharbia, John Wiley & Sons, pp. 269–310, 2017 and reprinted with permission from H. Tamura, Y. Hotta and H. Sato: Novel accurate bacterial discrimination by MALDI-Time-of-Flight MS based on ribosomal proteins coding in *S10-spc-alpha* operon at strain level *S10*-GERMS. *J. Am. Soc. Mass Spectrom.* **24**, 1185–1193 (2013), Copyright 2023, American Chemical Society.

genomics with proteomics reflects different evolutionary lineages for ribosomal proteins backed by the multi-gene sequence information (Fig. 2), the proteotyping using the *S10*-GERMS method may go bacterial identification into the next generation as follows; discrimination of genetically similar bacteria that are difficult to differentiate by16S rRNA gene sequencing, phylogenetic analysis at strain and/or serotype level, identification of mixed bacterial species.

4. The possibility of identifying residual bacteria based on mass spectrometry

Despite substantial efforts of the food industry, public health, and regulatory authorities to prevent their infections and/or outbreaks, it is hard to eradicate foodborne pathogens because of their ubiquitous presence in the environment and many potential avenues for an escape to invade the human body *via* the products, particularly in ready-to-eat (RTE) foods. The fact that bacteria in the bacterial pesticides are the same bacteria at the species level that cause food spoilage enforces to establish and/or standardize their analysis method of residual bacteria in the same way as for chemical pesticides as a food safety and product liability measure.

The sustainable agriculture requires a shift away from chemical pesticides to microbial pesticides such as *B. thuringiensis* which 16S rRNA gene sequence similarity to *B. cereus* is more than 99%, suggesting it is indistinguishable from *B. cereus*.[86] Moreover, a potential risk of foodborne illness associated with *B. thuringiensis* has been reported[87–89] and the *B. cereus* group carrying toxin genes has the conflicting issues of being human pathogens and causing food spoilage.[90,91]

Additionally, although *Pseudomonas fluorescence* is the well-known beneficial rhizobacteria in ISR,[28] *P. fluorescens* has also been one of the major sources of food spoilage in a wide range of food materials such as raw fish, raw vegetables, meat, and dairy products.[92,93]

A crucial key for food safety associated with both food loss and human health risk is the establishment of rapid and accurate methods to discriminate foodborne and soilborne bacteria at strain. However, physiological and pathobiochemical tests and 16S rRNA gene sequencing techniques have still been used widely for routine bacterial identification even though those methods are limited from family to species. Moreover, although a variety of molecular typing methods have been developed as follows; pulsed-field gel electrophoresis (PFGE), multilocus sequence-based typing (MLST), ribotyping and phage-typing as well as the use of the antiserums and/or antibody,[94–96] those classical methods take up to about one week to confirm results for bacteria responsible for an infection. The development of a fully satisfied discrimination method for foodborne bacteria has become one of the most challenging and state-of-the-art aspects in those fields.

With the arrival of mass spectrometry (MS) into bacterial identification by using unique signatures for bacterial characterization obtained from bacterial extracts and low molecular biomarkers,[97,98] this robust MS approach opened a new window for bacterial identification and has been paid much attention as the promising detection methods of microorganisms.

The fingerprint method for bacterial identification by MALDI-TOF MS was authorized by the US FDA in 2013 and has been burgeoning especially in routine clinical microbiology laboratories. However, the discrimination power of the fingerprint method is of growing concern owing to the inadequacy of

(a) Binary peak matching profile of *B. subtilis* strains.

Strains	Reference peaks																			
NBRC No.	L36	L34	**L30***	L32	L28	**S14***	L35	**L29***	S18	S20	S16	S15	**S19***	**L24***	**S10***	**L22***	**L18***	L20	S09	S12
13719^T	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
14415	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
14474	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
101588	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
101246	1	1	1	1	1	1	1	1	0	0	1	0	1	1	1	1	0	1	1	0
13722	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	0	1	1	1	1
101239	1	1	1	1	1	1	1	0	1	1	1	0	1	1	1	1	0	0	1	1
104440	1	0	1	1	1	1	1	1	0	0	0	1	0	1	1	0	0	0	1	0

*:selected 8 biomarkers

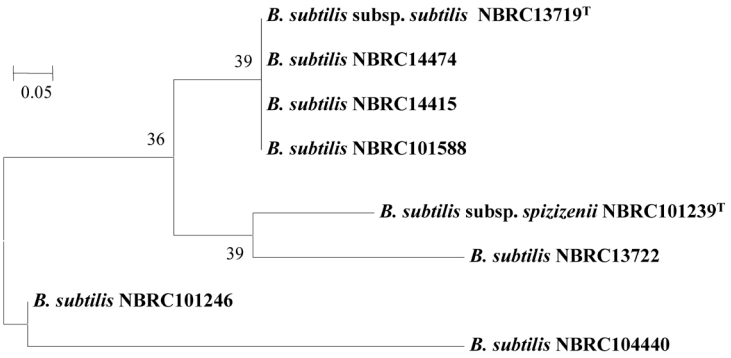

Fig. 3. Phylogenetic trees of *B. subtilis* strains based on the proteotyping using their ribosomal proteins. *B. subtilis* ssp. *subtilis* NBRC 13719^T was used as a reference strain. Reused with permission from Y. Hotta, J. Sato, H. Sato, A. Hosoda and H. Tamura: Classification of the genus *Bacillus* based on MALDI-TOF MS analysis of ribosomal proteins coded in *S10* and *spc* operons. *J. Agric. Food. Chem.* **59**, 5222–5230 (2011), Copyright 2023, American Chemical Society.

the methodological attribute of the conventional fingerprint approach for the typing of foodborne bacteria. Thus, proteotyping using the *S10*-GERMS method had a formidable challenge to overcome the limitations of MALDI-TOF MS fingerprint analysis.

In the genus *Bacillus*,[81] the eight ribosomal subunit proteins, *i.e.*, L18, L22, L24, L29, L30, S10, S14, and S19, were selected as reliable and reproducible biomarkers for rapid bacterial classification of *Bacillus* strains by the *S10*-GERMS method. Moreover, in *B. subtilis* there are three subspecies as follows: *B. subtilis* subsp. *subtilis*, *B. subtilis* subsp. *spizizenii*, and *B. subtilis* subsp. *inaquosorum*.[99,100] However, the 16S rRNA gene sequence identity between *B. subtilis* subsp. *subtilis* NBRC 13719^T and *B. subtilis* subsp. *spizizenii* NBRC 101239^T is 99.8% (1473/1475 bases), which shows 63% and 67% of DNA–DNA relatedness value.[99] Although this two-base difference in the 16S rRNA gene sequence makes it difficult to discriminate *B. subtilus* at the subspecies level, in the *S10*-GERMS method, the difference in the masses of eight biomarkers indicated the ability of discriminating *B. subtilis* at the subspecies level, according to the binary peak matching profile (Fig. 3).[81]

Since the eight ribosomal subunit proteins had a particularly limited role in the discrimination of psychrotolerant species of the *B. cereus* group Takahashi *et al.*, focused on finding new biomarkers to discriminate the *B. cereus* group, including psychrotolerant species.[101] Among the theoretically calculated masses of ribosomal subunit proteins based on actual sequencing data, the set of four biomarkers (*i.e.*, S10, S16, S20, and L30), of which S16 and S20 were present outside the *S10* and *spc* operons, were selected as promising biomarkers to discriminate psychrotolerant species, *e.g.*, *B. mycoides* and *B. weihenstephanensis*, of the *B. cereus* group after evaluation of the MALDI-TOF MS analysis. Moreover, the three selected biomarkers, S16, S20, and L30, also worked well in discriminating the psychrotolerant strains such

Table 3. The mass information (*m*/*z*) of selected biomakers for discrimination of *B. cereus* group. Reused with permission from N. Takahashi, S. Nagai, T. Tomimatsu, A. Saito, N. Kaneta, Y. Tsujimoto and H. Tamura: Simultaneous discrimination of cereulide-producing *Bacillus cereus* and psychrotolerant *Bacillus cereus* group by MALDI-TOF MS, *J. Food Protect.* **85**, 1192–1202 (2022)

Biomarker	*B. cereus*		*B. thuringiensis*	*B. mycoides*	*B. weihenstephanensis**
	Non cereulide-producing	Cereulide-producing			
Cereulide	a	1191.8	—	—	—
L30	6425.6	6425.6	6439.6	6425.6	6425.6
S16	9987.6	9987.6	9987.6	9972.6	9987.6
S20	9211.6	9211.6	9227.6	9271.6	9271.6

*: Synonym of *B. mycoides*. a: none.

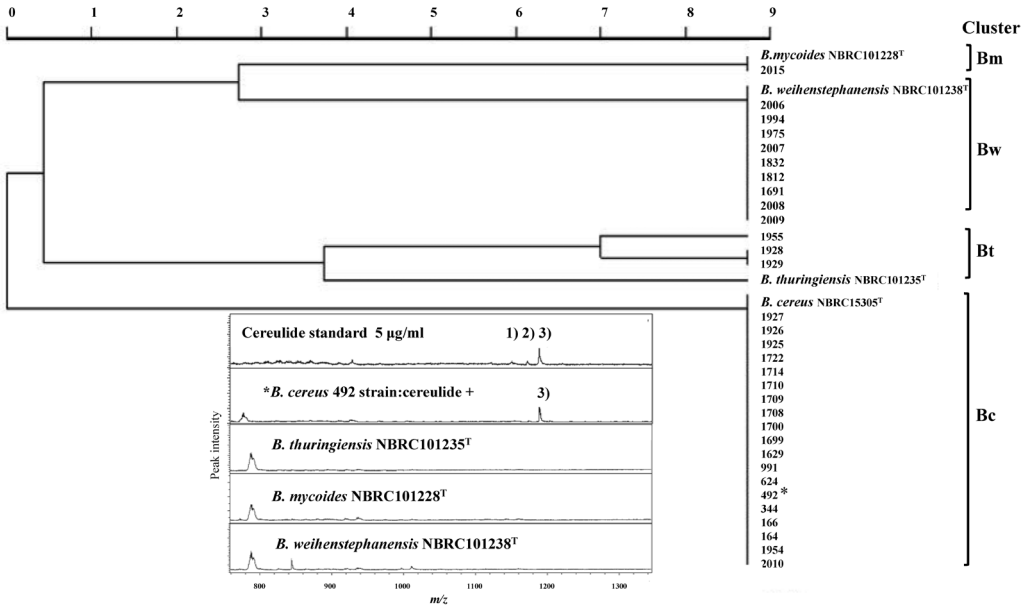

Fig. 4. Phylogenetic tree of *B. cereus group* strains based on proteotyping using their selected biomarkers and simultaneous representative detection of cereulide. Bm: *B. mycoides*; Bw: *B. weihenstephanensis*; Bt: *B. thuringiensis*; Bc: *B. cereus*. *: cereulide-producing *B. cereus*. Observed *m*/*z* of cereulides as follows; 1) $[M+H]^+=1153$; 2) $[M+Na]^+=1174$; 3) $[M+K]^+=1190$. Reused with permission from N. Takahashi, S. Nagai, T. Tomimatsu, A. Saito, N. Kaneta, Y. Tsujimoto and H. Tamura: Simultaneous discrimination of cereulide-producing *Bacillus cereus* and psychrotolerant *Bacillus cereus* group by MALDI-TOF MS, *J. Food Protect*. **85**, 1192–1202 (2022) and H. Tamura: "A MALDI-TOF MS Proteotyping Approach for Environmental, Agricultural and Food Microbiology" In Microbiological Identification using MALDI-TOF and Tandem Mass Spectrometry: Industrial and Environmental Applications, ed. by H. N. Shah, S. E. Gharbia, A. J. Shah, E. Y. Tranfield and K. C. Tom, John Wiley & Sons, pp. 147–182, 2023.

as *B. weihenstephanensis* in the *B. cereus* group. In addition, those biomarkers also showed the possibility for identification of *B. thuringiensis* from the *B. cereus* group, suggesting that by narrowing down the target bacteria, they can be discriminated more specifically by a few characteristic biomarkers (Table 3).[102] Manzulli *et al.* analyzed the *B. cereus* group using the species characteristic ion peaks, which were selected by the statistical method processed in the ClinPro Tools software.[103] They identified the specific signals as follows: 4637, 7324, and 9272 Da for *B. weihenstephanensis*; 5422 Da for *B. mycoides*; and 2956, 2968, and 3411 Da for *B. thuringiensis*. In comparison with the biomarkers selected by the *S10*-GERMS method, 9272 Da for *B. weihenstephanensis*, which was selected by the statistical method using ClinPro Tools software, may be assigned to the S20 ribosomal subunit protein. Theoretically, *B. weihenstephanensis* and *B. mycoides* have identical mass values of S20.[102] Therefore, caution is required in using characteristic ion peaks selected by the statistical method for the identification of microorganisms by MALDI-TOF MS analysis.

The emetic *B. cereus* group produces their toxin cereulide synthesized by a non-ribosomal enzyme encoded by the *ces* gene, which is a cyclic dodecadepsipeptide composed of three repeats of tetrapeptide units, D-Oxy-Leu-D-Ala-l-Oxy-Val-L-Val amino acids sequence, and acts as a potassium ionophore.[104,105] Recently, 18 cereulide variants were identified and have different toxicities in severity.[105] Therefore, while many advanced detection methods of the *B. cereus* group and its toxins have been developed,[106] the discrimination of a pathogenic strain from other non-pathogenic strains in the *B. cereus* group still needs a reliable, time-saving method for *in situ* analysis in clinical and food safety microbial laboratories. MALDI-TOF MS analysis was used to detect cereulide, and the peaks of its sodium (*m*/*z* 1174) and potassium (*m*/*z* 1187) adducts were observed.[107] Moreover, taking up the challenge of simultaneous detection of cereulide and a cereulide-producing strain in the *B. cereus* group,[108] bac-

a) Comparison of the 16S rRNA gene sequence similarities of *P. putida* at strain level

P. putida strains	NBRC 14671	NBRC 15366	NBRC 100988	JCM 13061
NBRC 14671	-	99.5 [a]	99.6	99.5
NBRC 15366	9/14 [b]	-	99.7	99.0
NBRC 100988	11/14	10/14	-	99.2
JCM 13061	11/14	8/14	10/14	-

a: values obtained by 16S rRNA sequencing similarities.
b: the top and bottom of fractions show the number of identical biomarkers and the number of total biomarkers, respectively.

b) Phylogenetic tree of *P. putida* based on proteotyping using their selected biomarkers

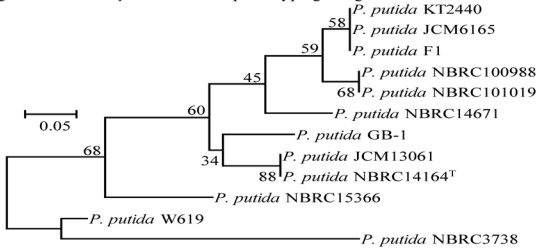

Fig. 5. The discrimination of *P. putida* isolates at strain level. a) Comparison of the 16S rRNA gene sequence similarities of *P. putida* at strain level; b) Phylogenetic tree of *P. putida* based on proteotyping using their selected biomarkers.

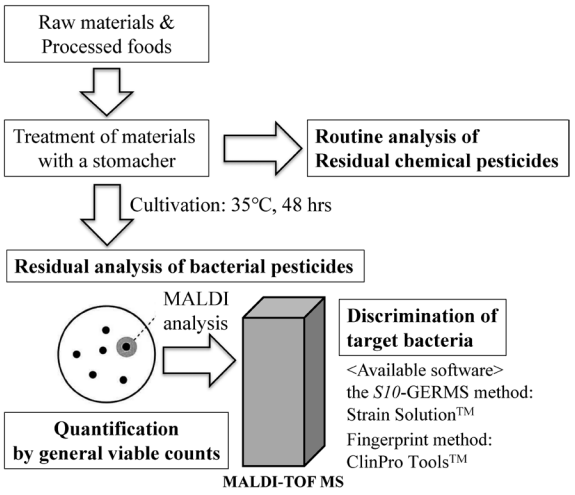

Fig. 6. Methodological perspective on simultaneous residue analysis of residual bacteria and chemical pesticides.

terial species were first identified by comparing the observed MALDI-TOF MS spectra with the conventional spectral database; if the sample was identified as *B. cereus*, the sample fraction was further analyzed by MALDI-TOF MS using the linear mode in the *m*/*z* region between 700 and 2000 Da. The *S10*-GERMS method combining cereulide detection and the selected biomarkers allowed a simultaneous discrimination of cereulide-producing *B. cereus* group strains from other non-cereulide-producing *B. cereus* group strains using bacterial strains cultured on standard agar plates,[102] suggesting that this simple, rapid, reliable, and one-step MALDI-TOF MS analysis thus has major potential as a valuable tool for ensuring human health and food safety (Fig. 4).[102,114]

In the genus of *Pseudomonas*, the *S10*-GERMS method successfully identified the following 10 species by using 14 ribosomal proteins (L18, L22, L23, L24, L29, L30, L36, S08, S10, S11, S13, S14, S17, S19) as biomarkers; *P. chlororaphis*, *P. fluorescens*, *P. putida*, *P. fulva*, *P. azotoformans*, *P. aeruginosa*, *P. mendocina*, *P. straminea*, *P. stutzeri* and *P. alcaligenes*. Furthermore, these 14 biomarkers were also effective in the discrimination of *P. putida* isolates at strain level (Fig. 5).[78] In addition, the 16 biomarkers with the addition of new biomarkers S12 and S16 could also discriminate *P. syringae* at the pathovar level.[79]

Therefore, the combination of bacterial quantification by the general viable bacterial count method with proteotyping of each colony by the *S10*-GERMS method is a promising method for identifying and quantifying bacterial residues similar to chemical pesticide residues (Fig. 6).

Conclusions and perspectives

In the environment, there are at least two types of bacteria. One is beneficial endophytic and rhizospheric bacteria to promote plant health, which is applied to improve plant nutrition and promote plant hormone production for ISR. Bacterial pesticides play a role in inducing ISR. The other is bacteria that threaten human health and food safety. In terms of SDGs, sustainable agriculture prefers biopesticides rather than chemical pesticides. With the increasing use of bacterial pesticides, the hazardous potential as foodborne bacteria such as foodborne illness and/or spoilage will increase because foodborne bacteria can use plants as alternative hosts temporarily. SDG12.3 sets a clear goal of halving global food loss by 2030, meaning that food loss must be reduced at all stages, from growers to consumers. The food loss is also closely related to SDG goals 1, 6, 13, 15. The bacteria involved in these contradictory actions are the same species that are autochthonous in the soil environment. The development of innovative crop protection technologies is an urgent issue, but it will also bring new challenges. It will become even more important to consider solutions to the potent issues before they become evident, to promote and expand the use of new technology. Shouldn't we standardize the analysis of residual bacteria for this purpose?

Biomarker-assignment approaches using MALDI-TOF MS proteotyping analysis play a pivotal role in the discrimination of microorganisms. The proteotyping method by a reliable MS technique may contribute to the development of a desired discrimination method for bacteria. However, the immediate need is to find effective biomarkers to construct accurate biomarker databases. Two methods can help us to meet this need: one is a statistical method using principal component analysis to determine useful biomarkers from characteristic ion peaks in observed mass spectra, *e.g.*, ClinProTools software[109]; and the other is the *S10*-GERMS method which requires no statistical analysis because the selected biomarkers have a genetically theoretical background. Although the big difference between the two methods is whether biomarkers have a theoretical background or not, the methods will contribute to rapid and simple identification of closely related bacteria with the following impacts: (i) appropriate risk management of public health and food hygiene by rapid and simple proteotyping; (ii) decreasing food waste in association with maintaining resources of food supply and avoiding unnecessary recalls, and reducing the risk of starvation; and (iii) microbiome research for clinical, agricultural, and environmental microbiology. Currently, the information of web-accessible whole genome sequence (WGS) database has proved effective for *in silico* prediction of foodborne pathogens at the serovar level[96,110,111] and is becoming the gold standard not only for bacterial discrimination but also for microbiome research in humans, animals, plants, and soil.[112,113] The artificial intelligence (AI) approach combined with WGS will also make possible the creation of a sophisticated method for establishing a database constructed with genetically theoretical target-specific biomarkers, leading to proteotyping of bacterial isolates at species, strain, and serovar levels. This advanced MALDI-TOF MS proteotyping method using specific biomarkers in combination with the selection medium for the target microorganism (Fig. 6) will pave the way for the transformation of the MALDI analysis from a qualitative identification method to one of the possible candidates for quantitative identification of microorganisms in the near future.

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References

1) https://www.maff.go.jp/j/syouan/syokubo/keneki/iyph/attach/pdf/iyph-1.pdf (Accessed 13 Nov. 2023).
2) https://ec.europa.eu/food/farm2fork_en (Accessed 13 Nov. 2023).
3) https://food.ec.eiropa.eu/plants/pesticides/sustainable-use-pesticides/farm-fork-target-progress-en (Accessed 13 Nov. 2023).
4) https://www.usda.gov/aia (Accessed 13 Nov. 2023).
5) https://www.maff.go.jp/e/policies/env/env_policy/attach/pdf/meadri_s.pdf (Accessed 13 Nov. 2023).
6) D. Šunjka and S. Mechora: An alternative source of biopesticides and improvement in their formulation-Recent advances. *Plants* **11**, 3172 (2022).
7) M. Kah, L. J. Johnston, R. S. Kookana, W. Bruce, A. Haase, V. Ritz, J. Dinglasan, S. Doak, H. Garelick and V. Gubala: Comprehensive framework for human health risk assessment of nanopesticides. *Nat. Nanotechnol.* **16**, 955–964 (2021).
8) G. E. Heimpel and N. Mills: Book summary. In "Biological Control—Ecology and Applications," Cambridge University Press, Cambridge, 2017.
9) OECD: OECD SERIES ON PESTICIDES Number 18 Guidance for Registration Requirements for Microbial Pesticides, 2003.
10) EU: REGULATION (EC) No 1107/2009.
11) US EPA: OPPTS 885.0001 Overview for Microbial Pest Control Agents.
12) J. C. van Lenteren, K. Bolckmans, J. Köhl, W. J. Ravensberg and A. Urbaneja: Biological control using invertebrates and microorganisms: Plenty of new opportunities. *BioControl* **63**, 39–59 (2018).
13) IRAC: https://irac-online.org/documents/moa-structures-poster-english/?ext=pdf (Accessed 13 Nov. 2023).
14) FRAC: https://www.frac.info/docs/default-source/publications/frac-code-list/frac-code-list-2022--final.pdf?sfvrsn=b6024e9a_2 (Accessed 13 Nov. 2023).
15) A. Bravo, S. S. Gill and M. Soberón: Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. *Toxicon* **49**, 423–435 (2007).
16) http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/ (Accessed 13 Nov. 2023).
17) R. Schünemann, N. Knaak and L. M. Fiuza: Mode of action and specificity of *Bacillus thuringiensis* toxins in the control of caterpillars and stink bugs in soybean culture. *ISRN Microbiol.* **2014**, 135675 (2014).
18) R. A. de Maagd, A. Bravo and N. Crickmore: How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world. *Trends Genet.* **17**, 193–199 (2001).
19) E. O. Fenibo, G. N. Ijoma and T. Matambo: Biopesticides in sustainable agriculture: A critical sustainable development driver governed by green chemistry principles. *Front. Sustain. Food Syst.* **5**, 619058 (2021).
20) S. Vero, G. Garmendia, E. Allori, J. M. Sanz, M. Gonda, T. Alconada, I. Cavello, J. R. Dib, M. A. Diaz, C. Nally, R. S. Pimenta, J. F. M. da Silva, M. Vargas, F. Zaccari and M. Wisniewski: Microbial biopesticides: Diversity, scope, and mechanisms involved in plant disease control. *Diversity (Basel)* **15**, 457 (2023).
21) M. Chandrasekaran, M. Paramasivan and J. J. Sahayarayan: Microbial volatile organic compounds: An alternative for chemical fertilizers in sustainable agriculture development. *Microorganisms* **11**, 42 (2023).
22) J. Köhl, R. Kolnaar and W. J. Ravensberg: Mode of action of microbial biological control agents against plant diseases: Relevance beyond efficacy. *Front. Plant Sci.* **10**, 845 (2019).
23) S. Compant, B. Duffy, J. Nowak, C. Clément and E. A. Barka: Use of plant growth-promoting bacteria for biocontrol of plant diseases: Principles, mechanisms of action, and future prospects. *Appl. Environ. Microbiol.* **71**, 4951–4959 (2005).
24) B. Mauch-Mani, I. Baccelli, E. Luna and V. Flors: Defense priming: An adaptive part of induced resistance. *Annu. Rev. Plant Biol.* **68**, 485–512 (2017).
25) U. Conrath, G. J. M. Beckers, C. J. G. Langenbach and M. R. Jaskiewicz: Priming for enhanced defense. *Annu. Rev. Phytopathol.* **53**, 97–119 (2015).
26) M. I. Mhlongo, L. A. Piater, N. E. Madala, N. Labuschagne and I. A. Dubery: The Chemistry of plant–microbe interactions in the rhizosphere and the potential for metabolomics to reveal signaling related to defense priming and induced systemic resistance. *Front. Plant Sci.* **9**, 112 (2018).
27) B. Lugtenberg and F. Kamilova: Plant-growth-promoting rhizobacteria. *Annu. Rev. Microbiol.* **63**, 541–556 (2009).
28) C. M. J. Pieterse, C. Zamioudis, R. L. Berendsen, D. M. Weller, S. C. M. Van Wees and P. A. H. M. Bakker: Induced systemic resistance by beneficial microbes. *Annu. Rev. Phytopathol.* **52**, 347–375 (2014).
29) L. Wiesel, A. C. Newton, I. Elliott, D. Booty, E. M. Gilroy, P. R. J. Birch and I. Hein: Molecular effects of resistance elicitors from biological origin and their potential for crop protection. *Front. Plant Sci.* **5**, 655 (2014).
30) Y. Yu, Y. Gui, Z. Li, C. Jiang, J. Guo and D. Niu: Induced systemic resistance for improving plant immunity by beneficial microbes. *Plants* **11**, 386 (2022).
31) J. Bigeard, J. Colcombet and H. Hirt: Signaling mechanisms in pattern-triggered immunity (PTI). *Mol. Plant* **8**, 521–539 (2015).
32) J. D. G. Jones and J. L. Dangl: The plant immune system. *Nature* **444**, 323–329 (2006).
33) Y. Deng, H. Chen, C. Li, J. Xu, Q. Qi, Y. Xu, Y. Zhu, J. Zheng, D. Peng, L. Ruan and M. Sun: Endophyte *Bacillus subtilis* evade plant defense by producing lantibiotic subtilomycin to mask self-produced flagellin. *Commun. Biol.* **2**, 368 (2019).
34) M. Guo, F. Tian, Y. Wamboldt and J. R. Alfano: The majority of the type III effector inventory of *Pseudomonas syringae* pv. *tomato* DC3000 can suppress plant immunity. *Mol. Plant Microbe Interact.* **22**, 1069–1080 (2009).
35) M. Yuan, Z. Jiang, G. Bi, K. Nomura, M. Liu, Y. Wang, B. Cai, J.-M. Zhou, S. Y. He and X.-F. Xin: Pattern-recognition receptors are required for NLR-mediated plant immunity. *Nature* **592**, 105–109 (2021).
36) Z. Q. Fu and X. N. Dong: Systemic acquired resistance: Turning local infection into global defense. *Annu. Rev. Plant Biol.* **64**, 839–863 (2013).
37) C. M. Pieterse, S. C. van Wees, E. Hoffland, J. A. van Pelt and L. C. van Loon: Systemic resistance in *Arabidopsis* induced by biocontrol bacteria is independent of salicylic acid accumulation and pathogenesis-related gene expression. *Plant Cell* **8**, 1225–1237 (1996).
38) M. Yasuda, A. Ishikawa, Y. Jikumaru, M. Seki, T. Umezawa, T. Asami, A. Maruyama-Nakashita, T. Kudo, K. Shiozaki, S. Yoshida and H. Nakashita: Antagonistic interaction between systemic acquired resistance and the abscisic acid-mediated abiotic stress response in Arabidopsis. *Plant Cell* **20**, 1678–1692 (2008).

39) M. Kusajima, M. Yasuda, A. Kawashima, H. Nojiri, H. Yamane, M. Nakajima, K. Akutsu and H. Nakashita: Suppressive effect of abscisic acid on systemic acquired resistance in tobacco plants. *J. Gen. Plant Pathol.* **76**, 161–167 (2010).
40) N. Li, X. Han, D. Feng, D. Yuan and L. J. Huang: Signaling crosstalk between salicylic acid and ethylene/jasmonate in plant defense: Do we understand what they are whispering? *Int. J. Mol. Sci.* **20**, 671 (2019).
41) M. Yuan, Y. Huang, W. Ge, Z. Jia, S. Song, L. Zhang and Y. Huang: Involvement of jasmonic acid, ethylene and salicylic acid signaling pathways behind the systemic resistance induced by *Trichoderma longibrachiatum* H9 in cucumber. *BMC Genomics* **20**, 144 (2019).
42) S. C. M. van Wees, E. A. M. de Swart, J. A. van Pelt, L. C. van Loon and C. M. J. Pieterse: Enhancement of induced disease resistance by simultaneous activation of salicylate- and jasmonate-dependent defense pathways in *Arabidopsis thaliana. Proc. Natl. Acad. Sci. U.S.A.* **97**, 8711–8716 (2000).
43) D. D. Niu, H. X. Liu, C. H. Jiang, Y. P. Wang, Q. Y. Wang, H. L. Jin and J. H. Guo: The plant growth-promoting rhizobacterium *Bacillus cereus* AR156 induces systemic resistance in *Arabidopsis thaliana* by simultaneously activating salicylate- and jasmonate/ethylee- dependent signaling pathways. *Mol. Plant Microbe Interact.* **24**, 533–542 (2011).
44) A. Schweighofer and I. Meskiene: Regulation of stress hormones jasmonates and ethylene by MAPK pathways in plants. *Mol. Biosyst.* **4**, 799–803 (2008).
45) OECD: OECD Environment, Health and Safety Publications Series on Pesticides, No.98 (2018).
46) Z. Li, M. Zheng, J. Zheng and M. G. Gänzle: *Bacillus* species in food fermentations: An underappreciated group of organisms for safe use in food fermentations. *Curr. Opin. Food Sci.* **50**, 101007 (2023).
47) J. Meerak, P. Yukphan, M. Miyashita, H. Sato, Y. Nakagawa and Y. Tahara: Phylogeny of γ-polyglutamic acid-producing *Bacillus* strains isolated from a fermented locust bean product manufactured in West Africa. *J. Gen. Appl. Microbiol.* **54**, 159–166 (2008).
48) F. Valerio, P. De Bellis, M. Di Biase, S. L. Lonigro, B. Giussani, A. Visconti, P. Lavermicocca and A. Sisto: Diversity of spore-forming bacteria and identification of *Bacillus amyloliquefaciens* as a species frequently associated with the ropy spoilage of bread. *Int. J. Food Microbiol.* **156**, 278–285 (2012).
49) A. Raio and G. Puopolo: *Pseudomonas chlororaphis* metabolites as biocontrol promoters of plant health and improved crop yield. *World J. Microbiol. Biotechnol.* **37**, 99 (2021).
50) P. Cornelis and S. Matthijs: Diversity of siderophore-mediated iron uptake systems in fluorescent pseudomonads: Not only pyoverdines. *Environ. Microbiol.* **4**, 787–798 (2002).
51) M. T. Ringel and T. Brüser: The biosynthesis of pyoverdines. *Microb. Cell* **5**, 424–437 (2018).
52) J. Chen, Z. Sun, J. Jin, F. Wang, Q. Yang, H. Yu, J. Yu and Y. Wang: Role of siderophore in *Pseudomonas fluorescens* biofilm formation and spoilage potential function. *Food Microbiol.* **109**, 104151 (2023).
53) E. Stackebrandt and J. Ebers: Taxonomic parameters revisited: Tarnished gold standards. *Microbiol. Today* **33**, 152–155 (2006).
54) E. Stackebrandt and B. M. Goebel: Taxonomic Note: A place for DNA–DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int. J. Syst. Evol. Microbiol.* **44**, 846–849 (1994).
55) S. Viulu, K. Nakamura, A. Kojima, Y. Yoshiyasu, S. Saitou and K. Takamizawa: *Geobacter sulfurreducens* subsp. *ethanolicus*, subsp. nov., an ethanol-utilizing dissimilatory Fe(III)-reducing bacterium from a lotus field. *J. Gen. Appl. Microbiol.* **59**, 325–334 (2013).
56) J. P. Anhalt and C. Fenselau: Identification of bacteria using mass spectrometry. *Anal. Chem.* **47**, 219–225 (1975).
57) H. N. Shah and M. D. Collins: Fatty acid and isoprenoid quinone composition in the classification of *Bacteroides melaninogenicus* and related taxa. *J. Appl. Bacteriol.* **48**, 75–87 (1980).
58) C. Fenselau and P. A. Demirev: Characterization of intact microorganisms by MALDI mass spectrometry. *Mass Spectrom. Rev.* **20**, 157–171 (2001).
59) A. Croxatto, G. Prod'hom and G. Greub: Applications of MALDI-TOF mass spectrometry in clinical diagnostic microbiology. *FEMS Microbiol. Rev.* **36**, 380–407 (2012).
60) A. Wieser, L. Schneider, J. Jung and S. Schubert: MALDI-TOF MS in microbiological diagnostics-identification of microorganisms and beyond. *Appl. Microbiol. Biotechnol.* **93**, 965–974 (2012).
61) M. Vargha, Z. Takáts, A. Konopka and C. H. Nakatsu: Optimization of MALDI-TOF MS for strain level differentiation of *Arthrobacter* isolates. *J. Microbiol. Methods* **66**, 399–409 (2006).
62) R. Dieckmann and B. Malorny: Rapid screening of epidemiologically important *Salmonella enterica* subsp. enterica serovars using Whole-Cell MALDI-TOF mass spectrometry. *Appl. Environ. Microbiol.* **77**, 4136–4146 (2011).
63) A. Grosse-Herrenthey, T. Maier, F. Gessler, R. Schaumann, H. Böhnel, M. Kostrzewa and M. Krüger: Challenging the problem of clostridial identification with matrix-assisted laser desorption and ionization-time-of-flight mass spectrometry (MALDI-TOF MS). *Anaerobe* **14**, 242–249 (2008).
64) E. Nagy, T. Maier, E. Urban, G. Terhes, M. Kostrzewa and ESCMID Study Group on Antimicrobial Resistance in Anaerobic Bacteria: Species identification of clinical isolates of Bacteroides by matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry. *Clin. Microbiol. Infect.* **15**, 796–802 (2009).
65) A. Cherkaoui, J. Hibbs, S. Emonet, M. Tangomo, M. Girard, P. Francois and J. Schrenzel: Comparison of two matrix-assisted laser desorption ionization-time of flight mass spectrometry methods with conventional phenotypic identification for routine identification of bacteria to the species level. *J. Clin. Microbiol.* **48**, 1169–1175 (2010).
66) M. Welker and E. R. Moore: Applications of whole-cell matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry in systematic microbiology. *Syst. Appl. Microbiol.* **34**, 2–11 (2011).
67) E. A. Idelevich, I. Schüle, B. Grünastel, J. Wüllenweber, G. Peters and K. Becker: Rapid identification of microorganisms from positive blood cultures by MALDI-TOF mass spectrometry subsequent to very short-term incubation on solid medium. *Clin. Microbiol. Infect.* **20**, 1001–1006 (2014).
68) A. Machen, T. Drake and Y. F. W. Wang: Same day identification and full panel antimicrobial susceptibility testing of bacteria from positive blood culture bottles made possible by a combined lysis-filtration method with MALDI-TOF VITEK mass spectrometry and the VITEK2 system. *PLoS One* **9**, e87870 (2014).
69) R. J. Arnold and J. P. Reilly: Observation of *Escherichia coli* ribosomal proteins and their posttranslational modifications by mass spectrometry. *Anal. Biochem.* **269**, 105–112 (1999).
70) F. J. Pineda, M. D. Antoine, P. A. Demirev, A. B. Feldman, J. Jackman, M. Longenecker and J. S. Lin: Microorganism identification by matrix-assisted laser/desorption ionization mass spectrometry and model-derived ribosomal protein biomarkers. *Anal. Chem.* **75**, 3817–3822 (2003).
71) K. Teramoto, H. Sato, L. Sun, M. Torimura, T. Tao, H. Yoshikawa, Y. Hotta, A. Hosoda and H. Tamura: Phylogenetic classification of

Pseudomonas putida strains by MALDI-MS using ribosomal subunit proteins as biomarkers. *Anal. Chem.* **79**, 8712–8719 (2007).
72) P. A. Demirev, Y. P. Ho, V. Ryzhov and C. Fenselau: Microorganism identification by mass spectrometry and protein database searches. *Anal. Chem.* **71**, 2732–2738 (1999).
73) P. A. Demirev, J. S. Lin, F. J. Pineda and C. Fenselaut: Bioinformatics and mass spectrometry for microorganism identification: Proteome-wide post-translational modifications and database search algorithms for characterization of intact *H. pylori*. *Anal. Chem.* **73**, 4566–4573 (2001).
74) C. Fenselau and P. A. Demirev: Characterization of intact microorganisms by MALDI mass spectrometry. *Mass Spectrom. Rev.* **20**, 157–171 (2001).
75) F. J. Pineda, M. D. Antoine, P. A. Demirev, A. B. Feldman, J. Jackman, M. Longenecker and J. S. Lin: Microorganism identification by matrix-assisted laser/desorption ionization mass spectrometry and model-derived ribosomal protein biomarkers. *Anal. Chem.* **75**, 3817–3822 (2003).
76) T. Coenye and P. Vandamme: Organisation of the *S10, spc* and *alpha* ribosomal protein gene clusters in prokaryotic genomes. *FEMS Microbiol. Lett.* **242**, 117–126 (2005).
77) B. Victoria, A. Ahmed, R. L. Zuerner, N. Ahmed, D. M. Bulach, J. Quinteiro and R. A. Hartskeerl: Conservation of the *S10-spc-alpha* locus within otherwise highly plastic genomes provides phylogenetic insight into the genus Leptospira. *PLoS One* **3**, e2752 (2008).
78) Y. Hotta, K. Teramoto, H. Sato, H. Yoshikawa, A. Hosoda and H. Tamura: Classification of genus *Pseudomonas* by MALDI-TOF MS based on ribosomal protein coding in *S10-spc-alpha* operon at strain level. *J. Proteome Res.* **9**, 6722–6728 (2010).
79) H. Tamura, Y. Hotta and H. Sato: Novel accurate bacterial discrimination by MALDI-Time-of-Flight MS based on ribosomal proteins coding in *S10-spc-alpha* operon at strain level *S10*-GERMS. *J. Am. Soc. Mass Spectrom.* **24**, 1185–1193 (2013).
80) H. Tamura: MALDI-TOF MS based on ribosomal protein coding in *S10-spc-alpha* operons for proteotyping. In "MALDI-TOF and TANDEM MS for Clinical Microbiology," ed. by H. N. Shah and S. E. Gharbia, John Wiley & Sons Ltd, pp. 269–310, (2017).
81) Y. Hotta, J. Sato, H. Sato, A. Hosoda and H. Tamura: Classification of the genus *Bacillus* based on MALDI-TOF MS analysis of ribosomal proteins coded in *S10* and *spc* operons. *J. Agric. Food Chem.* **59**, 5222–5230 (2011).
82) Y. Hotta, H. Sato, A. Hosoda and H. Tamura: MALDI-TOF MS analysis of ribosomal proteins coded in *S10* and *spc* operons rapidly classified *Sphingomonaceae* as alkylphenol polyethoxylate-degrading bacteria from the environment. *FEMS Microbiol. Lett.* **330**, 23–29 (2012).
83) H. Sato, M. Torimura, M. Kitahara, M. Ohkuma, Y. Hotta and H. Tamura: Characterization of the *Lactobacillus casei* group based on the profiling of ribosomal proteins coded in *S10-spc-alpha* operons as observed by MALDI-TOF MS. *Syst. Appl. Microbiol.* **35**, 447–454 (2012).
84) T. Ojima-Kato, N. Yamamoto, M. Suzuki, T. Fukunaga and H. Tamura: Discrimination of *Escherichia coli* O157, O26 and O111 from other serovars by MALDI-TOF MS based on the *S10*-GERMS method. *PLoS One* **9**, e113458 (2014).
85) T. Ojima-Kato, N. Yamamoto, Y. Iijima and H. Tamura: Assessing the performance of novel software Strain Solution on automated discrimination of *Escherichia coli* serotypes and their mixtures using matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J. Microbiol. Methods* **119**, 233–238 (2015).
86) C. T. Sacchi, A. M. Whitney, L. W. Mayer, R. Morey, A. Steigerwalt, A. Boras, R. S. Weyant and T. Popovic: Sequencing of 16S rRNA gene: A rapid tool for identification of *Bacillus anthracis*. *Emerg. Infect. Dis.* **8**, 1117–1123 (2002).
87) J. B. Kim, O. K. Choi, S. M. Kwon, S. H. Cho, B. J. Park, N. Y. Jin, Y. M. Yu and D. H. Oh: Prevalence and toxin characteristics of *Bacillus thuringiensis* isolated from organic vegetables. *J. Microbiol. Biotechnol.* **27**, 1449–1456 (2017).
88) S. Johler, E. M. Kalbhenn, N. Heini, P. Brodmann, S. Gautsch, M. Bağcioğlu, M. Contzen, R. Stephan and M. Ehling-Schulz: Enterotoxin production of *Bacillus thuringiensis* isolates from biopesticides, foods, and outbreaks. *Front. Microbiol.* **9**, 1915 (2018).
89) M. Ehling-Schulz, D. Lereclus and T. M. Koehler: The Bacillus cereus group: Bacillus species with pathogenic potential. *Microbiol. Spectr.* **7**, 0032 (2019).
90) A. Guérin, H. T. Rønning, C. Dargaignaratz, T. Clavel, V. Broussolle, J. Mahillon, P. E. Granum and C. Nguyen-The: Cereulide production by *Bacillus weihenstephanensis* strains during growth at different pH values and temperatures. *Food Microbiol.* **65**, 130–135 (2017).
91) K. Rouzeau-Szynalski, K. Stollewerk, U. Messelhäusser and M. Ehling-Schulz: Why be serious about emetic *Bacillus cereus*: Cereulide production and industrial challenges. *Food Microbiol.* **85**, 103279 (2020).
92) H. Kumar, L. Franzetti, A. Kaushal and D. Kumar: *Pseudomonas fluorescens*: A potential food spoiler and challenges and advances in its detection. *Ann. Microbiol.* **69**, 873–883 (2019).
93) Y. Wang, Y. Wang, J. Chen, S. Koseki, Q. Yang, H. Yu and L. Fu: Screening and preservation application of quorum sensing inhibitors of *Pseudomonas fluorescens* and *Shewanella baltica* in seafood products. *Lebensm. Wiss. Technol.* **149**, 111749 (2021).
94) J. Q. Chen, P. Regan, P. Laksanalamai, S. Healey and Z. Hu: Prevalence and methodologies for detection, characterization and subtyping of Listeria monocytogenes and *L. ivanovii* in food and environmental sources. *Food Sci. Hum. Wellness* **6**, 97–120 (2017).
95) P. Rajapaksha, A. Elbourne, S. Gangadoo, R. Brown, D. Cozzolino and J. Chapman: A review of methods for the detection of pathogenic microorganisms. *Analyst* **144**, 396–411 (2019).
96) S. Tang, R. H. Orsi, H. Luo, C. Ge, G. Zhang, R. C. Baker, A. Stevenson and M. Wiedmann: Assessment and comparison of molecular subtyping and characterization methods for *Salmonella*. *Front. Microbiol.* **10**, 1591 (2019).
97) J. P. Anhalt and C. Fenselau: Identification of bacteria using mass spectrometry. *Anal. Chem.* **47**, 219–225 (1975).
98) H. N. Shah and M. D. Collins: Fatty acid and isoprenoid quinone composition in the classification of *Bacteroides melaninogenicus* and related taxa. *J. Appl. Bacteriol.* **48**, 75–87 (1980).
99) L. K. Nakamura, M. S. Roberts and F. M. Cohan: Relationship of *Bacillus subtilis* clades associated with strains 168 and W23: A proposal for *Bacillus subtilis* subsp. *subtilis* subsp. nov, and *Bacillus subtilis* subsp. *spizizenii* subsp. nov. *Int. J. Syst. Evol. Microbiol.* **49**, 1211–1215 (1999).
100) A. P. Rooney, N. P. J. Price, C. Ehrhardt, J. L. Swezey and J. D. Bannan: Phylogeny and molecular taxonomy of the *Bacillus subtilis* species complex and description of *Bacillus subtilis* subsp. *inaquosorum* subsp. nov. *Int. J. Syst. Evol. Microbiol.* **59**, 2429–2436 (2009).
101) N. Takahashi, S. Nagai, A. Fujita, Y. Ido, K. Kato, A. Saito, Y. Moriya, Y. Tomimatsu, N. Kaneta, Y. Tsujimoto and H. Tamura: Discrimination of psychrotolerant *Bacillus cereus* group based on MALDI-TOF MS analysis of ribosomal subunit proteins. *Food Microbiol.* **91**,

103542 (2020).
102) N. Takahashi, S. Nagai, T. Tomimatsu, A. Saito, N. Kaneta, Y. Tsujimoto and H. Tamura: Simultaneous discrimination of cereulide-producing *Bacillus cereus* and psychrotolerant *Bacillus cereu*s group by MALDI-TOF MS. *J. Food Prot.* **85**, 1192–1202 (2022).
103) V. Manzulli, V. Rondinone, A. Buchicchio, L. Serrecchia, D. Cipolletta, A. Fasanella, A. Parisi, L. Difato, M. Iatarola, A. Aceti, E. Poppa, F. Tolve, L. Pace, F. Petruzzi, I. D. Rovere, D. A. Raele, L. Del Sambro, L. Giangrossi and D. Galante: Discrimination of *Bacillus cereus* group members by MALDI-TOF mass spectrometry. *Microorganisms* **9**, 1202 (2021).
104) A. Guérin, H. T. Rønning, C. Dargaignaratz, T. Clavel, V. Broussolle, J. Mahillon, P. E. Granum and C. Nguyen-The: Cereulide production by *Bacillus weihenstephanensis* strains during growth at different pH values and temperatures. *Food Microbiol.* **65**, 130–135 (2017).
105) K. Rouzeau-Szynalski, K. Stollewerk, U. Messelhäusser and M. Ehling-Schulz: Why be serious about emetic *Bacillus cereus*: Cereulide production and industrial challenges. *Food Microbiol.* **85**, 103279 (2020).
106) N. Ramarao, S. L. Tran, M. Marin and J. Vidic: Advanced methods for detection of *Bacillus cereus* and its pathogenic factors. *Sensors (Basel)* **20**, 2667 (2020).
107) S. Ulrich, C. Gottschalk, R. Dietrich, E. Märtlbauer and M. Gareis: Identification of cereulide producing *Bacillus cereus* by MALDI-TOF MS. *Food Microbiol.* **82**, 75–81 (2019).
108) J. Doellinger, A. Schneider, T. D. Stark, M. Ehling-Schulz and P. Lasch: Evaluation of MALDI-ToF mass spectrometry for rapid detection of cereulide from *Bacillus cereus* cultures. *Front. Microbiol.* **11**, 511674 (2020).
109) H. Y. Wang, F. Lien, T. P. Liu, C. H. Chen, C. J. Chen and J. J. Lu: Application of a MALDI-TOF analysis platform (ClinProTools) for rapid and preliminary report of MRSA sequence types in Taiwan. *PeerJ* **6**, e5784 (2018).
110) A. M. Fricker, D. Podlesny and W. F. Fricke: What is new and relevant for sequencing-based microbiome research? A mini-review. *J. Adv. Res.* **19**, 105–112 (2019).
111) S. Pornsukarom, A. H. M. van Vliet and S. Thakur: Whole genome sequencing analysis of multiple *Salmonella* serovars provides insights into phylogenetic relatedness, antimicrobial resistance, and virulence markers across humans, food animals and agriculture environmental sources. *BMC Genomics* **19**, 801 (2018).
112) S. Compant, A. Samad, H. Faist and A. Sessitsch: A review on the plant microbiome: Ecology, functions, and emerging trends in microbial application. *J. Adv. Res.* **19**, 29–37 (2019).
113) J. K. Jansson and K. S. Hofmockel: The soil microbiome—from metagenomics to metaphenomics. *Curr. Opin. Microbiol.* **43**, 162–168 (2018).
114) H. Tamura: A MALDI-TOF MS proteotyping approach for environmental, agricultural and food microbiology. In "Microbiological Identification using MALDI-TOF and Tandem Mass Spectrometry: Industrial and Environmental Applications," ed. by H. N. Shah, S. E. Gharbia, A. J. Shah, E. Y. Tranfield and K. C. Tom, John Wiley & Sons, pp. 147–182 (2023).