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New Techniques for the Detection, Separation and Identification of Microorganisms in Environmental Research

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GENERAL INTRODUCTION

At present, various methods are available for identifying microbes, *e.g.*different types of staining [1,2], specific antibodies [3], polymerase chain reaction (PCR) and DNA-typing based identification [4,5]. Moreover, mass spectrometry (MS), capillary electrophoresis (CE) and flow cytometry (FCM) can be used to analyse intact and lysed microbes [6-10]. The latter techniques represent interesting innovations, where MS is used as a common identification tool formicrobes [11,12], CE is an efficient high-throughput separation technique [13,14] and FCM is a powerful cell counting method, which is widely used for cell sorting [15]. The characteristics and the applications of these techniques are discussed as follows.

RAPID IDENTIFICATION OF MICROORGANISMS BY MATRIX-ASSISTED LASER/DESORPTION IONIZATION (MALDI)-MS

A novel method for the phylogenetic classification of microorganisms in environmental research using MALDI-MS was proposed in 1975 [16]. MALDI is a soft ionization technique that can be combined with MS to facilitate the analysis of biomolecules and large organic molecules, which tend to be fragile and they fragment when ionized using more conventional ionization

methods. The capabilities of MS have been utilized to characterize bacteria, bacterial spores and other types of microorganisms with minimal sample volumes and preparation procedures, but without fractionation or chromatography.

The mass spectral fingerprints obtained from whole cells that belong to unknown strains can be compared with those of different strains or a reference library of known strains [17-20]. This method can be used to classify bacterial strains simply and rapidly when combined with chemometric data analysis [21-23],but it has the following major disadvantages [24-28]: 1) the mass spectra have low reproducibility, which is affected greatly by the culture and measurement conditions; and 2) the uncertainty of the results because the observed peak components are unknown.

To overcome these disadvantages, Pineda [29]first proposed the use of ribosomal proteins as biomarkers for the identification of microorganisms. Ribosomes are the organelles responsible for protein synthesis, which comprise three types of RNA and about 50 types of ribosomal subunit proteins. Following the MALDI-MS characterization of ribosomal proteins isolated from *Escherichia coli*, Arnold and Reilly [30,31] confirmed that about half of the MALDI-MS spectral peaks from *E. coli*cells were related to ribosomal proteins. Ribosomal proteins are house-keeping proteins and the variations in their amino acid sequences reflect molecular evolution. The amino acid sequences of ribosomal proteins are highly conserved and they have unique masses at the species level, thereby providing biomarkers for species. Furthermore, there are differences in some of the amino acid sequences of ribosomal proteins among different strains of microorganisms, thereby providing biomarkers for strains. The molecular weights of ribosomal proteins are 4–30 KDa, which lie within the optimum mass range for modern MS analysis.

To evaluate this approach, the expressed ribosomal proteins of a genome-sequenced bacterium, *Lactobacillus plantarum*(NCIMB 8826), were characterized as a model. The 42 ribosomal subunit proteins in the isolated ribosome fraction were confirmed by two-dimensional gel electrophoresis combined with peptide mass fingerprinting. The observed masses of the proteins in the isolated ribosome fraction were then determined by MALDI-MS, where the observed masses matched with the calculated masses predicted from the amino acid sequence registered in protein databases [32].

Due to recent progress in the sequencing of complete microbial genomes, many amino acid sequences of ribosomal subunit proteins have been registered in public protein sequence databases such as Swiss-Prot, TrEMBL and NCBInr. Indeed, an Internet search engine is now available for identifying bacterial species based on the masses of ribosomal subunit proteins. Teramoto*et al.* [33] characterized the expressed ribosomal proteins of typical lactic acid bacteria strains of *L. plantarum*, *L. delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*based on comparisons with MALDI-MS data using publicly available protein sequence databases.

MALDI-MS has unique advantages as a technique for the phylogenetic classification of microorganisms, particularly its high sensitivity, accuracy and resolution. This method can even identify microorganisms at the strain level, while the analyses only require a small sample volume and the overall process is completed within a few minutes. Furthermore, the absolute molecular weight can be determined directly rather than the relative molecular weight and there is no requirement for standard compounds. Finally, there is no requirement for pure cultures of microorganisms, thus the samples can be applied directly to the MALDI-MS.

The discrimination of microorganisms by MALDI-MS has been applied in different fields. At present, commercial MALDI systems are available for biological research and for diagnostic applications in food, clinical medicine, biotechnology and industry [34]. For routine bacterial isolates, Bizzini and Greub reported that species were identified correctly by MALDI-MS in 84.1–93.6% of cases [35]. In 2013, C/IS MALDI-MS analysis was successfully employed for the characterization of *Penicilliumchrysogenum*cells, which were grown in a bioreactor for penicillin production [36]. In addition to bacteria, MALDI-MS has been applied to the identification of food-borne yeast isolates and performs far better than conventional methods in terms of speed, accuracy and efficiency

ANALYSIS OF MICROORGANISMS BY CE

Traditionally, CE has been used for the separation of molecules based on their mass-to-charge ratio. This technique has recently been explored in the analysis and characterization of microorganisms [38-45].Due to its unique attributes such as the speed of analysis, high efficiency, separation selectivity, small sample size, automation, low reagent consumption and the lack of any requirements for expensive dyes or biological reagents, CE has become an attractive approach for the analysis of colloidsand biological entities such as cells and microorganisms (including bacteria and viruses) [46-47].

The first application of capillary zone electrophoresis (CZE) to the analysis of microbes was reported by Hjerten*et al*. in 1987 [48], where they described how tobacco mosaic virus and *L. casei*were carried by an electroosmotic flow through a capillary. Subsequently, CE became an interesting tool for the analysis of biological particles. In 1993, Ebersole and McCormick [50] successfully separated *Streptococcus pyogenes*, *S. agalactiae*, *S. pneumoniae* and *Enterococcus faecalis* in TBE buffer. They also found that most of the bacteria (approximately 80% for*E. faecalis* and *S. pneumoniae*, and approximately 60% for *S. agalactiae*) remained viable after CZE separation, with the exception of *S. pyogenes*(only approximately 10% were viable).

Later, Torimura*et al*. [50] published an interesting study of the electrophoretic behaviour of nine bacteria, where they obtained negative electrophoretic mobilities for all of these microorganisms in phosphate buffer, and further studied the changes in the bacterial bands caused by colistinpretreatment. Two other groups (Pfetsch and Welsch and Glynn*et al*.) [51,52]

also studied bacterial mobility, where they found that bacterial mobility decreased with increasing ionic strength.

A common detection method used in CE for microbes is UV spectrometry. However, fluorescence detection (FLD) is a very powerful alternative because it is more sensitive and can also be used in many biological applications. In 2001, Armstrong's group showed that *L. acidophilus*, *Bifidobacteriainfantis* and *Saccharomycescerevisiae*remained viable after separation using propidium iodide (PI) and SYTO 9 staining with online FLD [53]. Later, Girod and Armstrong [54] monitored the migration of *B. infantis*, *L. acidophilus* and *S. cerevisiae*with a 488 nm argon-ion laser using SYTO 9 staining. Shintani*et al*. [55] used the same staining method for laser-induced fluorescence detection (LIF) of *Salmonella sp*. in TBE buffer.

The CZE analysis of microbes can also be applied to the quantification of microbes, *e.g*. Palenzuela*et al*. [56] described the quantification of bacterial contamination in foods. Lim *et al*. [57] also analysed *L. delbrueckii* and *Streptococcus thermophilus*in yoghurt samples.

Since its development, CE has been combined with other techniques such as PCR and fluorescence detection.For example, a method that combined CE with a Palm PCR was developed for the rapid onsite analysis of *influenza A (H1N1) virus* [58], while the usefulness of a CE-based multiplex PCR assay was demonstrated for the species-specific identification of *Candida spp.* [59]. A CE method based on whole-cell molecular labelling via fluorescent *in situ*hybridization (FISH) was developed for the detection of *Candida albicans*[60]. CE coupled with LIF has been used to obtain protein fingerprints to characterize two species of *Staphylococcus* [61].

USE OF FCM METHODS FOR SINGLE-CELL ANALYSIS IN ENVIRONMENTAL MICROBIOLOGY

FCM is a laser-based technology that is employed for cell counting, cell sorting, biomarker detection and protein engineering, where cells are suspended in a stream of fluid and passed through an electronic detection apparatus. FCM facilitates the simultaneous multiparametric analysis of physical and chemical characteristics at rates of up to thousands of particles per second. Fluorescence-activated cell sorting (FACS) is a specialized type of FCM, which is used for sorting a heterogeneous mixture of biological cells into two or more containers based on the specific light scattering and fluorescent characteristics of each cell. FCM is a useful scientific technique because it allows the rapid, objective and quantitative recording of fluorescent signals from individual cells, as well as the physical separation of cells of interest.

FCM has been a routine method in cellular biology since the 1970s. However, its application to prokaryotic cells has been limited mainly because of the difficulty of interpreting signals from very small objects, although FCM techniques have been applied increasingly in environmental microbiology in recent years. Indeed, it is now possible to analyse microbial communities by using two or three different fluorescent dyes to target specific biomolecules and physiological processes. Thus, the physiological states of cells, their taxonomic positions and expressed gene

functions can be assessed simultaneously, where the target cells can be separated and isolated for further study.

Community Structure and Spatial Distribution

At present, FACS is used for the analysis and separation of microalgae,where individual particles or cells scatter light, absorb the laser beam and emit fluorescence, which is detected using a set of highly sensitive detectors to obtain information related to the size, integrity and photosynthetic characteristics of cells [62,63]. This information is closely related to the morphology and photosynthetic characteristics of microalgae, which are conventionally used for their identification and classification. The usefulness of FACS has been demonstrated in many microalgae-related studies, including analyses of the developmental and growth-related properties of microalgal cultures [64], as well as monitoring phytoplankton communities in oceans, especially the pico- and nano-plankton fractions [65-67].

FCM as a Tool for Analysing Physiological States

One of the most promising applications of FCM is the characterization and distinction of different physiological states in microorganisms at the single-cell level [68,69].Differentiating between viable, intermediate and nonviable states can be achieved using fluorescent dyes that measure biological parameters such as the nucleic acid content, respiration rates, intracellular enzyme activity and the cytoplasmic and outer membrane integrity [68,70]. One widely used strategy is to analyse cell viability by double staining with nucleic acid-binding SYTO dyes (green fluorescence) and PI (red fluorescence) [71,72]. In this approach, all of the cells are assumed to incorporate SYTO and fluoresce green, but only 'dead' (with compromised membranes) cells are permeable to PI. PI binding reduces the fluorescence intensity of SYTO and thus the cells fluoresce red. Berney*et al*. [71] analysed a number of bacterial species that are important for drinking water systems, where different cytometric signatures were obtained for 'viable' (green) and 'non-viable' (red) cells.

Quantifying Microbial Growth in Oligotrophic Environments

FCM instruments can be very useful for studying growth in oligotrophic environments, which is usually difficult to assess. Hammes*et al*. [15,73] developed a quantitative method for the accurate counting of microbial cells at low cell densities. This method uses an instrument with volumetric hardware and nucleic acid staining of cells with SYBR Green to improve their detection, thereby allowing the reproducible quantification of cell numbers as low as 1000 cells/ml to determine cell growth or decline in different unit processes at drinking water treatment facilities. An approximate quantitative protocol for the rapid analysis of bloom-forming *Microcystis*cells and colonies in the sediment from Lake Taihu was also developed using a modified flow cytometer [74].

Sorting Based on Specific RNAs and Genes

Techniques have also been developed that utilize FISH probes to identify cells containing

specific nucleic acid targets before sorting these cells by FACS. Miyauchi*et al*. used FISH–FACS to sort polyphosphate-accumulating bacteria belonging to 'Candidatus*Accumulibacterphosphatis*' directly from awastewater reactor [75]. However, one problem whenapplying FISH–FACS to microbial communities is the possibility of sorting contamination whenthere are large numbers of events in complex systems,although recent studies have reported that the typicalefficiency exceeds 95% [75-78]. Another limitation isthe difficulty of sorting low abundance members when theyare discriminated inadequately from samplesduring the staining procedure. Thus,catalyzed fluorescent reporter disposition–FISH [78,79]and molecular beacons [80] are now being incorporated successfully into FISH–FACS strategies to boost the sensitivity of staining.

Sequencing the Unsequenced

With the development of whole genome amplification (WGA) techniques, FISH–FACS is now being used to target and sort previously uncultivated microorganisms from complex communities in genome analyses [76,81].For example, members of the uncultured bacterial TM7 division were sorted from a soil sample, where they comprised less than 2% of the total microbial community, and WGA was then performed with five sorted cells and 20% of the estimated genome was sequenced [81].

Culturing the Uncultured

FCM–FACS techniques are also being used to isolate and cultivate thousands of previously uncharacterized microorganisms from marine and terrestrial ecosystems. These techniques typically involve encapsulating cells in millimitre-sized agarosemicrodroplets[82,83]. The initial cell concentration is calibrated, where it is assumed that the majority of the microdroplets contain only a single cell. The microdroplets are then incubated in simulated natural conditions. Single microdroplets that contain growing or non-growing cells are then sorted by FCM–FACS into microtiter plates, and the outgrowth of the cells from the agarose beads allows the cells to be recovered in liquid cultures.

Conventional FISH techniques typically involve fixation steps that result in cell death. Thus, FISH–FACS sorted cells cannot be recovered in liquid cultures. However, Silverman and Cool developed an *in vivo*hybridization technique for characterizing living cells. This technique utilizes a novel quenched autoligation (QUAL) probe that increases the signal intensity upon hybridization [84].The probe was then introduced into living cells using a mild detergent treatment step. FCM was used to demonstrate that the probes could discriminate living cells of *E. coli*, *Salmonella enterica* and *Pseudomonas putida*even when the target region in the 16S rRNA differed by only a single nucleotide [85].In principle, this technique can target and recover any living microorganism from an environmental sample based only on sequence information. Moreover, the approach does not rely on QUAL probes. Any type of quenched probe where the fluorescence intensity increases upon hybridization could conceivably be used for isolating living organisms, such as molecular beacons [85] or thiazole orange 'Light-up' probes [86].

CONCLUSIONS

These latest techniques are their distinct characteristics and are summarized in Table 1.

Table 1: Comparison of the MALDI-MS, CE and FCM.

	MALDI-MS	CE	FCM
Pre-treatment	Not necessary	Sometimes necessary	Necessary
Analysis time	Several Minutes	Tens of minutes	Several hours
Identification level	Strain level	Species level	-
Cell recovery	No	Not yet available	Yes
Qualitative analysis	Yes	Yes	Yes
Quantitative analysis	No	Yes	Yes

MALDI-MS requires a very simple sample preparation process and the analytical process is completed within a few minutes. The identification of microbes is accurate and they can even be identified at the strain level. When a mixed sample is analysed, the target spectrum may be affected by other components present in the sample.

The high sensitivity of CE facilitates the detection of several cells in the same samples. In particular, CE is suitable for low density and uncultured microbes. The components of the sample can be separated and then recovered using an appropriate technique. The quantification of selected components is also possible. However, the repeatability is readily affected by the analytical conditions.

The major advantages of FCM are that targeted cells can be separated and sorted, while qualitative and quantitative analyses may be performed simultaneously. The throughput can reach 5000 cells/s, thereby separating 10^5 cells from a 1 g sample that contains only 1% of the target cells. FCM is a routine method in cellular biology, but the optimal conditions for microbial analysis still require further exploration.

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