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Laser desorption mass spectrometry for microbial DNA analysis

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Abstract

Recently, we demonstrated that a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF MS) can be used to determine the molecular weight of polymerase chain reaction (PCR) products of intact 16S rRNA regions and to profile their restriction digests. This is the first time that MALDI-TOF MS with ultraviolet (UV) photoionization has been used to analyze a PCR product of ~1600 nucleotides in length. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Matrix-assisted laser desorption/ionization; 16S rDNA; Time-of-flight mass spectrometry

1. Introduction

Recently, bioremediation has been used to reduce, eliminate or contain contaminants by microorganisms. Bioremediation has been gaining popularity and has been applied to organic solvents, polyaromatic hydrocarbon, polychlorinated biphenyl, hydrocarbon fuel, radionuclide and heavy metal contaminants (McCullough et al., 1999). Bioremediation processes usually involve either biotransformation to change the molecular structures of a compound or biodegradation to break down the large molecules into smaller and less harmful compounds. For bioremediation of metal and radionuclides, biotransformation generally can be applied. Microorganisms can interact with these contaminants and transform them from one chemical form to another, usually by changing their oxidation state. The change of oxidation state can often lead to a drastic change in the solubility, so that contaminants can be kept at the desired locations to reduce the risk of exposure to individuals. Since bacteria are usually the agents in most bioremediation processes and the efficiency depends on the presence of the appropriate microbes in the correct amounts and adequate environmental conditions, it is critically important to be able to know what kinds of microorganism and how much of them are in soil samples. However, the careful analysis of a microbial community is a very time-consuming and labor-intensive process. It usually involves cloning and DNA structure identification. In this work, we apply matrix-assisted laser desorption/ionization (MALDI) mass spectrometry for rapid DNA analysis of microbial samples.

The importance of characterization of bacterial communities has been well recognized for bioremediation. At present, isolation of microbial species and PCR amplification of specific sequences are the two major methods for revealing the composition of bacterial communities. Isolation and enrichment are the conventional processes for microbial community survey. However, different bacteria can grow with very different rates in culture. Indeed, it was estimated that less than 1% of community species can be cultured (Maymo-Gatell et al., 1997). Thus, DNA profiling can be a more reliable approach. PCR amplification of

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bacterial sequences with primers to the 16S rDNA or inter spacer region (ISR) between 16S and 23S (Robb et al., 1986) can identify a significant portion of a microbial community. In order to achieve a clear distinction among species, sequencing of 16S rDNA is often pursued. Instead of using time-consuming DNA sequencing, restriction fragment length polymorphism (RFLP) can be used following PCR to try to achieve the determination of the species profile based on 16S rRNA characterization. There are often many different types of bacteria in the soil samples, so a large number of RFLP samples need to be analyzed. A methodology for quick analysis of RFLP is highly preferred. In this work, we demonstrated that MALDI-MS can be used not only to measure the molecular weights of PCR products from the replication of 16S rDNA but also DNA fragments from RFLP for the identification of various types of bacteria. The molecular weights of PCR products with the size up to $\sim$1600 base pairs (bp) were successfully measured by MALDI MS. The results for RFLP measurements agree well with DNA gel electrophoresis data. However, the resolution can be significantly better than the results from agarose gel electrophoresis for RFLP analysis. No radioactive material or chromophore tagging is required.

Since the discovery of MALDI by Hillenkamp et al. for protein analysis, mass spectrometry has been broadly used for measurements of protein (Karas et al., 1987). The measurements of DNA were limited to very small DNA segments until the discovery of 3-hydroxypicolinic acid as a good matrix for MALDI TOF-MS of DNA (Wu et al., 1993). With the development of an instrument to give high ion energy and the use of new matrices, we (Tang et al., 1994) succeeded in using MALDI MS to detect single-stranded DNA the size of 500 nucleotides and double-stranded DNA of 500 base pairs (bp). Recently, Hillenkamp et al. also reported the detection of nucleic acid 2180 bp in size with mass accuracy of better than 1% (Berkenkamp et al., 1998). In this work, we report the detection of $\sim$1600 bp PCR products of 16S rDNA by MALDI mass spectrometry with ultraviolet laser desorption (UV-MALDI). To our knowledge, this is the largest DNA fragment detected by UV-MALDI TOF-MS. The recent successes in detecting large DNA fragments clearly indicate that MALDI-MS can be used for large DNA analysis. During the past few years, MALDI TOF-MS has been successfully used for diagnosis of base deletion and point mutation for cystic fibrosis as well as measuring a genetic polymorphism (Chang et al., 1995; Taranenko et al., 1996). More recently, success sequencing short DNAs by mass spectrometry was also reported (Koster et al., 1996; Rosky et al., 1996; Taranenko et al., 1997, 1998). MALDI was also used to identify bacteria by a few specific peaks in mass spectra (Gantt et al., 1999).

MALDI TOF-MS can be used to measure molecular weights of PCR products of 16S rDNA and RFLP to help to identify various microbes in the samples. Since 16S rRNA is highly conserved among various microbes, the use of primers complementary to those sequences for PCR amplification can yield products for different microorganisms. With different sequences between microbes, PCR amplification can produce products with different lengths. The number of different PCR products can give the first information on the minimum number of different microbes in the soil samples. The different patterns of RFLP can provide further information to distinguish different microbes with similar lengths of PCR products of 16S rDNA. If the size of PCR products and RFLP patterns are too similar to be distinguished by MALDI-TOF due to the limited mass resolution, DNA sequencing is required to confirm the microbial species in the samples.

The special advantage of using MALDI TOF-MS for microbial community assessment is the rapid analysis speed. For measurements of PCR or RFLP products, the analysis time for each sample by MALDI-MS is estimated to be less than 1 min instead of hours for gel electrophoresis. Thus, MALDI-MS is particularly suitable for the analysis of a large amount of DNA samples. Since it takes equal time to analyze PCR products, RFLP or DNA sequencing by MALDI mass spectrometry, the time saving can be even more significant for RFLP measurements and sequencing by MALDI-MS compared to gel electrophoresis.

2. Materials and methods

The major steps for microbial DNA analysis in this work include: (1) DNA extraction and amplification;

(2) gene cloning; (3) RFLP production; and (4) MALDI MS for DNA analysis.

2.1. DNA extraction and amplification

Bacteria DNAs were obtained from soil samples. Samples were typically frozen and transported to the laboratory with minimal delay. Microbial DNA was extracted from soil using a high temperature/salt/SDS-based lysis method (Zhou et al., 1996). Part of the crude DNA was purified by a gel-plus-minicolumn purification method (Zhou et al., 1996). The extracted soil DNA was subsequently used for small subunit rRNA gene amplification by PCR using primers fD1 and 1540R, which is similar to the processes described by Weisburg et al. (1991), except for the selection of primers. One microliter of the purified DNA ($\sim$ 10 ng) was used as a template in a 20-μl PCR reaction. The conditions for amplifying both eubacterial and archaeal 16S rRNA genes were described previously (Kwok and Higuchi, 1989; Duarte et al., 1998; Gregory and Sayler, 1998).

2.2. Small subunit rRNA gene cloning

The PCR-amplified 16S rDNA gene products were quantified by comparing the band intensity on agarose gels to the known concentrations of standard lambda DNA. The amplified PCR products were directly ligated to the pCR™ II vector from Invitrogen (San Diego, CA). Ligation and transformation were carried out according to the manufacturer's instructions. The ratios of inserts to vectors in ligation were 0.5–1:1. Two microliters of the ligation reaction mixture was transformed by heat pulse into *Escherichia coli* INV F′ ' competent cells supplied by Invitrogen. To speed up the cloning processes, a set of primers complementary to the polylinker of the vector pCR™ II was designed as described previously (Zhou et al., 1997). The cloned inserts were then amplified from transformant cells using these primers The size of the PCR amplified product was expected to be less than 100 bp if no fragment is inserted into the vector. The amplified PCR products with 16S rDNA inserts were analyzed by gel electrophoresis and mass spectrometry. We usually did 20 μl PCR reaction for each sample. This step can screen the clones with correct insertions (1600 bp).

2.3. RFLP of PCR products of 16S rDNA

For clones with correct size insertion, 5 μl of each PCR amplified product was digested with the combination of restriction enzymes *Msp*I and *Rsp*I. Another 5 μl was digested with the combination of *Hae*III and *Hha*I. Restriction products were separated by gel electrophoresis in Metaphor agarose (FMC Bioproducts, Rockland, ME). The Metaphor agarose gel was prepared as per manufacturer instructions. The gel was stained with 0.5 μg of ethidium bromide per ml and visualized by UV excitation. RFLP patterns were compared using the computer program GelCompare for comparison with the results from MALDI analysis. The clones showing different RFLP pattern based on two sets of restriction enzymes digestion were chosen for partial sequencing using primer 529R. The partial sequences were searched for similarity in a current database and used for phylogenic analysis.

2.4. MALDI TOF-MS for DNA analysis

For MALDI MS for DNA analysis, DNA samples amplified by PCR processes were purified using QIAquick spin columns (QIAGEN, Santa Clara, CA). The final product was then precipitated in alcohol, centrifuged, dried under vacuum and dissolved in 2–5 μl of sterile, deionized water. The sample for mass spectrometry analysis was prepared by mixing 1 μl of aqueous analyte solution with 1 μl of matrix solution. One microliter of this mixture was spotted on a stainless steel plate and dried by a forced nitrogen gas at ambient temperature. The dried sample was loaded into the time-of-flight mass spectrometer (TOF-MS) immediately without further exposure to the atmosphere. The matrix samples were prepared from a mixture of 0.3 M 3-hydroxypicolinic acid, 0.5 M picolinic acid and 0.3 M ammonium fluoride (molar ratio 9:1:1). The matrix materials were purchased from Aldrich and used without further purification. DNA samples from RFLP by enzyme digestion of PCR products were also purified through the above process. We also pursued phenol/chloroform extraction and alcohol precipitation for some DNA samples. The mass spectra results are similar as long as salts in the solution were eliminated.

A linear time-of-flight mass spectrometer was used for measuring the molecular weights of DNA frag-

ments. A pulsed nitrogen laser for desorption and ionization was used in obtaining mass spectra of DNA samples. The typical laser fluence was measured between 45 and 65 mJ cm^2. The acceleration voltage was about 30 KeV. The pressure of the TOF-MS vacuum chamber was typically at 1.5×10^{-7} Torr. The signal of DNA ions were collected and digitized by a digital oscilloscope (Tektronix 520A), which was controlled by a personal computer. A delayed pulse ion extraction was installed to improve mass resolution.

3. Results and discussion

Mass spectrum of DNA products to replicate 16S region is shown in Fig. 1. The mass resolution was estimated as ~ 80. The mass accuracy can not be determined since soil samples were used. These results indicate the quality of the spectra is comparable to the results of IR-MALDI. However, it is more convenient to use UV-MALDI than IR-MALDI. The

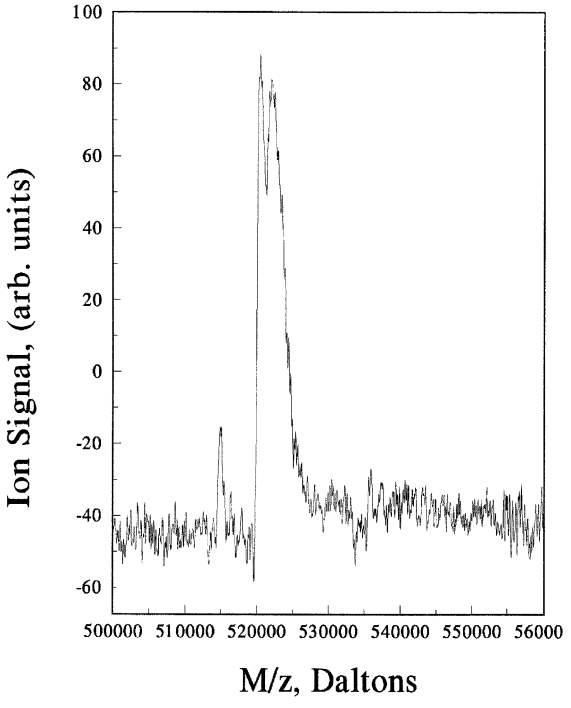

Fig. 1. Mass spectrum of MALDI of PCR products from replication of 16S rRNA. The primers used are FD1 and 1540R.

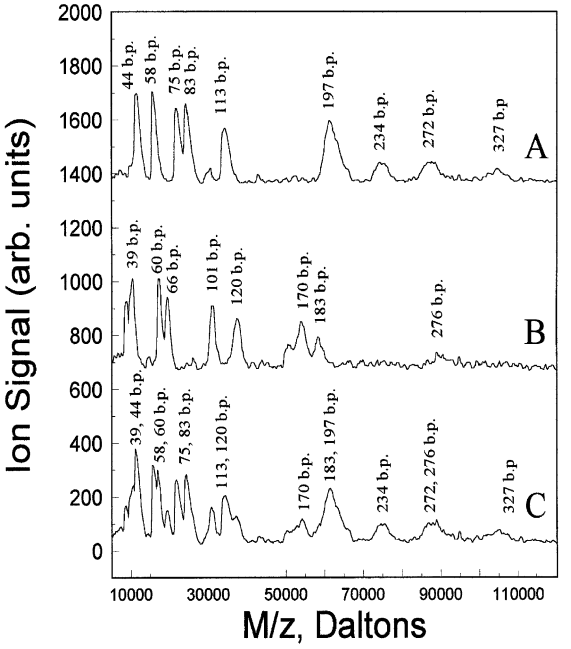

Fig. 2. MALDI spectra from enzyme digestion of PCR products of 16S rRNA.

result indicates UV-MALDI can be used to help to analyze 16S rDNA to help to identify the type of bacteria. To our knowledge, it is the largest DNA fragments detected by UV-MALDI with a time-of-flight mass spectrometer. It indicates that MALDI can become a convenient fast tool to measure the size of 16S rRNA. Since 16S rRNA and the region for the primers used for PCR are highly conserved, the use of complementary primers for PCR can yield different products for different microbes. For sequences within rRNA being different between different microbes, PCR products can have different lengths. The number of different PCR products can be used to estimate the minimum number of microbes in the sample.

Fig. 2A and B show the mass spectra of DNA fragments from RFLP with enzyme digestion by *Hha*I and *Has*III and *Msp*I and *Rsa*I, respectively, for a selected microbial sample. The pattern of fragmentation can be used to confirm the type of bacteria. The results agree well with results from agarose gel electrophoresis. Fig. 2C shows the results of DNA fragments from the mixture of two PCR products. Spectrum Fig. 2C is similar to the sum of Fig. 2A and

B. These results also indicate MALDI can be used to help the assessment of different types of bacteria by measuring DNAs from PCR and RFLP processes. If the mass resolution can be further improved, there is a potential that several different kinds of enzymes can be used simultaneously to obtain RFLP, which can still be resolved by MALDI mass spectrometry.

The special advantage of using MALDI mass spectrometry for microbial community assessment is rapid analysis. The time to get a mass spectrum is less than 1 min. However, the time to finish extraction and purification is much more time consuming. Nevertheless, purification with commercial spin columns can significantly reduce the purification time. Robotic array arrangement can further reduce the time for purification per sample. Our data demonstrate a potential for an efficient use of MALDI to achieve high sensitivity and high throughput in the analysis of bacteria DNA. Our results from MALDI have been confirmed by the results of gel electrophoresis. This indicates that MALDI mass spectrometry can be used for the detection of biomarkers, which can be specific to selected bacteria. Since MALDI-MS can be used to obtain the molecular weights of each DNA fragment, multiplexing can possibly be achieved by using multiple sets of primers to replicate various rDNA regions. However, better mass resolution is required for precisely identifying the size of DNA fragments. Various approaches to improve mass resolution are currently under evaluation.

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