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Phylogenetic analysis of microalgae based on highly abundant proteins using mass spectrometry

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ABSTRACT

The blooms of toxic phototrophic microorganisms, such as microalgae and cyanobacteria, which are typically found in freshwater and marine environments, are becoming more frequent and problematic in aquatic systems. Due to accumulation of toxic algae, harmful algal blooms (HABs) exert negative effects on aquatic systems. Therefore, rapid detection of harmful microalgae is important for monitoring the occurrence of HABs. Mass spectrometry-based methods have become sensitive, specific techniques for the identification and characterization of microorganisms. Matrix-assisted laser desorption/ionization (MALDI) with time-of-flight (TOF) mass spectrometry (MS) allows us to measure a unique molecular fingerprint of highly abundant proteins in a microorganism and has been used for the rapid, accurate identification of bacteria and fungi in clinical microbiology. Here, we tested the specificity of MALDI-TOF MS using microalgal strains (*Heterocapsa*, *Alexandrium*, *Nannochloropsis*, *Chaetoceros*, *Chlorella*, and *Dunaliella* spp.). Our research suggested that this method was comparable in terms of the rapid identification of microalgea to conventional methods based on genetic information and morphology. Thus, this efficient mass spectrometry-based technique may have applications in the rapid identification of harmful microorganisms from aquatic environmental samples.

1. Introduction

Harmful algal blooms (HABs), commonly called red tides [1], result from accumulation of various microalgae and are known to cause outbreaks of shellfish poisoning and death of fish, mammals, and sea plants [2]. HABs affect global coastal regions [1], and some algal toxins can cause diarrheic, paralytic, amnesic, and neurotoxic shellfish poisoning, thereby endangering human health and negatively affecting the tourism industry and fishery resources [3]. Most HABs are formed by blooming microscopic algae, phytoplankton, or specific cyanobacteria and occur when microalgae rapidly proliferate and when a large biomass of toxic or noxious microalgae accumulates in marine ecosystems or freshwater ecosystems [1]. Therefore, in order to respond quickly and appropriately to dissipate HABs, studies are needed to understand the ecology of microalgae as aquatic microbial eukaryotes and for enumeration and identification of harmful microalgae. Traditionally, identification of microalgae in HAB events has been achieved by light or electron microscopy examination. However, these methods may be difficult and time-consuming. Within the last decade, real-time polymerase chain reaction (PCR) assays [3] have been developed for rapid detection of microalgae in HABs. Molecular tools such as real-time PCR are known to be very useful for the detection of microorganisms [3]. However, exact identification of individual strains is nearly impossible using real-time PCR because of the limited availability of specific primer-probe sets.

Mass spectrometry (MS)-based methods have been established as an indispensable technology for interpretation of the information encoded in genomes, complementing DNA-based approaches [4].

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Currently, MALDI-time-of-flight (TOF) MS has been applied for the detection of ribosomal protein mass patterns for the confirmation of pathogens [5]. Additionally, MALDI-TOF MS provides identification of pathogen and microorganisms and can be used to determine microbial taxonomy [6]. Reproducibility and accuracy of taxonomic identification of microorganisms using MALDI-TOF MS were confirmed by the previous studies under different cultivation conditions [7,8]. In another study, identification of filamentous fungi through spores was carried out using MALDI-TOF MS [9]. Importantly, the use of MALDI-TOF MS can reduce costs and time required for identification of microorganisms [10] and elucidation of microbial taxonomy can be used to determine microbial diversity based on natural relationships without identification of functional taxonomy or isolation of microorganisms [11].

Taxonomic system mirroring 'order in nature' [12] is established by phylogenetic analysis that can identify unknown organisms and classification in taxonomic groups on the bases of relationships. In this study we focused on developing a MALDI-TOF MS-based method for the phylogenetic analysis of harmful microalgal strains belonging to the genus *Alexandrium*, *Chaetoceros*, and *Heterocapsa*, as well as strains of the genus *Nannochloropsis*, *Chlorella*, and *Dunaliella* and we compared identification using MALDI-TOF MS to classical morphological methods and molecular genetic methods based on ribosomal RNA (rRNA) genes.

2. Experimental

2.1. Cultures of microalgal strains

Eight microalgae (*Chlorella* sp. J31 and J37, *Dunaliella* sp. J33, *Chaetoceros* sp. J28 and J32, *Alexandrium tamarense* J18, *Heterocapsa* sp. J3, and *Nannochloropsis granulata* J34) were obtained from the University of Nagasaki in Japan. Each strain was maintained and cultured using Erd-Schreiber modified (ESM) medium in plant growth chambers (VS-3DM, Vision Scientific, Korea). The ESM medium contained the following: 120 mg/L $NaNO_3$, 5 mg/L K_2HPO_4, 0.1 mg/L vitamin B_1, 0.01 mg/L vitamin B_{12}, 0.001 mg/L biotin, 0.26 mg/L EDTA–Fe^{3+}, 0.33 mg/L EDTA–Mn^{2+}, and 1000 mg/L Tris dissolved in artificial seawater; the pH was adjusted to 8.0. The seawater used for ESM medium was prefiltered through a membrane filter with a 0.2-μm pore size made of cellulose acetate. The cultures were stored in sterilized 500-mL Erlenmeyer flasks and inoculated in a plankton chamber under controlled conditions at 26 °C with 60% humidity and a 12/12 h light/dark cycle. The cool-white fluorescent lamp used for incubation supplied approximately 45.8 W/m^2 of light intensity. Each species of microalgae was tested in the exponential growth phase.

2.2. Morphological analysis using microscopy

Morphologies and sizes of the eight microalgae strains were observed on slides using the phase-contrast mode of a fluorescence microscope (FSX100, OLYMPUS, JPN).

2.3. Phylogenetic analysis based on 18S rRNA gene sequences

For phylogenetic analysis based on the 18S rRNA gene sequences, microalgal DNA was extracted using a DNA extraction kit (DNeasy Blood & Tissue Kit, Qiagen, Netherlands) in order to remove PCR inhibitors from the microalgae [13]. Then, the 18S rRNA gene amplification was carried out using a PCR premix (AccuPower, Bioneer, Korea) and two eukaryotic primers (Ecol-7F, 5′-ACCTGGTTGATCCTGCCAG-3′ and Ecol-1534R, 5′-TGATCCTTCYGCAGGTTCAC-3′). The PCR protocol was as follows: initial denaturation for 10 min; 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 2 min, extension at 72 °C for 3 min, and a final extension for 5 min. The PCR products were sequenced using an automated DNA analyzer system (PRISM 3730xl DNA Analyzer, Applied Biosystems, USA) with a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) according to the manufacturer's instructions. The 18S rRNA gene sequences of microalgae, verified using the BLAST search program (http://blast.ncbi.nlm.nih.gov/Blast.cgi), were aligned by CLUSTAL_W. Phylogenetic dendrograms were constructed using MEGA5 and the neighbor-joining method with 1000 randomly selected bootstrap replicates.

2.4. Spectral fingerprinting analysis using MALDI-TOF MS

We carried out the direct transfer method to analyze protein profiling of whole microorganism cells by MALDI-TOF MS without protein extraction and inactivation steps [14]. MALDI-TOF MS samples in triplicate were air-dried and mixed with α-cyano-4-hydroxycinnamic acid (CHCA) in 33% acetonitrile (ACN) and 33% ethanol (EtOH) with 3% trifluoroacetic acid (TFA) at a 1:1 ratio. Prepared samples were then homogenized. Ion spectra providing protein profiles were obtained in linear mode and positive-ion mode (laser frequency of 1000 Hz) with a mass range of 2000–20,000 Da using an UltrafleXtreme TOF/TOF mass spectrometer (Bruker Daltonics, Germany). Extraction delay time, lens, and ion sources 1 and 2 were set to 150 ns, 6.47, 25, and 23 kV, respectively. Adjustment and analysis of obtained MALDI-TOF MS spectra data were carried out using Excel 2010 (Microsoft, USA) and MultiExperiment Viewer (MeV) 4.8.1. Ion spectra of protein profiles from 2000 to 20,000 Da (Fig. 1) for each microalga were used to construct dendrograms by hierarchical clustering analysis (HCA) in MeV. The ordering optimization was sample leaf order, the distance metric was Euclidean distance, and the linkage method was complete linkage clustering. The sensitivity of the MALDI-TOF MS approach was analyzed to determine the amount of cells required for mass spectra analysis using each microalgae strain of *Heterocapsa* sp. J3, *Chaetoceros* sp. J28, and *Dunaliella* sp. J33. We first established the number of cells present in dense cultures using a hemocytometer with direct viable counts of 10-fold serial dilutions. Based on these counts we prepared samples containing 10^1–10^6 cells which were then analyzed using MALDI-TOF MS as previously described.

3. Results and discussion

We rapidly identified a total of eight species of microalgae using MALDI-TOF MS and compared these results with those of other techniques, i.e., morphological observations and phylogenetic analyses. First, we observed microalgal samples using light microscopy to identify morphologies.

3.1. Morphology

The eight microalgae exhibited proper morphologies for each of their species (Fig. S1). However observation of morphology can be easily confusing and is not as accurate as other techniques, such as phylogenetic analysis for taxonomy. Moreover, strains J31, J34, and J37 exhibited similar morphologies; this may cause difficulties in correctly identifying such strains by morphological and taxonomical evaluations. While observation of the ultrastructural morphology of microalgae by transmission electron microscopy has been carried out and can be much more precise in taxonomy analysis, it is costly, time-consuming, and difficult.