The study of the molecular ecology of bacteria involved in a life cycle impact assessment of agricultural adjuvants#

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The life cycle impact assessment of chemicals in the environment is essential for establishing an environmentally conscious, material-recycling society. To understand the life cycle of chemicals through the environmental problems arising from the alkylphenol polyethoxylate (APEOₙ) issue, our research has focused as follows: (1) clarification of environmental fate based on interaction between bacteria and environmental factors; (2) establishment of a rapid and simple bacterial identification method.

Environmental elements, such as Ca²⁺, Mg²⁺ and Fe³⁺, may influence the activity and rate of APEOₙ biodegradation in the environment. A highly reliable method for phylogenetic analysis at the strain level, named the S10-GERMS (S10-spc-alpha operon gene encoded ribosomal protein mass spectrum) method, was developed using bacterial ribosomal subunit proteins as theoretical biomarkers encoded in the S10-spc-alpha operon with a validation procedure. © Pesticide Science Society of Japan

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Introduction

Although chemicals have contributed to the development of humans, the environmental pollution caused by chemicals has affected the ecosystem and human health. Therefore, life cycle impact assessment (LCIA) of chemicals in the environment is essential for establishing an environmentally conscious, material-recycling society. Since the environmental impact of chemicals is dependent on metabolic toxicants by environmental microorganisms, studying the molecular ecology of bacteria is extremely important for the LCIA of chemicals. The active ingredient in agrochemicals is a good example of appropriate management of each process in the LCIA. Although it is common in the application of agrochemicals to use a larger volume of agricultural adjuvants rather than the active ingredient itself to improve performance, scientific knowledge of their environmental fates through the LCIA has been insufficient; therefore, nonionic surfactant alkylphenol polyethoxylates (APEOₙ), which have been widely used as adjuvants of pesticides, were used as the model compounds for the study of the molecular ecology of bacteria involved in the LCIA of chemicals. To understand the life cycle of chemicals through the environmental problems arising from the APEOₙ issue, our research has focused as follows: (1) clarification of environmental fate based on interaction between bacteria and environmental factors; (2) establishment of a rapid and simple bacterial identification method.

1. Clarification of environmental fate based on interaction between bacteria and environmental factors

Harmless APEOₙ degraded to estrogen agonistic compounds that is AP, APEOₙ, with shorter EO chains, and the corresponding alkylphenol carboxylates (APECₙ). Our previous studies have revealed that AP, alkylphenol monoethoxylate (APEO₁) and alkylphenol diethoxylate (APEO₂) also had antiandrogenic activity. Their estrogenic and antiandrogenic activities increased as the EO chain length decreased. Accordingly, the detailed microbial degradation of APEOₙ was well characterized by the Pseudomonas putida strain S5 isolated from a Japanese paddy field, which proceeds via the successive exo-scission of the ethylene oxide chain accompanied by oxidation of the hydroxyl terminal side through their carboxylic intermediates. Since we had a clue to help us understand why various APEOₙ degradation products were detected in the environment, our research has focused on the effect of environmental elements on the APEOₙ biodegradation.

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Sphingomonas sp. strain BSN22, isolated from bean fields, degraded octylphenol monoethoxylate (OPEO\textsubscript{n}) to octylphenol (OP) under aerobic conditions. The biodegradation mechanism proceeded by the following 2-step degradation process: step 1—degradation of OPEO\textsubscript{n} to octylphenol triethoxylate (OPEO\textsubscript{3}), and step 2—degradation from OPEO\textsubscript{3} to OP via octylphenoxacyclic acid (OPEC\textsubscript{3}). Quantitative studies revealed that Mg\textsuperscript{2+} and Ca\textsuperscript{2+} were essential for the biodegradation of OPEO\textsubscript{n}. Furthermore, the rate of step-2 biodegradation was markedly accelerated by Fe\textsuperscript{3+}, and the accumulated amounts of endocrine active chemicals, such as OP, OPEO\textsubscript{n} and OPEC\textsubscript{3}, significantly increased in the presence of Fe\textsuperscript{3+}. Taken together, those environmental elements significantly influence the resultant ecotoxicity as well as the rate of their biodegradation in the environment because OP and OPEO\textsubscript{n} (n=1, 2) act as endocrine active substances. This study of the LCIA of OPEO\textsubscript{n} may play an important role in understanding and managing the safety of the environment, including the safety of drinking water.

2. Establishment of a rapid and simple bacterial identification method

Our recent evidence has shown that bacteria that can degrade APEO\textsubscript{n} to estrogenic and antiandrogenic metabolites exist fairly universally in paddy fields in Japan. Therefore, it is important to establish a rapid and simple bacterial identification method to monitor and/or regulate the environmental fate of APEO\textsubscript{n}. We proposed a simple and rapid bacterial identification method by MALDI-TOF MS analysis using only ribosomal subunit proteins as biomarkers. The ribosomal subunit proteins coded in the S10-spc-alpha operon were selected as especially reliable biomarkers for bacterial discrimination at the strain level because the S10-spc-alpha operon encodes half of the ribosomal subunit proteins and is highly conserved in eu-bacterial genomes. Our method was applied with the genus Pseudomonas as the model bacteria. It revealed that 14 reliable and reproducible ribosomal subunit proteins with less than \( m/z 15,000 \) except for L14—coded in the S10-spc-alpha operon were useful biomarkers for bacterial classification at the species and strain levels by MALDI-TOF MS analysis of the genus Pseudomonas strains. In particular, ribosomal subunits S14, L24, and S13 were significantly useful biomarkers for discrimination of \textit{P. putida} at the strain level. The phylogenetic tree based on 14 ribosomal subunit protein profile matching was consistent with that based on the genetic sequence (\textit{gyrB}). MALDI-TOF MS analysis using selected ribosomal proteins, named the S10-GERMS (S10-spc-alpha operon gene-encoded ribosomal protein mass spectrum) method, is a rapid, efficient, and versatile bacterial identification method with the validation procedure for the obtained results. The workflow of the S10-GERMS method is as follows: step 1—MALDI-TOF MS analysis of the genome-sequenced type strain or non-genome-sequenced type strain; step 2—sequencing and translating S10-spc-alpha operon of the type strain; step 3—constructing a theoretical \( m/z \) database by calculating the theoretical \( m/z \) of the ribosomal protein; step 4—constructing an accurate database by comparing the theoretical and observed data and selecting biomarker proteins.

3. Application of the S10-GERMS method for APEO\textsubscript{n}-degrading bacteria

In our studies, 16 strains isolated as APEO\textsubscript{n}-degrading bacteria were subjected to biodegradation tests. They were classified into 4 patterns based on final metabolic toxicants due to their degrading activity: (1) OP, OPEO\textsubscript{1}, and OPEC\textsubscript{1}; (2) OPEO\textsubscript{2} and OPEC\textsubscript{2}; (3) OPEO\textsubscript{3}; and (4) OPEC\textsubscript{3}, as main biodegradation products. Most isolated APEO\textsubscript{n}-degrading bacteria in our studies belonged to the \( \alpha \)-proteobacteria subclass, and diverse species of Sphingomonadaceae are ubiquitous bacteria that bio-degrade APEO\textsubscript{n} in the environment; therefore, the S10-GERMS method was applied to the classification of the environmental bacteria, Sphingomonadaceae. To construct a ribosomal protein database, the S10-spc-alpha operon of the type strains of Sphin-gomonadaceae and their related APEO\textsubscript{n}-degrading bacteria were sequenced using specific primers designed based on nucleotide sequences of genome-sequenced strains. The observed MALDI mass spectra of intact cells were compared with the theoretical mass of the constructed ribosomal protein database. The 9 selected biomarkers coded in the S10-spc-alpha operon could successfully distinguish the \textit{Sphingopyxis terrae} NBRC 15098\textsuperscript{T} and APEO\textsubscript{n}-degrading bacterium strain BSN20, despite only one base difference in the 16S rRNA gene sequence. APEO\textsubscript{n}-degrading bacteria assigned as \textit{M. thiogenteticum} and \textit{C. asaccharovorans} based on the 16S rRNA gene sequence have never been reported for the degradation of APEO\textsubscript{n}, although their homologies (\%) were lower than 98.5%; therefore, the S10-GERMS method was applied to them. The MALDI mass spectra of \textit{M. thiogenteticum} DSM 17097\textsuperscript{T} and APEO\textsubscript{n}-degrading bacterium strain BSN20, despite only one base difference in the 16S rRNA gene sequence have never been reported for the degradation of APEO\textsubscript{n}, although their homologies (\%) were lower than 98.5%; therefore, the S10-GERMS method was applied to them. The MALDI mass spectra of \textit{M. thiogenteticum} DSM 17097\textsuperscript{T} and BSN58 revealed a significant difference and those of \textit{C. asaccharovorans} DSM 6462\textsuperscript{T} and BSNN6 had no similarity at all. Therefore, the S10-GERMS method revealed that they may belong to new species, although further study is required to confirm them as new species. The S10-GERMS method could not predict the capability of bacterial APEO\textsubscript{n} biodegradation activity. However, it is important to discriminate APEO\textsubscript{n}-degrading bacteria for evaluating and monitoring the degrading activity of APEO\textsubscript{n}.

Conclusions

LCIA of chemicals would play an important role in fully understanding the environmental issues arising from the chemicals. This study advances the idea that external inputs, such as minerals in the environment, modulate a specific response of the microbe community by regulating the population size of a specific microbe involved in controlling the biodegradation of chemicals. Such microbial plasticity in the environment can weaken or strengthen communication between microbes and external inputs under the stress of the chemicals. Our results demonstrate the potential of the S10-GERMS method as a tool for accurately discriminating and/or typing bacteria at the strain level over the 16S rRNA gene sequencing identification techniques. Therefore,
establishing of a useful search engine for the S10-GERMS method will enable simple and rapid bacterial identification. In the future, the application of a rapid bacterial identification method such as the S10-GERMS method in environmental microbiology will be advantageous for the LCIA of chemicals.