論文の内容の要旨

応用生命化学専攻
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論文題目

Rhizoremediation of Dioxin-Contaminated Soil

（植物－根圏微生物共生系を用いたダイオキシンで汚染された土壌の修復に関する研究）

Polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are widespread in the environment. These well-known, toxic environmental contaminants are formed unintentionally as by-products during the manufacture of pesticides and herbicides or during municipal incineration. Once emitted, PCDD/F move from these sources, some react in the atmosphere, some deposit in the earth through wet and dry deposition, and some eventually accumulated in human tissue. Many of their congeners are considered to be environmental pollutants of major concern due to their persistence, recalcitrance and mutagenic properties. Studies have demonstrated their long-term accumulation in soils and sediments is related to the physicochemical characteristics of these molecules, and also adverse health effects in animals and humans are caused by progressive bioaccumulation in food chains. In order to clean up contaminated environments, numerous physicochemical techniques have been developed, such as air sparging, soil washing, solvent extraction, thermal desorption and thermal enhancement and so on. However, these methods are very expensive to perform and are not complete processes for remediation. Very often, they require secondary remediation processes for the extracted contaminants that are disruptive to the environment.

It has long been known that microorganisms exist in a variety of tissue types within numerous plant

species. Plant-microbe symbioses are ubiquitous in natural and most anthropogenically influenced soils. Currently, research efforts of many laboratories focus on the use of plants for degradation of various xenobiotic compounds. This technology, phytoremediation, is defined as a technology using green plants to remove, or render harmless environmental contaminants. Phytoremediation has been recognized as an alternative to physicochemical remediation technologies for removal of organic pollutants from soil, due to its potentially lower cost and simple. During recent years metabolism of organic contaminants such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), 2,4,6-trinitrotoluene (TNT) and other chlorinated compounds has been studied by using plants. Among these mechanisms, rhizoremediation has attracted considerable attention owing to the potential of the cooperative interaction between plant roots and their associated microorganisms involving rhizosphere effect and root-microbe symbioses. Plants provide a remediation strategy that utilizes solar energy. Root exudates provide microorganisms with a wide range of organic substrates for use as carbon and energy sources and also stimulate microbial growth in the rhizosphere. Rhizosphere microorganisms are able to biodegrade a wide variety of organic contaminants, such as PAHs, pesticides, chlorinated alkanes, etc. The latest study has demonstrated that depletion of polychlorinated biphenyls (PCBs) was enhanced using *Arabidopsis* root-associated microbes which can use plant secondary metabolites. However, so far the potential of plant-associated microorganisms for degradation of PCDD/F compounds has received little attention.

The ability of plant-microbe combinations to remedy PCDD/F-contaminated soil has not been sufficiently studied yet. With a focus on the practical use, it is necessary to extend the fundamental knowledge of phytoremediation. The object of this study is to investigate the feasibility of applying the plant-microbe combination to clean up dioxin-contaminated soil.

1. Plant selection with potential for remediation of contaminated soil

In contaminated soil, most of PCDD/F were found to distribute spatially in the top layer (0~10cm), owing to their high affinity to the soil matrix. Therefore, compared with the wood plants, herbaceous plants may be more appropriate to clean up the PCDD/F pollution due to their root morphology. Fibrous roots offer more root surface area for microbial colonization than other roots and result in a larger microbial population in the polluted soil. In this experiment, three grasses (Bermudagrass [*Cynodon dactylon*], Creeping Bentgrass [*Agrostis palustris* Huds.], Lawngrass [*Zoysia japonica*]) and a shallow-rooted legume (white clover [*Trifolium repens* L.] were planted into uncontaminated soil and dibenzofuran(DF)-contaminated soil. During two months of growth, the root biomass and heterotrophic microbial numbers were measured to evaluate the efficiency of remediation. Despite the contamination of DF, white clover had the highest root fresh biomass and dry biomass by comparing with those of other three grasses ($p \leq 0.05$). Among all plants, higher heterotrophic microbial numbers were found in rhizosphere soil than in bulk soil presumably in response to the root exudates. The microbial numbers in the rhizosphere of white clover roots were statistically larger than those of the other plants. Based upon the results above, white clover was selected for further investigations.

2. Isolation and characterization of DF-degrading *Comamonas* sp. strains.

Owing to the similarity of metabolism between DF and dibenzo-*p*-dioxin (DD) in the microorganisms, DF was used as a model substrate in enrichment culture. Three DF-degrading strains were newly isolated from roots of white clover and poplar trees grown in DF-contaminated soil samples. These strains designated KD2, KD7 and PD1, were identified as *Comamonas* sp. on the basis of the sequences of 16S rDNA and physiological characteristics. After 12 days incubation, strains KD2, KD7 and PD1 can reduce DF by 32%, 14% and 19% from the initial concentration, respectively. Among these strains, strain KD7, isolated from the inside of roots, showed the most efficient degradation of DF than the other strains. In the presence of DF, strain KD7 also showed the ability of co-metabolism to degrade some chlorinated DD.

The metabolites produced when strain KD7 was incubated with DF and DD were identified by gas chromatography-mass spectrometry (GC-MS) analysis. Interestingly, it was exhibited that strain KD7 was recognized to have two pathways for DF degradation, beginning with angular dioxygenation at carbons 4 and 4a, and lateral dioxygenation at carbons 1 and 2, respectively. Furthermore, strains KD2 and KD7 possessed not only efficient root colonization for clover but also a promotion effect on growth of clover. They are the first reported *Comamonas* sp. strains capable of utilizing DF as a sole source of carbon. It provided additional information on the diversity of DF-degrading bacteria.

3. Cloning and identification of genes involved in DF degradation from *Comamonas* sp. strain KD7.

A 4.3-kb *Pst*I DNA fragment was cloned in *Escherichia coli* from *Comamonas* sp. strain KD7 by shot-gun cloning. The *E. coli* colonies carrying this fragment were assayed for extradiol dioxygenase activity by spraying an aqueous solution of 2,3-dihydroxybiphenyl (DHB). The colonies exhibiting a yellow color were selected as positive clones. The database research at NCBI with nucleotide sequence of this fragment revealed a 99% identity to the *meta*-cleavage enzyme, 2,3-dihydroxybiphenyl-1,2-dioxygenase (BphC) of *Terrabacter* sp. strain DPO360. Furthermore, the gene encoding angular dioxygenase in *Comamonas* sp. strain KD7 was cloned by using a degenerate set of PCR primers designed by using conserved sequences of the dioxygenase alpha subunit genes. The result of homology search exhibited 96% similarity to the angular dioxygenase of *Terrabacter* sp. strain YK3. Interestingly, a gram-negative bacterium, *Comamonas* sp. strain KD7 carried very similar DF-degrading genes as gram-positive bacteria. Southern blot hybridization analysis showed that these DF-degradation genes exist in a linear plasmid isolated from strain KD7. These results suggest a possibility that *Comamonas* sp. strain KD7 obtained the genes responsible for DF-degradation via horizontal gene transfer.

4. Phytotoxicity of PCDD/F as indicator to assess the efficiency of plant-microbe combinations.

Plants used for rhizoremediation of polluted soils must be able to produce sufficient biomass and to maintain higher microbial populations. Furthermore, successful rhizoremediation of the contaminated soils requires the plants to tolerate the contaminants. Phytotoxicity test was developed to assess the

acute toxicity of chemical substances. Toxicity assessment of chemical substances serves as a tool for evaluating the stress tolerance of plants. In this thesis, the comparative inhibition of germination rate of white clover by selected PCDD/F compounds was investigated. The suitability of germination rate was evaluated to determine phytotoxicity endpoint. The dose-response relations for all tested compounds were observed. The statistical model based on quantitative structure-activity relationship (QSAR) for estimating phytotoxicity of PCDD/F was obtained. This model showed that the germination rate inhibition of PCDD/F compounds correlated with their logarithm of 1-octanol/water partition coefficient ($\log K_{ow}$) and energy of the lowest unoccupied molecular orbital (E_{lumo}). Moreover, after the inoculation with *Comamonas* sp. strain KD7, as a result, the germination rate inhibition on white clover could be greatly reduced. These results are indicating the efficiency of white clover inoculated by *Comamonas* sp. strain KD7 in rhizoremediation. The phytotoxicity of contaminants could serve as a novel indicator to estimate the potential of rhizoremediation.

5. Rhizoremediation experiments.

At first, the effects of the combination comprised of white clover and *Comamonas* sp. strain KD7 on the degradation of DF and 2,4,8-trichlorodibenzofuran (2,4,8-T_3CDF) were investigated by hydroponic cultures. White clovers were cultivated under hydroponic conditions in the presence/absence of sand. In the absence of sand, strain KD7-inoculating white clovers showed significant reductions of DF and 2,4,8-T_3CDF ($p \leq 0.05$) compared to the uninoculated plant after 2 weeks of growth. A similar result was obtained on the reduction of DF in the presence of sand.

Prior to the rhizoremediation experiments, it is necessary to develop a rapid, cost-effective and reliable extraction method for PCDD/F from soil. In the standard U.S. Environmental Protection Agency (EPA) method, soxhlet extraction, is labor intensive, and requires expensive equipment. An investigation was undertaken to evaluate the mechanical shaking ultrasonic method for the extraction of PCDD/DF from contaminated soil. Five solvents were chosen to represent a range of properties: hexane, dichloromethane, acetone, 1:1(v:v) methanol and hexane, and 1:1(v:v) acetone and dichloromethane. One extraction cycle consisted of 30min of ultrasonic extraction and 1hr of shaking period. Among these solvents, 1:1(v:v) acetone and dichloromethane exhibited the highest efficiency by contrast with soxhlet extraction. Although four-cycle extraction showed slightly higher efficiency than three-cycle extraction, however, their difference was not statistically significant ($p > 0.05$). Consequently, the three-cycle mechanical shaking ultrasonic method with 1:1(v:v) acetone and dichloromethane for the extraction of PCDD/DF was used in further rhizoremediation soil experiments. After 12 weeks of growth, compared to the control soil, only a significant reduction of DF was observed in white clover planted soil, however, in the soil planted with strain KD7 inoculated white clover, the enhanced degradation of DF, 2,8-DCDF, 2,4,8-TCDF, 1-CDD, 1,2,4-T_3CDD and 1,2,3,4-T_4CDD were confirmed.