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SUMMARY OF DOCTORAL THESIS

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Title:

Genetic and Ecological Studies on 2,4-Dichlorophenoxyacetic Acid (2,4-D)- and 2,4,5-Trichlorophenoxyacetic Acid (2,4,5-T)-Degrading Bacteria in Vietnamese Soils

ベトナム土壌における 2,4-ジクロロフェノキシ酢酸（2,4-D）および 2,4,5-トリクロロフェノキシ酢酸（2,4,5-T）分解菌の遺伝学的および生態学的研究

The phenoxyherbicides, 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), have been widely used for the control of broad-leaf weeds since 1940s. They were major components of the product Agent Orange, which was used extensively as a defoliant in the Vietnam conflict. These herbicides might have influenced soil microbial communities as well as ecosystem. Genetic and ecological study on 2,4-D- and 2,4,5-T-degrading bacteria in Agent Orange-contaminated and uncontaminated soils in Vietnam are examined.

A variety of 2,4-D- and 2,4,5-T-degrading bacteria were isolated country-wide from ten Vietnamese soils, with or without history of Agent Orange. The 353 degraders were phylogenetically grouped into three majors belonging to *Burkholderia* spp. (43.3% of all degraders), *Sphingomonas* spp. (40.2%), and *Ralstonia* spp. (15.3%) and two minors of *Bradyrhizobium* sp. (0.8%) and *Nocardioides* sp. (0.3%). The 2,4,5-T degraders, 65% of all degraders, were isolated from all soil samples and their 16S rRNA genes were the most homologous with that of *Sphingomonas* spp., *Burkholderia* spp., or *Bradyrhizobium* sp. The degraders of *Burkholderia* spp. were isolated only from central and south sites, while those of *Ralstonia* spp. were found only in the north sites with one exceptional strain. The *Sphingomonas* spp. degraders with same 16S rRNA genes were isolated country-wide, while four phylogenetically different groups were found in one site.

TfdA genes, encoding α–ketoglutarate-dependent 2,4-D dioxygenase, were found in *Burkholderia* spp., *Ralstonia* spp., and *Nocardioides* sp. strains. *TfdAα*, *tfdA* homolog, was found in *Bradyrhizobium* sp. strain. Absence of *tfdA* and *tfdAα* in *Sphingomonas* sp. strains were confirmed by PCR and Southern hybridization. 2,4-dichlorophenol hydroxylase genes (*tfdB*) were detected in all strains, and phylogenetically divided into three groups. The first one consisted of *Ralstonia* spp., *Nocardioides* sp., and *Burkholderia* spp., and the others were *Sphingomonas* spp. and *Bradyrhizobium* sp. strains. *CadA* genes, which have a nucleotide sequence similar to that of 2,4,5-T oxygenase gene (*tftA*), were found in *Bradyrhizobium* sp. and *Sphingomonas* sp. strains, and clearly separated between the two genera. The 2,4,5-T oxygenase gene and 2,4,5-trichlorophenol 4-monooxygenase gene (*tftC*) were found only

in 2,4,5-T-degrading *Burkholderia* sp. strain.

2,4-D-degrading *Bradyrhizobium* strains have been found as a new group of 2,4-D degraders in the environments that had no history of pesticide applications. In our study, 2,4,5-T-degrading *Bradyrhizobium* strain was isolated from contaminated soil and able to degrade both 2,4-D and 2,4,5-T, suggesting adaptation to the contaminated environments for its proliferation. However, substrate specificity for chlorophenols and phenol was the same in both strains. Nucleotide and deduced amino acid sequence of *tfdB* in 2,4,5-T-degrading *Bradyrhizobium* sp. strain were 59% and 64% identical to those of *tfdB* in 2,4-D-degrading *Bradyrhizobium* sp. strain, respectively. The *tfdB* gene of 2,4,5-T-degrading *Bradyrhizobiun* sp. was phylogenetically separated from that of 2,4-D-degraders, suggesting different origins of these genes. Based on codon usage patterns and GC content of the genes, *tfdB* genes in the *Bradyrhizobium* sp. strains were obtained through horizontal gene transfer. TfdB of 2,4-D-degrading *Bradyrhizobium* sp. strain expressed in *Escherichia coli* showed the highest activity for 2,4-dichlorophenol but narrower range of activity for the other chlorophenols than previously reported TfdBs. TfdB of 2,4,5-T *Bradyrhizobium* sp. has not been examined yet.

As *tfdA* and *tfdB* are almost identical among the isolates belonging to *Ralstonia* and *Burkholderia* strains, country-wide distribution of the genes by plasmid-mediated horizontal gene transfer was suggested. Presence of plasmids was observed in most of isolates except for those homologous with *Sphingomonas* spp., *Bradyrhizobium* sp., *Nocardioides* sp. and *Burkholderia sacchari* JS150 strains. At least four different plasmids that carried the *tfd* genes were found in the *Burkholderia* spp. and *Ralstonia* spp. degraders without relation to the sites and phylogenetic groups. These observations suggest that the *tfd* degradative genes have been distributed on diverse plasmids among the several species of the genera in Vietnamese soils.

Diverse 2,4-D- and 2,4,5-T degrading bacteria were isolated from Vietnamese soils by enrichment-cultivating method. The enrichment condition may have selected 2,4-D- and 2,4,5-T-degraders. Changes in bacterial community in the soil-water suspensions during the enrichment period were examined using denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA gene. Almost all major bands at degradation of 2,4-D and 2,4,5-T corresponded to the genes of 2,4-D- and 2,4,5-T-degrading isolates and successions of diverse 2,4-D- and 2,4,5-T-degrading bacterial community were demonstrated in DGGE profile. These results suggested that the 2,4-D- and 2,4,5-T-degrading isolates were responsible to the degradation of 2,4-D and 2,4,5-T in the soil-water suspensions. However, major bands of a non-degrader were found in some cases. Therefore, importance of combined approach of culture-dependent and independent methods is demonstrated for understanding the relationship between the structures and function of bacteria communities. Further research should be taken to clarify factors which determine behavior of the degrading bacteria in the soil environments.