ANALYSIS OF MICROBIAL COMMUNITY STRUCTURE IN HEAVY HERBICIDE/DIOXIN CONTAMINATED SOILS BY PCR-SSCP FINGERPRINT

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Abstract

The analysis of uncultured microbial community structure as well as composition and distribibution of 2,4-D gene fragments (*tfdA*) in nine heavy, long-term herbicide and dioxin contamined soils at US former military base in Danang, Vietnam by PCR-SSCP fingerprint and MPN techniques was carried out. Members of five different bacterial classes were found: *Actinobacteria*, *Acidobacteria*, α-, β- and γ- *proteobacteria* and *Burkholderiaceae* seems to be predominant family. *Acidocella*, *Acidiphillum*, *Acidobacterium*, *Rhodococcus* and *Mycobacterium* were also detected in various samples. There was no significant difference in bacterial community structure between heavily and slightly contaminated sites. *tfdA* gene that encoded an α-ketoglutarate dependent 2,4-D dioxygenase was found in all of nine soil samples and high number of *tfdA* gene copies of 1×10^5 to 6×10^6 per gram soil was observed. *tfdA* gene fragments of classes I and II in *Wautersia eutropha* JMP 134 (formerly *Ralstonia eutropha* JMP134) and *Burkholderia* sp. RASC were detected to be predominant, while *tfdAa*-genes, recently identified from *α-proteobacteria*, were only found in minor abundance in herbicide/dioxin contaminated soils.

Introduction

During Vietnam War, herbicides were used to defoliate the jungle canopy and destroy crops. An estimation, from 1961 to 1971 the United States Armed Forces in South Vietnam has used approximately 100 milion liters of herbicide [13]. Several types and mixture of chemicals were selected such as Agent White, Agent Blue, Agent Pink, Agent Green, Agent Purple and Agent Orange. The herbicides containing 2,4,5-T were Agent Green, Agent Purple and Agent Orange and only these Agents were contaminated with dioxins including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) with varying levels [13]. After 40 year, consequences of usage of Agent Orange and other herbides to US and foreingner veterans as well as Vietnamese who involved to the Vietnam War and natural environment are continuing. Persistently high levels of dioxins in Vietnam soil and biota, as well as in samples of Vietnamese serum, adipose tissue and breast milk have been reported and so-called “hot spots” identified [3].

In order to recovery soil ecology in highly herbicide contaminated areas – “hot spot”, several treatments were carried out such as isolation, landfill, chemical treatment and bio-treatment or combination of these approaches [2, 9]. Bio-treatment or bioremediation is the safety and low cost method althought time requirement. Indigenous microorganisms in xenobiotic contaminated environment were known as ability to degrade pollutants and play an important role to self cleanup and/or involve to bioremediation proccess. The evaluation of a polluted site before as well as during bioremediation often involves detection and enumeration of the quantity and activity of xenobiotic-degrading microorganisms [14]. However, by the cultivating method only 1% microbial number could be detected [6]. Several advanced molecular techniques were applying including fingerprint analysis. Among these fingerprints, PCR- single strand conformation polymorphisms (PCR-SSCP) is the used PCR-based method for point mutation detection and now being widely used in study diversity of microbial communities from rhizospheres, compost, analyze bulk soil from limed forests, bioaugmentation with groundwater microcosms, bacterial biofilms on stone surfaces, sludge and water samples from plant-sewage treatment systems and groundwater and aquifer material from contaminated sediments, aromatic hydrocarbon contaminated soil based on 16S rDNA gene and catabolic gene sequences. In order to have a new insight of

microbial structure and natural catabolic gene potential that existed in long term herbicide/dioxin contaminated soil, the PCR-SSCP fingerprint and MPN-PCR (most proable number-PCR) analyses were carried out.

Material and methods

Nine herbicide/dioxin contaminated soils were collected from an approximately area of 10,000 m^2 in an US former military base in Danang, Vietnam. Soils were sampled from a depth of 5-20 cm. Eight samples were collected from different sites in heavy contamination sites and designed as HDN1, HDN2, HDN3, HDN4, HDN5, HDN6, HDN7, and HDN8. Sample HDN9 was taken from slightly contaminated site that grass growing.

Total soil DNA was isolated and purified by the commercial Bio101 kit. Sequences of 16S rRNA genes (V4-V5 regions) and *tfdA* genes (catabolic gene family involved in 2,4-D degradation) were PCR amplified and subject to SSCP fingerprinting [12].

Sequences were obtained from the PCR-SSCP patterns, aligned by using Clustal X and compared with reference genes in RDP and GenBank databases.

Phylogenetic trees were constructed by using Neighbour-Joining method. Bootstrap values higher than 60 % are shown on branch positions. The partial sequences of 16S rDNA gene in this study were submitted and disposed in GenBank under accession numbers from DQ991255 to DQ9912300. The sequence fragments of *tfdA* gene were also disposed in GenBank under accession numbers EF600716, EF600718, EF600719, EF600720, EF600724- EF600728, EF600731- EF600735, EF600738- EF600742, EF600744, EF600752 and EF600753. Enumeration of *tfdA* gene copy numbers per gram of soil was carried out by MPN-PCR [1].

Results and discussion

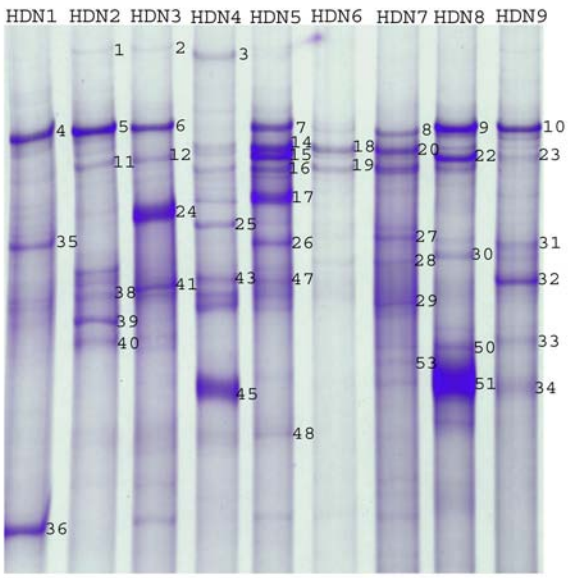

Fig. 1: Cultivation-independent PCR-SSCP profiles of bacterial communities in herbicide/dioxin contaminated soils. Bands which were successfully isolated and sequenced are shown in their corresponding positions.

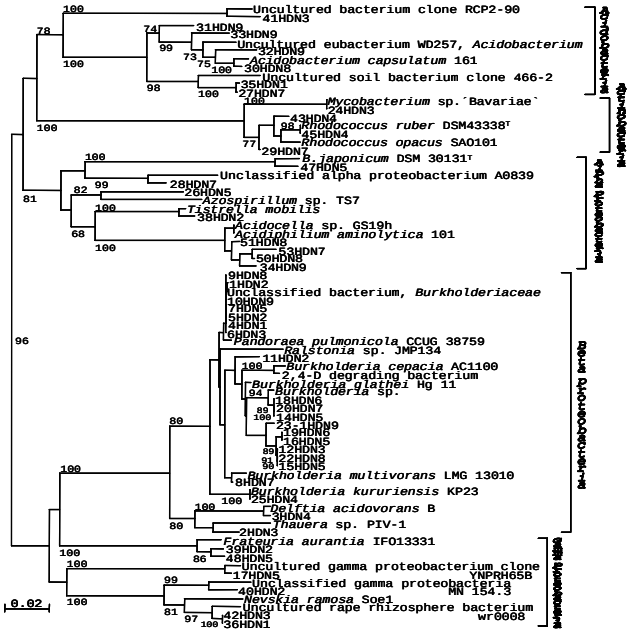

Fig. 2: Phylogenetic tree of sequences obtained from 16S rDNA amplification fragments separated by SSCP gel. and references. Bar, 2 substitute nucleotides per 100 nucleotides

The results in Fig.1 and Fig. 2 showed that members of five different bacterial classes were detected: *Actinobacteria*, *Acidobacteria*, α-, β- and γ- *proteobacteria*. *Burkholderiaceae* seems to be predominant family. Several *Burkholderiaceae* strains have previously been described to be capable of degrading 2,4-D or 2,4,5-T [5, 11]. *Acidocella*, *Acidiphillum* and *Acidobacterium* were also observed in various samples. Predominant bacterial

groups *Burkholderia*, acidophilic bacteria are similar to those observed in PCB and 2,4-D contaminated soils [7, 10]. *Rhodococcus* and *Mycobacterium* were also detected. These general are often reported to be capable of degrading chlorinated or polycyclic aromatics [4, 8]. There was no significant difference in bacterial community structure between heavily and slightly contaminated sites.

Table 1: Analysis of *tfdA* gene copy MPN in herbicide/dioxin contaminated soils

Samples	HDN1	HDN2	HDN3	HDN4	HDN5	HDN6	HDN7	HDN8	HDN9
tfdA	3.67 x 10^5	1.31 x 10^5	1.11 x 10^5	1.06 x 10^5	5.78 x 10^5	5.99 x 10^6	2.85 x 10^5	5.27 x 10^5	5.99 x 10^6

2,4-D and 2,4,5-T are two major contaminants that were detected with high content in herbicide/dioxin contaminated soils in Danang. The degradation pathway of 2,4-D had been found in the α-, β- and γ-*proteobacteria* with the involvement of genes located on mobile genetic elements (catabolic plasmids) and/ or within the chromosome [5]. 2,4-D is transformed into 2,4-Dichlorophenol (2,4-DCP) by an α-ketoglutarate dependent 2,4-D dioxygenase encoded by *tfdA* [5]. High number of *tfdA* gene copies of 1 x 10^5 to 6 x 10^6 per gram soil was observed (table 1).

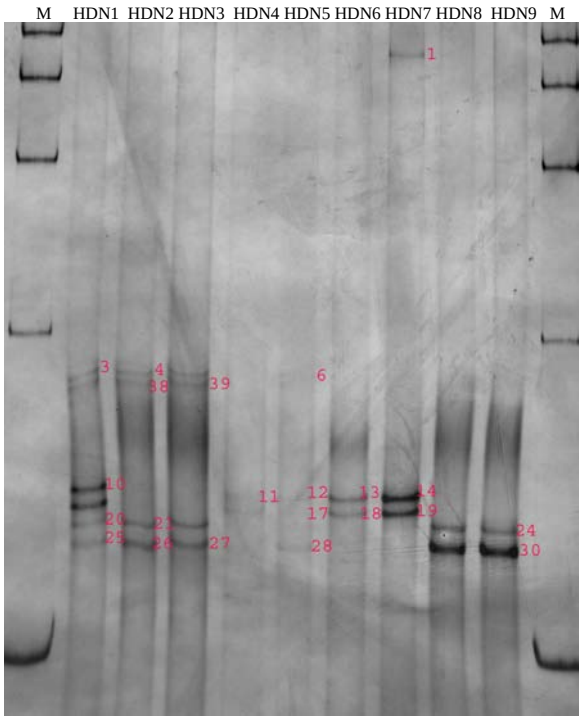

Fig. 3: Analysis of *tfdA* gene fragments from herbicide/dioxin contaminated soil DNA by PCR-SSCP with the single-strand removal method. Bands which were successfully isolated and sequenced are shown in their corresponding positions.

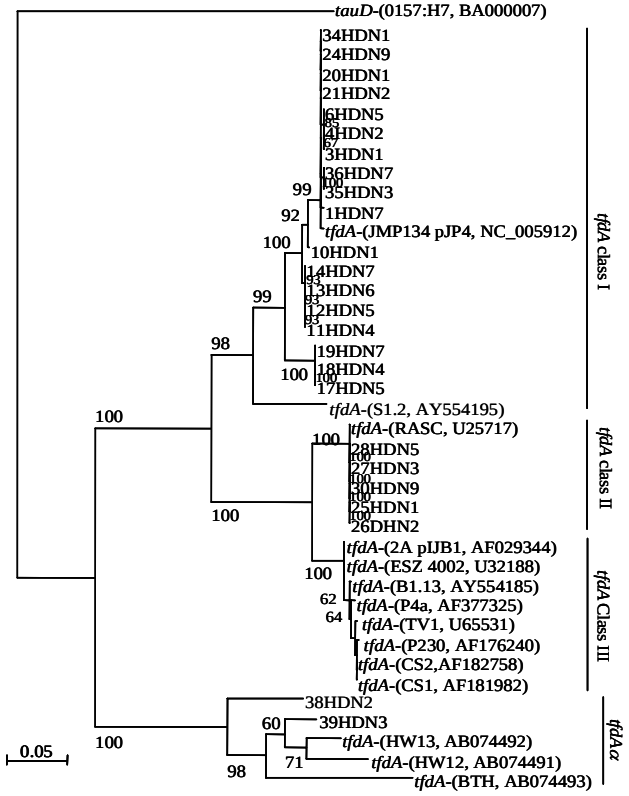

Fig. 4: Phylogenetic tree of *tfdA* gene sequences recovered of PCR-SSCP bands and representative *tfdA* genes. Bar, 5 substitute nucleotides per 100 nucleotides.

Classes **I** and **II** of *tfdA* gene of *Wautersia eutropha* JMP 134 (formerly *Ralstonia eutropha* JMP134) and *Burkholderia* sp. RASC were observed to be predominant, while *tfdAa*-genes, recently identified from *α-proteobacteria* [5], were only observed in minor abundance in herbicide/dioxin contaminated soils (fig.2).

Results from this study revealed that the existence of diverse bacteria groups in long-term herbicide/dioxin contaminated soils in Danang, Vietnam. Bioremediation treatments of this contaminated soil were successfully carried out [2, 9]. The homologous of uncultured bacterial clones in these soil and 2,4-D, 2,4,5-T, dioxin and dioxin-like compound degrader indicated that the native bacteria were play and importance role in the detoxification processes of chlorinated compounds during natural attenuation and bioremediation.

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