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Application of DNA Probe Method for Detection of 2,4-Dichlorophenoxyacetic Acid Degrading Bacteria in Soil

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Abstract : Total bacterial community DNA, which was extracted from microcosm soil and field soil after 2,4-D amendments, was analyzed on Southern blots, using the *tfdA* gene probe derived from plasmid pJP4 and the *Spa* probe from *Sphingomonas paucimobilis*. Southern blot analyses with total bacterial DNA extracted from soils inoculated with *Pseudomonas cepacia*/pJP4 revealed that DNA probe method could detect the 2,4-D degrading bacteria down to 10^5 cells/g dry soil. In the microcosm experiment, there was a good correlation between 2,4-D degradation and banding patterns in hybridization analyses performed after each 2,4-D treatment using the two probes. When bacterial DNA extracted from microcosm soil was hybridized with the *tfdA* probe, a change in the position of hybrid bands was observed over time in a Southern blot, suggesting that population change or possibly genetic rearrangement in 2,4-D degrading microbial populations occurred in this soil. With the *Spa* probe, one hybrid DNA band was persistently observed throughout the five 2,4-D additions. When bacterial DNA isolated from the field soil was probed with the *tfdA* and *Spa*, strong hybridization signal was observed in the 100 ppm-treated subplot, weak signal in the 10 ppm-treated subplot, and no significant signal in the 1 ppm-treated and control subplots. The data show that DNA probe analyses were capable of detecting and discriminating the indigenous 2,4-D degrading microbial populations in soil amended with 2,4-D under laboratory and field conditions.(Received June 25, 1996; accepted August 26, 1996)

Introduction

2,4-dichlorophenoxyacetic acid, a widely used herbicide, is rapidly degraded by soil microbial populations.[1-5] Repeated 2,4-D applications to soil cause a significant increase in the indigenous 2,4-D degrading populations.[6-9] However, it has not been easy to analyze population or genetic changes of microbial communities in natural environments in response to the herbicide due to the lack of the appropriate tools for detection of multiple populations in the community.

Traditionally, the isolation of pure cultures[10] and the fluorescent antibody techniques[11,12] have been used to confirm the presence of specific microorganisms in environmental samples, each of which is useful but limited to some aspects. The cultural method is selective for certain microorganisms which can be cultivated in particular laboratory media. Although the fluorescent antibody technique has high specificity even to the strain level, it is labor-intensive, relatively expensive and is not well-suited to routine screening for the presence of organisms.

As a new methodological approach, gene probes were developed to detect specific microorganisms in environmental samples by using colony hybridization.[13-15] But this method requires that microorganisms be cultivated in laboratory media prior to analysis. Since more than 90% of soil microorganisms are non-culturable in laboratory media,[16] the colony hybridization method would have limited utility for detecting these non-culturable microorganisms in soils.

On the other hand, a DNA probe method involving total bacterial community DNA extracted from soil was suggested to be able to detect microbial populations in natural environmental samples without requirement of cultivation.[17,18] The DNA probe method was useful in evaluating the response of 2,4-D degrading soil microbial population[19] and successfully used to distinguish and identify specific 2,4-D degrading bacteria in microcosm soil treated with 2,4-D.[20]

The limitation of this DNA probe method, however, is the limited sensitivity. This method generally requires a large number of targets in order to detect a signal, which means that the native target population should be amplified enough to have a detectable response. Another cause of limited sensitivity is the

Key words : DNA probe method, 2,4-D degrading bacteria, detection

divergence of the target DNA sequence in the native microbial population. Due to this divergence of the target sequence, it is difficult to detect and assess all of the responsive, diverse populations with a single gene probe.

In this study, we applied DNA probe method to detect and identify 2,4-D degrading bacteria in soil using two different DNA probes and evaluated its sensitivity and specificity under laboratory and field conditions.

Materials and Methods

Bacterial strains and media

Pseudomonas pickettii/p712 capable of utilizing 2,4-D as a sole carbon was isolated from the Gene Flow plot at the Long-Term Ecological Research (LTER) site at the Kellogg Biological Station (KBS) in Hickory Corners, Michigan.[21] *Pseudomonas cepacia*/pJP4 was obtained from J. M. Tiedje, Michigan State University. Peptone-tryptone-yeast extract-glucose medium[22] was used for strain purification and maintenance. For the isolation of plasmid, bacterial strains were cultivated in MMO mineral medium[23] containing 2,4-D at a concentration of 500 ppm at 30℃ and then plasmid DNA was extracted by using the method of Hirsch *et al.*[24]

Inoculation of *P. cepacia*/pJP4 in soil

Culture of *P. cepacia*/pJP4 grown in PTYG at 30℃ was harvested by centrifugation at 10,000×g for 10 min at 4℃ and washed twice with an equal volume of 15 mM sodium phosphate buffer (pH 7.0). The cells were resuspended in 0.1 volume of sodium phosphate buffer, kept in ice, and enumerated by using a counting chamber.

Soil (loam) with no history of 2,4-D treatment was taken from the LTER site of KBS, on which a plot has been subjected to microbial ecological research in response to 2, 4-D treatment as one of the LTER projects.[25] Soil was stored at field moisture levels at 4℃ until used. Soil samples (500 g) which had been sifted through a 2 mm sieve were adjusted to a water content of ca. 10% (wt/wt), transferred to polyethylene wide-mouth bottles, inoculated with *P. cepacia*/pJP4 at densities of ca. 1.0×10^4, 1.0×10^5, 1.0×10^6, 1.0×10^7, and 1.0×10^8 cells/g soil, respectively, and were thoroughly mixed with sterile spatulas. Two weeks after incubation at room temperature, each 50 g soil sample was used to isolate total bacterial DNA by the cell extraction method.[17]

2,4-D amendment of soil

The KBS soil samples (500 g) which had been sifted through a 2-mm sieve were transferred to sterile polyethylene wide-mouth bottles and the soil water content was adjusted to 10% by adding sterile distilled water. 2,4-D in phosphate buffer was added to the 10% moisture soil at the concentration of 250 ppm, thoroughly mixed, and then incubated at room temperature. Each control soil received only phosphate buffer without 2,4-D. The disappearance of 2,4-D in soil was monitored as described below and the soil was respiked for each of 5 cycles of degradation. At the end of each cycle of degradation, a 50 g subsamples were taken from the 2,4-D-treated and control soils and total soil bacterial DNA was extracted with cell extraction method.

DNA from Michigan field soil that had been treated with 2,4-D for three years and extracted by the same procedure[25] was used for evaluation of DNA probe method in the open environment.

Analysis of 2,4-D concentration in soil

For analysis of the concentration of 2,4-D in soil, 1 g soil samples were combined with 1 m*l* of distilled water in an eppendorf tube and vortexed for 1 min. The soil was pelleted by centrifugation in a microfuge (14,000 rpm, 5 min) and the supernatant was transferred into a clean eppendorf tube. This sample was filtered through a Millipore Millex-GS syringe filter and analyzed for 2,4-D with an Hewlett Packard series 1050 HPLC equipped with Lichrosorb RP-18 column (Anspec Co., Ann Arbor, MI) and a UV detector set at 230 nm, using methanol/0.1% phosphoric acid (60 : 40) as eluant.

Hybridization analysis

For Southern blot analysis, total soil bacterial DNA (1.5 ug) was digested with appropriate restriction endonucleases according to the manufacturers' specifications, size fractionated by electrophoresis through horizontal 0.7% agarose gels, and transferred to nitrocellulose hybridization membranes by the capillary technique.[26] The DNA was fixed to the membrane with a UV cross-linker and then hybridized with a *tfdA* gene probe subcloned from the well-known 2,4-D degradative plasmid pJP4 and also a *Spa* probe derived from *Sphingomonas paucimobilis* 1443.[21]

Prehybridization, hybridization and posthybridization washes were performed as described in the previous paper (14). Hybridization signals were visualized with a Betascope radioactive blot anaylzer (Betagen Corp., Waltham, Mass.) or by autoradiography with X-Omat AR film (Kodak, Rochester, N. Y.) exposed at −70℃, using a Quanta III intensifying screen (Sigma). The exposure time were 1 to 7 days depending on the intensity of the radioactive signal.

Results and Discussion

Sensitivity of DNA probe method

To analyze the detection limit of DNA probe method, *P. cepacia*/pJP4 was inoculated in the microcosm soils at

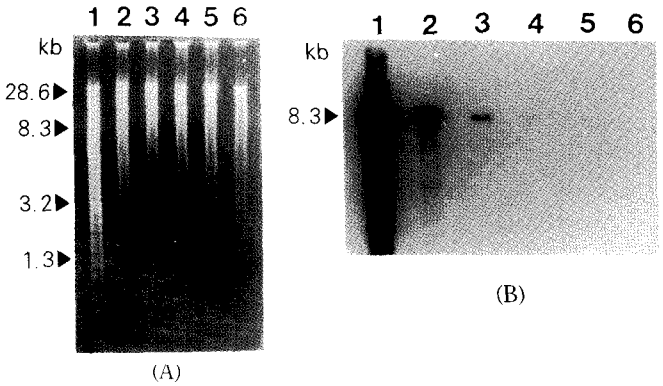

Fig. 1. Detection of inoculated *P. cepacia*/pJP4 in soil by Southern blot analysis. Soil bacterial DNA samples were digested with *Eco*RI, size-fractionated by agarose gel electrophoresis (A), transferred to nitrocellulose membrane, and then hybridized with the *tfdA* probe (B). 1, 2, 3, 4, 5, and 6, DNA samples from soils inoculated with *P. cepacia*/pJP4 at the densities of ~ 10^8, 10^7, 10^6, 10^5, 10^4, and 0 cells/g soil, respectively.

various densities and then total bacterial community DNAs were isolated from the soils by using the cell extraction method. The soil DNAs appeared to be readily digested to completion with *Eco*RI, showing DNA smears on agarose gel (Fig. 1-A). When the digested DNAs were subjected to Southern transfer and hybridized to the *tfdA* probe (Fig. 1-B), one band (8.3 kb) of hybridization was observed with DNA samples isolated from soils inoculated with *P. cepacia*/pJP4 at densities of ca. 10^8, 10^7, 10^6, and 10^5 cells/g soil. The band of 8.3 kb in size exactly corresponds to the *Eco*RI fragment of plasmid pJP4, suggesting that the bands came from the plasmid pJP4 of the inoculated strain *P. cepacia*/pJP4 in natural soil. Quantitative hybridization analysis using the Betascope radioactive blot analyzer (data not shown) indicated that the hybridization signal exhibited about 9-fold differences between any two adjacent bands. No hybridization was observed with the DNA samples isolated from soil inoculated at density of 10^4 cells/g soil and soil not inoculated with *P. cepacia*/pJP4. This result shows that DNA probe method in conjunction with Southern transfer is capable of detecting organisms down to 10^5 cells/g soil and quantifying their relative population levels in natural soil without cultivation of the organisms.

Degradation of 2,4-D in soil

Exposure of 2,4-D degrading populations of soil to 2,4-D for the first time resulted in slow degradation for first treatment of 2,4-D in the microcosm soils (data not shown), requiring about 3 weeks for the added 2,4-D to be degraded completely. The rate of degradation for subsequent additions of 2,4-D was greatly increased, requiring 1 week or less for the complete degradation for each of four more 2,4-D additions.

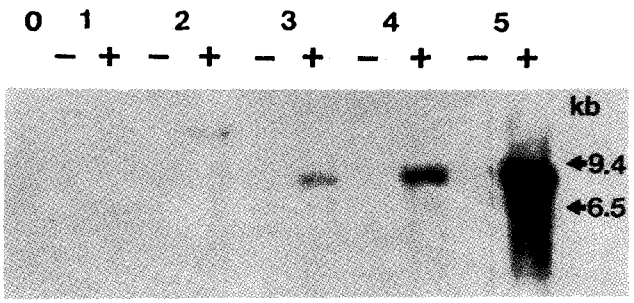

Fig. 2. Detection of indigenous 2,4-D-degrading microbial populations in microcosm soil by Southern blot analysis with the *tfdA* probe. 0, 1, 2, 3, 4, and 5, total soil bacterial community DNA isolated after zero, one, two, three, four, and five treatments with 2,4-D, respectively. +, 2,4-D treated ; −, no 2,4-D control. DNA samples were digested with *Bam*HI.

DNA probe hybridization with microcosm soil DNA

When total bacterial community DNAs extracted from the microcosm soils treated with 2,4-D were hybridized to the *tfdA* probe, a single band of hybridization was detected from the second through the fifth addition (Fig. 2). The location of the band of hybridization shifted between the second and third treatments and thereafter remained constant. A possible interpretation of this data is that the dominant 2,4-D degrading population present after two treatments was displaced by another 2,4-D degrading population that then stably dominated throughout the course of the experiment. Alternatively, it is possible that the sequence encoding the *tfdA* homology was somehow rearranged resulting in shift in the band of hybridization and that this derivative strain displaced the original population possibly because of its increased fitness. For 2,4-D free control soils, the total bacterial DNA had no detectable hybridization to the *tfdA* gene probe (Fig. 2).

To identify the responsible *tfdA*-hybridizing strain, all 2,4-D degrading bacteria isolated from this KBS soil were screened for homology with the *tfdA* probe. Among them, *P. pickettii* was expected to correspond to the hybridization band of the microcosm soil bacterial DNA. Plasmid DNA obtained from this strain was compared to total soil bacterial DNA of the fifth treatment of 2,4-D in Southern analysis using the *tfdA* probe (Fig. 3). Matching band patterns were obtained between these two DNA samples when digested with three different restriction enzymes, suggesting that the dominant 2,4-D degrading organism responsible for the hybridization signals observed in soil bacterial DNA from the third to fifth treatments of this microcosm experiment could be *P. pickettii*. Moreover, its population level was increasing with repeated additions of 2,4-D through the fifth treatment, as indicated by increasing band intensities on Southern blot (Fig. 2). Since each lane contained the

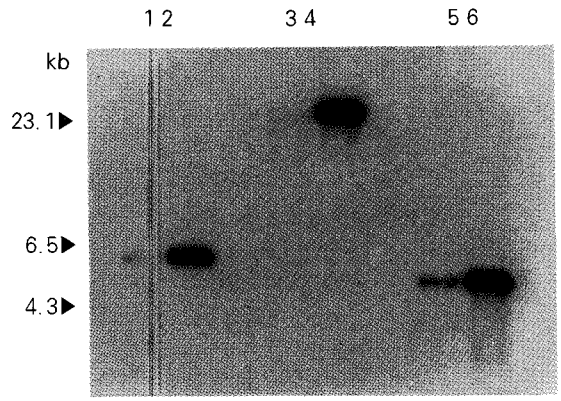

Fig. 3. Comparison of *tfdA* hybridization bands produced by total soil bacterial DNA and plasmid DNA digested with three different restriction enzymes. Lanes 1, 3, and 5, total soil bacterial DNA after the fifth treatment with 2,4-D ; lanes 2, 4, and 6, plasmid p712 DNA from *P. pickettii*/p712. DNA samples were digested with *Bgl*II (lanes 1 and 2), *Hind*III (lanes 3 and 4), or *Pst*I (lanes 5 and 6).

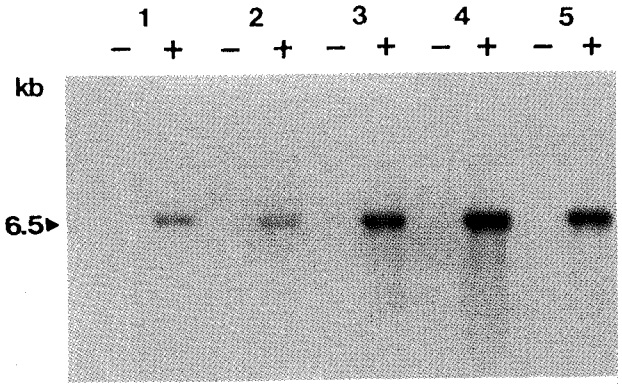

Fig. 4. Detection of indigenous 2,4-D-degrading microbial populations in microcosm soil by Southern blot analysis with the *Spa* probe. 0, 1, 2, 3, 4, and 5, DNA samples from soils after zero, one, two, three, four, and five treatments with 2,4-D, respectively. +, 2,4-D treated ; −, no 2,4-D control. Soil bacterial DNA samples were digested with *Bam*HI.

same amount of total DNA (1.5ug), the intensity of the hybridization for each time point is proportional to the amount of target DNA in each DNA sample. Quantitative hybridization analysis using the Betascope radioactive blot analyzer indicated that the hybridization signal exhibited a 5-fold increase when the third 2,4-D treatment was compared to the fifth treatment (data not shown), suggesting a five fold increase of the target sequence in the degrading population detected.

When total soil bacterial DNA was digested with *Bam*HI and hybridized with the *Spa* probe (Fig. 4), a single band (6.5 kb in size) of hybridization was detected throughout the five treatments of soil with 2,4-D. The position of the hybridization bands remained constant throughout the experiment, indicating that it represent a single, stably maintained population. Since the *Spa* probe was developed by cloning a 6.5 kb *Bam*HI fragment from *S. paucimobilis* 1443 that has been a dominant 2,4-D degrader in this soil,[21] the hybridization band of 6.5 kb in size appeared to have come from this strain. Again, there was no hybridization signal to the total bacterial DNA of 2,4-D free control soils (Fig. 4).

DNA probe hybridization with field soil DNA

To analyze the sensitivity and specificity of DNA probe method in field condition, total bacterial DNAs extracted from field subplots were hybridized to the *tfdA* probe after *Bam*HI digestion, gel separation, and Southern transfer (Fig. 5). Three clear bands (4.5 kb, 6.8 kb, 18.1 kb in size) were detected in the soil DNA sample of 100 ppm-treated subplot and weak hybridization signal was observed from 10 ppm-treated subplot. No significant hybridization signal was observed from 2,4-D free control subplot and from 1 ppm-treated subplot, where the 2,4-D degrading population was below the detection limit, 10^5 cells/g soil.[21] This result shows that there is an increasing correlation between hybridization signal and 2,4-D application rate and that DNA probe analysis requires 2,4-D application of at least 10 ppm to have a detectable hybridization signal in field condition.

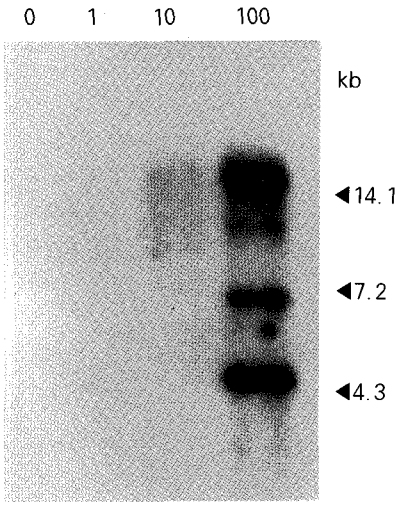

Fig. 5. Detection of indigenous 2,4-D-degrading microbial populations in field soil. Total soil bacterial community DNA was isolated from soil samples of four subplots which were treated with 0, 1, 10, and 100 ppm of 2,4-D, respectively. The DNA was digested with *Bam*HI, size-fractionated, transferred to nitrocellulose membrane, and then hybridized with the *tfdA* probe.

When total bacterial DNAs from field subplots were digested with *Hind*III and hybridized with the *Spa* probe (Fig. 6), three hybridization bands were commonly observed in all of the soil DNA samples, and two discrete bands (5.0 kb and 11.3 kb in size) were detected in 100 ppm- and 10 ppm-treated subplots. The latter two bands were shown to correspond to those of *S. paucimobilis* strain 1443,[25] which was the dominant 2,4-D

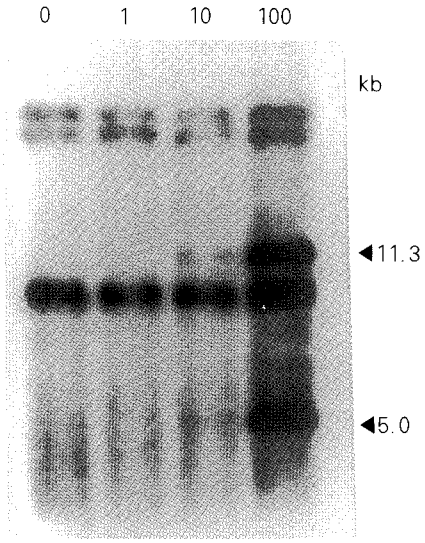

Fig. 6. Detection of indigenous 2,4-D-degrading microbial populations in field soil. Total soil bacterial community DNA was isolated from soil samples of four plots which were treated with 0, 1, 10, and 100 ppm of 2,4-D, respectively. The DNA was digested with *Hind*III, size-fractionated, transferred to nitrocellulose membrane, and then hybridized with the *Spa* probe.

degrader in this soil. However, the common three bands are not thought to correspond to a 2,4-D degrading population but to come from another indigenous microbial population, since the same banding pattern was observed in all four subplots including control subplot not treated with 2,4-D. The crop in field at this time was corn and its residues may have selected this new hybridizing population in all subplots. This nonspecific hybridization with the *Spa* probe was not observed in well-mixed, controlled, laboratory microcosm soils, suggesting that a more highly specific probe is necessary for DNA hybridization to the complex microbial community in natural field. The result with the *Spa* probe also shows that 2,4-D application of at least 10 ppm is necessary to have a detectable response in field.

These results show that the use of DNA probe method in conjunction with Southern transfer is analytically powerful, being able to detect multiple organisms, population changes, and genetic rearrangements and quantify the relative microbial population levels in natural soils.

Acknowledgment

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DNA probes에 의한 토양의 이사디 (2,4-D) 분해세균의 검출

가종억 (서울대학교 농생물학과)

초록 : 토양에서 세균군집의 DNA를 추출하여 이사디 분해세균의 밀도와 군집변화를 *tfdA* 유전자와 *Spa* probe를 이용하여 조사하였다. 이사디 분해균주인 *Pseudomonas cepacia*/pJP4을 토양에 여러 가지 밀도로 접종한 후 추출된 토양세균군집의 DNA를 Southern blot에서 분석한 결과, 본 실험에 사용된 DNA probe method에 의해 이 세균을 10^5 cells/g soil 수준까지 검출할 수 있는 것으로 나타났다. 이사디를 가해준 microcosm 토양에서 추출된 세균군집의 DNA를 분석한 실험에서는 *Pseudomonas pickettii*와 *Sphingomonas paucimobilis*가 우점종으로 검출되었고, 사용된 두가지의 DNA probes는 토양의 이사디 분해미생물에 대해 매우 높은 특이성을 가지고 있는 것으로 나타났다. 밭에 이사디를 장기적으로 가해준 후 추출된 토양세균군집의 DNA를 분석한 실험에서는 이사디를 최소한 10 ppm 이상 가해주어야 토양의 이사디 분해세균을 DNA probe method에 의해 검출할 수 있었고, *tfdA* 유전자는 실제의 밭토양에서도 높은 특이성을 나타냈으나 *Spa* probe는 일부의 토착세균에 비특이적으로 반응하는 것으로 나타났다. 토양에서 추출된 세균군집의 DNA를 분석하는 DNA probe method는 Southern blot과 함께 사용되었을 때 토양에 존재하는 이사디 분해미생물을 실험실 배지에 배양하지 않고 검출할 수 있었고, 이 미생물들의 밀도, 군집변화, 유전적 변화 등을 효과적으로 분석할 수 있는 것으로 나타났다.