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Monitoring 4-Chlorobiphenyl-Degrading Bacteria in Soil Microcosms by Competitive Quantitative PCR

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The competitive quantitative PCR method targeting *pcbC* gene was developed for monitoring 4-chlorobiphenyl(4CB)-degrading bacteria, *Pseudomonas* sp. strain DJ-12, in soil microcosms. The method involves extraction of DNA from soil contaminated with 4CB, PCR amplification of a *pcbC* gene fragment from the introduced strain with a set of strain-specific primers, and quantification of the electrophoresed PCR product by densitometry. To test the adequacy of the method, *Pseudomonas* sp. strain DJ-12 was introduced into both contaminated and non-contaminated soil microcosms amended with 4CB. *Pseudomonas* sp. strain DJ-12 was monitored and quantified by a competitive quantitative PCR in comparison with 4CB degradation and the result was compared to those obtained by using the conventional cultivation method. We successfully detected and monitored 4CB-degrading bacteria in each microcosm and found a significant linear relationship between the number of 4CB-degrading bacteria and the capacity for 4CB biodegradation. The results of DNA spiking and cell-spreading experiments suggest that this competitive quantitative PCR method targeting the *pcb*C gene for monitoring 4CB-degrading bacteria appears to be rapid, sensitive and more suitable than the microbiological approach in estimating the capacity of 4CB biodegradation in environmental samples.

Key words: 4-chlorobiphenyl degrading bacteria, soil microcosms, competitive quantitative PCR

Bioremidation is potentially a powerful technique for the cleanup of contaminated soil. In the case of chloroaromatics, usually the absence or the presence in insufficient numbers, of an acclimated microbial population capable of degrading the pollutant limits biodegradation. These deficiencies can be overcome by introduction of specific organisms into contaminated soils (bioaugmentation). This bioaugmentaion method is considered useful when effective pollutant-degrading populations are not present. Successful bioaugmentation depends mainly on the behavior of the inoculated strain in the environment where it is introduced. Therefore, the first criterion is good survival and retention of the strain in the system. The second criterion for successful bioaugmentation is the activity of the inoculum. However, the addition of specialized strains to enhance the removal of pollutants is not yet widely applied, because it is very difficult to monitor the survival and behavior of the specialized strains. Additional experiments need to be performed to investigate the population dynamics of bacterial strain introduced into a soil microcosm. When a bacterial strain is introduced into the environment, many parameters need to be fulfilled including the selection of strains suitable for each polluted site and distribution and frequency of specific indigenous or introduced microbial catabolic genotypes and activities. Microbiological characterization of contaminated soils is important to assess a particular bioremidation process. However, the spatial distribution of microorganisms in soil (Hattori, 1988) and the need to overcome a range of microbe-soil interactions (Stotzky, 1985) are serious limitations to quantitatively and representatively sample soil microorganisms. In this respect, it is desirable to establish laboratory evaluation methods for selecting the strains to be introduced into contaminated soils.

Competitive quantitative PCR is a highly sensitive technique that bypasses quantification problems caused by differences in the exponential PCR amplification of DNA by using a competitor (Ferre, 1992). A competitor is a competitive DNA template that shares two primers and thus is co-amplified in competition with specific DNA sequences in the sample. Because the lengths of the fragments differ, amplification products from the competitor

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and target DNAs are readily separated on a gel. Competitive quantitative PCR techniques have been used to measure numbers of plant pathogens (Hu *et al.*, 1995), fungal populations (Baek and Kenerley, 1998), and uncultivated bacterial strains in soils (Lee *et al.*, 1996). But soil is heterogeneous and consists of large amounts of inhibitory compounds, therefore reproducible results are more difficult to obtain. In order to obtain successful results in bioaugmentation of chloroaromatic-contaminated soil, it is a prerequisite to accurately monitor an introduced specific strain. More accurate understanding and monitoring of an exogenously supplied strain should be possible if a specific catabolic gene can be qualified. Greater catabolic gene copy numbers within a contaminated area (relative to those in uncontaminated soils) could be used as evidence of natural attenuation or of the effectiveness of exogenously supplied growth amendments in engineered bioremediation. In order to monitor the introduced strain, a 4-chlorobiphenyl-degrading *Pseudomonas* sp. strain DJ-12, competitive quantitative PCR techniques have been developed to target the *pcb* gene encoding the 2,3-dihydroxybiphenyl 1,2-dioxygenase.

In this study, we identified a single set of *pcb*C-specific primers and used them to detect and enumerate the *Pseudomonas* sp. strain DJ-12 to be introduced in soil microcosms. The aim of this study was to develop a *pcb*C-targeted competitive quantitative PCR method for monitoring the population dynamics of a bacterial strain to be introduced into soil microcosms.

Materials and Methods

Bacterial strain

Pseudomonas sp. strain DJ-12 (kindly provided by professor C.K. Kim), was used as the test strain to validate the competitive quantitative PCR in soil Pseudomonas sp. strain DJ-12 which has been previously described (Kim *et al.*, 1996). This strain is a natural isolate that can grow in biphenyl or 4-chlorobiphenyl as the sole carbon and energy source.

Soil collection and preparation of microcosms

Two surface soil samples (0~25 cm) were collected from a contaminated pear farm area (dark-colored) and from a forest area, with no history of contamination with chlorinated aromatics. The two soils were silt loam soils. They were sieved (2-mm nominal pore size) and stored in polyethylene bags at -20°C. Stored soils were incubated at 28°C for one day before amendments were added, which allowed the microbial populations to acclimate. Table 1 shows the chemical and physical properties of each soil. The soil microcosm was established using 200 g (dry weight) of pear farm silt loam soil or 200 g of a natural uncultivated silt loam forest soil. Two hundred gram of the soil sample was added to 10 g vermiculite (Aldrich Chemical Co., Milwaukee, WI). Acetone with 4-chlorobiphenyl was added to the soil to a final concentration of 500 ppm (g/g). After mixing and evaporation of the solvent, M9 medium (non-inoculated control microcosm) or bacterial suspension in M9 medium was added. Following addition of chemical and/or bacteria, the soil was brought to 20% of water-holding capacity with M9 buffer, and then this sample was placed into a 800 cm^3-size polypropylene bottle. The soil microcosm was incubated in the dark at 25°C for one month. Bacteria were grown in LB medium to get a sufficient number of cells. They were harvested in the mid-log phase, pelleted, resuspended in M9 medium, and starved for 48 h at room temperature (Van Elasas and Overbeek, 1993). Samples (one gram of each test) were taken periodically for microbiological and PCR testing and chemical analyses for 4CB degradation. Each experiment was performed in duplicate.

Extraction and analysis of soil samples

Microorganisms were extracted from one gram of soil sample by shaking with 19 ml of phosphate buffered saline on a rotary shaker for 2 h, then decanting the soil particles. The viability of soil-supplemented bacteria was monitored by plating soil extracts on LB plates containing ampicillin using antibiotic resistance of the strain DJ-12 as a marker and M9 plates containing both of 4CB (500 ppm) and ampicillin, respectively. Indigenous bacteria were estimated as CFU (cell forming unit) on LB plates containing cyclohexamide (100 μg/ml) to avoid fungal growth.

The 4CB was extracted similarly with ethyl acetate instead of phosphate buffered saline. After drying the extracts over anhydrous sodium sulfate, and the solvent was removed by a rotavaporator (Buchi R-114) at 50°C. The dark yellow residues were dissolved in 0.2 ml of methanol for gas chromatographic analysis. The degradation of 4CB was determined by using a Hewlett-Packard model 5890A GC apparatus equipped with an HP-5 cap-

Table 1. Sampling location and chemical characteristics of the soils used

Sampling location	Soil texture	Native vegetation	pH[a] (water)	Total Org. C (g kg^{-1} soil)	Total (mg kg^{-1} soil)					Moisture contents [%(vl/wt)]
					P_2O_5	Ca^{2+}	K^+	Mg^{2+}	Na^+	
Hwacheon	Silt loam	Forest	4.7	10.3	3.9	49.3	25.3	5.9	3.0	12.4
Toikewon	Silt loam	Pear farm	5.9	64.3	784	129	58.3	5.6	2.1	14.9

[a]pH was measured in a 1:1 soil-to-deionized water suspension using a glass electrode. All other analysis were conducted according to the determination of organic compounds in soils, sediments and sludges (Compton, 2000).

illary column (25 m × 0.2 mm × 0.5 m). The samples were injected into the gas chromatography at 50°C, and the oven temperature was programmed to reach 190°C at the rate of 15°C/min. Injector and detector temperatures were 250°C and 280°C, respectively.

DNA extraction

Total soil DNA was extracted and purified by a modification of the procedure described by Lee *et al.* (1996) as follows. One gram of soil sample was washed twice with washing solution (120 mM sodium phosphate buffer, pH 8.0) to remove extracellular DNA and to disperse the soil. Eight milliliters of lysis solution (0.15 M NaCl, 0.1 M EDTA [pH 8.0], 10 mg/ml lysozyme) was added to the sample and incubated at 37°C with occasional mixing for 2 h, and then 8 ml of lysis solution II (0.1 M NaCl, 0.5 M Tris-HCl [pH 8.0], 10% SDS) was added. The sample was frozen at -70°C for 30 min and thawed in a 65°C water bath for 30 min. The freezing and thawing cycle was repeated three times. The lysate was centrifuged at 7,500 × g for 10 min, and then filtered through a single sterile Kimwipe into a fresh tube. The lysate was brought to a final concentration of 1% CTAB (hexadecyltrimethyl ammonium bromide; Sigma Biochemical, St. Louis, Mo) and 0.7% NaCl. The lysate was mixed and incubated at 65°C for 10 min, followed by extraction with an equal volume of chloroform:isoamyl alcohol (24:1). An equal volume of 13% polyethylene glycol (molecular weight, 8,000) in 1.6 M NaCl was added to the upper phase and kept on ice for 2 h. The sample was then centrifuged at 12,000×g for 20 min, and the pellet was washed once with 70% ethanol, dried at room temperature, and dissolved in 750 μl TE buffer (pH 8.0). A 110 μl of 10 M ammonium acetate was added, and the sample was incubated at room temperature overnight. This mixture was centrifuged in a microcentrifuge at 12,000×g for 15 min, and the pellet was discarded. Two volumes of ethanol were added to the supernatant. This mixture was kept at -20°C for 2 h, and the precipitated DNA was recovered by centrifugation. The pellet was washed once with 70% ethanol and dried at room temperature. The dried pellet was dissolved in 40 μl TE buffer and the soil DNA was further purified by electrophoresis in 0.8% Seaplaque (FMC Bioproduct, Rockland, Marine) containing 0.2% polyvinylpyrrolidone (Sigma). High-molecular-weight DNA (greater than 20-kb) was excised and placed in preweighed microcentrifuge tubes. The same mass of water was added, and the sample was heated at 65°C for 10 min and used as a template for PCR amplification.

Selection of PCR primers for pcbC gene and construction of competitor

PCR primer was constructed based upon conserved regions (Kim *et al.*, 1996) using program Primer Express (PE Applied Biosystems), and targeted a 379-bp fragment of the *pcbC* gene are 5'-ATTCTTGGCTATGTGGTGAT-3'(corresponding to positions 1038 to 1057 of *pcbCD* gene) and 5'-AGAACGGCTGATTACTGTGA-3'(corresponding to the complement of positions 1416 to 1397 of *pcbCD* gene). The *pcb*C genes of strain DJ-12 were amplified by PCR with described primers. Sequencing of the amplification product obtained with *pcb*C gene primers confirmed that only *pcb*C gene sequences were amplified.

The *pcb*C genes of strain DJ-12 were amplified by PCR with the primers described above. PCR was performed on a DNA thermal cycler (Model480; Perkin-Eelmer, Norwalk, Conn.). PCR amplification was conducted in a total volume of 20 μl containing 0.5 μM of each primer, 200 μM dNTP, 1.5 mM $MgCl_2$, 1× *Taq* buffer, and 1U of *Taq* polymerase. The DNA template was first subjected to a denaturation step at 94°C for 2 min. The subsequent 30 cycles consisted of a 2 min denaturation step at 92°C, a 1 -min annealing step at 57°C, a 2 min primer extension step at 72°C, and a final extension at 72°C for 10 min. Negative controls with *E. coli* genomic DNA showed no amplification. PCR products were run on a low-melting-point Nusieve agarose gel (FMC Bioproducts Co., Rockland, ME) and viewed with ethiduim bromide (1 g/μl). The PCR-generated 379-bp product was cloned into a TA cloning vector pT7Blue (Novagen Inc., Madison, WI). One of the clones was sequenced and found 100% identical to the *pcbCD* gene sequence between 1038 and 1416 and was designated pCB.. A 41-bp fragment isolated from lambda bacteriophage DNA by digestion with *Sty* I was inserted into a *Sty* I restriction endonuclease site within pCB, yielding the pCB-C competitor for *pcbC* gene sequences. The *pcb*C gene competitor, pCB-C, was 41bp larger than the original clone, therefore the resulting PCR products for pCB and pCB-C were 379 and 420 bp, respectively.

Evaluation of competitive QPCR protocol

A single set of standard samples containing a known target DNA was prepared. Each reaction mixture was spiked with a constant amount of competitor, and all samples were amplified in triplicate PCR reactions. PCR products were separated by electrophoresis on a 3% Nusieve agarose gel containing ethiduim bromide. After the electrophoresis, the bands of target DNA and competitor in the same lane were quantified by Image analyzer (model BAS 2500, Fuji Photo Film Co., Tokyo, Japan).

In order to construct a calibration curve for each amplification, log ratios of target to competitor band intensities were plotted against the logarithm of the mass of input target DNA (Schneeberger *et al.*, 1995). This calibration curve was calculated by a least squares analysis to determine the target DNA in the microcosm soil samples by using the ratio of target DNA to competitor PCR product. The yield of two products is defined by the following

equation: $\log (Nn_1/Nn_2)=\log (No_1/No_2)+[n \times \log (eff_1/eff_2)]$, where Nn_1 and Nn_2 are concentrations of PCR products, No_1 and No_2 are concentrations of initial templates, n is number of PCR cycles, eff_1 and eff_2 are efficiencies of template amplification. If the efficiencies of amplification of the two templates are the same ($eff_1=eff_2$), the ratios of the yield of two products (Nn_1/Nn_2) depend directly on the ratio of the concentrations of the initial templates (No_1/No_2) present. Even if the eff_1/eff_2 ratios are not equal, the equation is valid if one assumes that the eff_1/eff_2 ratio is a constant value.

Results and Discussion

Detection of Pseudomonas sp. strain DJ-12 by PCR

Two PCR primers, 5'-ATTCTTGGCTATGTGGTGAT-3' and 5'-AGAACGGCTGATTACTGTGA-3', were used in this study to amplify the DJ-12 *pcb*C gene encoding the 2,3-dihydroxybiphenyl 1,2-dioxygenase. The advantage of using *pcb*C gene sequence as a strain-specific probe is the low sequence homology between *pcb*C and other corresponding dioxygenases. Thus we decided to examine the use of the *pcb*C gene for specific detection of strain DJ-12 in a complex microbial community, such as soil. The specificity and sensitivity of the PCR method using these primers were tested for detecting 4CB degraders in soil (Fig. 1).

To test the adequacy of the method, *Pseudomonas* sp. strain DJ-12 (0 to 1.0×10^6 cells per one gram of soil) was introduced into uncontaminated forest soil. Following extraction and purification of soil DNA according to our method, *pcb*C gene was amplified. Figure 1 shows the results of amplification of *pcb*C under these conditions. We obtained a significant linear relationship between the density counts of *pcbC* genes' PCR products and the number of colonies of 4CB-degradative *Pseudomonas* sp. strain DJ-12 ($r = 0.96$; $P \leq 0.001$). The detection limit of strain DJ-12 was about 1.0×10^2 CFU per gram of soil. As a preliminary study, the indigenous microflora of soil was tested for its ability to grow on 4-chlorobenzoate. No cul-

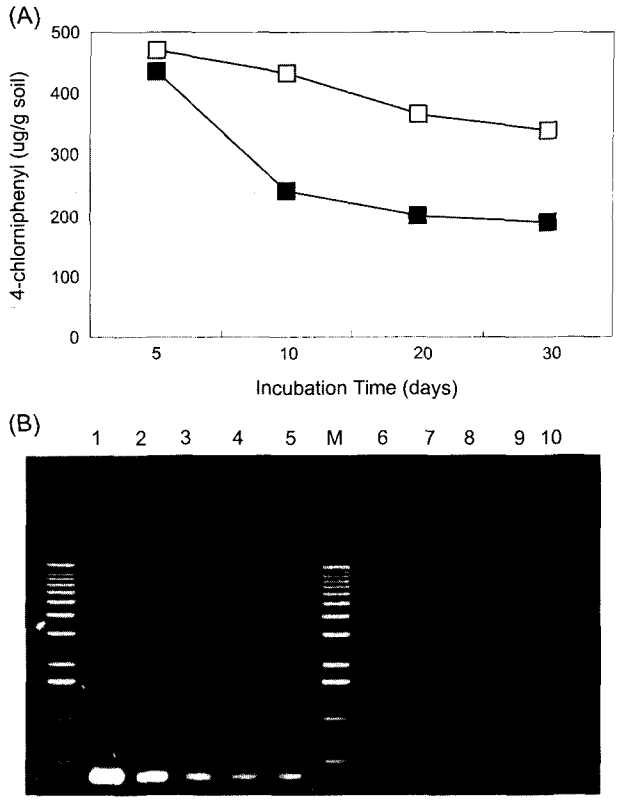

Fig. 2. Biodegradation of 4CB and tracking *pcb*C gene in uncontaminated forest soil microcosm amended with 4CB. (A) Biodegradation of 4CB in soil microcosm inoculated with (■) or without (□) *Pseudomonas* sp. strain DJ-12 (1.0×10^7 cells per gram of soil). (B) Tracking *pcbC* gene in soil microcosm inoculated with (Lanes 1 to 5) or without (Lanes 6 to 10) *Pseudomonas* sp. strain DJ-12 (1.0×10^7 cells per gram of soil). Lanes M, 1-kb DNA size marker; Lanes 1 and 6, PCR detection of *pcbC* gene from samples at 0 day; Lanes 2 and 7, at 5 days; Lanes 3 and 8, at 10 days; Lanes 4 and 9, at 20 days; Lanes 5 and 10, at 30 days.

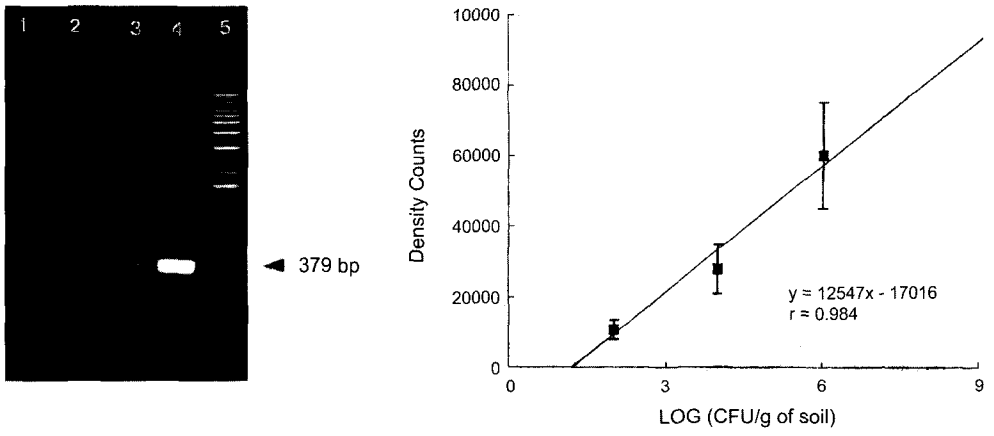

Fig. 1. Specificity and sensitivity of PCR method for detecting 4CB degrader, strain DJ-12, in uncontaminated forest soil. Lanes 1, 1.0×10^2 cells per one gram of soil; Lanes 2, 1.0×10^4 cells per one gram of soil; Lanes 3, 1.0×10^6 cells per one gram of soil; Lanes M, 1-kb DNA size marker.

spreading on M9 media containing 4-chlorobenzoate (data not shown). Amplification products in forest soil to which strain DJ-12 was not added were not observed, indicating that forest soil has no indigenous 4CB degrading bacteria containing *pcb*C gene (Fig. 2).

Relationship between PCR products of the biodegradative pcbC genes and 4CB degradation in soil microcosm

Pseudomonas sp. strain DJ-12 was introduced into both uncontaminated forest soil and contaminated pear farm soil microcosms, each amended with 4CB. *Pseudomonas* sp. strain DJ-12 of each microcosm was detected by PCR amplification using specific primers targeting *pcb*C gene for one month, and the result was compared to the 4CB degradation rate of each microcosm (Fig. 2 and Fig. 3). Fig. 2 shows that the density counts of *pcbC* gene PCR products in the uncontaminated forest soil microcosm with inoculation strain DJ-12 showed low values for 30 days. 4CB was slowly degraded in comparison with contaminated pear farm soil. While PCR products were not detected in the uncontaminated forest soil microcosm without inoculation strain DJ-12, the amount of 4CB decreased slightly. It is supposed that this phenomenon was caused by expected volatilization of some part of 4CB. Fig. 3 indicates that the density counts of *pcbC* genes PCR products in the contaminated pear farm soil microcosm inoculated with strain DJ-12 had high density values for 10 days. 4CB was rapidly degraded, and the density counts still had high values after 20 days when 4CB was completely degraded (Fig. 3). From these results, we find a linear relationship between the *pcbC* gene PCR products and the capacity of biodegradation of 4CB. Therefore, these results indicate that the numbers of the *pcbC* gene PCR-mediated cells in the four soil microcosms correlated with the capacity of biodegradation of 4CB. In addition, the effect of inoculation of *Pseudomonas* sp. strain DJ-12 into each microcosm was effective in the degradation of 4CB compared with those of non-inoculated microcosms.

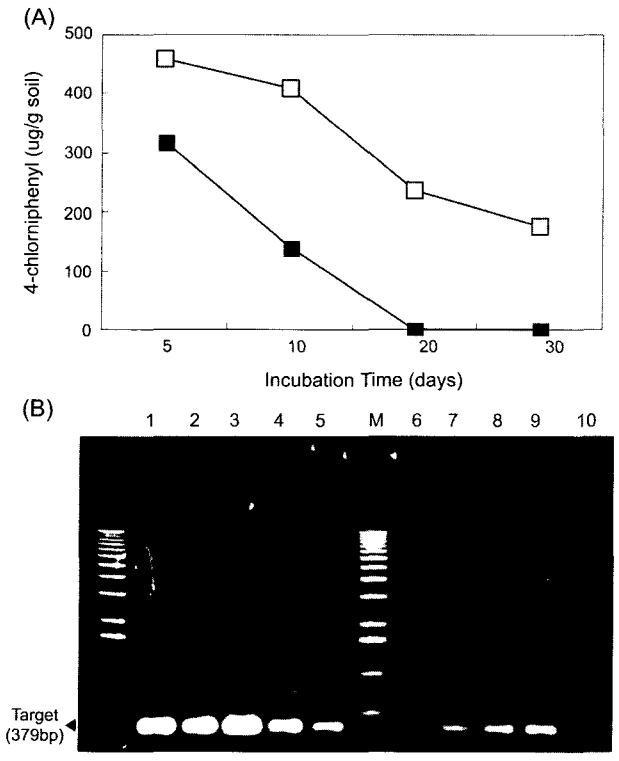

Fig. 3. Biodegradation of 4CB and tracking *pcb*C gene in contaminated soil (pear farm) microcosm amended with 4CB. (A) Biodegradation of 4CB in soil microcosm inoculated with (■) or without (□) *Pseudomonas* sp. strain DJ-12 (1.0×10^7 cells per gram of soil). (B) Tracking *pcbC* gene in soil microcosm inoculated with(Lanes 1 to 5) or without(Lanes 6 to 10) *Pseudomonas* sp. strain DJ-12 (1.0×10^7 cells per gram of soil). Lanes M, 1-kb DNA size marker; Lanes 1 and 6, PCR detection of *pcbC* gene from samples at 0 day; Lanes 2 and 7, at 5 days; Lanes 3 and 8, at 10 days; Lanes 4 and 9, at 20 days; Lanes 5 and 10, at 30 days.

Competitive quantitative PCR

Enumeration of any bacterial species by DNA-based method is a function of efficiencies of cell lysis and subsequent purification of DNA. Above 99.99% of *Pseudomonas fluorescens* in pure culture was lysed using the DNA-based method (Lee *et al.*, 1996). The recovery efficiency of our soil DNA isolation and purification method, previously reported (Lee *et al.*, 1996), was estimated to be $3.7\% \pm 0.1\%$. The recovery efficiency by our method is low, although DNA recovered was pure as judged by uninhibited PCR amplification (Fig. 4). Fig. 4 shows the amplification rates of strain DJ-12 genomic DNA added to forest soil DNA and pCB-C purified from pure culture. We selected the concentrations of the two templates and amplified each for 30 cycles. Fig. 4 also indicates that the amplification efficiencies of the target DNA that had been purified from soil and pCB-C obtained directly from pure culture are the same. This demonstrates that the amplification of *pcb*C gene from soil was not inhibited by soil

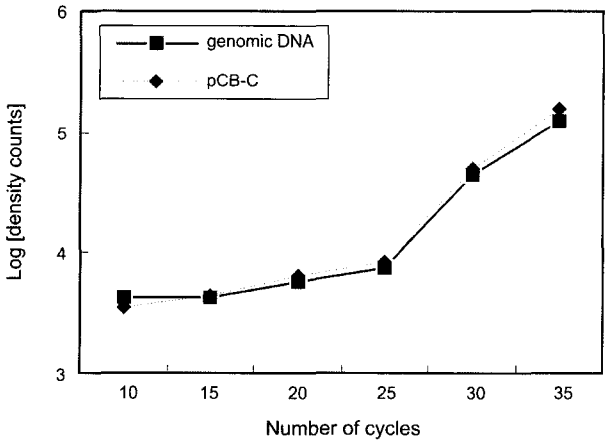

Fig. 4 Amplification rates of strain DJ-12 genomic DNA added to forest soil extract DNA and pCB-C purified from pure culture.

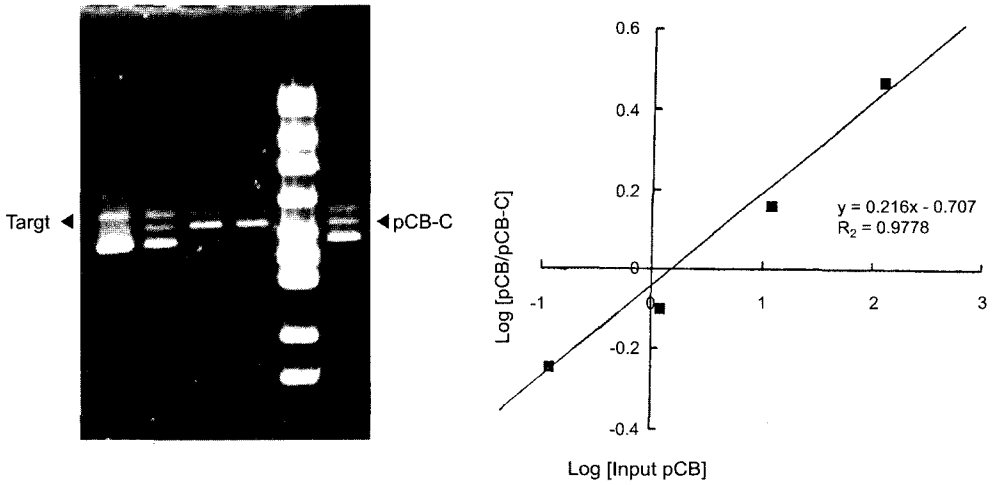

Fig. 5. Competitive QPCR of *pcbC* gene sequences. (A) Lanes 1 to 4, a range of masses of pCB were coamplified with 2.4 pg of competitor (pCB-C); Lanes 1, 120 pg of pCB; Lane 2, 12 pg of pCB; Lanes 3, 1.2 pg of pCB; Lanes 4, 0.12 pg of pCB; Lanes 5, ΦX174-*Hinc*II digest marker; Lanes 6, 10 μl of soil DNAs after 20 days were coamplified with 2.4 pg of competitor (pCB-C); Lanes 7, 10 μl of soil DNA before inoculation. (B) Calibration curve of competitive QPCR of *pcbC* gene sequences. Log ratios of target to competitor band intensities were plotted against the logarithm of the mass of input target DNA This calibration curve was calculated by a least squares analysis.

contaminants or any DNA from indigenous soil organisms.

Competitive quantitative PCR was performed with *pcb*C specific primer set. In order to adjust the concentration of competitors, the optimum concentration of the competitor was first determined by amplifying 10-fold dilution of a competitor plasmid pCB-C with the purified soil DNA extracts containing target *pcb*C gene. A fixed amount of pCB-C, 2.40 pg (corresponding to 2.87×10^5 copies of *pcb*C), was added to all PCR tubes. The concentration of *pcb*C of standard sample ranged from 0.12 pg (corresponding to 1.43×10^4 copies) to 120 pg (corresponding to 1.43×10^7 copies). Under these conditions a standard curve was established using the coamplification of the competitor and of *pcb*C genes extracted from a known number of cells. PCR under these conditions (with a constant amount of competitor) yielded amplification products that were detectable on ethidium bromide-stained gels. Fig. 5A shows the example of typical agarose gel electrophoresis from these experiments. From the intensity of the two bands, the 379-bp/420-bp fragment ratios were calculated and plotted against the number of target cells in the PCR tube. The standard curve was fitted by least squares method (Fig. 5B). The yield of two products is defined by the following equation (Schneeberger *et al.*, 1996): $\log (Nn_1/Nn_2)=\log (No_1/No_2)+[n \log (eff_1/eff_2)]$, where Nn_1 and Nn_2 are concentrations of PCR products, and No_1 and No_2 are concentrations of initial templates. If the efficiencies of amplification for the two templates are the same ($eff_1=eff_2$), the ratios of the yield of two products (Nn_1/Nn_2) depend directly on the ratio of the concentrations of the initial templates (No_1/No_2) present. Even if the eff_1/eff_2 ratios are not equal, assuming that the eff_1/eff_2 ratio is a constant value, this equation is valid. This competitive quantitative PCR using standard curve obtained by the above equation has the advantage that once the competitor has been constructed, multiple samples can be easily and accurately analyzed. In Fig. 5, we observe a third band. Schneeberger *et al.* (1995) reported these heteroduplexs are formed primarily during the later amplification, and target and competitor amplification products are affected equally. Ruano *et al.* (Ruano and Kiid 1992) reported that this kind of third band is commonly observed in competitive PCR and is attributed to the formation of heteroduplexes composed of target and competitor amplification products. As the concentrations of target and competitors DNA become similar, Lee *et al.* (1996) also observed a third band.

Validation of competitive QPCR with microcosm

We applied the competitive quantitative PCR method to monitoring introduced strain DJ-12 into contaminated pear farm (silt loam) soil for one month. The number of *pcb*C targets was immediately tested after adding strain DJ-12 and 4CB to soil and the ratio, log (*pcbC* target/pCB-C)=0.16, was obtained. Ten days, 20 days and 30 days later, the ratios were 0.31, -0.16, and -0.44, respectively. The amounts of *pcbC* gene sequences in 10 μl of purified soil DNA extract were estimated to be 9.04, 38.02, 0.43, and 0.03 pg, respectively. Since the total volume of all purified soil DNAs were 90, 88, 92, and 92 μl, respectively, the total amounts of *pcbC* gene sequences in the total purified soil DNA extract were estimated to be 81, 336, 4, and 0.3 pg, respectively. For final estimation of the number of *pcb*C copies in the samples, 3.7% of the recovery efficiency must be factored. Therefore, the number of *pcbC* gene copies per gram=(0.9) (81.36, 3.94, and 0.29 pg/g, respectively)/(0.0368)=1.989×10^{-9}, 8.221×10^{-9},

Table 2. Survival of *Pseudomoas* starin DJ-12 in contaminated pear farm soil microcosm

Incubation time of microcosm	[a]number of (cfu/g)		[b]number of target *pcb*C per g	[c]4-chloro-biphenyl
	4CB degrader	DJ-12		
Before inoculation	ND	ND	[d]6.2×10^3	ND
[e]After inoculation				
0 day	1.6×10^7	1.6×10^7	2.4×10^7	500 ppm
10 day	6.8×10^6	5.8×10^6	9.8×10^8	141 ppm
20 day	1.1×10^5	0.9×10^5	1.1×10^7	0
30 day	1.9×10^3	1.4×10^3	8.5×10^5	0

[a]Total cell number of 4CB degrading bacteria and strain DJ-12 were determined by plating soil extracts on M9 medium containing 4CB and M9 medium containing 4CB and ampicillin, respectively.
[b]The number of target *pcb*C was estimated by competitive quantitative PCR method targeting *pcb*C gene.
[c]4-chlorobipheny was added to soil to a final concentration of 500 ppm (g/g).
[d]The number of target *pcb*C before adding strain DJ-12 to soil.
[e]*Pseudomonas* strain DJ-12 ($2.0\sim3.0\times10^7$ cfu/g) was introduced into soil microcosm amended with 4CB.
N.D., not determined. All experiments were performed in duplicate and data are the average of two results.

9.636×10^{-11}, and 7.092×10^{-12} g/g, respectively. Assuming a mass of 8.38×10^{-18} g per copy of pCB, these corresponded to 2.4×10^7, 9.8×10^8, 1.1×10^7, and 8.5×10^5 copies of *pcbC* gene per gram of soil, respectively. Because there is only one copy of the *pcbC* gene in *Pseudomonas* sp. strain DJ-12, and thus *pcbC* gene in contaminated and inoculated silt loam soil microcosm was present at a level of 2.4×10^7 (0 day), 9.8×10^8 (10 days), 1.1×10^7 (20 days), and 8.5×10^5 cells (30 days) per gram (Table 2).

Deveriegher *et al.* (1995) reported that the treatment of soil with certain detergents resulted in 100- to 1,000-fold increases in the density of detergent degradative inoculum. After 10 days, the number of 4CB-degrading bacteria containing *pcb*C target gene was increased 41-fold in our research. The number of indigenous bacteria containing *pcb*C before adding strain DJ-12 and 4CB to the microcosm was 6.2×10^3 cfu per gram and they may also be increased in number, but it was approximately 10^4-fold lower than that of the inoculating cell number ($2.0\sim3.0\times10^7$ cfu/g). We observed that the 4CB biodegradation was proportional to the number of 4CB-degrading bacteria. The degradation of 4CB resulted in a 72% decrease in 4CB concentration after 10 days with a concomitant increase in cell number (Fig. 4, Table 2), and leading to completely decrease in 4CB after 20 days. The decrease in number of 4CB-degrading bacteria containing *pcb*C target gene in microcosm after 20 days may result from insufficient nutrients available for maintenance and replication, and sub-optimal environmental conditions, such as matric potential, pH, ionic strength and temperature (van Elsas and van Overbeek, 1993). Therefore, the catabolic activities of strain DJ-12 inoculated can be sustained at a high population density in inoculated contaminated soil sites and efficiently degrade 4CB completely.

We also estimated the number of strain DJ-12 by traditional cultivation method for 30 days. The cell number of strain DJ-12 immediately after the inoculation was approximately the same as the cell number estimated by a competitive QPCR, however, in contrast with QPCR based results, the strain DJ-12 values were decreased in number after 10 days. Blasco *et al.* (1997) presented strong evidence that if 4-chlorobenzoate, which is the dead-end metabolite of 4CB degradation pathway, is accumulated, it will converted to a toxic product, most probably protoanemonium, by indigenous soil microflora, and this toxic product has severe effects on cell viability of both the indigenous organisms and organisms that were supplimented to the soil. Kim *et al.* (1996) reported that *Pseudomonas* sp. strain DJ-12 degraded 4-chlorobipenyl to 4-chlorobenzoate, which was further degraded. Strain DJ-12 is thought to have an effective system for chlorinated biphenyl degradation without having to be coinoculated into the soil with a chlorobenzoate degrader. Therefore, 4-chlorobenzoate did not effect the survival of strain DJ-12 but it may be culture-ability on minimal medium of strain DJ-12. The enumerations of bacteria on minimal medium were the culturable ones. Manahan and Steck (1997) reported that many Gram-negative bacteria can enter the viable but non-culturable (VBNC) state. Roszak *et al.* (1984) reported that a viable but non-culturable state that could affect the recovery of the amount of bacteria strain introduced into soils. They proposed that this recovery depends on the physiological state of the bacteria introduced and the stress arising from contact with oligotrophic environment could explain the differences observed. The result of QPCR obtained in this study strongly supported the survival of strain DJ-12 in microcosm. We obtained the unexpected results from QPCR method and cell-cultivation experiments and may not explain these results as a VBNC of indigenous microorganisms (or stain DJ-12), it is suggested that this competitive quantitative PCR method targeting *pcbC* gene for monitoring 4CB-degrading bacteria appeared to be rapid, sensitive and more suitable than the microbiological approach to estimate the capacity of 4CB biodegradation in environmental samples. Competitive QPCR has the advantage that, once the competitor has been constructed, multiple samples can be analyzed relatively easily and with a higher degree of precision than would be allowed by using the viable plating counting method. It could be applied to monitor a specific strain in a polluted soil during the bioremidation process or the survival of genetically engineered microorganisms introduced into soils.

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