Biodegradation (2012) 23:263–276
DOI 10.1007/s10532-011-9505-x

ORIGINAL PAPER

Impacts of gene bioaugmentation with pJP4-harboring bacteria of 2,4-D-contaminated soil slurry on the indigenous microbial community

Daisuke Inoue · Yuji Yamazaki · Hirofumi Tsutsui · Kazunari Sei · Satoshi Soda · Masanori Fujita · Michihiko Ike

Received: 6 April 2011 / Accepted: 6 August 2011 / Published online: 18 August 2011

Abstract Gene bioaugmentation is a bioremediation strategy that enhances biodegradative potential via dissemination of degradative genes from introduced microorganisms to indigenous microorganisms. Bioremediation experiments using 2,4-dichlorophenoxyacetic acid (2,4-D)-contaminated soil slurry and strains of *Pseudomonas putida* or *Escherichia coli* harboring a self-transmissible 2,4-D degradative plasmid pJP4 were conducted in microcosms to assess possible effects of gene bioaugmentation on the overall microbial community structure and ecological functions (carbon source utilization and nitrogen transformation potentials). Although exogenous bacteria decreased rapidly, 2,4-D degradation was stimulated in bioaugmented microcosms, possibly because of the occurrence of transconjugants by the transfer of pJP4. Terminal restriction fragment length polymorphism analysis revealed that, although the bacterial community structure was disturbed immediately after introducing exogenous bacteria to the inoculated microcosms, it gradually approached that of the uninoculated microcosms. Biolog assay, nitrate reduction assay, and monitoring of the *amoA* gene of ammonia-oxidizing bacteria and *nirK* and *nirS* genes of denitrifying bacteria showed no irretrievable depressive effects of gene bioaugmentation on the carbon source utilization and nitrogen transformation potentials. These results may suggest that gene bioaugmentation with *P. putida* and *E. coli* strains harboring pJP4 is effective for the degradation of 2,4-D in soil without large impacts on the indigenous microbial community.

Keywords Gene bioaugmentation · pJP4 · Soil slurry · Microbial community structure · Carbon utilization potential · Nitrogen transformation potential

Introduction

Bioaugmentation, which introduces appropriate microorganisms with specific catabolic abilities into the contaminated environment to accelerate the degradation/removal of contaminants, is a promising and attractive approach to bioremediation of contaminated soil environments. In bioaugmentation, the degradative capability of the environment can be enhanced by the activity of the introduced whole cells (cell bioaugmentation) or via dissemination of the degradative genes from the introduced microorganisms to

Electronic supplementary material The online version of this article (doi:10.1007/s10532-011-9505-x) contains supplementary material, which is available to authorized users.

D. Inoue (✉) · Y. Yamazaki · H. Tsutsui · K. Sei · S. Soda · M. Fujita · M. Ike
Division of Sustainable Energy and Environmental Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan
e-mail: inoue@wb.see.eng.osaka-u.ac.jp

the indigenous population (gene bioaugmentation) (Pepper et al. 2002). Despite the usefulness of bioaugmentation, the intentional release of exogenous microorganisms and genetic materials into the environment may cause unexpected detrimental effects on the public health [e.g., increase of environmental pathogenicity by spread of antibiotic resistance among pathogenic bacteria (Keese 2008)] and on the structure and/or functions of natural ecosystems [e.g., disruption (stimulation/depletion) of normal biogeochemical cycles of carbon, nitrogen, sulfur etc. (Levin and Harwell 1986)]. Therefore, the potential benefits of bioaugmentation must be balanced against its potential to cause detrimental effects prior to its implementation. Since microorganisms introduced during bioaugmentation are considered to primarily influence the indigenous microbial populations, the assessment of possible negative effects on the indigenous microbial community would be a significant issue to be addressed.

To date, many studies have examined the impacts of introduced bacteria with high biodegradative ability on the structure/diversity of entire indigenous microbial communities in soil (Dejonghe et al. 2000; Jernberg and Jansson 2002; Gomes et al. 2005; Paul et al. 2006; Baek et al. 2007; Coppotelli et al. 2008; Niu et al. 2009). Because minor microbial populations often play indispensable roles in the ecological functions of the natural environment, the effects on specific functions or their responsible populations should also be monitored to enable assessment of the total impact of bioaugmentation. However, only a few studies have investigated the perturbations of ecological functions of the soil microbial community in response to bioaugmentation, and these studies have focused on the gross metabolic activity (Short et al. 1991; Coppotelli et al. 2008), specific substrate utilization potential (Coppotelli et al. 2008) and nitrogen transformation potential (Jones et al. 1991; Zhao et al. 2009). Indeed, there have been no studies conducted to evaluate the impact of gene bioaugmentation employing bacteria harboring a conjugative plasmid on the microbial functions, even though dissemination of the introduced plasmid may cause additional effects on the microbial community due to enhancement of the survival capability of the indigenous microorganisms that incorporate the plasmid.

In this study, we examined the effects of bioaugmentation of 2,4-dichlorophenoxyacetic acid (2,4-D)-contaminated soil slurry with exogenous bacteria harboring a self-transmissible plasmid pJP4 on the microbial community structure/diversity and ecological functions in the system. We selected *Pseudomonas putida* and *Escherichia coli* as the inoculum bacteria (plasmid donors) because strains of *Pseudomonas* spp. exert degradative capability against various xenobiotics and are promising candidates as bioaugmentation agents, while *E. coli* is the most popular host for genetic manipulations. Slurry bioaugmentation, which has been successfully applied for enhanced biodegradation of soil contaminants (Habe et al. 2002; Park et al. 2003; Arshad et al. 2008), possibly due to advantages of slurry such as improved accessibility to contaminants and enhanced physiological activity of degraders under a high water content condition, was applied in this study. The fates of the introduced bacteria, indigenous bacteria, and transconjugants in the system were monitored. To assess the effects on the indigenous microbial community, the eubacterial community structure, dynamics of the *amoA* gene of ammonia-oxidizing bacteria and *nirK* and *nirS* genes of denitrifying bacteria, and carbon source utilization and nitrate reduction potentials of the microbial community were examined.

Materials and methods

Bacterial strains and plasmid

Pseudomonas putida KT2440 (Bagdasarian et al. 1981) and *E. coli* HB101 (Boyer and Roulland-Dussoix 1969) harboring plasmid pJP4 (Don and Pemberton 1981), as described in our previous study (Tsutsui et al. 2010), were used as the inoculum strains. Plasmid pJP4 is an 80-kb IncP1β self-transmissible plasmid that encodes genes for the degradation of 2,4-D to 2-chloromaleylacetate and resistance to mercury (Hg). Neither *P. putida* KT2440(pJP4) or *E. coli* HB101(pJP4) were capable of degrading 2,4-D; thus, they were used as the carrier of plasmid pJP4 to soil microorganisms. These strains were routinely maintained on L-medium (Inoue et al. 2005). $HgCl_2$ at 10 mg-Hg/l and streptomycin at 20 mg/l were also added to the media when required.

Microcosm experiment

A soil sample was collected from a forest without significant contamination with chemicals on the Osaka University Suita Campus (Osaka, Japan). The sample was sieved through a 2.0-mm screen and stored at −20°C. The pH and water content of the soil sample were 5.8 and 23.3% (w/w), respectively. Prior to use, the soil sample was incubated at 28°C for 1 day for the restoration and restabilization of soil microbial community.

For microcosm experiments, soil slurry was prepared by adding basal salt medium (BSM) (Inoue et al. 2005) to the soil sample to prepare a slurry content of 30% (v/w). Four slurry microcosms (microcosms A–D) were prepared in 500-ml Erlenmeyer flasks containing 250-ml of the soil slurry. Microcosms A–C were amended with 200 mg-2,4-D/kg-dry soil. Overnight cultures of *P. putida* KT2440(pJP4) and *E. coli* HB101(pJP4) were harvested by centrifugation (8,500×*g*, 4°C, 10 min) and then washed twice with 50 mM phosphate buffer (pH 7.5). Next, the pellets of *P. putida* KT2440(pJP4) and *E. coli* HB101(pJP4) were suspended in 50 mM phosphate buffer and inoculated into microcosms A and B, respectively, to give an optical density of 1.0 at 600 nm (approximately 1.0×10^8 CFU/ml). All of the microcosms were then incubated at 28°C in the dark on a rotary shaker at 120 rpm. The microcosms were periodically sampled to monitor the introduced bacteria and indigenous microbial community, and for measurement of the 2,4-D concentration, and Biolog and nitrate reduction assays.

Enumeration of inoculated and indigenous bacteria by culturing methods

Pseudomonas putida KT2440(pJP4) and *E. coli* HB101(pJP4) inoculated into microcosms were enumerated on BSM containing glucose (1 g/l) and $HgCl_2$ (5 mg-Hg/l). Thiamine (50 mg/l), leucine (50 mg/l), proline (50 mg/l), and streptomycin (20 mg/l) were also added to the medium for *E. coli* HB101(pJP4). Total heterotrophic bacteria were counted on R2A agar (Merck, Darmstadt, Germany). 2,4-D-utilizing bacteria were detected using a 2,4-D indicator plate (Newby et al. 2000). Transconjugants were selected from the colonies that appeared on the 2,4-D indicator plate based on resistance to Hg and amplification of the *tfdB* gene by colony PCR as described below. Cycloheximide (100 mg/l) was added to all selective media to prevent fungal growth. All liquid media was amended with 1.7% (w/v) agar to prepare solid media.

Confirmation and characterization of indigenous transconjugants

The presence of pJP4 in transconjugant cells obtained in the selective plating was confirmed by colony PCR (Joshi et al. 1991) targeting the *tfdB* gene that encodes 2,4-dichlorophenol hydroxylase. PCR amplification was conducted using primers 591 and 592 (Neilson et al. 1992). Isolated transconjugant cells were morphologically and physiologically characterized as described previously (Inoue et al. 2008). Representative transconjugants were subjected to terminal restriction fragment length polymorphism (T-RFLP) analysis as described below to determine their terminal restriction fragments (T-RFs). Some of them were also identified by phylogenetic analysis based on their 16S rRNA gene sequence (Inoue et al. 2008). Furthermore, their potentials as plasmid donors were evaluated by broth mating (Inoue et al. 2005) using spontaneous rifampicin resistant mutants of *P. putida* BH (Hashimoto and Fujita 1987) as the recipient. The plasmid transfer frequency (the number of transconjugants per recipient) after 24 h was calculated as the index for the plasmid transfer potential.

DNA extraction

Bacterial DNA was extracted from 0.5 g-wet soil slurry using ISOIL for Beads Beating (Nippon Gene, Tokyo, Japan) and then purified with a MagExtractor-PCR&Gel Clean up kit (Toyobo, Tokyo, Japan) according to the manufacturers' instructions.

Quantification of introduced bacteria by real-time PCR

The *pcaG* gene, which encodes protocatechuate 3,4-dioxygenase alpha subunit, on the *P. putida* KT2440 chromosome (Nelson et al. 2002) and the *tbpA* gene, which encodes thiamin transporter subunit, on the *E. coli* HB101 chromosome (Blattner et al. 1997) were quantified by real-time PCR. The following specific PCR primers were designed for each target gene using the Primer Express software ver. 2.0

(Applied Biosystems, CA, USA): pca-F (5′-ATCCACTTCGCAACGCTTG-3′) and pca-R (5′-GTATTTCGATGATGAGGCCCA-3′) for the *pcaG* gene and tbp-F (5′-GCATCCATAGCAACAGACCCA-3′) and tbp-R (5′-CGCCACAAAGCCTGAAAGAA-3′) for the *tbpA* gene. The specificity of the designed primers was confirmed by BLAST similarity searches (Altschul et al. 1990) and by PCR amplification using a DNA template prepared from the soil that was used to construct the microcosms prior to the experiments (data not shown). Real-time PCR assays were conducted on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using the SYBR Green PCR Master Mix (Applied Biosystems). Primers were added at 200 and 500 nM to quantify the *tbpA* and *pcaG* genes, respectively. A thermal profile for quantification of the *tbpA* gene consisted of initial enzyme activation at 95°C for 10 min and then 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. The thermal profile for the *pcaG* gene quantification consisted of an initial enzyme activation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 61°C for 20 s, and extension at 72°C for 30 s. The results were obtained as CFU-equivalents/ml-slurry using DNA prepared from pure culture of introduced bacteria as the standard.

Quantification of 16S rRNA, *amoA*, *nirK* and *nirS* genes by MPN-PCR

The copy numbers of eubacterial 16S rRNA, *amoA*, *nirK*, and *nirS* genes in each sample were measured by the most probable number-polymerase chain reaction (MPN-PCR) as described elsewhere (Sang et al. 2008). The number of copies of the 16S rRNA gene was used as the index of total eubacteria, whereas the copy numbers of the *amoA* gene and the *nirK* and *nirS* genes were used as indices of ammonia-oxidizing bacteria and denitrifying bacteria, respectively. PCR amplifications of the 16S rRNA, *amoA*, *nirK*, and *nirS* genes were conducted using the following primers: EUBf-933 and EUBr-1387 (Iwamoto et al. 2000), *amoA*-1F and *amoA*-2R (Rotthauwe et al. 1997), NIRK-F and NIRK-R (Sang et al. 2008), and NIRS-F3 and NIRS-R3 (Sang et al. 2008), respectively. The gene copy numbers were determined as MPN-DNA copies/g-dry soil at the 95% confidence interval using Cochran's tables (Cochran 1950).

T-RFLP analysis

The eubacterial community structure was analyzed by T-RFLP targeting the eubacterial 16S rRNA gene as described previously (Matsuda et al. 2010) with minor modifications. The T-RFs with peak height of 200 fluorescence units were judged as positive peaks. The diversity of eubacterial community was evaluated using the Shannon–Weaver index (H') (Blackwood et al. 2007). The H' values were calculated from T-RFLP profiles according to the following equation:

$$H' = -\sum (Pi \times \ln Pi),$$

where Pi is the relative abundance of the targeted T-RF. Similarities in the T-RFLP profiles among samples were identified by cluster analysis employing the unweighted pair group method with arithmetic averages (UPGMA) of Dice's coefficients using PAST ver.1.3.4 (http://folk.uio.no/ohammer/past). Cluster analysis was performed with the presence/absence matrix of T-RFs.

Biolog assay

The carbon source utilization potential, namely the potential of soil microbial community for utilizing 95 different kinds of sole-carbon sources, was evaluated using Biolog GN2 plates (Biolog, Hayward, CA, USA). Briefly, aliquots (1 g-wet) of soil slurry were added to 9 ml of saline solution (0.85% (w/v) NaCl), vigorously mixed for 30 min, and then allowed to settle for 10 min. The supernatant was then diluted by tenfold with fresh saline solution, after which aliquots (150 µl) of the solution were added to the wells of a Biolog GN2 plate. The plates were then statically incubated at 28°C in the dark, after which the absorbance at 595 nm (A_{595}) was measured periodically during a 72-h period. The average well color development (AWCD) (Garland and Mills 1991) was used to evaluate the carbon source utilization potential of the microbial community.

Nitrate reduction assay

Aliquots (1 g-wet) of soil slurry were inoculated into 9 ml of sterilized standard nitrate solution [KNO_3 1 mM and $C_4H_4Na_2O_4{\cdot}6H_2O$ 1 mM (as the electron donor and carbon source)] in 25-ml glass vials and

then sealed with butyl rubber septa and aluminum crimp seals. After the headspace was replaced with N_2 gas, vials were incubated at 28°C on a rotary shaker at 120 rpm. At the indicated intervals, two vials were destructively sampled to determine the nitrate concentration.

Chemical analysis

Measurement of the 2,4-D concentration and detection of 2,4-D metabolites were performed by high-performance liquid chromatography (HPLC) as previously described (Newby et al. 2000). A Shimadzu LC-10A*vp* HPLC system (Shimadzu, Kyoto, Japan) equipped with a Shim-Pack VP-ODS column (150 × 4.6 mm [i.d.]; particle size, 5 μm; Shimadzu) and an SPD-10A*vp* UV/VIS detector (Shimadzu) was used for HPLC analysis. Prior to HPLC analysis, 2,4-D and its metabolites were extracted from soil slurry samples according to the method described by Newby et al. (2000). The recovery of 2,4-D from the soil slurry by the extraction method was 96.1 ± 2.9% ($n = 8$), and the detection limit of 2,4-D was 4.6 mg/kg-dry soil.

For the nitrate analysis, 1 ml aliquots of soil slurry sample were centrifuged (14,000×*g*, 4°C, 10 min), and the supernatants were then subjected to determination of the nitrate concentration by ion chromatography using a DX-300 IC system (Dionex, CA, USA) as previously described (Yamamura et al. 2003).

Nucleotide sequence accession numbers

The partial 16S rRNA gene sequences of transconjugant strains H6-1, K6-1, and K20-1 were registered in GenBank/EMBL/DDBJ as accession numbers AB578860, AB578861, and AB578862, respectively.

Results

2,4-D degradation

Figure 1 shows the 2,4-D degradation profiles of microcosms A–C. All of these microcosms contained 2,4-D-contaminated soil slurry, and microcosms A and B were inoculated with *P. putida* KT2440(pJP4) and *E. coli* HB101(pJP4), respectively. In microcosms A and B, which were inoculated with *P. putida* KT2440(pJP4) and *E. coli* HB101(pJP4), respectively, 2,4-D was completely degraded within 6 days after a lag phase of 3 days. In uninoculated microcosm C, a lag period of 6 days was observed prior to the onset of 2,4-D degradation, and 2,4-D was below the detection limit on day 20. No metabolites of 2,4-D were detected throughout the monitoring period, regardless of inoculation with pJP4-harboring bacteria.

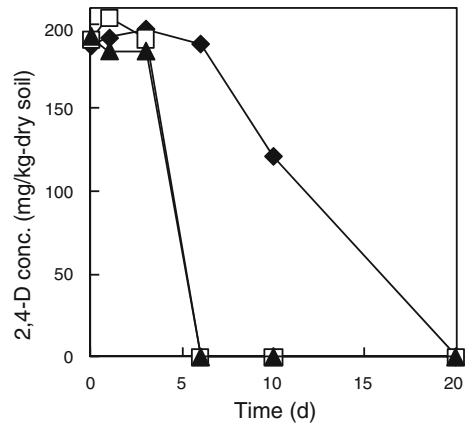

Fig. 1 2,4-D degradation profiles in microcosms A (2,4-D-contaminated soil slurry with introduction of *P. putida* KT2440(pJP4)) (*closed triangle*), B (2,4-D-contaminated soil slurry with introduction of *E. coli* HB101(pJP4)) (*open square*) and C (2,4-D-contaminated soil slurry without introduction of exogenous bacteria) (*closed diamond*)

Fate of introduced and indigenous bacteria

Cultivable cells of *P. putida* KT2440(pJP4) and *E. coli* HB101(pJP4) decreased rapidly in microcosms A and B, respectively, and were below the detection limit (3.3×10^3 and 1.9×10^6 CFU/ml-slurry, respectively) within 1 day (Fig. 2a, b). The high detection limit for *E. coli* HB101(pJP4) was due to its auxotrophic nature. The decline of chromosomal genes of the introduced bacteria was slower than that of cultivable introduced bacteria (Supplementary material—Fig. S1).

The abundance of total heterotrophic bacteria in microcosms A, B, C, and D did not change markedly except for the initial period (Fig. 2). The number of 2,4-D-utilizing bacteria was stable at around 10^6 CFU/ml-slurry in control microcosm D without 2,4-D-contamination and exogenous bacterial introduction throughout the monitoring period, while it

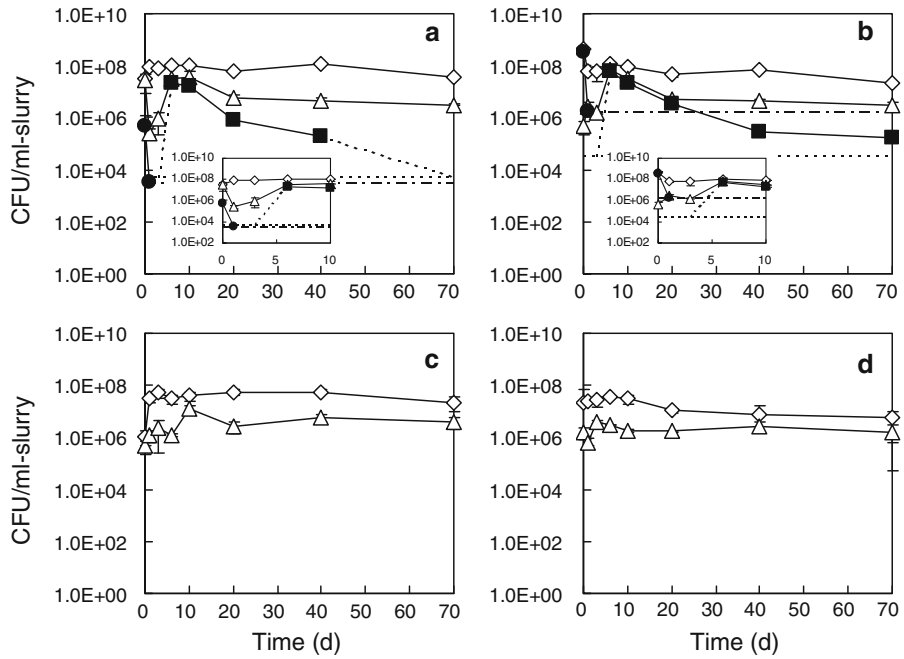

Fig. 2 Abundance of introduced bacteria (*closed circle*), heterotrophic bacteria (*open diamond*), 2,4-D-utilizing bacteria (*open triangle*) and transconjugants (*closed square*) in microcosms A (2,4-D-contaminated soil slurry with introduction of *P. putida* KT2440(pJP4)) (**a**), B (2,4-D-contaminated soil slurry with introduction of *E. coli* HB101(pJP4)) (**b**), C (2,4-D-contaminated soil slurry without introduction of exogenous bacteria) (**c**) and D (uncontaminated soil slurry) (**d**) analyzed by culturing methods. *Error bar* shows the 95% confidence interval. *Dashed* and *broken lines* represent the detection limit of introduced bacteria and transconjugants, respectively

gradually increased from 4.5×10^5 CFU/ml-slurry to 1.2×10^7 CFU/ml-slurry during the first 10 days in microcosm C (Fig. 2c, d). In microcosm A, 2,4-D-utilizing bacteria rapidly declined by two orders of magnitude from day 0 to day 1, after which they increased to 10^7 CFU/ml-slurry by day 6, and then decreased again to 10^6 CFU/ml-slurry by day 10 (Fig. 2a). In microcosm B, 2,4-D-utilizing bacteria increased from 4.6×10^5 CFU/ml-slurry to 6.9×10^7 CFU/ml-slurry from day 0 to day 6, after which they decreased to 5.0×10^6 CFU/ml-slurry by day 20 (Fig. 2b). After 20 days, the 2,4-D-utilizing bacteria remained stable at 10^6 CFU/ml-slurry in both microcosms. Increases in the number of 2,4-D-utilizing bacteria in microcosms A, B, and C in the initial period were well correlated with the 2,4-D degradation profile shown in Fig. 1.

In microcosms C and D, where exogenous bacteria harboring pJP4 were not inoculated, no 2,4-D-utilizing bacteria detected had both the Hg resistance and the *tfdB* gene, confirming that 2,4-D-utilizing bacteria with Hg resistance and *tfdB* gene in microcosms A and B were indigenous transconjugants that received pJP4. In microcosms A and B, transconjugants harboring pJP4 appeared on day 6 at 2.2×10^7 and 5.7×10^7 CFU/ml-slurry, respectively (Fig. 2a, b). Subsequently, they decreased to approximately 2×10^5 CFU/ml-slurry by day 40 in both microcosms. On day 70, the transconjugant population was below the detection limit (5.3×10^3 CFU/ml-slurry) in microcosm A, while it remained at 1.8×10^5 CFU/ml-slurry in microcosm B. The percentages of transconjugants in 2,4-D-utilizing bacteria in microcosms A and B were 66 and 82% on day 6, 46% and 72% on day 10, 13% and 64% on day 20, and 4.1 and 6.5% on day 40, respectively.

Characterization of pJP4-harboring transconjugants

A total of 35 transconjugants colonies (3 and 10 colonies on day 6, 1 and 7 colonies on day 10, 4 and 5 colonies on day 20, 2 and 3 colonies on day 40 from microcosms A and B, respectively) were isolated for

the physiological characterization. All the isolated transconjugants were gram-negative, rod-shaped, non-motile, catalase-positive, oxidase-negative bacteria, and their colonies were morphologically identical. Further, all of them were able to produce acid from glucose (oxidative in the oxidation/fermentation test). T-RFLP analysis of eight transconjugant colonies (K6-1, K10-1, K20-1 and K40-1 from microcosms A and H6-1, H6-7, H10-3 and H20-5 from microcosm B) showed the same length of T-RF (200 bp). Therefore, two transconjugant colonies (K6-1 and K20-1) from microcosm A and one colony (H6-1) from microcosm B were selected as the representatives for phylogenetic analysis based on their partial 16S rRNA gene sequence. Strain K6-1 had the greatest 16S rRNA gene similarity of 99.2% with *Burkholderia ubonensis* CIP107078^T (accession number: EU024179), while the closest phylogenetic relative of strains K20-1 and H6-1 was *Burkholderia unamae* MTI-641^T (accession number: NR_027569) with a sequence similarity of 98.2 and 98.1%, respectively. Thus, we identified strain K6-1 as *B. ubonensis* and strains K20-1 and H6-1 as *Burkholderia* sp.

The potential of eight transconjugants, which were used for the determination of T-RF length, as the secondary donor of pJP4 was evaluated by broth mating (Fig. 3). Transfer of pJP4 to the rifampicin resistant mutant of *P. putida* BH used as the recipient was detected in seven of eight transconjugants. The transfer frequency of the seven transconjugants ranged from 4.4×10^{-8} to 5.0×10^{-7}, and was lower than that of *P. putida* KT2440(pJP4) and *E. coli* HB101(pJP4).

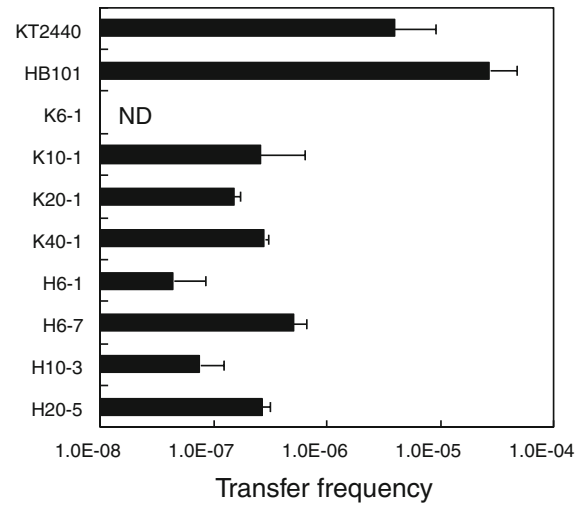

Fig. 3 Potential for transconjugants to act as the donor of plasmid pJP4. Spontaneous rifampicin-resistant *P. putida* BH was used as the recipient in the transfer experiments. The transfer frequency was calculated as transconjugants per recipient. *Error bars* indicate SD in triplicate experiments. *ND* not detected

Impact of bioaugmentation on the indigenous microbial community

Impact on the whole eubacterial community

Total number of eubacterial 16S rRNA genes was generally stable and did not change drastically in any of the microcosms, although it fluctuated slightly with time (Fig. 4). T-RFLP analysis of the eubacterial community revealed that T-RFs of the introduced bacteria (204 and 373 bp for *P. putida* KT2440 and *E. coli* HB101, respectively) were dominant in microcosms A and B on day 0 (Fig. 5). Consequently, the whole T-RF profiles in these microcosms were quite different from those in uninoculated microcosms C and D. The T-RFs of the introduced bacteria diminished with time, and entire T-RF profiles became similar in all four microcosms (Fig. 5). Calculation of Shannon–Weaver diversity index (H') based on the T-RF profiles revealed that the diversity of eubacterial community in microcosms A and B was lower than that in microcosms C and D on day 0 (Table 1). Thereafter, the community diversity in microcosms A and B increased and became similar to that in microcosms C and D. Cluster analysis based on the T-RF profiles showed that on day 0, the four microcosms shared less than 40% similarity although they were allocated in a cluster (Fig. 6). However, the similarity between the inoculated and uninoculated microcosms increased with time. On day 70, the eubacterial community in all four microcosms was allocated in a small cluster and shared greater than 75% similarity with each other.

Impacts on carbon source utilization potential

The AWCD of the microbial community in slurry microcosms on days 0 and 20 is shown in Fig. 7. On day 0, the AWCD profiles of microcosms C and D were similar to each other (Fig. 7a). The AWCD of microcosm B was slightly lower than that of

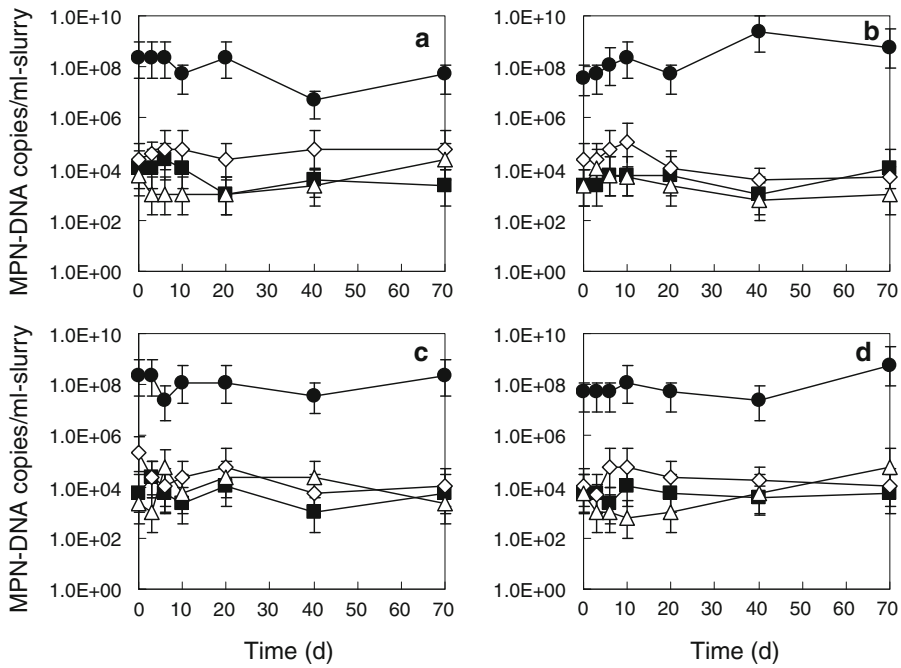

Fig. 4 Abundance of 16S rDNA (*closed circle*) and functional genes related to nitrogen cycling, i.e. *amoA* (*open triangle*), *nirK* (*closed square*) and *nirS* (*open diamond*) genes, in microcosms A (**a**), B (**b**), C (**c**) and D (**d**) as determined by MPN-PCR. *Error bar* shows the 95% confidence interval

microcosms C and D. Although the AWCD was highest in microcosm A during the early period, the increase in AWCD became slower than in the other microcosms during the later period, and at 72 h it was similar to that of microcosm B. The AWCD profile of microcosm D on day 20 was almost the same as that on day 0 (Fig. 7). In contrast, the AWCD values of microcosms A–C were higher than those of the corresponding microcosms on day 0. This was likely due to the activation of indigenous bacteria capable of utilizing 2,4-D and its metabolites in response to the 2,4-D contamination. Overall, the AWCD on day 20 was in the following order: microcosm C > microcosm A > microcosm B > microcosm D (Fig. 7b).

Impacts on nitrogen cycling function

The dynamics of the *amoA*, *nirK*, and *nirS* genes in four microcosms are shown in Fig. 4. Although the number of *amoA* genes increased by more than 50 folds from day 3 to day 6 in microcosm C (Fig. 4c), no noticeable stimulation or suppression was observed in response to the introduction of exogenous bacteria (Fig. 4a, b). Indeed, the variation in the *amoA* gene number in microcosm A was nearly identical to that in microcosm D, which did not contain 2,4-D contamination or exogenous bacteria (Fig. 4a, d). No great difference in the copy number of the *nirK* and *nirS* genes compared with that in microcosm D was observed in microcosms A, B, and C during the 70-day experimental period (Fig. 4).

The nitrate reduction potentials of the microbial community in the four microcosms on days 0 and 20 are shown in Fig. 8. On day 0, the nitrate reduction potential of microcosm C was similar to that of microcosm D (Fig. 8a). By contrast, the nitrate reduction in microcosms A and B was slower and faster than in microcosm D, respectively. The nitrate reduction potential on day 20 was lower than that on day 0 in microcosms B, C, and D, although the potential in microcosm A was similar on both investigated days (Fig. 8). Consequently, the nitrate reduction potential became relatively similar among all four microcosms (Fig. 8b).

Discussion

In this study, gene bioaugmentation experiments were conducted in 2,4-D contaminated soil slurry microcosms using *P. putida* KT2440 and *E. coli* HB101 as the carrier of pJP4. Cultivable cells of both exogenous bacteria declined very rapidly in the microcosms (Fig. 2a, b). Rapid reduction of exogenously

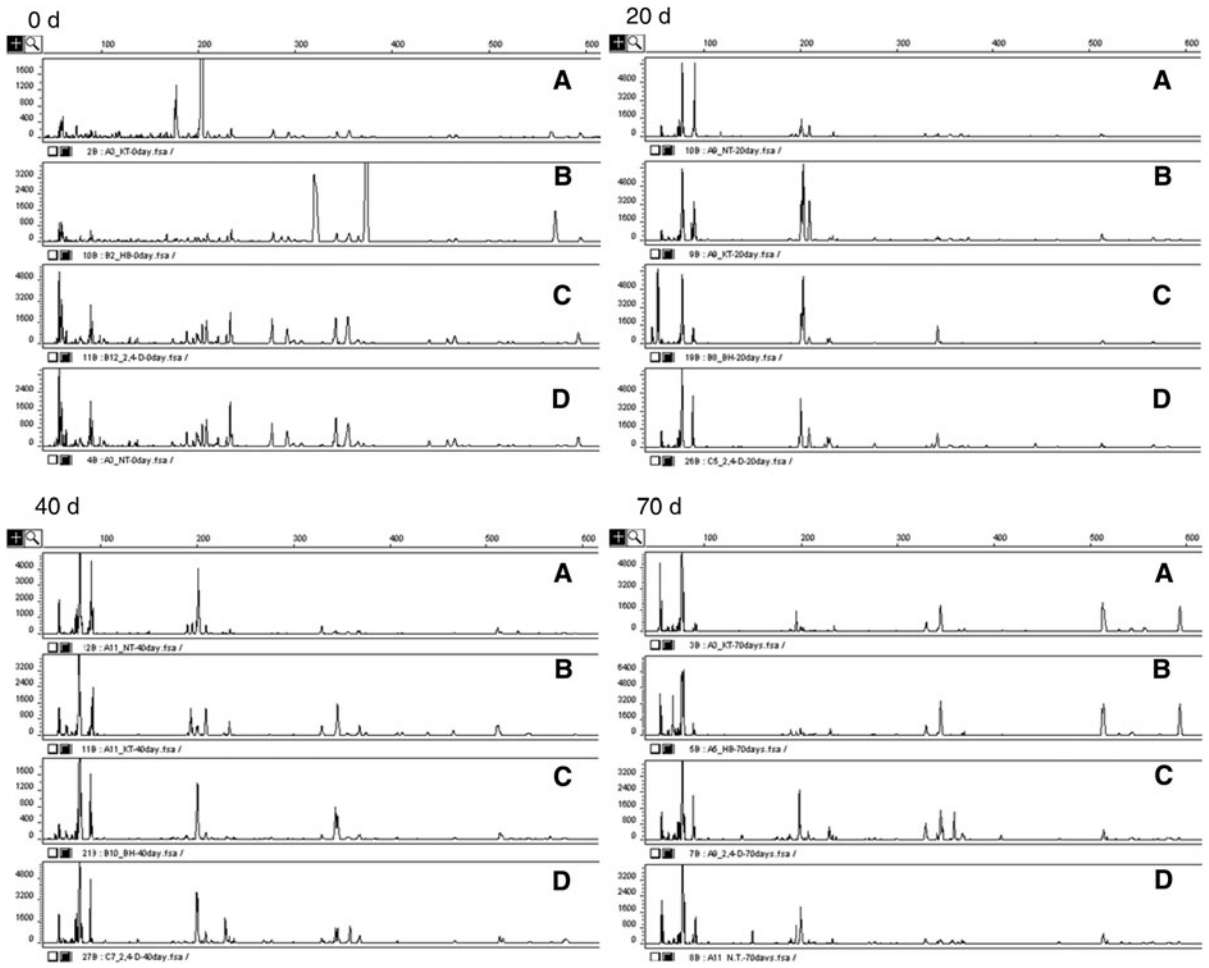

Fig. 5 T-RF profiles of the eubacterial community in microcosms A, B, C and D on days 0, 20, 40 and 70

Table 1 Shannon–Weaver diversity index (H') of eubacterial community in microcosms calculated from T-RF profiles

Time (days)	Microcosm			
	A	B	C	D
0	2.4	2.7	3.8	3.6
20	3.1	3.3	3.1	2.7
40	3.2	3.1	3.5	3.0
70	3.6	3.5	3.6	3.6

introduced bacteria in soil has been commonly observed in previous studies (Ramos et al. 1991; Recorbet et al. 1993). Such rapid reduction of exogenous bacteria is likely due to a combination of various abiotic (e.g., nutrient limitation and temperature and pH stresses) and biotic (competition with indigenous bacteria and predation by protozoa) factors (Goldstein et al. 1985; van Veen et al. 1997). Conversely, the decline in the chromosomal genes of introduced bacteria was much slower than that of introduced bacteria monitored by the culturing method (Figs. 2a, b, S1). This was likely because part of the introduced bacteria entered into a viable but nonculturable (VBNC) state (Bunker et al. 2004; Muela et al. 2008) or due to free DNA derived from the introduced bacteria persisting in the systems (Recorbet et al. 1993; Nielsen et al. 2007).

In general, a key factor of successful bioaugmentation (cell bioaugmentation) is the density and activity of the introduced microorganisms (van Veen et al. 1997); thus, bioaugmentation fails if the introduced bacteria survive poorly. However, despite the rapid reduction of introduced bacteria, the 2,4-D

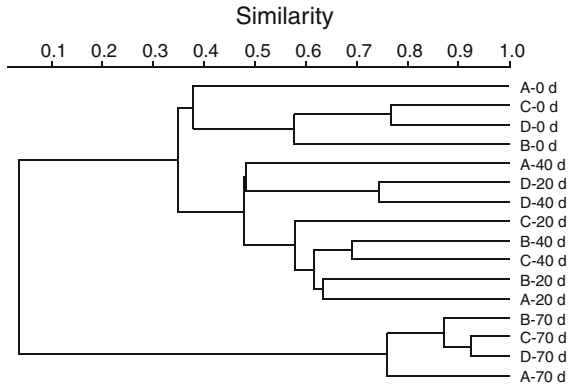

Fig. 6 Cluster analysis of T-RF profiles of the eubacterial community in microcosms. The dendrogram was constructed by the UPGMA method using Dice's coefficients

degradation in soil slurry was successfully enhanced (Fig. 1), as observed in previous studies in which pJP4-harboring bacteria were introduced into the soil (Dejonghe et al. 2000; Newby et al. 2000). The concomitant increase in the number of indigenous transconjugants with 2,4-D degradation ability that was observed in microcosms A and B (Fig. 2a, b) may suggest that these transconjugants would make a significant contribution to the 2,4-D degradation in these microcosms. Thus, during gene bioaugmentation, sufficient remediation effects can be obtained by transfer of the specific degradative gene/plasmid of introduced bacteria to indigenous bacteria, even if the introduced bacteria survive poorly. It has been observed that conjugative plasmid transfer can occur even when environmental conditions are unfavorable for survival of the original donor, which results in the original donor entering the VBNC state (Chandrasekaran et al. 1998) or declining rapidly (de Lipthay et al. 2001).

In this study, transconjugants appeared to be limited to the genus *Burkholderia*, which have frequently been found to be 2,4-D-degrading transconjugants of plasmid pJP4 in soil (DiGiovanni et al. 1996; Goris et al. 2002). Almost all of the isolated indigenous transconjugants can express the transfer function of pJP4 (Fig. 3). Thus, the transconjugant population may easily increase in response to the

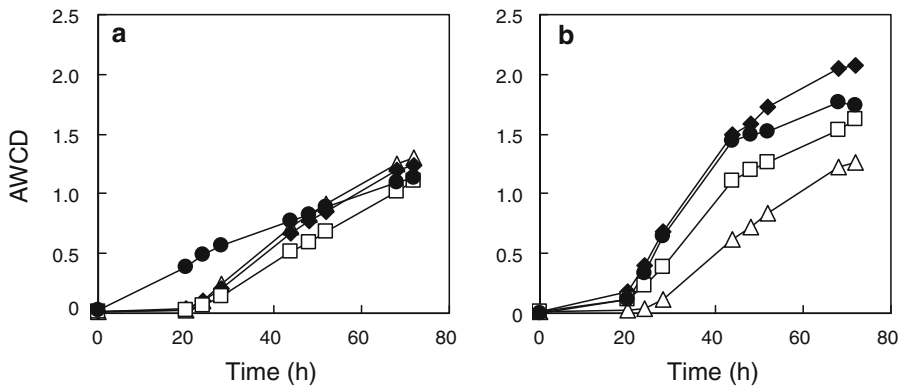

Fig. 7 Average well color development (AWCD) in Biolog GN2 microplates for microcosms A (*closed circle*), B (*open square*), C (*closed diamond*) and D (*open triangle*) on days 0 (**a**) and 20 (**b**). Data points are the means of duplicate experiments

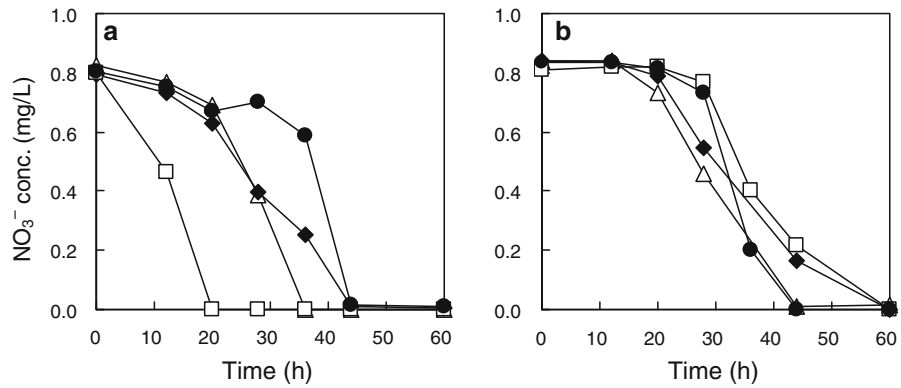

Fig. 8 Biological nitrate reduction potential for microcosms A (*closed circle*), B (*open square*), C (*closed diamond*) and D (*open triangle*) on days 0 (**a**) and 20 (**b**). Data points are the means of duplicate experiments

secondary conjugative transfer from initial transconjugants to plasmid-free indigenous bacteria, in addition to the vertical transfer of pJP4 through cell replication of generated transconjugants, once conjugative plasmid transfer from introduced bacteria to indigenous bacteria occurs. In addition, persistence of transconjugant population even without the selective pressure (i.e., 2,4-D) may suggest that plasmid pJP4 would be relatively stable in the soil microbial community.

In this study, possible impacts of gene bioaugmentation on the soil microbial community were also assessed by monitoring the whole eubacterial community and specific microbial populations related to the carbon and nitrogen transformations.

The numbers of total heterotrophic bacteria and eubacterial 16S rRNA genes did not differ greatly in response to 2,4-D contamination or introduction of exogenous bacteria, although the numbers of total heterotrophic bacteria on day 0 in microcosms A and B were slightly higher than those in microcosm D, while those in microcosm C were slightly lower than those in microcosm D (Figs. 2, 4). Conversely, T-RFLP analysis showed that, although 2,4-D contamination exhibited little impact on the structure and diversity of the indigenous bacterial community, introduction of exogenous bacteria suppressed the indigenous bacteria and reduced the complexity of the indigenous bacterial community immediately after their introduction (Figs. 5, 6; Table 1). Previous studies have also reported that the complexity of the bacterial community (genetic diversity) in soil was significantly reduced by bioaugmentation (Gomes et al. 2005; Coppotelli et al. 2008; Niu et al. 2009). Such a reduction in the complexity of the bacterial community might be caused by the numerical dominance of the introduced bacteria. Previous studies have also reported that disturbance of the indigenous bacterial community continues for a prolonged period due to the persistence of the introduced bacteria (Baek et al. 2007; Coppotelli et al. 2008). However, in this study, the bacterial community structure and diversity in microcosms A and B gradually approached that of microcosm D (Fig. 6). During the same period, introduced bacteria disappeared rapidly, while indigenous transconjugant populations that received plasmid pJP4 persisted in microcosms A and B (Fig. 2). Therefore, the additional impact on the bacterial community structure and diversity via plasmid dissemination might be considerably smaller than the impact of the introduced bacteria. Accordingly, the impact of gene bioaugmentation on the structure/diversity of the bacterial community may be almost equivalent to that of cell bioaugmentation if the remediation of contaminated soil is completed within a short period of time, as is the case in this study.

Previous studies have reported that the gross metabolic activity of the indigenous microbial community declined in soil amended with 2,4-D and inoculated with *P. putida* PPO301 harboring plasmid pRO103, a derivative of plasmid pJP4 that encodes genes for the degradation of 2,4-D to 2-chloromaleylacetate, due to the accumulation of 2,4-dichlorophenol, a toxic metabolite of 2,4-D (Doyle et al. 1991; Short et al. 1991). Conversely, in this study, the carbon source utilization potential was similar on days 0 and 20 in microcosm D, which had no 2,4-D contamination, while it increased from day 0 to day 20 in the other three microcosms, which had 2,4-D contamination, regardless of the introduction of pJP4-harboring bacteria (Fig. 7). That is, gene bioaugmentation with pJP4-harboring bacteria applied here might not induce a depressive effect on the overall carbon source utilization potential of the indigenous soil microbial community. Because 2,4-D was rapidly degraded and no metabolites accumulated in microcosms A–C, the toxic effects of 2,4-D metabolites on the indigenous microorganisms would be negligible; thus, the metabolic potential of the indigenous microorganisms would not be depressed. Moreover, enhancement of the carbon source utilization potential in microcosms A, B, and C was likely due to the activation of indigenous microorganisms by 2,4-D addition. Microcosm C showed higher carbon source utilization potential than microcosms A and B on day 20 (Fig. 7b). Although the limited transconjugant population receiving plasmid pJP4 seemed to mainly degrade 2,4-D and its metabolites in microcosms A and B, a variety of indigenous microorganisms with the ability to degrade 2,4-D and its metabolites would be activated in microcosm C, resulting in a higher gross metabolic potential of the indigenous microbial community.

Temporal variations in the *amoA*, *nirK*, and *nirS* genes did not differ greatly among the four microcosms (Fig. 4). Thus, the introduction of exogenous bacteria harboring the transmissible plasmid need not

have a drastic impact on the dynamics of microbial populations related to nitrification and denitrification in soil. Nitrate reduction assays revealed that the nitrate reduction potentials of the microbial community in microcosms A and B were respectively lower and higher than those in uninoculated microcosms immediately after introducing exogenous bacteria (day 0) (Fig. 8a). In microcosms A and B, the introduced bacteria were dominant on day 0. *E. coli* is well-known to be capable of dissimilatory reduction of nitrate (Bonnefoy and Demoss 1994), while *P. putida* is not. Thus, these findings suggest that the nitrate reduction potential of microcosms A and B was greatly affected by that of the introduced bacteria, with microcosm B showing a high nitrate reduction potential due to the nitrate reducing ability of the introduced *E. coli* HB101(pJP4). By contrast, no noticeable difference in the nitrate reduction potential was detected in the four microcosms after the introduced bacteria disappeared and 2,4-D degradation was completed (day 20) (Fig. 8b). The lowering of nitrate reduction potential in microcosm B would be due to the disappearance of introduced *E. coli* HB101(pJP4). These findings may suggest that inoculation with exogenous bacteria had no drastic effect on the nitrate reduction potential of the indigenous microbial community. Thus, the gene bioaugmentation applied here may not have an irretrievable effect on the microbial populations involved in nitrification and denitrification. Jones et al. (1991), who introduced five genetically engineered microorganisms (GEMs) harboring transmissible plasmids into soil, also reported that none of the introduced GEMs exhibited consistent and significant impacts on ammonification, nitrification, denitrification, or the microbial populations involved in the transformations. They suggested that one possible reason for these findings was that the introduced GEMs had no genetic information (presumably) affecting the nitrogen transformations or the responsible microbial populations. Plasmid pJP4 used in this study also has no genetic information that directly influences nitrogen dynamics (Trefault et al. 2004); thus, the predominance of pJP4-harboring transconjugants might not have shown any significant impacts on the microbial populations involved in nitrification and denitrification.

The results obtained in this study showed that gene bioaugmentation with *P. putida* and *E. coli* strains harboring pJP4 can enhance the 2,4-D degradation ability of soil microbial community, despite the poor survival of the introduced bacteria. It was also revealed that the dissemination of pJP4 among the indigenous microbial community would be less influential on the indigenous microbial community than the introduction of exogenous bacteria, and the impact of gene bioaugmentation with *P. putida* and *E. coli* strains harboring pJP4 on the indigenous soil microbial populations and their ecological functions related to the carbon and nitrogen transformations need not to be irretrievable and long-lasting. Overall, the results may suggest that gene bioaugmentation applied here is effective for the remediation of 2,4-D contaminated soil without drastic impacts on the indigenous microbial community. However, further studies with various combinations of introduced bacterial strain, plasmid, contamination type (contaminant and contamination scenario), and soil characteristics (indigenous microbial community and physical characteristics) should be performed to determine possible ecological impacts of gene bioaugmentation in more detail and verify its effectiveness for remediation of contaminated environments.

Acknowledgments This study was supported in part by a Grant-in-Aid for Scientific Research (B) (No. 15310055) and for the Encouragement of Young Scientists (A) (No. 18681013, 21681009) from the Ministry of Education, Culture, Sports, Science and Technology of Japan. This study was conducted as part of "The project for studies on risk management of recombinant organisms in industrial uses," which was sponsored by the New Energy and Industrial Technology Development Organization (NEDO) and the Japan Bioindustry Association (JBA).

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Arshad M, Hussain S, Saleem M (2008) Optimization of environmental parameters for biodegradation of alpha and beta endosulfan in soil slurry by *Pseudomonas aeruginosa*. J Appl Microbiol 104:364–370

Baek K-H, Yoon B-D, Kim B-H, Cho D-H, Lee I-S, Oh H-M, Kim H-S (2007) Monitoring of microbial diversity and activity during bioremediation of crude oil-contaminated soil with different treatments. J Microbiol Biotechnol 17:67–73

Bagdasarian M, Lurz R, Rückert B, Franklin FCH, Bagdasarian MM, Frey J, Timmis KN (1981) Specific-purpose plasmid cloning vectors. II. Broad host range, high copy number,

RSF1010-derived vectors, and a host-vector system for gene cloning in *Pseudomonas*. Gene 16:237–247

Blackwood CB, Hudleston D, Zak DR, Buyer JS (2007) Interpreting ecological diversity indices applied to terminal restriction fragment length polymorphism data: insights from simulated microbial communities. Appl Environ Microbiol 73:5276–5283

Blattner FR, Plunkett G III, Bloch CA, Perna NT, Burland V, Riley M, Collado-Vides J, Glasner JD, Rode CK, Mayhew GF, Gregor J, Davis NW, Kirkpatrick HA, Goeden MA, Rose DJ, Mau B, Shao Y (1997) The complete genome sequence of *Escherichia coli* K-12. Science 277:1453–1462

Bonnefoy V, Demoss JA (1994) Nitrate reductase in *Escherichia coli*. Antonie van Leeuwenhoek 66:47–56

Boyer HW, Roulland-Dussoix D (1969) A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J Mol Biol 41:459–472

Bunker ST, Bates TC, Oliver JD (2004) Effects of temperature on detection of plasmid or chromosomally encoded *gfp*- and *lux*-labeled *Pseudomonas fluorescens* in soil. Environ Biosaf Res 3:83–90

Chandrasekaran S, Venkatesh B, Lalithakumari D (1998) Transfer and expression of a multiple antibiotic resistance plasmid in marine bacteria. Curr Microbiol 37:347–351

Cochran WG (1950) Estimation of bacterial densities by means of the "most probable number". Biometrics 6:105–116

Coppotelli BM, Ibarrolaza A, Del Panno MT, Morelli IS (2008) Effects of the inoculant strain *Sphingomonas paucimobilis* 20006FA on soil bacterial community and biodegradation in phenanthrene-contaminated soil. Microb Ecol 55:173–183

de Lipthay JR, Barkay T, Sørensen SJ (2001) Enhanced degradation of phenoxyacetic acid in soil by horizontal transfer of the *tfdA* gene encoding a 2,4-dichlorophenoxyacetic acid dioxygenase. FEMS Microbiol Ecol 35:75–84

Dejonghe W, Goris J, El Fantroussi S, Höfte M, De Vos P, Verstraete W, Top EM (2000) Effect of dissemination of 2,4-dichlorophenoxyacetic acid (2,4-D) degradation plasmids on 2,4-D degradation and on bacterial community structure in two different soil horizons. Appl Environ Microbiol 66:3297–3304

DiGiovanni GD, Neilson JW, Pepper IL, Sinclair NA (1996) Gene transfer of *Alcaligenes eutrophus* JMP134 plasmid pJP4 to indigenous soil recipients. Appl Environ Microbiol 62:2521–2526

Don RH, Pemberton JM (1981) Properties of six pesticide degradation plasmids isolated from *Alcaligenes paradoxus* and *Alcaligenes eutrophus*. J Bacteriol 145:681–686

Doyle JD, Short KA, Stotzky G, King RJ, Seidler RJ, Olsen RH (1991) Ecologically significant effects of *Pseudomonas putida* PPO301(pRO103), genetically engineered to degrade 2,4-dichlorophenoxyacetate, on microbial populations and processes in soil. Can J Microbiol 37:682–691

Garland JL, Mills AL (1991) Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. Appl Environ Microbiol 57:2351–2359

Goldstein RM, Mallory LM, Alexander M (1985) Reasons for possible failure of inoculation to enhance biodegradation. Appl Environ Microbiol 50:977–983

Gomes NCM, Kosheleva IA, Abraham W-R, Smalla K (2005) Effects of the inoculant strain *Pseudomonas putida* KT2442 (pNF142) and of naphthalene contamination on the soil bacterial community. FEMS Microbiol Ecol 54:21–33

Goris J, Dejonghe W, Falsen E, De Clerck E, Geeraerts B, Willems A, Top EM, Vandamme P, De Vos P (2002) Diversity of transconjugants that acquired plasmid pJP4 or pEMT1 after inoculation of a donor strain in the A- and B-horizon of an agricultural soil and description of *Burkholderia hospital* sp. nov. and *Burkholderia terricola* sp. nov. Syst Appl Microbiol 25:340–352

Habe H, Ide K, Yotsumoto M, Tsuji H, Yoshida T, Nojiri H, Omori T (2002) Degradation characteristics of a dibenzofuran-degrader *Terrabacter* sp. strain DBF63 toward chlorinated dioxins in soil. Chemosphere 48:201–207

Hashimoto S, Fujita M (1987) Identification of three phenol-degrading microorganisms isolated from activated sludge and their characteristics. J Jpn Sew Wks Assoc 9:655–660 (in Japanese)

Inoue D, Sei K, Soda S, Ike M, Fujita M (2005) Potential of predominant activated sludge bacteria as recipients in conjugative plasmid transfer. J Biosci Bioeng 100:600–605

Inoue D, Hara S, Kashihara M, Murai Y, Danzl E, Sei K, Tsunoi S, Fujita M, Ike M (2008) Degradation of bis(4-hydroxyphenyl)methane (bisphenol F) by *Sphingobium yanoikuyae* strain FM-2 isolated from river water. Appl Environ Microbiol 74:352–358

Iwamoto T, Tani K, Nakamura K, Suzuki Y, Kitagawa M, Eguchi M, Nasu M (2000) Monitoring impact of in situ biostimulation treatment on groundwater bacterial community by DGGE. FEMS Microbiol Ecol 32:129–141

Jernberg C, Jansson JK (2002) Impact of 4-chlorophenol contamination and/or inoculation with the 4-chlorophenol-degrading strain, *Arthrobacter chlorophenolicus* A6L, on soil bacterial community structure. FEMS Microbiol Ecol 42:387–397

Jones RA, Broder MW, Stotzky G (1991) Effects of genetically engineered microorganisms on nitrogen transformations and nitrogen-transforming microbial populations in soil. Appl Environ Microbiol 57:3212–3219

Joshi AK, Baichwal V, Ames GFL (1991) Rapid polymerase chain reaction amplification using intact bacterial cells. BioTechniques 10:42–44

Keese P (2008) Risks from GMOs due to horizontal gene transfer. Environ Biosaf Res 7:123–149

Levin SA, Harwell MA (1986) Potential ecological consequences of genetically engineered organisms. Environ Manag 10:495–513

Matsuda M, Inoue D, Anami Y, Tsutsui H, Sei K, Soda S, Ike M (2010) Comparative analysis of DNA-based microbial community composition and substrate utilization patterns of activated sludge microorganisms from wastewater treatment plants operated under different conditions. Water Sci Technol 61:2843–2851

Muela A, Seco C, Camafeita E, Arana I, Orruño M, López JA, Barcina I (2008) Changes in *Escherichia coli* outer membrane subproteome under environmental conditions inducing the viable but nonculturable state. FEMS Microbiol Ecol 64:28–36

Neilson JW, Josephson KL, Pillai SD, Pepper IL (1992) Polymerase chain reaction and gene probe detection of the 2,4-dichlorophenoxyacetic acid degradation plasmid, pJP4. Appl Environ Microbiol 58:1271–1275

Nelson KE, Weinel C, Paulsen IT, Dodson RJ, Hilbert H, Martins dos Santos VAP, Fouts DE, Gill SR, Pop M, Holmes M, Brinkac L, Beanan M, DeBoy RT, Daugherty S, Kolonay J, Madupu R, Nelson W, White O, Peterson J, Khouri H, Hance I, Chris Lee P, Holtzapple E, Scanlan D, Tran K, Moazzez A, Utterback T, Rizzo M, Lee K, Kosack D, Moestl D, Wedler H, Lauber J, Stjepandic D, Hoheisel J, Straetz M, Heim S, Kiewitz C, Eisen J, Timmis KN, Düsterhöft A, Tümmler B, Fraser CM (2002) Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. Environ Microbiol 4:799–808

Newby DT, Gentry TJ, Pepper IL (2000) Comparison of 2,4-dichlorophenoxyacetic acid degradation and plasmid transfer in soil resulting from bioaugmentation with two different pJP4 donors. Appl Environ Microbiol 66:3399–3407

Nielsen KM, Johnsen PJ, Bensasson D, Daffonchio D (2007) Release and persistence of extracellular DNA in the environment. Environ Biosaf Res 6:37–53

Niu G-L, Zhang J-J, Zhao S, Liu H, Boon N, Zhou N-Y (2009) Bioaugmentation of a 4-chloronitrobenzen contaminated soil with *Pseudomonas putida* ZWL73. Environ Pollut 157:763–771

Park C, Kim T-H, Kim S, Lee J, Kim S-W (2003) Bioremediation of 2,4,6-trinitrotoluene contaminated soil in slurry and column reactors. J Biosci Bioeng 96:429–433

Paul D, Pandey G, Meier C, van der Meer JR, Jain RK (2006) Bacterial community structure of a pesticide-contaminated site and assessment of changes induced in community structure during bioremediation. FEMS Microbiol Ecol 57:116–127

Pepper IL, Gentry TJ, Newby DT, Roane TM, Josephson KL (2002) The role of cell bioaugmentation and gene bioaugmentation in the remediation of co-contaminated soils. Environ Health Perspect 110(suppl 6):943–946

Ramos JL, Duque E, Ramos-Gonzalez M-I (1991) Survival in soils of an herbicide-resistant *Pseudomonas putida* strain bearing a recombinant TOL plasmid. Appl Environ Microbiol 57:260–266

Recorbet G, Picard C, Normand P, Simonet P (1993) Kinetics of the persistence of chromosomal DNA from genetically engineered *Escherichia coli* introduced into soil. Appl Environ Microbiol 59:4289–4294

Rotthauwe J-H, Witzel K-P, Liesack W (1997) The ammonia monooxygenase structural gene *amoA* as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Appl Environ Microbiol 63:4704–4712

Sang NN, Soda S, Sei K, Ike M (2008) Effect of aeration on stabilization of organic solid waste and microbial population dynamics in lab-scale landfill bioreactors. J Biosci Bioeng 106:425–432

Short KA, Doyle JD, King RJ, Seidler RJ, Stotzky G, Olsen RH (1991) Effects of 2,4-dichlorophenol, a metabolite of a genetically engineered bacterium, and 2,4-dichlorophenoxyacetate on some microorganism-mediated ecological processes in soil. Appl Environ Microbiol 57:412–418

Trefault N, De la Iglesia R, Molina AM, Manzano M, Ledger T, Pérez-Pantoja D, Sánchez MA, Stuardo M, González B (2004) Genetic organization of the catabolic plasmid pJP4 from *Ralstonia eutropha* JMP134 (pJP4) reveals mechanisms of adaptation to chloroaromatic pollutants and evolution of specialized chloroaromatic degradation pathways. Environ Microbiol 6:655–668

Tsutsui H, Anami Y, Matsuda M, Inoue D, Sei K, Soda S, Ike M (2010) Transfer of plasmid pJP4 from *Escherichia coli* and *Pseudomonas putida* to bacteria in activated sludge developed under different sludge retention times. J Biosci Bioeng 110:684–689

van Veen JA, van Overbeek LS, van Elsas JD (1997) Fate and activity of microorganisms introduced into soil. Microbiol Mol Biol Rev 61:121–135

Yamamura S, Ike M, Fujita M (2003) Dissimilatory arsenate reduction by a facultative anaerobe, *Bacillus* sp. strain SF-1. J Biosci Bioeng 96:454–460

Zhao S, Ramette A, Niu G-L, Liu H, Zhou N-Y (2009) Effects of nitrobenzene contamination and of bioaugmentation on nitrification and ammonia-oxidizing bacteria in soil. FEMS Microbiol Ecol 70:315–323