Running head: An autochthonous bioaugmentation technique

Isolation and characterization of bacteria from soil contaminated with diesel oil, and the possible use of these in autochthonous bioaugmentation

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Summary

Two bacterial species (isolates N and O) were isolated from a paddy soil microcosm that had been artificially contaminated with diesel oil to which extrinsic *Pseudomonas aeruginosa*, strain WatG, had been added exogenously. One bacterial species (isolate J) was isolated from a similar soil microcosm that had been biostimulated with Luria–Bertani (LB) medium. Isolates N and O, which were tentatively identified as *Stenotrophomonas* sp. and *Ochromonas* sp., respectively, by sequencing of their 16 S rRNA genes had no ability to degrade diesel oil on their own in any liquid medium. When each strain was cocultivated with *P. aeruginosa* strain WatG in liquid mineral salts medium containing 1% diesel oil, isolate N enhanced the degradation of diesel oil by *P. aeruginosa* strain WatG, but isolate O inhibited it. In contrast, isolate J, which was tentatively identified as a *Rhodococcus* sp., degraded diesel oil contained not only in liquid LB and mineral salts media, but also in paddy soil microcosms supplemented with LB medium. The bioaugmentation capacity of isolate J in soil microcosms contaminated with diesel oil was much higher than that of *P. aeruginosa* strain WatG. The possibility of using isolate J for autochthonous bioaugmentation is discussed.

Keywords Autochthonous bioaugmentation · Biostimulation · Diesel oil degradation · Soil intrinsic bacteria · Soil microcosms.

Abbreviations DGGE, denaturing gradient gel electrophoresis; LB, Luria-Bertani medium; LB-BS, LB-added biostimulation; MSM, mineral salts medium; WatG-BA, *P. aeruginosa* strain WatG-added bioaugmentation; TPH,total petroleum hydrocarbons

Introduction

As industrialization expands, petroleum hydrocarbons become a greater potential source of contaminants in the water and soil environments (Margesin and Schinner 2001). Recently, more and more oil spill accidents have been reported. Chang and Lin (2006) reviewed 242 accidents with storage tanks in industrial facilities over the last 40 years. They reported that 74% of accidents occurred in petroleum refineries, oil terminals, or storage facilities. To remediate petroleum contaminants in these environments, biostimulation and bioaugmentation are generally considered as environmentally friendly techniques. However, the use of extrinsic microorganisms is unlikely to be acceptable to the public.

Biostimulation is a technique that relies on increasing the activity of the resident bacteria by adding the factors that are limiting activity, such as nutrients or air. This technique seems to be effective because the indigenous bacteria are likely to be better adapted to the soil environment requiring treatment (Rahman et al. 2003). However, biostimulation sometimes does not work well and may take longer because bacteria with the ability to degrade xenobiotics may be scarce at contaminated sites or because the high-concentration of xenobiotics reduces the activity of degrading microorganisms.

Bioaugmentation is a promising and low-cost bioremediation method in which an effective bacterial isolate (s) or microbial consortium capable of degrading xenobiotics is administered to contaminated sites (Gentry et al. 2004). The number of petroleum-degrading microbial isolates available for bioaugmentation is increasing (Van Hamme et al. 2003; Singer et al. 2005). However, the soil environment is very complicated and the degrading ability of exogenously added microorganisms tends to be affected by the physicochemical and biological features of the soil environment. Sometimes, the administration of petroleum-

degrading microorganisms leads to a failure of bioaugmentation (Vogel 1996; Gentry et al. 2004). The two bioremediation treatments can be performed *ex situ* and *in situ*. Compared with *ex situ* bioremediation, in which contaminated soils have to be transferred to a specialized facility and treatment plants have to be constructed, *in situ* bioremediation is a relatively cost-effective technique for remediation of soil and groundwater contaminated with petroleum hydrocarbons (Bouwer et al. 1994).

Previously, we investigated the microbial community structure of diesel-oil-contaminated local paddy soil microcosms that had been biostimulated with Luria–Bertani medium (LB-BS) or bioaugmented with a petroleum-degrading bacterium, *Pseudomonas aeruginosa* strain WatG (WatG-BA; Wongsa et al. 2004; Ueno et al. 2006a), by using denaturing gradient gel electrophoresis (DGGE) and monitoring diesel oil degradation (see Fig. 1A; Ueno et al. 2006b). The degree of degradation in the microcosms containing WatG-BA was higher than that in LB-BS during the first two weeks. The microbial community in the microcosm containing WatG-BA, which was markedly dominated by *P. aeruginosa* strain WatG, was much simpler than that in LB-BS, where six or more hydrocarbon degraders occurred with time lags of 3–7 days after the addition of diesel oil. However, the intensity of the DGGE band of *P. aeruginosa* strain WatG decreased after prolonged cultivation (60 days; see Fig. 1A). Sequencing of 16S rRNA genes of each DGGE band showed that no strains were common to the two soil microcosms. The clustering profiles of the DGGE banding patterns of the two soil microcosms were only 12% similar (Ueno et al. 2006b). These results suggest that the microbial community structure was influenced not only by physicochemical soil conditions, but also by the bacteria that had been administered for bioaugmentation.

For degradation to occur, the right microbes should be utilized in the right place with the right environmental factors (Boopathy 2000). We consider that the *in situ* bioaugmentation-mediated technique should be tailored specifically to each polluted site, because each site is thought to have inherent characteristics. In this paper, a new concept of bioaugmentation,

"autochthonous bioaugmentation", will be proposed that uses petroleum degraders isolated from the contaminated sites to be remediated.

Materials and methods

Bacteria, culture media and soil conditions

Pseudomonas aeruginosa strain WatG (Wongsa et al. 2004) was precultivated to stationary phase in Luria–Bertani (LB) medium (pH 7.0) at 20°C with rotary shaking at 180 rpm. Strains isolated from biostimulated and bioaugmented paddy soil microcosms (see below) were cultivated in the same way as *P. aeruginosa* strain WatG.

Mineral salts medium (MSM) containing diesel oil was prepared as described previously (Ueno et al. 2006a,b). A soil extract medium was prepared according to the DSM catalog provided by the German Collection of Microorganisms and Cell Cultures [http://www.dsmz.de/]. This contained (per liter) 5.0 g of peptone, 3.0 g of beef extract, and 250 ml of soil extract. The pH was adjusted to 7.0 with 5.0 M NaOH. A soil extract was prepared by sterilizing 400 g of soil that was then suspended in 1,000 ml of distilled water for 1 h at 121°C. Soil particles were removed by centrifugation at 1,000 × *g* for 20 min, and the supernatant was passed through Whatman® 3MM paper.

Tests of the degradation of diesel oil in liquid media were carried out using 2 ml of MSM, LB medium, and a soil extract medium, with each medium containing 1% diesel oil in test tubes, as described previously (Wongsa et al. 2004). Bacteria were precultivated in LB medium at 20°C overnight and inoculated to the above media to a final concentration of 0.1 at OD_{600} (Ueno et al. 2006a; 2006b).

Biostimulation and bioaugmentation tests using local paddy soil (Briones et al. 2003) were carried out, as described previously (Ueno et al. 2006b). Briefly, 2 g of dried soil were

put into 15 ml porous silica-capped (aerated) test tubes. When necessary, soil was autoclaved twice at 121°C for 30 min to sterilize it completely. In this study, LB medium was used as the fertilizer for soil (Ueno et al. 2006a,b). Then, 500 µl portions of the preculture of *P. aeruginosa* strain WatG or isolates J, N, or O in LB medium or fresh LB medium were added to 2 g (approximately 2 ml) of the soils containing 1% (w/w) diesel oil. The isolates J, N, and O correspond to the DGGE bands J, N, and O, respectively (see Fig. 1A; Ueno et al. 2006b). Soil microcosms supplemented with a culture of *P. aeruginosa* strain WatG plus LB medium, and also with LB medium alone were designated *P. aeruginosa* strain WatG-bioaugmented (WatG-BA) and LB-biostimulated (LB-BS) soil microcosms, respectively (Ueno et al. 2006a). The WatG-BA and LB-BS soil microcosms were left at 20°C for up to 2 months. The optimum growth temperature of strains tested here has not been examined. Considering the practical use of them in soils, the temperature of 20°C was selected.

Isolation and characterization of soil intrinsic bacteria

To isolate those intrinsic soil bacteria capable of degrading petroleum hydrocarbons from WatG-BA and LB-BS soil microcosms, 20 g of paddy soil was used. Diesel oil at 1% (w/w) and LB medium at 25% (v/w) were added to the soil. In the case of WatG-BA, a preculture of *P. aeruginosa* strain WatG was added to the soil microcosm and then cultured up to for 2 months. After incubation, portions of 1 g of soil were taken from the soil microcosms, suspended with 10 ml of 0.85% (w/v) NaCl by shaking manually, and the suspension was left for 30 min at room temperature. Serial dilutions (10^{-3} to 10^{-5}) of the suspension prepared in 0.85% (w/v) NaCl were surface spread onto LB or soil extract agar medium plates, and individual colonies were isolated. Bacteria were tentatively identified by the sequencing of 16S rRNA genes (see below).

DNA procedure

For PCR amplification of 16S rRNA genes, bacteria were cultivated overnight in 3 ml of LB medium at 20°C, with rotary shaking at 180 rpm. Bacterial cells were collected by centrifugation at 15,000 × *g* for 5 min. Genomic DNA was isolated by the method of Marmur (1961).

PCR was performed using a Mastercycler® ep gradient Thermal Cycler (Eppendorf AG, Hamburg, Germany) in a total volume of 50 µl using TaKaRa *Ex Taq* DNA polymerase (TaKaRa Shuzo, Shiga, Japan) in the supplied buffer. For the amplification of the full length of 16S rRNA genes, 27F (5′-GAGTTTGATCCTGGCTCAG-3′) and 1541R (5′-AAGGAGGTGATCCAGCC-3′) were used. PCR was carried out according to the following program: denaturation at 94 ºC for 5 min; then denaturation at 94ºC for 1 min, annealing at 55ºC for 1 min and extension at 72ºC for 2 min for the amplification of the full length of 16S rRNA genes up to 30 cycles; finally, conclusion at 72ºC for 5 min. PCR products were resolved by electrophoresis on 1.5% agarose gels and visualized by a UV transilluminator after staining with ethidium bromide (Nippon Gene, Tokyo, Japan).

Amplified 16S rRNA gene fragments were ligated into the pCR®2.1-TOPO® TA cloning vector by following the instructions given by the manufacturer (Invitrogen, CA, USA). Plasmids were transformed into *Escherichia coli* DH5 competent cells. Recombinant transformants were selected by blue/white colony screening. Individual white colonies were grown at 37ºC overnight with rotary shaking in 3 ml of LB medium. Plasmid DNA was extracted according to the ordinary alkaline-SDS minipreparation method (Sambrook and Russell 2001). After plasmid preparation, 2 µl (out of 100 µl) of each sample was treated with restriction enzyme, *Eco*RI (TOYOBO, Japan), to check for the presence of insert DNA. Only plasmids containing the expected 1,540 bp inserts were used for the subsequent nucleotide sequencing analysis.

Cycle sequencing reaction was carried out using the same primers described above with a BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA), and each sample was subjected to DNA sequencing analysis using an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems).

Analysis of total petroleum hydrocarbons

Total petroleum hydrocarbons (TPH) were analyzed by gas–liquid chromatography, as described previously (Ueno et al. 2006a,b).

Statistical data analysis

The group mean values for residual total petroleum hydrocarbons were analyzed using the Kruskal–Wallis test, with a $P < 0.05$ level of significance (Breslow 1970). If significant, the two-sample *t*-test was used to analyze the differences at the $P < 0.05$ level of significance.

Nucleotide sequence accession numbers

Sequences corresponding to the 5′ and 3′ ends of the 16S rRNA genes from isolates J, N, and O have been deposited in the DNA Data Bank of Japan under accession numbers of AB272075, AB272076, and AB272074, respectively.

Results

Isolation and identification of intrinsic soil bacteria from WatG-BA soil microcosms

A large number of colony isolates were obtained from WatG-BA soil microcosms on agar media containing LB medium and soil extracts. The purity of the bacteria was confirmed by DGGE analysis of the 16S rRNA genes. Figure 1B shows DGGE profiles of isolates from WatG-BA soil microcosms after an incubation period of 3 weeks. Almost all the isolates obtained were *P. aeruginosa* strain WatG. The mobility of the DGGE bands of the remaining isolates was the same as that of the band N or band O. The isolates were designated isolates N and O. The band O was detected in only one isolate sample. In other soil samples left for 1 month, no isolates other than *P. aeruginosa* were obtained.

The whole 16S rRNA genes of isolates N and O with 1,536 bp and 1,476 bp, respectively, were sequenced. BLAST search results revealed that the isolate N had 98% identity with *Stenotrophomonas maltophilia* VUN 10,003 (AF10073) and *Stenotrophomonas* sp. Ellin 162 (AF409004), and the isolate O had 99% identity with *Ochrobactrum anthropi* (AB120120) and *O. intermedium* (AJ242583). Based on these results, the isolates N and O were identified as *Stenotrophomonas* sp. and *Ochrobactrum* sp., respectively.

Diesel oil degradation by isolates N and O under liquid conditions

The degradation of diesel oil by isolates N and O was tested at 20ºC in liquid LB medium, MSM, and soil extract medium containing 1% diesel oil. Bacterial growth was observed in both LB and soil extract media, but not in MSM (data not shown). No degradation of diesel oil by these isolates was observed, whether in MSM or in LB and soil extract media, suggesting isolates N and O has no direct capacity to utilize the hydrocarbons of diesel oil.

P. aeruginosa strain WatG was cocultivated with either isolate N or isolate O in MSM containing 1% (w/w) diesel oil at 20°C for 1 week. Significant decreases of diesel oil were observed in the cocultivation of *P. aeruginosa* strain WatG and isolate N (62.6 ± 0.9%, mean ± SD). This value was higher than that in the single cultivation of *P. aeruginosa* strain WatG (57.0

± 3.6%) (Fig. 2). On the other hand, the decrease of diesel oil was inhibited (48.5 ± 1.4%), when *P. aeruginosa* strain WatG was cocultivated with isolate O (Fig. 2).

Isolation and tentative identification of the intrinsic soil bacteria from LB-BS soil microcosms

Thirteen visibly distinct colonies were isolated using LB and soil extract agar media containing diesel oil from LB-BS soil microcosms left for 2 months. Five randomly selected colony isolates were all capable of degrading diesel oil in MSM. Their nucleotide sequences of 1,511 bp were identical (data not shown). Among them, one colony isolate was used in the subsequent experiments. The nucleotide sequence of the 16S rRNA gene of this colony isolate included a sequence identical with that of DGGE band J. This colony isolate, which was classified as a species of the genus *Rhodococcus* (data not shown), was designated isolate J.

Diesel oil degradation by isolate J under liquid conditions and in soil microcosms

Isolate J was grown in MSM and LB medium containing 1% (w/v) diesel oil at 20°C for 1 week. The residual TPH in the culture of isolate J was 42.1 ± 4.6% in MSM and 44.7 ± 6.0% in LB medium (Fig. 3). These values were significantly lower than that of the control (89.4 ± 4.8%; Fig. 3) and demonstrated that isolate J has the ability to degrade diesel oil in both LB medium and MSM.

Bioaugmentation experiments were conducted using isolate J in paddy soil microcosms supplemented with MSM. When unsterilized paddy soil containing 1% (w/w) diesel oil was inoculated with isolate J or *P. aeruginosa* strain WatG, no significant difference in TPH decrease level was observed between the two (Fig. 4). On the other hand, residual TPH were lower in soil microcosms containing isolate J (55.6 ± 5.6%) than in those containing *P.*

aeruginosa strain WatG (65.0 ± 5.0%), when sterilized soils were used (Fig. 4). This difference was significant at the $P < 0.05$ level of significance.

Discussion

The analysis of microbial community structure by PCR–DGGE of biostimulated and bioaugmented soil microcosms makes it possible to specify the bacteria involved in the degradation of petroleum hydrocarbons, and the occurrence of degraders and their fate are easily monitored by this method. We showed previously that the population of exogenously added extrinsic *P. aeruginosa* strain WatG was completely lost when it was administrated to unsterilized paddy soil that had been artificially contaminated with diesel oil, whereas it was maintained for more than 3 months in the sterilized soil (see Fig. 1A and Ueno et al. 2006b). A similar tendency was also observed when *P. aeruginosa* strain WatG was administrated to weathered soils contaminated with crude oil (Ueno 2006). These results suggest that extrinsic bacteria would encounter severe competition with bacteria intrinsic to the soils. Therefore, for more efficient bioaugmentation of xenobiotics, including petroleum hydrocarbons, avoidance or reduction of such competition between microorganisms intrinsic and extrinsic in soils must be considered.

We obtained isolates N and O, which corresponded to DGGE bands N and O, respectively (see Fig. 1B), from soil microcosms to which *P. aeruginosa* strain WatG had been added (WatG-BA). Since isolate N was cooperative with *P. aeruginosa* strain WatG in the degradation of diesel oil under liquid culture conditions and isolate O inhibited it (Fig. 2), the previous observation that diesel oil degradation was clearly higher in WatG-BA soil microcosms using unsterilized soil than in those using sterilized soil (Ueno et al. 2006a,b) could be explained by the cooperative participation of inherent petroleum-degrading bacteria such as isolate N.

How *P. aeruginosa* strain WatG and isolate N cooperate is not known. One possibility is

that the cooperation may be mediated by rhamnolipids produced by *P. aeruginosa* strain WatG, which has an ability to secrete such substances under liquid and soil cultivation conditions (Ueno et al. 2006a). The observation that isolate N grew in liquid LB and soil extract media, but not in MSM, containing diesel oil (data not shown) suggests that diesel oil becomes available because of emulsification by the biosurfactant rhamnolipids secreted by *P. aeruginosa* strain WatG. Coinoculation of biosurfactant-producing bacteria is known to enhance the degradation of hydrocarbons such as phenanthrene in soils (Dean et al. 2001). However, it is quite clear that the bioaugmentation capacity of extrinsic microorganisms (*P. aeruginosa* strain WatG in this study) is hindered by soil intrinsic microorganisms such as isolate O, even if *P. aeruginosa* strain WatG was coinoculated with isolate N to the paddy soil. To overcome the limited capacity of exogenously added extrinsic petroleum-degrading bacteria in the bioaugmentation process, we considered using bacterial isolates or bacterial consortia (intrinsic individual isolates or bacterial consortia) that had inhabited the soils requiring renovation. The combined use of *P. aeruginosa* strain WatG and isolate N is not practical and the single use of isolates such as isolate J would be more preferable.

In LB-BS, at least six strains (DGGE bands E to J) appeared after the addition of diesel oil to paddy soil (see Fig. 1A; Ueno et al. 2006b). Among these, only isolate J was isolated using nutrient media such as LB and soil extracted media. This difficulty in isolating soil intrinsic bacteria coincides with the general finding that most soil intrinsic bacteria are hard to cultivate in ordinary nutrient media (Daniel 2005). However, bacterial strains corresponding to DGGE bands E to J were surely “cultivated” in soils supplanted with LB medium. Therefore, we would probably be able to isolate and cultivate them by modifying the traditional methods. Furthermore, we could use not only isolates, but also cultivated soil microcosms, even if individual strains could not be isolated, as degrader(s) for bioaugmentation of xenobiotics or contaminants (Ueno 2006). Our present isolation and use of isolate J from LB-BS microcosms of paddy soil and the successful use of this isolate for bioaugmentation of the paddy soil

artificially contaminated with diesel oil affords one instance of this mode of bioaugmentation. To this type of technology, the name “autochthonous bioaugmentation” was given (Fig. 5). According to D’Annibale et al. (2006), several isolated autochthonous fungal species from an aged and heavily contaminated soil were administered to the same oil-contaminated soil, and a marked removal of naphthalene, dichloroaniline, *o*-hydroxybiphenyl, and 1,1′-binaphthalene was achieved. This is another example of autochthonous bioaugmentation. However, no previous report of the use of autochthonous bacterial isolates for bioaugmentation of the treatment of petroleum hydrocarbons is available. Autochthonous isolates and/or bacterial consortia would be more useful and effective inoculants in *in situ* bioremediation.

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Figure legends

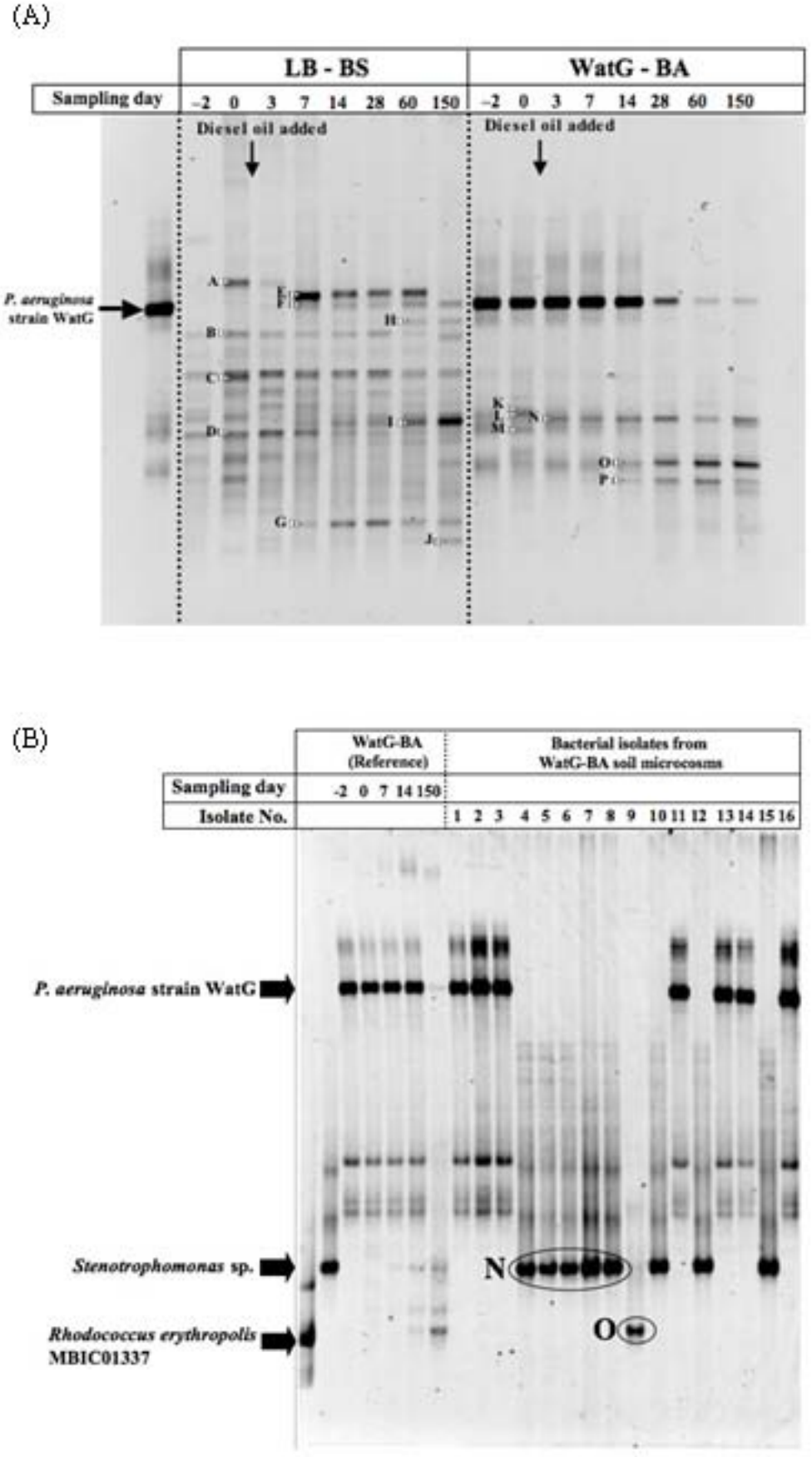
Fig. 1 (A) PCR–DGGE profiles of soil microcosms in LB-BS and WatG-BA over the 150 days of the experiment. The large arrow indicates the band corresponding to *P. aeruginosa* strain WatG. The days of sampling are given above each lane. Day –2 refers to the day on which nutrients (LB) or WatG were added. Day 0 refers to the day on which diesel oil was added. Upper case letters indicate the DNA fragments that were sequenced. This figure was cited in Ueno et al. (2006b). (B) PCR-DDGE profiles of DGGE isolates derived from the WatG-BA soil microcosms left for 3 weeks. Individual isolates appeared on the agar plates of the soil extract medium.

Fig. 2 Diesel oil decrease caused by cocultivation of *P. aeruginosa* strain WatG with isolates N and O derived from a WatG-BA soil microcosm in liquid medium. Diesel oil degradation was examined in 2 ml of MSM containing 1% (w/v) diesel oil. Each bar represents a mean value % with the standard deviation % (n = 5). *P. aeruginosa* strain WatG and DGGE isolates N and O were precultivated in LB medium at 20 °C overnight and inoculated to the medium. Cultivation for diesel oil degradation was conducted at 20 °C for 1 week. TPH was measured by GLC as described in Materials and Methods.

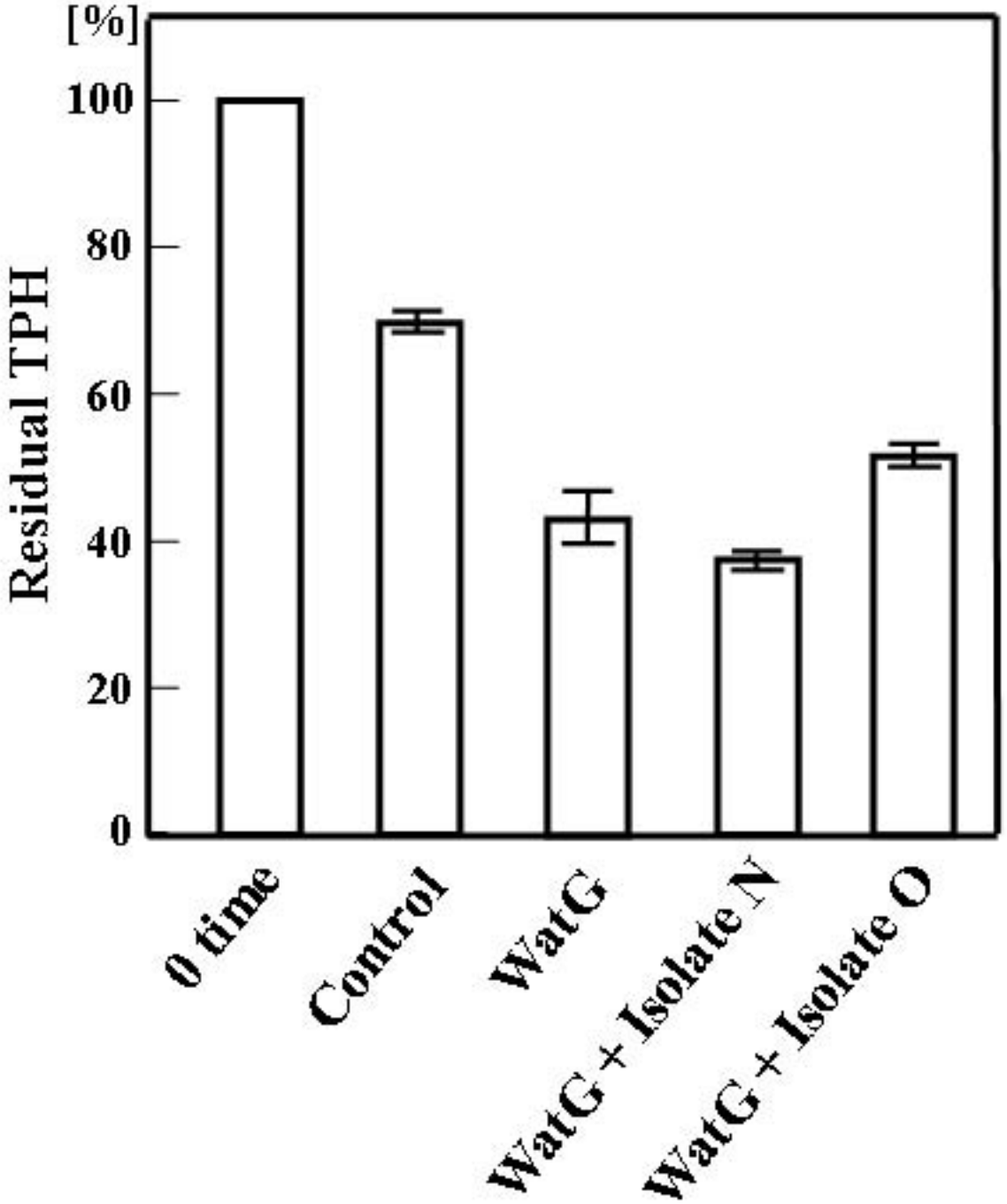
Fig. 3 Diesel oil decrease caused by isolate J in liquid media. Diesel oil degradation ability was examined in LB medium and MSM containing 1% (w/v) diesel oil. Cultivation for diesel oil degradation was conducted at 20 °C for 1 week. Isolate J precultivated in LB medium at 20 ºC overnight was used as the inoculant. The ability to degrade diesel oil of this isolate was compared with a control sample in which only spontaneous evaporation was observed. Each bar represents the mean value with the standard deviation (n = 5).

Fig. 4 Autochthonous bioaugmentation using isolate J in paddy soil microcosms. Isolate J was inoculated to sterilized (ste.) and unsterilized (unste.) soil microcosms consisting of 2 g of paddy soil contaminated with 1% (w/w) diesel oil. *P. aeruginosa* strain WatG was used as a control bacterium. All the soil microcosms were left at 20 °C for 1 week. LB medium was used to supply nutrients. The percentage of residual TPH was compared with the zero time samples. Each bar represents the mean value with the standard deviation ($n = 5$). Stars indicate a significant difference at the $P < 0.05$ level of significance.

Fig. 5 Concept of autochthonous bioaugmentation. Petroleum-degrading bacteria exist ubiquitously. We are able to isolate petroleum-degrading bacteria and/or consortia from soils artificially contaminated with petroleum hydrocarbons prior to accidental oil spills. Once soils are contaminated by an oil spill, the petroleum-degrading bacteria and/or consortia that have been prepared in advance can be administrated to the oil-polluted soils. These bacteria and consortia are expected not only to remove petroleum hydrocarbons effectively, but also to receive ready public acceptance in terms of biosafety.



Ueno et al. Fig. 1



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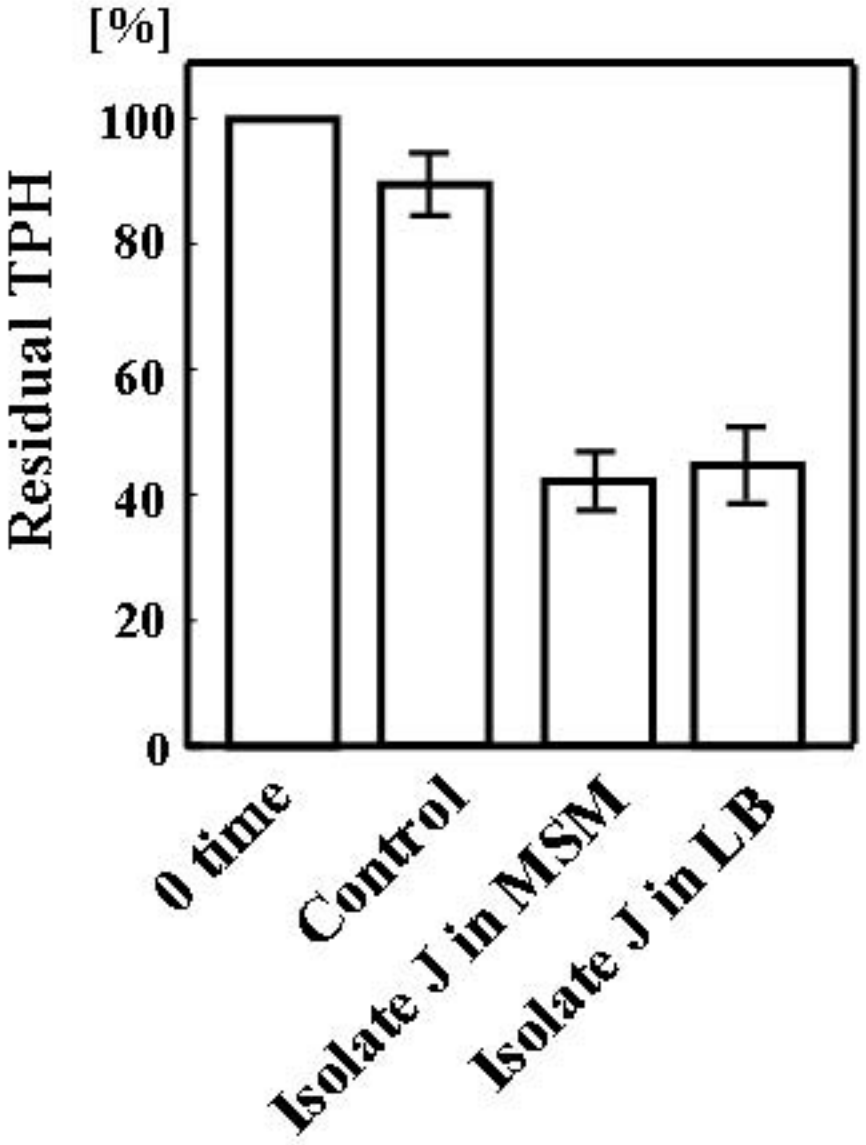

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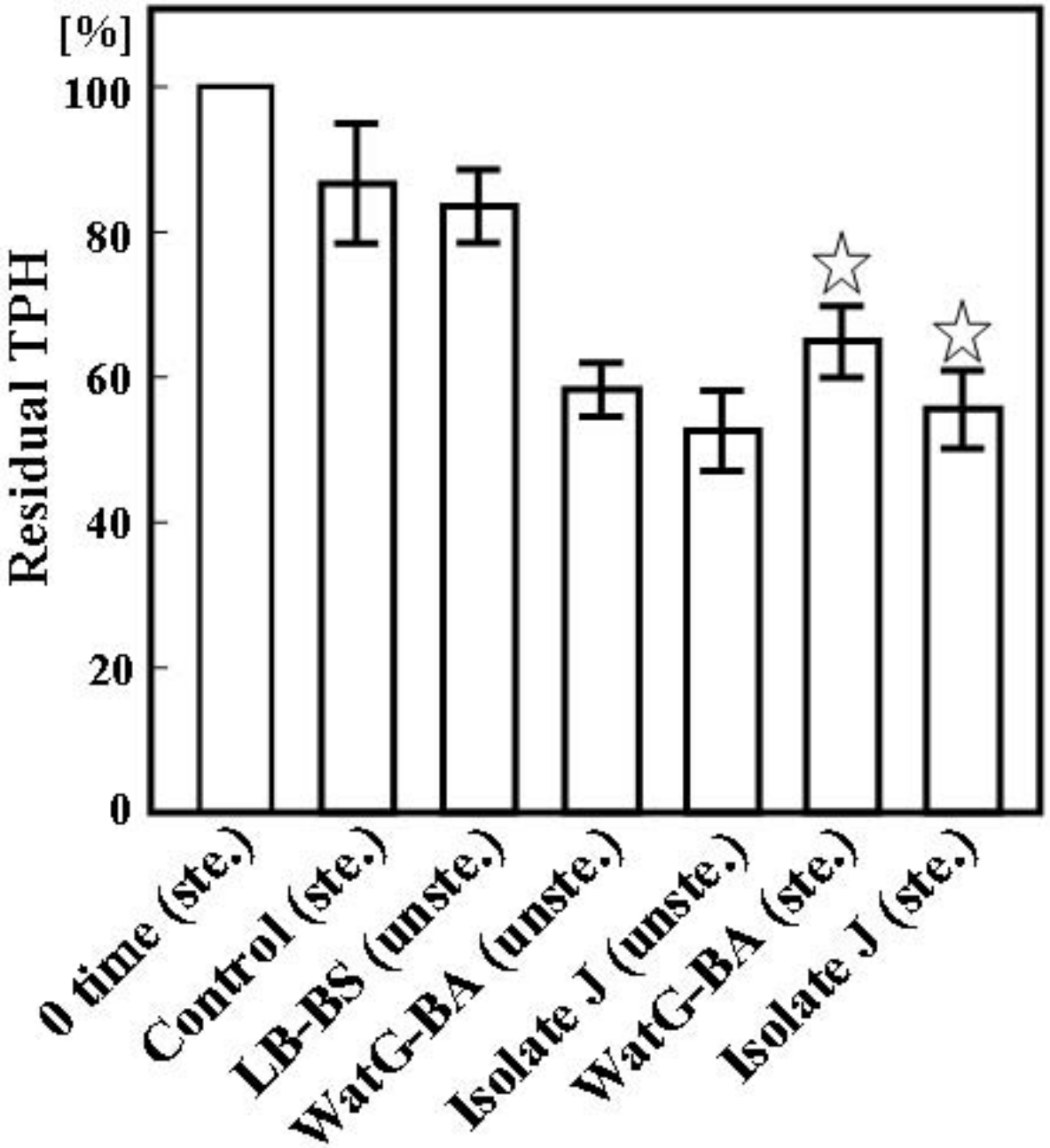

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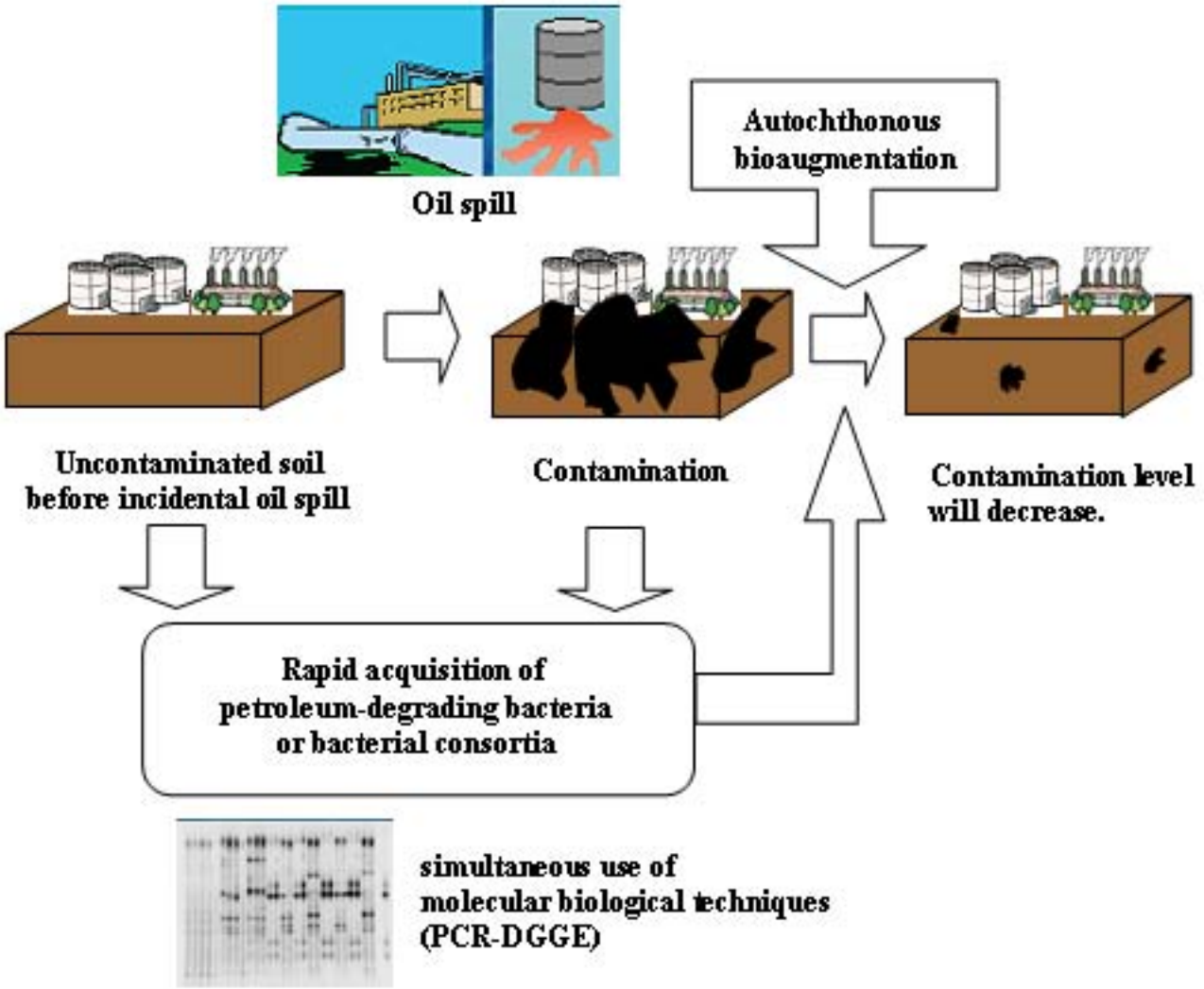

Ueno et al. Fig. 5