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A turbine oil-degrading bacterial consortium from soils of oil fields and its characteristics

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Abstract

A microbial consortium capable of degrading turbine oil (TuO), which consisted mainly of recalcitrant cycloalkanes and isoalkanes, was obtained from a soil sample collected from oil fields using repeated enrichment. When this consortium, named Atsuta A, was cultured in mineral salts medium containing 0.5% (w/v) TuO, it degraded 90% of TuO at 30 °C and pH 7 over 5 days. Although nine bacterial strains were isolated from the Atsuta A consortium, TuO degradation by the individual isolates and by a mixture of them was negligible. The community structure of the consortium, which was investigated by PCR–denaturing gradient gel electrophoresis (DGGE) targeting 16S rRNA genes, changed significantly during the degradation of TuO. Four major bands (F, K, N and T) out of at least 23 visible DGGE bands significantly increased in intensity over time during incubation. The DGGE bands F, K and N corresponded to those of previously isolated species. However, DGGE band T did not correspond to any isolated strain. The 16S rRNA gene sequence collected from band T was 98% homologous to that of an unculturable strain belonging to the γ-Proteobacteria. The degradation of TuO in the consortium may occur by cooperation between the unculturable species corresponding to band T and other strains in the consortium, including species corresponding to bands F, K and N.

Keywords: Bacterial consortia; Oil field; Turbine oil (TuO); Turbine Oil-degrading bacteria; Unculturable bacteria

1. Introduction

Anthropic activities are highly reliant on petroleum products. However, the widescale production, transport, usage and disposal of petroleum products have caused major contamination of the environment. Of the physical, chemical and biological methods available to remediate oil-polluted environments, the biological method (bioremediation) is considered an environment-friendly and relatively cost-effective technology.

Crude oil contains a huge number of chemical compounds (hydrocarbons). They are usually grouped

into four fractions: aliphatics, aromatics, resins and asphalthenes (Goto et al. 1994). Resins and asphalthenes are generally solvent-insoluble and resistant to biodegradation. Aliphatic hydrocarbons consist of normal alkanes (*n*-alkanes), branched alkanes (isoalkanes) and cyclic alkanes (naphthenes). *n*-Alkanes are mostly biodegradable and there are an enormous number of reports on *n*-alkane-degrading microorganisms and their application to bioremediation (Di Cello et al. 1997; van Beilen et al. 2003). Isoalkanes, naphthenes and aromatics are much less biodegradable than *n*-alkanes (Gough and Rowland 1990). According to Perry (1984), the susceptibility of hydrocarbons to microbial attack is ranked in the following order: *n*-alkanes > isoalkanes > low molecular weight aromatics > naphthenes. Although naphthene-degrading (Beam and Perry 1974; Koma et al. 2005) and isoalkane-degrading (Solano-Serena et al. 2000; Namio et al. 2005) microorganisms have been isolated, the number of such strains is limited compared with the number of strains that degrade *n*-alkanes (Göbel et al. 2002; Sarma et al. 2004; Hunter et al. 2005)**.**

Lubricating oils consist of a 'base oil' mixture consisting mainly of hydrocarbons such as isoalkanes and naphthenes, with some additives (Komiya 1997). The base oil used as lubricating oil is generally refined by several processes: distillation (at 400 °C or much higher) for evaporating light fractions, dewaxing for removing the solid wax fraction and hydrogen refining for making the oil resistant to oxidation. Throughout these processes, naphthenes and isoalkanes remain in the base oil. Turbine oils (TuOs), which are used for lubrication and the control of gas and steam turbine systems, are typically composed of 95%–99.5% (w/w) highly refined base oil and 0.5%–5% (w/w) additives (Vittoria 2005). Considering their general chemical composition, lubricating oils including TuO are assumed relatively recalcitrant to microbial degradation. However, the biodegradability of TuO has scarcely been investigated. To our knowledge, Zvyagintseva et al. (2001) reported the only study in which TuO degraders were isolated from oil-polluted soils and in which their abilities to degrade TuO were described. The degradation of TuO by bacterial consortia and the isolate *Rhodococcus erythropolis* strain 100 was no more than 38% (Zvyagintseva et al. 2001).

The aims of this study were (1) to screen samples of oil-polluted soil for TuO-degrading microorganisms, (2) to characterize these microorganisms by their physiology, microbiology and molecular biology and (3) to identify characteristics of these strains that may allow them to be used for the bioremediation of TuO. Various soil samples collected from oil-polluted sites were used for enrichment

cultivation using minimal salts medium (MSM) containing TuO as the sole carbon and energy source.

2. Materials and Methods

2.1. Soil samples and reference bacterial strains

Six soil samples in total were collected from two oil-polluted sites, Atsuta and Mourai oil fields in Atsuta-ku, Ishikari-shi, Hokkaido prefecture, Japan. Four samples (named Atsuta A and B, and Mourai A and B) were collected from sites that were lightly polluted by crude oil, where there was some vegetation. Two samples (named Atsuta H and Mourai H) were collected from heavily polluted sites, where crude oil pooled and where there was no vegetation.

Two hydrocarbon degraders were used as reference bacterial strains: *R. erythropolis* MBIC01337 obtained from the Marine Biotechnology Institute Culture Collection (Kamaishi, Iwate, Japan), and the laboratory *Pseudomonas aeruginosa* strain WatG (Wongsa et al. 2004).

2.2. Petroleum products and media

TuO (type FBK turbine SH), a product of Nippon Oil Corporation (Tokyo, Japan), was gifted from Hokkaido Electric Power Co., Inc., car engine oil (type Castrol XLX; abbreviated as EO) was obtained from Castrol Company (Tokyo) and diesel oil (type ZOA excellent 10W-30; abbreviated as DO) was obtained from Nippon Oil Corporation. The chemical composition of the TuO was an industrial secret of Nippon Oil Corporation, so the only information available on this oil was that approximately 65% (w/w) of the base oil was naphthene (by private communication). The TuO and EO were sterilized by autoclaving at 121 °C for 20 min. The DO was sterilized by filtration using a filter (type Millex, pore size of 0.2 μm; Millipore, Bedford, MA, USA). In some cases, used TuO that had been spilled from turbines into pit water was used after being concentrated. Each petroleum product was added to MSM (Ueno et al. 2006a) at a final concentration of 0.5% (w/v) to be used as an energy source. Unless otherwise stated, the MSM was at pH 7. Isolation of bacterial cells was carried our on MSM plates containing 1.5% (w/v) agar. A 50 μl

aliquot of TuO was spread onto the MSM agar plates.

Yeast glucose medium (YGM) consisting of 1% (w/v) Bacto™ yeast extract (BD, Franklin Lakes, NJ USA) and 1% (w/v) glucose (Nacalai Tesque, Kyoto, Japan) was used to cultivate *R. erythropolis* MBIC01337. Luria-Bertani (LB) medium was used to cultivate *P. aeruginosa* WatG, the Atsuta A consortium, isolates from the consortium and a mixture of Atsuta A isolates.

2.3. Enrichment of TuO-degrading bacterial consortia

To enrich microorganisms capable of utilizing TuO, soil samples (5 g of each) were added to a 3 l flask containing 1 l of MSM supplemented with 0.5% (w/v) TuO. The culture was incubated at 20 °C on a rotary shaker (160 rpm) for 1 month. A 100 µl aliquot of the culture was transferred to a 50 ml flask containing 10 ml of MSM and 0.5% (w/v) TuO, which was then incubated at 20 °C and 160 rpm for 2 weeks. During the cultivation of Atsuta A and B samples, bacterial cells formed aggregates. A small block of these bacterial aggregates (approximately 2 mm × 4 mm × 1 mm) was collected using an inoculation loop and transferred to fresh medium of the same composition. Total hydrocarbons were extracted from these cultures after each cultivation (see below).

2.4. Degradation tests

A block of bacterial aggregates from the Atsuta A consortium, which was collected from cultures grown for 2–4 weeks, was directly inoculated into 10 ml MSM supplemented with 0.5% (w/v) of one of the petroleum products. In some cases, the bacterial aggregates were transferred to 10 ml LB medium and then cultivated at 20 °C for 1 day before being used as an inoculum (see below). Individual isolates from the Atsuta A consortium were cultivated in LB medium for 1 day at 20 °C and a mixture of them, which contained equal quantities of each isolate, was also used as an inoculum. *R. erythropolis* MBIC01337 and *P. aeruginosa* WatG were cultured at 20 °C for 1 day in 10 ml YGM and LB media, respectively. Cultures of the isolates, a mixture of them, the Atsuta A consortium and the reference strains were transferred to MSM containing 0.5% (w/w) of one of the petroleum products being tested (TuO, EO and DO) so that the final OD was 0.1 at 600 nm. These cultures were incubated at 30 °C with shaking for the indicated time and the

petroleum product remaining after that time was measured (see below). When necessary, cultivation was carried out in the presence of the antibiotics ampicillin (50 μg ml^{-1}), chloramphenicol (30 μg ml^{-1}), kanamycin (50 μg ml^{-1}), streptomycin (30 μg ml^{-1}) or tetracycline (10 μg ml^{-1}).

2.5. Analytical procedures

Hydrocarbons were extracted directly from the cultures using a mixture of chloroform and methanol. Briefly, cultures (10 ml each) were transferred by decantation to 50 ml glass centrifuge tubes containing 50 mg of stearic acid, which was used as an internal standard. The flask was then washed twice with a mixture of 5 ml of chloroform and 2 ml of methanol. The wash fluids were mixed with the original culture in the 50 ml centrifuge tubes. After shaking vigorously for 10 min, aqueous and organic phases were separated by centrifugation at 2000 rpm for 5 min. The organic phase (hydrocarbons) was transferred to a glass vial and kept at –30 °C until analysis using an Iatroscan (see below). The hydrocarbon fraction analyzed by gas chromatography (GC) was extracted from cultures containing no internal standard, as described above.

Extracted hydrocarbons were analyzed by thin-layer chromatography–flame ionization detection (TLC–FID) on an Iatroscan (type MK-6, Mitsubishi Kagaku Iatron, Tokyo, Japan) using rods precoated with an active silica thin layer (type Chromarods-SIII; Mitsubishi Kagaku Iatron). One microliter of sample was manually spotted onto the origin of each rod and then Chromarods were developed with a hexane/diethyl ether/acetic acid mixture (80:20:1 by volume) for 15 min. After being dried at room temperature for 5 min, the Chromarods were scanned under the following conditions: hydrogen flow rate 160 ml min^{-1}, air flow rate 2 l min^{-1} and scan speed 30 s $scan^{-1}$. Data were recorded and analyzed on an Iatrocorder TC-21 (Mitsubishi Kagaku Iatron).

Calibration curves were constructed in order to estimate the amount of petroleum product (TuO, EO and DO) present. Various amounts (0.5–5 μg) of each petroleum product containing 5 μg of stearic acid as internal standard were loaded onto the Chromarods and analyzed as described above. Regression equations were constructed of the relationship between the ratio of the area of each petroleum product to that of stearic acid (x) and the concentration of each petroleum product (y), as $y = a + bx$. The percentage biodegradation was calculated from the formula: Degradation (%) = (concentration of TuO in the control – y)/(concentration of TuO in the control) × 100 (Okumura et al. 1998).

The GC analysis of TuO was carried out using a GL Sciences GC-353B gas chromatograph equipped with a capillary column, TC-1 (30 m length, 0.25 mm ID, 0.10 μm film thickness, GL Sciences Inc., Tokyo, Japan) and FID with nitrogen as the carrier gas. The temperature of the GC oven was initially 80 °C, increasing to 350 °C at a rate of 10.0 °C min^{-1} and then remaining at that temperature for 30 min. Injector and detector temperatures were set at 322 °C.

2.6. Isolation of bacterial strains from the Atsuta A consortium

After the fourth enrichment, bacterial strains were isolated from the Atsuta A consortium. A bacterial cell aggregate was streaked onto MSM agar plates on which TuO was spread. The plates were incubated at 20 °C. Colonies were streaked onto TuO-spread agar plates at least four times. All isolates were Gram stained.

2.7. DNA procedures

Cells of the consortium (cell aggregates and culture fluids) and individual isolates were harvested by centrifugation at 6000 rpm for 10 min. The cell pellets were freeze-dried for 1 day and then stored at −30 °C. In order to extract genomic DNA, cells were rehomogenized in 2 ml buffer consisting of 20 mM Tris-HCl buffer (pH 8) and 250 mM EDTA. A 200 μl aliquot of homogenates was transferred to a 2 ml sterile screw-capped tube with O-ring (Sarstedt, Nümbrecht, Germany), which contained 300 μl of suspending buffer, 500 μl of stabilizing reagent, 400 μl of a mixture of phenol-chroloform-isoamyl alcohol (25:24:1 by volume) and 500 mg of glass beads (105–150 μm diameter; Polysciences Inc., Warrington, PA, USA). The stabilizing reagent consisted of 100 mM Tris-HCl (pH 8), 20 mM EDTA, 1.4 M NaCl and 2% (w/v) cetyltrimethyl ammonium bromide. The mixture was homogenized (using bead beating) with a Mini-Beadbeater™ (Wakenyaku, Kyoto, Japan) at 5000 rpm for 1 min, and heated at 65 °C for 15 min. After the sample was cooled, it was subjected again to bead beating at 5000 rpm for 1 min, then centrifuged at 13 000 rpm for 1 min. A 400 μl sample of the supernatant was transferred to a sterile Eppendorf tube and precipitated with ethanol. The genomic DNA was finally dissolved in 500 μl of TE. The DNA solution was

stored at –20 °C.

Primers used for PCR were 9F (5′–GAG TTT GAT CCT GGC TGA G–3′) and 1541R (5′–AAG GAG GTG ATC CAG CC–3′), corresponding to positions 9–27 and 1525–1541, respectively, of the 16S rRNA gene sequence of *Escherichia coli* (Brosius et al. 1978). The set of primers used for amplification of the whole 16S rRNA gene and its fragments were GC-338F (5′–CGC CGG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG–3′) and 518R (5′–ATT ACC GCG GCT GCT GG–3′). The PCR reaction mixture (final volume of 50 μl) included one unit of *Ex Taq* DNA polymerase (TAKARA BIO, Kyoto, Japan), 200 nM dNTP mixture, 25 pM of each primer, 1 × PCR reaction buffer (10 mM Tris-HCl [pH 8.0], 50 mM KCl, 1.5 mM $MgCl_2$ and 0.01% Triton X-100) and each genomic DNA as template. PCR–denaturing gradient gel electrophoresis (DGGE) was performed in a Mastercycler® ep thermal cycler (Eppendorf AG, Hamburg, Germany) using the following program: an initial denaturation at 72 °C for 2 min followed by 30 cycles of 1 min at 94 °C, 1 min at 55 °C and 2 min at 72 °C, concluding with a 5-min extension at 72 °C for the amplification of the 16S rRNA gene, and then a second series of steps involving an initial denaturation for 5 min at 94 °C followed by 30 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C, concluding with a 5-min extension at 72 °C. The DNA was electrophoresed using 1.5% agarose gel in 0.5 × TAE buffer at 100 V for 30 min. Each DNA sample was mixed with 6 × loading buffer (TOYOBO, Tokyo, Japan). After electrophoresis, the gel was stained with ethidium bromide for 30 min, and DNA bands were visualized on a UV transilluminator at 254 nm.

PCR products were ligated to the TOPO® vector using the TOPO TA cloning® Kit (Invitrogen, Japan, Tokyo) according to the manufacturer's protocol. A 100 ng sample of each product was used to transform *E. coli* DH5α (Ueno et al. 2006b).

2.8. DGGE

DGGE was performed on the D-code™ Universal Mutation Detection system (Bio-Rad Laboratories, Tokyo, Japan). In total, 10 μl of each PCR product was dissolved in 2 μl of alkaline gel-loading buffer containing 300 mM NaOH, 6 mM EDTA, 18% (w/v) Ficoll and 0.25% (w/v) xylene cyanol. This was then loaded onto an acrylamide gel containing a gradient ranging from 20% to 60% of the denaturant mix

urea-formamide. A 100% concentration of the denaturants was defined as 7 M urea and 40% (v/v) formamide (Muyzer et al. 1993). Electrophoresis was performed at a constant voltage of 200 V for 3 h at a constant temperature of 60 °C. After running the DGGE, the gels were stained for 30 min in 0.5 × TAE containing 1 × SYBR Green (BioWhittaker, Walkersville, MD, USA) and photographed under UV illumination.

2.9. DNA sequencing

The DNA inserted into the TOPO® plasmid (Invitrogen) was amplified using the BigDye® terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Tokyo, Japan) following the manufacturer's instructions, and the nucleotide sequences were analyzed by an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems), as described previously (Ueno et al. 2006b). The nucleotide sequences were analyzed by searching GenBank using the Blast program (http://www.ncbi.nlm.nih.gov/BLAST/).

In order to sequence the DGGE bands, small pieces of the bands were punched from the gel using sterile pipette tips. Each piece was transferred to a microfuge tube containing 500 μl of TE buffer and incubated overnight at 4 °C to allow diffusion of the DNA into the buffer. Acrylamide residues were pelleted after a low-speed centrifugation step. The DNA in the supernatant was precipitated by ethanol precipitation and used for the reamplification of 16S rRNA gene fragments. To verify that bands on a DGGE gel were equivalent to bands of the cloned inserts, PCR products using the primer with GC clamps were analyzed by DGGE and compared with the original nested PCR–DGGE profile.

2.10. Chemicals.

All antibiotics except for streptomycin were purchased from Wako Chemicals (Kyoto, Japan). Streptomycin was the product of Sigma-Aldrich Japan (Tokyo). All organic solvents from Kanto Chemical (Tokyo, Japan) were of analytical grade. dNTP, agarose, and all other chemicals for PCR were products of TaKaRa Bio (Kyoto, Japan). Urea and formamide for DGGE were purchased from Kanto Chemical and Sigma-Aldrich Japan, respectively. All other reagents were of analytical grade.

3. Results

3.1. Analysis and components of TuO

TuO was initially analyzed by GC. The GC profile of TuO extracted at time zero showed only one irregular broad peak like a 'hump' with some minor sharp peaks, and the base line of the GC profile varied inconsistently (Fig. 1A). The irregular broad peak and minor sharp peaks were considered to be derived mostly from naphthenes with high boiling temperatures in the base oil and from *n*-alkane contaminants (Koma et al. 2003), respectively. When a TuO-degrading bacterial consortium was incubated with the TuO (see below), the area of the broad peak in the extracted TuO fraction appeared to decrease (Fig. 1B), suggesting that biodegradation of TuO may have occurred. However, it was impossible to quantify the amount of TuO present using GC because of the inconsistent base line in the GC chromatograms. In TLC–FID analysis, all TuO components were detected in the saturated fraction, which decreased over time relative to the internal standard when cultivated with the Atsuta A consortium (Fig. 1C and see below). Minor peaks close to the origin that appeared after day 5 would be those of cellular lipids.

3.2. Enrichment cultivation of TuO-degrading bacterial consortia

Small aliquots of six soil samples were inoculated into MSM containing 0.5% (w/v) TuO and incubated at 20 °C for 1 month. An aliquot of each culture was transferred to fresh TuO containing MSM and cultivated at 20 °C for 2 weeks. The culture was then used as inoculum for the next enrichment. The process of enrichment at 20 °C for 2 weeks was repeated four or more times. The degradation of TuO by six samples at the fourth enrichment is shown in Fig. 2. The Atsuta A sample exhibited the highest degradation of 79%. Approximately 60% degradation was observed in the Atsuta B and Mourai A samples. The degradation observed in the four remaining samples was less than 40%. Since the cultures after enrichment were considered to consist of bacterial consortia, each bacterial culture was named as a consortium, for example Atsuta A consortium. Degradation of TuO by the Atsuta A consortium after the sixth enrichment increased to as high as 90%. This level of TuO degradation by the Atsuta A consortium was maintained throughout each subsequent enrichment step (i.e. after the sixth cultivation). No

evaporation of TuO from the culture was observed throughout cultivation.

3.3. Characterization of the Atsuta A consortium

The Atsuta A consortium formed bacterial aggregates during cultivation in MSM containing TuO (data not shown). The aggregates were too sticky to be suspended homogeneously in MSM and water. Some of the aggregates attached to the walls of the flask, while others floated as small blocks in the medium. One block (approx. 2 mm × 4 mm × 1 mm) of bacterial aggregates was used as inoculum unless otherwise stated.

Maximum degradation (approx. 64%) over 3 days was observed at 30 °C (Fig. 3A). Almost no degradation occurred below 20 °C or above 35 °C over 3 days. The TuO in MSM could maintain its fluid state above 15 °C. Fig. 3B shows the relationship between the degradation of TuO and the initial pH of the medium. The Atsuta A consortium degraded TuO optimally at pH 7. Degradation over 3 days at pH 7 was 56% and that at pH 6 was 37%. At pH 4−5 and pH 8−9, only low degradation (less than 10%) was observed, and no degradation was observed at pH 3 and pH 10.

The time-course of TuO degradation by the Atsuta A consortium was examined under optimal temperature and pH conditions (Fig. 4). No degradation was observed at day 1. Degradation started after day 1 and reached up to 90% at day 5. The calculated degradation rate of TuO by the Atsuta A consortium over 4 days from day 1 to day 5 was 1125 mg day^{-1} l^{-1} of culture. In contrast, the degradation of TuO by *R. erythropolis* MBIC01337, which is known to be an efficient degrader of petroleum products (Komukai-Nakamura et al. 1996), was 11% at day 7. The TuO degradation rate of *R. erythropolis* MBIC01337 was calculated to be 92 mg day^{-1} l^{-1}. *P. aeruginosa* WatG, which is able to degrade petroleum products in DO and in automobile EO (Wongsa et al. 2004), exhibited no degradation of TuO for up to 7 days. The Atsuta A consortium degraded waste TuO that had been used in a hydroelectric power station for more than 8 years, in a similar manner to that of the degradation of fresh TuO. However, the degradation of waste TuO at day 3 was 24% (data not shown).

Degradation of TuO by the Atsuta A consortium was tested in the presence of various antibiotics. In the absence of antibiotics, 52% of TuO in MSM was degraded over 3 days at 30 °C. However, the degradation of TuO was completely inhibited by the addition of chloramphenicol, kanamycin, streptomycin or

tetracycline. Only 10% of TuO was degraded in the medium containing ampicillin. These results suggest no involvement of eukaryotic microorganisms in TuO degradation.

The Atsuta A consortium degraded other petroleum products such as EO and DO (data not shown). Degradation of EO was 83% within 21 days and that of DO was 95% within 10 days at 30 °C. In contrast to TuO, EO in MSM was partially solidified even at 30 °C, while DO was in a liquid state under the same conditions. The EO was not evaporated throughout incubation, but 12% of DO was evaporated over 10 days.

3.4. Isolation of individual bacterial strains from the Atsuta A consortium

Nine isolates were obtained from the Atsuta A consortium. Their purity was ascertained by plating four times. The 16S rRNA genes of all isolates were sequenced (Table 1). Tentative identification of each strain was based on the identity of their 16S rRNA gene sequence with those of published strains. The sequences of isolates A1 and A7 and isolates A4 and A8 had 99% identity with those of *Pseudomonas* sp. WAI-21 and *Comamonas* sp. D1, respectively. Isolates A6 and A9 had 100% identity with *Buttiauxella noackiae* and *Pseudomonas* sp. pDL01, respectively. Isolates A2 and A5 had 99% identity with *Sphingobacterium* sp. MG2 and *R. erythropolis* strain EPWF, respectively. Isolate A3 was 98% identical to *Cytophaga* sp. An36. Isolates A1, A7 and A9, and isolates A4 and A8 were regarded as examples of two different strains belonging to *Pseudomonas* and *Comamonas*, respectively, based on different characteristics of their colonies.

3.5. Degradation of TuO by isolates from the Atsuta A consortium and a mixture of these isolates

The degradation of TuO by each isolated strain was much lower than the degradation by the Atsuta A consortium (data not shown). Isolates A5, A7, A8 and A9 degraded approximately 14% or less of the TuO during 7 days at 30 °C. No growth or TuO degradation was observed for the other isolates (A1–A4 and A6). The mixture of all nine isolates, which consisted of the same amount of each isolate, degraded only 7% of the TuO. Although the mixture of isolates was transferred repeatedly onto fresh MSM containing TuO in order to enrich TuO degrading isolates, TuO degradation did not increase (Table 2). The amount of TuO

degraded by the Atsuta A consortium decreased to 12% when the consortium was initially cultivated in LB medium. However, TuO degradation increased back up to 82% after repeated cultivation in MSM containing TuO (Table 2).

3.6. PCR–DGGE analysis of the Atsuta A consortium during the degradation of TuO

Fig. 5A shows the change in the banding profile of 16S rRNA genes after PCR–DGGE analysis during the cultivation of the Atsuta A consortium. In this experiment, template DNA from each sample was diluted equally and then subjected to PCR–DGGE. At least 23 bands were observed in total and each band was labeled (from band A to band W). The band profile at day 1, when many new bands appeared, was very different from profiles at day 0 and after day 3. Bands F, K, N and T were dominant after day 3. The intensity of bands F, K and N increased transiently (at day 3 and/or at day 5) and then decreased gradually with time. Band T was consistently dominant after day 5. Bands B, G and J were dominant only at day 1 when no degradation of TuO occurred.

The mobilities of DGGE bands derived from the nine isolates were compared with that of bands derived from the consortium (Fig. 5B). Bands from isolates A1, A7 and A9 (tentatively identified as three different *Pseudomonas* spp.) had the same mobility as band G. Bands F, K and N each had the same mobility as bands from isolates A2, A3 and A6, respectively, and these isolates were tentatively identified as *Sphingobacterium* sp., *Cytophaga* sp. and *Buttiauxella* sp., respectively. Band E had the same mobility as the bands of isolates A4 and A8, and these isolates were tentatively identified as *Comamonas* sp. The mobility of band V was close to that of isolate A5, which was tentatively identified as *Rhodococcus* sp. However, none of the isolates corresponded to band T. The 16S rRNA gene sequence of band T had 98% identity with that of an uncultured bacterium belonging to γ-Proteobacteria (data not shown). This bacterium was reported as clone LTUG00514 (Christopher and Christopher 2004).

4. Discussion

All soil samples tested in this study were able to degrade TuO after the fourth enrichment, and much higher degradation (more than 50%) was observed in the soil samples collected from lightly polluted sites

(Atsuta A and B, and Mourai A) compared with those collected from heavily polluted sites. Although the samples were not characterized in detail, it is considered that lightly polluted soils with vegetation would have a much more abundant and diverse bacterial population than heavily polluted soils because the soil would be relatively rich in nutrients and would have lower toxic effects from the petroleum hydrocarbons. A similar trend was observed for sites contaminated with mercury; that is, a more diverse microbial community was detected in lightly contaminated soils where there was some vegetation than in heavily contaminated soils (Müller et al. 2001).

The repeated enrichment of the Atsuta A consortium increased TuO degradation, which reached approximately 90% by the sixth enrichment. This high capacity of the Atsuta A consortium to degrade TuO was maintained consistently after the sixth enrichment. This result suggests that the Atsuta A bacterial consortium became more adapted to MSM containing TuO and attained an optimized state. The formation of bacterial aggregates in the flask might have facilitated the generation of a bacterial community structure that degraded TuO efficiently in the liquid medium. Although we were not able to identify individual hydrocarbon components of the base oil of TuO by gas chromatography–mass spectrometry because of their poor resolution in GC (see Fig. 1A, B), the Atsuta A consortium is assumed to be a mixture of bacteria capable of degrading naphthenes and isoalkanes. The degradation of TuO by the Atsuta A consortium was never more than 90%, implying that the TuO used in this study included compounds recalcitrant to biodegradation by this bacterial consortium.

Degradation of EO by the Atsuta A consortium was less than that of TuO (data not shown). The degradation rate of EO (198 mg day^{-1} l^{-1}) by the Atsuta A consortium was less than one sixth that of TuO. This low degradation was probably due to the presence of hydrocarbon components with relatively high melting temperatures in EO, as EO was partially solidified in MSM at 30 °C, and solidified EO attached to the wall of the flask during incubation. Another possible explanation of the inefficient degradation of EO by the Atsuta A consortium is that the bacterial community did not adapt to EO. On the other hand, the degradation of DO by the Atsuta A consortium was comparable with that of TuO, suggesting that the consortium has a high potential to degrade *n*-alkanes, as more than 50% of DO is made up of *n*-alkanes with 10–20 carbon atoms (Ueno et al. 2006a).

Although at least 23 distinct DGGE bands were detected in a DGGE analysis of the Atsuta A consortium (Fig. 5A), only nine bacterial strains were isolated and, unexpectedly, all isolates grown

individually exhibited very low or negligible TuO degradation (data not shown). In addition, the degradation of TuO by a mixture of the nine isolates was 7%. Therefore, it is most likely that the highest performing degrader(s) were not isolated from the consortium. As shown in Fig. 4, the degradation of TuO began after day 1 (at day 3) and increased with time. Thus, the degradation of TuO by the Atsuta A consortium may depend strongly on bacterial strains corresponding to bands F K, N and T, because the intensity of these four bands was higher than that of the other bands and increased transiently or consistently with time (Fig. 5A). Since the intensity of band T was the highest of all the bands after day 5, the bacterium corresponding to this band may be the one mostly involved in the degradation of TuO by the Atsuta A consortium. Another possibility is that TuO might be cooperatively degraded by various strains including the unculturable and culturable species in the consortium. The bacteria corresponding to DGGE bands F, K, and N and DGGE band T may be involved in the early and late phases, respectively, of the incubation. Unfortunately, no bacterial strain corresponding to band T was isolated despite repeated attempts (Fig. 5B). This coincides with the finding that a partial sequence (180 bp) of the 16S rRNA gene of band T had a high homology with an uncultured bacteria belonging to γ-Proteobacteria (data now shown). Even so, it should be recognized that the strain corresponding to band T is not totally unculturable as it grew as part of the consortium in MSM containing TuO (Fig. 5A). It is unclear whether bacteria corresponding to bands B, G and J that appeared transiently at day 1 were involved in TuO degradation by the consortium during subsequent incubation. Although no evidence is available, such bacteria might use an unknown mechanism(s) to facilitate the activity of the TuO-degrading strains that appeared after day 3.

The observation of little or no degradation of TuO by the nine isolates and a mixture of them might be due to their being cultivated in LB medium. According to Pattanasupong et al. (2004), the addition of readily utilizable carbon sources decreased the degradability of methyl-2-benzimidazole carbamate by a bacterial consortium. Therefore, one or more of the nine isolates might be a high-performance degrader of TuO, but its (or their) capability may have become latent by being cultivated in LB medium. While this explanation is consistent with observations of the Atsuta A consortium, it is not consistent with the observed behavior of the mixture of isolates (Table 2). Therefore, it is most likely that none of the isolates was a high-performance degrader of TuO.

It should be noted that two or more enrichments were necessary to obtain the Atsuta A consortium with maximum degradation capacity (approximately 90% over 5 days; see Table 2). According to Bastos et al.

(2002), the highly developed structure of a microbial consortium that degraded 1,3-dichloro-2-propanol efficiently was established only by repeated (or prolonged) enrichment. This would apply to the Atsuta A consortium. It is also true that the mature microbial structure that allowed TuO degradation could be destroyed easily by cultivating the consortium in a rich medium or, probably, in MSM supplemented with carbon sources other than petroleum hydrocarbons.

Band T from the DGGE profile is very interesting as it was one of the major bands from the Atsuta A consortium. Its intensity increased during incubation almost in parallel with increases in the degradation of TuO (Figs. 4 and 5A). The difficulties encountered in cultivating the bacterial strain corresponding to band T may have been due to the possibility that this strain could grow only in liquid MSM containing TuO, but not on agar plates. If this were true, then it would be impossible to isolate it by the plating method. According to Kirk et al. (2004), some bacterial strains cannot grow on plates as, in this environment, they are exposed to greater stresses from molecular oxygen and/or dessication than in liquid media. It is also possible that slow-growing bacteria are outcompeted by fast-growing bacteria on agar plates. Another possible explanation for not being able to cultivate bacteria from band T is that the strain may be able to grow only where certain compounds essential for its growth are provided by other bacteria. Sonderkamp et al. (2001) reported that the growth of a bacterial community could be promoted (accelerated) by the secretion of exoenzymes such as lipase. In their report, a microbial consortium degraded trimethylpropaneoleate, a synthetic fatty acid ester, only when minor enzyme producers secreted lipase. Therefore, an investigation of mutual relationships and the roles of various members of the Atsuta A consortium is indispensable.

In conclusion, a microbial consortium named Atsuta A consortium degraded TuO most efficiently (90% degraded) at 30 °C and pH 7 over 5 days. The oils EO and DO were also degraded by the consortium. Although nine different strains were isolated from the Atsuta A consortium, neither of the individual isolates nor a mixture of them degraded TuO to the same extent as the consortium. A DGGE analysis of 16S rRNA genes of the Atsuta A consortium suggested that it consisted of at least 23 different strains and that four major strains (represented by bands F, K, N and T) increased their population during cultivation in MSM containing TuO. Of these four strains, one strain that was not isolated and that corresponded to DGGE band T might be a high-performance degrader of TuO. Even if no high-performance TuO degraders were isolated from the consortium, consortia such as Atsuta A could be used immediately for

bioaugmentation of waters or soils contaminated by TuO or other lubricating oils.

Acknowledgement

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Figure legends

Figure 1. Analysis of TuO by GC and TLC–FID on an Iatroscan. The Atsuta A consortium was cultivated in MSM containing 0.5% (w/v) TuO and incubated at 30 °C for 0 (A) and 5 (B) days on a rotary shaker (160 rpm). The chloroform fraction (hydrocarbons) extracted from the cultures was analyzed by GC. In (C) the Atsuta consortium was cultivated under similar conditions for 14 days. The hydrocarbon fraction was analyzed with an Iatroscan. Peaks a and b are those of saturated hydrocarbons and stearic acid (internal standard), respectively. The arrow indicates the origin in TLC.

Figure 2. Degradation of TuO by various soil samples after the fourth enrichment. Each soil sample was incubated in MSM containing 0.5% (w/v) TuO and incubated at 20 °C for 2 weeks on a rotary shaker (160 rpm). Atsuta A and B, and Mourai A and B samples were collected from sites that were lightly polluted with crude oil. Atsuta H and Mourai H samples were collected from heavily polluted sites.

Figure 3. Effects of temperature and pH on the degradation of TuO by the Atsuta A consortium in MSM. The Atsuta A consortium was cultivated in 0.5% (w/v) TuO containing MSM (pH 7) at various temperatures (A) on a rotary shaker (160 rpm) for 3 days. In (B) the consortium was cultivated in the same medium at various initial pH values and incubated at 30 °C for 3 days.

Figure 4. Degradation of TuO by the Atsuta A consortium, *R. erythropolis* MBIC 01337 and *P. aeruginosa* WatG in MSM. The 50 ml flasks that contained 10 ml MSM with 0.5% (w/v) petroleum products were incubated at 30 °C for 7 days on a rotary shaker (160 rpm).

Figure 5. DGGE band profiles of the Atsuta A consortium and nine isolates obtained from it during cultivation in MSM containing 0.5% (w/v) TuO. PCR–DGGE was carried out using genomic DNA that was diluted equally in each culture as template (A). (B) illustrates the DNA band profiles of the Atsuta A consortium (left) and those of the nine isolates during cultivation. Vertical lines have been drawn to allow comparison of the mobility of bands from the consortium and isolates. The position of line V* did not agree with that of DGGE band V.

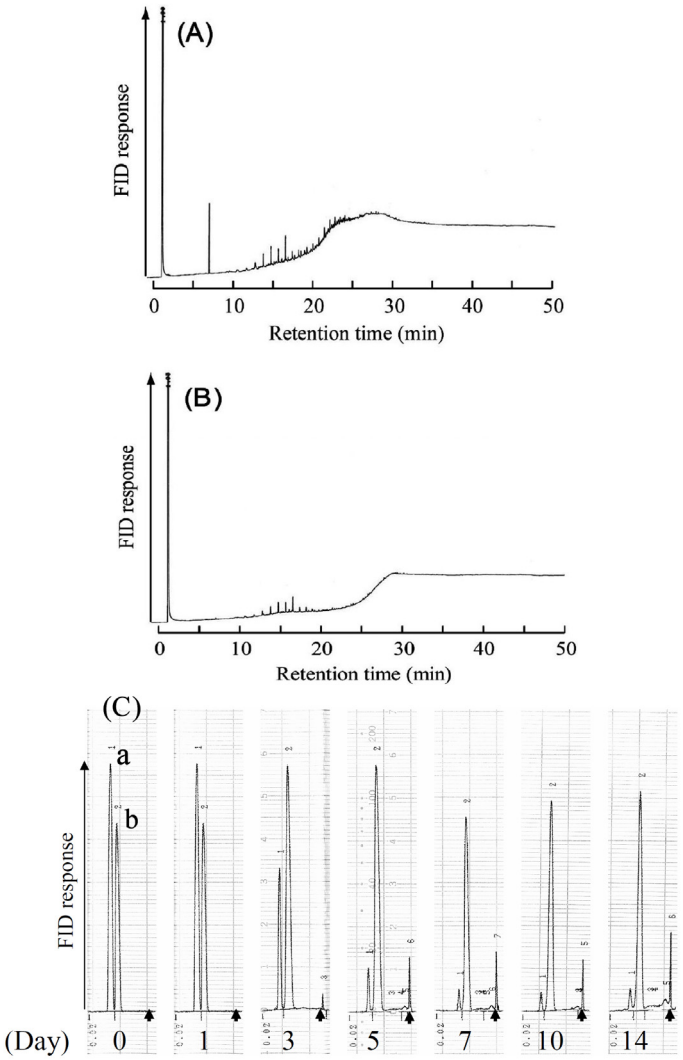

(A)
FID response
0
10
20
30
40
50
Retention time (min)
(B)
FID response
0
10
20
30
40
50
Retention time (min)
(C)
a
b
FID response
(Day)
0
1
3
5
7
10
14

Figure 1.

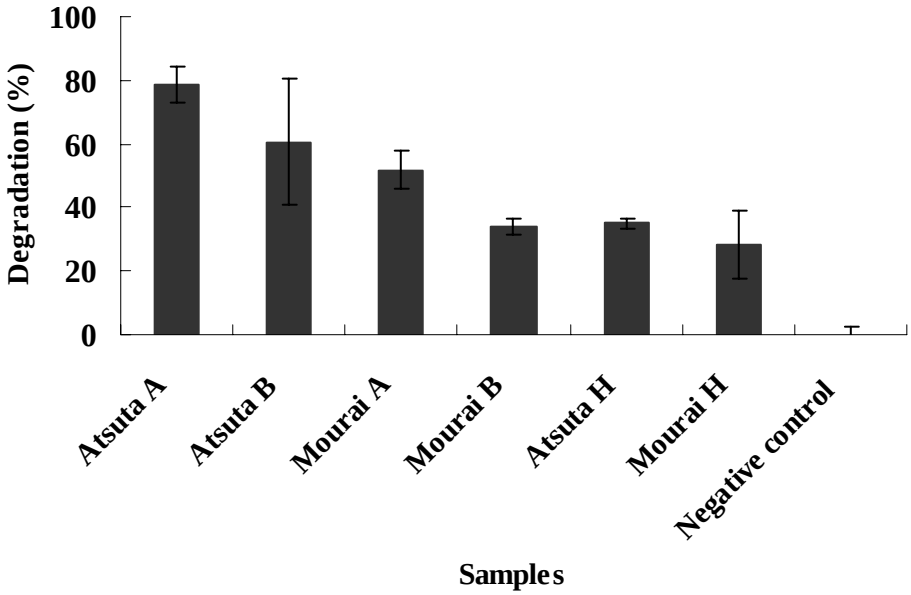
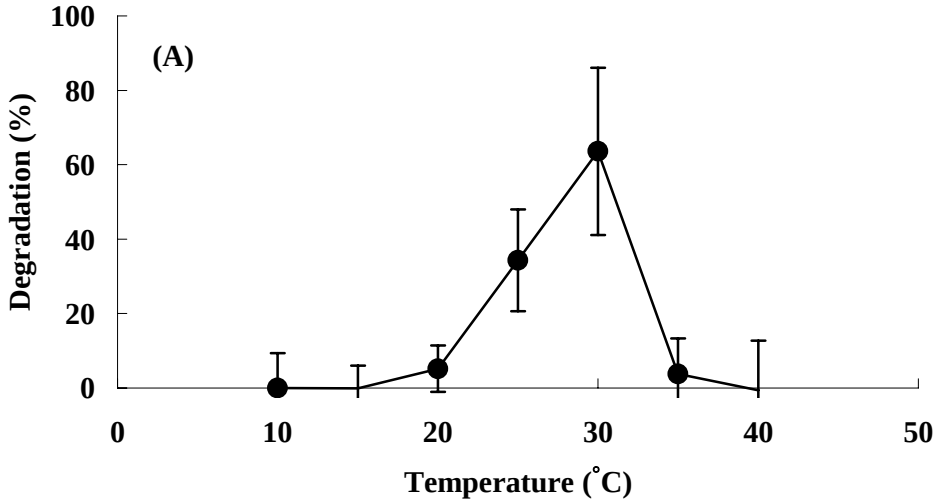

100
80
60
40
20
0
Degradation (%)
Atsuta A
Atsuta B
Mourai A
Mourai B
Atsuta H
Mourai H
Negative control
Samples

Figure 2


(A)
Degradation (%)
100
80
60
40
20
0
0
10
20
30
40
50
Temperature (˚C)

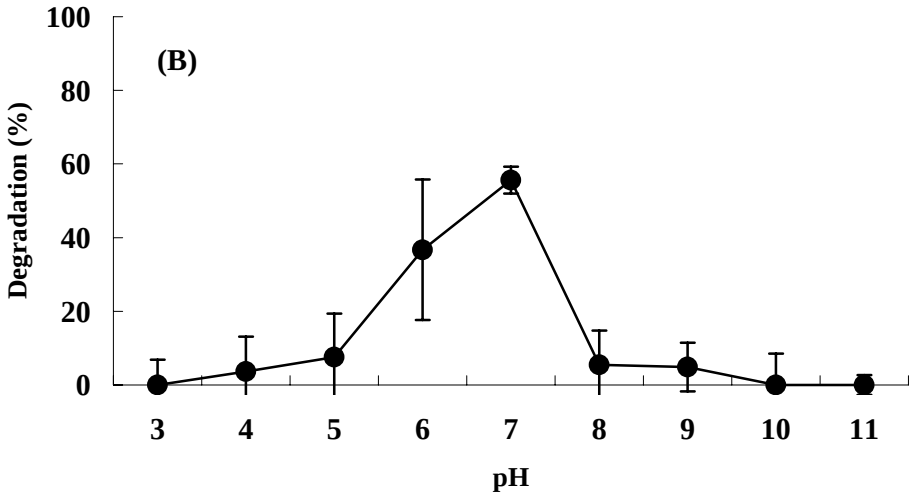

(B)
Degradation (%)
100
80
60
40
20
0
3
4
5
6
7
8
9
10
11
pH

Figure 3

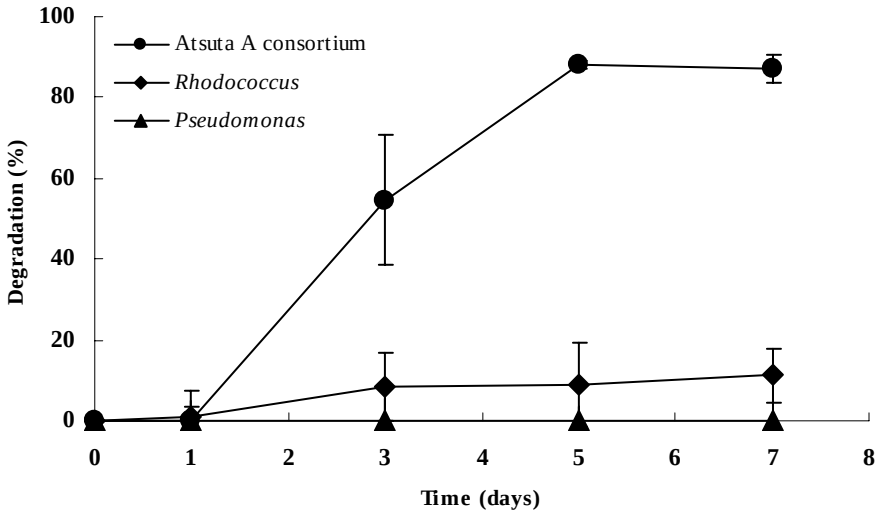

100
80
60
40
20
0
Degradation (%)
Atsuta A consortium
Rhodococcus
Pseudomonas
0
1
2
3
4
5
6
7
8
Time (days)

Figure 4

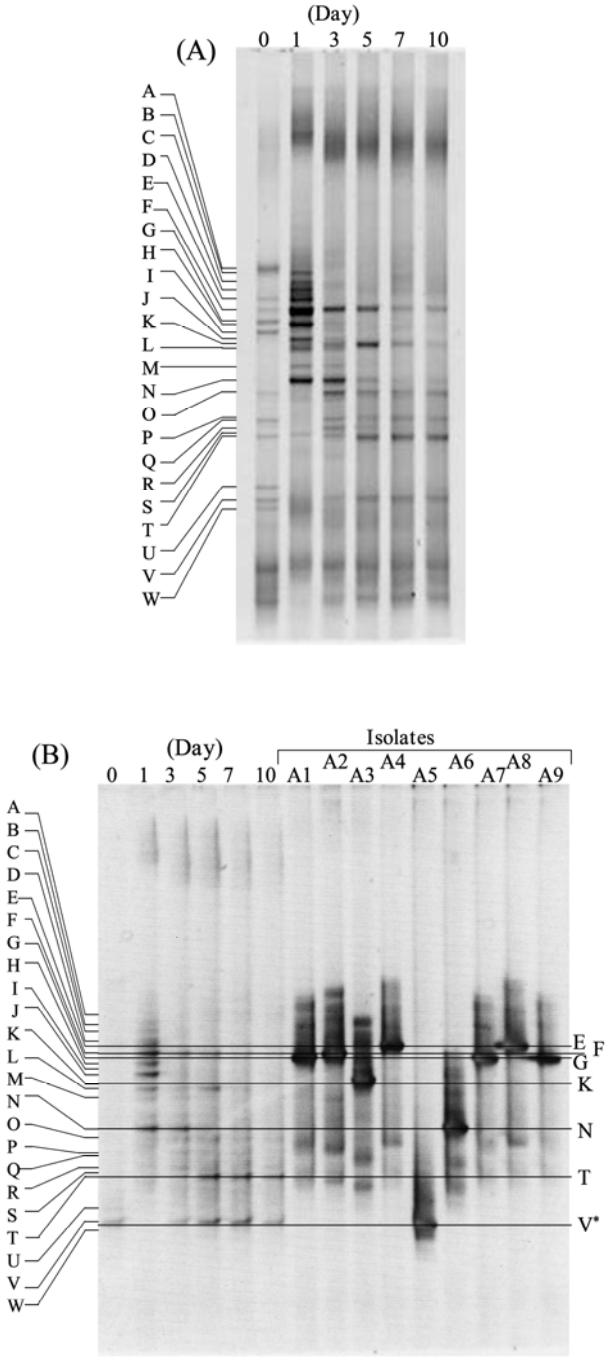

(A)
(Day)
0 1 3 5 7 10
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
W
(B)
(Day)
0 1 3 5 7 10
Isolates
A1 A2 A3 A4 A5 A6 A7 A8 A9
E F
G
K
N
T
V*

Figure 5

Table 1
Characteristics of isolates from Atsuta A consortium and their tentative identification

Isolates	Characteristics of colony		Gram stain	Closest strain in 16S rRNA gene sequence (% identity) [a] Tentative identification	Corresponding DGGE bands in Fig. 5
	Form Margin Elevation	Color Clarity			
A1	Irregular Undulate Raised	Cream Semitransparent	-	*Pseudomonas* sp. WAI-21 (99) *Pseudomonas* sp. A1	G
A2	Circle Entire Raised	Cream Semitransparent	-	*Sphingobacterium* sp. MG2 (99) *Sphingobacterium* sp. A2	F
A3	Irregular Undulate Flat	Yellow Transparent	-	*Cytophaga* sp. An36 (98) *Cytophaga* sp. A3	K
A4	Irregular Undulate Flat	White Semitransparent	-	*Comamonas* sp. D1 (99) *Comamonas* sp. A4	E
A5	Circle Entire Raised	Cream Opaque	+	*Rhodococcus erythropolis* EPWF (99) *Rhodococcus* sp. A5	V[b]
A6	Irregular Undulate Flat	Rainbow cream Semitransparent	-	*Buttiauxella noackiae* (100) *Buttiauxella* sp. A6	N
A7	Irregular Entire Raised	Rainbow cream Semitransparent	-	*Pseudomonas* sp. WAI-21 (99) *Pseudomonas* sp. A7	G
A8	Circle Entire Raised	Cream Semitransparent	-	*Comamonas* sp. D1 (99) *Comamonas* sp. A8	E
A9	Irregular Undulate Flat	Cream Semitransparent	-	*Pseudomonas* sp. pDL01 (100) *Pseudomonas* sp. A9	G

[a]Identified by the GenBank Blast search (http://www.ncbi.nlm.nih.gov/BLAST)

[b]The mobility of DGGE band of isolate A5 was close to that of band V but not the same.