Oceanobacter-related bacteria are important for the degradation of petroleum aliphatic hydrocarbons in the tropical marine environment

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Petroleum-hydrocarbon-degrading bacteria were obtained after enrichment on crude oil (as a 'chocolate mousse') in a continuous supply of Indonesian seawater amended with nitrogen, phosphorus and iron nutrients. They were related to *Alcanivorax* and *Marinobacter* strains, which are ubiquitous petroleum-hydrocarbon-degrading bacteria in marine environments, and to *Oceanobacter kriegii* (96.4–96.5 % similarities in almost full-length 16S rRNA gene sequences). The *Oceanobacter*-related bacteria showed high n-alkane-degrading activity, comparable to that of *Alcanivorax borkumensis* strain SK2. On the other hand, *Alcanivorax* strains exhibited high activity for branched-alkane degradation and thus could be key bacteria for branched-alkane biodegradation in tropical seas. *Oceanobacter*-related bacteria became most dominant in microcosms that simulated a crude oil spill event with Indonesian seawater. The dominance was observed in microcosms that were unamended or amended with fertilizer, suggesting that the *Oceanobacter*-related strains could become dominant in the natural tropical marine environment after an accidental oil spill, and would continue to dominate in the environment after biostimulation. These results suggest that *Oceanobacter*-related bacteria could be major degraders of petroleum n-alkanes spilt in the tropical sea.

INTRODUCTION

A wide variety of micro-organisms are known to degrade petroleum hydrocarbons (Head *et al.*, 2006; Prince, 2005). Most hydrocarbonoclastic bacteria metabolize either aliphatic or aromatic hydrocarbons, although some bacteria such as strains of *Pseudomonas* (Whyte *et al.*, 1997) and *Rhodococcus* (Andreoni *et al.*, 2000) have been shown to degrade both types of hydrocarbons. Among hydrocarbonoclastic bacteria, *Alcanivorax* (Hara *et al.*, 2003; Kasai *et al.*, 2001; Roling *et al.*, 2004; Yakimov *et al.*, 1998, 2005) and *Cycloclasticus* (Dyksterhouse *et al.*, 1995; Kasai *et al.*, 2002a; Maruyama *et al.*, 2003) strains have been identified as key micro-organisms in the degradation of aliphatic and aromatic hydrocarbons, respectively, in marine environments (Harayama *et al.*, 2004). *Alcanivorax* strains are distributed in natural marine environments around the world (Golyshin *et al.*, 2005). The ability of *Alcanivorax*

strains to use branched alkanes is high (Hara *et al.*, 2003; McKew *et al.*, 2007), and this could be one of the reasons why these strains predominate in crude-oil-impacted temperate marine environments (Cappello *et al.*, 2007; Hara *et al.*, 2003; Kasai *et al.*, 2001; Roling *et al.*, 2002, 2004; Yakimov *et al.*, 2005). *Thalassolituus oleivorans* has been reported to degrade aliphatic hydrocarbons (Yakimov *et al.*, 2004), and *Thalassolituus* strains have recently been shown to dominate in n-alkane-containing temperate seawater microcosms (McKew *et al.*, 2007; Yakimov *et al.*, 2005) and in crude-oil-containing temperate estuarine seawater microcosms (Coulon *et al.*, 2007; McKew *et al.*, 2007).

In addition to these 'professional' hydrocarbonoclastic bacteria, many 'non-professional' hydrocarbonoclastic bacteria such as *Marinobacter hydrocarbonoclasticus* (Gauthier *et al.*, 1992) have also been isolated. *Marinobacter* strains (Gauthier *et al.*, 1992; Hedlund *et al.*, 2001; Huu *et al.*, 1999; Sproer *et al.*, 1998) are of increasing interest as many marine hydrocarbonoclastic isolates from various marine environments, including pristine areas, have been suggested to be classified into this genus (González & Whitman, 2006).

Abbreviations: rep-PCR, repetitive extragenic palindromic sequence PCR; T-RF, terminal restriction fragment; T-RFLP, terminal restriction fragment length polymorphism.

The GenBank/EMBL/DDBJ accession numbers for the sequences determined in this work are listed in Table 1.

Variation in seawater temperature has been indicated to select the composition of hydrocarbonoclastic bacteria (Coulon *et al.*, 2007). However, relatively few studies have been conducted on petroleum-hydrocarbon-degrading bacteria in tropical marine environments (Brito *et al.*, 2006; Chaillan *et al.*, 2004; Harwati *et al.*, 2007; Zhuang *et al.*, 2003; Zinjarde & Pant, 2002). We therefore decided to focus on the petroleum-hydrocarbon-degrading bacteria in the tropical marine environment.

Batch culture techniques have most commonly been used to isolate bacteria that are capable of degrading hydrocarbons of interest. However, this method is highly selective, resulting in the enrichment of a few species with a selective growth advantage under laboratory conditions (Dunbar *et al.*, 1996, 1997; Watanabe *et al.*, 1998). On the other hand, by using alternative methods, such as continuous flow-through culture, dominant bacterial populations in actual environments have successfully been isolated (Kasai *et al.*, 2001, 2002b; Stach & Burns, 2002; Watanabe *et al.*, 1998). Thus, in the present study, petroleum-hydrocarbon-degrading bacteria in Indonesian seawater were enriched in flow-through cultures containing 'chocolate-mousse' crude oil. We report here on the hydrocarbonoclastic bacteria isolated from these cultures.

METHODS

Seawater sample. The seawater samples used for isolating petroleum-hydrocarbon-degrading bacteria were collected in June 2006 at Pari Island (5.86° S, 106.62° E) located off Jakarta, Indonesia.

Preparation of crude oil and of its chocolate mousse. Arabian light crude oil was treated at 214 °C for 10 h to remove the volatile fraction (30 % in volume), and used in this study. Chocolate-mousse crude oil was prepared by mixing the crude oil and fresh seawater collected at Pari Island in a ratio of 1 : 5 (w/w), followed by vigorous and continuous shaking for 1 day. The resultant chocolate mousse was stable for several weeks.

Strains. The reference strain *Alcanivorax borkumensis* SK2 (ATCC 700651; Yakimov *et al.*, 1998) was obtained from the ATCC; *Oceanobacter kriegii* strain NBRC 15467 was from NBRC (NITE Biological Resource Center). The strains isolated in this study were deposited in NBRC under numbers NBRC 105758 to NBRC 105769, and also in BTCC (Biotechnology Culture Collection, LIPI, Cibinong, Indonesia) under numbers BTCC B-675 to BTCC B-686.

Continuous-flow culture. One litre of non-sterilized seawater collected at Pari Island was supplemented with 1 g NH₄NO₃, 0.2 g K₂HPO₄ and 12 mg FeCl₃ (SW medium), and incubated in a glass basin with gentle shaking at room temperature (around 25 °C). Three grams of chocolate-mousse crude oil (comprising 0.5 g crude oil and 2.5 g seawater) was applied to one side of a pumice stone and floated on the SW medium with the coated side down. SW medium was continuously supplied to the glass basin at a rate of 200 ml per day, while the same amount of the medium was pumped out from the glass basin to maintain the culture volume at 1 l. Such continuous-flow cultures were conducted in two different modes to isolate hydrocarbon-degrading bacteria of wider diversity. In the first type (culture 1), non-sterilized SW medium prepared with fresh seawater was supplied for the first 10 days, and sterilized SW medium,

prepared by autoclaving, was supplied for the next 6 days. In the second type (culture 2), non-sterilized SW medium prepared with fresh seawater was supplied for the first 3 days, and sterilized SW medium was supplied for the next 13 days. On the 16th day after starting the cultivation, the surface of the chocolate-mousse oil and the aqueous phase of the culture were spread onto an SW medium plate [1.5 % (w/v) agar; 9 cm in diameter] covered with 30 μl crude oil. The plates were incubated at 18 °C for up to 6 weeks. The bacterial colonies that appeared on the crude-oil-covered SW medium plates were purified at room temperature (around 25 °C) on dMB plates containing 0.5 % (w/v) pyruvate. The dMB plate medium comprised (per litre) 15 g agar, 0.9 l seawater, 0.1 l distilled water and 3.74 g Marine broth 2216 (Difco).

Analysis of 16S rRNA genes. Bacterial 16S rRNA gene fragments were amplified using primers 27F (5′-AGRGTTTGATCMTGG-CTCAG-3′; Vergin *et al.*, 1998) and 1492R (5′-GGYTACCTTGTT-ACGACTT-3′; Lane, 1991). The PCR was performed in a total volume of 25 μl containing 1.25 U *Taq* DNA polymerase (Ex Taq; Takara), Mg²⁺-containing buffer supplied with the polymerase (2 mM Mg²⁺ at the final concentration), 1 M betaine (Sigma), 1.25 μl DMSO (Sigma), 200 μM of each deoxynucleoside triphosphate, 1 μM of each primer, and a small amount of bacterial cells that were transferred using a toothpick from a colony that appeared on a plate. The amplification programme was as follows: (i) 5 min at 94 °C; (ii) 10 touchdown cycles consisting of denaturation for 1 min at 94 °C, annealing for 1 min at 65 °C with the temperature dropping by 1 °C each cycle, and elongation for 2 min at 72 °C; (iii) 25 cycles consisting of denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C and elongation for 2 min at 72 °C; and (iv) a final extension for 7 min at 72 °C. The PCR products were purified with a Qiaquick PCR purification kit (Qiagen) or AMPure (Agencourt). The PCR products were sequenced using a DNA sequencing kit (Dye Terminator Cycle Sequencing kit; Perkin-Elmer) with primers 339F (5′-CTCCTACGGGAGGCAGCAG-3′), 536R (5′-GTATTACCGCG-GCTGCTG-3′), 907F (5′-AAACTCAAAGGAATTGACGG-3′), 1510R (5′-GGCTACCTTGTTACGACTT-3′) and 27F (5′-AGAGTTTGAT-CCTGGCTCAG-3′) according to the manufacturer's instructions, and the sequences were determined with a model 3730xl DNA analyser (Applied Biosystems). These sequences were assembled using the ATGC program (version 4.3.0; Genetyx), and compared to those in the GenBank database by using BLAST (Altschul *et al.*, 1990). The alignment of the sequences was done using CLUSTAL_X (version 1.83; Thompson *et al.*, 1997). The distance matrices for the aligned sequences, including all gaps, were calculated, and a neighbour-joining tree (Saitou & Nei, 1987) was constructed using the NJPlot software in the CLUSTAL_X program.

Rep-PCR. Genomic fingerprints of bacteria were obtained by repetitive extragenic palindromic sequence PCR (rep-PCR) performed with primers REP1R-I and REP2-I (de Bruijn, 1992). The PCR was performed in a total volume of 25 μl containing 1.25 U *Taq* DNA polymerase (AmpliTaq Gold; Perkin-Elmer), 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001 % (w/v) gelatin, 1 M betaine (Sigma), 1.25 μl DMSO (Sigma), 200 μM of each deoxynucleo-side triphosphate, 1 μM of each primer, and a small amount of bacterial cells that were transferred using a toothpick from a colony that appeared on a plate. The PCR conditions used were as follows: 10 min of polymerase activation at 94 °C, followed by 40 cycles consisting of 1 min at 94 °C, 1 min at 40 °C and 8 min at 65 °C, and finally 10 min of extension at 72 °C. The PCR products were analysed using a 2100 Bioanalyser (Agilent Technologies). The rep-PCR analysis was conducted twice to determine the reproducibility of the method.

Degradation of crude oil and GC-MS analysis. The SW and dMB media used for characterizing bacteria were formulated using seawater collected from the Pacific Ocean, 300 km off the coast of

Tokyo, Japan. Cells of each bacterium freshly grown on a dMB plate supplemented with 0.5 % (w/v) pyruvate at room temperature (approx. 25 °C) were collected and suspended in filter-sterilized seawater to an OD_{600} of 0.5. Then 150 µl of this bacterial suspension was inoculated into 3 ml sterilized SW medium supplemented with 3 µl crude oil, and this culture was incubated for the indicated period with shaking (200 r.p.m.) at 25 or 30 °C to examine the crude-oil-emulsifying and -degrading abilities. Non-inoculated tubes were similarly incubated and served as controls. Hydrocarbons were extracted twice from the cultures by shaking vigorously with an equal volume of dichloromethane for 1 min. Sodium sulfate was added to the dichloromethane extracts to dehydrate them, and the supernatants were concentrated to approximately 100 µl by N_2 purging. The concentrated extracts were subjected to GC-MS using a 6890A gas chromatograph with a 5973 mass-selective detector (Agilent Technologies) equipped with an Ultra 2 fused silica capillary column (25 m in length, 0.2 mm in diameter and 0.33 µm in film thickness; HP 19091B-102, Agilent Technologies). The temperatures of the injection port, transfer line, MS source and quadrupole were maintained at 250, 250, 230 and 150 °C, respectively. The column temperature was increased from 80 °C to 320 °C at a rate of 5 °C min^{-1} for the first 48 min, and then kept at 320 °C for the next 20 min. Helium was used as the carrier gas at a constant pressure of 20 p.s.i. (138 kPa). To investigate whether a strain degraded alkanes, aromatic compounds, or both, all peak areas for hydrocarbons obtained with the GC-MS selected ion monitoring were normalized by dividing by the peak area for $17\alpha(H),21\beta(H)$-hopane (Prince *et al.*, 1994). For bacteria that could not degrade aromatic hydrocarbons, methyldibenzothiophene instead of $17\alpha(H),21\beta(H)$-hopane was used as the internal standard. The emulsification of crude oil was investigated by visual inspection of crude-oil-containing cultures that had been incubated for 2 weeks.

Substrate utilization analysis. The utilization of carbon sources was tested on ONR7a medium (Dyksterhouse *et al.*, 1995) plates [1.5 % (w/v) Agar Noble (Difco)] supplemented with an appropriate carbon source. The carbon sources were filter-sterilized and added at the final concentration of 0.2 % (w/v for solid compounds and v/v for liquid compounds). The plates were incubated at 30 °C for 3 weeks, and the growth was checked by visual inspection.

The GN2 MicroPlate system (Biolog) was used to test for the oxidation of 95 different carbon sources. Cells of each bacterium freshly grown on dMB plates supplemented with 0.5 % (w/v) pyruvate at room temperature were collected and suspended in sterilized artificial seawater consisting of 38.39 g l^{-1} of artificial sea salt, Marine Art SF-1 (Tomita Pharmaceutical). The cell density was adjusted to 20–25 % transmittance by using a Biolog absorbance meter, and 150 µl aliquots were dispensed into each of the 96 wells of the GN2 MicroPlates. The plates were incubated at 30 °C for 1 week, and the colour change in each well, containing a single carbon source, was scored by visual inspection. Two independent assays were performed for each strain.

PCR detection of alkane monooxygenase gene. The amplification of DNA fragments homologous to *alkB* and *cyp153* was carried out by using the primer sets alkB-1f plus alkB-1r (Kloos *et al.*, 2006) and P450fw1 plus P450rv3 (van Beilen *et al.*, 2006), respectively. The reaction mixture was prepared in the same way as described for the analysis of 16S rRNA genes. The amplification programme was as follows: (i) 5 min at 94 °C; (ii) 40 cycles consisting of 1 min at 94 °C, 1 min at 52 °C and 1 min at 72 °C; and (iii) a final extension for 7 min at 72 °C. The amplified DNA fragments were precipitated by ethanol (Sambrook *et al.*, 1989), ligated to the pCR 2.1-TOPO vector (TOPO TA cloning kit; Invitrogen), and introduced into *Escherichia coli* DH5α (Toyobo) according to the manufacturer's instructions. Plasmids were isolated from *E. coli* with the QIAprep Spin Miniprep kit (Qiagen). Sequences of the DNA fragments were determined using

M13 primers as described for the analysis of 16S rRNA genes. The DNA sequences were assembled using Sequencher, version 4.8 (Gene Codes Corporation), and compared to those in the GenBank database by using BLAST (Altschul *et al.*, 1990).

Design and operation of beach-simulating microcosms. Microcosms were designed to simulate oil-contaminated sand on a beach. Five hundred grams of sand and 500 ml of seawater collected at Pari Island were placed in each of four stainless steel trays (length, 27 cm; width, 18 cm; depth, 8 cm), and left for 1 day. The seawater was then discarded from the trays as much as possible, 50 g chocolate-mousse crude oil was loaded onto the sand in each of the trays, and the day 0 sample was taken. One or two types of slow-release fertilizers were then evenly distributed onto the sand in three of the trays, while the fertilizers were not added in the 'control' tray. Fresh seawater (500 ml) was resupplied to each of the trays to start the microcosm experiments. The fertilizers used were either 2 g per tray of Osmocote 14-14-14 (granules containing 14 % N, 6.1 % P and 11.6 % K; Scotts); 2 g per tray of Sumicoat 42-80 (granules containing 42 % N; Sumitomo Chemical); or 2 g per tray of Super IB (Super IBS562; granules containing 15 % N, 7 % P and 10 % K; Mitsubishi Chemical Agri) plus 0.4 g per tray of Linstar 30 (granules containing 13.1 % P; Mitsubishi Chemical Agri). The seawater in the trays was exchanged with fresh seawater every day by discarding as much as possible of the seawater from the trays and adding 500 ml fresh seawater. These microcosms were maintained at 26–27 °C. N and P concentrations in the seawater just before the exchange were measured every 3 to 5 days with a Hach DR/890 portable colorimeter (Hach Company) using Nitrogen, Total Reagent Set, Test 'N Tube (product no. 2672245) and Phosphorus, Total, Test 'N Tube Reagent Set (product no. 2742645), respectively. In the microcosm without fertilizer, total N in seawater was 0.5 ± 0.2 p.p.m. ($\pm$SD), while total P was 0.06 ± 0.03 p.p.m. throughout the experiment (until day 38). In the Osmocote-supplemented microcosm, total N dissolved in the seawater was 12 p.p.m. on day 1 and 1.5 ± 0.6 p.p.m. from day 4 to day 38, while total P was 0.55 ± 0.31 p.p.m. throughout the experiment. In the Sumicoat-supplemented microcosm, total N was 50 p.p.m. on day 1 and 6.1 ± 3.5 p.p.m. from day 4 to day 38, while the level of total P was almost the same as that in the microcosm without fertilizer. In the Super IB- plus Linstar-supplemented microcosm, total N was 208 p.p.m. on day 1, and 32.4 p.p.m. on day 4, and this level decreased almost linearly from 10.7 p.p.m. on day 7 to 0.5 p.p.m. on day 29, while total P was 178 p.p.m. on day 1, 24.4 p.p.m. on day 4 and 3.79 ± 2.47 p.p.m. from day 7 to day 38. For each sampling, 0.5 g of the crude-oil-covered sand was taken for analysis of the bacterial community structure, and 3–5 g of the crude-oil-covered sand was taken for the GC-MS analysis using $17\alpha(H),21\beta(H)$-hopane as the internal standard.

Terminal restriction fragment length polymorphism (T-RFLP) analysis. DNA was extracted with 'ISOIL for Beads Beating' (Nippon Gene) from crude-oil-covered sand in the above-mentioned microcosm. Bacteria-specific primers $1492R_{MOD}$ (5′-TACGGYTACCTT-GTTAYGACTT-3′) and 5′ fluorescently labelled (with 6-carboxy-fluorescein; Applied Biosystems) $27F_{MOD}$ (5′-AGRGTTTGATCM-TGGCTCAG-3′) (Vergin *et al.*, 1998) were used to amplify 16S rRNA gene fragments. The PCR mixture contained, in a total volume of 25 µl, 0.63 U PrimeSTAR HS DNA Polymerase (Takara), Mg^{2+}-containing buffer supplied with the polymerase, 200 µM of each deoxynucleoside triphosphate, 0.2 µM of each primer and 35 ng of the extracted DNA. The reactions comprised 30 cycles of 10 s at 98 °C, 5 s at 55 °C and 90 s at 72 °C followed by a final extension of 5 min at 72 °C. Amplification of the PCR products with the expected size was confirmed by electrophoresis through a 1 % (w/v) agarose gel (LO3; Takara) with 1× TAE buffer (40 mM Tris, 20 mM acetate, 1 mM EDTA, pH 8.0). PCR products were purified with GenElute PCR Clean-Up kit (Sigma).

The PCR products (~100 ng) for the T-RFLP analysis were digested with 4 U *Hha*I (Takara) in a total volume of 10 µl for 3 h at 37 °C with the reaction buffer supplied with the enzyme. The restriction endonuclease was inactivated by heating at 65 °C for 20 min. Fluorescently labelled terminal restriction fragments (T-RFs) were separated on the basis of size by using an ABI PRISM 3130 genetic analyser (Applied Biosystems) with an internal size standard (GENESCAN-1200 LIZ; Applied Biosystems). T-RFLP electropherograms were analysed by peak area integration of each T-RF (GeneMapper 3.5 software; Applied Biosystems). The percentage fluorescence intensities represented by single T-RFs relative to the total fluorescence intensity of all T-RFs were calculated to obtain a measure of relative 16S rRNA gene abundance. The method for phylogenetic assignment of T-RFs is described in the next section.

Clone library analysis. To analyse the microbial community structure in the microcosm described in the previous section, DNA was extracted with ISOIL for Beads Beating from crude-oil-covered sand in the microcosm with Osmocote on day 29. The $27F_{MOD}$ and $1492R_{MOD}$ primers were used to amplify 16S rRNA gene fragments. The PCR mixture contained, in a total volume of 25 µl, 0.63 U *Taq* DNA polymerase (Ex Taq), Mg^{2+}-containing buffer supplied with the polymerase, 200 µM of each deoxynucleoside triphosphate, 0.2 µM of each primer, 1.25 µl DMSO (Sigma), 20 µg BSA and 10 ng of the extracted DNA. The PCR conditions were as follows: 3 min at 94 °C, followed by 15 cycles consisting of 1 min at 94 °C, 1 min at 50 °C and 3 min at 72 °C, and a final extension for 10 min at 72 °C. Amplification of PCR products was confirmed by electrophoresis through 1 % (w/v) agarose gel with 1× TAE buffer. PCR products were purified with the GenElute PCR Clean-Up kit, and the purified PCR products (~10 ng) were cloned into TOPO TA cloning vector (pCR4-TOPO; Invitrogen) and used to transform competent *E. coli* Mach1-T1 cells (Invitrogen) according to the manufacturer's instructions.

Eighty-two clones were randomly selected from the transformants, and the cloned 16S rRNA gene fragments were amplified using M13 forward (5′-GTAAAACGACGGCCAG-3′) and M13 reverse (5′-CAGGAAACAGCTATGAC-3′) primers. The PCR mixture contained, in a total volume of 25 µl, GoTaq Master Mixes (Promega), 1 µM of each primer and a small amount of bacterial cells that were transferred from a colony by using a toothpick. The PCR conditions were as follows: 3 min at 95 °C, followed by 35 cycles consisting of 30 s at 95 °C, 30 s at 55 °C and 2 min at 72 °C, and a final extension for 7 min at 72 °C. These PCR-amplified 16S rRNA gene fragments were purified with AMPure, and their nucleotide sequences were determined by using the DNA sequencing kit with primers 350F (5′-TACGGGAGGCAGCAG-3′) and 1100R (5′-AGGGTTGCGCT-CGTTG-3′), and the 3730xl DNA analyser according to the manufacturer's instructions. Two sequences obtained with the 350F and 1100R primers were assembled using the ATGC program and the resultant sequences were compared to the GenBank database using BLAST (Altschul *et al.*, 1990). T-RFLP analysis was also performed with each of the representative clones to link their 16S rRNA gene sequences with T-RFs.

RESULTS AND DISCUSSION

Isolation of petroleum-hydrocarbon-degrading bacteria enriched on crude oil

Petroleum-hydrocarbon-degrading bacteria were enriched on chocolate-mousse crude oil in a flow-through system in which Indonesian seawater supplemented with nitrogen, phosphorus and iron nutrients was continuously supplied.

After 16 days of cultivation, bacteria were isolated either from the surface of the chocolate-mousse crude oil or from the aqueous phase. Colonies of different morphologies were selected; a total of 60 and 70 isolates were obtained from the surface of the chocolate-mousse crude oil of cultures 1 and 2, respectively, while 20 isolates each were obtained from the aqueous phase of the two cultures. The isolates were grown in SW medium supplemented with 0.1 % (v/v) crude oil for GC-MS analysis to examine the degradation of petroleum hydrocarbons. The isolates exhibiting significant n-alkane-degrading activity and the isolates exhibiting both n-alkane-degrading and oil-emulsifying activities, a total of 40 isolates, were chosen for further analysis.

Taxonomy of the petroleum-aliphatic-hydrocarbon-degrading isolates

The 40 isolates were subjected to a rep-PCR analysis for strain typing, and the almost full-length 16S rRNA gene sequence of each of the strains exhibiting distinct rep-PCR patterns was determined (Table 1). In addition to strains of *Alcanivorax* and *Marinobacter*, which are ubiquitous petroleum-hydrocarbon-degrading bacteria in marine environments (Golyshin *et al.*, 2005; González & Whitman, 2006), bacteria related to *Oceanobacter kriegii* (96.4–96.5 % similarity) were obtained. Since petroleum-hydrocarbon-degrading activity in the genus *Oceanobacter* has not to our knowledge been reported previously, the activity in *O. kriegii* NBRC 15467[T] was examined by incubating the strain for 2 weeks at 30 °C in SW medium supplemented with 0.1 % (v/v) crude oil. No degradation of petroleum hydrocarbons was detected by GC-MS analysis (data not shown).

A phylogenetic analysis showed that the *Alcanivorax* strains isolated from Indonesian seawater were not closely related to *Alcanivorax borkumensis* strain SK2 (Fig. 1). This result was in contrast to that obtained with Japanese seawater using the same enrichment method and temperature conditions: *Alcanivorax* strains from Japanese seawater are closely related to *A. borkumensis* SK2 (M. Teramoto and others, unpublished data). This observation may suggest that the SK2-type *Alcanivorax* strains prefer to inhabit temperate seawater, while tropical seawater is mainly populated by other types of *Alcanivorax*. Although the genus *Alcanivorax* is found universally (Golyshin *et al.*, 2005), the geographical distribution of the type of *Alcanivorax* seems to be biased.

Petroleum-aliphatic-hydrocarbon-degrading abilities of the isolates

Alkane-degrading activities of the Indonesian isolates were compared at 30 °C together with *A. borkumensis* strain SK2 (Fig. 2). Crude-oil-emulsifying activity was detected with all the *Alcanivorax* strains examined but not with the other alkane-degrading strains examined. The *Oceanobacter*-related isolates showed high n-alkane-degrading activities

Table 1. Taxonomic affiliation of alkane-degrading bacteria obtained in this study, based on almost full-length 16S rRNA gene sequences

Isolate* (accession no.)	Closest GenBank relative† (accession no.)	Similarity (%)
2A17 (AB435641)	*Alcanivorax* sp. K3-3 (AB055205)	98.8
2A75 (AB435642)	*Alcanivorax* sp. Mho1 (AB053124)	100.0
2A44 (AB435643)	*Alcanivorax* sp. Haw1 (AB053127)	99.9
2*l*A10 (AB435644)	*Alcanivorax* sp. CBF L53 (AB166953)	100.0
2M3 (AB435645)	*Marinobacter hydrocarbonoclasticus* (Y16735)	100.0
2M46‡, 2M48‡ (AB435646)	*Alteromonadaceae* bacterium LA50 (AF513454)	99.9
2M49‡ (AB435647)	*Alteromonadaceae* bacterium LA50 (AF513454)	99.7
2M26‡ (AB435648)	*Alteromonadaceae* bacterium LA50 (AF513454)	99.8
2O1§ (AB435649)	*Oceanospirillum* sp. ME101 (AJ302699)	98.2
1O14§ (AB435650)	*Oceanospirillum* sp. ME101 (AJ302699)	98.2
1O18§ (AB435651)	*Oceanospirillum* sp. ME101 (AJ302699)	98.4

*Another isolate is also given when the isolates have the same sequence of the 16S rRNA gene fragment but show different rep-PCR patterns. The first number (1 or 2) corresponds to the culture number. The *l* after the first number shows that the isolate was obtained from the liquid phase, while the others were from the oil surface.
†All closest GenBank relatives belonged to the *γ-Proteobacteria*.
‡The validly described bacterial species with the highest 16S rRNA gene sequence similarity was *Marinobacter sediminum* (AJ609270; 97.1–97.3 % similarity).
§The second-closest GenBank relative was *Oceanobacter kriegii* NBRC 15467^{T} (AB006767; 96.4–96.5 %).
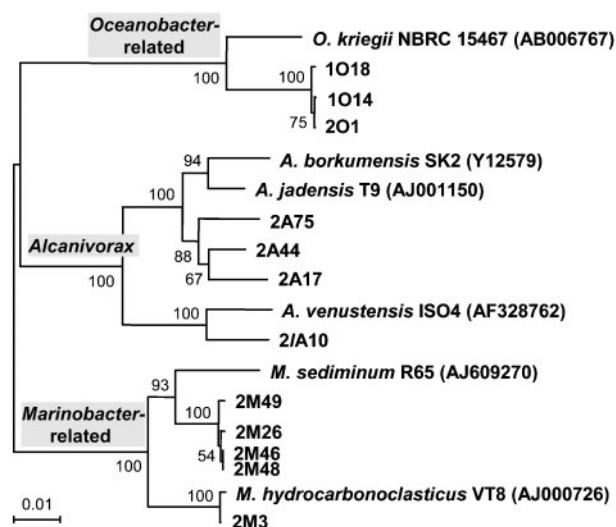
that were comparable to *A. borkumensis* SK2. *Alcanivorax* strains isolated in this study, except strain 2*l*A10, also exhibited n-alkane-degrading activities as high as *A. borkumensis* SK2. *Oceanobacter*-related strains, as well as *Alcanivorax* strains, could thus be important n-alkane degraders in the tropical sea. *Marinobacter* sp. 2M3 showed the lowest n-alkane-degrading activity, but *Marinobacter*-

Fig. 1. Phylogenetic relationships based on almost full-length 16S rRNA gene sequences of the alkane-degrading strains obtained in this study, their phylogenetically closest type strains and the well-known alkane-degrading strain *Alcanivorax borkumensis* SK2. Bootstrap values are indicated at the nodes. The scale bar indicates 0.01 substitutions per site.

related (*Alteromonadaceae*) isolates (2M26, 2M46, 2M48 and 2M49) showed higher n-alkane-degrading activities than *Alcanivorax* sp. 2*l*A10.

Concerning the activity for degrading branched alkanes, all the *Alcanivorax* strains showed significant activity (Fig. 2). In particular, *A. borkumensis* strain SK2 showed exceptionally high activity for the degradation of branched alkanes. This activity was not significant in *Oceanobacter*-related or *Marinobacter*-related strains. Therefore, *Alcanivorax* strains could be key bacteria for branched-alkane degradation in the tropical sea as well as in the temperate sea (Hara *et al.*, 2003; McKew *et al.*, 2007). Alkane-degrading activities in these strains were also determined at 25 °C (data not shown). Their activities at 25 °C were either similar to or lower than those at 30 °C shown in Fig. 2, and the comparative observations of their activities at 25 °C were similar to those at 30 °C described above.

Further characterization of the novel alkane degraders, *Oceanobacter*-related strains

In general, the first step in the biodegradation of n-alkanes is catalysed by one of two major alkane-monooxygenase families: AlkB-related integral-membrane diiron proteins and the CYP153 subfamily of cytochrome P450s. Thus, the distribution of *alkB* and *cyp153* in the three *Oceanobacter*-related strains 2O1, 1O14 and 1O18 was examined. Although no PCR product was amplified with CYP153-specific primers, 548 bp DNA fragments homologous to *alkB* were PCR-amplified from the three *Oceanobacter*-related strains. Their deduced amino acid sequences showed 79 % identity to that of *alkB2* from *Pseudomonas aeruginosa* strain DSM1128 (Q6H936).
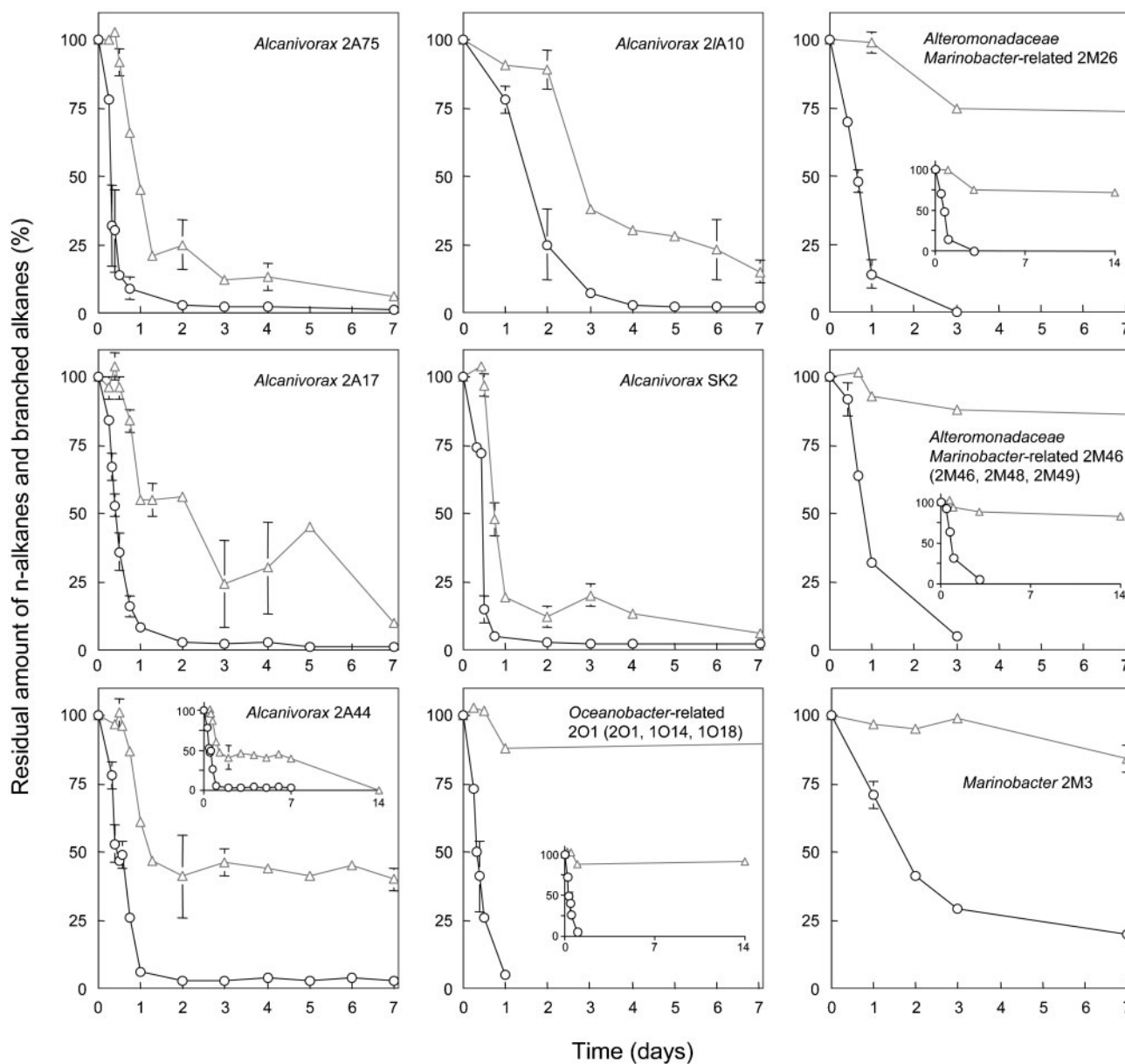
Fig. 2. Degradation of n-alkanes and branched alkanes by the alkane-degrading strains at 30 °C. Colonies from each strain grown on dMB plates supplemented with pyruvate were suspended in filter-sterile seawater until the OD_{600} was 0.5. Then 150 μl of this bacterial suspension was inoculated into 3 ml SW medium containing 3 μl crude oil, and the culture was incubated for the indicated period at 30 °C. Non-inoculated sterile samples were similarly incubated and served as controls (100 %). Oil was extracted from the cultures as described in Methods. The n-alkanes (C_{12-22}; ○) and branched alkanes (pristane and phytane; △) were quantified. The names of strains that showed similar biodegradation profiles are indicated in parentheses, and the profile of the representative strain is shown. The degradation by *Alcanivorax* strain SK2 is also shown as a reference. The inset figures show the same data for 14 days incubation. Each value is the mean ± SE from two independent experiments.

Oceanobacter-related strains 2O1, 1O14 and 1O18 were able to grow on acetate, pyruvate and propionate but were unable to grow on D-glucose, D-fructose, formate, L-arabinose, ribose, glycolate, citrate, DL-lactate, aspartate, L-asparagine or L-proline as a single source of carbon and energy. The *Oceanobacter*-related strains were shown to

oxidize a restricted range of carbon sources by the Biolog GN2 microplate test. Of the 95 different carbon sources, only Tween 40 was oxidized by all three strains. β-Hydroxybutyric acid, γ-hydroxybutyric acid and L-glutamic acid were also oxidized by strains 2O1 and 1O14; Tween 80 was oxidized only by strain 2O1. These results

indicate that *Oceanobacter*-related strains 2O1, 1O14 and 1O18 are professional hydrocarbonoclastic bacteria.

Dominance of *Oceanobacter*-related bacteria on crude oil in beach-simulating microcosms using Indonesian seawater

Crude-oil-contaminated sand was submerged in Indonesian seawater to simulate oil-polluted sand on a beach. Different slow-release fertilizers were added to three such microcosms, while another microcosm was not supplemented with fertilizer. Indonesian seawater in the four microcosms was daily exchanged with fresh seawater for 38 days. The fertilizers used were Osmocote (supplying N and P), Sumicoat (supplying N) and Super IB/Linstar (supplying N and P/supplying P).

The T-RFLP analyses of crude oil in these microcosms indicated that a 362 bp T-RF became dominant in all the four microcosms, although this T-RF was minor at day 0 (Fig. 3). The comparison between T-RFs from the microcosms (Fig. 3) and the 16S rRNA gene clone library (Table 2) showed that the dominant 362 bp T-RF band corresponded to *Oceanobacter*-related strains. These results suggest that *Oceanobacter*-related strains could dominate in the natural tropical marine environment after an accidental oil spill, and would continue to dominate in the environment after biostimulation. Together with the observation that *Oceanobacter*-related strains showed high n-alkane-degrading activity (Fig. 2), these findings strongly suggest that *Oceanobacter*-related bacteria could be major degraders of petroleum n-alkanes accidentally spilt in tropical seas. On the other hand, *Alcanivorax* (Cappello *et al.*, 2007; Hara *et al.*, 2003; Kasai *et al.*, 2001; Roling *et al.*, 2002, 2004; Yakimov *et al.*, 2005) and *Thalassolituus* (Coulon *et al.*, 2007; McKew *et al.*, 2007) strains have been reported to dominate in crude-oil-containing temperate seawater and temperate estuarine seawater, respectively. *Oceanobacter*-related bacteria may prefer oil surfaces, while *A. borkumensis* was dominantly present on heavy oil remaining on a Japanese beach after the *Nakhodka* tanker oil-spill accident (Kasai *et al.*, 2001). Thus, the type of bacteria important for the bioremediation of oil-polluted marine environments would be different depending on the climate. Rich microbial resources including hydrocarbon-degrading bacteria must be harboured in tropical marine environments (Harwati *et al.*, 2007). Accordingly, other aliphatic-hydrocarbon-consuming bacteria would be able to enter the game in tropical marine environments and this may have caused the difference in bacterial types which are important for the bioremediation of petroleum hydrocarbons in marine environments.

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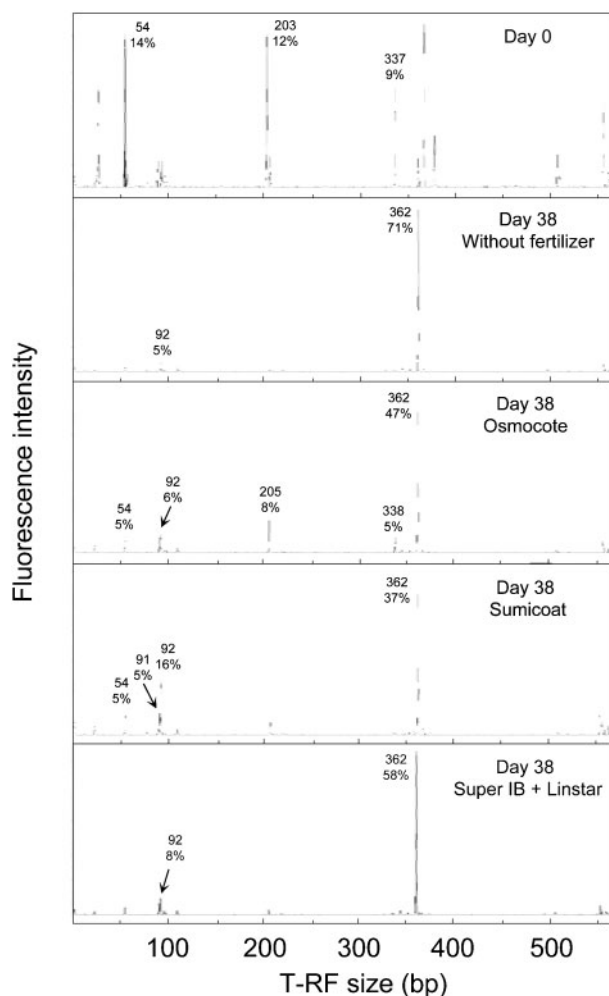
Fig. 3. T-RFLP profiles from microcosms simulating oil-polluted sand on a beach. Three microcosms were supplemented with one or two kinds of slow-release fertilizers (Osmocote, Sumicoat and Super IB/Linstar: see text for details), while one microcosm was not treated with fertilizer. Profiles of day 0 samples from each of the four microcosms were similar, and one representative profile is shown. Sizes of T-RFs with a fragment frequency of 5 % or more and identified in the 16S rRNA gene clone library (Table 2) are indicated. The residual amount of n-alkanes (C_{11-30}) of the crude oil on day 38 was about 75 % in the microcosm without fertilizer, 25 % with Osmocote, 55 % with Sumicoat and 55 % with Super IB/Linstar, with day 0 samples as the controls (100 %).

Table 2. Distribution of the bacterial phylotypes in the 16S rRNA gene clone library from the Osmocote-supplemented microcosm on day 29, and their corresponding T-RFs

No. of clones*	Closest GenBank relative		Similarity (%)†	Size of T-RF (bp)
	Phylogenetic group	Strain, species, or clone (accession no.)		
31 (38 %)‡	γ-Proteobacteria	*Oceanospirillum* sp. ME101 (AJ302699)	96.0	362
6 (7 %)§	α-Proteobacteria	Marine strain B50 (AB302372)	100.0	54
4 (5 %)	α-Proteobacteria	*Donghicola eburneus* (DQ667965)	100.0	338
3 (4 %)	α-Proteobacteria	*Rhodobacteraceae* strain UST061013-083 (EF588012)	97.6	337
2 (2 %)	γ-Proteobacteria	*Marinobacter vinifirmus* (DQ768655)	100.0	205
1 (1 %)	γ-Proteobacteria	*Alteromonas* sp. 46Xb1 (EU440052)	100.0	203
1 (1 %)	δ-Proteobacteria	*Desulfospira joergensenii* (X99637)	95.4	92
1 (1 %)	δ-Proteobacteria	*Desulfobacterium zeppelinii* (EF442983)	94.7	92
1 (1 %)	δ-Proteobacteria	Marine sulfate-reducing strain WN (U51844)	97.0	92
1 (1 %)	δ-Proteobacteria	Marine sulfate-reducing strain oXyS1 (Y17286)	94.8	92
1 (1 %)	δ-Proteobacteria	Sulfate-reducing clone 126I-1 (AF328857)	97.8	92
1 (1 %)	*Bacteroidetes*	*Lewinella nigricans* (AM295255)	86.8	92
1 (1 %)	δ-Proteobacteria	*Desulfospira joergensenii* (X99637)	96.0	91
1 (1 %)	δ-Proteobacteria	*Desulfobacterium cetonicum* (AJ237603)	93.9	91
1 (1 %)	δ-Proteobacteria	*Desulfocapsa* sp. La4.1 (AF228119)	95.6	91
1 (1 %)	α-Proteobacteria	*Roseobacter* sp. NT N37 (AB166982)	97.2	54
1 (1 %)	α-Proteobacteria	*Shimia marina* (AY962292)	99.2	54

*Eighty-two clones were analysed in total. Information on the clones with a T-RF with fragment frequency of 5 % or more detected by T-RFLP analysis is provided. Each of the remaining 24 clones, which are not on the list, constituted less than 4 % of the library.

†Identity values are based on 602–684 sequenced base pairs.

‡The second-closest GenBank relative was *Oceanobacter kriegii* NBRC 15467[T] (AB006767; 95.3 %).

§The validly described bacterial species with the highest 16S rRNA gene sequence similarity was *Thalassobius mediterraneus* (AJ878874; 98.3 % similarity).

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