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Bacteria Belonging to the Genus *Cycloclasticus* Play a Primary Role in the Degradation of Aromatic Hydrocarbons Released in a Marine Environment

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To identify the bacteria that play a major role in the aerobic degradation of petroleum polynuclear aromatic hydrocarbons (PAHs) in a marine environment, bacteria were enriched from seawater by using 2-methylnaphthalene, phenanthrene, or anthracene as a carbon and energy source. We found that members of the genus *Cycloclasticus* became predominant in the enrichment cultures. The *Cycloclasticus* strains isolated in this study could grow on crude oil and degraded PAH components of crude oil, including unsubstituted and substituted naphthalenes, dibenzothiophenes, phenanthrenes, and fluorenes. To deduce the role of *Cycloclasticus* strains in a coastal zone oil spill, propagation of this bacterial group on oil-coated grains of gravel immersed in seawater was investigated in beach-simulating tanks that were 1 m wide by 1.5 m long by 1 m high. The tanks were two-thirds filled with gravel, and seawater was continuously introduced into the tanks; the water level was varied between 30 cm above and 30 cm below the surface of the gravel layer to simulate a 12-h tidal cycle. The number of *Cycloclasticus* cells associated with the grains was on the order of 10^3 cells/g of grains before crude oil was added to the tanks and increased to 3×10^6 cells/g of grains after crude oil was added. The number increased further after 14 days to 10^8 cells/g of grains when nitrogen and phosphorus fertilizers were added, while the number remained 3×10^6 cells/g of grains when no fertilizers were added. PAH degradation proceeded parallel with the growth of *Cycloclasticus* cells on the surfaces of the oil-polluted grains of gravel. These observations suggest that bacteria belonging to the genus *Cycloclasticus* play an important role in the degradation of petroleum PAHs in a marine environment.

An oil spill is one of the most serious disasters that can occur in a marine environment (22, 23). In the early stage of an oil spill, the light fraction of the oil evaporates, while the heavier fraction is slowly removed by photooxidation and biodegradation (11). Microorganisms, especially bacteria, play an important role in the biodegradation of the spilled oil (28). However, the growth of oil-degrading bacteria in seawater and the resulting biodegradation of oil in seawater are limited by nutritional requirements (2, 3). Addition of nitrogen and phosphorus fertilizers has been shown to enhance the biodegradation of oil released in a marine environment (21, 38, 43).

Oil is a complex mixture made up of hundreds of compounds, and these compounds are classified into four groups, namely, saturates, aromatics, resins, and asphaltenes (12). Aromatics are the second most abundant hydrocarbons in crude oil. Benzene, naphthalene, and phenanthrene and their alkyl-substituted derivatives represent typical aromatics (47). Although the biodegradation of such simple aromatics as benzene, toluenes, xylenes, naphthalene, and phenanthrene has been extensively characterized (19), the biodegradation of alkyl-substituted polynuclear aromatic hydrocarbons (PAHs) has scarcely been studied (10, 30, 34).

One group of bacteria capable of degrading aromatics in a marine environment is the members of the genus *Cycloclasticus*. These bacteria have been isolated from several locations, including Resurrection Bay (Alaska), Puget Sound (Washington), and the Gulf of Mexico (7, 13, 17). They utilize aromatic hydrocarbons, including biphenyl, naphthalene, phenanthrene, toluene, and xylenes, as sole sources of carbon and energy, while they cannot utilize sugars and amino acids.

In the present study, we investigated the roles of bacteria belonging to the genus *Cycloclasticus* in the biodegradation of petroleum PAHs in a marine environment. We found that members of this genus were the most abundant PAH-degrading bacteria associated with oil-polluted grains of gravel and that the bacterial density reached 10^8 cells/g of grains after preparations were supplemented with nitrogen and phosphorus.

MATERIALS AND METHODS

Enrichment and isolation of the PAH-degrading microorganisms. Seawater was collected from a depth of 15 m in Kamaishi Bay. Each 100 ml of seawater was placed into a 500-ml baffled flask and supplemented with 100 mg of NH_4NO_3, 20 mg of K_2HPO_4, 2 mg of ferric citrate, and 100 mg of a PAH (2-methylnaphthalene, phenanthrene, or anthracene). Three cultures were prepared for each PAH substrate, and they were incubated at 20°C on a rotary shaker operating at 90 rpm for several weeks until a yellowish orange or reddish brown color developed. Such a color change was an indication of ring cleavage of the aromatic compounds (18, 33).

Bacteria were isolated from the enrichment cultures containing phenanthrene by using the most-probable-number technique (9, 16). Serial 10-fold dilutions of the phenanthrene enrichment cultures were made with ONR7a artificial seawater medium (13) supplemented with 1 mg of phenanthrene per ml as the sole source of carbon and energy, and the mixtures were incubated at 20°C in the dark for about 4 weeks. The most diluted cultures that exhibited a color change were spread onto ONR7a plates overlaid with an opaque layer of 0.8% agarose containing 1 mg of phenanthrene per ml (5). The surfaces of the plates were opaque, and the colonies surrounded by a clear zone were purified further by restreaking on the same type of plates.

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The purified colonies were subjected to repetitive extragenic palindromic sequence PCR (rep-PCR) analysis to identify identical strains, as described previously (8). The rep-PCR analysis was repeated several times to determine the reproducibility of the method.

Biodegradation of crude oil in beach-simulating tanks. The beach-simulating tank system used consisted of a tank (1 by 1.5 m; depth, 1 m), a reservoir, and a level-controlling device (31). Seawater was collected at a depth of 15 m from Kamaishi Bay and was filtered through layers of coarse and fine sand. It was introduced into the reservoir at a constant flow rate of 60 liters/h. In the reservoir, the seawater was aerated by bubbling, and the temperature was kept at approximately 20°C. The seawater was subsequently introduced at a flow rate of 60 liters/h into the tank, which was two-thirds filled with 1 m^3 of gravel grains which were 2 to 8 mm in diameter, were collected from a river in Kamaishi, and were washed several times with seawater to remove dirt and debris. The room temperature was kept at 20 ± 2°C, while the temperature of the seawater in the tank varied between 19.1 and 23.5°C. The tidal level in the tank was regulated so that it oscillated, with two cycles per day, between the low-tide mark, which was 20 cm above the bottom of the tank, and the high-tide mark, which was 80 cm above the bottom of the tank (between 30 cm above and 30 cm below the top of the gravel layer). Excess seawater was drained from the bottom of the tank. One thousand grams of heat-treated Arabian Light crude oil was poured on the surface of the seawater at the high-tide level. This oil spread on the surface, forming a slick, and stuck to the gravel when the tide level fell. Several hours after the crude oil was added, 300 g of Super IB (containing 64 g of N; Mitsubishi Chemicals) and 60 g of Linstar 30 (containing 13.2 g of P_2O_5; Mitubishi Chemicals) were uniformly distributed in one tank (tank 2), while no fertilizer was added to the other tank (tank 1) as a control. Three independent experiments were conducted with a pair of the beach-simulating tanks. The duration of each experiment was 63 days.

The total bacterial counts in seawater samples from the beach-simulating tanks were determined by fluorescence microscopy after the samples were stained with 4,6-diamidino-2-phenylindole (DAPI) (48). To analyze changes in the microbial population and degradation of crude oil components, approximately 2 liters of seawater and approximately 40 g of oil-coated grains were collected every week at the high-tide level and at the low-tide level, respectively.

Extraction of DNA. The microorganisms in approximately 10 ml of a liquid culture or in a 2-liter sample of seawater from a beach-simulating tank were collected on a GV membrane (Millipore) by filtration, and DNA was extracted by the Marmur procedure (32). DNA of the microorganisms attached to grains of gravel was extracted by putting approximately 20 g of a sample into 13.5 ml of DNA extraction buffer (100 mM Tris-HCl [pH 8.0], 100 mM EDTA [pH 8.0], 100 mM sodium phosphate [pH 8.0], 1.5 M NaCl, 1% [wt/vol] hexadecyltrimethylammonium bromide), to which 0.1 ml of 1% (wt/vol) proteinase K was added. This suspension was incubated at 37°C for 30 min with horizontal shaking, after which 1.5 ml of a 20% (wt/vol) sodium dodecyl sulfate solution was added and the mixture was incubated for 1 h at 65°C while it was gently inverted end over end every 20 min. The suspension was then extracted three times with a phenol-chloroform (1:1) solution, and the aqueous solution was recovered. Then 0.6 volume of isopropyl alcohol was added to the aqueous solution, and after gentle mixing the solution was left at room temperature for 1 h. Nucleic acids were precipitated by centrifugation at 20,000 × *g* for 20 min, washed with 5 ml of a 70% (vol/vol) ethanol solution, and dissolved in 0.1 ml of Tris-EDTA buffer containing 100 mg of RNase A. This solution was incubated at 37°C for 1 h, and the DNA was recovered by ethanol precipitation (41). The purity and quantity of the DNA were determined by recording its UV absorption spectrum (41).

DGGE of the PCR products. About 50 ng of DNA extracted from enrichment cultures, seawater, or grains was used as the template in a PCR. PCR primers P2 and P3 (35) were used to amplify the V3 region of bacterial 16S ribosomal DNA (rDNA) (corresponding to positions 341 to 534 in the *Escherichia coli* rRNA sequence) connected to the GC clamp. PCR was performed as described previously (48), and amplification of the PCR products to obtain the expected sizes was confirmed by electrophoresis through a 1.5% (wt/vol) agarose gel (LO3; Takara) with TBE buffer (89 mM Tris-borate [pH 8.3], 2 mM Na_2EDTA [41]). Denaturing gradient gel electrophoresis (DGGE) (36) was performed with a DCode instrument (Bio-Rad) as described previously (25). For each sample, three independent PCR-DGGE analyses were performed to verify the reproducibility of the technique. To determine a DNA sequence, a gel slice containing a DNA band was excised and processed as described previously (48). A search of the GenBank nucleotide library for sequences similar to the sequences obtained was performed by using BLAST (1) through the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST/).

Sequencing of 16S rDNA and *gyrB* and phylogenetic analysis. The 16S rDNA of isolates were amplified by PCR with primers 27f and 1492r (corresponding to positions 8 to 1510 in the *E. coli* rRNA sequence) (49). The PCR conditions used were 35 cycles consisting of 1 min at 94°C, 1 min at 58°C, and 2 min at 72°C. After the last cycle, the preparation was incubated at 72°C for an additional 10 min. The PCR products were electrophoresed through a 0.8% (wt/vol) agarose gel with TBE buffer (41) and then were purified with a QIAquick gel extraction kit (QIAGEN). The nucleotide sequences of the PCR products were then determined by using a *Taq* DyeDeoxy terminator cycle sequencing kit and a model 377 sequencer (Applied Biosystems). The nucleotide sequences obtained were aligned by using CLUSTAL W, version 1.7 (46), with several reference sequences of members of the γ subdivision of the *Proteobacteria*. A phylogenetic tree was constructed from the evolutionary distance data (27) by the neighbor-joining method (40). The bootstrap resampling method of Felsenstein (14) was used with 1,000 replicates to evaluate the robustness of the branches of the inferred tree.

Fragments of the gene for the DNA gyrase β subunit (*gyrB*) of isolates were PCR amplified with two degenerate primers, UP-1E and UP-2r, as described previously (52). The *gyrB* PCR product was purified and sequenced by using the protocol described above for the 16S rDNA genes. The *gyrB* sequences were aligned by using CLUSTAL W, version 1.7 (46), with reference sequences of members of the γ subdivision of the *Proteobacteria* compiled in the Identification and Classification of Bacteria database (24). A phylogenetic tree based on *gyrB* was constructed as described above for the 16S rDNA-based tree.

Ability to degrade crude oil. Arabian Light crude oil was heated at 230°C for 4 h to remove the light fraction (11), and the ability of *Cycloclasticus* strains A5 and H4 isolated in this study to degrade hydrocarbons in the crude oil was examined in 10 ml of ONR7a medium supplemented with 1 mg of heat-treated Arabian Light crude oil per ml. Colonies of strains A5 and H4 were used to inoculate ONR7a medium supplemented with 1 mg of phenanthrene per ml and were grown at 25°C with shaking to the late exponential growth phase. The cultures were not shaken for several minutes in order to let the phenanthrene crystals settle, and samples of the supernatants were removed and used to determine cell densities after DAPI staining. Approximately 10^3 cells of the *Cycloclasticus* strains were used to inoculate triplicate 10-ml portions of media in 50-ml tubes fitted with Teflon-lined caps, and the tubes were incubated at 25°C on a reciprocating shaker at 90 rpm for 4 weeks. Sterile samples were incubated similarly and served as controls. Cell growth and degradation of the crude oil components after 4 weeks were examined as described below.

Analysis of the oil. Oil was extracted from cultures or from grains of gravel as described previously (11) and was analyzed by gas chromatography-mass spectrometry (GC-MS) (GC/MS-QP5000; Shimadzu) in order to quantify a series of *n*-alkanes (C_{13-33}), C_{0-4}-alkylnaphthalenes (C_0 indicates a nonsubstituted aromatic compound, while the number in C_n indicates the number of carbons in the side chains), C_{0-3}-alkyldibenzothiophenes, C_{0-7}-alkylphenenthrenes, C_{0-2}-alkylfluorenes, and the biomarker 17α(H),21β(H)-hopane. GC-MS was performed by the method of Wang et al. (47). All values obtained with the instrument were normalized by dividing by the value obtained for 17α(H),21β(H)-hopane (39).

q-cPCR. The primers used for quantitative competitive PCR (q-cPCR) were designed by comparing the *gyrB* sequences of the *Cycloclasticus* strains with those of the strains most closely related to *Cycloclasticus*, namely, *Marinospirillum minutulum* ATCC 19193 and *Marinospirillum megaterium* JCM 10129. PCR primers pCG-f (5′-CGGATGAGCGCACAGCAA-3′) and pCG-r (5′-CCGATGTTGTCACCTTCTG-3′) were used to specifically detect the genus *Cycloclasticus*. Competitor fragments were produced by using a competitive DNA construction kit (Takara Shuzo), and the sizes of the target fragments and the competitors were 318 and 286 bp, respectively. Amplification was performed with a Progene thermal cycler (Techne) by using a 50-μl mixture containing 1.25 U of *Taq* DNA polymerase, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (wt/vol) gelatin, each deoxynucleoside triphosphate at a concentration of 200 μM, 50 pmol of each primer, 50 ng of DNA, and an appropriate amount of a competitor. The PCR conditions used were as follows: 10 min of polymerase activation at 94°C, followed by 35 cycles consisting of 30 s at 94°C, 1 min at 60°C, and 30 s at 72°C and a final 10-min extension at 72°C. Ten microliters of PCR product was electrophoresed through a 3.0% (wt/vol) agarose gel with TBE buffer. The gel was photographed after it had been stained with SYBR Gold. The copy number of a target sequence in the PCR mixture was determined by comparing the band intensity of the target fragment with that of the competitor. The number of bacterial cells was considered to be equal to the copy number of the *gyrB* sequence (4). The q-cPCR analysis was repeated three times to verify the reproducibility of the method.

Nucleotide sequence accession numbers. The nucleotide sequences reported in this paper have been deposited in the DDBJ, EMBL, and GenBank libraries under accession no. AB080091 to AB080110.

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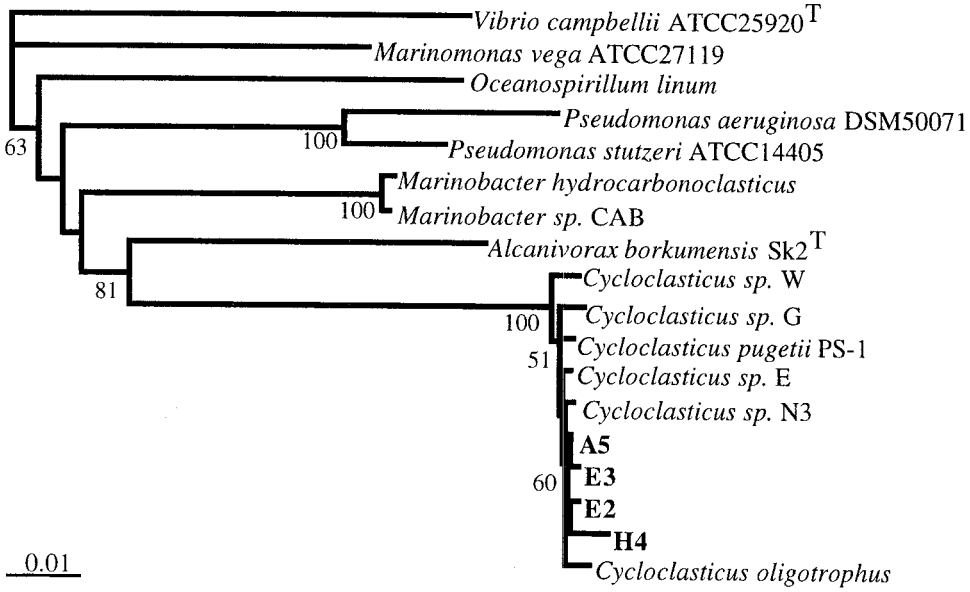

FIG. 1. Phylogenetic relationships based on 16S rDNA sequences of strains A5, E2, E3, and H4 and representative members of the γ subdivision of the *Proteobacteria*. Bootstrap values greater than 50% are indicated at the nodes. Scale bar = 0.01 substitution per site.

RESULTS

Isolation of *Cycloclasticus* strains. Enrichment cultures of PAH-degrading bacteria were obtained as described in Materials and Methods. DNA was extracted from the original enrichment cultures, and the bacterial populations in the cultures were analyzed by DGGE. The most intense bands of all the samples migrated similarly on the DGGE gels, and sequencing of these bands revealed that their sequences were closely related to the 16S rDNA sequence of *Cycloclasticus pugetii* strain PS-1 (data not shown). Thus, bacteria related to the genus *Cycloclasticus* were the bacteria most frequently found in the original enrichment cultures regardless of the growth substrate used (2-methylnaphthalene, phenanthrene, or anthracene). Bacteria grown on phenanthrene were further screened by using the most-probable-number technique and phenanthrene as the sole source of carbon and energy, and they were purified

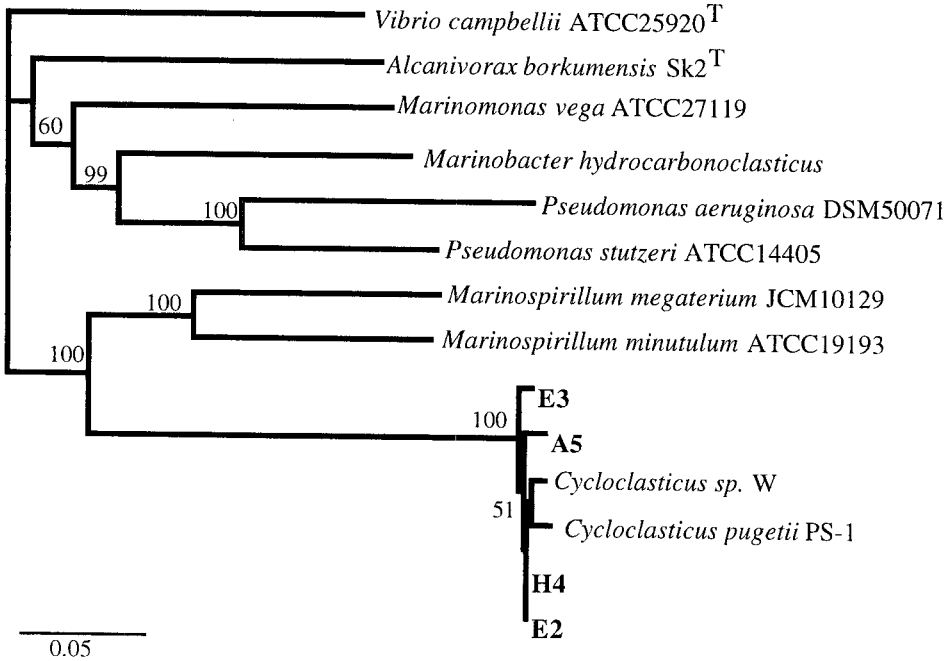

FIG. 2. Phylogenetic relationships based on *gyrB* sequences of strains A5, E2, E3, and H4 and representative members of the γ subdivision of the *Proteobacteria*. Bootstrap values greater than 50% are indicated at the nodes. Scale bar = 0.05 substitution per site.

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TABLE 1. Biodegradation of crude oil components by *Cycloclasticus* strains

Oil component	Residual amt (SE) (%)[a]	
	Strain A5	Strain H4
n-Alkanes	121.6 (20.1)	109.4 (20.3)
C_0-naphthalene	0.5 (0.6)	0.4 (0.3)
C_1-naphthalene	2.4 (1.2)	2.9 (1.5)
C_2-naphthalene	0.9 (0.4)	1.2 (0.4)
C_3-naphthalene	1.2 (0.6)	1.1 (0.4)
C_4-naphthalene	13.1 (1.5)	9.6 (1.9)
C_0-dibenzothiophene	7.3 (2.2)	6.5 (1.1)
C_1-dibenzothiophene	5.0 (0.8)	5.7 (0.7)
C_2-dibenzothiophene	35.8 (3.5)	23.2 (4.9)
C_3-dibenzothiophene	100.7 (7.4)	93.5 (5.7)
C_4-dibenzothiophene	116.7 (8.4)	115.1 (7.3)
C_0-phenanthrene	24.7 (9.7)	42.7 (12.6)
C_1-phenanthrene	4.3 (1.3)	5.9 (1.4)
C_2-phenanthrene	29.7 (2.6)	17.7 (4.1)
C_3-phenanthrene	105.1 (5.7)	93.6 (4.7)
C_4-phenanthrene	98.1 (6.9)	100.1 (5.0)
C_5-phenanthrene	93.4 (4.5)	96.0 (4.6)
C_6-phenanthrene	100.8 (5.0)	98.5 (4.0)
C_7-phenanthrene	104.0 (3.8)	112.7 (3.0)
C_0-fluorene	10.8 (3.7)	5.8 (2.9)
C_1-fluorene	6.4 (2.8)	7.7 (2.6)
C_2-fluorene	16.0 (5.1)	15.1 (4.8)

[a] *Cycloclasticus* cultures were incubated aerobically at 25°C for 4 weeks. Sterile samples were incubated similarly and served as controls. Oil was extracted from the cultures as described in Materials and Methods.

on plates containing phenanthrene. DNA of the purified clones was isolated, and the rDNA fragments were PCR amplified and used for DGGE analysis. The rDNA fragments from 35 isolates migrated to the same position as the *Cycloclasticus* rDNA, suggesting that all 35 clones belonged to the genus *Cycloclasticus*. These isolates were divided into four distinct groups, designated A5 (21 isolates), E2 (5 isolates), E3 (1 isolate), and H4 (1 isolate), by rep-PCR (8). One strain was selected from each of these four groups, and the 16S rDNA and *gyrB* sequences of the four strains selected (A5, E2, E3, and H4) were determined and used for phylogenetic analyses.

Phylogenetic analyses. Approximately 1,430-bp 16S rDNA fragments and approximately 1,200-bp *gyrB* fragments of strains A5, E2, E3, and H4 were sequenced and aligned before the neighbor-joining trees were constructed (Fig. 1 and 2). All four isolates formed a cluster with previously described *Cycloclasticus* strains.

Degradation of hydrocarbons in crude oil. Cell growth and degradation of the crude oil components after 4 weeks were examined with triplicate samples by using q-cPCR and GC-MS. The numbers of strain A5 and H4 cells increased to approximately 5×10^5 cells per ml. The concentrations of crude oil components were normalized by using the concentrations of 17α(H),21β(H)-hopane, and the normalized values obtained in three independent experiments were averaged. The average concentration of each component in the control crude oil samples was defined as 100%. Both *Cycloclasticus* strains degraded more than 80% of $C_{0\text{-}4}$-alkylnaphthalenes, $C_{0\text{-}1}$-alkyldibenzothiophenes, C_1-alkylphenanthrene, and $C_{0\text{-}2}$-alkylfluorenes. They also degraded almost 60 to 70% of C_2-alkyldibenzothiophenes and C_0- and C_2-alkylphenanthrenes. The levels of degradation of *n*-alkanes, $C_{3\text{-}4}$-alkyldibenzothiophenes, and $C_{3\text{-}7}$-alkylphenanthrenes were not significant (less than 10%) (Table 1).

Biodegradation of crude oil and growth of *Cycloclasticus* in the beach-simulating tanks. Three independent experiments were conducted to examine the biodegradation of crude oil in the beach-simulating tanks that mimicked an oil-polluted beach. Natural seawater was continuously introduced into two

TABLE 2. Degradation of aromatic hydrocarbons in crude oil attached to grains of gravel placed in the beach-simulating tanks

Oil component	Residual amt (SE) (%)[a]					
	Control tank			Fertilizer-containing tank		
	Day 14	Day 28	Day 56	Day 14	Day 28	Day 56
n-Alkane	92.8 (2.0)	74.9 (14.3)	26.7 (9.7)	61.9 (12.4)	22.3 (12.0)	2.1 (0.0)
C_1-naphthalene	25.9 (21.0)	12.4 (10.7)	0.0 (0.0)	14.4 (17.7)	0.0 (0.0)	0.0 (0.0)
C_2-naphthalene	38.8 (23.9)	28.7 (19.3)	7.6 (6.2)	23.4 (20.8)	0.7 (0.8)	0.0 (0.0)
C_3-naphthalene	56.5 (27.6)	46.3 (26.2)	24.9 (15.9)	46.5 (26.3)	4.9 (4.7)	0.0 (0.0)
C_4-naphthalene	70.7 (18.5)	63.9 (30.8)	48.9 (23.0)	66.9 (22.5)	25.8 (11.4)	0.6 (0.4)
C_0-dibenzothiophene	50.1 (26.7)	37.3 (21.0)	13.7 (7.5)	32.3 (19.1)	3.6 (4.4)	0.1 (0.2)
C_1-dibenzothiophene	70.0 (22.1)	56.4 (27.4)	42.1 (20.5)	57.9 (19.9)	19.0 (9.6)	0.4 (0.3)
C_2-dibenzothiophene	93.4 (6.2)	81.1 (18.5)	71.8 (17.3)	85.1 (10.8)	50.4 (9.4)	9.5 (2.4)
C_3-dibenzothiophene	96.5 (4.4)	93.1 (8.3)	87.5 (8.9)	92.9 (0.6)	73.7 (4.5)	23.7 (5.1)
C_4-dibenzothiophene	98.4 (2.1)	95.3 (2.8)	84.9 (2.7)	96.3 (2.7)	87.1 (1.3)	39.3 (8.1)
C_0-phenanthrene	42.7 (23.8)	28.0 (17.9)	8.7 (10.7)	24.6 (16.5)	2.6 (3.2)	0.0 (0.0)
C_1-phenanthrene	65.4 (26.5)	51.0 (25.9)	35.6 (18.3)	53.9 (22.2)	16.8 (9.8)	0.0 (0.0)
C_2-phenanthrene	85.6 (6.1)	72.3 (18.0)	62.0 (16.8)	76.9 (8.0)	43.7 (8.0)	6.5 (0.7)
C_3-phenanthrene	91.5 (3.6)	89.2 (10.7)	78.0 (8.5)	113.1 (27.1)	76.8 (13.1)	19.1 (3.4)
C_4-phenanthrene	110.1 (0.8)	95.8 (1.5)	98.7 (6.6)	100.5 (8.8)	87.5 (4.3)	37.2 (12.9)
C_5-phenanthrene	106.9 (0.5)	96.3 (0.6)	97.0 (6.5)	98.3 (7.5)	87.8 (7.1)	41.9 (6.2)
C_6-phenanthrene	104.9 (2.9)	102.6 (2.3)	98.7 (3.2)	101.4 (5.6)	94.0 (2.3)	57.4 (7.9)
C_7-phenanthrene	107.7 (8.6)	108.4 (4.5)	103.4 (3.9)	111.2 (5.5)	104.7 (3.8)	79.8 (9.0)
C_0-fluorene	40.9 (27.0)	37.0 (22.8)	5.1 (6.2)	17.6 (21.6)	0.0 (0.0)	0.0 (0.0)
C_1-fluorene	69.1 (16.0)	49.6 (24.1)	33.8 (20.9)	59.4 (12.9)	6.4 (7.8)	0.0 (0.0)
C_2-fluorene	94.3 (16.2)	81.2 (29.6)	74.0 (32.1)	77.1 (11.1)	34.1 (8.7)	1.6 (2.0)

[a] The initial concentration of each component at zero time was defined as 100%.

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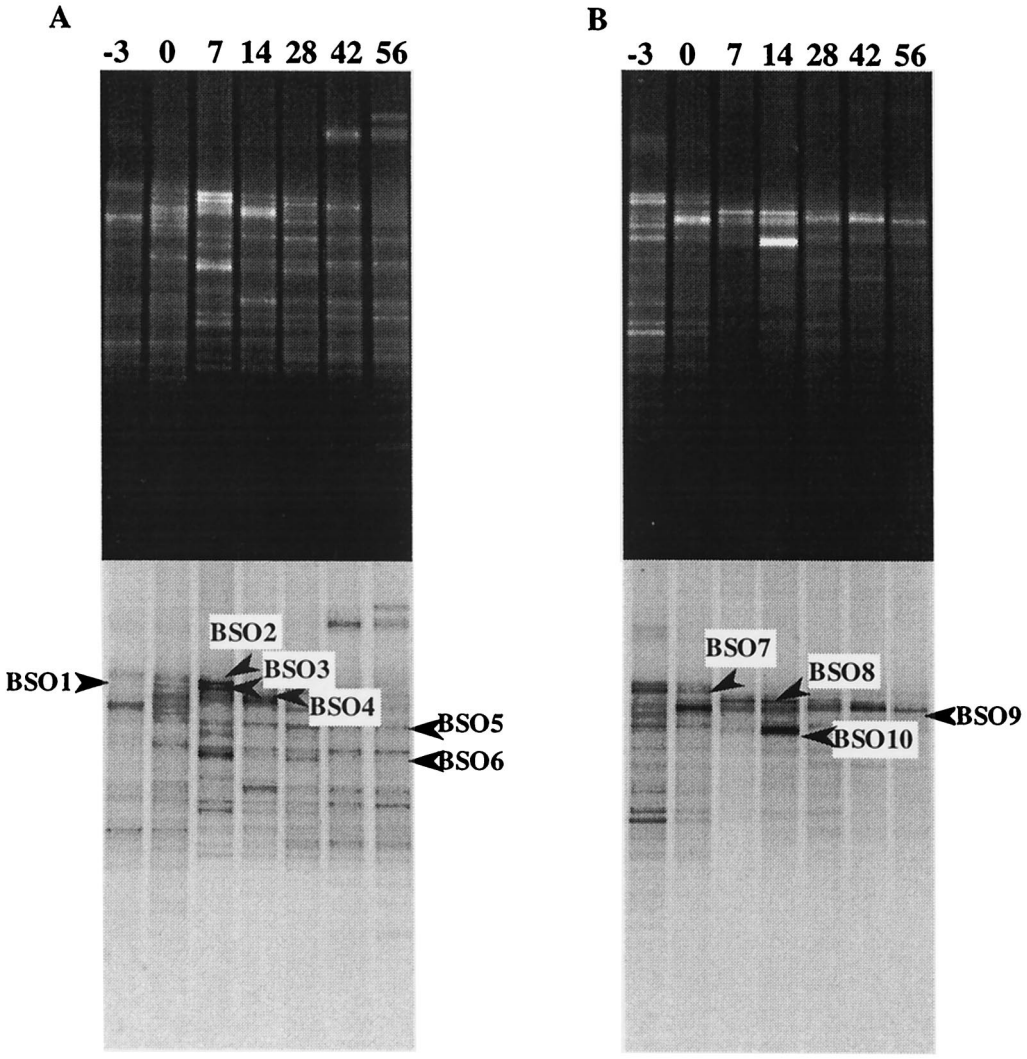

FIG. 3. DGGE profiles of partial 16S rDNA fragments, showing major bacterial populations attached to the surfaces of oil-polluted grains of gravel. The lower panels show bands that were excised and used for the DNA sequencing analysis. Fresh seawater was continuously introduced into two beach-simulating tanks, and at zero time crude oil was poured into the tanks. In one tank no fertilizer was added (A), while in the other tank slow-release nitrogen and phosphorus fertilizers were added at zero time (B). Samples of the oil-polluted grains of gravel were collected on days −3, 0, 7, 21, 28, 42, and 56, as indicated at the top.

tanks containing oil-polluted grains of gravel, and nitrogen and phosphorus fertilizers were added to tank 2 but not to tank 1. Samples of the grains of gravel were removed, the crude oil attached to the grains was extracted, and the degradation of aromatic hydrocarbons in the crude oil was analyzed by GC-MS. The concentrations of crude oil components were normalized by using the concentrations of 17α(H),21β(H)-hopane, and the normalized values in three independent experiments were averaged. The concentration of each component in the initial crude oil sample was defined as 100% (Table 2).

The patterns of degradation of crude oil by a bacterial community in the natural seawater were the same in the three experiments. When no fertilizer was added, $C_{4\text{-}7}$-alkylphenanthrenes were not degraded, and only 20 to 30% of the C_4-alkylnaphthalene, 20 to 30% of the $C_{2\text{-}4}$-alkyldibenzothiophenes, 20 to 30% of the C_3-alkylphenanthrene, and 20 to 30% of the C_2-alkylfluorene were degraded in 2 months. On the other hand, fertilizers stimulated the biodegradation of crude oil; in the presence of fertilizers, *n*-alkanes, $C_{0\text{-}4}$-alkylnaphthalenes, $C_{0\text{-}2}$-alkyldibenzothiophenes, $C_{0\text{-}2}$-alkylphenanthrenes, and $C_{0\text{-}2}$-alkylfluorenes were completely degraded, more than 60% of the $C_{3\text{-}4}$-alkyldibenzothiophenes and $C_{3\text{-}5}$-alkylphenanthrenes was degraded, and more than 40% of the C_6-alkylphenanthrene was degraded in 2 months (Table 2).

The total bacterial count in seawater at zero time was $1.6 \times 10^5 \pm 6.1 \times 10^4$ cells per ml. Addition of the fertilizers resulted in an increase in the total bacterial count in seawater to $1 \times 10^7 \pm 2.9 \times 10^6$ cells per ml in 21 days, while no such increase in the total bacterial count was apparent in the seawater from tank 1, in which the count on day 21 was $7.9 \times 10^4 \pm 4.0 \times 10^4$ cells per ml (data not shown).

Twenty-gram samples of oil-polluted grains of gravel were periodically removed, and DNA was extracted from the microorganisms attached to the grains and used for PCR-DGGE

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TABLE 3. 16S rDNA sequences most closely related to those of the major bacterial populations detected by PCR-DGGE

DGGE band (accession no.)	Most closely related sequence (accession no.)	% Sequence identity
BSO1 (AB080091)	*Flavobacterium xanthum* (AF030380)	97
BSO2 (AB080092)	*Alcanivorax borkumensis* (Y12579)	100
BSO3 (AB080093)	*Cycloclasticus pugetii* PS-1 (L34955)	100
BSO4 (AB080094)	*Marinobacter hydrocarbonoclasticus* (X67022)	100
BSO5 (AB080095)	*Sphingomonas subarctica* KF1 (X94102)	100
BSO6 (AB080096)	*Sphingomonas subarctica* KF1 (X94102)	99
BSO7 (AB080097)	*Flavobacterium xanthum* (AF030380)	97
BSO8 (AB080098)	*Alcanivorax borkumensis* (Y12579)	100
BSO9 (AB080099)	*Cycloclasticus pugetii* PS-1 (L34955)	100
BSO10 (AB080100)	*Aeromonas popoffii* LMG 17543 (AJ223181)	91
BSS1 (AB080101)	Uncultured marine eubacterium MBE4 (AF191755)	98
BSS2 (AB080102)	Uncultured *Roseobacter* strain NAC1-4 (AF245617)	100
BSS3 (AB080103)	Unidentified alpha-proteobacterium BD1-8 (AB015520)	94
BSS4 (AB080104)	*Bdellovibrio* sp. strain AQ (AF084855)	94
BSS5 (AB080105)	Uncultured bacterium NoosaAW93 (AF269026)	89
BSS6 (AB080106)	Uncultured *Roseobacter* strain NAC1-4 (AF245617)	100
BSS7 (AB080107)	*Cycloclasticus pugetii* PS-1 (U57920)	100
BSS8 (AB080108)	*Alcanivorax borkumensis* (Y12579)	100
BSS9 (AB080109)	*Oceanospirillum* sp. strain ME113 (AJ302700)	98
BSS10 (AB080110)	Uncultured Banisveld landfill bacterium BVB94a (AYO13660)	98

analysis in order to monitor the change in the bacterial population associated with grains of gravel (Fig. 3). Some major bands were excised, and their DNA sequences were determined. Table 3 shows the sequence in the databases that exhibited the highest level of nucleotide identity to each DGGE band. In tank 1 without any added fertilizer, *Alcanivorax* and *Cycloclasticus* were detected after 7 days, while *Marinobacter* was detected between days 0 and 15. In tank 2, *Alcanivorax* was detected on days 7 and 15, while *Cycloclasticus* was detected between days 0 and 42. A band distantly related to *Aeromonas popoffii* was visible on day 14. Similar results were obtained in independent experiments; the predominance of *Alcanivorax* and *Cycloclasticus* among the bacteria attached to the oil-polluted grains of gravel was always observed when the fertilizers were added. The band distantly related to *A. popoffii* was not detected in any other experiment.

The bacterial populations in seawater were also analyzed by PCR-DGGE, and phylogenetic affiliations of major bands were inferred, as shown in Table 3. The results of one of three experiments are shown in Fig. 4. The pattern of bacterial populations in tank 1 was relatively constant, and *Roseobacter* and *Bdellovibrio*, bacteria that are common in marine environments, were detected. Hydrocarbon degradation by bacteria belonging to these groups is not well known. In tank 2, *Alcanivorax* and *Cycloclasticus* were detected together with *Oceanospirillum*, which is commonly found in various marine environments. However, in two other experiments, *Cycloclasticus* was not detected in seawater samples from tank 2, to which the crude oil and fertilizers were added.

The size of the *Cycloclasticus* population attached to the grains was determined by q-cPCR (Fig. 5). Almost 10^3 *Cycloclasticus* cells were detected on 1 g of grains before crude oil was introduced into the tanks. After the crude oil was introduced, the number of *Cycloclasticus* cells increased to 3×10^6 to 4×10^6 cells/g in 1 day, even without addition of fertilizers. The size of the *Cycloclasticus* population continued to increase to 10^8 cells/g in 2 weeks when the fertilizers were added, while the number of cells stayed at $4.2 \times 10^6 \pm 6.4 \times 10^5$ cells/g when no fertilizer was added.

DISCUSSION

Bioremediation of spilled oil is a powerful tool for in situ cleaning of oil-polluted beaches. Application of fertilizers promotes the growth of oil-degrading bacteria and hence the rate of biodegradation of crude oil (6, 21, 43). Changes in microbial populations during bioremediation of crude oil have been analyzed by molecular techniques (26, 29), and *Alcanivorax* (51) has been identified as the bacterium that was mainly responsible for the degradation of alkanes in an oil-contaminated marine environment (26). On the other hand, the bacteria responsible for the degradation of aromatic compounds in an oil-contaminated marine environment have not been identified yet.

Some marine bacteria have previously been reported to be PAH degraders; these bacteria include members of the genera *Cycloclasticus* (13, 16, 17), *Flavobacterium* (37, 42), *Marinobacter* (15), *Moraxella* (45), *Pseudomonas* (42, 45), *Sphingomonas* (53), and *Vibrio* (50). However the activities of these organisms in the natural environment remain unknown. In this study we analyzed the major players in the bioremediation of petroleum PAHs by using beach-simulating tanks that are more similar to the natural environment.

PCR-DGGE analyses revealed that populations of *Alcanivorax* and *Cycloclasticus* were two major populations on the surfaces of oil-coated grains of gravel when nitrogen and phosphorus fertilizers were added (Fig. 3 and 4). We have recently reported that *Alcanivorax* is a major player in biodegradation of alkanes in a petroleum-contaminated marine environment (20, 26, 44) and does not effectively degrade aromatic compounds (26). Therefore, the other bacteria, members of the genus *Cycloclasticus*, were expected to be involved in the degradation of aromatic compounds.

Cycloclasticus strains degrade aromatic compounds, includ-

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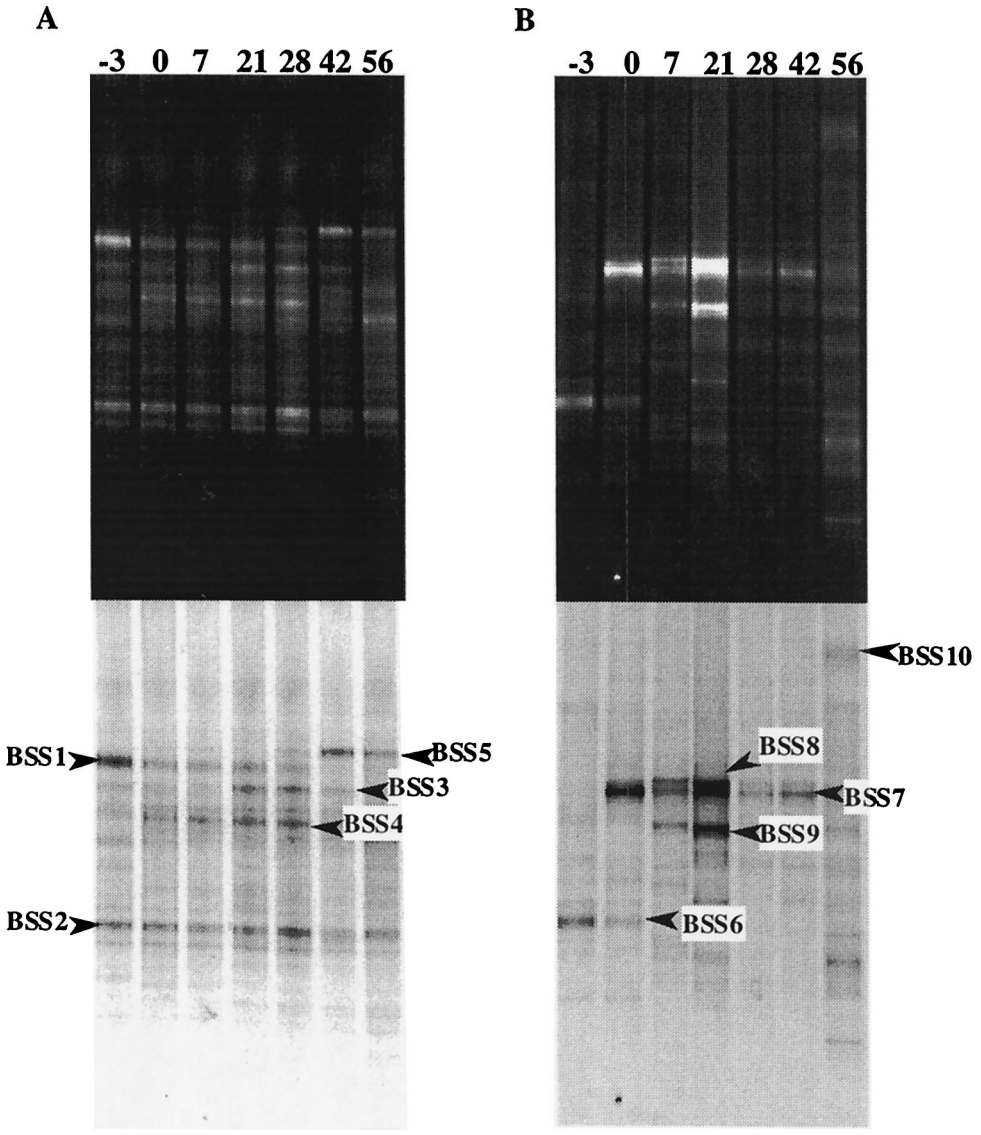

FIG. 4. DGGE profiles of partial 16S rDNA fragments, showing major bacterial populations in seawater. The experimental conditions were the same as those described in the legend to Fig. 3. The lower panels show bands that were excised and used for the DNA sequencing analysis. (A) Samples from the fertilizer-free tank; (B) samples from the tank to which fertilizer was added. The seawater samples were collected on days −3, 0, 7, 21, 28, 42, and 56, as indicated at the top.

ing naphthalene, alkylnaphthalene, biphenyl, phenanthrene, fluorene, and anthracene (13, 17). Our *Cycloclasticus* isolates also degraded these compounds. Furthermore, they degraded alkyl-substituted PAHs in crude oil (Table 1). The extent of degradation of $C_{1\text{-}2}$-alkyl aromatic hydrocarbons by *Cycloclasticus* was extremely high compared with the extents of degradation by other PAH degraders, such as *Marinobacter*, *Pseudomonas*, and *Sphingomonas* strains isolated from beach-simulating tanks (data not shown).

We determined the *gyrB* sequences of several *Cycloclasticus* strains, and based on the sequences, a q-cPCR method was developed to estimate the numbers of *Cycloclasticus* cells in environments. The number of *Cycloclasticus* cells associated with grains of gravel in the beach-simulating tank increased in 14 days to 10^8 cells/g when the nitrogen and phosphorus fertilizers were added (Fig. 5). This high density led us to expect that *Cycloclasticus* strains play a major role in the degradation of $C_{0\text{-}3}$-alkyl aromatic hydrocarbons in a marine environment, especially when nitrogen and phosphorus nutrients are added. This notion is supported by the observation that the rate of degradation of the $C_{0\text{-}3}$-alkyl aromatic hydrocarbons in crude oil was strongly accelerated by addition of the fertilizers between days 14 and 28, when the density of *Cycloclasticus* cells increased to 10^8 cells/g (Table 2).

The beach simulation experiments indicated that degradation of substituted aromatic hydrocarbons having more than three carbons in the side chain did occur (Table 2). *Cycloclasticus* spp. could not degrade any of these aromatic hydrocarbons except alkylnaphthalene. We therefore believe that some other bacterial populations were involved in the degradation of

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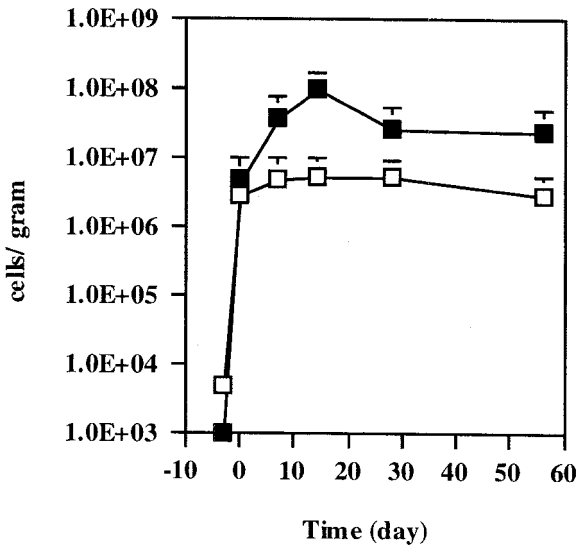

FIG. 5. Propagation of *Cycloclasticus* on the surfaces of oil-polluted grains of gravel. The number of *Cycloclasticus* cells was estimated by q-cPCR. Symbols: □, cell number in the control tank; ■, cell number in the tank to which fertilizer was added.

such aromatic hydrocarbons. It is important to identify these bacterial populations and to analyze their ability to degrade hydrocarbons.

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