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博　士　論　文

Studies on marine bacteria-mediated bioremediation after petroleum spill in specific reference to the outer membrane protein involved in the uptake of environmental chemicals

（原油汚染後の海洋性細菌群によるバイオレメディエーションと環境中の化学物質除去に関与する細胞外膜タンパク質の解析）

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Chapter I

General Introduction

Petroleum-based products are major power sources of industries and civil life in the current society. Petroleum is also a raw material for many chemical products such as plastics, paints, and cosmetics. The consumption of petroleum increases every year, and in 1998, approximately 41-billion m^3 of petroleum were consumed in the whole world. Oil fields are not uniformly distributed around the globe but rather localized in limited areas such as the Persian Gulf region. The world production of crude oil is more than 3 billion tons per year about a half of which is transported by sea. In Japan, approximately 2.5-billion m^3 of petroleum are consumed in a year, and almost 87% of the petroleum was imported from the Middle East. Consequently, the international petroleum transportation by tankers is frequent. Tanker shipping takes on ballast water, which contaminates marine environment upon its discharge. More importantly, tanker accidents exemplified by that of the T/V *Exxon Valdez* in Prince William Sound, Alaska, severely affect local marine environments. In these days, the offshore drilling is common to explore new oil resources. This is another source of the petroleum pollution. However, the largest source of marine contamination is caused by petroleum runoff from land. Annually, more than 2 million tons of petroleum are estimated to end up in the sea. In Table I-1, the large oil-spill accidents that have recently occurred in the world are listed.

Once petroleum is released into the marine environment organisms are affected in many ways (Hose and Brown, 1998; Hester and Mendelssohn, 2000). Many petroleum components are known to be mutagenic, and are suspected carcinogens to marine organisms and may be transferred to human through seafood consumption (Malins et al., 1985; Meador et al., 1995; Sikkema et al., 1995; Stegeman, 1981). Fortunately, petroleum released into the sea seems to be degraded either biologically or abiotically (Readman et al., 1992).

Table I-1 The big oil-spill accident of late years

Year	Place of accident	Cause of accident	Outflow (t)
1989	Prince Williams Bay, Alaska	Standing of the Exxon Valdez	34,850
1991	Persian Gulf	The Gulf war	202,000 - 540,000
1992	La Cornia Bay, Spain	Standing of the Aegean Sea	73,000
1993	Shetland Island, England	Standing of the Braer	85,000
1993	The Sea of West Andaman, Sumatra	Collision of the Maersk Navigator	25,000
1995	Southeast shore of Shori Island, Korea	Standing of the Sea Prince	81,600
1996	Milford Heaven, Wales	Standing of the Sea Empress	50,000 - 70,000
1997	Japan sea	Standing of the Nakhodka	5,304
1997	Tokyo bay	Standing of the Diamond Grace	1,317
1997	Straits of Singapore	Collision of the Evoikos	28,463
1999	The offshore of France	Standing of the Erika	11,000

Petroleum characteristics

All the petroleum products are derived from crude oil, complex mixture of hydrocarbons of varying molecular weights and structures ranging from alight gas (methane) to heavy solids. Hydrogen and carbon are the most important and prevalent elements, comprising up to 98% of crude oil and 100% of many refined products. Petroleum components are separated into four fractions, the saturated, aromatic, resin and asphaltene fractions, by means of the absorption chromatography (Fig. I-1). Each of these fractions contains a large number of different compounds (Karlsen and Larter, 1991).

Saturated hydrocarbons contain no double bonds. They are further classified according to their chemical structures into alkanes (paraffins) and cycloalkanes (naphthenes). Alkanes have either branched or unbranched (normal) carbon chain(s). They have a general formula of C_nH_{2n+2}, and are the major constituents of natural gas and petroleum. Alkanes containing less than 5 carbon atoms per molecule are usually gaseous at room temperature (such as methane), those having between 5 and 15 carbon atoms are usually liquid, and straight chain alkanes having more than 15 carbon atoms are solid. Small carbon number alkanes can cause cell damage and death in a variety of organisms at high concentrations. Higher carbon alkanes are not generally toxic, but may interfere with normal metabolic processes and communication in some organisms. Cycloalkanes (naphtenes) have one or more rings of carbon atoms (mainly cyclopentanes and cyclohexanes), and have a general formula of C_nH_{2n}. They are insoluble in water and generally boil at temperatures 10 to 20 °C higher than alkanes of the corresponding carbon numbers. A majority of cycloalkanes in crude oil have alkyl substitution(s) (Fig. I-2).

Aromatic hydrocarbons have one or more aromatic rings with or without alkyl substitution(s). Benzene is the simplest of all (Fig. I-2). They are the most toxic hydrocarbons present and are present in crude oil and in practically all petroleum products. Many aromatic hydrocarbons are soluble in water to some extent, increasing their toxicity to aquatic organisms. Certain aromatic hydrocarbons are considered long-term poisons and often exert carcinogenic effects. Alkyl-substituted aromatic hydrocarbons are generally more abundant than non-substituted ones in crude oil (Mater and Hatch, 1994).

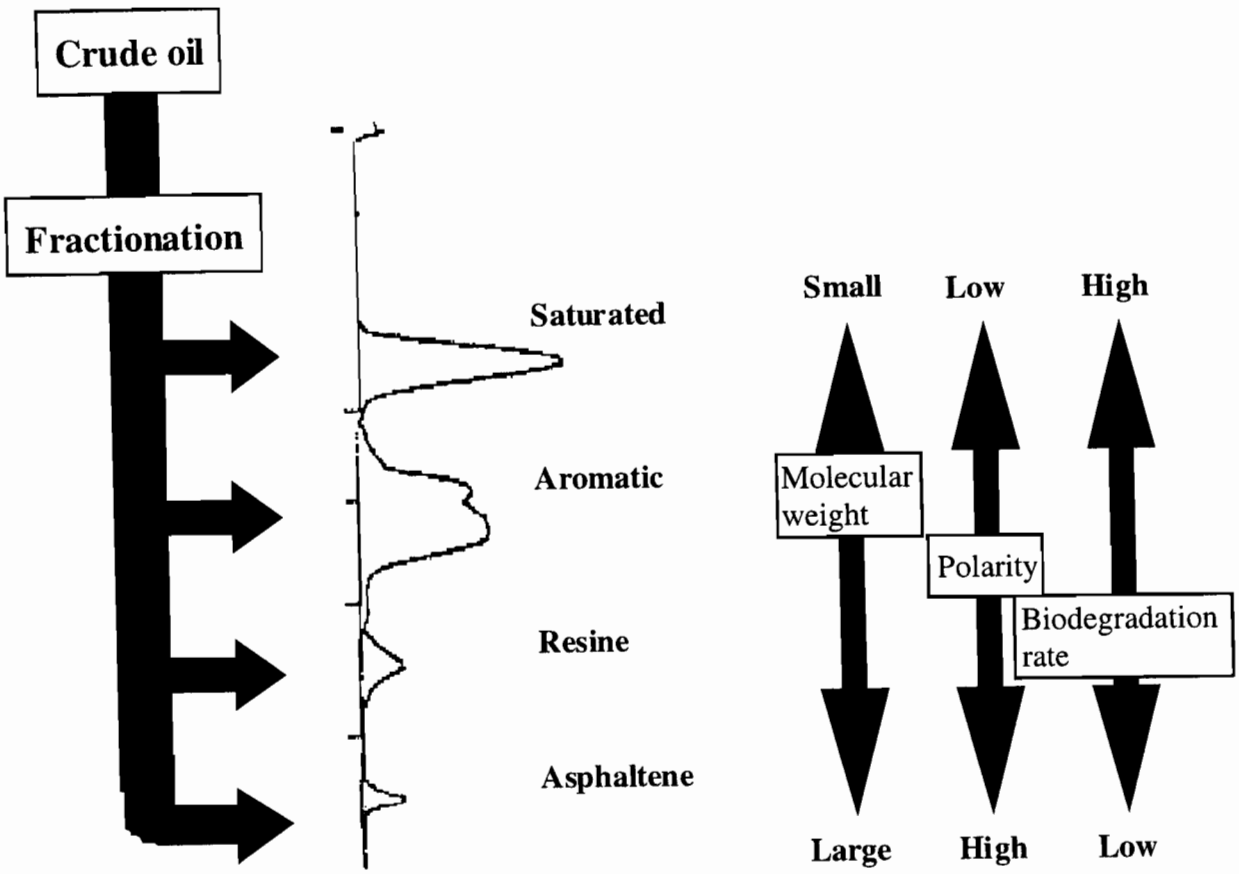
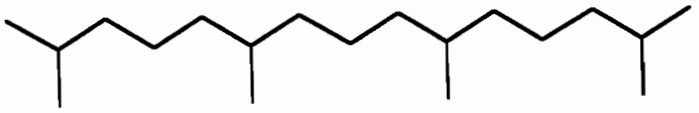
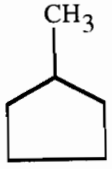


Fig. I-1. Four fraction of petroleum and their chemical characteristics

Tetradecane

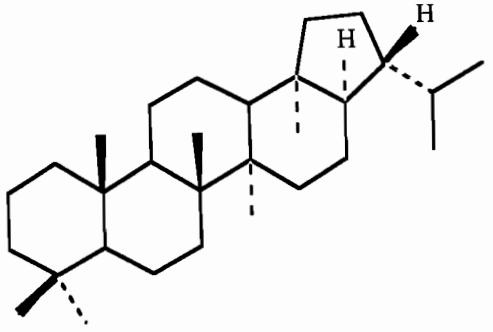


Pristane



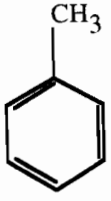
Methylcyclopentane

Cyclohexane

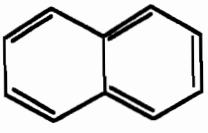


17α(H), 21β(H)-hopane

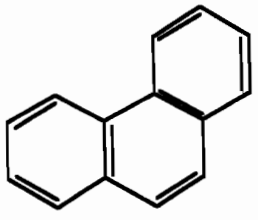
Benzene



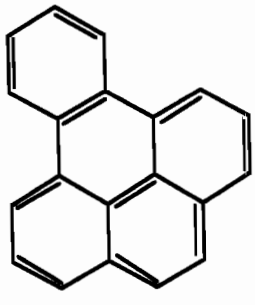
Toluene



Naphthalene



Phenanthrene



1,2-Benzopyrene

Fig. I-2. Representative hydrocarbons. Tetradecane (an n-alkane), pristane (a branched alkane), and methylcyclopentane, cyclohexane and 17α[H],21β[H]-hopane (cycloalkane compounds) are present in the saturated fraction of crude oil. The other compounds shown in this figure are present in the aromatic fraction.

In contrast to the saturated and aromatic fractions, both the resin and asphaltene fractions contain non-hydrocarbon polar compounds. They contain, in addition to carbon and hydrogen, trace amounts of nitrogen, sulfur or oxygen. These compounds often make complexes with heavy metals. Asphaltenes consist of high-molecular-weight compounds that are not soluble in solvent such as *n*-heptane while resins are polar molecules soluble in *n*-heptane. Resins contain heterocyclic compounds, acids and sulfoxides.

The components of petroleum in crude oil have been analyzed mainly by using gas chromatography in combination with mass spectrometry (GC/MS). Therefore, the chemical structures of higher molecular weight components (heavy fractions) that cannot be analyzed by GC are mostly unknown. Furthermore, the compositions of many branched alkanes and alkyl cycloalkanes are not determined as they have numerous isomers that cannot be resolved by GC (Killops and Al-Juboori, 1990; Gough and Rowland, 1990). Therefore, a multitude of detection techniques such as flame ionization detection, infra-red and UV-absorption spectrometry, NMR and elemental analysis in combination with appropriate separation methods such as various chromatography methods and/or chemical derivatisation methods is necessary to characterize petroleum especially its heavy fractions.

Behavior of petroleum in marine environments

When petroleum is spilled into the sea, it stays at the water surface because of its light gravity (approximately 0.85), and then drifts and spreads over the water surface. It is subjected to many modifications, and changes its composition with time. This process is called weathering, caused mainly by evaporation of low molecular weight fractions, dissolution of water-soluble components, mixing of oil droplets with seawater, photochemical oxidation, and biodegradation.

Evaporation is the most important weathering process during the first 24 to 48 hours of an oil spill with regard to mass transfer and to removal of the more toxic, lower molecular weight components with boiling points below 250°C from the spilled oil. Therefore, the content of *n*-alkanes whose chain length being shorter than C14 is reduced by the weathering. The contents of aromatic hydrocarbons with the same boiling point range are also reduced as

they are subjected to both evaporation and dissolution. The rates and magnitudes of loss by evaporation are heavily dependent on the oil composition and on the environmental conditions. For example, approximately 90% of gasoline and diesel fuel evaporate in one to two days in mild climate (Fingas, 1994).

Mixing of oil with seawater occurs in several forms. Dispersion of oil droplets into water column occurs by the action of waves. Water-oil emulsification occurs when the petroleum contains polar components that act as emulsifiers. Water-oil emulsion containing more than 70% of seawater becomes quite viscous and is called 'chocolate mousse' from its appearance. After the evaporation of light fractions, heavy residues of petroleum can aggregate and form tar balls whose diameter ranges from microscopic sizes to several tenth cm.

Under the sunlight, petroleum discharged at sea receives photochemical modifications. Increase of the polar fraction and decrease of the aromatic fraction can also be observed. Aliphatic components do not absorb sufficient solar energy, and are by themselves photochemically inert. However, they can be degraded by photosensitized oxidation. The aromatic or polar components of petroleum and anthraquinone yielded in seawater can provoke the degradation of *n*-alkanes into terminal *n*-alkenes (carbon-carbon double bond at position 1) and low molecular weight carbonyl compounds (Ehrhardt and Weber, 1991).

The water-soluble components of petroleum exhibit toxic effect to marine organisms. In general, aromatic compounds are more toxic than aliphatic compounds, and smaller molecules are more toxic than larger ones of the same series. Solar irradiation affects oil toxicity. Surface films become less toxic due to the loss of polycyclic aromatic hydrocarbons but the toxicity of the water-soluble fraction increases as its concentration increases (Nicodem *et al.*, 1997).

After the *Exxon Valdez* accident, the fate of spilled oil was investigated (Wolfe et al., 1994). It was reported that 84% of spilled oil was found to diminish by evaporation, photochemical modification, recovery by man power and by biodegradation, and 16% of spilled oil remained in the marine environment even after four years (Wolfe et al., 1994).

Biodegradation of petroleum components

Saturate hydrocarbons

n-Alkanes, a major group in crude oil, are readily biodegraded in the marine environment. They are aerobically degraded by several pathways. The terminal oxidation pathway is most common. In this pathway, the methyl group of *n*-alkanes is oxidized to alcohol and fatty acid, with subsequent entry to the β-oxidation pathway (Fig. I-3; van Beilen et al., 1994). The subterminal oxidation pathway yielding secondary alcohols has also been reported. In this pathway, *n*-alkanes are oxidized to secondary alcohols, then to ketones, and finally to fatty acids (Fig. I-3; Markovetz and Kallio, 1971; Whyte et al., 1998). *n*-Alkanes degradation via alkyl hydroperoxides may also occur. In this pathway, *n*-alkanes are transformed to *n*-alkyl peroxides, and these molecules would be metabolized to the corresponding aldehyde (Fig. I-3; Maeng et al., 1996). Catabolic pathways for the degradation of branched alkanes have been elucidated for a few bacteria. such as *Rhodococcus* strain BPM 1613 for norpristane (2,6,10-trimethylhexadecane), norpristane (2,6,10-trimethylpentadecane) and farnesane (2,6,10-trimethyldodecane) via β-oxidation (Nakajima et al., 1985).

Cycloalkanes including condensed cycloalkanes are degraded by a co-oxidation mechanism. The formation of a cyclic alcohol and ketone has been observed (Fig. I-3). The degradation of substituted cycloalkanes seems to be less difficult than that of unsubstituted cycloalkanes (Morgan and Watkinson, 1994).

Aromatic hydrocarbons

A multitude of catabolic pathways for the degradation of aromatic compounds have been elucidated. Bacteria degrade toluene in five different pathways. (Fig. I-4; Johnson and Olsen, 1997). For example, through the pathway carried out by enzymes encoded by TOL plasmid, toluene is successively degraded to benzyl alcohol, benzaldehyde and benzoate, which is further transformed to the TCA cycle intermediates.

Simple polynuclear aromatic hydrocarbons (PAHs) such as naphthalene, biphenyl and phenenthrene are readily degraded aerobically. The degradation of these compounds is generally initiated by dihydroxylation of one of the polynuclear aromatic rings, followed by

cleavage of the dihydroxyalted ring. The carbon skeleton produced by the ring-cleavage reaction is then dismantled, before cleavage of the second aromatic ring (Fig. I-5; Saito et al., 1999; Harayama et al., 1992).

PAHs possessing four or more fused aromatic rings have very low water solubility and tend to be adsorbed to a solid surface. These characteristics constitute a major constraint for biodegradation. *Mycobacteria* and *Sphingomonas* have been isolated as bacteria that are able to degrade PAHs possessing four or more fused aromatic rings. Some *mycobacteria* mineralize (degrade into CO_2 and H_2O) pyrene, fluoranthene and benzo[*a*]pyrene (Harayama, 1997).

Asphaltenes and resins

Asphaltenes and resins are considered to be recalcitrant to biodegradation. Rontani et al. (1985) have reported that about half of the asphaltene components were co-metabolically oxidized by a microbial consortium in the presence of C12 to C18 *n*-alkanes. These observations should be reinvestigated as the preparation of pure fractions of resins and asphaltenes is difficult and these fractions almost always contain saturated and aromatic hydrocarbons (Myhr et al., 1989).

Anaerobic degradation

Petroleum components that are trapped in sediment tend to persist under anaerobic conditions. Nevertheless, ecological studies have demonstrated that certain hydrocarbons can be oxidized under anaerobic conditions when either nitrate reduction, sulfate reduction, methane production, Fe(III) reduction or photosynthesis is coupled to the hydrocarbon oxidation (Evans and Fuchs, 1988). It is known that many hydrocarbons, such as alkanes, alkenes, and aromatic hydrocarbons are anaerobically degraded (Bregnard et al., 1997). However, the rate of anaerobic degradation is generally lower than equivalent aerobic degradation (Coates et al. 1997; Caldwell et al., 1998).

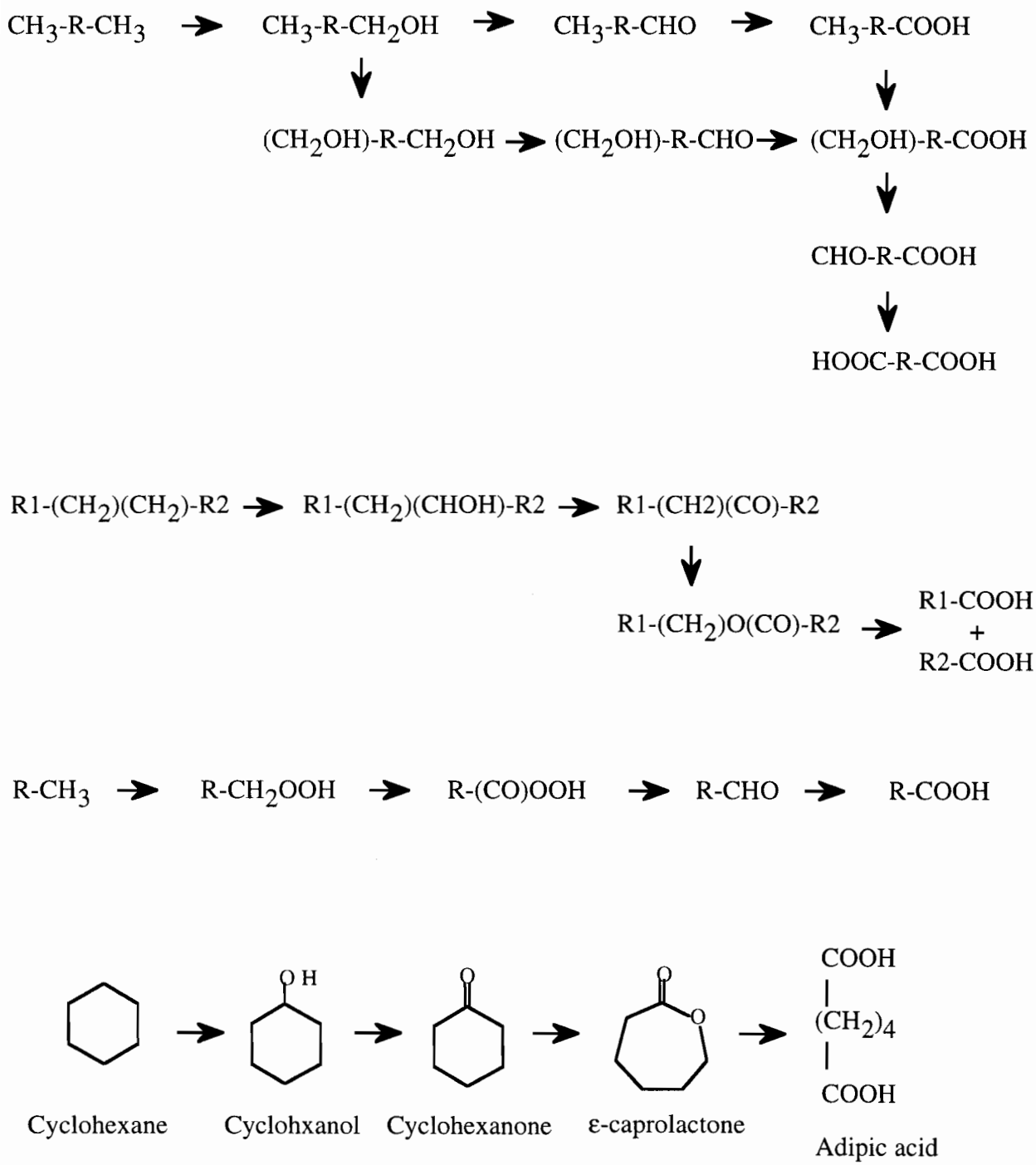


Fig. I-3. Alkane degradative pathways.[A] Terminal oxidation of *n*-alkanes. α- and ω-hydroxylation is catalyzed by the same set of enzymes; [B] Subterminal oxidation of *n*-alkanes; [C] *n*-Alkane degradation via alkyl hydroperoxides; [D] Degradation of cyclohexane.

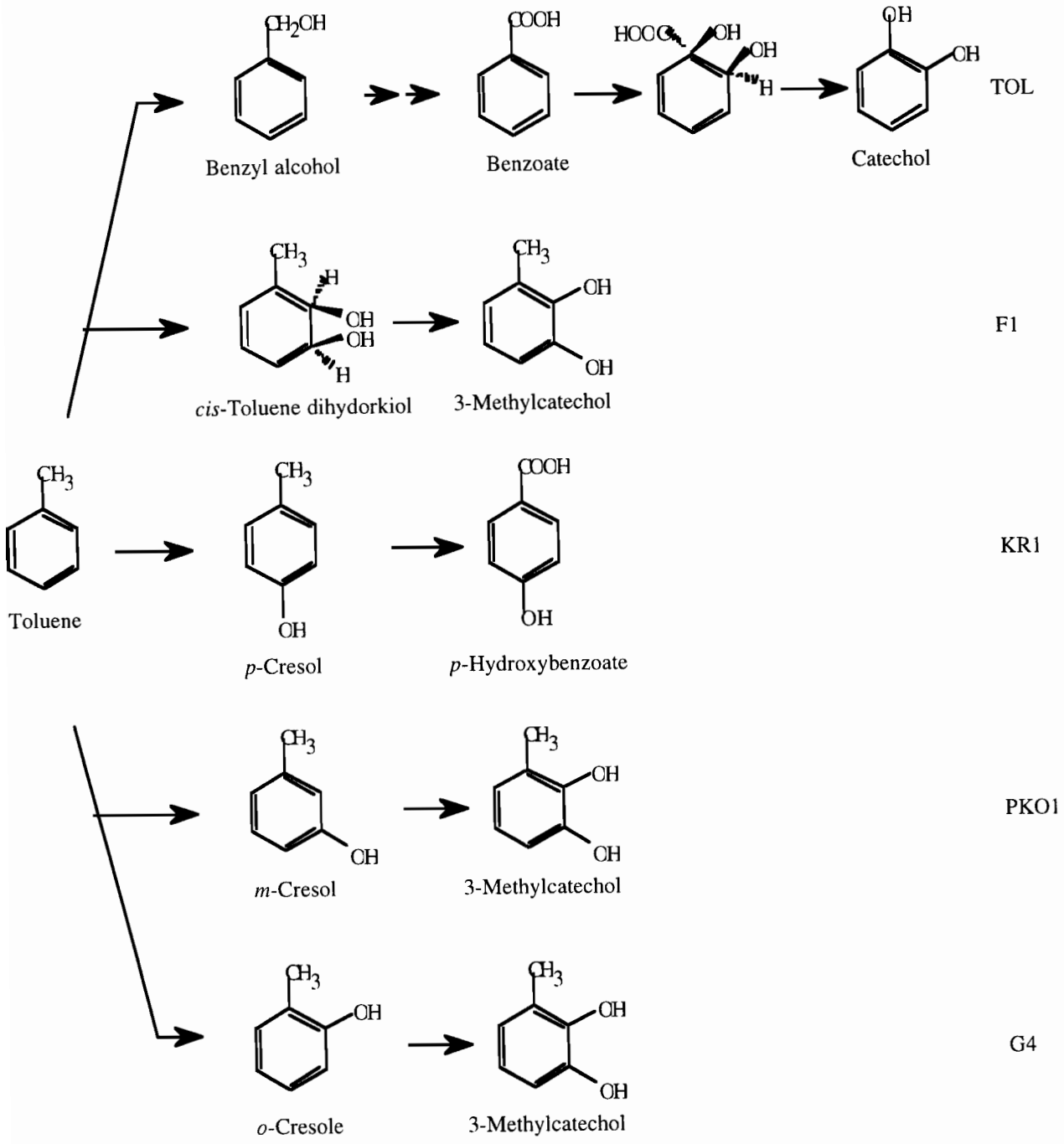


Fig. I-4 Degradation pathways for the aerobic degradation of toluene

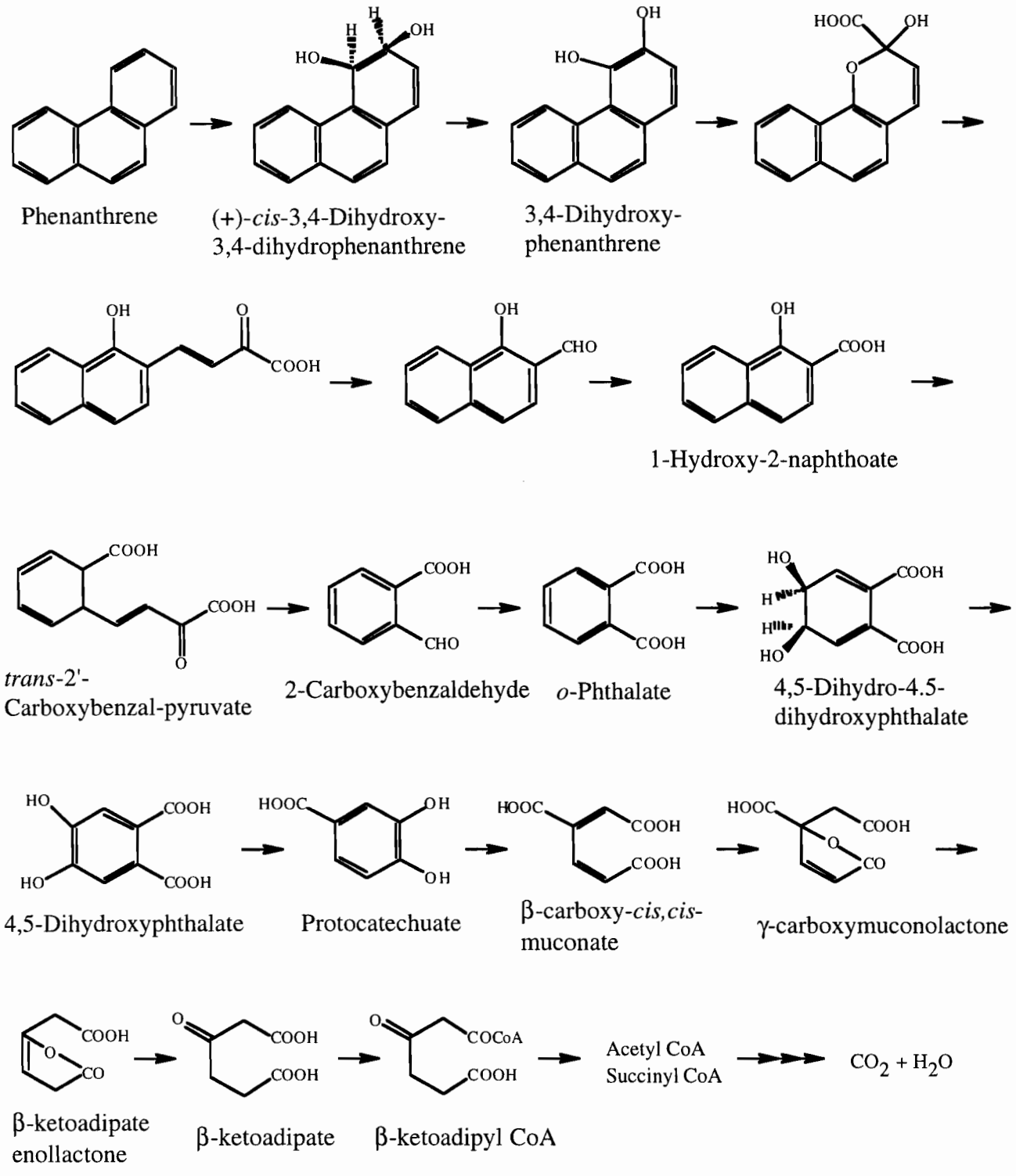


Fig. I-5. Catabolic Pathway for the Degradation of Phenanthrene with *Nocardioides sp.* KP7
PAHs possessing four or more fused aromatic rings have very low water solubility and tend to be adsorbed to a solid surface. These characteristics constitute a major constraint for biodegradation. *Mycobacteria* and *Sphingomonas* have been isolated as bacteria that are able to degrade PAHs possessing four or more fused aromatic rings. Some *mycobacteria* mineralize (degrade into CO2 and H2O) pyrene, fluoranthene and benzo[*a*]pyrene (Harayama, 1997).

Petroleum bioremediation in marine environments

While accidental releases may contribute to only a small percentage of the oil released into marine environment, large accidental oil spills have attracted public attention to the fate of petroleum hydrocarbons. In response to this concern, analysis of the biodegradation of petroleum in the natural environments has been intensified. The pioneering studies by Atlas, Bartha and colleagues (Atlas and Bartha, 1993; Prince, 1993) have demonstrated that the available concentrations of nitrogen and phosphorous in seawater are limiting factors for the growth of hydrocarbon-degrading microorganisms. Thus, addition of nitrogen and phosphorous fertilizers stimulates the biodegradation of petroleum (bioremediation). In general, small hydrocarbon molecules are more easily biodegraded than larger ones, and aromatic hydrocarbons are degraded at a much slower rate than that of alkanes in marine environments (Oudot, 1984; Kennicut, 1988; Ishihara et al., 1995; Sugiura et al., 1997; Sasaki et al., 1998; Wang et al., 1998).

Many oil spills in the sea cause extensive contamination of the shoreline, despite efforts to treat the oil at sea. Cleaning up a polluted coastline by enhancing microbial activities was first attempted in 1989 after the spill from the *Exxon Valdez*. The initial measure taken after this accident was physical washing with high-pressure water. Subsequently, fertilizers were applied to the polluted beaches to accelerate the growth and activities of petroleum-degrading microorganisms. Two to three weeks later, pebbles on the beaches that were treated with fertilizers became significantly cleaner than those in the control area (Pritchard and Casta, 1991). Quantitative changes in the oil composition were evaluated by the ratios of total GC detectable hydrocarbons against five-ring cycloalkane, 17α(H),21β(H)-hopane (Fig. I-2; Bragg et al., 1992). Inspection of the change in the ratio of GC detectable hydrocarbons to hopane suggested that oil were removed within three months after applying a sufficient amount of fertilizers (Bragg et al., 1994).

Purpose and composition of this thesis

The bioremediation studies after the oil spill from the *Exxon Vardez* demonstrated that this technology is quite promising for the cleanup of petroleum-contaminated shorelines. However,

there are few examples of the field operations of petroleum bioremediation by reason of insufficient data on influences on a marine environment. As described before, crude oil is composed of thousands of components and, therefore, countless microorganisms must be involved in the degradation of crude oil. The microbial diversity and community dynamics within contaminated and bioremediated ecosystems remain unknown.

For these reasons, I analyzed in chapter II of this thesis the marine microbial populations after an actual oil spill to know the long-term influence of spilled oil on the microbial ecosystem. In chapters III and IV, I analyzed the microbial population dynamics during bioremediation to identify the microorganisms important for bioremediation in offshore sediments, and analyzed their petroleum degradation abilities.

In a natural environment, the concentrations of polluted chemicals are generally low due to their poor water solubility and adsorption to colloidal particles in the surface water or to the matrix or organic phase of the sediment (Meijers and van der Leer. 1976; McCarty et al., 1981; Frank et al., 1990). Therefore, microorganisms that utilize such chemicals as carbon and energy sources may have some mechanisms that are indispensable for the survival of the population of microorganisms important for bioremediation. In chapter V, I analyzed the gene encoded by the TOL plasmid of *Pseudomonas putida* to know the mechanisms by which uptake of low concentration substrates in the natural environment is achieved.

Chapter II

Molecular detection of the marine bacterial population on the beaches contaminated by the tanker *Nakhodka*'s oil-spill accident

II-1. Introduction

Many oil spill accidents are reported every year, and the biggest oil spills almost always occur at sea (Swannell et al., 1996). After the Alaskan oil spill from *Exxon Valdez*, extensive monitoring has been conducted (Bragg et al., 1994), and a number of factors that influence the speed of biodegradation of spilled oil have been elucidated. In these studies, the microorganisms were analyzed as the total number of heterotrophs and/or oil degraders by using culture-dependent methods such as a plate count or most probable number (Lee et al., 1991; Lindstrom et al., 1991; Bragg et al., 1994).

Molecular approaches, mainly involving 16S rRNA frameworks (Olsen et al., 1986; Pace et al., 1986; DeLong, 1992), have been developed for analyzing natural microbial populations without cultivation. These have been proven to be powerful tools in microbial ecology, showing that natural microbial populations are much more diverse than previously thought from cultured microorganisms (Ward et al., 1990; Barns et al., 1994; Amann et al., 1995). Unfortunately, no molecular approach has yet been applied to analyze marine microbial populations after an actual oil spill. This present study applies the rRNA approach by the denaturing gradient gel electrophoresis of PCR-amplified 16S rDNA fragments (PCR/DGGE) (Ferris et al., 1997) to analyze the bacterial populations on oiled beaches resulting from the *Nakhodka* accident. The data obtained in this chapter will provide useful information about the long-term influence of spilled oil on the marine environment and the extent of natural attenuation in this environment.

II-2. Materials and Methods

Sampling

All the samples used in this study were collected in November 1997, July 1998, November 1998 and June 1999. The atmospheric temperature ranged from 15°C to 30°C, while the temperature of the seawater was from 15°C to 27°C at the sampling sites. The location of the sampling sites is presented in Fig. II-1. Oil spilled from the tanker *Nakhodka* did not reach Ajigasawa, and no recent pollution episode has been reported in this region. Therefore, we considered this place to be pristine. The oil-removal efforts have been conducted most intensively at the Antoh beach, and no oil was visible on the surface of this beach in November 1997. In contrast, a large amount of oil was found on the Taura beach where no effort has been made to recover the oil.

Analyses of the seawater samples

DOC was measured with a TOC meter (TOC-5000, Shimadzu) after filtering a seawater sample through a GV membrane (pore size of 0.22 mm, Millipore) (Watanabe et al., 1998). The total nitrogen and phosphorous concentrations in the seawater were measured according to the methods described in JIS K0102 (Japanese Industrial Standard Committee, 1986). The total bacterial count in the seawater was determined by fluorescence-microscopy after staining with 4',6-diamidino-2-phenylindole (DAPI) (Watanabe et al., 1998). The colony forming unit in the seawater was determined on one fifth strength Marine Agar plates after incubation at 20°C for 7 days. The TPH content in 4 l of seawater was extracted with 4-chloromethylene and measured according to EPA method 418.1 (Environmental Protection Agency, 1983).

DNA extraction from the seawater samples

The microorganisms in 2 l of seawater were collected on a GV membrane (Millipore) by filtration, and DNA was extracted by the Marmur procedure (Marmur, 1961). The purity and quantity of the DNA preparation were examined by measuring the UV absorption spectrum (Sambrook et al., 1989).

DNA extraction from the oil

Approximately 1 g of oil paste or oily sand was put in 1 ml of a cell suspension buffer (Marmur, 1961), to which 1 ml of a lysing solution (Marmur, 1961) and 0.5 ml of an emulsifier (Cactus Clean L-10A, Tesuko) were then added. This suspension was thoroughly mixed to disperse the oil in the buffer, and then incubated at 55°C for 30 min. Next, the suspension was extracted 3 times with a phenol/chloroform solution (Sambrook et al., 1989), and the aqueous solution was recovered. Two volumes of ethanol were added to the aqueous solution and, after gently mixing, it was cooled at -20°C for 30 min. Nucleic acids were precipitated by centrifuging at 20,000 × *g* for 10 min, washed with 1 ml of a 70% (v/v) ethanol solution, and dissolved in 0.2 ml of a TE buffer (Sambrook et al., 1989) containing 100 mg of RNase A. This solution was incubated at 37°C for 1 h and finally subjected to separation in a QIA quick-spin column (QIAGEN). The purity and quantity of the DNA were examined by recording its UV absorption spectrum (Sambrook et al., 1989).

PCR/DGGE

PCR primers P2 and P3 (Muyzer et al., 1993) were used to amplify the variable V3 region of bacterial 16S rDNA (corresponding to positions 341 to 534 in the *Escherichia coli* rRNA sequence) connected to the GC clamp. PCR was performed as described previously (Watanabe et al., 1998). Amplification of the PCR products into the expected sizes was confirmed by electrophoresis through 1.5% (w/v) agarose gel (LO3 agarose, Takara) in a TBE buffer (Sambrook et al., 1989).

DGGE was performed with a DCode^{TM} instrument (Bio-Rad) according to the manufacturer's instructions. Gels were made by using a gradient of denaturants between 38% (containing 2.66 M urea and 15.2% formamide) and 58% (containing 4.06 M urea and 23.2% formamide). Ten ml of the PCR-amplified mixture was subjected to electrophoresis in 10% (w/v) polyacrylamide gel at 200 V for 3.5 h at a running temperature of 58°C. After the electrophoresis, the gel was stained with SYBR Green I (FMC Bioproducts) for 30 min according to the manufacturer's instructions.

A gel slice containing a DNA band was excised, and its DNA sequence was determined

as described previously (Watanabe et al., 1998). The resulting sequences were compared with the compilation of 16S rDNA genes available in the GenBank nucleotide library by a BLAST search (Atschul et al., 1990) through the U. S. National Institute of Healths Internet site.

Competitive PCR/DGGE (cPCR/DGGE)

To determine the density of the bacterial population in an oil paste sample, a known number of cells of *Pseudomonas putida* PaW94 (Worsey et al., 1978) was added to the oil paste in the cell-suspension buffer. DNA was then extracted from the mixture and subjected to a PCR/DGGE analysis, the gel being stained with SYBR green I before being photographed. The resulting DGGE bands were quantified by image processing software (NIH IMAGE ver. 1.60, National Institute of Health), and the intensity of each band was compared with that of the *P. putida* band in order to determine the apparent population density (*P. putida* equivalent density).

Biodegradation in seawater of heavy oil spilled from the tanker Nakhodka

To 800 ml of non-sterilized seawater sampled off a beach in Ishikawa prefecture, 200 ml of an autoclaved solution (pH 7.6) containing 5 g/l of NH4NO3, 1 g/l of K2HPO4, and 0.1 g/l of ferric citrate was added. Oil paste sampled from the same beach was then added at a concentration of 1 g/l as the carbon source. The medium was cultivated at 20°C while constantly shaking (100 strokes/min) for three months. The oil was extracted and analyzed as described previously (Dutta and Harayama, 2000).

Nucleotide sequence accession number

The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases with the following accession numbers: AB034995 to AB035039.

II-3. Results

Analyses of coastal seawater samples

On January 2, 1997, the Russian tanker *Nakhodka* carrying approximately 20,300 tons of heavy fuel oil was wrecked and sank off Oki Island in the Japan Sea, resulting in a spill of more than 5,000 tons of heavy fuel oil. Rough sea conditions broke off the bow section of the hull which then drifted ashore near the Antoh beach in Fukui prefecture. The spilled oil drifted with the Tsushima current, reaching the shoreline mainly in Hyogo, Kyoto, Fukui and Ishikawa prefectures. The spilled oil contaminated more than 500 km of the coastline. The Antoh beach was most heavily contaminated by a large amount of oil spilled from the broken bow section. Most of the cleanup efforts utilized mechanical recovery, and bioremediation was not attempted, except in experimental cases on a small scale. The oil had almost completely been removed from the surface of many beaches by the summer of 1997, although some oiled beaches that were hardly accessible to construction machinery remained untreated. Seawater was sampled at the beaches indicated in Fig. II-1 in November 1997, July 1998, November 1998 and June 1999. The dissolved organic carbon (DOC) values of the samples were between 0.7 and 3.2 mg/l, while the total nitrogen and phosphorus concentrations ranged from 0.08 to 0.35 mg/l and from 0.03 to 0.38 mg/l, respectively (Table II-1). A positive correlation was apparent between the total nitrogen and total phosphorus concentrations, the squared correlation coefficient (*R2*) being 0.42 (Fig. II-2C). A weaker correlation was also apparent between the total nitrogen concentration and the DOC value (the *R2* value was 0.38) (Fig. II-2A), while there was no significant correlation between the total phosphorus concentration and DOC value (the *R2* value was 0.08) (Fig. II-2B).

The oil dissolved in the seawater was extracted, and its total petroleum hydrocarbon (TPH) value was measured. The oil concentration in mg of oil/l of each seawater sample is shown in Table II-1. Petroleum hydrocarbons were the major constituents of DOC in the samples collected in November 1997, accounting for 10% of DOC. However, the TPH values were lower in the samples collected later.

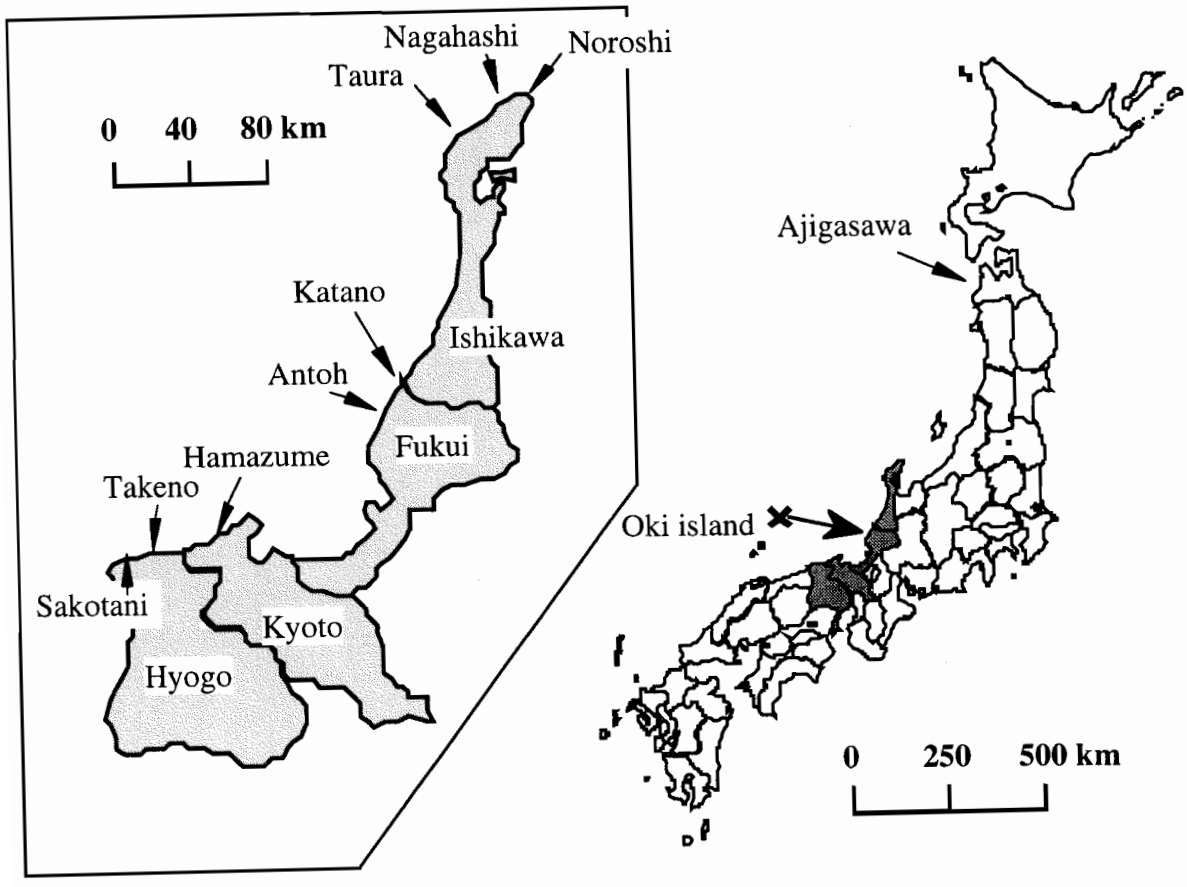


Fig. II-1. Map of the sampling sites. The illustration on the right shows a map of Japan, and the four prefectures indicated by a gray color are enlarged in the illustration on the left. X indicates the location of the *Nakhodka* tanker wreckage, and the arrow is the direction of drift of the bow section of the tanker toward the Antoh beach. The sampling sites in the Ishikawa, Fukui, Kyoto and Hyogo prefectures are indicated in the illustration on the left.

Table II-1. The chemical analyses in the seawater samples. The sampling sites are indicated in Fig. II-1.

Sampling date	Chemichals (mg/l)	Site								
		Noroshi	Nagahashi	Taura	Katano	Antho	Hamazume	Takeno	Sakotani	Mean (SD)
1997. 11	DOC	1.19	1.16	1.11	1.10	1.12	2.40	0.70	0.83	1.20 (0.51)
	TN	0.08	0.08	0.27	0.11	0.14	0.12	0.08	0.08	0.12 (0.06)
	TP	0.004	0.011	0.005	0.004	0.005	0.004	0.003	0.007	0.005 (0.0025)
	TPH	ND	ND	ND	ND	ND	ND	ND	ND	
1998. 7	DOC	0.85	0.68	0.99	1.06	0.98	0.90	0.98	0.96	0.93 (0.12)
	TN	0.25	0.16	0.23	0.15	0.16	0.12	0.09	0.08	0.16 (0.06)
	TP	0.026	0.012	0.026	0.015	0.015	0.009	0.006	0.006	0.014 (0.008)
	TPH	0.11	ND	0.08	0.10	0.15	0.09	ND	0.11	0.11 (0.02)
1998. 11	DOC	1.54	1.13	1.31	3.22	0.92	0.84	0.80	0.70	1.31 (0.82)
	TN	0.15	0.16	0.25	0.41	0.12	0.20	0.13	0.10	0.19 (0.10)
	TP	0.026	0.015	0.038	0.023	0.012	0.014	0.013	0.009	0.019 (0.01)
	TPH	0.06	ND	0.07	0.04	0.02	0.07	ND	0.06	0.053 (0.02)
1999. 6	DOC	2.30	0.70	0.80	0.90	1.30	1.40	1.10	0.80	1.16 (0.52)
	TN	0.35	0.12	0.18	0.14	0.15	0.15	0.18	0.12	0.17 (0.07)
	TP	0.024	0.005	0.014	0.013	0.006	0.011	0.007	0.009	0.011 (0.006)
	TPH	0.05	ND	0.03	0.02	0.02	0.06	ND	0.03	0.035 (0.016)

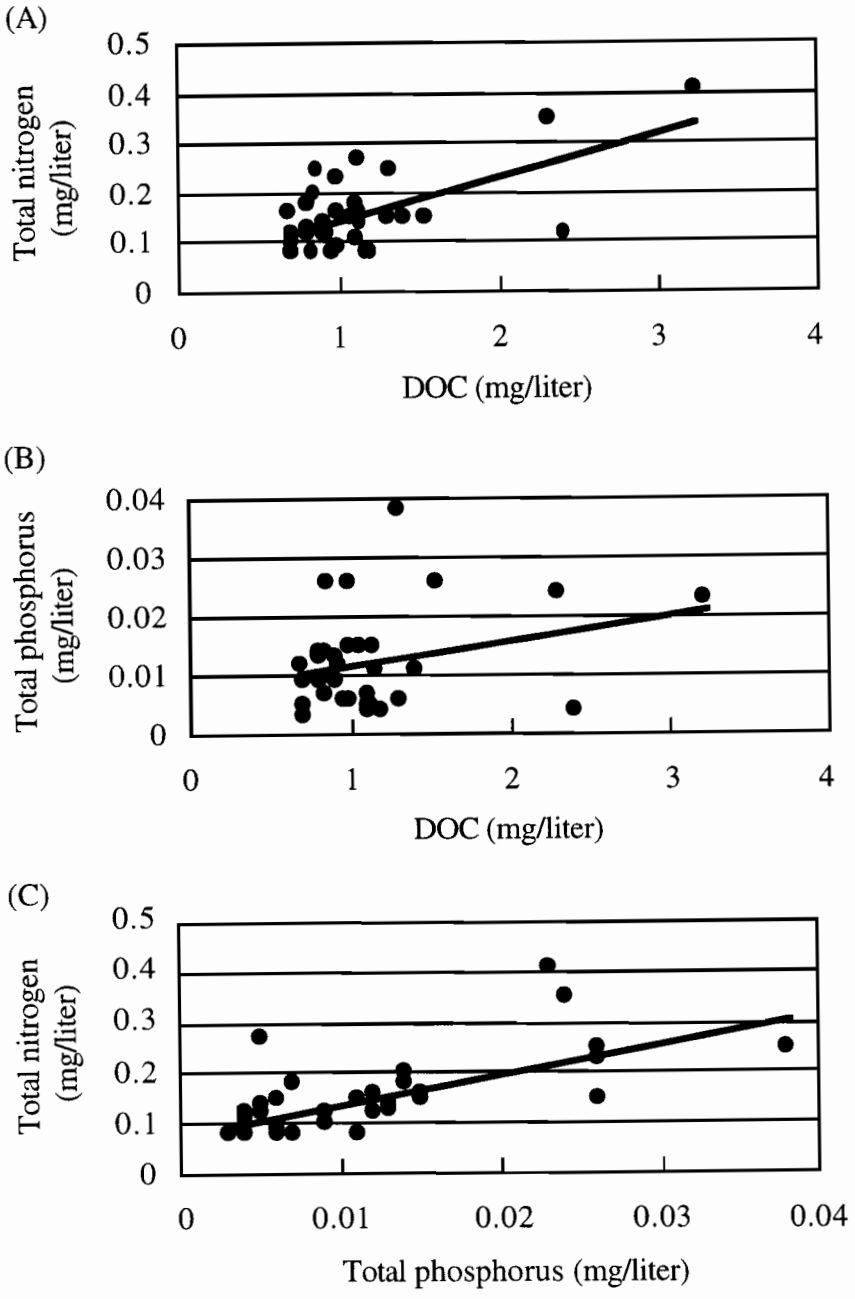


Fig. II-2. Correlation between DOC, total nitrogen concentration and total phosphorus concentration. The values for DOC, total nitrogen concentration and total phosphorus concentration in the samples obtained from eight different sites on four different occasions were used for the analysis.

A) Correlation between DOC and the total nitrogen concentration. The *R2* value was 0.38.

B) Correlation between DOC and the total phosphorus concentration. The *R2* value was 0.08.

C) Correlation between the total nitrogen concentration and total phosphorus concentration. The *R2* value was 0.42.

The values for the total bacterial count are shown in Table II-2. The total bacterial counts for the samples collected in July 1998 from three sites, Hamazume, Takeno and Sakotani, were exceptionally high, being almost 10^8/ml; otherwise, they were in the range of 1.3×10^5/ml to 7.1×10^6/ml, with an average value of 1.4×10^6/ml. The CFU values were approximately one thousandth of the total bacterial counts (data not shown).

There was no significant correlation between the total bacterial count and the other parameters, namely DOC, TPH, total nitrogen concentration and phosphorus concentration (data not shown). Bacterial growth in a marine environment may be more limited by the availability of nitrogen and phosphorus, rather than by the supply of organic carbon (Elser et al., 1995). The P limitation is generally imposed when the N/P ratio is more than 10, while the N limitation is imposed when the N/P ratio is less than 10. Thus, the total nitrogen concentration of a sample was divided by 10, and this value (N/10) and the value for the total phosphorus concentration (P) of the same sample were compared to obtain the smaller of the two values (this smaller value is referred as "N/10 or P"). Interestingly, a positive correlation was apparent between the total bacterial count and the "N/10 or P" value (R2 = 0.24) suggesting that either nitrogen or phosphorus could often be a limiting nutrient in the Japan Sea (Fig. II-3).

Dominant bacteria in the seawater samples

DGGE profiles of 16S rDNA amplified from the seawater samples were obtained (Fig. II-4), and some of the major bands were excised and sequenced. Among the different samples, the DGGE bands at equivalent positions exemplified by SNR2 and SN3 or by SA1, SH1, STak1 and SS1 were identical in their DNA sequences. Table II-3 lists a sequence in the databases exhibiting the highest nucleotide identity to each DGGE band. *Cytophaga sp.* and unknown α-proteobacterium were most frequently found.

Dominant bacteria associated with the residual oil samples

Paste samples of heavy oil remaining on the beaches were collected, the paste containing approximately 80% (w/w) of water. DNA was extracted from the paste, and 16S rDNA

Table II-2.Total bacterial counts in the seawater samples

Sampling site	November 1997	July 1998	November 1998	June 1999
Ajigasawa	1.1×10^6	8.4×10^5	ND	ND
Noroshi	7.1×10^5	7.1×10^6	6.0×10^5	7.0×10^5
Nagahashi	5.9×10^5	3.8×10^6	6.5×10^5	5.4×10^5
Taura	1.2×10^6	3.4×10^6	6.1×10^5	5.0×10^5
Katano	1.1×10^6	2.3×10^6	4.2×10^6	1.3×10^5
Antoh	4.0×10^5	1.3×10^6	6.1×10^5	1.3×10^6
Hamazume	6.7×10^5	6.1×10^7	3.0×10^6	2.1×10^5
Takeno	8.1×10^5	6.9×10^7	2.1×10^6	4.0×10^5
Sakotani	7.7×10^5	8.0×10^7	1.8×10^6	4.8×10^5

The standard error for each determination was less than 10%.

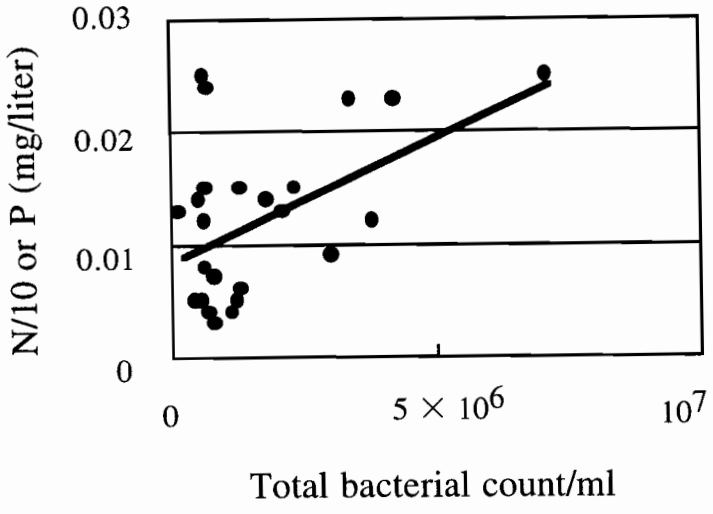


Fig. II-3. Correlation between the total bacterial count and the concentration of limiting nutrients. The total nitrogen concentration of a sample was divided by 10, and this value (N/10) and the value for the total phosphorus concentration (P) of the same sample were compared to identify a limiting nutrient. The smaller value is referred as "N/10 or P," and the correlation between this value and the total bacterial count is shown in this figure. The *R2* value was 0.24.

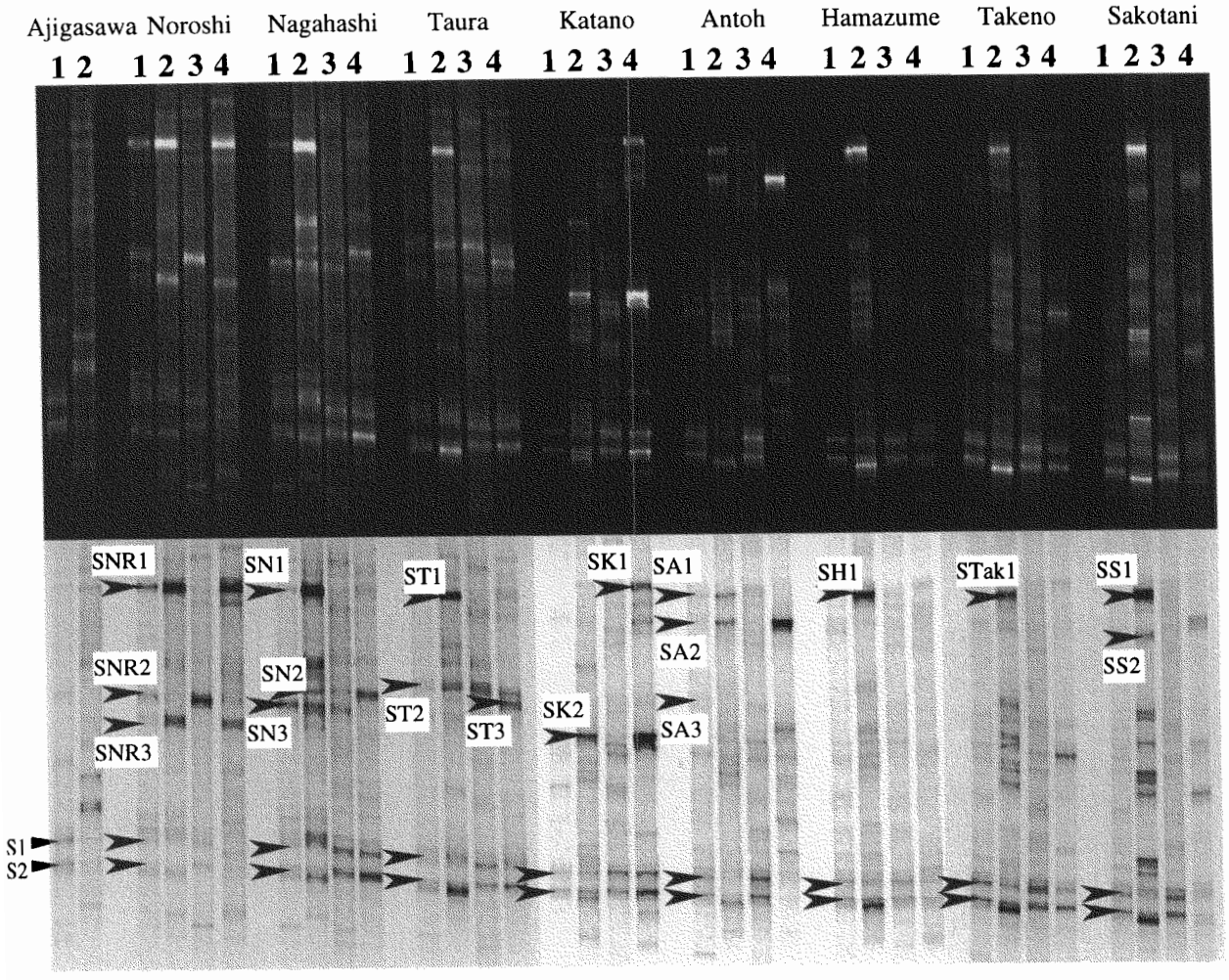


Fig. II-4. 16S rDNA DGGE profiles of the seawater samples. The lower part shows bands excised for the DNA sequencing analysis. Numbers above the DGGE profiles indicate the sampling time. Lane 1, November 1997; lane 2, July 1998; lane 3, November 1998; lane 4, June 1999.

DGGE profiles of DNA from the residual oil samples were obtained (Fig. II-5). Some of the major bands indicated in Fig. II-5 were excised and sequenced. The results indicate that the oil-associated bacterial populations were different from those in the seawater. Table II-3 lists the sequence in the databases most closely related to the sequence of each DGGE band. Bacteria closely related to *Hydrogenophilus thermoluteolus* TH-1, corresponding to bands ON4, OT4, OT5 and OS4, were most frequently found in the oil samples.

While the density of each major bacterium in the seawater population detected by PCR/DGGE could roughly be estimated from the total bacterial count data, the density of the bacterial populations in the oil samples could not be effectively evaluated by traditional microbiological methods. Therefore, competitive PCR (cPCR) in combination with DGGE, which has been proven to be useful for estimating the population density of uncultured marine bacteria (Watanabe et al., 1998), was applied to evaluate the apparent density of each major bacterium in the oil paste sampled from the Taura beach (Fig. II-6). A comparative densitometric analysis gave apparent population densities for O2-1, O2-2, O2-3 and O2-4 of 3.4×10^5, 3.5×10^5, 1.6×10^6 and 1.2×10^6 cells per gram of oil paste, respectively.

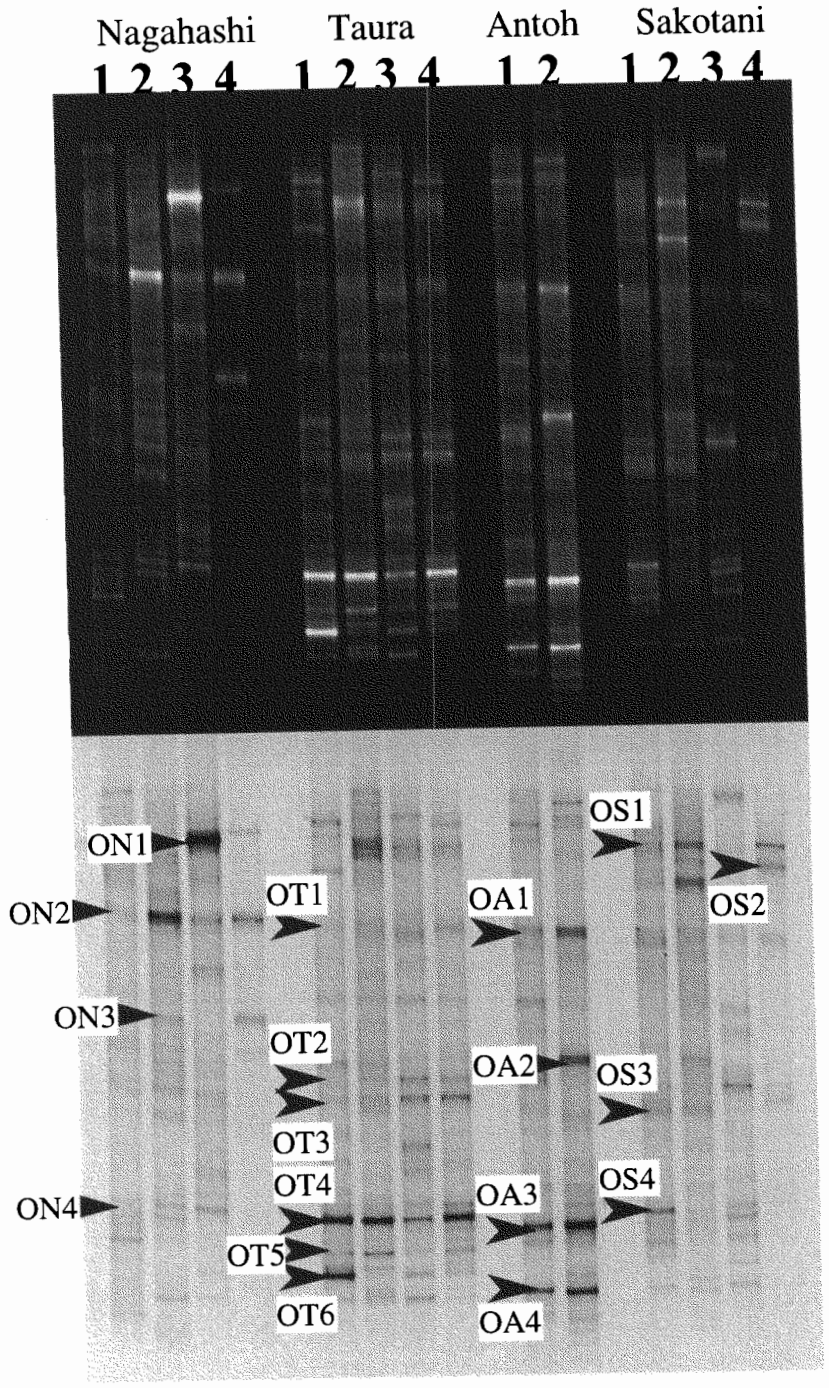


Fig. II-5. 16S rDNA DGGE profiles of the oil samples. The lower part shows bands excised for DNA sequencing. Numbers above the DGGE profiles indicate the sampling time. Lane 1, November 1997; lane 2, July 1998; lane 3, November 1998; lane 4, June 1999

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

Sample	DGGE band	Phylogenetic group	Closest sequence	Homology (%)
SW	SN2	Cyanobacteria	*Synechococcus* sp. AB015058	98
	ST3		Uncultured cyanobacterium ATT6 AF125333	99
	SK2		*Synechococcus* sp. AB015058	98
	SNR1	CFB phylum	*Cytophaga* sp. strain JTB244 AB015262	95
	SNR2, SN3		*Cytophaga* sp. strain JTB244 AB015262	96
	SN1		*Cytophaga* sp. strain JTB244 AB015262	94
	ST1		Unidentified bacterium HOS19 Z88577	100
	SK1		*Cytophaga* sp. strain JTB244 AB015262	94
	SA1, SH1, SS1, Stak1		Unidentified bacterium HOS19 Z88577	96
	SA2		*Cytophaga* sp. strain JTB244 AB015262	94
	SS2		*Cytophaga* sp. strain JTB244 AB015262	93
	S1	α-proteobacteria	Unknown α-proteobacterium clone PLY43 U13159.1	100
	S2		Unidentified α-proteobacterium OCS12 U75252	100
	SNR3	γ-proteobacteria	*Alteromonas macleodii* Y18228.1	95
	ST2		*Alteromonas macleodii* Y18228.1	97
	SA3		*Alcanivorax borkumensis* AF062642	100
Oil	ON3	CFB phylum	Marine psychrophile IC164 AF001372	94
	ON2, OA1, OS3	α-proteobacteria	*Sphingomonas surbactica* X94102	100
	OT3		Alpha proteobacterium MBIC1474 AB024595.1	99
	ON4, OT4, OS4	β-proteobacteria	*Hydrogenophilus thermoluteolus* TH-1 AB009828.1	92
	OT5		*Hydrogenophilus thermoluteolus* TH-1 AB009828.1	90
	ON1, OS1	γ-proteobacteria	*Alcanivorax borkumensis* AF062642	100
	OT1		*Stenotrophomonas maltophilia* strain LMG 10857 AJ131117	99
	OT2		Unidentified bacterium AJ001271	93
	OT6		Metal-contaminated soil clone K20-18 AF145821	92
	OA2		*Alkalispirillum mobilis* AF114783	92
	OA3		Unidentified gamma proteobacterium AB015254	91
	OA4		*Thiorhodovibrio winogradskyi* MBIC2776T AB016986	93
	OS2		*Alteromonas macleodii* Y18228.1	92

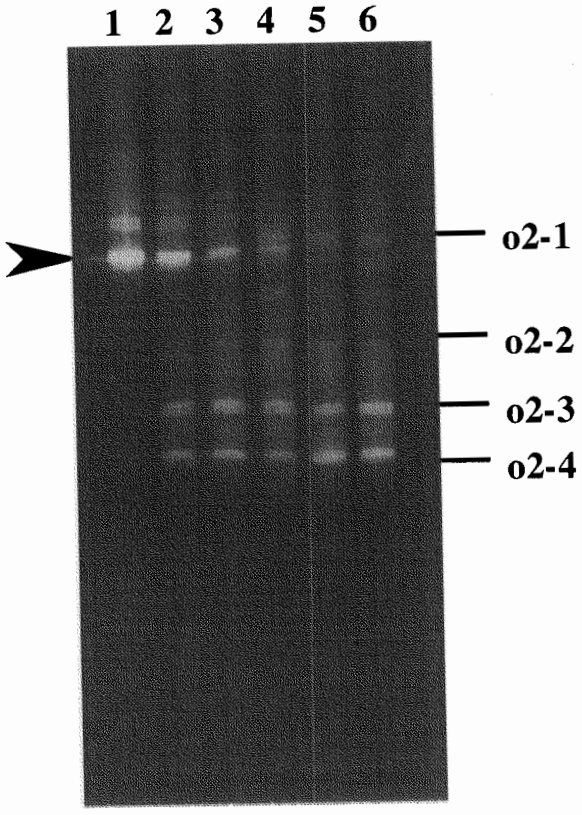


Fig. II-6. Competitive PCR/DGGE analysis for estimating the density of each major bacterial population in a Taura oil paste sample. The arrowhead indicates the band of the competitor (*P. putida*). Lanes 1 to 6, 1×10^9 to 1×10^4 cells of *P. putida* mixed per gram of oil paste.

II-4. Discussion

The water of the Japan Sea generally has a low nutrient concentration. The concentrations of total nitrogen and phosphorus were respectively about 0.1 mg/l and 0.01 mg/l as expected. While these values and those of DOC varied between the sampling sites, the fluctuation of one value was not independent of the others in each sample: a positive correlation was apparent between the total nitrogen and phosphorus concentrations, and between DOC and the total nitrogen concentration. Such correlations have been observed in other ecosystems too (Elser et al., 1995).

The total bacterial count for the July 1998 samples from the coast of Hyogo prefecture, namely the Hamazume, Takeno and Sakotani sites, was quite high, being almost 10^8/ml. However, the concentrations of carbon, nitrogen and phosphorus were not high enough to stimulate the growth of indigenous bacteria to this density, and we do not know why such a high population density of bacteria was observed at these sites in July 1998. An outbreak of red tide is one of the possibilities, as an increase in bacterial density has been observed in the early stage of red tide blooms (Romalde et al., 1990). I did not find any sign of red tide in these samples, although an outbreak of jellyfish was reported at these sites during this period. When these quite high total bacterial count data were not plotted, a positive correlation was observed between the total bacterial count and either the nitrogen or phosphorus concentration.

Although I did not measure the oil concentration in each sample before July 1998, the oil concentrations in seawater sampled in February 1997 (one month after the accident) from 11 beaches in Fukui prefecture were determined to be between 0.04 and 0.17 mg/l (Project team for the environmental protection of Fukui Prefecture, 1999). These values are of the same order of magnitude as those of our samples collected in July 1998. The oil concentration in the seawater, however, decreased gradually to approach the detection limit (0.02 mg/l) 29 months after the accident. Thus, the natural attenuation of the *Nakhodka* oil spill proceeded slowly, although I do not know the extent of the respective contributions of biodegradation, diffusion and other factors to this process.

The PCR/DGGE analysis of 16S rDNA fragments has been applied to detect specific microbial populations and to describe the overall structure of the microbial communities in various environments (Giovannoni et al., 1990; Choi et al., 1994). In this chapter, PCR/DGGE was used to analyze the major members of the bacterial population in seawater and those associated with the residual oil after the heavy-oil pollution. Different DGGE patterns in different samples were not PCR artifacts, as these patterns were reproducible in independent PCR/DGGE analyses. Two members (marked S1 and S2 in Fig. 4) that were closely related to α-proteobacterium clone PLY43 and to α-proteobacterium strain OCAS12, respectively, were ubiquitous in all the seawater samples. Otherwise, different bacterial populations were observed in the samples collected from different areas. Some local bacterial populations exhibited reproducible seasonal changes as have previously been observed (Pinhassi et al., 1997). The dominant members were related to the *Cytophaga-Flavobacter-Bacteroides* (CFB) phylum (Table II-3). This observation is consistent with previous reports on bacteria belonging to the CFB phylum (Delong et al., 1993; Bowman et al., 1997; Field et al., 1997) and α-proteobacteria (Field et al., 1997) being the major bacterial members in a marine environment.

It seems that the oil pollution have not strongly affected to the major marine bacterial members for a long time. This conclusion was drawn from three independent observations 10 months after the accident. First, two ubiquitous bacterial populations in oil-contaminated seawater (S1 and S2 in Fig. II-4) were found in non-contaminated seawater (Ajigasawa, Fig. II-4). Second, no population except one (SA3 in Fig. II-4) changed its size in response to the oil concentration. Third, phylogenetic characterization of the major bands indicated that they do not belong to genera containing members of the hydrocarbon-degrading bacteria such as *Sphingomonas*, *Ralstonia*, *Burkholderia*, *Pseudomonas* and *Mycobacterium*.

A bacterium closely related to *Alcanivorax borkumensis*, which is known as an alkane-degrading and surfactant-producing γ-proteobacterium (Yakimov et al., 1998), was detected by PCR/DGGE in the Antoh beach seawater sample (Fig. II-4). This was the only case of a hydrocarbon-degrading bacterium being detected in a seawater sample as a distinct DGGE band. TPH was a significant component of DOC (11% mean value) in the seawater samples

of July 1998. However, oil-degrading bacteria remained in a minority, probably because factors other than carbon availability were limiting for bacterial growth, and most hydrocarbon-degrading bacteria were not competitive with other marine bacteria under such conditions.

The major bacterial members detected in the oil samples were different from those in the seawater samples (Fig. II-5). This observation suggests that pore water in the oil paste was environmentally very different from a seawater column, and that specific bacteria were enriched in it. In fact, several members of the bacterial population detected in the oil samples were related to such hydrocarbon-degrading bacteria as *Sphingomonas spp.* (Fredrickson et al., 1995; Nohynek et al., 1996), *A. borkumensis* (Yakimov et al., 1998) and *Stenotrophomonas maltophilia* (Boonchan et al., 1998), an unidentified bacterium (AJ001271) and α-proteobacterium MBIC 1474. Among them, a bacterium closely related to *H. thermoluteolus* TH-1 was most frequently detected in the oil samples. *H. thermoluteolus* TH-1 is a thermophilic, facultatively chemolithoautotrophic, hydrogen-oxidizing bacterium that has been isolated from a hot spring (Hayashi et al., 1999). Hydrocarbon degradation by this bacterium is not yet known.

Although the residual oil samples contained several species of hydrocarbon-degrading bacteria as major components, their population sizes were relatively small, being less than 10^7/ml. This bacterial density was not sufficient for efficient degradation of the oil. However, when nitrogen and phosphorus fertilizers were supplemented to the seawater, the speed of biodegradation was strongly promoted (Table II-4). The application of nitrogen and phosphorus fertilizers to beaches contaminated by the *Nakhodka* accident would thus promote the degradation of residual oil.

MacNaughton *et al.* (1999) have recently conducted a population analysis of the bacteria in sand contaminated by an artificial oil spill in Delaware. They observed the development of several bacteria, most being α-proteobacteria. These bacteria were not identical to those detected in my study. I obviously cannot directly compare their results with mine, as the environmental conditions were quite different between these two studies. The degree of artificial pollution in Delaware was significant, but much smaller than that in the Japan Sea.

The seawater off a Delaware beach contained a much higher concentration of nitrogen (0.8 mg of N/l) than the Japan Sea. The experiment in Delaware was conducted for three months after the oil spill, while I started my observation 10 months after the oil spill and continued it for the next 19 months.

Chapter III

Predominant growth of *Alcanivorax* strains in the oil-contaminated seawater supplemented with nutrients

III-1. Introduction

A large oil spill is one of the most serious disasters to the marine environment. Oil released into a marine environment affects marine organisms in many ways (Hose and Brown, 1998; Hester and Mendelssohn, 2000), but it is gradually removed from the environment by the action of oil-degrading microorganisms, especially bacteria. The growth of these microorganisms in oil-contaminated seawater is, however, limited by nutritional requirements (Atlas, 1981; Atlas, 1988), and the addition of nitrogen and phosphorus fertilizers has been shown to be effective for the *in situ* cleanup of oil-contaminated beaches (Prince, 1993; Swannell *et al.*, 1996; Head and Swannell, 1999).

Many oil-degrading bacteria have been isolated and their degradation potential investigated. However, most of these studies have been carried out by using pure cultures, and the roles of these bacteria in a natural environment remain substantially unknown (Harayama *et al.*, 1999). Strains belonging to *Alcanivorax* were first isolated from the North Sea as biosurfactant-producing and *n*-alkane-degrading marine bacteria. These Gram-negative bacteria cannot use carbohydrates and amino acids as growth substrates (Yakimov *et al.*, 1998), but their growth on *n*-alkanes produces biosurfactants which have been shown to be glucose lipids (Abraham *et al.*, 1998).

In this chapter, I analyzed bacterial populations grown in oil-contaminated seawater. I found that a group of bacteria belonging to the genus *Alcanivorax* became the major bacterial population in oil-contaminated seawater when nitrogen and phosphorus nutrients were supplemented.

III-2. Materials and Methods

Culture conditions

In January 1997, the tanker *Nakhodka* sank in the Japan Sea, and more than 5,000 tons of heavy oil were released. The spilled oil contaminated more than 500 km of the coastline of the Japan Sea. Seawater, oil paste and oil-contaminated cobblestones were sampled from a beach in Ishikawa prefecture in November 1997, 10 months after the *Nakhodka* accident. The oil paste samples contained approximately 80% (w/w) of seawater. To each flask containing 100 ml of seawater collected from the same beach, oil paste (5 g) and/or two types of autoclaved fertilizers [0.5 g of a slow-release solid granular nitrogen fertilizer (Super IB, Mitsubishi Chemicals) and 0.1 g a slow-release solid granular phosphorous fertilizer (Linstar 30, Mitsubishi Chemicals)] were added, and the flasks were incubated on a reciprocating shaker at 60 rpm at 15°C for one month. Super IB contains 35% (w/w) nitrogen as isobutylidene diurea (IBDU) while Linstar 30 contains approximately 10% (w/w) phosphorous as calcium and/or magnesium phosphate salts.

Determination of bacterial cell number

The total bacterial count in the seawater was determined by fluorescence-microscopy after staining with 4',6-diamidino-2-phenylindole (DAPI) (Watanabe *et al.*, 1998). Colony-forming units (CFUs) were determined after incubating at 20°C for 7 days on agar plates containing one-fifth strength of Marine Broth (Difco) and 1.5% (w/v) of agar. The *Alcanivorax* strains formed flat and transparent colonies on the plates, and could thus be differentiated from the colonies of other strains. A good correlation between CFU and the direct cell count by fluorescent *in situ* hybridization (Syutsubo *et al.*, 2001) was obtained for *Alcanivorax* cells growing in seawater-based medium supplemented with nitrogen and phosphorus fertilizers (Syutsubo, unpublished data).

Analysis of the oil samples

Oil from the liquid cultures, grains of gravel and oil paste was extracted three times with

dichloromethane (DCM). DCM was then removed from each extract with a rotary evaporator. The dried oil sample was then dissolved in chloroform, transferred into a tube of known tare weight and dried again to measure the weight of the oil.

The oil samples were also analyzed by gas chromatography-mass spectrometry (GC-MS; GC/MS-QP5000, Shimadzu) to quantify a series of *n*-alkanes (C_{13-33}), C_{1-4}-alkylnaphthalenes, C_{0-4}-alkylfluorenes, C_{0-4}-alkyldibenzothiophenes and C_{0-7}-alkylphenanthrenes (where C_0 indicates a non-substituted compound), and the biomarker, 17α(H),21β(H)-hopane. GC-MS was done according to the method of Wang *et al.* (1998), all values obtained by the instrumental analyses being normalized to that of 17α(H),21β(H)-hopane (Prince *et al.*, 1994).

Extraction of DNA

Bacterial cells in the liquid culture were collected either by centrifugation or on a GV membrane (Millipore) by filtration, and DNA was extracted by the Marmur procedure (Marmur, 1961). DNA was extracted from the oil paste samples by putting approximately 1 g of a sample into 1 ml of a cell suspension buffer (10 mM Tris-HCl at pH 8.0, 1 mM EDTA, and 0.35 M sucrose), to which 1 ml of a lysing solution (50 mM Tris-HCl at pH 7.5, 25 mM EDTA, and 300 mM NaCl) and 0.5 ml of an emulsifier (Cactus Clean L-10A, Tesuko) were added. This suspension was thoroughly mixed to disperse the oil into the buffer, and then incubated at 55°C for 30 min. The suspension was then extracted 3 times with a phenol/chloroform (1:1) solution, and the aqueous solution was recovered. Two volumes of ethanol were added to the aqueous solution and, after gentle mixing, the solution was cooled at –20°C for 30 min. Nucleic acids were precipitated by centrifuging at 20,000 × *g* for 10 min, washed with 1 ml of a 70% (v/v) ethanol solution, and dissolved in 0.2 ml of a TE buffer containing 100 mg of RNase A. This solution was incubated at 37°C for 1 h and finally separated in a QIA-quick Spin-column (QIAGEN). The purity and quantity of the DNA were examined by recording its UV absorption spectrum.

Denaturing gradient gel electrophoresis of the PCR products

PCR primers P2 and P3 (Muyzer *et al.*, 1993) were used to amplify the variable V3 region of bacterial 16S rDNA (corresponding to positions 341 to 534 in the *Eschirichia coli* rRNA sequence) connected to the GC clamp. PCR was performed as described previously (Watanabe *et al.*, 1998). Amplification of the PCR products of expected sizes was confirmed by electrophoresis through 1.5% (w/v) agarose gel (LO3, Takara) in a TBE buffer.

Denaturing gradient gel electrophoresis (DGGE) (Muyzer *et al.*, 1996) was performed with a DCode^{TM} instrument (Bio-Rad) according to the manufacturer's instructions. Gels were made by using a gradient of denaturants between 38% [containing 2.66 M urea and 15.2% (w/v) formamide] and 58% [containing 4.06 M urea and 23.2% (w/v) formamide]. Ten ml of the PCR-amplified mixture were subjected to electrophoresis in 10% (w/v) polyacrylamide gel at 200 V for 3.5 h at a running temperature of 58°C. The gel was then stained with SYBR Green I (FMC Bioproducts) for 30 min according to the manufacturer's instructions.

A gel slice containing a DNA band was excised, and its DNA sequence was determined as described previously (Watanabe *et al.*, 1998). The resulting sequences were compared with the compilation of 16S rDNA genes available in the GenBank nucleotide library by a BLAST search through the National Center for Biotechnology Information (NCBI) Internet site (http://www.ncbi.nlm.nih.gov/BLAST/).

Sequence determination of 16S rDNA

Partial nucleotide sequences of 16S rDNA corresponding to positions 37-1370 of the *Escherichia coli* rRNA sequence were amplified according to the method of Edwards *et al.* (1989). The sequence of the 16S rDNA was determined with a Dye Terminator sequencing kit (Applied Biosystems), and the product was analyzed with an ABI Prism DNA sequencer (Applied Biosystems).

Crude oil biodegradation in columns

The crude oil used was Arabian light that had been heated at 230°C for 10 h to remove volatile fractions in it. Fresh seawater was collected from Kamaishi Bay at a depth of 15 m

and was filtered through a layer of fine sand to remove any debris. Grains of gravel (130 g) of a diameter between 2.4 and 4.8 mm were first immersed in seawater, and the wet grains were mixed with the heat-treated Arabian light crude oil. Extraction of the oil adhering to the gravel grains indicated that 1.788 ± 0.072 g of crude oil had been attached to 130 g of grains. The grains were packed in columns of 30 cm in length and 3 cm in diameter. Four grams of the Super IB nitrogen fertilizer and 0.3 g of the Linstar 30 phosphorous fertilizer were added on top of the gravel layer in one set of columns, while fertilizer was not added in the other (control) set of columns. The seawater was continuously supplied through a peristaltic pump to the top of each column at a flow rate of 25 ml/h, the water level in the column being adjusted to remain 4 cm above the top of the gravel layer.

Biodegradation of heavy oil in beach simulation tanks

A pair of tanks (1.5-m long by 1-m wide by 1-m deep) was set up to simulate the biodegradation of oil in the inter-tidal zone (Maki *et al.*, 1999). Each tank was partially filled with 1 m^3 of gravel grains (2 - 8 mm in diameter). Oil-polluted cobblestones sampled from a beach contaminated by the *Nadhodka* tanker accident were then put on top of the gravel layer. Seawater was continuously added to each tank at a flow rate of 60 l/h, the level of the seawater being adjusted by a level-controlling device to create the tidal cycle of 12 h with a range from 20 cm to 80 cm above the bottom of the tank, or approximately 30 cm above and 30 cm below the level of the oil-polluted cobblestones. Excess seawater was discharged from the level-controlling device. Four days after starting the seawater flow, 300 g of Super IB nitrogen fertilizer and 60 g of Linstar 30 fertilizer were uniformly spread in one tank but not in the other (control) tank.

Nucleotide sequence accession number

The nucleotide sequence data reported in this paper have been submitted to the DDBJ, EMBL and GenBank libraries, and listed in Table III-1.

III-3. Results

Alcanivorax predominated in batch cultures of oil-containing seawater when nitrogen and phosphorus fertilizers were supplemented

Heavy-oil paste containing approximately 20% (w/w) of oil and 80% (w/w) of water was collected from a heavy-oil-contaminated beach of the Japan Sea, 10 months after an oil-spill accident. From the same beach, seawater was also collected. Using these samples, three sets of seawater-based cultures, each in triplicate, were prepared. In one set, a non-sterilized oil paste (5 g), a sterilized nitrogen fertilizer (0.5 g) and a sterilized phosphorous fertilizer (0.1 g) were added to 100 ml of non-sterilized seawater; while in the second and third sets, the oil paste alone and the fertilizers alone, respectively, were added to the seawater. These cultures were incubated at 15°C for one month being constantly shaken to promote the growth of oil-degrading microorganisms indigenous to the oil paste and/or seawater. An increase in cell number was observed when the seawater was supplemented with the fertilizers. The cell number reached to approximately 3×10^7 cells per ml in the seawater supplemented only with the fertilizers, while the growth up to 3×10^8 cells per ml was observed in the seawater containing both the oil paste and the fertilizers. On the contrary, no significant growth was observed without the fertilizers even in the seawater supplemented with the oil paste (Fig. III-1).

Bacterial populations in these cultures were then analyzed by the denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rDNA fragments (Fig. III-2). Major DNA bands on the DGGE gels were excised and their DNA sequences were determined. It was found that the sequence of the intensive DNA band in lane 4 was 100% identical to that of *Alcanivorax borkumensis* (Yakimov *et al.*, 1998). Thus, bacteria closely related to *Alcanivorax* became predominant in the seawater containing both the oil paste and the fertilizers. The outgrowth of *Alcanivorax* was however not apparent in the control cultures where either the fertilizers or the oil paste were omitted.

Heavy oil remaining in these cultures was extracted, and its components were analyzed by gas chromatography/mass spectrometry (GC/MS). Without the fertilizers, the degradation

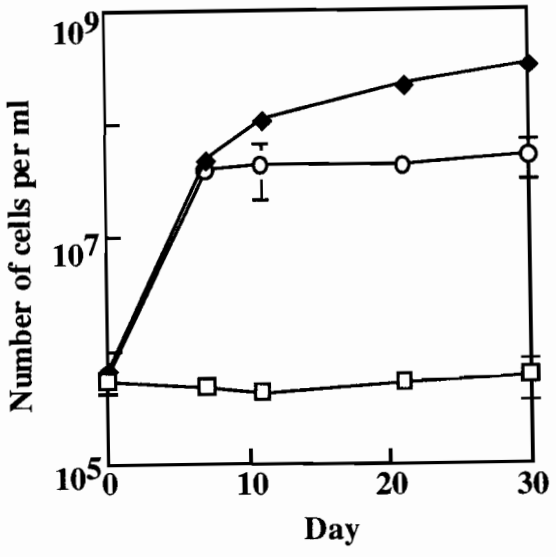


Fig. III-1. Bacterial growth in batch cultures of seawater supplemented with heavy-oil paste and/or fertilizers. Bacterial numbers in seawater supplemented only with the fertilizers (open circles), only with heavy-oil paste (open squares) or with both the fertilizers and the heavy-oil paste (closed squares) were determined after the DAPI staining of bacterial cells. The values are averages of three independent experiments. Error bars are not shown if the standard deviations were less than 20% of the averages

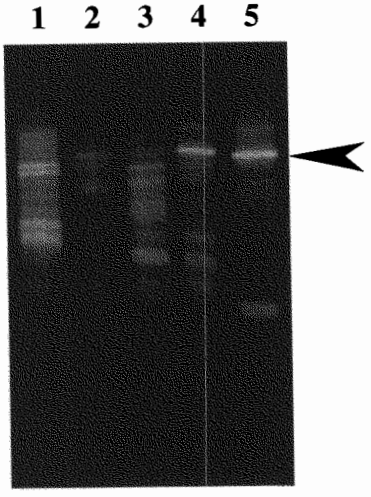


Fig. III-2. DGGE profiles of partial 16S rDNA fragments showing bacterial populations grown in seawater-based media. Lane 1: bacteria in a seawater sample used to prepare cultures of lanes 2 – 4. Lane 2: bacteria grown in the seawater supplemented only with heavy-oil paste. Lane 3: bacteria grown in the seawater supplemented only with the nitrogen and phosphorus fertilizers. Lane 4: bacteria grown in the seawater containing heavy-oil paste and the fertilizers. The arrow indicates the bands corresponding to *Alcanivorax*.

of crude oil was not significant, but the addition of the fertilizers promoted the degradation of certain components of crude oil: more than 90% of *n*-alkanes (C_{15}-C_{30}) and more than 60% of (alkyl)naphthalenes were degraded in 30 days, while the degradation of three-ring aromatics (phenanthrene, anthracene, fluorene and their alkyl-substituted derivatives) was less extensive being between 30 and 40% (Fig. III-3).

Alcanivorax was a predominant population of bacteria colonized on the surface of crude oil-polluted gravel

The biodegradation of crude oil attached to grains of gravel was studied by using the column system described in the Experimental procedures section. Seawater was continuously introduced into the columns at 20°C, and the number of bacteria in the effluent was determined by plating an appropriate amount of the effluent on 1/5 strength Marine Broth plates. In the effluent from the control column, to which no fertilizer had been added, the concentration of bacteria was in the order of $3 - 6 \times 10^6$ colony forming unit (CFU) per ml. In contrast, the CFU in the effluent increased with incubation time when the nitrogen and phosphorus fertilizers were added. Colonies with a diverse range of morphology developed on the plates, but transparent colonies hardly visible with naked eyes became predominant in effluent from the fertilized column within two weeks, although no such colony had been detected at day 0 (Fig. III-4). The transparent colonies were detected in effluent from the control column at a frequency less than 1%. The morphology of the transparent colonies was typical of *Alcanivorax* strains, and 16S rDNA sequences of several transparent colonies isolated in this study were very similar (>99% identity) to that of *Alcanivorax borkumensis* (Yakimov *et al.*, 1998).

After incubating for 30 days, the grains of gravel were immersed in sterile seawater and then sonicated in an ultrasonic cleaning bath for 2 min to release the grain-associated bacteria into the seawater. In the fertilizer-amended samples, *Alcanivorax*-type colonies dominated the population (9×10^8 CFU per gram of gravel), while the CFU value for other bacteria were in the order of 2×10^8/g. Such domination by *Alcanivorax*-type colonies was not apparent in the control column (5×10^4 CFU/g of *Alcanivorax*-type colonies versus 2×10^6 CFU/g of other

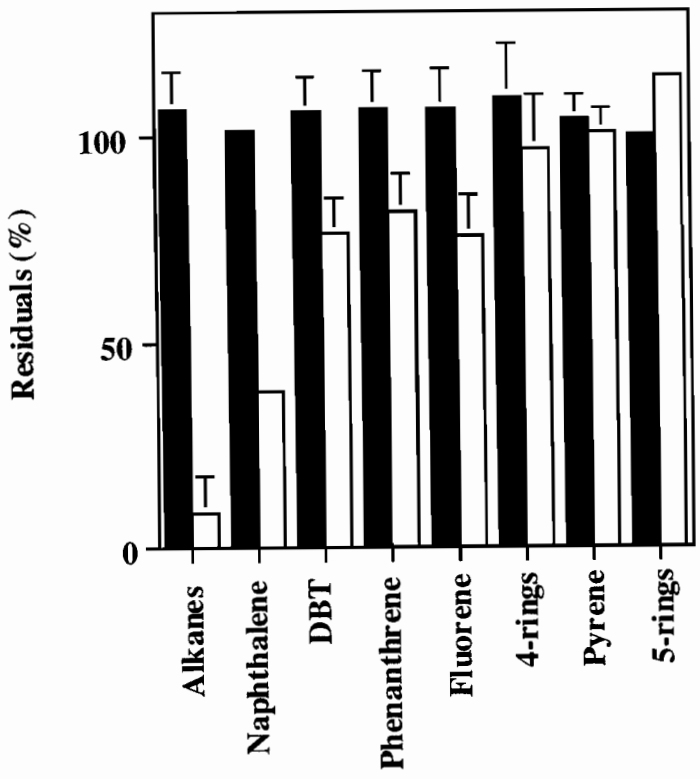


Fig. III-3. Biodegradation of GC/MS-detectable components of heavy oil in batch cultures. Biodegradation of heavy oil in the cultures shown in Fig. III-1 was examined. The concentrations of heavy oil components were determined in the cultures containing no fertilizers (solid bars) and those containing the nitrogen and phosphorus fertilizers (open bars) after the cultivation for 30 days. Each value was normalized by 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 original heavy oil sample was taken as 100%. Error bars are not shown if the standard deviations were less than 5% of the averages.

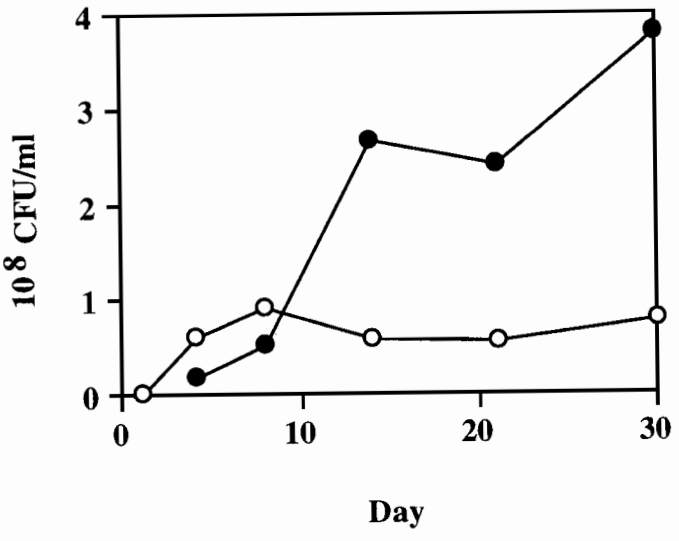


Fig. III-4. CFU of bacteria in the effluent from a column packed with oiled gravel and applied with the slow-release fertilizers. Numbers of *Alcanivorax*-type colonies (closed circles) and others (open circles).

bacteria) (Fig. III-4). CFU of bacteria in effluent from a column containing no fertilizer were 3 - 6 $\times 10^6$ per ml, and *Alcanivorax*-type colonies were less than 1%.

Similar results were obtained in independent experiments. Thus, *Alcanivorax* could efficiently colonize and grow on the surface of the oiled gravel when nitrogen and phosphorus nutrients were supplemented.

Gravimetric measurements indicated that 21% of the crude oil has been degraded after 30 days of incubation in the fertilizer-supplemented column, while only 3 - 4% of it has been degraded in the control column.

Alcanivorax became predominant in heavy-oil pastes attached to cobblestones

Heavy-oil-polluted cobblestones with sizes of 10 – 20 cm were sampled 10 months after an oil-spill accident from the oil-contaminated beach as described in Materials and methods. These cobblestones were placed in two beach simulation tanks (1.5-m long, 1-m wide and 1-m deep) undergoing tidal cycles with seawater. The nitrogen and phosphorus fertilizers were added to one tank, but not to the control tank. The temperature of seawater in the beach-simulation tanks was between 19.8°C and 22.8°C. Aliquots of oil paste on the surface of the cobblestones was periodically sampled, and oil components in the samples were analyzed by GC/MS. Furthermore, DNA was extracted from the paste, and the bacterial populations established in these samples were analyzed by PCR/DGGE.

Several major DNA bands on the DGGE gels were sequenced, and Table III-1 lists a sequence in the databases exhibiting the highest identity to the sequence of each DGGE band. In the fertilizer-amended tank, the bacterial population within oil paste was predominated by *Alcanivorax* in one week after the addition of the fertilizers, while no outgrowth of *Alcanivorax* was observed in the control tank (Fig. III-5).

The biodegradation of heavy-oil components was stimulated when the fertilizers were supplemented. More than 80% of *n*-alkanes, more than 70% of (alkyl)naphthalenes and more than 40% of three-ring aromatics (phenanthrene, anthracene, fluorene and their alkyl-substituted derivatives) were degraded in three months in the fertilizer-amended tank, while the biodegradation without the fertilizers was at a level of 30% degradation for *n*-alkanes and

(alkyl)naphthalenes and less than 5% degradation for three-ring aromatics.

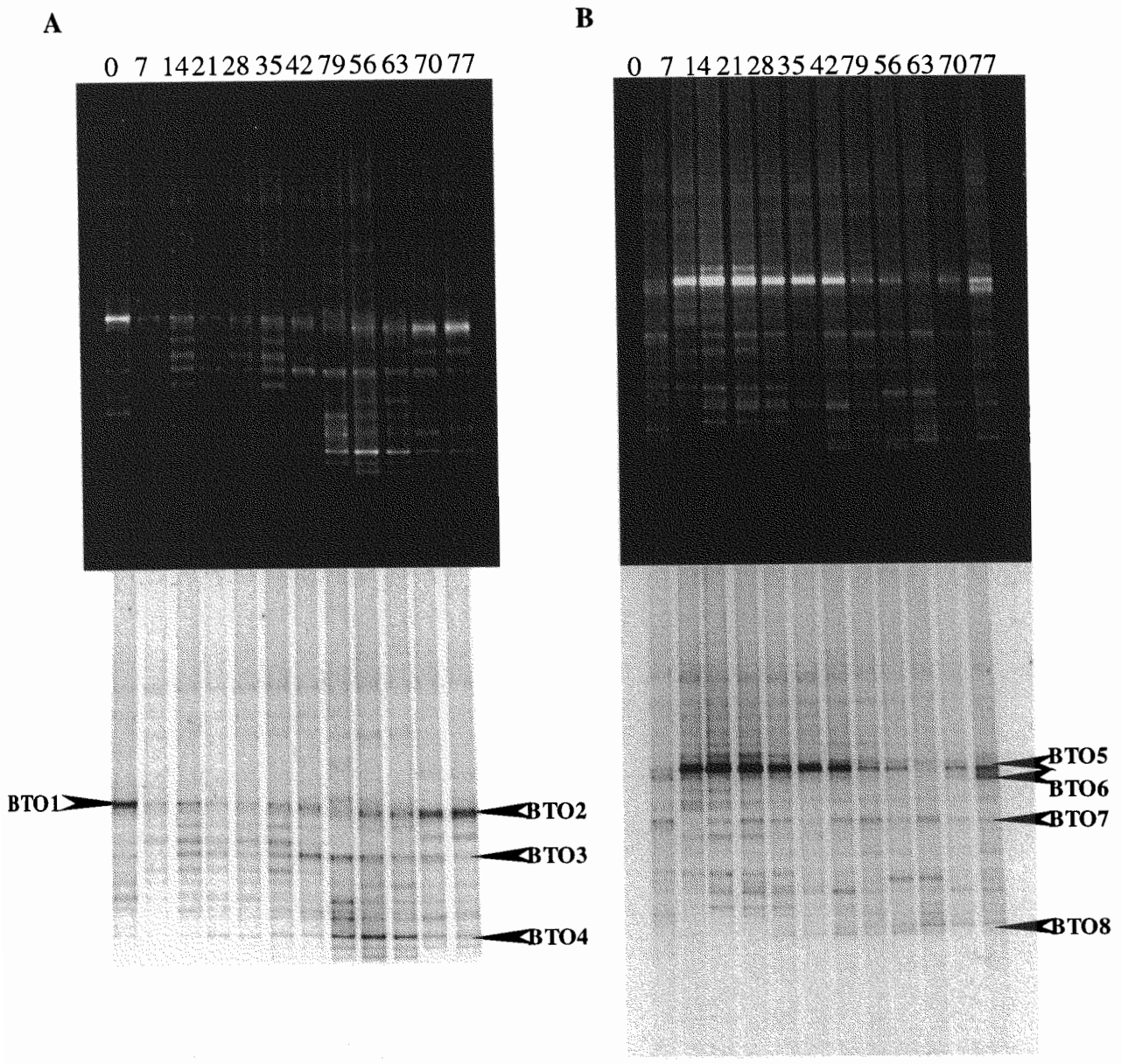


Fig. III-5. DGGE profiles of partial 16S rDNA fragments showing shifts in the major bacterial populations in heavy oil paste in response to the addition of the nitrogen and phosphorus fertilizers. Heavy-oil-polluted cobblestones sampled from an oil-contaminated beach were placed in two beach simulation tanks, and fresh seawater was continuously introduced into the tanks. In one tank, no fertilizer was added (A), while in another tank, the slow-release nitrogen and phosphorus fertilizers were added on days 0, 63 and 98 (B). Oil samples were collected on days 0, 7, 14, 21, 28, 35, 42, 49, 56, 63, 70 and 77 as indicated. The lower part shows bands excised for the DNA sequencing analysis.

Table III-1. 16S rDNA sequences most closely related to those of the major bacterial populations detected by PCR/DGGE

DNA band (accession number)	Most closely related organism (accession number)	Sequence identity (%)
BTO1 (AB074311)	*Bartonella bacilliformis* (Z70003)	97
BTO2 (AB074312)	*Cycloclasticus pugetii* (U12624)	100
BTO3 (AB074313)	*Sphingomonas subarctica* (X94104)	100
BTO4 (AB074314)	Uncultured marine bacterium OT5 (AB035030)	98
BOT5 (AB074315)	*Alcanivorax borkmensis* (AF062642)	100
BTO6 (AB074316)	*Cycloclasticus pugetii* (U12624)	100
BTO7 (AB074317)	*Sphingomonas subarctica* (X94104)	100
BTO8 (AB074318)	Bacterium Km4 (AF367848)	100

III-4. Discussion

After the *Exxon Valdez* tanker accident occurred, bioremediation of the oiled beaches was attempted on a large scale. The idea behind this operation was that the ability of indigenous microorganisms in degrading the oil is limited by the lack of available nitrogen and phosphorus nutrients, and therefore that the application of fertilizers to the beaches should increase the biodegrading capacity. Detailed analyses of the bioremediation demonstrated that the rate of biodegradation was increased two- to three-fold by the application of fertilizers (Bragg *et al.*, 1994). This study as well as others (e.g. Rosenberg *et al.*, 1996), however, did not identify the major players in the bioremediation of oil-contaminated marine environments.

In this chapter, I found that bacteria belonging to *Alcanivorax* predominated in seawater containing crude oil or heavy oil when nitrogen and phosphorus nutrients had adequately been supplemented. The predominance of *Alcanivorax* was observed in several experimental systems: (i) in batch cultures of seawater containing heavy oil, (ii) on the surface of crude-oil-polluted gravel, which has been packed in bench-scale columns undergoing a continuous seawater flow, and (iii) in heavy-oil pastes adhered on the surface of cobblestones, which have been placed in large-scale tidal flux reactors. Thus, the present study confirmed and extended the preliminary observation that *Alcanivorax* is a dominant population in a batch culture of seawater containing crude oil and appropriate nutrients (Syutsubo *et al.*, 2001). I do not yet know why *Alcanivorax* predominated under such conditions regardless of the presence of diverse oil-degrading bacteria in the marine environment. The growth of *Alcanivorax* on crude or heavy oil was not particularly rapid in comparison with other oil-degrading bacteria inhabiting in seawater, and currently my colleague is testing two working hypotheses to explain the predominance of *Alcanivorax*: (i) *Alcanivorax* can utilize broader hydrocarbon substrates than other oil-degrading bacteria; (ii) glucose lipid biosurfactants produced by *Alcanivorax* increases the bioavailability of petroleum hydrocarbons to this organism, but reduces it to many other oil-degrading bacteria.

In chapter II, I analyzed bacterial populations in heavy-oil pastes sampled from beaches contaminated by a tanker accident. Several bacteria related to hydrocarbon degraders,

including *Alcanivorax,* were detected, although *Alcanivorax* was not a predominant population. The concentrations of the nitrogen and phosphorus nutrients in seawater samples from the beaches were low, and this could explain why *Alcanivorax* was not a major population in these samples.

Field experiments simulating a coastal oil spill have been conducted, and *Alcanivorax* was not detected as the major population in core samples from oil-contaminated plots (MacNaughton *et al.*, 1999). In this study, the samples were collected only 8 and 14 weeks after the start of the experiments. Earlier samplings might detect an outbreak of *Alcanivorax*, because my experiments using the beach simulation tanks indicated that the predominance of *Alcanivorax* occurred only between one and six weeks after the addition of the fertilizers.

Chapter IV

Cycloclasticus Plays a Primary Role in the Degradation of Aromatic Hydrocarbons Released in a Marine Environment

VI-1. Introduction

An oil spill is one of the most serious disasters that can occur in a marine environment (Hose and Brown, 1998; Hester and Mendelssohn, 2000). In the early stage of an oil spill, the light fraction of the oil evaporates, while its heavier fraction is slowly removed by photo-oxidation and biodegradation (Dutta and Harayama, 2000). Microorganisms, and especially bacteria, play an important role in the biodegradation of the spilled oil (Leahy and Colwell, 1990). However, the growth of oil-degrading bacteria in seawater, and the resulting biodegradation of the oil in seawater, is limited by nutritional requirements (Atlas, 1981, Atlas, 1988). The addition of nitrogen and phosphorus fertilizers has been shown to enhance the biodegradation of oil released in a marine environment (Prince, 1993; Swannell et al., 1996; Head and Swannell, 1999).

Oil is a complex mixture made up of hundreds of compounds, and these are classified into four groups, namely saturates, aromatics, resins and asphaltenes (Dutta and Harayama, 2001). Aromatics are the second most abundant hydrocarbons in crude oil. Benzene, naphthalene, phenanthrene and their alkyl-substituted derivatives represent typical aromatics (Wang et al., 1998). Although the biodegradation of such simple aromatics as benzene, toluenes, xylenes, naphthalene and phenanthrene has been extensively characterized (Harayama et al., 1992), the biodegradation of alkyl-substituted polynuclear aromatic hydrocarbons has scarcely been studied (Miyachi et al., 1993; Mahajan et al., 1994; Dutta et al., 1998).

One group of bacteria capable of degrading aromatics in a marine environment is *Cycloclasticus*. These bacteria have been isolated from several locations, namely Resurrection Bay (Alaska), Puget Sound (Washington) and Gulf of Mexico (Button et al., 1993; Dyksterhouse et al., 1995; Geiselbrecht et al., 1998). They utilize aromatic hydrocarbons

including such aromatic hydrocarbons as biphenyl, naphthalene, phenanthrene, toluene and xylenes as sole sources of carbon and energy, while they cannot utilize sugar and amino acids.

In the present chapter, I investigated the roles of *Cycloclasticus* in the biodegradation of petroleum PAHs in a marine environment. I found that *Cycloclasticus* was the most abundant PAH-degrading bacterium associated with oil-polluted grains of gravel, the bacterial density reaching 10^8/g of grains after supplementing with nitrogen and phosphorus.

VI-2. 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 the sampled seawater was distributed 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 polynuclear aromatic hydrocarbon (PAH: 2-methylnaphthalene, phenanthrene or anthracene). The mixture was 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 is the sign of ring cleavage of the aromatic compounds (Gurin and Jones, 1988; Menn et al., 1993).

From the enrichment cultures with phenanthrene, bacteria were isolated using the most-probable-number (MPN) technique (de Man, 1975; Geiselberecht et al., 1996). Serial 10-fold dilutions of the phenanthrene-enrichment cultures were made with ONR7a artificial seawater medium (Dyksterhouse et al., 1995) supplemented with 1 mg/ml of phenanthrene as the sole source of carbon and energy, and the mixtures were incubated at 20°C in the dark for about four weeks. The most diluted cultures giving a color change were spread onto ONR7a plates overlaid with an opaque layer of 0.8% agarose containing 1 mg/ml of phenanthrene (Bogardt and Hemmingsen, 1992). The surface of the plates was opaque, and those colonies surrounded by a clear zone were further purified by restreaking on the same type of plates.

The purified colonies were subjected to repetitive extragenic palindromic sequence PCR (rep-PCR) to identify identical strains, as described previously (de Bruijin, 1992). The rep-PCR analysis was repeated several times to determine the reproducibility of the method.

Biodegradation of crude oil in the beach-simulating tanks

The beach-simulating-tank system consists of a tank (1 m by 1.5 m and 1 m in depth), a reservoir and a level-controlling devise (Maki et al., 1999). Seawater was collected from a 15-m depth of Kamaishi Bay, and filtered through layers of coarse and fine sand. It was introduced into the reservoir at a constant flow rate of 60 l/h. In the reservoir, seawater was aerated by bubbling and temperature-controlled to approximately 20°C. The seawater was

subsequently introduced, at a flow rate of 60 l/h, into the tank which was filled two-thirds with 1 m^3 of gravel grains of 2 to 8 mm in diameter. The room temperature was controlled at 20 ± 2°C while the temperature of seawater in the tank varied between 19.1 and 23.5°C. The "tidal level" in the tank was regulated to oscillate at two cycles per day between the low tide mark of 20 cm and the high tide mark of 80 cm above the bottom of the tank, or 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 were 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 adding the crude oil, 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 (No. 2), while no fertilizer was added to the other (No. 1) tank as a control. Three independent experiments were conducted using a pair of these beach-simulating tanks.

The total bacterial count in the seawater sampled from the beach-simulating tank was determined by fluorescence-microscopy after staining with 4,6-diamidino-2-phenylindole (DAPI) (Watanabe et al., 1998). To analyze the microbial population shift and the degradation of crude oil components, approximately 2 l of seawater and approximately 40 g of oil-coated grains were periodically collected at high tide level and at low tide level, respectively.

Extraction of DNA

The microorganisms in an approximately 10 ml of liquid culture or in a 2 l of seawater sampled from the beach-simulating tank were collected on a GV membrane (Millipore) by filtration, and DNA was extracted by the Marmur procedure (Marmur, 1961). DNA of the microorganisms attached to grains of gravel was extracted by putting approximately 20 g of a sample into 13.5 ml of a DNA extraction buffer [100 mM Tris-HCl (pH8.0), 100 mM ethylenediamine tetraacetic acid (pH8.0), 100 mM sodium phosphate (pH8.0), 1.5 M NaCl, and 1% (wt/vol) hexadecyltrimethylammonium bromide (CTAB)], to which 0.1 ml of 1% (wt/vol) proteinase K was added. This suspension was incubated at 37°C for 30 min while shaking horizontally, 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 gently inverting it, 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. A 0.6 volume of isopropyl alcohol was added to the aqueous solution, and after gently mixing, the solution was left at room temperature for 1 h. Nucleic acids were precipitated by centrifuging 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 a Tris-EDTA (TE) buffer containing 100 mg of RNase A. This solution was incubated at 37°C for 1 h, and DNA was recovered by ethanol precipitation (Sambrook et al., 1989). The DNA purity and quantity were determined by recording its UV absorption spectrum (Sambrook et al., 1989).

Denaturing gradient gel electrophoresis of the PCR products

About 50 ng of DNA extracted from enrichment cultures, seawater, or grains were used as the template in a PCR. PCR primers P2 and P3 (Muyzer et al., 1993) were used to amplify the V3 region of bacterial 16S rDNA (corresponding to positions 341 to 534 in the *Escherichia coli* rRNA sequence) connected to the GC clamp. PCR was performed as described previously (Watanabe et al., 1998), and amplification of the PCR products into the expected sizes was confirmed by electrophoresis through 1.5% (wt/vol) agarose gel (LO3, Takara) in a TBE buffer [89 mM Tris-borate (pH 8.3), 2 mM Na_2EDTA; Sambrook et al., 1989). Denaturing gradient gel electrophoresis (DGGE) (Muyzer et al., 1996) was performed with a DCode™ instrument (Bio-Rad) as described previously (Kasai et al., 2001). To determine DNA sequence, gel slice containing a DNA band was excised and processed as described previously (Watanabe et al., 1998). A search of the GenBank nucleotide library for sequences similar to the determined sequences was made by using BLAST (Atschul et al., 1990) through the National Center for Biotechnology Information (NCIB) Internet site (http://www.ncbi.nlm.nih.gov/BLAST/).

Sequencing of 16S rDNA and *gyrB*, and phylogenetic analysis

The 16S rRNA genes (rDNA) were amplified by PCR with primers 27f and 1492r

(corresponding to positions 8 to 1510 in the *Escherichia coli* rRNA sequence) (Weisburg et al., 1991). 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 incubation at 72°C was continued for 10 min. These PCR products were electrophoresed through a 0.8% (wt/vol) agarose gel with TBE buffer (Sambrook et al., 1989), and then 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 determined nucleotide sequences were aligned by CLUSTAL W version 1.7 (Thompson et al., 1994) with several reference sequences of the g subdivision of *Proteobacteria*. A phylogenetic tree was constructed from the evolutionary distance data (Kimura, 1980) by the neighbor-joining method (Saitou and Nei, 1987). The bootstrap resampling method of Felsenstein (Felsenstein, 1985) 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*) were PCR-amplified with the two degenerate primers, UP-1E and UP-2r, as described previously (Yamamoto and Harayama, 1995). The *gyrB* PCR product was purified and sequenced by the protocol already described for the 16S rDNA genes. The *gyrB* sequences were aligned by CLUSTAL W version 1.7 (Thompson et al., 1994) with reference sequences of the g subdivision of *Proteobacteria* compiled in the ICB database (Kasai et al., 1998). A phylogenetic tree based on *gyrB* was constructed as described for the 16S rDNA-based tree.

Crude oil degradation ability

Arabian Light crude oil was heated at 230°C for 4 h to remove the light fraction (Dutta and Harayama, 2000), and the ability of *Cycloclasticus* strains A5 and H4 to degrade hydrocarbons in the crude oil was examined in 10 ml of ONR7a medium supplemented with 1 mg/ml of heat-treated Arabian Light crude oil. Approximately 10^3 cells of the *Cycloclasticus* strains were used to inoculate the triplicate 10-ml 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 four weeks. Sterile samples were similarly incubated and served as controls. The cell growth and

degradation of the crude oil components after four weeks were examined as described later.

Analysis of the oil

Oil was extracted from the cultures or from the grains of gravel as described previously (Dutta and Harayama, 2000), and analyzed by gas chromatography-mass spectrometry (GC-MS; GC/MS-QP5000, Shimadzu) to quantify a series of *n*-alkanes (C_{13-33}), C_{0-4}-alkylnaphthalenes (C_0 indicates a non-substituted aromatic compound while 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 conducted according to the method of Wang et al. (Wang et al., 1998). All values obtained by the instrumental analyses were normalized by dividing by the value for 17α(H),21β(H)-hopane (Prince et al., 1994).

Quantitative competitive PCR

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), the sizes of the target fragments and competitors being 318 and 286 bp, respectively. Amplification was performed with a Progene thermal cycler (Techne) by using a 50 ml 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 deoxynucleotide triphosphate at a concentration of 200 mM, 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 sec at 94°C, 1 min at 60°C and 30 sec at 72°C, and a final 10 min extension at 72°C. Ten μl of PCR product was electrophoresed through 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 is considered to be equal to the copy number of the *gyrB* sequence (Blattner et al., 1997).

Nucleotide sequence accession numbers

The nucleotide sequence data reported in this paper have been submitted to the DDBJ, EMBL and GenBank libraries under accession no. AB080091 to AB080110.

VI-3. Results

Isolation of the *Cycloclasticus* strains

The enrichment cultures of PAH-degrading bacteria were obtained as described in the Materials and Methods section. DNA was extracted from the original enrichment cultures, and the bacterial populations in the cultures were analyzed by DGGE. The most intense bands on the DGGE gels had sequences closely related to the 16S rDNA sequence of *Cycloclasticus pugetii* strain PS-1 (data not shown). Thus, bacteria related to the genus *Cycloclasticus* were most frequently found in these enrichment cultures regardless of the growth substrates used (2-methylnaphthalene, phenanthrene or anthracene). The PAH-degrading bacteria were further screened by applying the most-probable-number technique, using phenanthrene as the sole source of carbon and energy, and purified on plates containing phenanthrene. DNA of the purified clones was isolated, and their rDNA fragments were PCR-amplified for the DGGE analysis. The rDNA fragments from 35 isolates migrated to the same position as that of the *Cycloclasticus* band suggesting that all the 35 clones belonged to the genus *Cycloclasticus*. They were divided into four distinct groups called A5 (21 isolates), E2 (5 isolates), E3 (1 isolate) and H4 (1 isolate) by rep-PCR (8). One strain from each of these four groups was selected, and the 16S rDNA and *gyrB* sequences of these four selected strains A5, E2, E3 and H4, were determined for phylogenetic analyses.

Phylogenetic analysis

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

Degradation of hydrocarbons in crude oil

The cell growth and degradation of the crude oil components after four weeks were examined in triplicate samples by q-cPCR and GC-MS. The cell numbers of strains A5 and H4

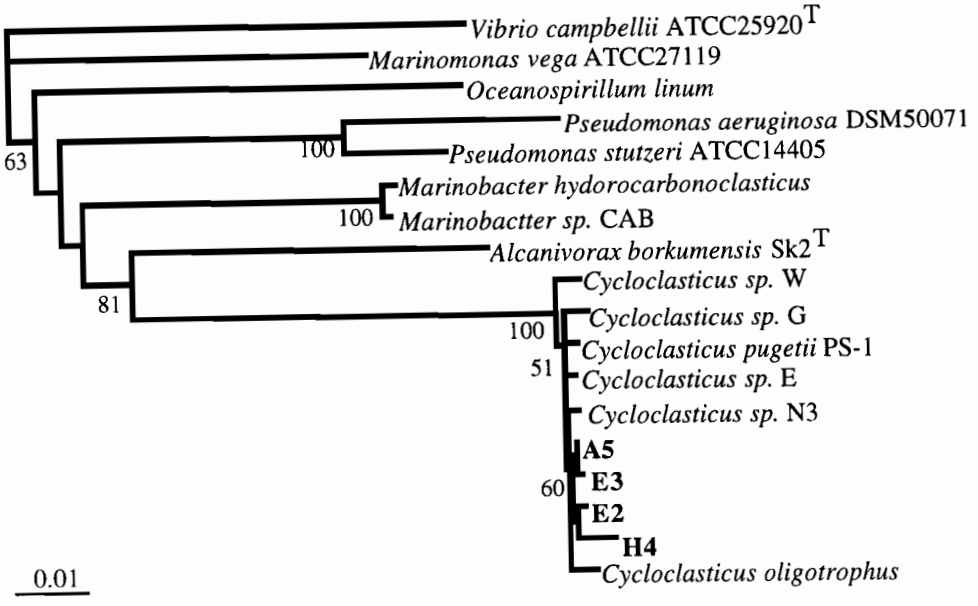


Fig. IV-1. Phylogenetic relationships based on 16S rDNA sequences among strains A5, E2, E3, H4 and representative members of the γ subdivision of *Proteobacteria*. Bootstrap values greater than 50% are indicated at the nodes. The scale bar indicates 0.01 substitution per site.

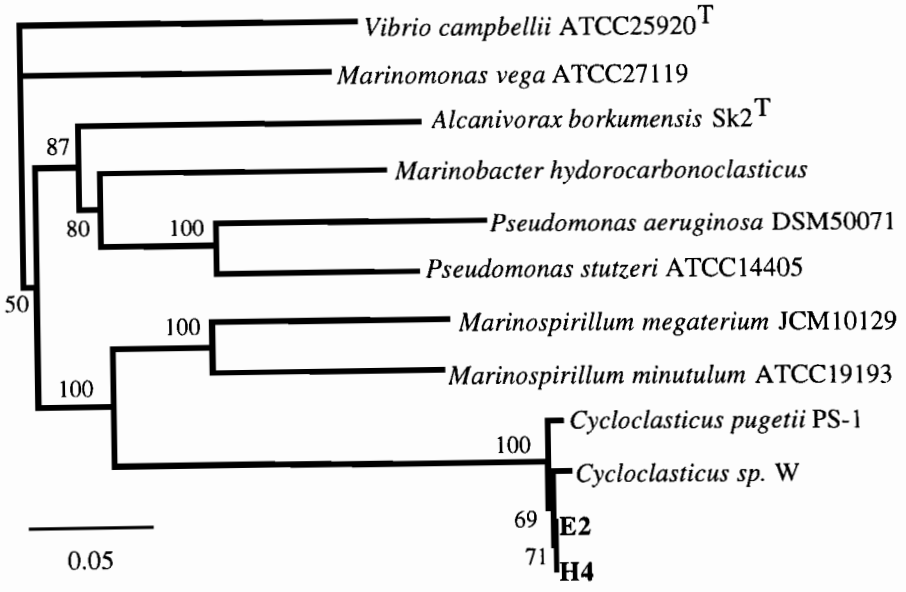


Fig. IV-2. Phylogenetic relationships based on *gyrB* sequences among strains E2, H4 and representative members of the γ subdivision of *Proteobacteria*. Bootstrap values greater than 50% are indicated at the nodes. The scale bar indicates 0.05 substitution per site.

increased to approximately 5×10^5 cells per ml. The concentrations of crude oil components were normalized by the concentrations of 17α(H),21β(H)-hopane, and the normalized values in three independent experiments were averaged. The average concentration of each component in the control crude oil samples was taken as 100%. Both *Cycloclasticus* strains degraded C_{0-4}-alkylnaphthalenes, C_{0-1}-alkyldibenzothiophenes, C_1-alkylphenanthrene and C_{0-2}-alkylfluorenes at levels greater than 80%. They also degraded almost 60-70% of C_2-alkyldibenzothiophenes and C_0 and C_2-alkylphenanthrenes. The degradation levels of *n*-alkanes, C_{3-4}-alkyldibenzothiophenes, and C_{3-7}-alkylphenanthrenes were not significant being less than 10% (Table IV-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 mimic an oil-polluted beach. Natural seawater was continuously introduced into two tanks containing oil-polluted grains of gravel, and nitrogen and phosphorus fertilizers were added to the No. 2 tank, but not to the No. 1 tank. The grains of gravel were sampled, 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 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 taken as 100% (Table IV-2).

The degradation patterns of crude oil by a bacterial community in the natural seawater were the same in the three experiments. When no fertilizer was added, C_{4-7}-alkylphenanthrenes were not degraded, and C_4-alkylnaphthalene, C_{2-4}-alkyldibenzothiophenes, C_3-alkylphenanthrene and C_2-alkylfluorene were degraded by only 30% and 20%, respectively, in two months. On the other hand, the supply of fertilizers stimulated the biodegradation of crude oil: *n*-alkanes, C_{0-4}-alkylnaphthalenes, C_{0-2}-alkyldibenzothiophenes, C_{0-2}-alkylphenanthrenes and C_{0-2}-alkylfluorenes were completely degraded, C_{3-4}-alkyldibenzothiophenes and C_{3-5}-alkylphenanthrenes were degraded by more than 60%, and C_{6-7}-alkylphenanthrenes were degraded by more than 20% in two months (Table IV-2).

Table IV-1. Biodegradation of crude oil components by *Cycloclasticus* spp.

Oil component	Residual amount[a] (SE)[b] (%)			
	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-DBT [c]	7.3	(2.2)	6.5	(1.1)
C_1-DBT	5.0	(0.8)	5.7	(0.7)
C_2-DBT	35.8	(3.5)	23.2	(4.9)
C_3-DBT	100.7	(7.4)	93.5	(5.7)
C_4-DBT	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 aerobically incubated at 25°C for four weeks. Sterile samples were similarly incubated and served as controls. Oil was extracted from the cultures as described in the materials and methods section.

[b] Standard error

[c] Dibenzothiophene

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

Oil component	Residual amount [a] (S.E.) (%)					
	Day (control tank)			Day (fertilizer-added tank)		
	14	28	56	14	28	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-DBT	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-DBT	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-DBT	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-DBT	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-DBT	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] Initial concentration of each component at time 0 taken as 100%

The addition of the fertilizers resulted in an increase in the total bacterial count in seawater up to 1×10^7 cells per ml in 21 days, while no such increase in the total bacterial count was apparent in the seawater from the No. 1 tank, the count fluctuating between 3×10^5 and 1×10^6 cells per ml (data not shown).

Twenty grams of oil-polluted grains of gravel were periodically sampled, and DNA was extracted from the microorganisms attached to the grains for a PCR/DGGE analysis to monitor the change in the bacterial population associated with grains of gravel (Fig. IV-3). Some major bands were excised and their DNA sequences were determined. Table IV-3 lists a sequence in the databases exhibiting the highest nucleotide identity to each DGGE band. In the No. 1 tank without any added fertilizer, *Alcanivorax* and *Cycloclasticus* were detected after 7 days, while *Marinobacter* was detected between days 0 and 15. In the No. 2 tank, *Alcanivorax* was detected on days 7 and 15, while *Cycloclasticus* was detected between day 0 and day 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 experiments.

The bacterial populations in seawater were also analyzed by PCR/DGGE, and the results from one of three experiments are shown in Fig. IV-4. The pattern of bacterial populations in the No. 1 tank was relatively constant, and such bacteria *Roseobacter* and *Bdellovibrio* that are common in a marine environment were detected. Hydrocarbon degradation by bacteria in these groups is not well known. In the No. 2 tank, *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 the No. 2 tank to which the crude oil and fertilizers were added.

The population size of *Cycloclasticus* attached to the grains was determined by q-cPCR (Fig. IV-5). Almost 10^3 *Cycloclasticus* cells were detected on 1 g of grains before the introduction of crude oil to these tanks. After the crude oil had been introduced, the cell number of *Cycloclasticus* increased to 3-4 $\times$ 10^6 cells/g in one day, even without the addition of

fertilizers. The size of the *Cycloclasticus* population continued to increase to 10^8 cells/g in two weeks when the fertilizers were added, while the cell number stayed at a level of 3×10^6 cells/g when no fertilizer was added.

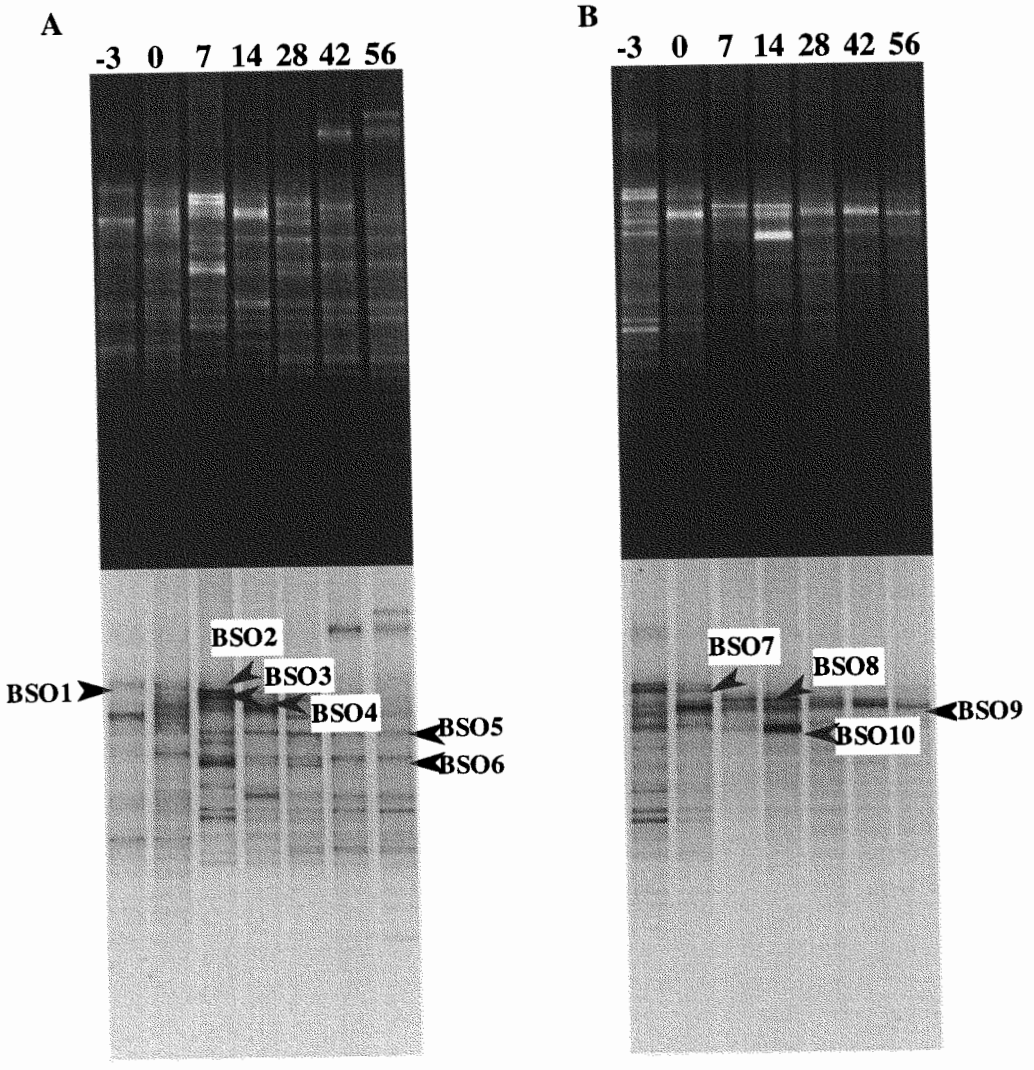


Fig. IV-3. DGGE profiles of partial 16S rDNA fragments showing major bacterial populations attached to the surface of oil-polluted grains of gravel. The lower part shows bands excised for the DNA sequencing analysis. Fresh seawater was continuously introduced into two beach-simulating tanks, and at day 0, 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 on day 0 (B). The oil-polluted grains of gravel samples were collected on days -3, 0, 7, 21, 28, 42 and 56 as indicated.

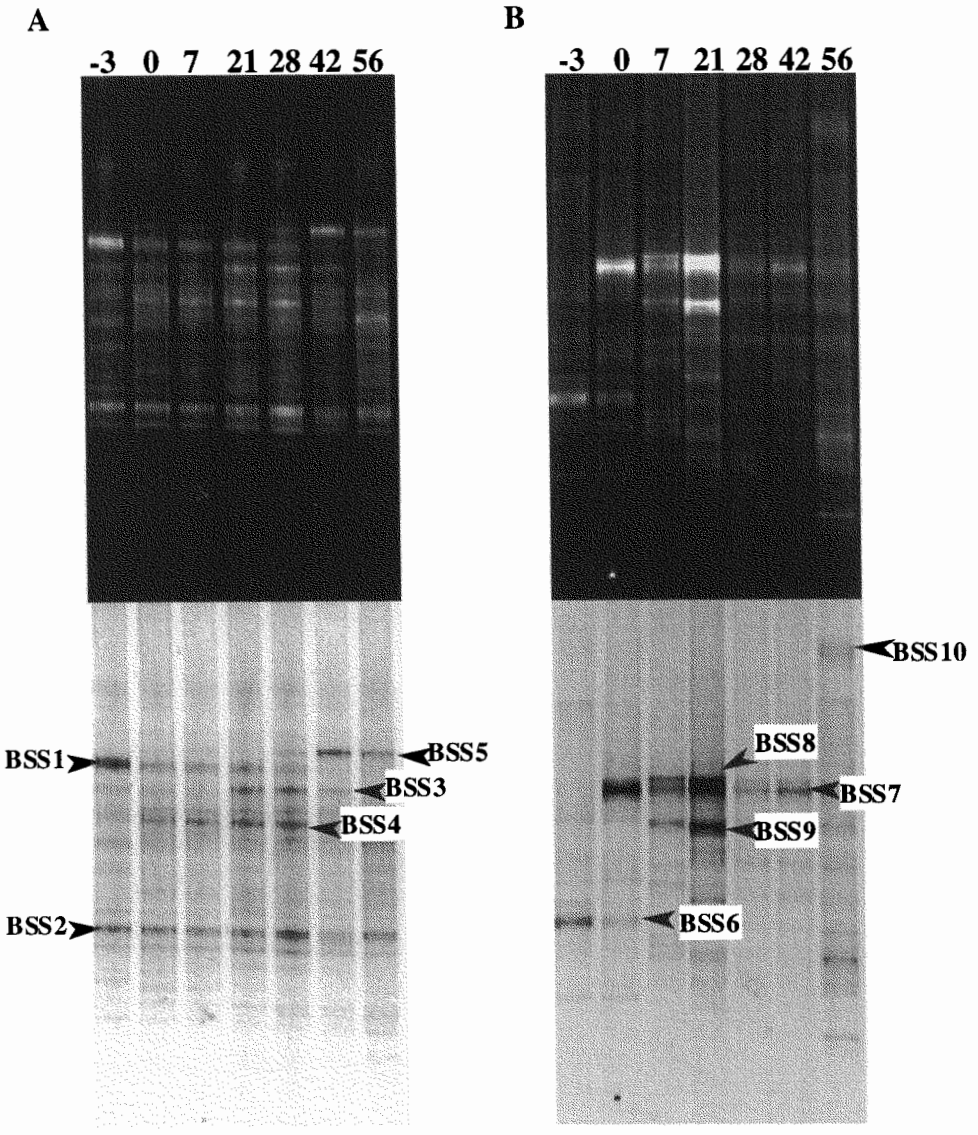


Fig. IV-4. DGGE profiles of partial 16S rDNA fragments showing major bacterial populations in seawater. The experimental conditions were the same as those described for Fig. IV-3. The lower part shows bands excised for the DNA sequencing analysis. Samples from the fertilizer-free tank (A); samples from the fertilizer-added tank (B). The seawater samples were collected on days -3, 0, 7, 21, 28, 42 and 56 as indicated.

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

DGGE band (accession number)	Closest sequence (accession number)	Sequence identity (%)
BSO1 (AB080091)	*Flavobacterium xanthum* (AF030380)	97
BSO2 (AB080092)	*Alcanivorax borukmensis* (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 borukmensis* (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* NAC1-4 (AF245617)	100
BSS3 (AB080103)	Unidentified alpha proteobacterium BD1-8 (AB015520)	94
BSS4 (AB080104)	*Bdellovibrio sp.* AQ (AF084855)	94
BSS5 (AB080105)	Uncultured bacterium NoosaAW93 (AF269026)	89
BSS6 (AB080106)	Uncultured *Roseobacter* NAC1-4 (AF245617)	100
BSS7 (AB080107)	*Cycloclasticus pugetii* PS-1 (U57920)	100
BSS8 (AB080108)	*Alcanivorax borkumensis* (Y12579)	100
BSS9 (AB080109)	*Oceanospirillum sp.* ME113 (AJ302700)	98
BSS10 (AB080110)	Uncultured Banisveld landfill bacterium BVB94a (AYO13660)	98

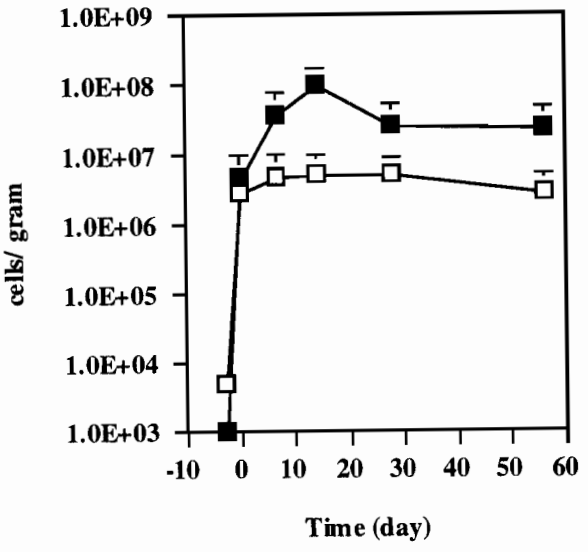


Fig. IV-5 Propagation of *Cycloclasticus* on the surface of oil-polluted grains of gravel. The number of *Cycloclasticus* cells was estimated by q-cPCR. Cell number in the control tank (open squares); cell number in the fertilizer-added tank (closed squares).

IV-4. Discussion

The bioremediation of spilled oil is a powerful tool for *in situ* cleaning of oiled beaches. The application of fertilizers has promoted the growth of oil-degrading bacteria, and hence the rate of biodegradation of crude oil (Bragg et al., 1994; Swannell et al., 1996; Head and Swannell, 1999). Changes in microbial populations during the bioremediation of crude oil have been analyzed by molecular techniques (MacNaughton et al., 1999; Kasai et al., 2002), and *Alcanivorax* (Yakimov et al., 1998) has been identified as a bacterium mainly responsible for the degradation of alkanes in an oil-contaminated marine environment (Kasai et al., 2002). On the other hand, the bacteria responsible for the degradation of aromatic compounds in an oil-contaminated marine environment have not yet been identified.

Some marine bacteria have previously been reported as PAH degraders; these include the genera *Cycloclasticus* (Dyksterhouse et al., 1995; Geiselberecht et al., 1996; Geiselbrecht et al., 1998), *Flavobacterium* (Shiraris and Cooney, 1983; Okpokwasili et al., 1984), *Marinobacter* (Gauthier et al., 1992), *Moraxella* (Tagger et al., 1990), *Pseudomonas* (Shiraris and Cooney, 1983; Tagger et al., 1990), *Sphingomonas* (Zylstra et al., 1997) and *Vibrio* (West et al., 1984). However their activities in the natural environment have remained unknown. In this chapter I analyzed the major players in the bioremediation of petroleum PAHs by using beach-simulating tank that is closer to natural environment.

PCR/DGGE analyses revealed that *Alcanivorax* and *Cycloclasticus* were two major populations on the surface of the oil-coated gravel grains when the nitrogen and phosphorous fertilizers were added (Fig. IV-3, Fig. IV-4). In chapter 3, I have reported that *Alcanivorax* is a major player of biodegradation of alkanes in a petroleum-contaminated marine environment, and is not effective for degrading aromatic compounds. Therefore, the other bacteria, *Cycloclasticus*, were expected to be involved in the degradation of aromatic compounds.

Cycloclasticus strains degrade aromatic compounds including naphthalene, alkylnaphthalene, biphenyl, phenanthrene, fluorene and anthracene (Dyksterhouse et al., 1995; Geiselbrecht et al., 1998). My *Cycloclasticus* isolates also degraded these compounds. Furthermore, they degraded alkyl-substituted PAHs in crude oil (Table IV-1). The extent of

the degradation of C_{1-2}-alkylaromatic hydrocarbons by *Cycloclasticus* was extreme high compared with those by other PAH degraders such as *Marinobacter*, *Pseudomonas* and *Sphingomonas* isolated from beach-simulating tanks (data not shown).

I determined the *gyrB* sequences of several *Cycloclasticus* strains, and based on the sequences, a quantitative competitive PCR method was developed to estimate the number of *Cycloclasticus* cells in environments. The number of *Cycloclasticus* cells associated to 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. IV-5). This high density leads me to expect that *Cycloclasticus* strains play a major role in the degradation of C_{0-3}-alkylaromatic hydrocarbons in a marine environment, especially when nitrogen and phosphorus nutrients are supplemented. This notion is supported by the observation that the degradation rate of the C_{0-3}-alkylaromatic hydrocarbons in crude oil was strongly accelerated by the addition of the fertilizers between day 14 and day 28 when the density of *Cycloclasticus* increased to 10^8 cells/g (Table IV-2).

The beach simulation experiments indicate that the degradation of substituted aromatic hydrocarbons having more than three carbons in the side chain did occur (Table IV-2). *Cycloclasticus* spp. could not degrade these aromatic hydrocarbons, except for alkylnaphthalene. It is therefore expected that some other bacterial populations were involved in the degradation of such aromatic hydrocarbons. It is important to identify these bacterial populations and analyze their hydrocarbon degradation ability.

Chapter V

The *xylN* gene product from TOL plasmid pWW0 of *Pseudomonas putida* is involved in m-xylene uptake

V-1. Introduction

TOL plasmids in *Pseudomonas putida* encode the metabolic pathways for the degradation of toluene, xylenes and their alcohol and carboxylate derivatives (Assinder and Williams, 1990). The TOL degradation pathways generally consist of two parts: an upper pathway that converts toluene and xylenes to their carboxylic acid derivatives (Harayama et al., 1989) and a lower (or *meta*-cleavage) pathway that transforms the carboxylic acids to the precursors of Krebs cycle intermediates (Harayama and Rekik, 1990). Genetic studies have shown that the genes for these catabolic enzymes are organized into two operons: one encoding enzymes for the upper pathway (the upper operon) and the other encoding enzymes for the *meta*-cleavage pathway (the *meta* operon) (Nakazawa et al., 1980; Franklin et al., 1981; Harayama et al., 1984; Harayama et al., 1986).

The upper operon of TOL plasmid pWW0 contains seven genes in the order of *xylU-xylW-xylC-xylM-xylA-xylB-xylN* in a region of about 8 kilobases (kb) (Harayama et al., 1989; Williams et al., 1997). The *xylU* and *xylW* genes are not required for toluene/xylene catabolism (Williams et al., 1997). The *xylC* gene encodes benzaldehyde dehydrogenase (Lebens and Williams, 1985; Inoue et al., 1995), the *xylM* and *xylA* genes encode subunits of xylene monooxygenase (Suzuki et al., 1991; Shaw and Harayama, 1992; Shaw and Harayama, 1995), while the *xylB* gene encodes benzyl alcohol dehydrogenase (Shaw et al., 1993). The *xylN* gene, the last gene of the upper operon, synthesizes a 52-kilodalton (kDa) protein which is processed to a 47-kDa polypeptide (Harayama et al., 1989); the physiological role of the *xylN* gene product has remained unknown. I investigate in this chapter the function of the *xylN* gene product.

V-2. Materials and Methods

Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used in this study are listed in Table V-1. pWW0-161 is a Tn*401*-insertion derivative of TOL plasmid pWW0, and confers on *Escherichia coli* hosts the resistance to ampicillin (Franklin et al., 1981). The Tn*401* insertion occurs outside the catabolic genes in pWW0-161. In this study, an *xylN*::Km^r mutant of pWW0-161 was isolated and named pWW0-161-*xylN*. *E. coli* JM110 was used for routine genetic manipulation. *E. coli* cells containing recombinant plasmids were maintained on Luria-Bertani (LB) plates (Sambrook et al., 1989) supplemented with appropriate antibiotics. The concentrations of the antibiotics used were 50 μg/ml for ampicillin and streptomycin, and 20 μg/ml for chloramphenicol, while the kanamycin concentration was either 50 μg/ml for *E. coli* harboring a high-copy-number kanamycin-resistant plasmid or 20 μg/ml for *E. coli* harboring pWW0-161-*xylN*. *P. putida* PaW94 (Worsey et al., 1978) harboring pWW0-161 was maintained on an M9 minimal medium (Sambrook et al., 1989) containing 5 mM *m*-toluate while that harboring pWW0-161-*xylN* was maintained on M9 medium containing 5 mM *m*-toluate and 50 μg/ml of kanamycin. To induce the upper and *meta* operons on pWW0-161, cells of PaW94 harboring pWW0-116 (hereafter referred to as the wild type) or those harboring pWW0-116-*xylN* (hereafter indicated as the *xylN* mutant) were grown for 16 h at 30°C while shaking in 100 ml of M9 minimal medium containing 5 mM benzyl alcohol. *m*-Xylene vapor was supplemented to the cultures when indicated. The simultaneous addition of benzyl alcohol and *m*-xylene vapor to the culture assured the reproducible induction of TOL catabolic enzymes.

DNA sequence analysis

DNA sequencing with double-stranded DNA was carried out by using a dye terminator cycle sequencing kit (Applied Biosystems) according to the manufacture's instructions, and the products were analyzed by a 377 DNA sequencer (Applied Biosystems). The computer-assisted sequence analysis was made with the PC/GENE software package (IntelliGenetics).

Table V-1. Bacterial strains and plasmids used in this study

Bacterial strain	Genotype or phenotype	Reference
P. putida		
KT2440		Harayama et al., 1986
PaW94	Benzoate-1,2-dioxygenase-negative mutant	Williams et al., 1997
E. coli		
JM110	*rpsL* (Str^r), *thr*, *leu*, *thi-1*, *lacY*, *galK*, *galT*, *ara*, *tonA*, *tsx*, *dam*, *dcm*, *supE44* *D*(*lac-proAB*), [F' *traD36* $proA^+B^+$ $lacI^q$ ZDM15]	
M15	F^-, Nal^S, Str^S, Rif^S, Thi^-, Lac^-, Ara^+, Gal^+, Mtl^-, $RecA^+$, Uvr^+, Lon^+	
Plasmids		
pFC121	$xylB^+N^+$, ColE1 replicon	
pSHG398	Cm^r, ColE1 replicon	
pWW0-161	Tol^+ Ap^r (Tn*401*)	Feilmeier et al., 2000
pWW0-161-*xylN*	Tol^+ Ap^r (Tn*401*) *xylN*::Km^r	this work
pXN960	Cm^r	this work
pXN961	Cm^r Km^r	this work
pUC4K	Amp^r Km^r	Suzuki et al., 1991

Tol^+ indicates that the cells grow on *m*-xylene and toluene. Nal^S, Str^S and Rif^S stand for sensitivity to nalidixic acid, streptomycin and rifampisin while Ap^r, Cm^r and Km^r stand for resistance to ampicillin, chloramphenicol and kanamycin, respectively.

Fractionation of membranes

Cells of the wild-type strain were induced as described above, harvested by centrifugation, washed with a 50 mM sodium phosphate buffer (pH 8.6), and resuspended in the same buffer. Cells were disrupted on ice using a sonifier (Branson, Model 250) at 100 W output by three 30-s bursts of sonication interspersed with a 30-s cooling. Non-disrupted bacteria and large cell debris were removed by centrifugation at 8,000 × *g* for 5 min. The supernatant was removed and centrifuged at 138,000 × *g* for 1 h. After this step, the supernatant was used as the soluble cytoplasmic fraction. The inner and outer membranes were prepared as described by Feilmeier et al. (Feilmeier et al., 2000) with slight modifications. Cells were suspended in 20% (wt/vol) sucrose in 10 mM Tris (pH 8.0) and disrupted by passing the cell suspension through a French pressure cell (Ohtake) at 137 MPa. After removing non-lysed bacteria and cell debris, the clarified lysate was layered onto a 60 to 70% (wt/vol) discontinuous sucrose gradient and centrifuged at 247,000 × *g* for 18 h. The inner-membrane fraction was recovered at the top of the 60% (wt/vol) sucrose layer while the outer membrane fraction was collected at the top of the 70% (wt/vol) sucrose layer. The recovered samples were diluted three-fold with distilled water. Each fraction was layered on a second 60 to 70% (wt/vol) discontinuous sucrose gradient and centrifuged as described above. The membrane fractions were again diluted as described above, and centrifuged at 100,000 × *g* for 2 h to pellet the membranes. The pellets were washed once with 1 M KCl, followed by an additional centrifugation step at 100,000 × *g* for 2 h to recover the membranes. The protein concentration was estimated with a DC protein assay kit (Bio-Rad Laboratories).

Western blot analysis

A *xylN* gene fragment was amplified by PCR using a set of PCR primers, pXN1 (5'-GCGGATCCATGAAAATAAAAAATTTA) and pXN2 (5'-GCAGATCTGAATGAATAATTATAGGC) (the introduced *Bam*HI and *Bgl*II restricion sites are underlined). The 1,411 bp PCR product was separated by electrophoresis on a 0.8% (wt/vol) low-melting-point agarose gel and purified with a QIAquick gel extraction kit (QIAGEN). The amplified DNA was subsequently digested with *Bam*HI and *Bgl*II, and

cloned into pQE-60 (QIAGEN). The constructed plasmid was transferred to *E. coli* M15(pREP4). The recombinant XylN with a tail of six histidine residues at the N-terminal end was expressed in the transformant, and purified using Ni-NTA spin column (QIAGEN) according to the manufacture's protocol.

For the Western blot analysis, proteins in the soluble, inner-membrane and outer-membrane fractions were separated by a 10% (wt/vol) polyacrylamide gel electrophoresis by the method of Laemmli (Laemmli, 1970), and transferred to a PVDF membrane by electroblotting using a Hoefer electroblotter according to the manufacturer's instructions. Prestained protein molecular weight standards (New England BioLabs) were used for molecular mass estimation. Blots were incubated for 1 h with 10,000-fold diluted rabbit anti-XylN serum, then with horseradish peroxidase-conjugated anti-rabbit donkey immunoglobulin G (Amersham Pharmacia Biotechnology) for 1 h. Detection of the XylN protein was carried out using ECL Plus Western blotting detection reagents (Amersham Pharmacia Biotechnology) according to the manufacture's protocol.

Inactivation of *xylN*

Plasmid pFC121 is a pUC18 derivative containing a 4-kb *Sac*I-*Pst*I fragment that incorporates the intact *xylN* gene. This plasmid was digested with *Pvu*II and *Cla*I, and the resulting 2.8-kb *Pvu*II-*Cla*I DNA fragment was extracted after electrophoretic separation in 0.8% (wt/vol) agarose gel to be subcloned into the *Sma*I site of pHSG398 (Takeshita et al., 1987), after converting its sticky end to a blunt end with a DNA blunting kit (Takara Shuzo). The constructed plasmid was called pXN960. The *xylN* gene was inactivated by excising the 1.3-kb fragment containing a kanamycin-resistant gene from plasmid pUC4K (Taylor and Rose, 1988) by *Bam*HI and inserting it into the *Dra*III site of *xylN* on pXN960 to yield pXN961.

The pWW0-161 plasmid in *P. putida* KT2440 (Harayama et al., 1989) was transferred to *E. coli* JM110 by conjugation as previously described (K^hler et al., 1989), and ampicillin-resistant derivatives of JM110 were selected on LB plates containing ampicillin and streptomycin. JM110(pWW0-161) thus constructed were subsequently transformed with pXN961, and those transformants resistant to kanamycin and chloramphenicol were selected.

One of the transformants, JM110(pWW0-161, pXN961), was conjugated with *P. putida* PaW94, and kanamycin-resistant transconjugants capable of growing on *m*-toluate were selected on M9 plates containing 5 mM *m*-toluate and 50 μg/ml of kanamycin.

PCR for the detection of *xylN*::Km^r

PCR for amplifying the DNA fragment containing *xylN* or *xylN*::Km^r was carried out in a volume of 50 μl with a GeneAmp kit (Perkin-Elmer). The oligonucleotides used as PCR primers were XYLU7191 (5'-GTTCACTTGATGCCAAGTGGAC-3') and XYLU3 (5'-CCGCTGTAACAGTCCCCTTC-3').

Growth inhibition by *m*-xylene

TOL catabolic enzymes were induced in cells of the wild-type strain and the *xylN* mutant as already described, harvested by centrifugation, washed twice with a 50 mM potassium phosphate buffer (pH 7.4), and resuspended in the same buffer. Four-hundred ml of the suspension containing 6×10^6 cells were then used to inoculate 100-ml bottles containing 20 ml of the M9 medium supplemented with 5 mM benzyl alcohol with or without 2.5 mM *m*-xylene. The bottles were tightly sealed with a Teflon-lined rubber septum and a crimped aluminum seal to prevent the evaporation of *m*-xylene. The cultures were grown at 30°C while shaking, and the turbidity at 600 nm of the cultures was periodically measured.

Enzyme assays

Cells grown under inducing conditions were harvested at the late-exponential-growth phase by centrifugation, resuspended in 1/10 volume of a 10 mM ethylene diamine buffer (pH 7.4) containing 10% (vol/vol) isopropanol, and disrupted by sonication. After the centrifugation at $20,000 \times g$ for 30 min, the supernatants were used as the cell-free crude extracts. The protein concentration of each crude extract was estimated with a protein assay kit (Bio-Rad Laboratories). Assays for benzyl alcohol dehydrogenase were carried out at 25°C in a 100 mM glycine/NaOH buffer (pH 9.4) containing 1 mM NAD^+, 0.1 mg/ml of bovine serum albumin and 200 mM benzyl alcohol as a substrate. NADH formation was determined

spectrophotometrically at 340 nm (Inoue et al., 1998). Catechol 2,3-dioxygenase was assayed at 25°C in a 100 mM potassium phosphate buffer (pH 7.5) with 33 μM catechol as the substrate; the amount of the reaction product (2-hydroxymunoic semialdehyde) was determined spectrophotometrically at 375 nm (Nozaki et al., 1970).

_m_-Xylene-dependent oxygen consumption by _P. putida_ cells

Cells of the wild-type strain and *xylN* mutant were cultivated in the presence of benzyl alcohol and *m*-xylene vapor as described previously, harvested, washed twice with a 50 mM potassium phosphate buffer (pH 7.4), and resuspended in the same buffer. The cells were adjusted to a turbidity of 1.0 at 600 nm and incubated for 5 h at 30°C while shaking. For oxygen consumption assays, cells were placed in a Clark-type oxygen electrode (model 5/6 Oxygraph, Gilson), and the oxygen consumption rates in response to the addition of various concentrations of *m*-xylene were determined. The apparent kinetic constants, K_s and V_{max}, were determined by reciprocal plots.

Chemicals and reagents

All the chemicals used in this study were of the highest purity commercially available. *m*-Xylene was purchased from Tokyo Kasei Kogyo, the other chemicals being purchased from Wako Pure Chemical Industries, Difco, Aldrich Chemical and Gibco BRL. The enzymes and reagents used for nucleic acid manipulation were purchased from Takara Shuzo.

Nucleotide sequence accession number

The nucleotide sequence data reported in this paper have been submitted to the DDBJ, EMBL and GenBank libraries under accession number D63341.

V-3. Results and Discussion

Nucleotide sequence analysis of *xylN*

Harayama et al. (Harayama et al., 1989) have reported that *xylN* was the last gene of the upper operon on the TOL plasmid pWW0 and that its product was a 52-kDa protein which was processed to a 47-kDa polypeptide. In this chapter, the nucleotide sequence of *xylN* was determined. The gene encoded a 465-amino-acid-long polypeptide with an estimated molecular mass of 49 kDa. A comparison of the deduced amino acid sequence of XylN with translated sequences from the GenBank database suggested that XylN had significant homology with a group of proteins in hydrocarbon-degrading bacteria, including PorA from *Pseudomonas* sp. strain Y2 (Velasco et al., 1998), CumH from *Pseudomonas fluorescens* IP01 (Habe et al., 1996), IpbH from *P. putida* RE204 (GenBank accession no. AF006691), CymD from *P. putida* F1 (Eaton, 1997), TodX from *P. putida* F1 (Wang et al., 1995), TbuX from *Ralstonia picketti* PKO1 (Kahng et al., 2000) and PhlX from *Rastonia eutropha* JMP134 (GenBank accession no. AF06589) (Table V-2). These proteins are homologous with FadL from *E. coli* (Black, 1991) that is involved in the transport of long-chain fatty acid across the outer membrane (Kumar and Black, 1993).

Location of the *xylN* product in the outer membrane of *P. putida* cells

The signal sequence was predicted at the N-terminal region of XylN by using both SignalP (version 1.1; Center for Biological Sequence Analysis: http://www.cbs.dtu.dk) (Nielsen et al., 1997) and PSORT (http://psort.nibb.ac.jp) (Nakai and Kanehisa, 1991). These programs suggested the predicted cleavage site to be at position either 24, 25 or 26, and the estimated molecular mass of the mature polypeptide to be 47 kDa. This result agrees with that of maxicell experiments: a 52-kDa XylN product was processed to a 47-kDa polypeptide (Harayama et al., 1989). PSORT (Nakai and Kanehisa, 1991) predicted XylN to be an outer membrane protein. The computer program for predicting bacterial outer membrane β-strand proteins (Faculty of Biology, University of Konstanz: http://loop8.biologie.uni-konstanz.de) (Diederichs et al., 1998) also produced this result. The existence of the signal sequence

Table V-2 Membrane-associated proteins sharing homology with XylN

Gene product	Bacterial strain	Identity (%)	Reference
CumH	*Pseudomonas fluoresens* IP01	49.7	Gibson et al., 1970
CymD	*P. putida* F1	38.7	Eaton, 1997
TodX	*P. putida* F1	37.8	Taylor and Rose, 1988
IpbH	*P. putida* RE204	49	GenBank accession no. AF006691
PorA	*Pseudomonas* sp. strain Y2	50.9	Takeshita et al., 1987
PhlX	*R. eutropha* JMP134	42.2	GenBank accession no. AF065891
TbuX	*R. eutropha* PKO1	41.8	Isken and deBont, 1996
FadL	*E. coli*	21.0	Black, 1991

together with the sequence similarity to the outer membrane protein FadL suggested XylN to be an outer membrane protein.

To identify the localization of XylN, cells of the wild-type strain were cultivated under conditions to induce the upper operon, and fractionated into cytoplasm, inner membrane and outer membrane. Western blot analysis detected XylN in the outer membrane (Fig. V-1). No XylN was detected among cytoplasmic proteins and inner membrane proteins.

Construction of a *xylN* mutant of pWW0-161

To analyze the possible role of the *xylN* product, PaW94 harboring pWW0-161-*xylN* was constructed by inserting the DNA fragment containing a kanamycin-resistant gene in *xylN*. To confirm the mutation in this plasmid, total DNAs were extracted from the wild-type strain and *xylN* mutant, and partial *xylN* fragments were amplified from the total DNAs by PCR. As expected, a 1.5-kb-long DNA fragment was amplified from the DNA that had been isolated from the *xylN* mutant, while a 0.3-kb-long fragment was amplified from the wild-type strain.

Growth inhibition of *P. putida* by *m*-xylene

Growth inhibition of the wild-type strain but not the *xylN* mutant was observed by 2.5 mM *m*-xylene as described below. Cells grown in the presence of benzyl alcohol and *m*-xylene vapor were used to inoculate an M9 medium containing 5 mM benzyl alcohol with or without 2.5 mM *m*-xylene in tightly stoppered bottles. They were cultivated at 30°C while shaking, and their growth was monitored by determining the turbidity of the cultures at 600 nm (Fig. V-2). When the wild-type strain and *xylN* mutant were grown on the M9 medium containing 5 mM benzyl alcohol without *m*-xylene, the exponential growth was observed without apparent lag phase and the specific growth rate constants (m) for these two strains were almost the same, namely 0.20 h^{-1} and 0.19 h^{-1}, respectively. However, when 2.5 mM *m*-xylene was added to the culture of the wild-type strain, a lag phase of about 40 h followed by the exponential growth phase (μ = 0.12 h^{-1}) was observed. On the other hand, the growth of the *xylN* mutant occurred without significant lag, and the growth rate was similar to that in the absence of *m*-xylene (μ = 0.19 h^{-1}). The same results were obtained when *o*-xylene was added to the cultures (data

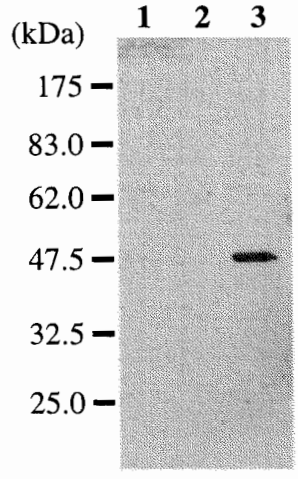


Fig. V-1. Localization of XylN in the outer membrane. The cytoplasmic, inner-membrane and outer-membrane fractions were prepared from cells of the wild-type strain grown under conditions to induce the upper operon. XylN was immuno-detected with antibodies raised against XylN. Lanes: 1, cytoplasmic fraction; 2, inner-membrane fraction; 3, outer-membrane fraction. The positions of the molecular weight markers are shown.

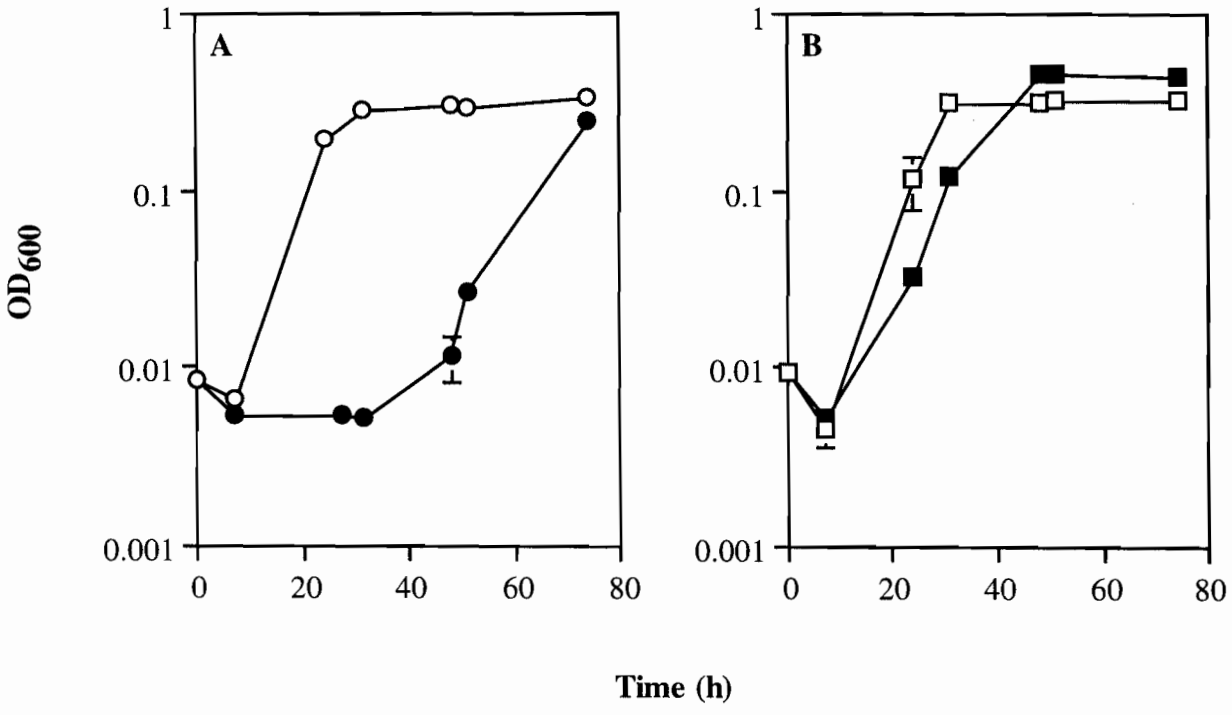


Fig. V-2. Effect of *m*-xylene on the growth of the wild-type strain (circle) and the *xylN* mutant (square). Cells were grown in an M9 medium containing 5 mM benzyl alcohol at 30°C with (solid) and without (open) 2.5 mM *m*-xylene. Growth was determined by monitoring the turbidity at 600 nm, the values are averages of 3 independent experiments. The standard deviation was less than 35% of the averages.

not shown). My result with the wild-type TOL plasmid agrees with a previous observation: it has been reported that the direct addition of aromatic hydrocarbons to a culture medium prevented the growth of *P. putida* harboring the TOL plasmid, while growth of this strain was observed when these compounds were supplied in the vapor phase (Gibson et al., 1970; Worsey and Williams, 1975).

Xylenes, like toluene, are toxic to microorganisms, even at a subsaturated concentration in water (Sikkema et al., 1995). It has been suggested that the accumulation of such organic solvents as toluene and xylenes in the cytoplasmic membrane disturbs its structural and functional properties (Sikkema et al., 1995). Accumulated aromatic compounds may reduce the integrity of the cytoplasmic membrane, and increase its permeability to proteins and ions. Some *P. putida* strains are tolerant to such organic solvents by active efflux of the solvents from the cytoplasm and the cytoplasmic membrane (Isken and de Bont, 1996; Ramos et al., 1997; Nikaido, 1998) or by the solvent-impermeable outer membranes (Heipieper et al., 1992; Weber et al., 1994; Pinkart et al., 1996; Ramos et al., 1997). Considering the location of XylN in the outer membrane, I propose that the resistance to xylenes of the *xylN* mutant was due to a reduction in the outer membrane permeability of xylenes caused by the inactivation of the XylN outer membrane porin.

_m_-Xylene-dependent oxygen consumption by whole cells

Cells of the wild-type strain and the *xylN* mutant were grown under inducing conditions and resuspended in a 50 mM potassium phosphate buffer (pH 7.4) with the turbidity of each suspension being adjusted to 1.0 at 600 nm. The suspensions were incubated for 5 h at 30°C to reduce the endogenous energy sources. The stimulation of oxygen consumption by whole cells upon the addition of various concentrations of *m*-xylene was then determined (Fig. V-3). The apparent K_s value for *m*-xylene oxidation in the *xylN* mutant (6.7 ± 1.9 μM) was 4-fold higher than that of the wild-type strain (1.7 ± 1.2 μM), while the apparent V_{max} value for the *xylN* mutant [0.12 ± 0.02 μmol sec^{-1} (g dry cells)$^{-1}$] was 70% of that for the wild-type strain [0.17 ± 0.05 μmol sec^{-1} (g dry cells)$^{-1}$].

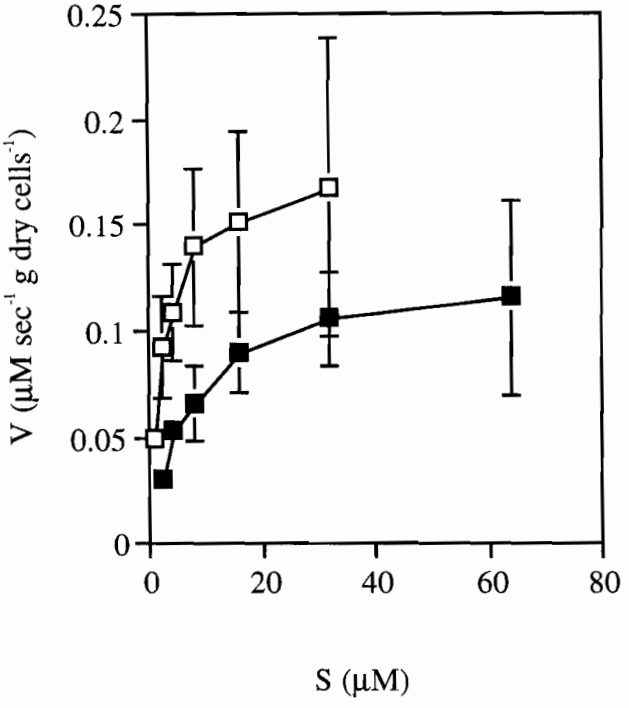


Fig. V-3. *m*-Xylene-dependent oxygen consumption by the wild-type strain (open squares) and the *xylN* mutant (solid squares). The values were averages of 3 independent experiments. Each vertical bar represents one standard deviation. The bars appears only above the average values of the data for the wild-type strain to make the graph clearer.

To confirm that the upper and *meta* operons of TOL plasmids pWW0-161 and pWW0-161-*xylN* had been induced at the same level, the activities of benzyl-alcohol dehydrogenase and catechol 2,3-dioxygenase in PaW94 harboring these two plasmids were determined. The activities of benzyl-alcohol dehydrogenase (nmol min^{-1} mg^{-1}) in these two strains were 31 ± 11 and 32 ± 10, respectively, while the activities of catechol 2,3-dioxygenase (μmol min^{-1} mg^{-1}) were 1.4 ± 0.5 and 1.4 ± 1.0, respectively.

Although the activities of TOL catabolic enzymes in the wild-type strain and the *xylN* mutant were not very different, the oxygen consumption rate of the *xylN* mutant was lower than that of the wild-type strain. This oxygraph data supports the interpretation that XylN is outer membrane porin involved in *m*-xylene uptake.

Since XylN renders cells sensitive to their growth substrates, this protein disadvantages host cells when the substrate is available at a high concentration. However, in most natural environments, the concentrations of available substrates may be very low, and the XylN function may be indispensable for the survival of the host in a natural environment. In fact, the growth rate of the *xylN* mutant was only one third of that of the wild-type strain when *m*-xylene was supplied in the vapor phase (data not shown).

Chapter VI

General Discussion

Natural processes by microorganisms will eventually cause substantial degradation of spilled-oil even if no bioremediation is performed. In other words, bioremediation is only expected to enhance natural biodegradation processes. Recently, the US Superfund Act has come to face a dead-end and almost lost its effect. Therefore, use of natural processes in combination with an efficient remedial procedure has received significant support from responsible parties, particularly to avoid the high costs of bioremediation. One of such remedial technology, called "monitoring natural attenuation" (MNA, USEPA_SAB, 2001), can be regarded as a remedial approach based on the understanding and quantitative documenting of the naturally occurring processes that protect the ecological system including human from exposure to hazardous contaminants at contamination sites. Responsible organizations must continue monitoring the petroleum degradation until they can announce that the clean-up has been achieved when the safety is confirmed according to the risk assessment standard by MNA.

Natural remediation processes are in general dependent on microbial diversity and dynamism within the contaminated ecosystems. Some microbiologists investigated the microbial communities that take part in situ hydrocarbon biodegradation activities (Lee and Levy, 1991; Lindstrom et al., 1991; Bragg et al., 1994). In these studies, microorganisms were expressed as the total number of heterotrophs and/or oil degraders as determined through culturing and plate counting. However, because almost 99% of marine bacteria are difficult to culture as evidenced by their inability to form colonies when current laboratory-based culture techniques are used (Rollins and Colwell, 1986; Rozsak and Colwell, 1987; Wilkinson, 1988), it is considered that the results do not reflect the real natural microbial communities. Moreover, few hydrocarbon-degrading microorganisms isolated by the culture-depended methods have turned out to be important for the actual petroleum biodegradation in the natural environment (Harayama et al., 1999).

For these reasons, in this thesis work I decided to perform analysis of the microbial communities on beaches that were heavily contaminated by a large amount of the spilled-oil

from the tanker Nakhodka. I adopted culture-independent molecular biological techniques for fingerprinting the 16S rDNA of the proliferating microorganisms within the seawater and oil samples. Most of the bacteria in the seawater samples were found to be classified in the *Cytophaga-Flavobacterium-Bacteroides* phylum as well as α-proteobacteria and cyanobacteria, which suggested that the oil pollution did not strongly affect the major marine bacterial population for a long time. The probable reason for this observation could be that the influence of the pollution became lighter after 10 months of the accident due to dilution of the spilled oil by seawater. On the other hand, some hydrocarbon-degrading bacteria were found to have predominated in the oil paste samples, although the size of the major bacterial population in the oil paste samples was less than 2×10^6 bacteria per gram of oil paste, which would explain the slow rate of natural attenuation.

The extent of remediation by natural processes is likely to become almost the same as that of active bioremediation after tens of years. Moreover, the former is more desirable from an ecological point of view. However, people in the contaminated area keenly wish the contaminants to be eliminated as soon as possible. Therefore, powerful bioremediation methods should be developed to solve undesirable oil pollution problems several-fold faster with minimum human interventions.

Major limiting factors of the aerobic biodegradation of pollutant are the low concentrations of available nutrients and soluble oxygen. Therefore, both nutrients and air are supplied for *in situ* bioremediation. To dig the ground and/or to set up pipe lines for air injection to enhance biodegradation are rather expensive and take much time. Moreover, these mechanical setups would cause serious problems to the environment. In the coastal zone, it is generally not necessary to supply oxygen because of the action of waves. The only important points that remain for effective bioremediation would be that nutrients should remain in contact with the oil-contaminated material, and that the nutrient concentrations are sufficiently high to support the maximal growth rate of the hydrocarbon-degrading bacteria throughout the clean-up operation. In the tidal zone, water-soluble nutrients are generally rapidly diluted into the surrounding seawaters and thus removed from the oil-water interface where the biodegradation occurs. To overcome this problem, slowly dissolving solid

fertilizers were selected to elongate the time for the nutrients to diffuse and to maintain optimum concentration of nitrogen and phosphorous in seawater as long as possible.

In this thesis, I have used a coastal simulator and attempted to evaluate the stimulation of the degradation of heavy oil C released form the tanker Nakhodka by adding slowly dissolving solid fertilizers under the conditions closer to the tidal zone. Under these conditions, *Alcanivorax* and *Cycloclasticus* groups that are widely distributed in marine environment predominated in the heavy oil-contaminated seawater and on the surface of heavy oil-polluted gravels, and materials such as *n*-alkanes and substituted low-molecular-weight PAHs were degraded significantly. Furthermore, the heavy oil sticking on cobble stones were observed to come off. Although the conditions for simulating the natural ecosystems were not the same as those existing in the actual 'natural oil spill', the results obtained in my thesis work were expected to be an acceptable compromise between laboratory experiments and the simple observation of uncontrolled phenomena in the natural environment.

The bioremediation strategy adopted in my thesis work has several advantages. (1) Since it dose not utilize any mechanical means for enhancement of oxygen mass transfer, damages of the marine environment are expected to be minimal. (2) The remediation proceeded to a considerable extent, despite that the strategy was simple and economical. (3) The biosurfactant-producing bacteria, *Alcanivorax* group, always predominated under the bioremediation conditions employed, and converted petroleum into many small oil droplets. Since biosurfacteants are known to be much less hazardous to living organisms than artificial detergents, they are expected to be useful as effective cleansing agents at oil-contaminated sites (Banat, 1995; Desai J. D., and I. M. Banat, 1997; Banat et al., 2000). Bacterial cells are often associated on the surface of oil-emulsion, and such contact may facilitate the assimilation of petroleum components into the cells. Zhang and Miller (1992) reported the effect of biosurfactant that glycolipid produced by *Pseudomonas aeruginosa* enhanced the biodegradation of alkanes. The observation suggests that enrichment of *Alcanivorax* bacteria promotes the degradation of petroleum components.

On the other hand, there are some disadvantages as well. (1) Because the oxygen supply depends on waves' energy, the bioremediation rate is likely to be reduced on beaches where waves are interrupted by the coastal geography. (2) Because oxygen supply is vital for bioremediation, the biodegradation rate of petroleum in deeper sediment layers is also likely to be reduced. (3) The biodegradation rate will also be low in regions where the indigenous bacteria are incompetent to degrade petroleum components, because the remediation depends on their hydrocarbon-degrading abilities.

Under the conditions in which oxygen supply is insufficient, anaerobic petroleum degradation should be taken into consideration. Various investigations have shown that in the absence of oxygen, hydrocarbon-degradation is usually linked with iron-, manganese- or sulfate-reduction and with the fermentative oxidation process with fumarate as a terminal electron acceptor (Coates et al., 1997; Caldwell et al., 1998; Lovley, 2000; Cervantes et al., 2001; Tor and Lovley, 2001). Therefore, the application of such electron acceptor is expected to promote anaerobic biodegradation of petroleum.

Bioaugmentation or seeding has been expected to significantly enhance the clean-up process at contaminated sites where natural activities are low. However, in most cases it is likely that introduced species will disappear in a short time by dilution (Tagger et al., 1983). Therefore, if hydrocarbon-degrading bacteria can be maintained at contaminated sites by embedding into some carriers, the clean-up process will become more effective.

In natural environmental settings, petroleum degradation often dose not occur even in the presence of hydrocarbon-degrading bacteria. In such a case, the 'bioavailabily' of hydrocarbons is said to be low because of their aqueous insolubility (Alexander, 1999). Therefore, it is necessary to increase their bioavailabily by facilitating the assimilation of hydrocarbons into bacterial cells. For this purpose, two ways are conceivable: (1) application of biosurfactants to convert petroleum into small droplets, and (2) to increase the hydrocarbon-permeability of the bacterial outer membrane. In this thesis work, I have found that the outer membrane protein XylN of *Pseudomonas putida* facilitates transport of *m*-xylene and its analogues into cells. Since a group of proteins in hydrocarbon-degrading bacteria posses significant homology with the deduced amino acid sequence of XylN, these

proteins are expected to play their roles in substrate transport. Recombinant hydrocarbon-degrading bacteria harboring and expressing the membrane protein gene such as *xylN* are expected to enhance transport of hydrocarbons. As mentioned earlier, application of the fertilizers stimulate the growth of biosurfactant-producing bacteria, *Alcanivorax*, and the bioavailability of petroleum becomes higher by emulsification. Therefore, the combination of application of fertilizers and seeding of appropriate recombinant bacteria is expected to increase petroleum remediation rate.

Petroleum bioremediation has advantages such that it can remove the oil stuck in crevices of rocks and accumulated behind big boulders. It is much less harmful to natural environment than use of chemical dispersants (Swannell et al., 1996). Since bioremediation cannot be practiced without public consent, more scientific data for cost-effectiveness should accumulate and risk should be discussed more openly. The possibility of mass-increase of marine algae, oxygen depletion by the addition of fertilizers or generation of toxic metabolites for humans and the marine biota are a few of such examples.

Until recently, the majority of bioremediation strategies towards ameliorating marine oil spills have assumed that the principal mechanism of hydrocarbon removal is aerobic respiration. Although a wide range of microorganisms have been discovered that are able to degrade petroleum components such as alkanes and aromatic hydrocarbons, still many pollutants persist in the environment the degradation pathways of which remain to be elucidated (Harayama et al., 1999). In future, a wide range of bioremediation strategies including anaerobic biodegradation and co-metabolism of highly stable components, along with careful monitoring of the treated site to obtain basic experimental data, should be developed to cope with various marine oil pollution problems.

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