Isolation and Characterization of oil degradablee microorganisms from heavy oil-spilled marine shores

ISOLATION AND CHARACTERIZATION OF OIL DEGRADABLE MICROORGANISMS FROM HEAVY OIL-SPILLED MARINE SHORES

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ABSTRACT

We have investigated the presence of remained or buried heavy oils in the marine shores of Hokuriku (Ishikawa area) by the field research with the portable boring machine (SCSC type boring machine). Our investigation revealed the existences of Nakhodka's heavy oil layered in the coastal sand of beach, or deeply adhered to the seashore structures and environments at the stages when passed about one year since the heavy oil spill accident. Furthermore, the alkane components degradable *Pseudomonas* species gram negative bacteria and gram positive microorganisms that ascribed as *Pimelobacter* (previouly *Nocardioides*) *simplex* have been isolated from these heavy-oil-contaminated coastal environments. This result suggested to contribute to the aerobic biodegradation of the light components in the heavy oil due to native microorganisms such as our isolates. However, our isolates could not use the heavy components like PAHs (poly aromatic hydrocarbons) as sole carbon source for aerobic growth. Aerobically PAH (over 3 rings of aromatic hydrocarbon) degradable microorganism has not been found in our investigated fields. These facts suggested that it would be effective for bioaugmentation with PAHs degradable microorganisms that obtained from other natural environments such as natural oil-producing wells or ponds in the bioremediation of Nakhodka's heavy oil contaminated marine shoreline.

INTRODUCTION

Contamination of petroleum hydrocarbons in harbor sediments and seashore environments from shipping activity, heavy oil spills, and runoffs is recently becoming a great concern due to the toxicity and recalcitrant of the fuel components. In particular, pollution due to heavy oil (diesel fuels) has received increased attention because of the presence of toxic and carcinogenic compounds called "heavy components" such as aromatics (PAHs), resins and asphaltens. Several reports have revealed the toxic effects

and significant damage to marine creatures and ecosystems due to exposure of spilled crude oil contained PAHs components (Thomas *et al*, 1999a, 1999b, Heintz *et al*, 1999, Carls *et al*, 1999). Therefore, the field research for the detection of heavy components is necessary in order to evaluate toxic effects to ecosystems and organisms that damaged by the spilled oil as a first step of the bioremedition .

So far, there have been many reports showing that marine microorganisms play important roles in the biodegradation of spilled oil in ecosystem (Madsen, 1991). It is suggesting that the artificially enhanced degradation of crude oil using marine microorganisms could be practical way to reduce damage to marine ecosystem caused by spilled oil (Prichard *et al.*, 1991, Venosa *et al.*, 1996). As to the bioremediation, what has been demonstrated in these studies is that already present (native) or/and external microorganisms can provide the effectiveness of biostimulation or/and bioaugmentation for oil-damaged marine environments.

Thus, in this paper we report the field research of Nakhodka's heavy oil contaminated marine shoreline with a portable boring machine for the investigation of hidden heavy oil after about one years since the accident. Besides, we also repot here the isolation and characterization of oil-degradable microorganisms that obtained from the heavy oil spilled marine shores in Ishikawa and natural oil producing environments in Niigata in order to use biomaterials for bioremediation.

MATERIALS and METHODS

Sampling and field research

Field investigations were carried out to research the presence of Nakhodka's heavy oil after about one years (4, 15 and 21 months later) since the accident in Ishikawa marine shores. A portable boring machine (SCSC type boring machine: SCSC Geotech Co. Ltd., Tokyo) was used to obtain boring core sample contained heavy oil hidden underground of sandy marine environments (Figure 1 A, B). This machine can dig out the layered boring-core sample in two-three meters in depth below the ground.

Core samples, beach sand, tide pool seawater and marine sediments were collected from Nakhodka's heavy oil contaminated seashore environments in Ishikawa during 4 –21 months later the accident. Collected samples were used as the inoculum for enrichment and isolation of oil-degradable microorganisms.

Isolation and screening of microorganisms

For isolation of microorganisms capable of degrading heavy oil components, the isolation medium was prepared. The isolation medium contained (per 1 liter of distilled

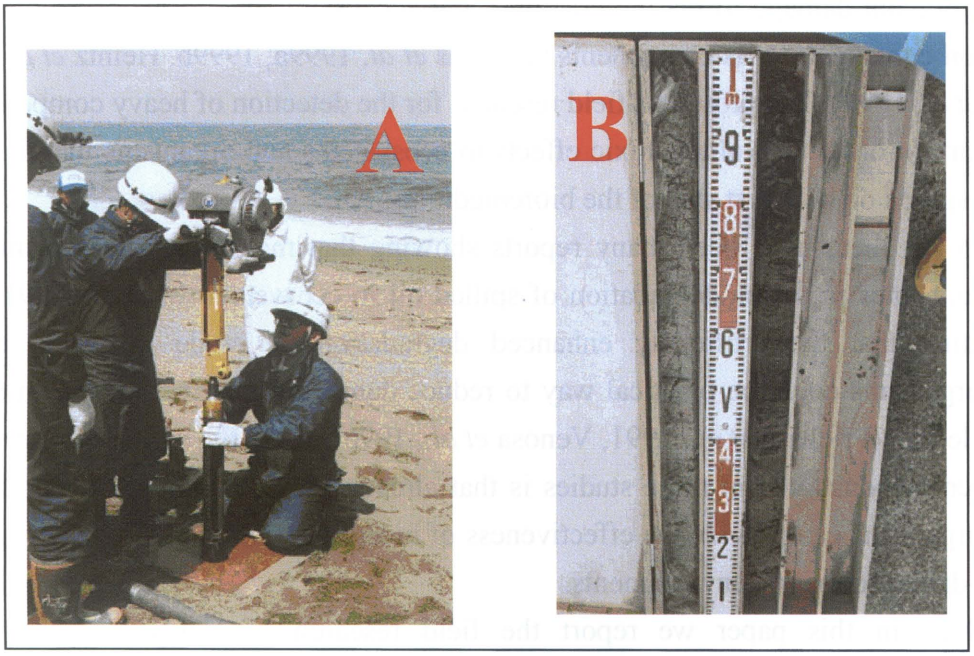

Figure 1 A potable boring machine ;SCSC type boring machine(A) and the obtained core sample (B). Sampling was performed in KATANO beach on 15 th July, 1998.

water): 0.4g potassium dihydrogen phosphate, 0.2g ammonium chloride, 0.05g yeast extract, 0.05g cystein HCl, 0.1g sodium sulfate, and 2.0 ml mineral solution (Sakaguchi, *et al.*, 1993). The medium was adjusted to pH 7.0 with NaOH before sterilization. After sterilization, various carbon compounds sterilized with membrane filter (pore size: 0.2 μm) was added to the medium as sole carbon source. For enrichment of microorganisms, the isolation medium which was inoculated the aquatic sample solution was incubated at 10°C or 25°C. After enrichment, isolate was purified by the colony formation method with the agar plate which was spread the sterilized carbon source solution on the surface. These experimental procedures were also carried out for the isolation of PAH-degradable microorganisms from natural oil-producing environments in Niigata.

Characterization and genetic analysis of microorganisms

Isolated and purified microorganisms were observed with a transmission electron microscope (TEM). Biochemical characteristics of isolated pure strains of microorganisms, such as gram stain, oxidase and catalase activities, were investigated. For the phylogenetic analysis based on 16SrDNA sequence, chromosome DNA extracted from the cells. Sequence samples were prepared by dye deoxy terminator kit (Perkin Elmer) and the sequence was performed by a DNA sequencer (ABI 310). DNA sequence was searched similarity and homology with other microorganisms by FAST analysis, and the phylogenetic tree was made by Clustal W algorithm program.

Growth experiment was carried out in grass serum bottles (20 ml) contained 10 ml medium at between 4 and 37 °C. Cell growth was determined by directly counting cell number with a heamacytometer.

PCR amplification of *alkB* (Kok *et al.*1989) and *ndoB* (Kurkela *et al.*,1988), genes was performed to indicate the potential abilities of alkane and simple aromatic compounds catabolization by using designed DNA primers and reaction conditions shown in Table 1.

Table **1** DNA primers used for PCR amplification of catabolic genes from strain K1D

Catabolic gene	Primer sequence	Predicated PCR fragment size	Microorganism and reference
alkB	Forward: 5'-TGGCCGGCTACTCCG ATGATCGGATCTGG-3' (position 703-732) Reverse: 5'-CGCGTGGTGATCCGA GTGCCGCTGAAGGTG-3' (position 1543-1572)	870 bp	*Pseudomonas oleovorans* ATCC 29347 Kok *et al.* , J. Biol. Chem. 264, 5435-5441, 1989
ndoB	Forward: 5'-CACTCATGATAGCCT GATTCCTGCCCCCGGCG-3' (position 622-653) Reverse: 5'-CCGTCCCACAACACA CCCATGCCGCTGCCG-3' (position 1234-1264)	642 bp	*Pseudomonas putida* ATCC 17484 Kurkela *et al.* , Gene, 73, 355-362, 1988

PCR reaction : (94°C, 3 min. denature → 55°C, 30 sec. primer binding → 72°C, 90 sec. propagation) x 25 cycles

RESULTS and DISCUSSION

Field research and sampling in heavy oil polluted marine shores

Our field investigations after about one years (4, 15 and 21 months later) since the accident revealed the presence of large amount of remained Nakhodka's heavy oil over wide range of unnoticed seashore line in Ishikawa (Figure 2 A~D). A large amount of adhered heavy oil on/around rocks (A) and artificial marine structures (B) in northern Noto area near Wajima city was observed. The damage extended to approximately 4~10 km along the seashore line. At the present time (2003 August), the appearance of the heavy oil damage is almost recovered and the adhered heavy oils have disappeared as elsewhere. However, heavy oil pollution in these areas had been remained for about two years since the accident. Heavy components of the spilled oil were mainly observed in our sampling points. Furthermore, our field research with a potable boring machine (4,15 and 21 months later the accident) showed that there was

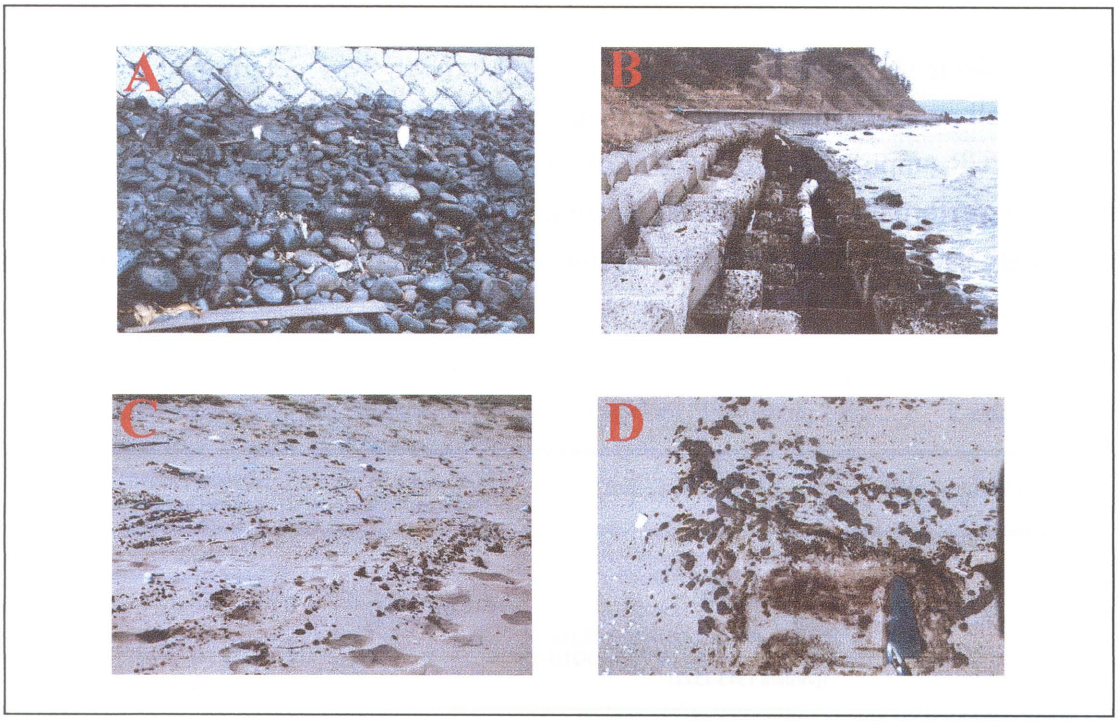

Figure 2 Field investigation of seashores damaged by Nakhodka's heavy oil.
A: typical seashore of Nebuta-onsen (northern Noto, near Wajima city, this picture was taken on January 1998).
B: marine artificial structures (concrete wave protectors) along seashore line in eastern Wajima city (northern Noto peninsula, this picture was taken on March 1998).
C and D: sandy beach contained heavy oil lump in seashore of western Kaga (Katano beach, these photographs were taken on September 1998).

still buried heavy oil in several sandy beaches (Figure 1 B, Figure 2 C and D). The heavy oil lumps were found in about 10 cm to 1.0 meter below the ground (Figure 1 B, Fugure 2 D). Almost buried heavy oil lumps distributed in layers 10 cm to 30 cm thickness (Figure 1 B, Fugure 2 D). These findings suggest that it may be difficult to degrade the heavy components by biocatabolate due to only native microorganisms in our investigated area, and that there is the possibility that naturally undegradable heavy oil components will be gathered in specific layer or space in the sands or bottom of sea by natural strong wave motions when occur in winter storm or typhoon season.

Isolation, characterization, genetic analysis of microorganisms from heavy oil polluted marine shores

Pseudomonas species gram negative bacteria (strains K1D, NL1and N2C showed in Figure 3) and gram positive microorganisms (strains N2D and N2B1 showed in Figure 4) that ascribed as Nocardioides simplex capable of growing with n-decane as

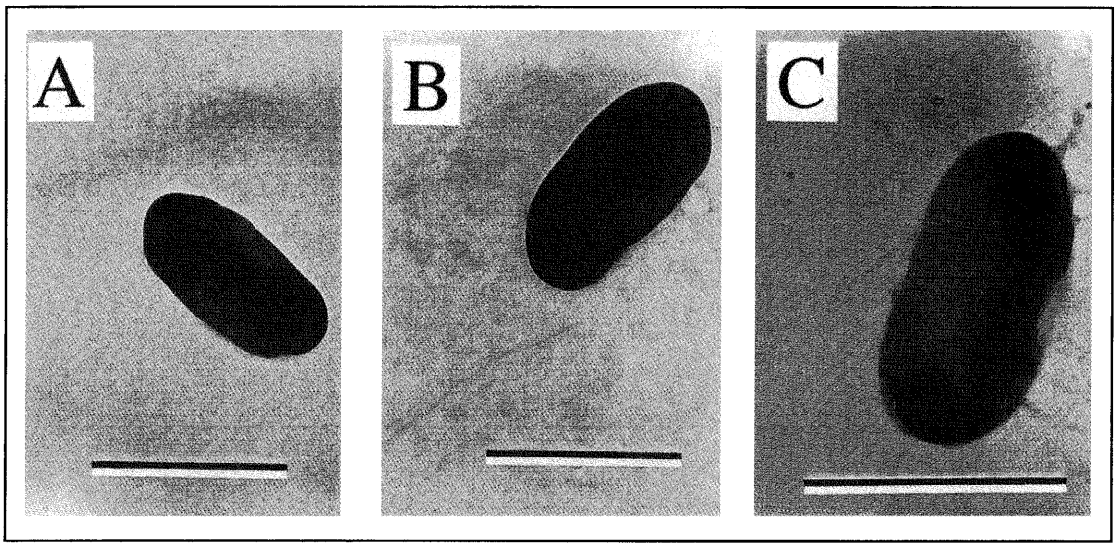

Figure 3 Transmission electron micrographs of cold–acitive decane degradable bacteria strains K1D (A), NL1(B) and N2C (C). These strains were isolated from Katano beach (Kaga), Mikuni (Shioya) beach (near Fukui) and Ninohama beach (Noto peninsula) respectively.
Cells were negatively stained with 1% sodium phosphotungustate solution.
Bars indicate 1.0 μm.

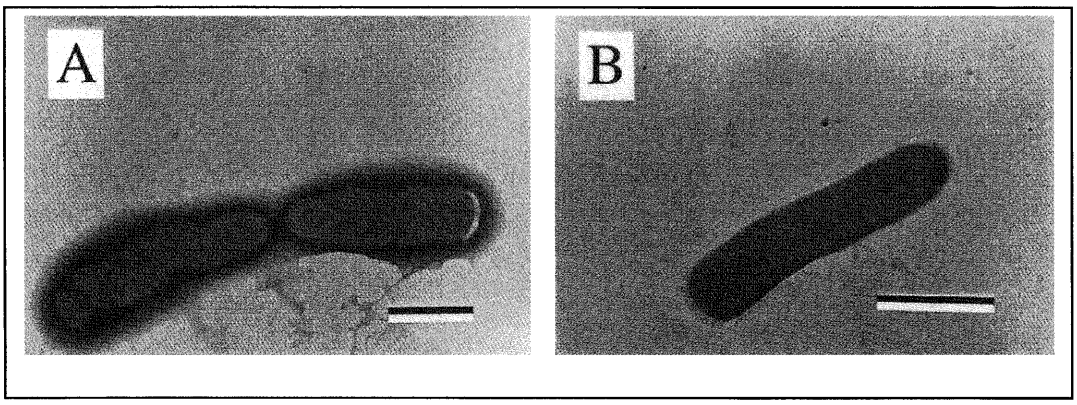

Figure 4 Transmission electron micrographs of cold–acitive decane degradable microorganisms strains N2B1 (A) and N2D (B). These strains were isolated from Katano beach (Kaga).
Cells were negatively stained with 1% sodium phosphotungustate solution.
Bars indicate 1.0 μm.

sole carbon source, have been isolated from enrichments that incubated at 10 °C. They were all cold-active microorganisms capable of growing at between 10 °C to 30 °C with n-decane as sole carbon source (Figure 5), and they could grow aerobically with the light components in the heavy oil such as simple aromatics and alkane like hydrocarbons. Although we have isolated totally over 300 strains of microorganisms from various samples, isolates could not use PAH (over 3 rings of aromatic

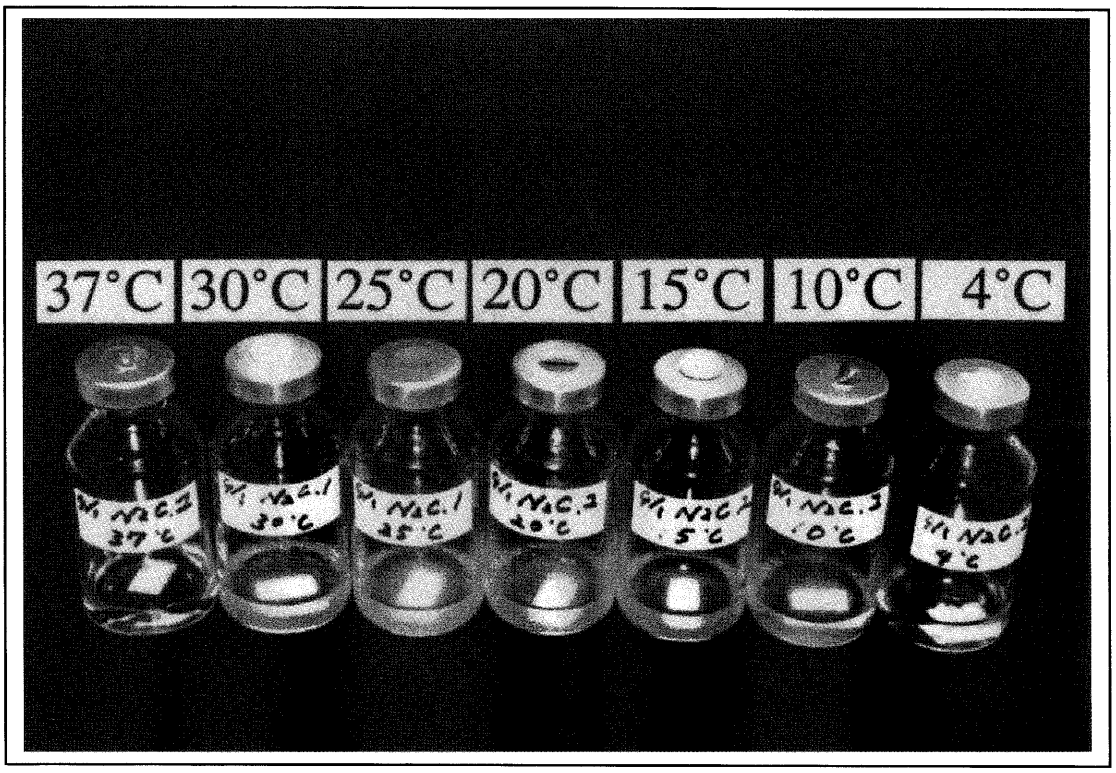

Figure 5 Cultures of strain N2C at various growth temperatures.
n-decane (10 μl) was added to 3 ml of the medium in 20 ml grass vials.
7 days growth.

hydrocarbon) aerobically and then we have not found PAHs degradable microorganisms in our investigated fields. These results suggested that native microorganisms such as our isolates was able to contribute to the aerobic biodegradation of the light components (alkane like chemicals and simple aromatics) of the heavy oil in the marine coastal areas under cold conditions in winter seasons of Hukuriku, and that the heavy components will be remained for long times in the marine ecosystem.

Phylogenetic analysis indicated that strains NL1, K1D and N2C had high similarity of the 16SrDNAwith genus *Pseudomonas* bacteria that were isolated from natural mineral water, spring water and deep sea water (Figure 6). Additionally, the phylogenetic position of strains NL1 and K1D was closest to *Pseudomonas veronii* (Elomari *et al.*, 1996), and strain N2C was to *Pseudomonas migulae* (Verhille *et al.*, 1999) (Figure 6). On the other hands, the sequence of strain N2D and N2B1 was identical to that of a gram positive actinobacterium, *Pimelobacter* (previouly *Nocardioides*) *simplex* (Yoon *et al.*, 1997). These results suggested that *Pseudomonas* bacteria and gram positive actinobacteria like *Pimelobacter* (previouly *Nocardioides*) *simplex,* may contribute to degrade the light components of Nakhodka's heavy oil in marine shore environments in Hokuriku.

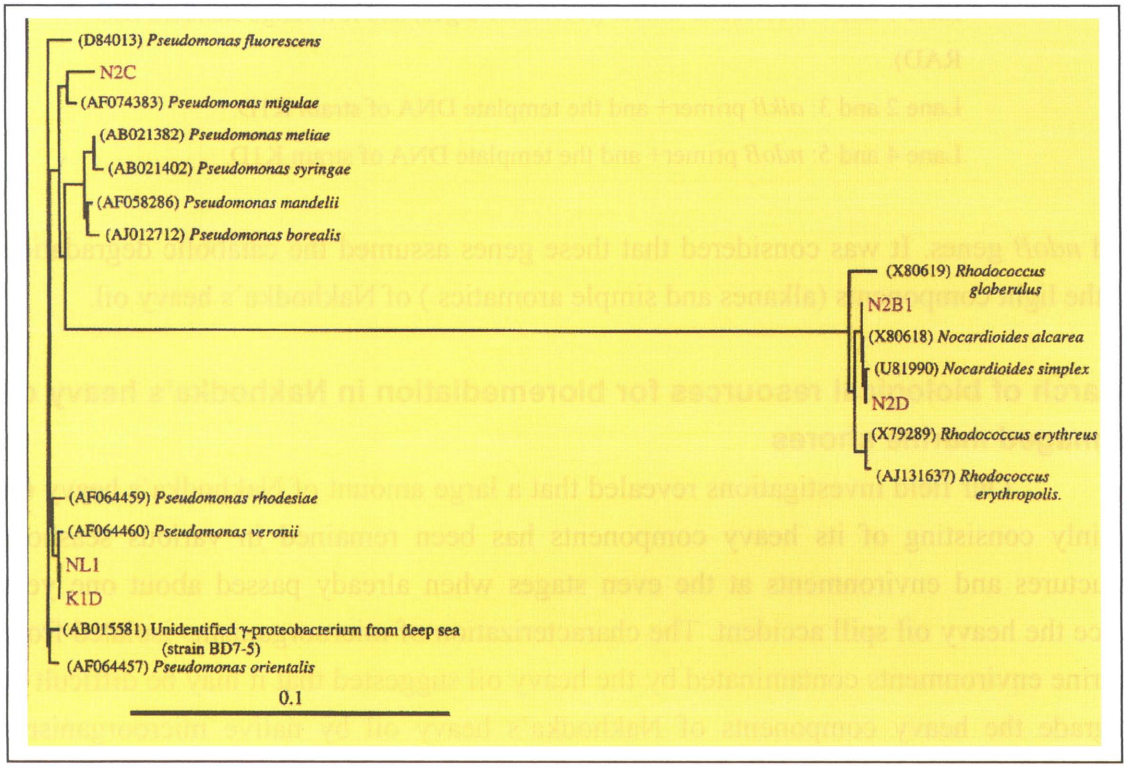

Figure 6 Phylogenetic tree based on 16SrDNA from strains NL1, K1D, N2C, N2B1 and N2D.

The agarose electrophoresis in Figure 7 showed that DNA bands predicated size (Table 1) were detected by PCR amplification with the designed primers for *alkB*

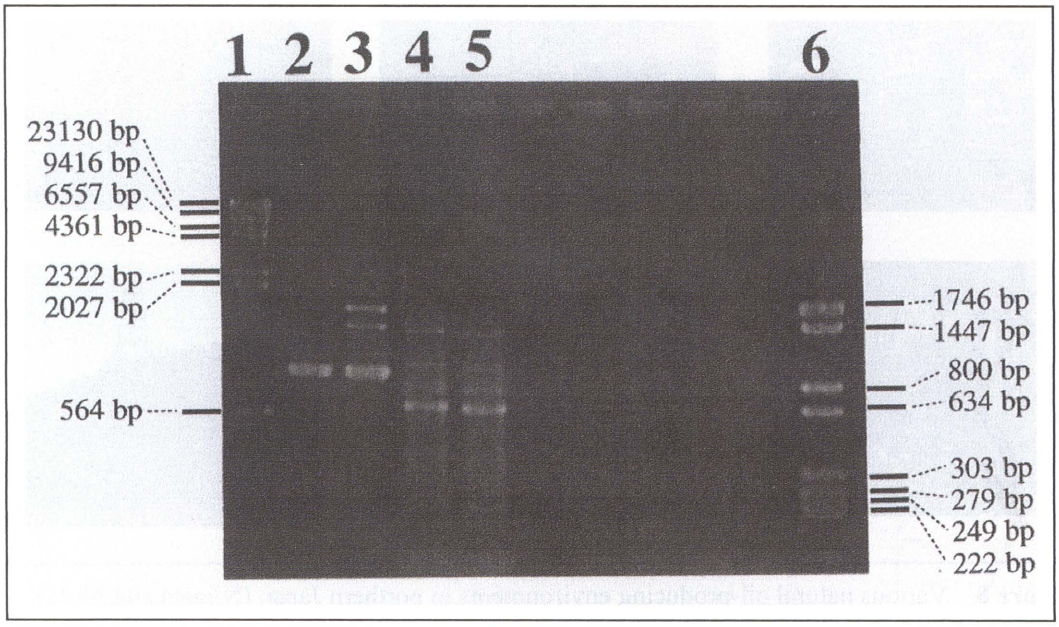

Figure 7 Detection of alkB and ndoB genes by PCR analysis in strain K1D.

Lane 1 and 6: Molecular maker (λ-*HindIII* digest and low range standard BIO-RAD).

Lane 2 and 3: *alkB* primer+ and the template DNA of strain K1D.

Lane 4 and 5: *ndoB* primer+ and the template DNA of strain K1D.

and *ndoB* genes. It was considered that these genes assumed the catabolic degradation of the light components (alkanes and simple aromatics) of Nakhodka's heavy oil.

Search of biological resources for bioremediation in Nakhodka's heavy oil damaged marine shores

Our field investigations revealed that a large amount of Nakhodka's heavy oil mainly consisting of its heavy components has been remained in various seashore structures and environments at the even stages when already passed about one year since the heavy oil spill accident. The characterization of microorganisms isolated from marine environments contaminated by the heavy oil suggested that it may be difficult to degrade the heavy components of Nakhodka's heavy oil by native microorganisms (originally living in the habitat). These facts suggested that it would be effective for bioaugmentation with external PAHs degradable microorganisms that obtained from other natural environments such as natural oil-producing wells or ponds (for example, shown in Figure 8) in the bioremediation of Nakhodka's heavy oil contaminated marine

Figure 8 Various natural oil-producing environments in northern Japan (Niigata and Akita).
A: Oil-producing wells in Sekiu-no-sato (Petroleum village) in Kanazu Niitsu, Niigata.

B: A natural oil-producing pond in Shinkleton memorial park in Kurokawa, Niigata.

C: A natural oil-producing pond in Toyokawa (Kusozu), Showa, Akita.

D: An oil producing well in Toyokawa (Kusozu), Showa, Akita.

E: Natural oil pumping up machines in operation in the suburb of Akita city, Akita.

F: A n oil producing well in Kurokawa Showa, Akita.

shoreline. In conclusion, we therefore would like to propose the bioaugmentation by using PAHs degradable microorganisms (like shown in Figure 9) that isolated from natural oil-producing environments in Japan such as shown in Figure 8, when heavy oil

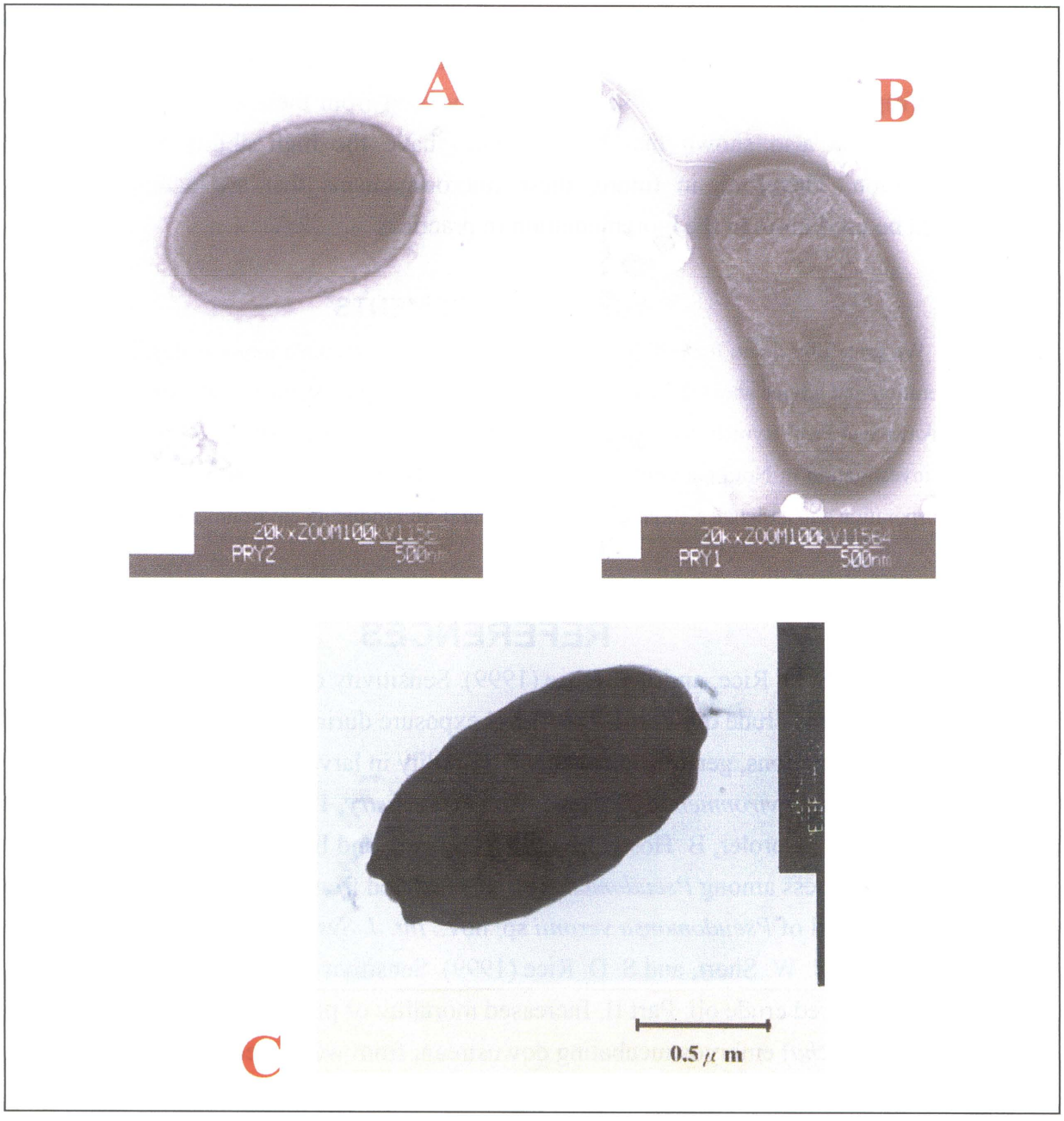

Figure 9 Transmission electron micrographs of PAHs degradable microorganisms isolated from natural oil-producing environments in Niigata.
Cells were negatively stained with 1% sodium phosphotungustate solution.
A and B: Pyrene degradable microorganisms strains PYR 2 and PYR1.
C: Benzo(a)pyrene degradable microorganism strain B2-1.

pollution (like Nakhodka's heavy oil spill accident) occurred in sea or ground environments of Japan. Because these environments have been always natural oil-spilling conditions for long time, many kinds of PAHs and/or oil components degradable microorganisms have been enriched naturally. In fact, we have succeeded to obtain many bacterial isolates that can grow using PAH component such as pyrene and benzo(a)pyrene as sole carbon source in the medium from these environments (Figure 9). We have also shown that these isolates have the high ability to toxic PAH degradation. Therefore, in future, these microorganisms that are natural treasures, should be used more to the bioremediation in practical.

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