Current Microbiology Vol. 49 (2004), pp. 415–422
DOI: 10.1007/s00284-004-4347-y

Current Microbiology
An International Journal
© Springer Science+Business Media, Inc. 2004

Isolation and Characterization of Novel Strains of *Pseudomonas aeruginosa* and *Serratia marcescens* Possessing High Efficiency to Degrade Gasoline, Kerosene, Diesel Oil, and Lubricating Oil

Patcharaporn Wongsa,[1,2] Makiko Tanaka,[1,3] Akio Ueno,[1,2] Mohammad Hasanuzzaman,[1,2] Isao Yumoto,[3] Hidetoshi Okuyama[2]

[1]ROM Co. Ltd., Minami 4, Nishi 9, Chuo-ku, Sapporo 064-0804, Japan
[2]Laboratory of Environmental Molecular Biology, Graduate School of Environmental Earth Science, Hokkaido University, Kita-ku, Sapporo 060-0810, Japan
[3]Institute for Biological Resource and Function, National Institute of Advanced Industrial Science and Technology, Toyohira-ku, Sapporo 062-8517, Japan

Received: 16 March 2004 / Accepted: 11 June 2004

Abstract. Bacteria possessing high capacity to degrade gasoline, kerosene, diesel oil, and lubricating oil were screened from several areas of Hokkaido, Japan. Among isolates, two strains, WatG and HokM, which were identified as new strains of *Pseudomonas aeruginosa* and *Serratia marcescens* species, respectively, showed relatively high capacity and wide spectrum to degrade the hydrocarbons in gasoline, kerosene, diesel, and lubricating oil. About 90–95% of excess amount of total diesel oil and kerosene added to mineral salts media as a sole carbon source could be degraded by WatG within 2 and 3 weeks, respectively. The same amount of lubricating oil was 60% degraded within 2 weeks. Strain HokM was more capable than WatG in degrading aromatic compounds in gasoline. This strain could also degrade kerosene, diesel, and lubricating oil with a capacity of 50–60%. Thus, these two isolates have potential to be useful for bioremediation of sites highly contaminated with petroleum hydrocarbons.

The use of enormous amounts of petroleum products contributes highly to environmental pollution. Spills of hydrocarbons occur from several causes, including blowouts, leakage from tanks, and dumping of waste petroleum products. The elevated loading of petroleum hydrocarbons in soil causes a significant decline in soil quality, and these soils have become unusable. To control the environmental risks caused by petroleum products, various new regulations have been introduced and, at the same time, research focusing on remediation of contaminated soils has increased.

Correspondence to: Hidetoshi Okuyama; *email:* hoku@ees.hokudai.ac.jp

Biodegradation by natural populations of microorganisms, or in situ attenuation [9], is a primary mechanism by which petroleum hydrocarbons could be eliminated from contaminated sites [2, 4, 14, 15]. To maximize pollution abatement, the following approaches have been exploited. Biostimulation, a process in which intrinsic hydrocarbon-degrading bacteria are stimulated with appropriate nutrient supplements to ensure that the microbial growth is sustained, and that the ratios of carbon (i.e., petroleum hydrocarbons) to other nutrients are limited only by carbon [19, 22]. Addition of biosurfactants increases the availability of long chain hydrocarbons to microbes and renders them more accessible to microbial enzyme systems for utilization [1, 3, 10, 11, 13]. In certain cases when intrinsic bacteria are unable to meet the required demand, specific de-

grader(s) that have been selected for degrading various hydrocarbons [6, 22, 25] are used (bioaugmentation). However, inhibition of inoculum growth due to nutrient limitation and competition with indigenous microorganisms, poor bioavailability of contaminants (because of their low water-solubility), and the difficulties of mixing the inoculated organisms with the contaminated soil [25] are the pitfalls of bioaugmentation. The combination of bioaugmentation with biostimulation might result in efficient bioremediation of soil. The strains able to degrade various petroleum hydrocarbon contaminants efficiently and grow cooperatively with normal indigenous soil microorganisms are those needed for bioaugmentation.

The present work has focused on this approach, aiming to isolate novel bacterial strains capable of petroleum degradation. In this study, we report isolates capable of efficiently degrading a wide spectrum of hydrocarbons found in refined petroleum.

Materials and Methods

Petroleum hydrocarbons. Refined fossil fuels (petroleum hydrocarbons) used throughout this study were gasoline, kerosene, diesel oil, and lubricating oil obtained from Nippon Oil Corporation (Tokyo, Japan).

Isolation of bacteria, media composition and culture conditions. Samples to be screened were collected from different areas of Hokkaido Prefecture, Japan. They comprised hot spring water, slightly contaminated with crude oil; water from an old kerosene tank of a residence, Sapporo City; and soils and mud from Hokkaido University campus, Sapporo. Media used throughout this study were LB or mineral salts medium (MSM) consisting of 0.4% NH_4NO_3, 0.47% KH_2PO_4, 0.0119% Na_2HPO_4, 0.001% $CaCl_2 \cdot 2H_2O$, 0.1% $MgSO_4 \cdot 7H_2O$, 0.001% $MnSO_4 \cdot 4H_2O$, and 0.0015% $FeSO_4 \cdot 4H_2O$, pH 7.0.

For isolation of oil degrader, 1 mL of sample was transferred to a 50-mL flask containing 10 mL of MSM supplemented with 0.1% yeast extract, and incubated at 30°C in a rotary shaker at 150 rpm until turbid growth was observed. The bacterial culture was diluted and spread on MSM agar plates containing kerosene (10 g L^{-1}) as carbon source for selective isolation of petroleum degrader. Plates were sealed with vinyl tape and left at 30°C until appearance of several colonies. Individual colonies were purified by repeated streaking on MSM-agar plates containing 10 g L^{-1} of kerosene, and a degradation test was conducted with purified isolates. The optimal temperature for growth was determined in LB broth in a range of 4–60°C.

Degradation test and GC analyses of petroleum products. Bacterial cells precultured overnight at 30°C in LB medium were transferred to a 50-mL screw-capped flask (to prevent loss of volatile hydrocarbon components) containing 10 mL of MSM plus one of the petroleum products (e.g., gasoline, kerosene, diesel oil, or lubricating oil) at a concentration of 10 g L^{-1}. Cells were grown for 4 weeks at 20°C with shaking at 150 rpm, and petroleum hydrocarbons remaining in the culture medium were determined. Cultured media were extracted every week with equal volume of dichloromethane (Wako Chemical Co., Osaka, Japan), and the extract was analyzed with a gas-liquid chromatograph (GC; model GC 353B, GL Science, Tokyo, Japan) equipped with a flame ionization detector (FID) and a capillary column (TC-1, length 30 m, ID 0.25 mm, film thickness 0.1 μm) obtained from GL Science. The carrier gas was helium. The injector and detector temperatures were set at 250°C and 300°C, respectively. For gasoline analysis, column temperature was first held at 35°C for 5 min, then raised to 220°C. For kerosene analysis, the column temperature was first set at 50°C, then raised to 220°C. For diesel oil analysis, the column temperature was first set at 50°C; then it was ramped to 270°C. In all above cases, temperatures were raised at the rate of 5°C min^{-1}. In case of lubricating oil, both injector and detector temperatures were set at 322°C. The column temperature was first set at 100°C; then it was ramped to 320°C at the rate of 10°C min^{-1} and held at least for 15 min. The percentage of total hydrocarbons remaining after one test was calculated by comparing area of their peaks with that of the corresponding peaks shown by a control that had been subjected to the same experimental conditions as the samples, except for the absence of a bacterial culture. The values so obtained were normalized against the internal standard. *normal*-Dodecane (nC_{12}) (Kanto Chemical Co., Tokyo) was used at a concentration of 3 g L^{-1} as internal standard.[1]

GC-MS analysis. Petroleum components were analyzed according to the method reported by Tzing et al. [24] with a Varian gas chromatograph-mass spectrometer (GC-MS; model CP-3800 gas chromatograph and Saturn 2200 mass spectrometer, Varian Technologies Japan, Inc.) equipped with the same column as described above. The temperature was programmed as described above. Resulting chromatograms were analyzed by Saturn Software GC/MS Workstation Version 5.52 to identify petroleum components. All analyses were carried out with the split ratio of 20:1. Helium was used as the carrier gas with a flow rate of 0.8 mL min^{-1}. Injector temperature was set at 250°C (322°C for lubricating oil). The MS conditions for electron-bombardment ionization-mass spectrometry (EI-MS) analysis were set as follows: m/z range of 10–500, 1 s scan time, ion trap temperature at 100°C, 10 μA emission current, 70 eV ionization voltage, and 25,000 automatic gain control. Acetonitrile was used as chemical ionization-mass spectrometry (CI-MS) reagent. The MS conditions for CI-MS analysis were as follows: m/z range of 10–500, 1 s scan time, ion trap temperature at 100°C, 100 μs ionization time, 2000 μs CI maximum ionization time, 120 ms CI maximum reaction time, 15 m/z CI ionization storage level, 75 m/z CI background mass, and 7.5 V reagent ion ejection amplitude.

Physiological and biochemical tests for strain identification. Phenotypic and biochemical characterization was performed as described by Yumoto et al. [26].

DNA base composition and DNA–DNA hybridization. DNA was isolated from bacterial cells as described by Marmur [16]. The DNA content of mol% G + C (G + C content) was determined according to Tamaoka and Komagata [21]. The levels of DNA relatedness were determined fluorometrically using photo-biotin-labeled DNA probes and microplates [8]. *Pseudomonas aeruginosa* JCM5962^T and *Serratia macescens* JCM 1239^T obtained from Japan Collection of Microorganisms (JCM; Wako, Japan) were used as reference strains.

Phylogenetic analysis using 16S rRNA gene sequence comparison. Almost the full length of 16S rRNA genes of bacteria was amplified by PCR with following sets of primers 5′-GAGTTTGATCCTGGCTCAG-3′ and 5′-AAGGAGGTGATCCA GCC-3′ corresponding to the positions 9 to 27 and 1525 to 1541,

[1]Note: Hydrocarbon with x carbon atoms is presented as C_x. normal-Aliphatic hydrocarbon with x carbon atoms is abbreviated as nC_x.

Table 1. Isolated petroleum-degrading bacterial strains and their growth temperatures (growth was followed by measuring the optical density of cultures at 595 nm)

Source	Strain	Optimal temperature (°C)	Temperature range (°C)
Hot spring water contaminated	HooP	45	4–50
with crude oil	TooY	45	4–50
Water in old kerosene tank	WatG	30	4–50
Soil from Hokkaido University campus	HokS	30	4–<50
Mud from Hokkaido University campus	HokM	30	4–<50

Table 2. Growth and degradation capacity of gasoline, kerosene, diesel oil, or lubricating oil at 10 g L^{-1} at 20°C. Growth is shown as the maximum optical density of cultures at 595 nm throughout 2 weeks of incubation. The initial optical density was 0.1. Degradation is percentage of the total amount of hydrocarbon remaining in 2-week cultures to that remaining in control cultures with no inoculum. All values in table are from the average of two or three independent experiments. Hydrocarbons with x carbon atoms are presented as C_x.

		Gasoline $\sim C_{10}$		Kerosene C_8–C_{18}		Diesel oil C_9–C_{23}		Lubricating oil C_{11}–C_{35}	
Strain	Source	Growth	Degradation (%)	Growth	Degradation (%)	Growth	Degradation (%)	Growth	Degradation (%)
WatG	Water	0.3 ± 0.1	30.0 ± 10.0	1.4 ± 0.1	70.0 ± 13.0	1.4 ± 0.1	90.8 ± 6.2	1.2 ± 0.1	57.0 ± 3.0
HokM	Mud	0.3 ± 0.1	44.0 ± 5.0	0.6 ± 0.1	55.0 ± 23.0	0.8 ± 0.5	67.0 ± 17.6	1.1 ± 0.5	50.0 ± 30.0
HooP	Hot spring	0.2 ± 0.0	24.0 ± 1.0	0.5 ± 0.1	28.5 ± 13.5	0.4 ± 0.1	32.0 ± 22.0	0.8 ± 0.1	66.0 ± 3.0
TooY	Hot spring	0.1 ± 0.0	0.0 ± 0.0	0.6 ± 0.0	60.0 ± 12.0	0.4 ± 0.1	62.0 ± 32.0	0.8 ± 0.4	60.0 ± 20.0
HokS	Soil	0.1 ± 0.0	0.0 ± 0.0	0.3 ± 0.1	20.5 ± 20.5	0.3 ± 0.1	40.0 ± 35.0	0.0 ± 0.0	0.0 ± 0.0

respectively, in the 16S rRNA gene sequence of *Escherichia coli* [5]. PCR products were sequenced directly using ABI PRISM Big Dye Terminator Cycle Sequencing Kit on an ABI 3100 DNA sequencer following the manufacturer's instruction. Multiple alignments of the sequences were performed, and a neighbor-joining phylogenetic tree [12, 18] was constructed using the latest version (ver. 1.8) of the CLUSTAL W program [23]. Similarity values of the sequences were calculated by using the GENETYX computer program (Software Development, Tokyo, Japan).

Results

Bacterial candidates. Mixtures of bacteria were obtained during enrichment using 0.1% yeast extract in MSM. Screening on an agar plate containing kerosene resulted in isolation of several candidates from the oil tank: hot spring water, soil, and mud samples. They all were able to grow at a wide range of temperatures of 4–50°C, although specific isolates showed different optimal growth temperatures. Strains WatG, HokM, and HokS showed optimal growth at 30°C. However, HooP and TooY isolates from the hot spring showed an optimal temperature of 45°C. The above results are summarized in Table 1.

Degradation capacity of petroleum products by various isolates. The growth and hydrocarbon degradation capacity of selected isolates in MSM supplemented with each refined fossil fuel are summarized in Table 2. No evaporation of any petroleum product from the screw-capped flasks was observed throughout the experiments. All isolates showed the ability to utilize kerosene; however, the efficiency of utilization varied among strains, with WatG being the best (70% removal within 2 weeks). HokM and TooY were able to degrade kerosene by more than 50%, whereas HooP and HokS could degrade about 20%. In the case of diesel oil, WatG was able to degrade diesel oil about 90%. HokM and TooY were able to degrade more than 60%, while HooP and HokS could degrade about 40%. One reference strain *P. aeruginosa* JCM 5962^T used as negative control could only degrade approximately 7% of diesel oil under the same conditions (data not shown). The results on the assessment for degradation capacity were similar to those of growth (Fig. 1). In gasoline-supplemented MSM, a small degree of growth was observed only in cases of strains WatG, HokM, and HooP. Gasoline was utilized to the extent of 30% for WatG, 44% for HokM, and 24% for HooP. Only WatG, HokM, HooP, and TooY could grow in MSM containing lubricating oil. WatG, HooP, and TooY have utilized about 60% of lubricating oil, whereas HokM utilized about 50%. Taken together, it suggests that strain WatG was the most efficient among the isolates and was able to

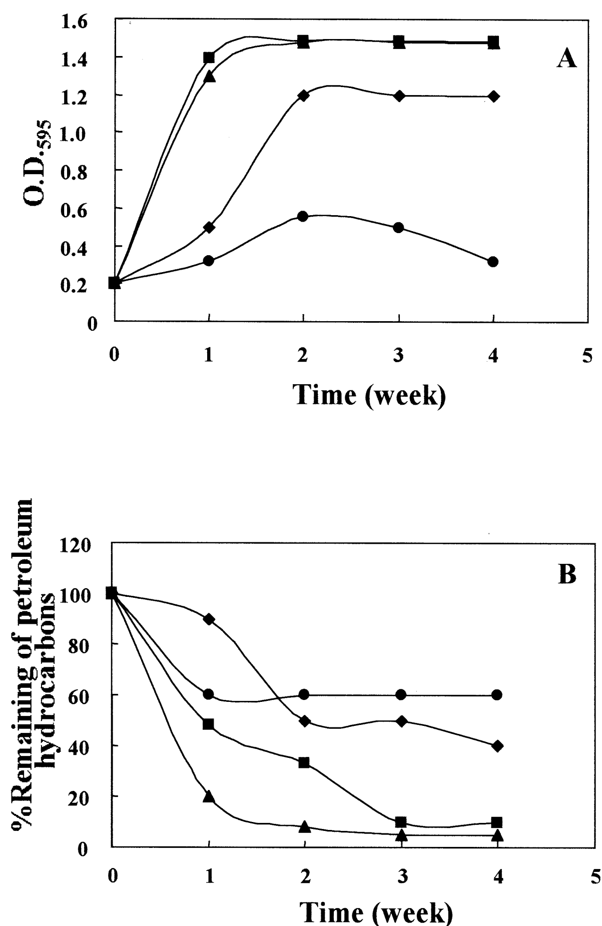

Fig. 1. Growth of strain WatG and its degradation of various petroleum products. WatG was grown in MSM containing either gasoline (●), kerosene (■), diesel oil (▲), or lubricating oil (♦) at 10 g L^{-1}. (A) Growth monitored by measuring the optical density of cultures at 575 nm; (B) remaining petroleum hydrocarbons estimated by the method described in Materials and Methods. The values are the average of two independent experiments. The maximum standard deviation is within ±0.1 for A and ±20 for B.

degrade all four types of petroleum products tested. The second best was strain HokM.

Degradation rate and preference of hydrocarbon utilization by strain WatG. Strain WatG was selected for closer examination of degradation rates. WatG in MSM supplemented with kerosene or diesel oil started to grow immediately and reached a stationary phase within a week (Fig. 1A). In contrast, the growth curves of WatG with gasoline and lubricating oil showed initial lag times, and 2 weeks were required to reach the stationary growth phase. Growth of WatG on gasoline was comparatively poor, but it grew rather well on lubricating oil. WatG degraded diesel oil the most rapidly, with the total amount of remaining diesel oil declined to 20% within 1 week (80% removal), and reached the maximum of 95% in 2 weeks (Fig. 1B). In the case of kerosene, WatG was able to degrade it by 50% in 1 week and about 90% in 3 weeks. About 30% of the total amount of gasoline was utilized after 1 week. However, no further change was observed even in the case of longer incubation. Lubricating oil appeared to be resistant to degradation by WatG at early stage, but degradation (40%) was observed during the second week of incubation.

The GC profile of the remnant compounds of gasoline, kerosene, diesel oil and lubricating oil in WatG culture was compared with that of the control. The major component of gasoline was aromatic hydrocarbons including toluene (C_7), dimethylbenzene (C_8), and trimethylbenzene (C_9). Aliphatic hydrocarbons such as nC_{10} and alicyclic hydrocarbons including cyclopentane (C_5) and cyclohexane (C_6) were minor components. Kerosene consisted of major components from C_8 to C_{18} *n*-alkanes. Figure 2A shows the GC profile of the control culture (2 weeks) with diesel oil, which included a mixture of hydrocarbons from C_9 to C_{23} consisting of *n*-alkanes, *iso*-alkanes, and cycloalkanes together with aromatic hydrocarbons having less than 12 carbon atoms (indicated by the solid bars in the figure). Lubricating oil contained minor *n*-alkanes of C_{11}–C_{20} and major unidentified long chain hydrocarbons from C_{20} to C_{35} (Fig. 3A).

All components in diesel oil were highly reduced by WatG, and particularly WatG degraded *n*-alkanes from C_9 to C_{23} almost completely (Fig. 2B). However, the degradation of hydrocarbons shorter than C_{12} was not so significant. This tendency was also observed in degradation of kerosene by WatG (data not shown). Figure 3B shows the GC profile of the remnant compounds of lubricating oil, in which most components were generally degraded by WatG, although less effectively than those of diesel oil and kerosene. In the case of gasoline, there was a common reduction of all components including aromatic, alicyclic, and aliphatic hydrocarbons (data not shown). These findings suggest that strain WatG had the substrate preference as follows: diesel oil > kerosene > lubricating oil > gasoline.

Effect of pH on growth of strain WatG and its utilization of petroleum hydrocarbons. To test cell growth and hydrocarbon degradability at different pH, cells of strain WatG were grown in MSM supplemented with 0.5% glucose or 1% diesel oil. In both conditions, WatG showed the ability to grow at a pH range from 5.5 to 8.0. The growth in glucose-supplemented medium was faster than that in diesel-oil-supplemented medium, and the optimal growth was

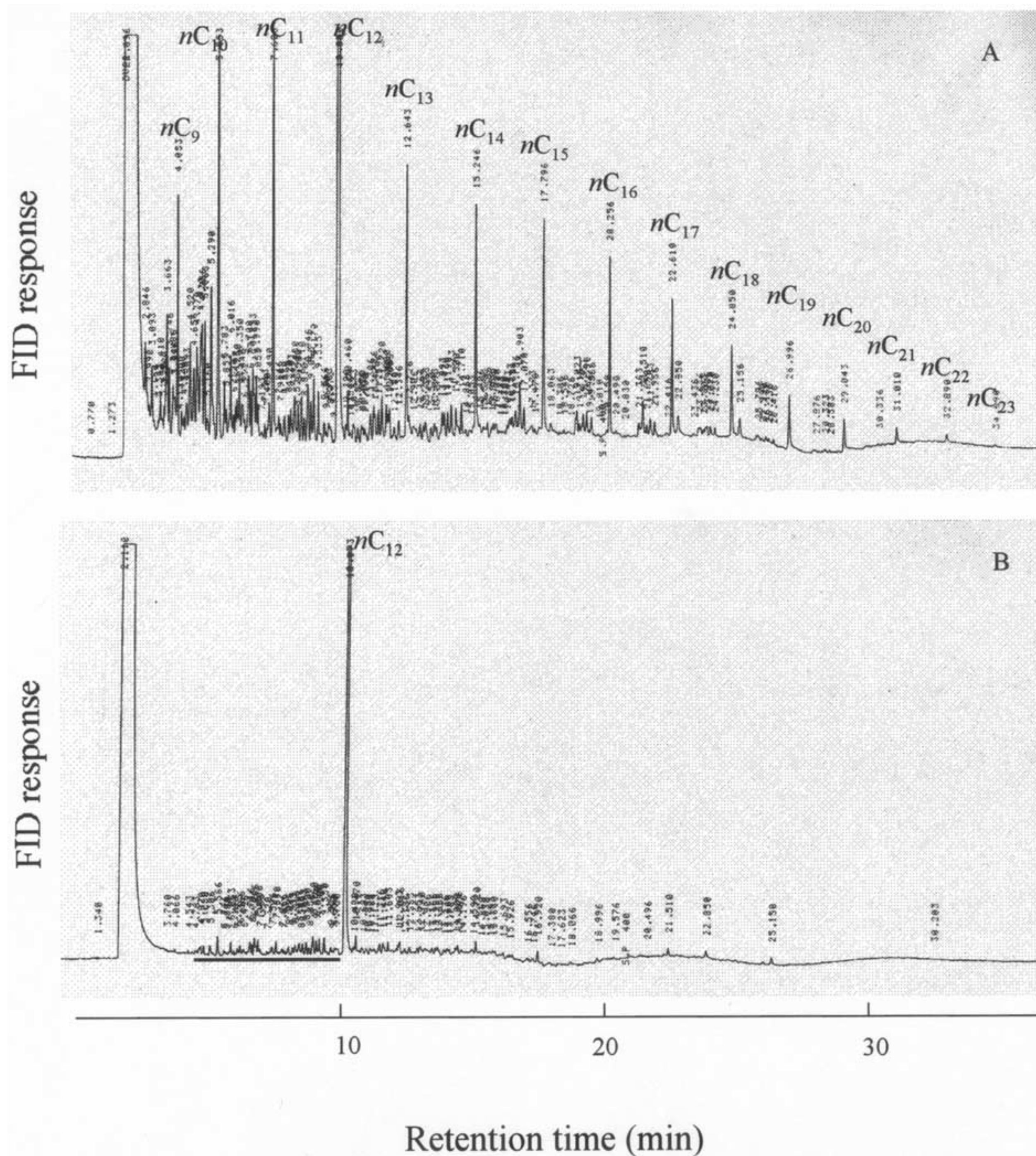

Fig. 2. GC profiles of diesel oil remaining in the MSM after incubation without (A) and with (B) strain WatG at 20°C for 2 weeks. *n*-Dodecane (nC_{12}) was used as the internal standard. nC_9 to nC_{23} indicate *n*-alkanes with the number of carbon atoms from 9 to 23. Minor peaks between two *n*-alkanes include those of *iso*-alkanes and cycloalkanes. The solid bar (B) indicates the area in which aromatic compounds were detected.

obtained at pH 7.5 (data not shown). However, in diesel-oil-supplemented medium, both bacterial growth and petroleum hydrocarbon consumption were optimal at pH 6.5 (data not shown).

Identification of strains WatG and HokM. The results of 16S rDNA sequence alignment and phylogenetic tree analysis revealed that 16S rDNA sequence of strain WatG was 100% identical to that of

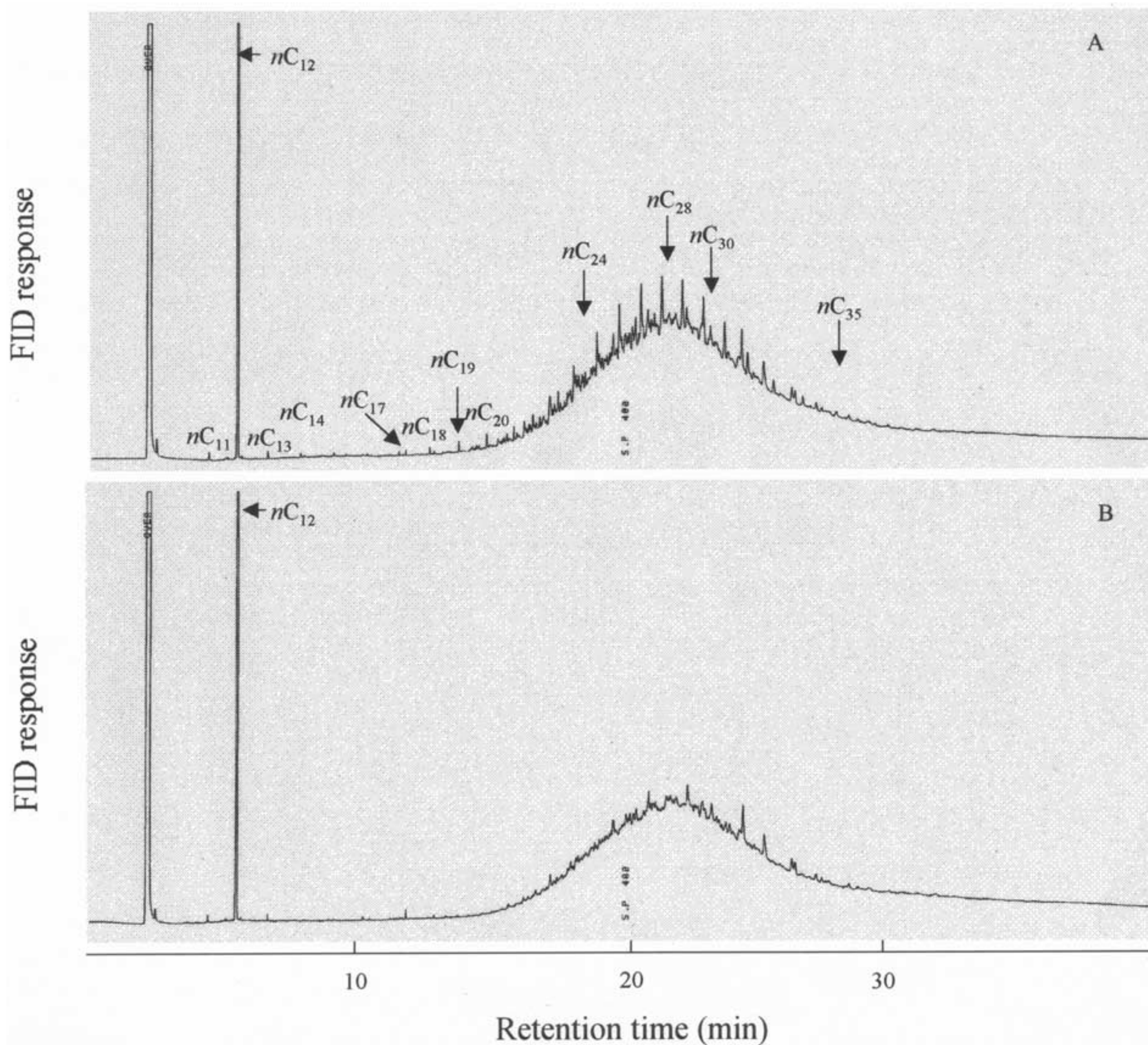

Fig. 3. GC profiles of lubricating oil remaining in the MSM after incubation without (A) and with (B) strain WatG at 20°C for 2 weeks. nC_{12} was used as the internal standard. *normal*-Alkanes of nC_{11} to nC_{20} are components of lubricating oil. Remaining components have not been identified. The positions of nC_{24}, nC_{28}, and nC_{30}, and nC_{35} are indicated (A) to show their relative retention time.

P. aeruginosa (Fig. 4A). The DNA–DNA hybridization between strain WatG and a reference strain *P. aeruginosa* JCM 5962^T was 96%. The taxonomic characteristics of strain WatG were mostly the same as those of *P. aeruginosa* JCM 5962^T, that is, tests for production of catalase and oxidase, reduction of NO_3 to NO_2, and hydrolysis of casein and gelatin are positive, but *o*-nitrophenyl-β-D-galactopyranoside (ONPG) test and hydrolysis of starch were negative for the both strains. However, strain WatG was able to hydrolyze neither lipids (supplied as tributyrin), maltose nor D-mannose, all of which were hydrolyzed by *P. aeruginosa* JCM 5962^T. The G+C content of WatG was found to be 73.6%, whereas it was 67.2% for *P. aeruginosa* JCM 5962^T, suggesting that strain WatG is a novel strain of this species.

The results of taxonomic tests on strain HokM indicated that this strain had similar characteristics to *S. marcescens* JCM 1239^T, that is, both strains were positive for production of catalase, hydrolysis of casein and DNA, and Voges-Proskauer and citrate (Simons's medium) tests but were negative for the production of oxidase and indole and for the methyl red test. The ONPG test was positive for strain HokM, but it was

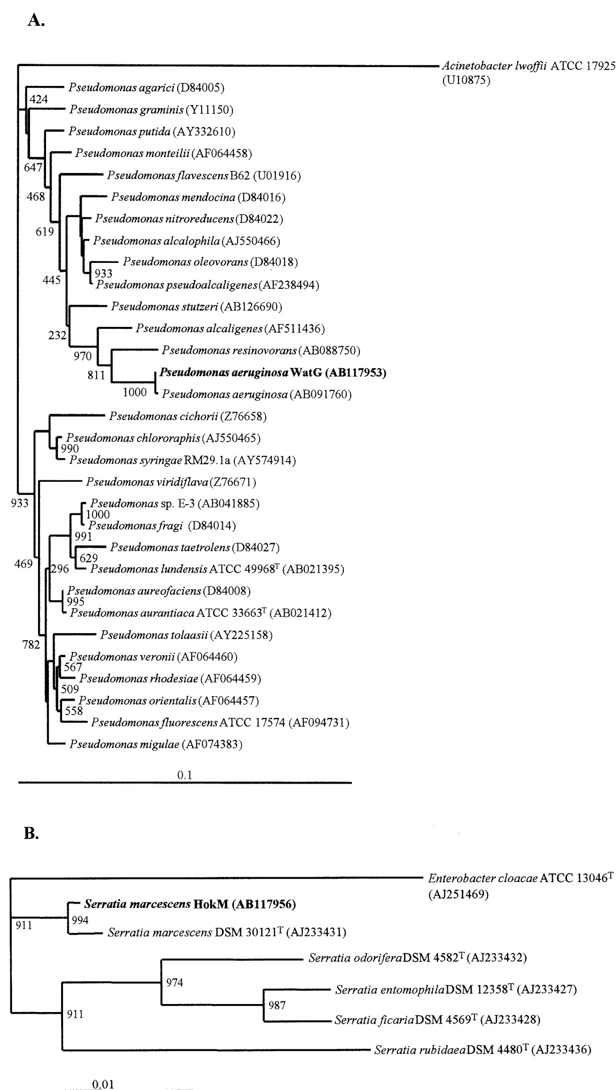

Fig. 4. Evolutionary distance tree based on 16S rDNA sequences of *P. aeruginosa* strain WatG (A) and *S. marcescens* strain HokM (B) with their references. Bar, nucleotide difference per sequence position. The accession numbers of the sequences are given in brackets.

negative for *S. marcescens* JCM 1239^T. The G+C content of HokM and *S. marcescens* JCM 1239^T was 60.2% and 57.5%, respectively. Strain HokM grew at 0°C, whereas *S. marcescens* $JCM1239^T$ did not grow below 4°C. The homology of 16S rDNA sequence between HokM and *S. marcescens* JCM 1239^T was 99.5% and the DNA–DNA hybridization between the strains was 96%. These results suggest that HokM is a novel strain of *S. marcescens*.

The nucleotide sequences of 16S rDNA of WatG and HokM have been deposited in the GenBank database under accession numbers AB117953 and AB117954, respectively.

Discussion

To carry out the bioaugmentation successfully, it would be necessary to select bacteria having a high capacity and the versatility to degrade the many components of petroleum products. Kerosene was known to contain short to middle size hydrocarbons in the range of C_8 to C_{18}, which include some aromatic groups as well as *n*-alkanes, *iso*-alkanes, and cycloalkanes [2, 20]. Consequently, bacteria able to grow on this carbon source would easily acquire the ability to degrade a wide variety of hydrocarbon components of different petroleum products. Therefore, bacterial screening was conducted using kerosene as a sole carbon source.

Among five isolated candidates, strains WatG and HokM appeared to be two best degraders (Table 2). However, in the present study, WatG was selected for detailed examination because of its characteristics in terms of hydrocarbon degradation ability and degradation rate (Table 2). It was also observed that it worked best at neutral or near neutral pH range (data not shown), and degradation efficiencies were dependent on the chain length and structure of hydrocarbons (Table 2). This strain was capable of degrading an excess amount of kerosene or diesel oil (10 g L^{-1}) present in the media in an efficient manner, and *n*-alkanes from C_9 to C_{23} in them were best degraded (Fig. 2B). Although it does it slowly, strain WatG is able to degrade longer recalcitrant hydrocarbons present in lubricating oil (Fig. 3B).

Strain HokM was identified as a new strain of *S. marcescens*. There are limited reports describing the involvement of *Serratia* in biodegradation of hydrocarbons [7, 17], and they are mostly degraders of aromatic compounds. In this study, it was observed that strain HokM utilized not only aromatic compounds but also long-chain alkanes (data now shown). As such, HokM would be a unique strain of *S. marcescens* possessing the ability to degrade a wide spectrum of hydrocarbons.

In this study, two highly efficient and wide-spectrum hydrocarbon degraders, strains WatG and HokM, were successfully isolated and identified as new strains of *Pseudomonas aeruginosa* and *Serratia marcescens*, respectively, by their taxonomic studies. They would have great application in bioremediation of hydrocarbon-contaminated sites. Bioaugmentation treatment with such a wide spectrum of degraders would be the preferable choice of treatment over many others. Currently, evaluation of WatG regarding its ability to decontaminate soils from petroleum hydrocarbons at laboratory scale is in progress.

ACKNOWLEDGMENTS

This work was partially supported by Northern Advancement Center for Science & Technology.

Literature cited

1. Banat IM, Makkar RS, Cameotra SS (2000) Potential commercial applications of microbial surfactants. Appl Microbiol Biotechnol 53:495–508
2. Bartha R (1986) Biotechnology of petroleum pollutant biodegradation. Microbial Ecol 12:155–172
3. Bouchez-Naïtali M, Blanchet D, Bardin V, Vandecasteele J (2001) Evidence for interfacial uptake in hexadecane degradation by *Rhodococcus equi*: the importance of cell flocculation. Microbiology 147:2537–2543
4. Boulton CA, Ratledge C (1984) The physiology of hydrocarbon utilizing microorganisms. In: Wiseman A (ed) Enzyme and fermentation biotechnology. New York: Wiley, pp. 11–77
5. Brosius J, Palmer JL, Kennedy JP, Noller HF (1978) Complete nucleotide sequence of a 16S ribosomal gene from *Escherichia coli*. Proc Natl Acad Sci USA 75:4801–4805
6. Dejonghe W, Boon N, Seghers D, Top EM, Verstraete W (2001) Bioaugmentation of soils by increasing microbial richness: missing links. Environ Microbiol 3:649–657
7. De la Fuente G, Perestelo F, Rodriguez Perez A, Falcon MA (1991) Oxidation of aromatic aldehides by *Serratia marcescens*. Appl Environ Microbiol 57:1275–1276
8. Ezaki T, Hashimoto Y, Yabuuchi E (1989) Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. Int J Syst Bacteriol 39:224–229
9. Gallego JLR, Loredo J, Llamas JF, Vázquez F, Sánchez J (2001) Bioremediation of diesel-contaminated soils: evaluation of potential *in situ* techniques by study of bacterial degradation. Biodegradation 12:325–335
10. Hommel RK (1994) Formation and functions of biosurfactants for degradation of water-insoluble substrates. In: Ratledge C (ed) Biochemistry of microbial biodegradation. Dordrecht: Kluwer, pp. 63–87
11. Iwabuchi N, Sunairi M, Urai M, Itoh C, Anzai H, Nakajima M, Harayama S (2002) Extracellular polysaccharides of *Rhodococcus rhodochrous* S-2 stimulate the degradation of aromatic components in crude oil by indigenous marine bacteria. Appl Environ Microbiol 68:2337–2343
12. Kimura M (1980) A simple method for estimating evolutionary rates base substitution through comparative studies of nucleotide sequences. J Mol Evol 16:111–120
13. Lang S, Wullbrandt D (1999) Rhamnose lipids-biosynthesis, microbial production and application potential. Appl Microbiol Biotechnol 51:22–32
14. Leahy JG, Colwell RR (1990) Microbial degradation of hydrocarbons in the environment. Microbiol Rev 54:305–315
15. Margesin R, Schinner F (2001) Biodegradation and bioremediation of hydrocarbons in extreme environments. Appl Microbiol Biotechnol 56:650–663
16. Marmur J (1961) A procedure for the isolation of deoxyribonucleic acid from microorganisms. J Mol Biol 3:208–218
17. Rojas-Avelizapa NG, Cervantes-Gonzalez E, Cruz-Camarillo R, Rojas-Avelizapa LI (2002) Degradation of aromatic and asphaltenic fractions by *Serratia liquefasciens* and *Bacillus* sp. Bull Environ Contam Toxicol 69:835–842
18. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic tree. Mol Biol Evol 4:406–425
19. Seklemova E, Pavlova A, Kovacheva K (2001) Biostimulation-based bioremediation of diesel fuel: field demonstration. Biodegradation 12:311–316
20. Solomons TWG (1990) Alkanes and cycloalkanes conformation analysis. In: Solomons TWG (ed) Fundamentals of organic chemistry, 3rd edn. New York: John Wiley & Sons Inc, pp. 93–143
21. Tamaoka J, Komagata K (1984) Determination of base composition by reversed-phase high-performance liquid chromatography. FEMS Microbiol Lett 25:125–128
22. Thomassin-Lacroix EJM, Eriksson M, Reimer K, Mohn WW (2002) Biostimulation and bioaugmentation for on-site treatment of weathered diesel fuel in Arctic soil. Appl Microbiol Biotechnol 59:551–556
23. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence weighing, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680
24. Tzing SH, Chang JY, Ghule A, Chang JJ, Lo B, Ling YC (2003) A simple and rapid method for identifying the source of spilled oil using an electronic nose: confirmation by gas chromatography with mass spectrometry. Rapid Commun Mass Spectrom 17:1873–1880
25. Vogel TM (1996) Bioaugmentation as a soil bioremediation approach. Curr Opin Biotechnol 7:311–316
26. Yumoto I, Kusano T, Shingyo T, Nodasaka Y, Matsuyama H, Okuyama H (2001) Assignment of *Pseudomonas* sp. strain E-3 to *Pseudomonas psychrophila* sp. nov., a new facultatively psychrophilic bacterium. Extremophiles 5:343–349