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Phylogenetic analysis of long-chain hydrocarbon-degrading bacteria and evaluation of their hydrocarbon-degradation by the 2,6-DCPIP assay

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Abstract Thirty-six bacteria that degraded long-chain hydrocarbons were isolated from natural environments using long-chain hydrocarbons (waste car engine oil, base oil or the *c*-alkane fraction of base oil) as the sole carbon and energy source. A phylogenetic tree of the isolates constructed using their 16S rDNA sequences revealed that the isolates were divided into six genera plus one family (*Acinetobacter*, *Rhodococcus*, *Gordonia*, *Pseudomonas*, *Ralstonia*, *Bacillus* and Alcaligenaceae, respectively). Furthermore, most of the isolates (27 of 36) were classified into the genera *Acinetobacter*, *Rhodococcus* or *Gordonia*. The hydrocarbon-degradation similarity in each strain was confirmed by the 2,6-dichlorophenol indophenol (2,6-DCPIP) assay. Isolates belonging to the genus *Acinetobacter* degraded long-chain normal alkanes (*n*-alkanes) but did not degrade short-chain *n*-alkanes or cyclic alkanes (*c*-alkanes), while isolates belonging to the genera *Rhodococcus* and *Gordonia* degraded both long-chain *n*-alkanes and *c*-alkanes.

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Introduction

Environmental pollution by petroleum hydrocarbons has become a serious problem all around the world. Large-scale incineration plants have been developed, and incineration of hydrocarbon pollutants is carried out to clean up hydrocarbon-contaminated sites. The treatment time is short, but the system requires huge machines and large amounts of heavy oils (Matsumiya and Kubo 2007). Biological treatments for hydrocarbon-degradation have also been investigated. For the construction of such bioremediation systems, many kinds of hydrocarbon-degrading bacteria have been isolated and analyzed (Pritchard et al. 1992; Komukai-Nakamura et al. 1996; Lal and Khanna 1996; Korda et al. 1997; Vidali 2001; Iwamoto and Nasu 2001; Dua et al. 2002; Aislabie et al. 2006). Long-chain hydrocarbons, especially long-chain cyclic alkanes (*c*-alkanes), are difficult for bacteria to degrade (Perry 1979, 1984). Therefore, isolation of long-chain hydrocarbon-degrading bacteria is important for bioremediation of hydrocarbons.

Various screening and evaluation methods have been developed for isolating hydrocarbon-degrading bacteria. 2,6-Dichlorophenol indophenol (2,6-DCPIP)

is an oxidation–reduction indicator that detects the oxidation of NADH to NAD^+, which is related to hydrocarbon-degradation by bacteria. The 2,6-DCPIP assay has been used for rapid and simple isolation of hydrocarbon-degrading bacteria (Hanson et al. 1993). This assay has also been used for estimating hydrocarbon-degrading bacteria in soil (van Hamme et al. 2000).

In previous studies, we isolated three kinds of long-chain hydrocarbon-degrading bacteria (*Acinetobacter* sp. ODDK71, *Rhodococcus* sp. NDKK48 and *Gordonia* sp. NDKY76A) for bioremediation of hydrocarbons (Koma et al. 2001, 2003a, b, 2005). Strain ODDK71 used long-chain *n*-alkanes as a sole carbon and energy source, but did not use *c*-alkanes (Koma et al. 2001). This strain did degrade *c*-alkanes under limited conditions, such as co-metabolism with *n*-alkanes (Koma et al. 2003a). On the other hand, strains NDKK48 and NDKY76A used *n*-alkanes and *c*-alkanes as sole carbon and energy sources (Koma et al. 2003b), but the metabolic pathways for *c*-alkanes differed between the two strains (Koma et al. 2003a, 2005). Since the hydrocarbon-degrading abilities seem to differ among genera, exhaustive analyses of hydrocarbon-degradation will be important for selecting microorganisms for bioremediation of hydrocarbons. The present paper describes the isolation and characterization of long-chain hydrocarbon-degrading bacteria, and analysis of their hydrocarbon-degrading abilities by the 2,6-DCPIP assay.

Materials and methods

Screening of long-chain hydrocarbon-degrading bacteria

Car engine oil-degrading bacteria were isolated using W medium (per liter: 2 g $(NH_4)_2SO_4$, 14.3204 g Na_2HPO_4, 5.4436 g KH_2PO_4, 0.5 g NaCl, 0.2465 g $MgSO_4$, 2.78 mg $FeSO_4 \cdot 7H_2O$, 14.7 mg $CaCl_2 \cdot 2H_2O$, 2.01 mg $ZnSO_4 \cdot 7H_2O$, 0.15 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.2 mg $CuSO_4 \cdot 5H_2O$, 0.4 mg $CoCl_2 \cdot 6H_2O$ and 1.49 mg $MnSO_4 \cdot 5H_2O$) (Koma et al. 2003a) containing 1% (w/v) car engine waste oil, car engine base oil (SAE10; Shin-nihonsekiyu Co., Tokyo, Japan) or the *c*-alkane fraction from the base oil as a sole carbon and energy source. The car engine base oil contains 75% *c*-alkanes (carbon numbers: above 16) (Konishi and Ueda 1992), and the *c*-alkane fraction was prepared by the urea adduct method (Lappas et al. 1997). Each soil suspension or water sample was added to 5 ml of the medium, and cultivated at 30°C and 100 rpm for 72 h. Cultures showing an optical density at 660 nm of >1.0 were spread on Luria-Bertani (LB)-agar medium (per liter: 10 g peptone, 5 g yeast extract, 5 g NaCl and 20 g agar). In the same environmental samples, the similar shaped colonies were omitted. Isolated microorganisms were pre-cultivated in 5 ml of LB broth at 30°C and 160 rpm overnight. Each pre-culture was inoculated into 5 ml of W medium containing 1% (w/v) oil, and cultivated at 30°C and 100 rpm for 72 h. One-ml of the culture was centrifuged at 4,000*g* for 5 min, and the cells were re-suspended into 1 ml of W medium. Bacteria showing an optical density at 660 nm of >1.0 were selected as candidates for hydrocarbon-degrading bacteria. The hydrocarbon-degradation abilities of the candidates were investigated using a previously reported method (Koma et al. 2003a).

Identification and phylogenetic analysis of isolated microorganisms

Isolated strains were identified by their 16S ribosomal DNA (16S rDNA) sequences. The DNA extraction method for 16S rDNA sequencing and sequence data analysis were described previously (Sanpa et al. 2006). The 16S rDNA of the isolates was amplified by polymerase chain reaction (PCR) with primers 20F (5′-TGTAA TCGTC GGCCA GTAGA GTTTG ATCCT GGCTC -3′) and 1510R (5′-CAGGA AACAG CTATG ACCGG CTACC TTGTT ACGAC T-3′). The reaction was carried out for 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 1 min. The amplified DNA was purified using a PCR product purification kit (PCR-M Clean-Up System; Viogene, Taipei, Taiwan). The 16S rDNA sequences (500 nt) of the isolated bacteria were determined with an SQ5500E DNA sequencer (Hitachi, Tokyo, Japan) using a sequencing primer (5′-TTGCG CTCGT TGCGG GACT-3′), and the data were compared with the data in the GenBank database using BLAST (version 3.0), which is available at http://www.ncbi.nlm.nih.gov/blast/. A phylogenetic tree was constructed by the neighbor-joining method. The 16S rDNA sequences of the isolated bacteria were aligned by the software Clustal X, and the root phylogenetic tree was drawn by the software njplot (Arenskötter et al. 2001).

Measurement of the degradation ratio of long-chain hydrocarbons

The strains *Acinetobacter* sp. ODDK71, *Rhodococcus* sp. NDKK48 and *Gordonia* sp. NDKY76A were pre-cultivated in 5 ml of LB broth at 30°C and 160 rpm for 24 h. Each pre-culture was inoculated into 100 ml of W medium in 500-ml baffled flasks containing 1% (w/v) car engine base oil or 0.1% (w/v) hydrocarbon (decane (C_8), hexadecane (C_{16}), eicosane (C_{20}) or dodecylcyclohexane) and cultivated at 30°C and 120 rpm for 72 h (Model AT-12R; Thomas, Tokyo, Japan). After the cultivation, residual oil was extracted by the chloroform-methanol extraction method and measured by gas-chromatography (Koma et al. 2001).

Estimation of hydrocarbon-degrading abilities by the 2,6-DCPIP assay

The 2,6-DCPIP assay was modified as follows. Isolated strains were pre-cultured in 5 ml of LB broth at 30°C and 160 rpm until the optical density at 660 nm became >1.0. After centrifugation at 4,000*g* for 5 min and washing with 0.9% saline, the cell density was adjusted to 1.0 according to the optical density at 660 nm. After sterilization, 750 µl of W medium (Fe-free), 50 µl of $FeCl_3 \cdot 6H_2O$ solution (150 µg/ml) and 50 µl of 2,6-DCPIP solution (37.5 µg/ml) were added to a 1.5-ml microtube. Subsequently, 80 µl of cell suspension and 5 µl of sterilized substrate (various petroleums or hydrocarbons) were added to the medium, and the cells were cultivated at 30°C and 100 rpm for 48 h. Subsequently, the color of the medium was observed, and evaluated as positive for microbial hydrocarbon-degrading ability if colorless (degraded) and negative for microbial hydrocarbon-degrading ability if blue (not degraded).

Results

Isolation and identification of long-chain hydrocarbon-degrading bacteria

Long-chain hydrocarbons are difficult to be degraded by microorganisms in nature (Perry 1979, 1984), in fact long-chain *n*- and *c*-alkanes are often detected in the hydrocarbon-contaminated soils. In order to enhance the efficiency of bioremediation for hydrocarbon-contaminated soils, various kinds of bacteria were isolated using three types of long-chain hydrocarbons (waste car engine oil, base oil or the *c*-alkane fraction of the base oil). About 400 samples were used for the isolation of long-chain hydrocarbon-degrading bacteria. Thirty-six strains showing high cell densities (optical density at 660 nm of >1.0) in the hydrocarbon-containing media were isolated (13 strains from waste oil; 6 strains from base oil; and 17 strains from the *c*-alkane fraction). The genera of the isolates were analyzed by BLASTN using the variable region of 16S rDNA registered in GenBank (Table 1), and a phylogenetic tree of the isolates based on their 16S rDNA sequences was constructed (Fig. 1).

The 36 strains were classified into 4 groups: actinobacteria (17 strains); γ-proteobacteria (12 strains); β-proteobacteria (4 strains); and firmicutes (3 strains). Thirteen strains among the actinobacteria isolates (NDMI114, NDKK1, NDKK2, NDKK5, NDKK6, NDKK7, NDKK48, ODNM2B, NDMI54, NDKY82A, ODNM1C, NDKY3D and NDKY72A) belonged to the genus *Rhodococcus*, and the remaining strains (NDK Y2B, NDKY2C, NDKY76A and NDKK46) belonged to the genus *Gordonia*. Ten strains among the γ-proteobacteria isolates (ODDK71, ODYM1, ODYM2, ODYM3, ODYM5, A132, ODMI29, NDMI78, NDM I119 and ODNM6) belonged to the genus *Acinetobacter*. In this screening, 3/4 isolates were identified as the genus *Rhodococcus*, *Acinetobacter* or *Gordonia*. The genera *Rhodococcus* and *Gordonia* were mainly isolated using the *c*-alkane fraction of base oil, while the genus *Acinetobacter* was mainly isolated when waste oil or base oil was used as the sole carbon and energy source.

Analysis of the hydrocarbon-degrading abilities of strains ODDK71, NDKK48 and NDKY76A by the 2,6-DCPIP assay

The 2,6-DCPIP solutions have been used for estimating hydrocarbon-degrading microorganisms in soil (van Hamme et al. 2000). To investigate hydrocarbon-degrading abilities of 36 strains exhaustively, the accuracy and sensitivity of the 2,6-DCPIP assay for hydrocarbon-degradation was tested. The

Table 1 Homology searches of isolated car engine oil-degrading bacteria[a]

Strain	Homology with[b]	Identified as
A132	*Acinetobacter* sp. SMCC B0208 (100)	*Acinetobacter* sp.
NDMI119	*Acinetobacter* sp. SMCC B0208 (99.8)	*Acinetobacter* sp.
NDMI78	*Acinetobacter* sp. SMCC B0208 (99.2)	*Acinetobacter* sp.
ODDK71	*Acinetobacter* sp. ATCC 11171 (100)	*Acinetobacter* sp.
ODMI29	*Acinetobacter* sp. SMCC B0208 (99.6)	*Acinetobacter* sp.
ODNM6	*Acinetobacter* sp. SMCC B0208 (99.8)	*Acinetobacter* sp.
ODYM1	*Acinetobacter haemolyticus* LH168 (100)	*Acinetobacter* sp.
ODYM2	*Acinetobacter haemolyticus* LH168 (100)	*Acinetobacter* sp.
ODYM3	*Acinetobacter* sp. SMCC B0208 (100)	*Acinetobacter* sp.
ODYM5	*Acinetobacter haemolyticus* LH168 (100)	*Acinetobacter* sp.
ODMI79	*Achromobacter xylosoxidans* (99.8)	Alcaligenaceae
	Alcaligenes sp. TS-MOSK-4 (99.4)	
ODNM5	*Alcaligenes* sp. J12 (99.8)	Alcaligenaceae
	Achromobacter xylosoxidans (99.8)	
F31	*Bacillus thuringiensis* 2000031482 (100)	*Bacillus* sp.
ODMI57	*Bacillus subtilis* GL (99.4)	*Bacillus* sp.
ODNM4	*Bacillus anthracis* 2000031664 (100)	*Bacillus* sp.
NDKK46	*Gordonia terrae* COE-O1 (100)	*Gordonia* sp.
NDKY2B	*Gordonia terrae* COE-O1 (100)	*Gordonia* sp.
NDKY2C	*Gordonia terrae* COE-O1 (100)	*Gordonia* sp.
NDKY76A	*Gordonia terrae* COE-O1 (100)	*Gordonia* sp.
F721	*Pseudomonas aeruginosa* PAO1 (100)	*Pseudomonas* sp.
F722	*Pseudomonas aeruginosa* PAO1 (100)	*Pseudomonas* sp.
ODMI90	*Ralstonia eutropha* HAMBI2380 (100)	*Ralstonia* sp.
ODNM1AB	*Ralstonia taiwanensis* BHU1(100)	*Ralstonia* sp.
ODNM2B	*Rhodococcus* sp. Lact1 (100)	*Rhodococcus* sp.
NDKK1	*Rhodococcus* sp. Lact1 (100)	*Rhodococcus* sp.
NDKK2	*Rhodococcus* sp. Lact1 (100)	*Rhodococcus* sp.
NDKK48	*Rhodococcus* sp. Lact1 (100)	*Rhodococcus* sp.
NDKK5	*Rhodococcus* sp. Lact1 (100)	*Rhodococcus* sp.
NDKK6	*Rhodococcus* sp. Lact1 (100)	*Rhodococcus* sp.
NDKK7	*Rhodococcus* sp. Lact1 (100)	*Rhodococcus* sp.
NDKY3D	*Rhodococcus ruber* AS 4.1038 (100)	*Rhodococcus* sp.
NDKY72A	*Rhodococcus ruber* AS 4.1038 (100)	*Rhodococcus* sp.
NDKY82A	*Rhodococcus* sp. Lact1 (100)	*Rhodococcus* sp.
NDMI144	*Rhodococcus* sp. Lact1 (99.8)	*Rhodococcus* sp.
ODMI54	*Rhodococcus* sp. Lact1 (100)	*Rhodococcus* sp.
ODNM1C	*Rhodococcus* sp. Lact1 (99.8)	*Rhodococcus* sp.

[a] Homology searches were performed by BLASTN using the 16S rDNA data registered in GenBank

[b] The values in parentheses indicate the percentage similarities

hydrocarbon-degradation abilities of strains ODD K71, NDKK48 and NDKY76A were compared with both the 2,6-DCPIP assay and GC analysis (Table 2). The degradation ratio of 0.1% (w/v) *n*-hexadecane by strain ODDK71 was >90% after 48 h of cultivation as evaluated by GC analysis, and the color of the 2,6-DCPIP solution changed from blue to colorless. In contrast, the strain did not degrade alkylcyclohexanes according to the GC analysis, and the color of the 2,6-DCPIP solution remained blue. On the other hand, *c*-alkane-degrading bacteria, strains NDKK48 and NDKY76A, degraded dodecylcyclohexane as evaluated by GC analysis, and the color of the 2,6-DCPIP solution changed from blue to colorless.

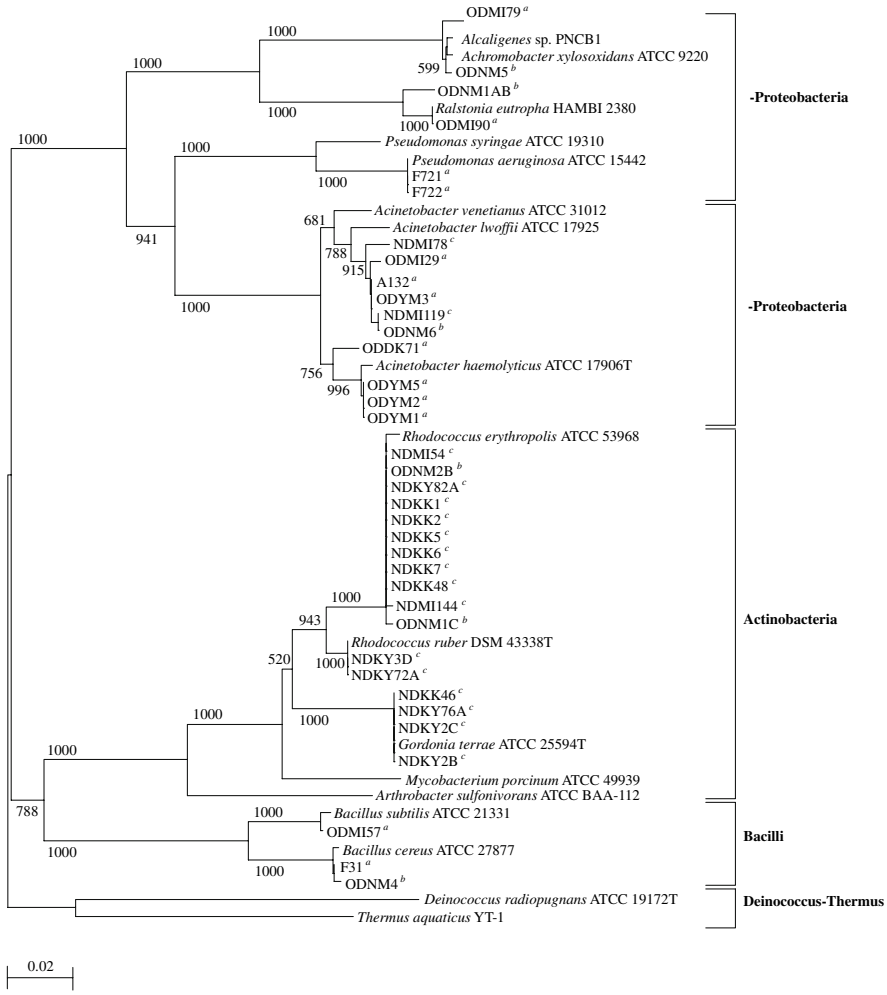

Fig. 1 Phylogenetic analysis of isolated car engine oil-degrading bacteria using partial sequences of their 16S rDNAs (500 nt). The kinds of oils used as carbon and energy sources are indicated by superscript letters: *a*, waste oil; *b*, base oil; *c*, *c*-alkane fraction of base oil. The 16S rDNA sequences of authentic strains were obtained from GenBank, and their accession numbers are as follows: *Alcaligenes* sp. PNCB1 (AY090020); *Achromobacter xylosoxidans* ATCC 9220 (AF411021); *Ralstonia eutropha* HAMBI 2380 (AF501365); *Pseudomonas syringae* ATCC 19310 (AJ308316); *Pseudomonas aeruginosa* ATCC 15442 (AF094718); *Acinetobacter venetianus* ATCC 31012 (AJ295007); *Acinetobacter lwoffii* ATCC 17925 (U10875); *Acinetobacter haemolyticus* ATCC 17906T (Z93437); *Rhodococcus erythropolis* ATCC 53968 (AY281112); *Rhodococcus ruber* DSM 43338T (X80625); *Gordonia terrae* ATCC 25594T (X81922); *Mycobacterium porcinum* ATCC 49939 (AY012580); *Arthrobacter sulfonivorans* ATCC BAA-112 (AF235091); *Bacillus subtilis* ATCC 21331 (AB018487); *Bacillus cereus* ATCC 27877 (Z84581); *Deinococcus radiopugnans* ATCC 19172T (Y11334); and *Thermus aquaticus* YT-1 (L09663)

These results indicate that color change of the 2,6-DCPIP solution and hydrocarbon-degradation by GC analysis corresponded completely (Table 2). Thus, the 2,6-DCPIP assay can evaluate microbial hydrocarbon-degradation abilities in an accurate, sensitive and simple manner.

Characterization of hydrocarbon-degrading bacteria by the 2,6-DCPIP assay

Exhaustive analysis of hydrocarbon-degradation can be evaluated by measuring the growth of each strain using many types of hydrocarbons, however growth

Table 2 Analysis of hydrocarbon-degradation by major strains using gas-chromatography and the 2,6-DCPIP assay

Substrate	*Acinetobacter* sp. ODDK71		*Rhodococcus* sp. NDKK48		*Gordonia* sp. NDKY76A	
	GC[a]	2,6-DCPIP[b]	GC[a]	2,6-DCPIP[b]	GC[a]	2,6-DCPIP[b]
n-Decane (C_{10})	0.0 ± 0.0	−	21.9 ± 2.6	+	95.4 ± 4.6	+
n-Hexadecane (C_{16})	90.2 ± 1.8	+	82.3 ± 5.9	+	95.0 ± 4.9	+
n-Eicosane (C_{20})	3.9 ± 2.2	+	20.2 ± 5.2	+	34.2 ± 5.6	+
Dodecylcyclohexane	0.0 ± 0.0	−	27.9 ± 2.7	+	98.4 ± 0.4	+
Car engine base oil	10.6 ± 2.8	+	14.6 ± 0.5	+	25.5 ± 0.2	+

[a] The values indicate the degradation ratio of 0.1% (w/v) of the respective hydrocarbons or 1% (w/v) car engine base oil after cultivation for 48 h (ODDK71) or 72 h (NDKK48 and NDKY76A)

[b] The symbols + and − indicate that the solution was colorless (degraded) or blue (not degraded), respectively

rate and cell size etc. influence the optical density. The 2,6-DCPIP assay can sensitively detect the primary oxidation of hydrocarbon, therefore the assay is suitable for exhaustive investigation of hydrocarbon-degradation of each isolate. Hydrocarbon-degradation by the isolated bacteria was analyzed by the 2,6-DCPIP assay (Table 3), and characterization of the isolates was carried out. All isolates belonging to the respective genera degraded base oil and heavy crude oils (types A and C). Regarding *n*-alkane-degradation, the long-chain *n*-alkanes hexadecane (C_{16}) and eicosane (C_{20}) were also degraded by all isolates. All strains of the genera *Gordonia* and *Pseudomonas* degraded short- and middle-chain *n*-alkanes, namely hexane (C_6), octane (C_8) and decane (C_{10}), while several strains of the genus *Rhodococcus* degraded hexane (4 of 13 strains), octane (4 of 13 strains) and decane (10 of 13 strains). However, almost all strains of the genus *Acinetobacter* (9 of 10 strains) did not degrade short-chain *n*-alkanes.

On the other hand, *c*-alkanes were mainly degraded by strains belonging to the genera *Rhodococcus* and *Gordonia*. Strains belonging to the genera *Rhodococcus* (5 of 13 strains) and *Gordonia* (4 of 4 strains) degraded hexylcyclohexane, and almost all strains belonging to the genera *Rhodococcus* (10 of 13 strains) and *Gordonia* (4 of 4 strains) degraded dodecylcyclohexane. However, strains belonging to the genera *Rhodococcus* (2 of 13 strains) and *Gordonia* (1 of 4 strains) found it difficult to degrade methylcyclohexane. These results indicate that strains belonging to the genera *Rhodococcus* and *Gordonia* can efficiently degrade *c*-alkanes with long alkyl side-chains.

Regarding aromatic hydrocarbon-degradation, dodecylbenzene (length of alkyl side-chain: 12) was degraded by many strains throughout all the genera, whereas no strains in the genera degraded benzene. For polycyclic hydrocarbons, decalin, naphthalene and anthracene were degraded by several strains belonging to 5 genera, although tetralin was difficult to degrade. Strains of the genus *Rhodococcus* degraded more kinds of polycyclic hydrocarbons compared to the other genera, since decalin, naphthalene and anthracene were degraded by 6, 8 and 7 strains of the genus *Rhodococcus*, respectively.

n-Alkanes were degraded by all strains of the respective genera, and the degradable substrates were similar for each genus (Table 3). *c*-Alkanes were mainly degraded by strains of the genera *Rhodococcus* and *Gordonia*, and almost all strains of the other genera did not degrade *c*-alkanes. These results indicate that class Actinobacteria may have a specific function for the degradation of *c*-alkanes.

Discussion

To investigate the characteristics of hydrocarbon-degrading genera, the hydrocarbon-degradation abilities of 36 isolates were exhaustively analyzed by the 2,6-DCPIP assay. Long-chain *n*-alkanes were degraded by all strains of the respective genera, whereas short-chain *n*-alkanes were mainly degraded by strains belonging to the genera *Pseudomonas* and *Gordonia*. The evaluation of *n*-alkane-degradation by each genus obtained by the 2,6-DCPIP assay is almost in agreement with data in previous studies (Rehm and Reiff 1981; Asperger and Singh 1991; Sakai et al. 1994; Throne-Holst et al. 2007).

Table 3 Evaluation of hydrocarbon-degradation by long-chain hydrocarbon-degrading bacteria by the 2,6-DCPIP assay[a]

Class	Genus or family	Strain	Petroleum[b]			*n*-Alkane[b]					*c*-Alkane[b]				Aromatics[b]		Polycyclic hydrocarbons[b]		
			BO	HO-A	HO-C	C_6	C_8	C_{10}	C_{16}	C_{20}	CH C_6	MC C_7	HC C_{12}	DC C_{18}	BE C_6	DB C_{18}	DE	NP	AN
Betaproteobacteria	Alcaligenaceae	ODMI79	+	+	+	−	+	+	+	+	−	−	−	−	−	+	−	−	−
	Alcaligenaceae	ODNM5	+	+	+	−	−	+	+	+	−	−	−	−	−	−	−	+	−
	Ralstonia	ODNM1AB	+	+	+	−	−	−	+	+	+	−	−	−	−	+	−	−	−
	Ralstonia	ODMI90	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−
	Pseudomonas	F721	+	+	+	+	+	+	+	+	−	−	−	−	−	+	−	−	−
	Pseudomonas	F722	+	+	+	+	+	+	+	+	−	−	−	−	−	+	−	−	−
Gammaproteobacteria	*Acinetobacter*	NDMI78	+	+	+	−	+	+	+	+	−	−	−	−	−	+	−	−	−
	Acinetobacter	ODMI29	+	+	+	−	−	−	+	+	−	−	−	−	−	−	−	−	−
	Acinetobacter	A132	+	+	+	−	−	−	+	+	−	−	−	−	−	+	−	−	−
	Acinetobacter	ODYM3	+	+	+	−	−	−	+	+	−	−	−	−	−	+	−	−	−
	Acinetobacter	NDMI119	+	+	+	−	−	−	+	+	−	−	−	−	−	+	−	−	−
	Acinetobacter	ODNM6	+	+	+	−	−	−	+	+	−	−	−	−	−	+	−	+	−
	Acinetobacter	ODDK71	+	+	+	−	−	−	+	+	−	−	−	−	−	−	−	−	−
	Acinetobacter	ODYM5	+	+	+	−	−	−	+	+	−	−	−	−	−	+	−	−	−
	Acinetobacter	ODYM2	+	+	+	−	−	−	+	+	−	−	−	−	−	+	−	−	−
	Acinetobacter	ODYM1	+	+	+	−	−	−	+	+	−	−	−	−	−	+	−	−	−
Actinobacteria	*Rhodococcus*	NDMI54	+	+	+	−	−	−	+	+	−	−	−	+	−	−	−	+	−
	Rhodococcus	ODNM2B	+	+	+	−	−	+	+	+	−	−	−	−	−	+	−	−	−
	Rhodococcus	NDKY82A	+	+	+	−	−	−	+	+	+	−	−	−	−	+	+	+	+
	Rhodococcus	NDKK1	+	+	+	−	−	+	+	+	+	−	−	+	−	+	+	+	+
	Rhodococcus	NDKK2	+	+	+	−	−	+	+	+	+	−	−	+	−	+	+	+	+
	Rhodococcus	NDKK5	+	+	+	−	−	+	+	+	+	+	−	−	−	+	+	+	+
	Rhodococcus	NDKK6	+	+	+	−	−	+	+	+	+	−	−	+	−	+	+	+	+
	Rhodococcus	NDKK7	+	+	+	+	+	+	+	+	−	−	+	+	−	+	−	−	−
	Rhodococcus	NDKK48	+	+	+	+	+	+	+	+	−	−	+	+	−	+	−	−	−
	Rhodococcus	NDMI144	+	+	+	−	−	−	+	+	+	−	−	+	−	+	+	−	+
	Rhodococcus	ODNM1C	+	+	+	+	+	+	+	+	+	+	+	+	−	+	−	+	+
	Rhodococcus	NDKY3D	+	+	+	−	−	+	+	+	−	−	+	+	−	+	−	−	−
	Rhodococcus	NDKY72A	+	+	+	+	+	+	+	+	−	−	+	+	−	−	−	+	−
	Gordonia	NDKK46	+	+	+	+	+	+	+	+	+	−	+	+	−	+	−	+	−
	Gordonia	NDKY76A	+	+	+	+	+	+	+	+	−	−	+	+	−	+	−	−	−
	Gordonia	NDKY2C	+	+	+	+	+	+	+	+	−	+	+	+	−	+	−	−	−
	Gordonia	NDKY2B	+	+	+	+	+	+	+	+	−	−	+	+	−	+	−	+	−
Bacilli	*Bacillus*	ODMI57	+	+	+	+	+	+	+	+	−	−	−	−	−	+	−	−	−
	Bacillus	F31	+	+	+	−	−	−	+	+	−	−	−	−	−	−	−	−	−
	Bacillus	ODNM4	+	+	+	−	−	−	+	+	−	−	−	−	−	+	−	+	−

[a] The symbols + and − indicate that the solution was colorless (degraded) or blue (not degraded), respectively

[b] The abbreviations represent the kinds of hydrocarbons or mineral oils as follows: BO, base oil; HO-A, heavy crude oil (type A); HO-C, heavy crude oil (type C); CH, cyclohexane; MC, methylcyclohexane; HC, hexylcyclohexane; DC, dodecylcyclohexane; BE, benzene; DB, dodecylbenzene; DE, decalin; NP, naphthalene; AN, anthracene

On the other hand, *c*-alkanes were degraded by strains of the genera *Rhodococcus* and *Gordonia*, which belong to class Actinobacteria, in the present study. In previous studies, strains of the genera *Rhodococcus* and *Gordonia* degraded *c*-alkanes (Schumacher and Fakoussa 1999; Kummer et al. 1999; Kostichka et al. 2001; Whyte et al. 1998), and strains of these genera carry the hydrocarbon oxidation gene *alkB* (Vomberg and Klinner 2000; van Beilen et al. 2002, 2003, 2007; Whyte et al. 2002). Several hydrocarbon-degrading bacteria belonging to the genus *Pseudomonas* or other genera carry *alkB* gene, however the strains did not degrade *c*-alkanes. The *c*-alkane-degrading bacteria belonging to the genera *Rhodococcus* and *Gordonia* may have the different type(s) of *alkB* gene relating to *c*-alkane-degradation.

In the present study, the genus *Acinetobacter* was mainly isolated when car engine waste oil or car engine base oil was used as a substrate for isolation of hydrocarbon-degrading bacteria. Since strains of the genus *Acinetobacter* degrade *c*-alkanes with co-oxidation (Perry 1979; Ko and Lebeault 1999; Koma et al. 2003a) and the growth rates of strains of the genus *Acinetobacter* are faster than those of strains of the genera *Rhodococcus* and *Gordonia*, strains of the genus *Acinetobacter* appeared to become dominant strains in medium containing car engine waste oil or car engine base oil. On the other hand, when the *c*-alkane fraction of car engine base oil was used for the isolation of hydrocarbon-degrading bacteria, *c*-alkane-degrading and long-chain *n*-alkane-degrading bacteria belonging to the genera *Rhodococcus* and *Gordonia* were isolated, and these genera could degrade *c*-alkanes without co-oxidation.

Long-chain hydrocarbons remain in hydrocarbon-contaminated soils for long periods of time (Stroud et al. 2007; Throne-Holst et al. 2007), and *c*-alkanes are especially difficult for microorganisms to degrade in nature (Matsumiya and Kubo 2007). The genera *Acinetobacter*, *Rhodococcus* and *Gordonia* would contribute to the bioremediation of long-chain hydrocarbons. The 2,6-DCPIP assay has been applied to the enumeration of hydrocarbon-degrading microorganisms in mixed cultures (van Hamme et al. 2000). This assay has also been applied to the selection of autochthonous microorganisms that can degrade hydrocarbon contaminants in contaminated soils, since these microorganisms would contribute to the cleaning process for hydrocarbon-contaminated soils. Development of bioremediation systems using hydrocarbon-degrading bacteria and the 2,6-DCPIP assay is currently in progress.

References

Aislabie J, Saul DJ, Foght JM (2006) Bioremediation of hydrocarbon-contaminated polar soils. Extremophiles 10:171–179

Arenskötter M, Baumeister D, Berekka MM, Potter G, Kroppenstedt RM, Linos A, Steinbuchel A (2001) Taxonomic characterization of two rubber degrading bacteria belonging to the species *Gordonia polyisoprenivorans* and analysis of hyper variable regions of 16S rDNA sequenced. FEMS Microbiol Lett 205:277–282

Asperger O, Singh HD (1991) The biology of *Acinetobacter*. Plenum Press, New York, pp 323–350

Dua M, Johri AK, Singh A, Sethunathan N (2002) Biotechnology and bioremediation: successes and limitations. Appl Microbiol Biotechnol 59:143–152

Hanson KG, Desai JD, Desai AJ (1993) A rapid and simple screening technique for potential crude oil degrading microorganisms. Biotechnol Tech 7:745–748

Iwamoto T, Nasu M (2001) Current bioremediation practice and perspective. J Biosci Bioeng 92:1–8

Ko SH, Lebeault JM (1999) Effect of a mixed culture on co-oxidation during the degradation of saturated hydrocarbon mixture. J Appl Microbiol 87:72–79

Koma D, Hasumi F, Yamamoto E, Ohta T, Chung SY, Kubo M (2001) Biodegradation of long-chain *n*-paraffines from waste oil of car engine by *Acinetobacter* sp. J Biosci Bioeng 91:94–96

Koma D, Hasumi F, Chung SY, Kubo M (2003a) Biodegradation of *n*-alkylcyclohexanes by co-oxidation via multiple pathways in *Acinetobacter* sp. ODDK71. J Biosci Bioeng 95:641–644

Koma D, Sakashita Y, Kubota K, Fujii Y, Hasumi F, Chung SY, Kubo M (2003b) Degradation of car engine base oil by *Rhodococcus* sp. NDKK48 and *Gordonia* sp. NDKY76A. Biosci Biochem Bioeng 67:1590–1593

Koma D, Sakashita Y, Kubota K, Fujii Y, Hasumi F, Chung SY, Kubo M (2005) Degradation pathways of cyclic alkanes in *Rhodococcus* sp. NDKK48. Appl Microbiol Biotechnol 67:1590–1593

Komukai-Nakamura S, Sugiura K, Yamauchi-Inomata Y, Toki H, Venkateswaran K, Yamamoto S, Tanaka H, Harayama S (1996) Construction of bacterial consortia that degrade Arabian light crude oil. J Ferment Bioeng 82:570–574

Konishi S, Ueda R (1992) Junkatsuyu no kiso to ouyou (in Japanese). Corona Publishing, Tokyo, pp 27–35

Korda A, Santas P, Tenente A, Santas R (1997) Petroleum hydrocarbon bioremediation: sampling and analytical techniques, *in situ* treatments and commercial microorganisms currently used. Appl Microbiol Biotechnol 48:677–686

Kostichka K, Thomas SM, Gibson KJ, Nagarajan V, Cheng Q (2001) Cloning and characterization of a gene cluster for

cyclododecanone oxidation in *Rhodococcus ruber* SC1. J Bacteriol 183:6478–6486

Kummer C, Schumann P, Stackebrandt E (1999) *Gordonia alkanivorans* sp. nov., isolated from tar-contaminated soil. Int J Syst Bacteriol 118:394–399

Lal B, Khanna S (1996) Degradation of crude oil by *Acinetobacter calcoaceticus* and *Alcaligenes odorans*. J Appl Bacteriol 81:355–362

Lappas AA, Patiaka D, Ikonomou D, Vasalos A (1997) Separation and characterization of paraffins and naphthenes from FCC feedstocks. Ind Eng Chem Res 36:3110–3115

Matsumiya Y, Kubo M (2007) Bioprocess handbook (in Japanese). NTS Publishing, Tokyo pp 638–652

Perry JJ (1979) Microbial cooxidations involving hydrocarbons. Microbiol Rev 43:59–72

Perry JJ (1984) Microbial metabolism of cyclic alkanes. Atlas, R. M., New York, pp 61–98

Pritchard PH, Mueller IJG, Rogers JC, Kremer FV, Glaser JA (1992) Oil spill bioremediation: experiences, lessons and results from the Exxon Valdez oil spill in Alaska. Biodegradation 3:315–335

Rehm H, Reiff I (1981) Mechanism and occurrence of microbial oxidation of long chain alkanes. Adv Biochem Eng 19:175–215

Sakai Y, Maeng JH, Tani Y, Kato N (1994) Use of long-chain *n*-alkanes (C_{13}-C_{44}) by an isolate, *Acinetobacter* sp. M-1. Biosci Biotechnol Biochem 58:2128–2130

Sanpa S, Sumiyoshi S, Kujira T, Matsumiya Y, Kubo M (2006) Isolation and characterization of a Bluegill-degrading microorganism, and analysis of the root hair-promoting effect of the degraded products. Biosci Biotechnol Biochem 70:340–347

Schumacher JD, Fakoussa RM (1999) Degradation of alicyclic molecules by *Rhodococcus ruber* CD4. Appl Microbiol Biotechnol 52:85–90

Stroud JL, Paton GI, Semple KT (2007) Microbe-aliphatic hydrocarbon interactions in soil: implications for biodegradation and bioremediation. J Appl Microbiol 102:1239–1253

Throne-Holst M, Wentzel A, Ellingsen TE, Kotlar H-K, Zotchev SB (2007) Identification of novel genes involved in long-chain *n*-alkane degradation by *Acinetobacter* sp. strain DSM 17874. Appl Environ Microbiol 73:3327–3332

van Beilen JB, Smits THM, Whyte LG, Schorcht S, Röthlisberger M, Plaggemeier T, Engesser KH, Witholt B (2002) Alkane hydroxylases homologues in Gram-positive strains. Environ Microbiol 4:676–682

van Beilen JB, Li Z, Duetz WA, Witholt B (2003) Diversity of alkane hydroxylase systems in the environment. Oil Gas Sci Technol 58:427–440

van Beilen JB, Funhoff EG (2007) Alkane hydroxylases involved in microbial alkane degradation. Appl Microbiol Biotechnol 74:13–21

van Hamme JD, Odumeru JA, Ward OP (2000) Community dynamics of a mixed-bacterial culture growing on petroleum hydrocarbons in batch culture. Can J Microbiol 46:441–450

Vidali M (2001) Bioremediation. A overview. Pure Appl Chem 73:1163–1172

Vomberg A, Klinner U (2000) Distribution of *alkB* genes within *n*-alkane-degrading bacteria. J Appl Microbiol 89:339–348

Whyte LG, Hawari J, Zhou E, Bourbonniére L, Inniss W, Greer CW (1998) Biodegradation of variable-chain-length alkanes at low temperatures by a psychrotrophic *Rhodococcus* sp. Appl Environ Microbiol 64:2578–2584

Whyte LG, Smits THM, Labbe D, Witholt B, Greer CW, van Beilen JB (2002) Gene cloning and characterization of multiple alkane hydroxylase systems in *Rhodococcus* strains Q15 and NRRL B-16531. Appl Environ Microbiol 68:5933–5942