Acinetobacter sp. Ud-4 Efficiently Degrades Both Edible and Mineral Oils: Isolation and Characterization

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Abstract   A novel *Acinetobacter* strain, Ud-4, possessing a strong capacity to degrade edible, lubricating, and heavy oil was isolated from seawater in a fishing port located in Toyama, Japan. It was identified by morphological and physiological analyses and 16S rDNA sequencing. This strain could utilize five types of edible oils (canola oil, olive oil, sesame oil, soybean oil, and lard), lubricating oil, and C-heavy oil as the sole carbon source for growth in M9 medium. The strain grew well and heavily degraded edible oils in Luria–Bertani medium during a 7-day culture at 25°C; it also degraded all kinds of oils in artificial seawater medium for marine bacteria. Furthermore, this strain was capable of degrading almost all C10–C25 *n*-alkanes in C-heavy oil during a 4-week culture. Oligonucleotide primers specific to two catabolic genes involved in the degradation of *n*-alkanes (*Acinetobacter* sp. *alkM*) and triglyceride (*Acinetobacter* sp. *lipA*) allowed amplification of these genes in strain Ud-4. To our knowledge, this is the first report on the isolation of a bacterium that can efficiently degrade both edible and mineral oils.

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Introduction

Various kinds of oils—edible oil, lubricating oil, and heavy oil—cause environmental pollution. Lipids are major components of domestic and industrial wastewater, including edible vegetable oils and animal fats, especially from restaurants and food processing industries [27, 37]. The amount of lipid-rich wastewater increases every year due to urbanization. Such wastewater can form large aggregates that obstruct drainage lines. Lubricating oil contaminates the soil and groundwater during daily use and via leaks and spills from industrial machines [36, 41]. Petroleum oil spills are one of the most serious problems that can occur in a marine environment [10, 11], endangering birds, fish, shells, and algae. Oil slicks in coastal regions, apart from destroying scenery, cause considerable economic damage to the fishery industry. In Japan, a heavy oil spill from the Russian tanker Nakhodka in 1997 received much attention [3, 23, 24, 34]. The main fraction of lubricating oil and heavy oil is a complex mixture of hydrocarbons, linear and branched paraffins, cyclic alkanes, and aromatic hydrocarbons [7, 38].

Physical and chemical technologies have been developed for the remediation of oil-contaminated environments. However, they are expensive for large-scale application, and are rarely completely successful. Recently, bioremediation techniques using microorganisms have attracted great interest [2, 35]. For bioremediation (bioaugmentation) to succeed, it is necessary to select microorganisms that can degrade contaminated materials under various temperatures, pH, salinities, and nutrient concentrations. Many kinds of microorganisms have been isolated for improved bioremediation of contaminated environments with lipids or petroleum hydrocarbons [3, 9, 15, 21,

31, 41]. However, most studies have focused on biodegradation of only one type of oil by each microorganism.

In the present study, we isolated a novel bacterium that degrades various kinds of oils. The bacterium showed the ability to efficiently degrade edible oil, lubricating oil, and heavy oil. Furthermore, the catabolic genes of the isolated strain were investigated.

Materials and Methods

Isolation, Media, and Culture Conditions

Seawater samples containing oil film were collected from the Uozu fishing port on the coast of Toyama Bay, Toyama, Japan. The culture media used in this study were Luria–Bertani (LB) medium (1% NaCl, 0.5% yeast extract, and 1% tryptone), M9 medium (0.68% Na_2HPO_4, 0.3% KH_2PO_4, 0.05% NaCl, 0.1% NH_4Cl, 2 ml of 24.6% $MgSO_4 \cdot 7H_2O$, and 0.1 ml of 11.1% $CaCl_2$ per liter), and artificial seawater (ASW) medium (artificial seawater 800 ml, NH_4NO_3 1 g, K_2HPO_4 0.02 g, yeast extract 0.5 g, and deionized water 200 ml, pH 7.8). To isolate the oil degrader, 1 ml of a sample was transferred to a glass tube containing 10 ml of ASW medium with 0.5% (w/v) C-heavy oil (Cosmo Oil Co., Tokyo, Japan), and incubated at 25°C in a rotary shaker at 120 rpm until emulsification was observed. Part of the culture solution containing the proliferated bacteria was then spread on ASW agar plates (1.5% agar) containing 0.5% (w/v) heavy oil. The colonies that formed transparent and irregular zones were isolated and transferred into the ASW liquid medium containing 0.5% (w/v) heavy oil and were shaken for 5–10 days. The bacterial clones that emulsified the oil were selected as candidate heavy-oil-degrading strains. Then, to find bacteria that could degrade other oils, the heavy-oil-degrading bacteria were examined by halo assay using agar plates with 1% (w/v) edible oil or lubricating oil. One strain, designated as Ud-4, was confirmed to degrade not only heavy oil, but also edible oils and lubricating oil. This strain was selected for further study.

Morphological and Physiological Analysis of the Microorganism

The morphology and motility of the bacteria were first observed by a phase microscope (Olympus BH-2, Tokyo, Japan). Electron microscopy samples were negatively stained with 1% phosphotungstic acid (pH 3–9), and then observed with a JEM-1200EXII electron microscope (JEOL, Tokyo, Japan). Micrographs were taken at an accelerating voltage of 80 kV. Physiological characterization of the microorganism was performed by an API 20NE

system (BioMerieux, Marcy, l'Etoile, France). In addition, gram staining (FAVOR-G SET-S, Nissui Pharmaceutical, Tokyo, Japan), catalase, and oxidase tests were conducted.

16S rDNA Sequencing

Almost the full length of the bacterial 16S rDNA was amplified by PCR using the primers 27f (5′-AGAGT TTGATCCTGGCTCAG-3′) and 1525r (5′-AAAGGAG GTGATCCAGCC-3′). The amplified products were purified with a QIAquick PCR purification kit (Qiagen, Tokyo, Japan), and then sequenced directly using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA), an ABI Prism 3130xl genetic analyzer (Applied Biosystems), and the following sequencing primers: r1L (5′-GTATTACCGCGGCTGCTGG-3′), r2L (5′-CATCGTTTACGGCGTGGAC-3′), r3L (5′-TTGCGC TCGTTGCGGGACT-3′), r4L (5′-ACGGGCGGTGTGTA CAAG-3′), f1L (5′-GAGTTTGATCCTGGCTCAG-3′), f3L (5′-GTCCCGCAACGAGCGCAAC-3′), and 926f (5′-AAA CTCAAAGGAATTGACGG-3′). The DNA sequence was searched by the BLAST program on the NCBI website (http://www.ncbi.nlm.nih.gov). The 16S rDNA sequence of strain Ud-4 was deposited in GenBank/EMBL/DDBJ under accession number AB488778.

Degradation of Edible Oil, Lubricating Oil, or Heavy Oil by Strain Ud-4

The oil degradation rate was examined according to previously described methods [7, 15, 24]. Bacterial cells precultured overnight at 25°C in LB medium were transferred to silicone-capped test tubes containing 6 ml of each medium with one of the edible oils (e.g., canola oil, olive oil, sesame oil, soybean oil, and lard) or lubricating oil (Noritake Cut; Noritake Co., Limited, Aichi, Japan) at a concentration of 1% (w/v), i.e. 60 mg. The cells were inoculated at a cell density of approximately 10^7 cells per ml and grown at 25°C by shaking at 120 rpm, and the remaining oil in each medium was extracted with *n*-hexane after 7 days. The extract was dehydrated with Na_2SO_4, and then dried at 30°C. The dry weight of extracts was measured using an electronic microbalance accurate to 0.1 mg in three sets of samples, with and without bacterial inoculation (negative control). The degradation rate of edible oils and lubricating oil was assessed from the average dry weight difference. The oil degradation rate was calculated as follows: percentage oil degradation (% per 7 days) = [(oil weight in negative control) − (oil weight in sample)/(oil weight in negative control)] × 100. For the degradation test of heavy oil, culture media with 0.5% (w/v) type C heavy oil were used. The remaining heavy oil in each medium was extracted with chloroform, and the degradation rate of heavy oil was calculated using a method

similar to that used for edible oils and lubricating oil. The extraction efficiency for control was 85–100% in edible oils, 40–45% in lubricating oil, and 85–90% in heavy oil.

Gas chromatography (GC) was performed with a gas chromatograph (Shimadzu GC-14A; Tokyo, Japan) and a capillary column (OV-1, 50 m × 0.25 mm i.d.; film thickness, 1.5 μm). Helium was the carrier gas. The injector and detector temperatures were set at 280°C. The oven temperature program was 70–160°C at 10°C/min, 160–280°C at 2°C/min, and held at 280°C for 30 min.

Detection of Catabolic Genes by PCR

PCR primers were used to detect catabolic genes as follows: *Pseudomonas putida* GPo1 *alkB*, *Acinetobacter* sp. ADP-1 *alkM* and *Rhodococcus* sp. Q15 *alkB1* and *alkB2* for alkane monooxygenase and alkane degradation [40]; *P. putida* ATCC 33015 *xylE* for catechol-2,3-dioxygenase, xylene, and toluene degradation; *P. putida* ATCC 17484 *ndoB* for naphthalene dioxygenase and naphthalene degradation [39]; and *Mycobacterium* sp. PYR-1 *nidA* for pyrene dioxygenase and PAH (pyrene) degradation [20]. For *Acinetobacter* sp. *lipA*, which encodes a lipase that catalyzes the hydrolysis of triglycerides, primers Ac lipA-F (5′-TGCHMARACMAARTATCC-3′) and Ac lipA-R (5′-AYYTGATTSACTTCATC-3′) were derived from regions of high DNA sequence identity from four lipase structural gene sequences from *A. calcoaceticus* RAG-1 [32] (GenBank accession number AF047691), *Acinetobacter* sp. SY-01 [8] (AF518410), *A. baumannii* BD5 [26] (EU078176), and *A. calcoaceticus* BD413 [18] (X80800). The degenerate bases used were H = A/C/T, M = A/C, R = A/G, Y = C/T, and S = G/C. In all cases, the primers were designed toward a sequence located inside the open reading frame. The expected size of PCR products was 870 bp for *alkB*, 496 bp for *alkM*, 629 bp for *alkB1*, 552 bp for *alkB2*, 834 bp for *xylE*, 642 bp for *ndoB*, 323 bp for *nidA*, and approximately 770 bp for *lipA*. All PCR amplifications were carried out as previously described [39] for 30 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, with final extension at 72°C for 3 min. The PCR products were analyzed by electrophoresis on 2% (w/v) agarose gel and visualized by ethidium bromide staining. Amplified PCR fragments were sequenced as described at the beginning of this section for 16S rDNA sequencing.

Results

Identification of Strain Ud-4

Morphological and physiological properties presented by strain Ud-4 were consistent with its assignment to the
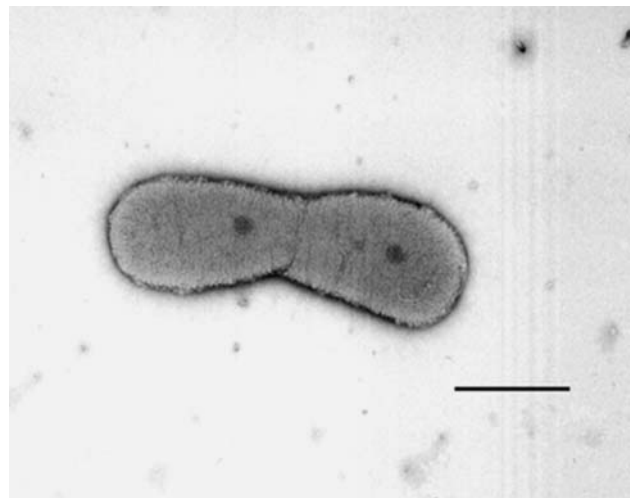

Fig. 1 Negatively stained cells of *Acinetobacter* sp. Ud-4 (*Bar*, 1 μm) from electron micrograph

genus *Acinetobacter*. The bacterium was gram-negative, non-spore forming, and non-motile. Phase microscopy demonstrated that the cells predominantly grew in pairs at logarithmic growth phase. Observation by electron microscope revealed that the paired cells were short rods in shape, 1.0–2.0 μm long, and 0.8–1.0 μm wide; peritrichate fibrils [12] could be observed on the cells (Fig. 1). Colonies were formed as circular and white on LB agar 2–3 days after inoculation. They grew well in media supplemented with 0–3% NaCl, but not in media with salinity exceeding 5%. Growth occurred at temperatures ranging from 5 to 35°C, with an optimum at 25°C. Catalase and oxidase reactions were positive and negative, respectively. In addition to these properties, the physiological characteristics (using the API 20NE system) are summarized in Table 1. Analysis with the api computer program indicated that it belongs to the genus *Acinetobacter*.

The 16S rDNA sequence of strain Ud-4, 1493 bp in length, was determined for phylogenetic analysis. It showed high sequence similarity to the *Acinetobacter* species, with the highest degree of similarity attained at 99% with *Acinetobacter psychrotolerans* (GenBank accession number AB207814), described as the *Acinetobacter* sp. strain Ths [43]. A phylogenic tree constructed by the neighbor joining method demonstrated that strain Ud-4 closely clusters with genus *Acinetobacter* (data not shown).

Degradation of Edible Oil, Lubricating Oil, and Heavy Oil by Strain Ud-4

To study the oil degradation potential in various environments, three different liquid media were used. The degradation of five different kinds of edible oils and mineral oils, such as lubricating oil and C-heavy oil, by strain Ud-4 was

Table 1 Physiological characteristics of strain Ud-4 and related *Acinetobacter* species

| Characteristics	Ud-4	Ths[a]
Nitrate reduction	−	−
Indole production	−	NR
Acid production from glucose	−	−
Arginine dihydrolase	−	NR
Urease	−	−
Hydrolysis of		
Esculin	−	NR
Gelatin	+	+
p-nitro-phenyl-β-D-galactopyranoside	−	NR
Assimilation of		
Glucose	−	−
Arabinose	−	−
Mannose	−	−
Mannitol	−	−
N-acetyl-glucosamine	−	NR
Maltose	−	−
Gluconate	−	NR
Caprate	+	NR
Adipate	−	NR
Malate	+	+
Citrate	−	+
Phenyl-acetate	−	NR
Oxidase	−	−
Catalase	+	+

+ positive, − negative, *NR* not reported

[a] Data are cited from [43]

Table 2 Oil degradability of strain Ud-4[a]

Type of oil	Oil degradation (%) in three media		
	LB medium	M9 medium	ASW medium[b]
Edible oil			
Canola oil	96.3 ± 1.2	16.9 ± 3.5	18.7 ± 6.7
Olive oil	48.5 ± 2.0	34.4 ± 4.2	10.9 ± 9.1
Sesame oil	60.3 ± 8.2	10.9 ± 1.5	15.5 ± 5.4
Soybean oil	60.2 ± 4.3	14.6 ± 4.2	3.2 ± 0.2
Lard	67.4 ± 1.0	63.0 ± 8.9	13.2 ± 1.3
Mineral oil			
Lubricating oil	67.6 ± 7.0	34.7 ± 9.6	31.7 ± 11.8
C-heavy oil	6.5 ± 1.6	9.6 ± 3.2	6.9 ± 2.7

[a] Data are cited as percentages

[b] ASW denotes artificial seawater

examined (Table 2). The results showed that strain Ud-4 could degrade all kinds of edible oils (canola oil, olive oil, sesame oil, soybean oil, and lard) in each medium after cultivation at 25°C for 7 days; however, the level of edible
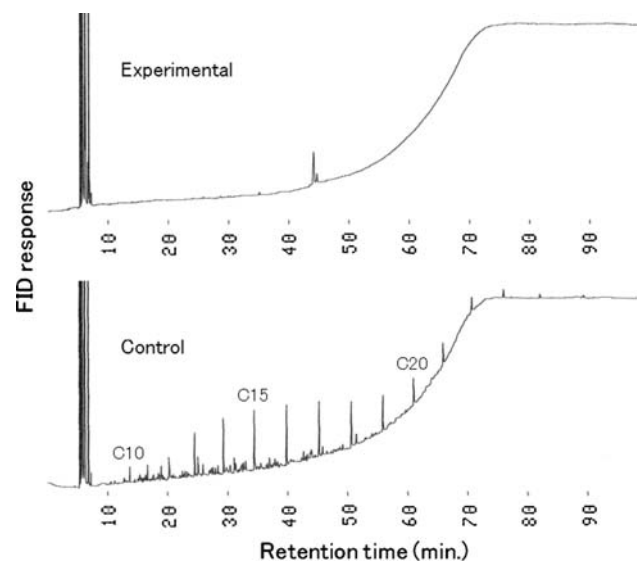
Fig. 2 Detection of degradation of *n*-alkanes in C-heavy oil by gas chromatography. C-heavy oil remaining in the ASW medium after incubation at 25°C for 4-wk period with strain Ud-4 and without inoculation (control) were examined. The *n*-alkanes are labeled C(*n*) where *n* is the number of carbons

oil degradation varied with different oils or media. The degradation of edible oils in LB medium occurred more efficiently than those in M9 medium or ASW medium. In particular, the degradation of canola oil in LB medium reached up to 96.3%. This strain degraded lubricating oil in LB, M9, and ASW mediums up to 67.6% (LB), 34.7% (M9), and 31.7% (ASW). It also degraded C-heavy oil in each medium up to 6.5% (LB), 9.6% (M9), and 6.9% (ASW).

The GC profile of the remnant *n*-alkanes of C-heavy oil was compared with those of the control (Fig. 2). At the end of the incubation period (4 weeks), the *n*-alkanes from C10 to C25 were almost completely degraded by strain Ud-4. This finding is consistent with the presence of alkane monooxygenase gene (*alk M*) in this strain as described below. However, one compound was not appreciably degraded. Since the peak is found near C17 *n*-alkane, it is possible that the compound is pristane, which is a highly branched alkane and is biodegraded slowly [1, 22].

Detection of Catabolic Genes

A novel bacterial strain, Ud-4, possessing high capacity to degrade edible oil, lubricating oil, and heavy oil was examined for the presence of known catabolic genes (*alkB*, *alkM*, a*lkB1*, *alkB2*, *xylE*, *ndoB*, *nidA*, and *lipA*) by PCR using oligonucleotide primers derived from these genes (Fig. 3). This strain showed PCR-positive for *alkM* (496 bp) and *lipA* (approximately 770 bp), involved in the degradation of *n*-alkanes and triglyceride, and showed
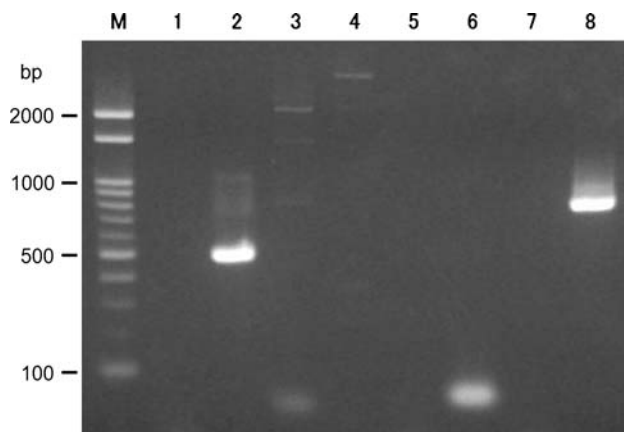
Fig. 3 Detection of catabolic genes in *Acinetobacter* sp. Ud-4 by PCR analysis with specific primers for *alkB* (*Lane 1*), *alkM* (*Lane 2*), *alkB1* (*Lane 3*), *alkB2* (*Lane 4*), *xylE* (*Lane 5*), *ndoB* (*Lane 6*), *nidA* (*Lane 7*), and *lipA* (*Lane 8*), with 100 bp ladder size standard shown in *Lane M*

PCR-negative for *alkB*, *alkB1*, *alkB2*, *xylE*, *ndoB*, and *nidA*. Sequence analysis showed that the obtained fragment of *alkM* gene had 85% similarity with that from *Acinetobacter* sp. M-1 (GenBank accession number AB049410). Meanwhile, a fragment of *lipA* had 77% homology with that from *Acinetobacter* sp. SY-01 (AF518410).

Discussion

Strain Ud-4 was identified as a new strain of the genus *Acinetobacter*. The 16S rDNA sequence of strain Ud-4 was 99% identical to that of *Acinetobacter psychrotolerans* (strain Ths), which has not yet been validated as an *Acinetobacter* species in the list of prokaryotic names with standing in nomenclature [6]. The results of physiological characterization of *Acinetobacter* sp. strain Ths [43] show that strain Ud-4 is slightly different in the utilization of citrate (Table 1) and in the hydrolysis of lipids. Therefore, strain Ud-4 may be a member of *Acinetobacter psychrotolerans*. *Acinetobacter* species are ubiquitous in geographical distribution, and are known to be involved in the bioremediation of alkanes and aromatic hydrocarbons as well as in the production of high-molecular-weight heteropolysaccharides that act as powerful emulsifiers, with many having high commercial potential [16, 25, 29]. Moreover, several *Acinetobacter* strains are known to produce extracellular lipase [17, 30, 33], which can degrade lipids. In this study, we observed that strain Ud-4 emulsified C-heavy oil to fine droplets during the initial 1-week period, and hence, probably produced a bioemulsifier. In preliminary experiments, we also observed that strain Ud-4 showed lipase activity in the culture supernatant.

In general, lard is persistent to biodegradation because of its high melting temperature (28–48°C) and fatty acid composition [5, 31]. Lubricating oils are also assumed to be relatively recalcitrant to biodegradation due to their high content of recalcitrant cycloalkanes and isoalkanes [13, 41]. Therefore, it is interesting that strain Ud-4 showed a strong ability to degrade lard and lubricating oil, as shown in Table 2. Interestingly, all kinds of edible oils were more efficiently degraded in LB medium than in ASW or M9 medium. LB medium contains a high nutrient content and enhances cell growth. From the results of the culture using M9 medium, we found that this strain could use edible oil, lubricating oil, or heavy oil as the sole carbon source. The culture using ASW medium showed that strain Ud-4 was able to degrade various kinds of oils in a medium containing high salinity as well as seawater. Thus, we expect that this strain certainly has the ability to cleanup oil contamination in the sea, wastewater with high salinity, and oligotrophic environments. Then, strain Ud-4 was capable of degrading almost all components of C10–C25 *n*-alkanes in C-heavy oil during 4-week culture. Since lubricating oil also contains *n*-alkanes [41], it is supposed that these components were degraded by strain Ud-4.

The use of specific primers to amplify catabolic genes is a very useful tool for characterizing microorganisms possessing a variety of biodegradative capabilities and for examining their catabolic pathways [39]. Therefore, we carried out PCR detection of the genes involved in *n*-alkane, aromatic, and triglyceride degradation pathways. This strain possessed *Acinetobacter* sp. *alkM*, involved in the initial oxidation of the alkane substrate to 1-alkanol [42] and *Acinetobacter* sp. *lipA*, involving the hydrolysis of triglyceride to glycerol and fatty acids [14], respectively. Thus, initial reactions involved in the degradation of *n*-alkane and triglyceride are different, but both metabolites are finally oxidized via beta-oxidation of fatty acids [4, 19, 28]. In addition to oil-degradation experiments, molecular detection of catabolic genes by PCR could provide additional information rapidly and accurately.

To our knowledge, this is the first report on the isolation of a bacterium that can efficiently degrade both edible and mineral oils. Therefore, this strain could be useful for application to bioremediation technology. However, further investigation is still needed to evaluate the performance in situ, because a variety of factors can affect the ability of microorganisms to degrade contaminated oils in natural environments.

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