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Isolation, Identification, and Characterization of a Novel, Oil-Degrading Bacterium, *Pseudomonas aeruginosa* T1

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Abstract. A novel, oil-degrading bacterium (strain T1) was isolated from a hot spring in Hokkaido, Japan. It efficiently degrades different types of fats and oils, including edible oil waste. When grown in a mineral salt medium containing 1% triacylglycerol (as salad oil), hydrolysis products were 1,3- and 1,2-diacylglycerols, monoacylglycerol, and free fatty acid. However, these products were almost completely consumed during cultivation at 30°C for 5 days, indicating that extracellular lipase acts randomly at different *sn*-positions of acylglycerols and that strain T1 has a high capacity to utilize free fatty acids. Secreted lipase activity was induced by salad oil and oleic acid. This strain was a Gram-negative straight rod shaped, aerobic, with a polar flagellum, capable of growing in temperature ranges between 15°C and 55°C. The 16S rRNA gene sequence analysis and DNA-DNA hybridization revealed it as a new strain of *Pseudomonas aeruginosa.* The type strain was T1.

Pseudomonas species, ubiquitous in soil and water, are of considerable scientific and technological importance and comprise a taxon of metabolically versatile organisms capable of utilizing a wide range of simple and complex organic compounds. They are known to be involved in biodegradation of natural or man-made toxic chemical compounds [11]. Furthermore, the bacterial genus *Pseudomonas* is a prolific producer of a number of extracellular enzymes, including lipase.

Lipases are important enzymes because of their ability to catalyze a number of reactions. Consequently, they are receiving considerable interest from both academia and industry. Fundamentally, lipases are esterases that are able to hydrolyze water-insoluble esters such as long-chain triacylglycerols. These enzymes also catalyze the formation of esters (esterification) and the exchange of ester bonds (transesterification) when present in non-aqueous media [1, 7, 15, 16, 31]. Lipases display a high degree of specificity and enantioselectivity for esterification and transesterification reactions [25]. That characteristic renders them potentially useful for myriad industrial functions. That property is used extensively in trans- and interesterification reactions in organic solvents to produce useful acylglycerols.

These potential industrial applications have been an important driving force for lipase research during the last several years, and in particular for the study of lipases produced by microorganisms. Microbial lipases are the most important group of biocatalysts used for a variety of different biotechnological applications [20, 28]. Microbial lipases have been used for production of desirable flavors in cheese and other foods, and for the interesterification of fats and oils to produce modified acylglycerols, which cannot be obtained by conventional chemical interesterification.

Among bacterial lipases, attention has usually been focused on particular classes of enzymes such as lipases from the genus *Pseudomonas* [10, 36]. *Pseudomonas* lipases are very interesting because they display special biochemical characteristics that are not common among lipases produced by other microorganisms, such as their

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thermoresistance and activity at alkaline pH [32]. These lipases play an important role in biotechnology both as hydrolases for detergent additives and as synthases for catalyzing the kinetic resolution of racemic compounds [27]. Each application requires unique properties with respect to specificity, stability, temperature, and pH dependence or ability to catalyze synthetic ester reactions in organic solvents. As each industrial application requires specific properties of lipases, additional lipases that could be used in new applications continue to garner interest.

In addition, lipase as well as oil-degrading bacteria could play a vital role in bioremediation of domestic or restaurant lipid wastes. These lipid wastes can create severe environmental hazards such as oil films on water surfaces, which prevent oxygen diffusion from air into water, killing many forms of aquatic flora and fauna. Aggregates formed by oil droplets and other particles that are present in wastewater can obstruct water drainage. Bioaugmentation of lipid-rich wastes has long been undertaken in both aquatic aerobic and anaerobic systems [2, 3, 35]. Furthermore, many reports have described the use of lipolytic enzymes in wastewater treatment [5, 30, 40]. A good source of large-scale production of lipase is microorganisms. In recent years, owing to strict waste disposal rules, concerns on bioremediation of lipid wastes prior to disposal into the environment have increased.

This study isolated a novel, oil-degrading bacterium belonging to the genus *Pseudomonas.* It produces inducible lipase that efficiently degrades all fats and oils tested, including some lipid wastes.

Materials and Methods

Isolation, media, and culture conditions. Water samples were taken from a hot spring located in Toyotomi (45° 1′N and 141° 50′E), Hokkaido, Japan. This hot spring has a temperature of around 40°C and has a natural smell of unknown petroleum. During isolation, 1 mL of the water sample was transferred aseptically into a 500-mL Erlenmeyer flask containing 100 mL sterile mineral salt (MS) medium with the following components: 0.4% NH_4NO_3, 0.47% KH_2PO_4, 0.0119% Na_2HPO_4, 0.001% $CaCl_2 \cdot 2H_2O$, 0.0015% $FeSO_4 \cdot 7H_2O$, 0.1% $MgSO_4 \cdot 7H_2O$, 0.001% $MnSO_4 \cdot 4H_2O$, and 0.01% yeast extract (pH 7.0). Sterilized salad oil (1%) was added to the flask and was incubated at 25°C with shaking at 180 rpm for 2 weeks. An aliquot of the culture (0.1 mL) was spread on PYA plates (1% peptone, 1% yeast extract, and 1.5% agar; pH 7.8) containing emulsified salad oil (1% vol/vol) and was incubated at 25°C. Colonies that appeared after 3 days of incubation were picked off and transferred on PYA with salad oil (1% vol/vol). Pure cultures were obtained by repeated streaking on fresh LB plate. Vegetable oils (salad and olive) and animal fat (lard) used in this experiment were obtained commercially. Three types of oil wastes were obtained from three different food-processing industries in Japan. Fatty acid composition of their lipid components was not determined.

Electron microscopy. For examination of cells under a transmission electron microscope (TEM), strain T1 cells were grown on nutrient agar (0.5% meat extract, 1.0% peptone, 0.5% NaCl, and 1.5% agar) at 27°C for 1 day and were suspended in physiological saline solution. A small drop of suspension was placed on a carbon-coated grid, and cells were negatively stained with 1% phosphotungstic acid for observation under the TEM (model H-800; Hitachi, Ltd., Tokyo, Japan).

Physiological and biochemical tests. Phenotypic and biochemical characterization of strain T1 was performed as described by Yumoto et al. [39].

DNA base composition and DNA-DNA hybridization. DNA was isolated from bacterial cells as described by Marmur [22]. The G + C content of the DNA from strain T1 was determined by high performance liquid chromatography (HPLC) analysis of hydrolyzed DNA, according to Tamaoka and Komagata [37]. The levels of DNA relatedness were determined fluorometrically according to the method described by Ezaki et al. [8] by using photobiotin-labeled DNA probes and black microplates. *Pseudomonas aeruginosa* JCM 5962^T obtained from JCM (Japanese Collection of Microorganisms) was used as reference strain.

Phylogenetic analysis by using 16S rRNA gene sequence comparison. Almost the entire length of the 16S rRNA gene of strain T1 was amplified by PCR with the following set of primers (5′-AGAGTTTGATCCTGGCTC-3′) and (5′-AAGGAGGTGATCCAGCCGCA-3′), corresponding to positions 8–24 and 1,521–1,540, respectively, in the 16S rRNA gene sequence of *Escherichia coli* [4]. This PCR product was isolated, cloned into a plasmid vector pT7Blue (Novagen, USA), and sequenced with an ABI PRISM Big Dye Terminator Cycle Sequencing Kit on an Applied Biosystems model 310 DNA sequencer following the manufacturer's instructions. Multiple alignments of the sequence were performed, and a neighbor-joining phylogenetic tree [17, 29] was constructed with the CLUSTAL W program [38]. Similarity values of the sequences were calculated with the GENETYX computer program (Software Development, Tokyo). The partial 16S rRNA gene sequence has been deposited with GenBank/EMBL/DDBJ with the accession number AB119535.

Lipolytic activity analysis. The ability to grow and degrade salad oil of purified cultures was tested on PYA or MS medium with 1% (vol/vol) salad oil. One of the potential isolates (we termed it as strain T1) was selected for further characterization.

The ability of strain T1 to degrade other oils was determined in MS medium containing 1% (vol/vol) vegetable oils (olive or salad) or lard as a sole source of carbon and energy. Three kinds of waste oils that were provided by different food processing companies were also used to determine the oil-degrading ability of strain T1 for waste oil disposal. These were from rapeseed wastes, sandwich wastes, and potato wastes. Hydrolysis products were analyzed by thin layer chromatography (TLC). Samples were extracted with an equal volume of *n*-hexane followed by centrifugation for 30 min at 6,000 *g*. The hexane extraction step was repeated at least two times. All hexane extracts were collected together, concentrated under nitrogen, and were resolved on a precoated silica gel with hexane-diethyl ether-acetic acid (80:30:1, vol/vol) as a developing solvent. Resolved lipid components were visualized under ultraviolet light after being sprayed with 0.005% primuline in acetone-water (4:1, vol/vol). Resolved lipid components were identified by comparing with the standards.

Lipase induction. Bacteria were cultivated in a 300-mL Erlenmeyer flask containing 60 mL of MS medium supplemented with 0.5% glucose. Incubation was at 30°C with shaking at 180 rpm. Aliquots of samples were withdrawn at indicated times and centrifuged to collect cells as pellets. Supernatants were saved at 4°C as the source for lipase

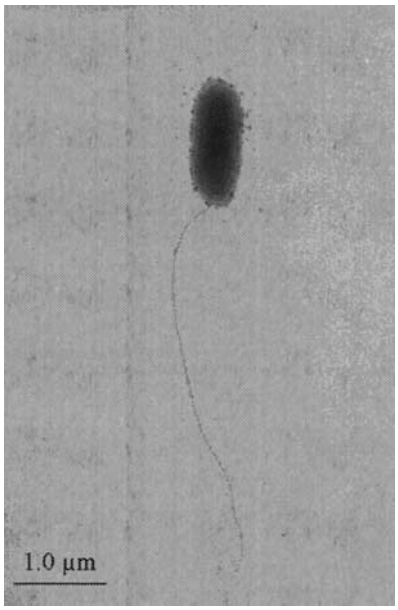

Fig. 1. Electron micrograph of a negatively stained cell of *P. aeruginosa* T1. Bar, 1 μm.

assay. Pellets were washed twice with 50 mM Tris-HCl (pH 7.5) and dissolved into a known amount of the same buffer before taking absorbance at 600 nm (A_{600}). Salad oil or oleic acid (Wako Pure Chemical Industries Ltd., Osaka, Japan) was added, to final concentrations of 1.0% or 0.2%, respectively, to the grown cultures at a stationary phase just before its A_{600} started to decline.

Lipase assay. Lipase was assayed by a standard procedure using *p*-nitrophenyl-stearate (*p*-NPS) as substrate purchased from Sigma-Aldrich, USA. To test tubes, each containing 500 μL of 100 mM Tris-HCl (pH 8.0), an aliquot of enzyme solution was added. The total volume of 900 μL was achieved with deionized water. This solution was mixed well and incubated at 37°C for 2 min. One hundred microliters of 2.5 mM *p*-NPS (dissolved in 2-propanol) was added to the above mixture, mixed well, and incubated at 37°C for 5 min. After this incubation, the reaction mixture was cooled quickly in an ice-water bath, and the reaction was terminated with addition of 100 μL of 0.5 M KOH. Lipase activity was determined as a function of increased absorbance of liberated *p*-nitrophenol (*p*-NP) monitored by a Beckman DU-65 spectrophotometer at 405 nm. One unit of activity was expressed as micromoles of *p*-NP liberated per minute.

Results

Morphological and physiological characterization of strain T1. The cells were Gram-negative, straight rods, 1.3–1.7 μm long and 0.6–0.8 μm wide, and motile by means of a polar flagellum (Fig. 1). Colonies were circular and slightly yellowish on LB agar. Catalase and oxidase reactions were positive. They grew well in media supplemented with 0–3% NaCl, but not in media with salinity exceeding 5%. Growth occurred at temperatures between 15°C and 55°C. Urease and nitrate reduction tests were positive; however, methyl red, *O*-nitro-phenyl-β-D-galactopyranoside (ONPG), arginine dihydrolase, and Voges-Proskauer tests were negative, as well as tests for indole and H_2S production. The strain T1 hydrolyzed casein, gelatin, DNA, alginic acid, acetamide, lipids such as olive oil, salad oil, and tributyrin, and Tween-20, -40, -60, and -80. Table 1 summarizes the characteristics of T1.

DNA composition, 16S rRNA analysis, and DNA-DNA hybridization. The DNA G + C content of strain T1 was 65.7%. Comparative ribosomal RNA gene sequence analysis supported a strong relationship between strain T1 and members of genus *Pseudomonas.* Particularly, the 16S rRNA gene sequence from strain T1 (1491 nucleotides determined) possessed the high sequence (among the type strains of validly published species) similarity of 100% with that of *P. aeruginosa* PAO1 (AE004844). It demonstrated slightly less similarity to sequences of *P. alcaligenes* (97.6%, Z76653), *P. resinovorans* (98.6%, AB021373), and *P. anguilliseptica* (96.1%, AF439803).

A phylogenetic tree constructed by the neighbor-joining method demonstrated that strain T1 clustered with "*Pseudomonas aeruginosa* and *P. resinovorans*" lineages (data not shown). The branching order suggests that strain T1 comprises the same phylogenetic "lineage" within the *P. aeruginosa* intrageneric cluster [23]. The level of DNA relatedness between strain T1 and *P. aeruginosa* was more than 83.5% (data not shown).

Vegetable oil, lard, and waste oil degradation by strain T1. Visible lipolytic activity of strain T1 was observed by formation of a distinct clearance halo around the bacterial colony after growth for 3 days on an agar plate made turbid with emulsified salad oil, indicating the presence of an extracellular lipase (Fig. 2). Although detailed data are not presented here, analyzed lipase hydrolysis products of this bacterial strain were 1,3- and 1,2-diacylglycerols (1,3-, and 1,2-DG), monoacylglycerol (MG), and free fatty acid (FFA). The extracellular lipase secreted on the medium by strain T1 hydrolyzed the triacylglycerol (TG) component of salad, olive, lard, and waste oils (sandwich and potato waste oils). Hydrolysis products such as DG and MG were also degraded, and the FFA released was further metabolized. However, it was observed that degradation was more efficient and rapid with salad or olive oils as substrate. In the case of rapeseed waste oil, FFA was metabolized, but 1,3-DG was not hydrolyzed (data not shown).

Induction of lipase. When cultivated in the presence of glucose as sole source of carbon in MS medium, low levels of lipase activity were detected throughout bacterial growth (Fig. 3). After addition of salad oil, lipase

Table 1. Differential characteristics of strain T1 in comparison with related *Pseudomonas* species[a]

Characteristic	Strain T1	*Pseudomonas aeruginosa*	*Pseudomonas resinovorans*	*Pseudomonas alkaligenes*	*Pseudomonas anguilliseptica*
Cell length (μm)	1.3–1.7	1.5–3.0	2.0–2.5	2.0–3.0	2
Production of					
Catalase	+	+	NR	NR	+
Oxidase	+	+	+	+	+
Arginine dihydrolase	−	−	NR	+	NR
DNA G+C content (mol %)	65.7	66.0	NR	64–68	NR
Hydrolysis of:					
Gelatin	+	+	−	D	+
Starch	−	−	W	−	−
Acid production from:					
Arabinose	+	+	−	N-R	−
Fructose	+	+	−	−	−
Maltose	−	−	−	−	−
Mannitol	+	+	−	−	−
Mannose	+	+	−	NR	−
Galactose	+	+	−	NR	−
Glucose	+	+	−	−	−
Lactose	−	−	−	NR	−
Rhamnose	−	−	−	NR	−
Trehalose	−	−	NR	−	NR
Sucrose	−	−	−	−	−
Xylose	+	+	−	−	−
Raffinose	−	−	−	NR	−
Inositol	−	−	−	NR	−
Reference	Present study	Present study	26	18, 26	26

[a] +, 90% or more strains are positive; −, 90% or more strains are negative; NR, not reported; W, weakly positive; D, differs among strains.

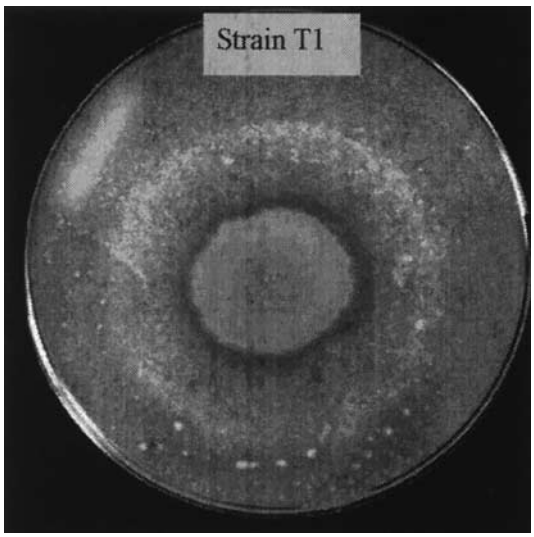

Fig. 2. Clearance halo formation by *P. aeruginosa* T1 on agar plate made turbid by emulsified oil.

activity increased clearly from the basal level (Fig. 3a) concomitant with the increase in biomass production. Lipase was detected in two peaks at 31 h and 84 h of growth (7 h or 60 h after addition of salad oil). Similar results were obtained in the case of oleic acid (Fig. 3b). Activity showed the first peak at about 36 h of growth (12 h after addition), whereas the second peak reached its highest level at about 51 h of growth (27 h after addition).

Discussion

The G+C content of genomic DNA of strain T1 was 65.7%, within the range of 59–68% measured in species of *Pseudomonas* [26]. As bacteria sharing 16S rRNA sequence identity with less than 97.5% are not likely to exhibit reassociation of genomic DNA greater than 60–70% [33], the strains with 16S rRNA identity of less than 97.5% would most likely represent distinct species. On this basis, isolate T1 is not a strain of *P. anguilliseptica.* However, strain T1 shares more than 97.5% 16S rRNA sequence identity with *P. alcaligenes* (97.6%), *P. resinovorans* (98.6%), and *P. aeruginosa* (100%), respectively. Nevertheless, phenotypic data (Table 1) reveal that strain T1 differs from *P. alcaligenes* and *P. resinovorans.* Although the strain T1 has the ability to grow at 55°C, *P. resinovorans, P. alcaligenes,* and *P. anguilliseptica* cannot tolerate such temperatures [26]. Strain T1

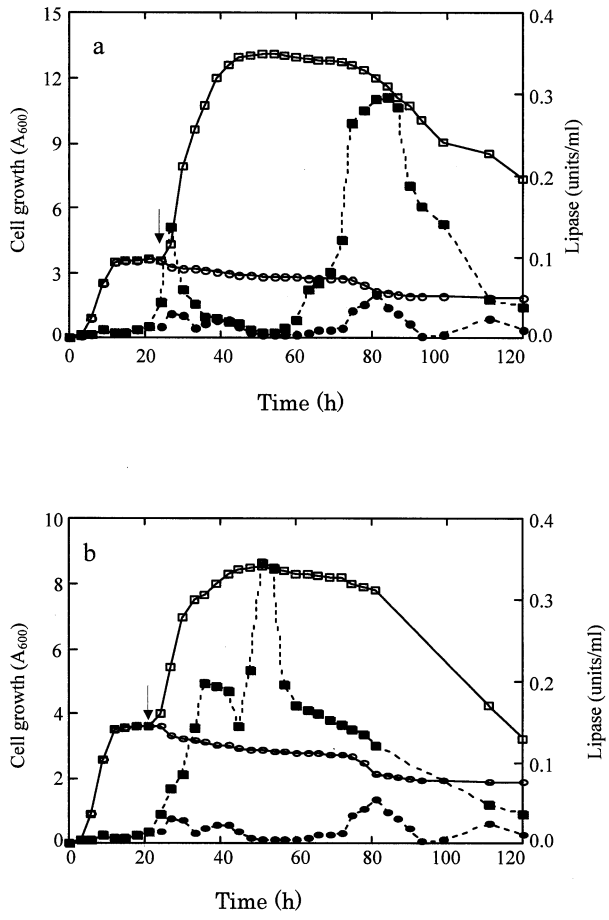

Fig. 3. Growth and lipase activity induction profiles of *P. aeruginosa* T1. Lipase was induced by salad oil (a) or oleic acid (b). Salad oil or oleic acid was added at the stationary phase of growing culture of T1 cells indicated by the arrow. Solid lines, bacterial growth; broken lines, lipase activity. Open symbols show bacterial growth without (circle) and with inducer (square). Closed symbols show lipase activity without (circle) and with inducer (square). Experimental details are described in the text.

is closely related phenotypically with *P. aeruginosa* (Table 1). Because the 16S rRNA sequence and phenotypic data were insufficient to conclude whether a strain belongs to existing species or a new species [9, 34], DNA-DNA hybridization became necessary. The high DNA reassociation values between strain T1 and *P. aeruginosa* (83.5–84%) with high identity in rRNA clearly indicate that strain T1 represents a strain of the species *Pseudomonas aeruginosa.* The type strain is strain T1.

Strain T1 has the ability to efficiently degrade different vegetable and animal fats or oils that also include edible-lipid wastes. Strain T1 had active lipase to hydrolyze TG totally and further degrade the resulting hydrolyzed products (DGs and MG) (data not shown); therefore, it is considered that the enzyme has random specificity towards each *sn*-position of the glycerol backbone of the substrates. This strain has the capacity to utilize FFA very efficiently, because most of the released FFA disappeared in cultures at 25°C or 30°C (data not shown). Regarding rapeseed waste oil, the 1,3-DG component was not hydrolyzed. This may be caused by the presence of other contaminants that would interrupt the reaction.

Strain T1 produced high extracellular lipase activity when grown in the presence of salad oil or oleic acid (Fig. 3). Similar results were obtained when salad oil was added at earlier phases of growth (data not shown). Olive oil was also a good inducer of lipase (data not shown). Irrespective of inducing compounds added to the medium, both lipase activity and bacterial growth were increased when lipids were added to the medium, indicating that they not only act as lipase inducers, but can also be utilized as energy and carbon sources. Although two lipase activity peaks were observed in both cultures, whether or not each peak contains the same lipase remains to be clarified.

There are some reports that microbial lipase production is induced by the presence of lipids such as TG [6, 12, 21] and FFA [12], and by the presence of cyclohexane [24] in the medium. Marcin et al. [21] reported olive oil-induced lipase production in *Pseudomonas aeruginosa* MB 5001. According to Ito et al. [12], organic solvent-tolerant *Pseudomonas aeruginosa* LST-03 produced extracellular lipase when grown in a synthetic medium containing some lipids as the sole carbon source, as was the case with strain T1. Interestingly, in *P. aeruginosa* LST-03, free saturated fatty acids such as stearic and palmitic acids and TGs that were esterified with those saturated fatty acids were effective at inducing lipase. However, in contrast with strain T1, the *P. aeruginosa* LST-03 lipase production was induced by neither olive oil nor oleic acid [12]. Strain T1 shows two-peak lipase activity profiles in cultures containing either salad oil or oleic acid (Fig. 3), while such phenomena were not observed in *P. aeruginosa* LST-03. Although no biochemical data on extracellular lipase(s) of strain T1 and *P. aeruginosa* LST-03 are available, differences in lipase-inducing molecular species of lipids between these two strains might be caused by differences in their lipase induction systems, including specificities of membrane fatty acyl transporter to free fatty acids rather than those in lipase proteins themselves.

Results of our experiments indicate that strain T1 secretes an efficient oil/fatty acid-inducible extracellular lipase degrading different kinds of oils, including waste oils, and that it would be very valuable not only for industrial applications, but also for disposal of problematic waste oils. Isolation of new oil-degrading *Pseudo-*

monas species like strain T1 offers interesting implications because they are considered to be the most important group of biocatalysts used for a variety of different biotechnological applications [13, 14]. Moreover, there is considerable industrial interest in producing these enzymes on a large scale. It has been noted that the importance of lipases lies in their potential, rather than current, level of usage [19]. It would be interesting to identify less expensive, stable lipases for industrial application from newly examined microorganisms, especially those that might have unique selectivity and high activity. Moreover, because of the ability of strain T1 to utilize lipids as sources of carbon and energy, the use of strain T1 in treatment of wastewater containing lipids prior to disposal seems to be promising.

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