# Isolation and characterization of thermophilic and psychrophilic bacteria from oil fields

新規高温/低温油田細菌の単離と諸性質の解析

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# CHAPTER 1

# General Introduction

## 1.1. Bioremediation

Bioremediation is a technology to settle environmental problems by utilizing biological activities (Brubaker et al. 1988, White et al. 1998, Brim et al. 2000, Lee et al. 1998, Coates et al. 1998, De Schrijver et al. 1999, Chen et al. 1999, Zwillich et al. 2000). This technology takes advantage of biological reaction or in other words enzymatic reaction which proceeds slowly but free from non-degradable wastes and by-products causing impact on the earth. In fact, life has been maintained on the earth over than 3,000,000,000 years (Reid et al. 2000). Recently, more and more attention has been paid for bioremediation because environmental pollution is considered as serious global troubles. Regardless of our recognition, contaminants such as carbon dioxide, hydrocarbons, and chemicals originated and synthesized from petroleum have been released and discharged into the nature quantitatively and qualitatively over the capacity of natural attenuation and then a part of life cycle are being broken. This happens not only by accident in the process of transportation like tanker accident or improper disposal, but also by continuously using of fine-chemicals such as detergents, solvents, pesticides, and etc in our daily life. Of course, leakage of petroleum hydrocarbons, organic and inorganic compounds at underground storage tanks and pipelines causes a serious scatter of pollutants that immediately threaten drinking water.

## 1.2. Microbial diversity

Microorganisms represented by "bacteria" consist of single cells. Because the structure of bacterial cells are much simpler than those of higher living organisms, growth rates of microorganisms are generally high and therefore they have high adaptation ability to various environments (Krulwich et al. 1990, Huber et al. 2000, Price 2000, Richard 1997). When we look over the nature, the microbial ecosystem could be symbolized by a saying "The right man in the right place." Although petroleum hydrocarbons are very useful source of energy and chemical compounds, they turn to be one of the most popular and annoying contaminants in case of sudden spills and leaks. Because so many kinds of hydrocarbons including aliphatic-, aromatic-, branched-, and cyclic-hydrocarbons are contained in the crude oil, oil field is expected be a suitable place to isolate microorganisms that degrade various hydrocarbons (Leahy et al. 1990). Moreover, thermophilic and psychrophilic oil bacteria valuable for bioremediation would propagate in high temperature and low temperature oil fields, respectively. Former

bacterial group would be applied for biodegradation of high molecular weight hydrocarbons whose melting temperature is higher than ambient. The latter group might be useful for cleaning spilled oil at low temperature site such as winter-sea. Oil-spill bioremediation methods aim at providing favorable conditions of oxygens, temperature and nutrients to maximize biological hydrocarbon breakdown. Although such methods have been applied successfully in part to restoring polluted seashores, airports, military operations, power plants, etc, demands are increasing for supplying microorganisms which are capable of degrading harmful pollutants with high velocity and high selectivity. Piles of dangerous soils that are contaminated by high concentration of toxic pollutants, such as dioxine, are simply removed by shovels and are now waiting for the treatment in the storehouse. Commercially available bacteria and fungal strains used for bioremediation of petroleum hydrocarbon are listed in Table 1-1 (Korda et al. 1997).

## 1.3. Hydrocarbon degradation mechanisms of bacteria

Although many bacterial genera have been reported to degrade aliphatic and aromatic hydrocarbons, *Pseudomonas* sp. is the most extensively studied one for its genes responsible for degradation of alkane (*alk* operon), naphthalene (*nah* operon), and toluene (*xyl* operon). All of the genes form operons and are encoded on large plasmids, OCT, NAH, and TOL plasmids, respectively (Sayler et al. 1990).

### 1.3.1. Alkane degradation

Degradation ability of alkane is a widespread trait among bacteria. Extensive biochemical and genetic studies on alkane degradation have been conducted for *P. oleovorans*, which is able to grow on medium-chain-length alkanes (C6-C12) (Van Beilen et al. 1994). The *alk* genes are located in two different regions on OCT plasmid, *alkBFGHJKL* and *alkST*. *alkS* encodes a transcriptional regulator which activates expression of *alkBFGHKJL* operon in the presence of alkane (Canosa et al. 1999, Canosa et al. 2000). This operon encodes an alkane hydroxylase, two rubredoxins, an aldehyde dehydrogenase, an alcohol dehydrogenase, an acyl-CoA synthetase, and an outer membrane protein whose function remains unknown (Van Beilen et al. 1994). In the genus *Acinetobacter*, genes encoding alkane hydroxylase (AlkM) and its regulator protein (AlkR) has been isolated from its genomic DNA. Recently, a novel alkane oxygenase with putative dioxygenase activity has also been isolated and characterized in another strain of *Acinetobacter* sp.

Four kinds of initial oxidation mechanism are known for alkane under aerobic condition.

(i) Terminal oxidation pathway in *Pseudomonas* sp. (May et al. 1990) and others.

[ $RCH_3$ → $RCH_2OH$ → RCHO → RCOOH ]

(ii) Diteminal oxidation pathway in fungi and others (Rehm et al. 1981).

[ $CH_3RCH_3$ → $CH_3RCH_2OH$ → $HOCH_2RCH_2OH$ → HOOCRCOOH ]

(iii) Subterminal oxidation pathway in *Nocardia* sp. and others (Markovetz 1971).

[ $RCH_2CH_3$ → $RCH(OH)CH_3$ → $RC(O)CH_3$ ]

(iv) Hydroxyperoxide pathway in long-chain-alkane degrading *Acinetobacter* sp. (Maeng et al. 1996, Finnerty 1988)

[ $RCH_3$ → $RCH_2OOH$ → RCHO → RCOOH ]

An oxidation pathway is also proposed for alkane under anaerobic condition.

(v) Terminal oxidation pathway in *Pseudomonas* sp. (Parekh et al. 1977), sulfate reducing strain AK-1 (So et al. 1999), and strain HD-1 (Morikawa et al. 1996)

[ $RCH_3$ → $RCH{=}CH_2$ → $RCH_2OH$ → RCHO → RCOOH ]

Most of microbial degradation of alkane is initiated by monooxygenase or dioxygenase reaction which requires molecular oxygen as electron acceptor. Little is known about the biodegradation of alkanes under anoxic conditions, where oxygen-initiated reactions cannot occur.

### 1.3.2. Aromatic hydrocarbon degradation

Benzene, toluene, ethylbenzene, and xylene are abreviated as BTEX, which are of concern in aquifer water and sediments where leakage and spill of petroleum hydrocarbon occurred. Catechol plays a key role in aerobic degradation pathway of these aromatic hydrocarbons. Catechol is further catabolized into central metabolisms such as TCA cycle by way of *meta* cleavage or *ortho* cleavage reaction. In *meta* cleavage reaction, catechol is converted to 4-hydroxy-2-ketovalerate by action of two enzymes catechol 2,3-dioxygenase and hydro-lyase. In *ortho* cleavage reaction, catechol is transformed to 3-oxoadipate by four enzymes catechol 1,2-dioxygenase, muconate cycloisomerase, muconolactone isomerase, and 3-oxoadipate enol-lactone hydrolase. A number of denitrifying toluene degraders have been isolated (Altenschmidt et al. 1991, Chee-Sanford et al. 1992, Dolfing et al. 1990, Evans et al. 1991, Fries et al. 1994, Schocher et al. 1991). They usually catabolize toluene both under aerobic and anaerobic conditions. Anaerobic degradation of toluene has also been observed under Fe(III)-reducing (Lovely et al. 1990), methanogenic (Edwards et al. 1994, Grbic-Galic 1990, Grbic-Galic et al. 1987, Wilson et al. 1986), and sulfidogenic (Edwards et al. 1994, Beller et al. 1992) conditions, demonstrating a wide range of electron acceptors are available for anaerobic

degradation of BTEX compounds. Recently, Coschigano *et al.* (1997) have reported for the first time cloning of genes involved in anaerobic toluene degradation pathway.

## 1.4. Subterranean biosphere

Subsurface environment had long been believed an abiotic sphere. There are only a few reports available on the research for subterranean biosphere (Godsy 1980, Fredrickson et al. 1989, Pheips et al. 1989). In 1995, subsurface lithoautotrophic microbial ecosystem (SLiME) was found in deep basalt aquifers from 100 to 1,500 subsurface (Kaiser 1995, Stevens et al. 1997). Lithoautotrophic bacteria reduce carbon dioxide with hydrogen to produce methane or acetic acid, which are metabolized by heterotrophic ones. This indicates that there exist unique ecosystems based on lithoautotrophs in subsurface sphere (Stevens et al. 1997, Dojka et al. 1998, L'Haridon et al. 1995). More and more attention has been paid for microbial ecology in subterranean environment, because these microorganisms would have an infinite potential for degrading various contaminants. In fact, subsurface bacteria in groundwater and unsaturated subsurface sediments have been reported to metabolize a variety of organic contaminants including aromatic hydrocarbon components in gasolin (Ghiorse et al. 1988, Major et al. 1988), surfactants (Federle et al. 1998), and aliphatic chlorinated solvents (Wilson et al. 1987). Deep subsurface environment, especially hot condition, is also attractive from a view point of research on the latest common ancestor of life and evolution of life because subterranean conditions would have been less changed than surface conditions on the earth (Glansdorff 2000, Stetter 1995). These situations prompted us to isolate and characterize microorganisms that survive and live at subterranean oil fields (at high or low temperature).

Since most microorganisms isolated from subterranean petroleum reservoir are strangely unrelated to synthesis and degradation of hydrocarbons (Beeder et al. 1995, Cayol et al. 1995, Cochrane et al. 1988, Fardeau et al. 1996, Fardeau et al. 1997, Jeanthon et al. 1995, Nilsen et al. 1996a, 1996b, Ravot et al. 1995, Rees et al. 1995, Stetter et al. 1993), the ability for the degradation of hydrocarbons and ester compounds related to petroleum were used as a key factor for selecting microorganisms. The materials from which microorganisms were screenedwere taken from following two place; (1) deep subterranean hot oil production water from 1,000 to 2,000 m subsurface in Akita and Niigata Japan, in May 1997 and (2) cold oil sands from 25 to 75 m subsurface at Ft. McMurray, Alberta, Canada, in March 1999. In Ft. McMurray, average atmospheric temperature is below ice temperature in a year.

**Table 1-1** Available microbial strains used in bioremediation

| Name | Description | Supplier | Reference |
|---|---|---|---|
| HYDROBAC | Bacterial preparation specific for petroleum hydrocarbon materials | Polybac Corporation | Huesemann et al. 1994 |
| *Pseudomonas, Rhodococcus, Arthrobacter* | Biosurfactant-producing bacteria | | Hunt et al. 1994 |
| *Pseudomonas putida* | Naphthalene-degrading bacteria | ATCC30015 | Churchill et al. 1995 |
| *Pseudomonas oleovorans* | Bacterial species | ATCC29347 | |
| Unknown | | ATCC15075 | |
| *Acinetobacter calcoaceticus* MM5 | Bacterial species | | Marin et al. 1995 |
| *Pseudomonas fluorescens* 2a | Bacterial species | TsMPM B2556 | Kozhanova et al. 1991 |
| *Alteromonas, Pseudomonas, Deleya, Moraxella, Bacillus, Flavobacterium, Micrococcus, Mycobacterium* and *Vibrio* , or mixture of above | Marine aerobic bacteria | | Kaushanskii et al. 1994 |
| *Candida* sp. | Fungus | FERM P-11023 | Anonymous 1995 |
| S1EW1 | | FERM P-13871 | |
| S2EW1 | | FERM P-13872 | |
| S1OW5 | | FERM P-13873 | |
| *Candida tropicalis* VSB-637 and *Mycococcus lactis* VSB-D5 (pair) | Bacterial and fungal species | | Bitteeva et al. 1994 |
| *Mycococcus lactis* VSB-574 and VSB-D5 (pair) | | | |
| *Acinetobacter oleovorum* subsp. *paraphinicum* VSB-576 and *Candida guilliermondii* subsp. *paraphinicum* VSB-638 (pair) | Bacterial and fungal species | | Bitteeva et al. 1994 |
| *Candida* sp. M23-2 | Fungus | FERM P-13540 | Anonymous 1994a |
| *Trichoderma* sp. AP-5 | Fungus | FERM P-13541 | Anonymous 1994b |
| *Rhodococcus erythropolis* | Bacterial species | BKM Ac-1339D | Anonymous 1993a |
| BB-232 | Petroleum-degrading bacterium | | Cubitto et al. 1994 |
| *Bacillus* sp. | Petroleum-degrading bacterium | BKPM B-5467 | Anonymous 1992 |
| *P. putida* , and *Geotrichum candidum* | Mixed bacteria/fungi culture | CCM 4307 CCM 8170 | Anonymous 1993b |
| *Pseudomonas alkaligenes* or *Alcaligenes* sp. ER-RL3 | Bacterial species | NCIMB 40464 | Anonymous 1993c |
| *Pseudomonas* sp. ER-RL4 | | NCIMB 40465 | |
| *Pseudomonas* sp. ER-RT | | NCIMB 40466 | |
| *Acinetobacter calcoaceticus* ER-RLD | | NCIMB 40506 | |
| *Acinetobacter calcoaceticus* ER-RLX | | NCIMB 40507 | |

# CHAPTER 2

## Isolation and characterization of psychrotrophic bacteria from oil reservoir and oil sands

### 2.1 Introduction

Utilizing biological catabolic activity for the elimination of environmental pollutants, so called bioremediation, is of great interest, as it has a less negative impact on ecological systems compared to chemical strategies (Leahy et al. 1990). Extensive genetic and biochemical studies aimed at understanding the biodegradation pathways of various xenobiotic compounds have been carried out (Johri et al. 1999). Gene technology, combined with a solid knowledge of catabolic pathways, has enabled the development of new or improved microbial degradation activities for hazardous pollutants (Timmis et al. 1999, Kumamaru et al. 1998). Most bioremediation research has focused on mesophilic bacteria such as *Pseudomonas* and *Acinetobacter* strains (Beilen et al. 1994, Ratajczak et al. 1998), which are metabolically inactive at temperatures lower than 10°C. However, natural biodegradation of organic compounds, such as petroleum hydrocarbons or esters, proceeds even at low temperatures in soil, water, and marine systems (Whyte et al. 1996, Margesin et al. 1997). This is mainly due to the activity of indigenous psychrophilic and psychrotrophic microorganisms. These cold-adapted microorganisms are metabolically poorly understood, despite their enormous importance for *in situ* bioremediation technologies (Mohn et al. 1997, Gerday et al. 2000).

Microorganisms that are able to grow under low-temperature conditions are grouped into psychrophiles and psychrotrophs. Psychrophiles are defined as microorganisms incapable of growing at temperatures above 20°C (Morita 1975). They are often difficult to maintain in culture owing to the thermo-instability of cell-building macromolecules, such as lipids and proteins. Psychrotrophs are defined as microorganisms that can grow at 5°C, and most psychrotrophs have optimum growth temperatures between 15 and 30 °C. Although biological strategies for the adaptation of psychrophiles and psychrotrophs to cold-temperature conditions have been studied (Kim et al. 1999, Annous et al. 1997), there is very limited knowledge available concerning the distribution of these cold-adapted bacteria and their catabolic activities in oil fields, which contain various kinds of toxic hydrocarbons. In order to optimize the biodegradation activity of psychrophiles and psychrotrophs towards toxic hydrocarbon or esters at hazardous waste sites, it is essential to develop a basic understanding of their physiology and ecology as well as the genetics and biochemistry of their catabolic pathways.

These considerations prompted me to isolate and characterize cold-adapted bacteria obtained from oil fields. Here, I describe the isolation and characterization of psychrotrophic *Shewanella* sp. and *Arthrobacter* sp. from oil-reservoir water in Japan and oil sands in Canada, respectively.

## 2.2 Material and Methods

### 2.2.1 Isolation procedures

Production water samples from an oil-reservoir tank at the Shibugaki petroleum-gathering plant (Teikoku Oil, Niigata, Japan), and oil-sand samples from Fort McMurray (Syncrude Canada Ltd., Alberta, Canada) were the sources for isolation of the bacteria. Enrichment cultures were prepared by inoculating samples into its respective culture medium on ice and incubating at 4℃ under either aerobic or anaerobic conditions ($CO_2$:$H_2$:$N_2$ = 10:10:80, in an EAN-10 anaerobic incubator, Tabai espec, Osaka, Japan). The cultures were serially diluted and spread onto solid medium (containing 1.5% agar) for single-colony isolation.

### 2.2.2 Culture media

Various kinds of media described in the literature were used to isolate psychrophilic and psychrotrophic bacteria (Krieg et al. 1994). Strains SIB1, SIC1, and SIS1 were isolated in B-medium ( 0.5% peptone, 0.5% glycerol, 0.2% yeast extract, 0.2% meat extract, 0.1% $KH_2PO_4$, 0.1% $K_2HPO_4$, 0.01% $MgSO_4 \cdot 7H_2O$, 0.005% $CaCl_2$, trace biotin, pH 7.2), C-medium ( meat extract 0.3%, polypeptone 0.5%, pH 7.0), and S-medium ( 1.5% polypeptone, 0.1% glycerol, 0.2% $K_2HPO_4$, 0.1% $KH_2PO_4$, 0.01% $MgSO_4 \cdot 7H_2O$, 3% NaCl, pH 7.2), respectively. Strain CAB1 was isolated and cultivated in B-medium. When the cells were grown at temperatures under 4℃, 5% glycerol was added to each medium in order to avoid freezing.

### 2.2.3 Scanning electron microscopy

Cells grown at 4℃ for 50 h were fixed with 2.5% glutaraldehyde in 0.1 M $K_2HPO_4/KH_2PO_4$ buffer, pH 7.2, for more than 2 h. They were then washed with the buffer and dehydrated in acetone on cellulose-filter membranes. After freeze-drying with an ES-2030 freeze dryer (Hitachi, Tokyo, Japan), specimens were coated with gold-palladium using an E-1030 ion sputter coater and observed with a Hitachi S-4700 scanning electron microscope.

### 2.2.4 Cloning and sequencing of the 16S rRNA gene

PCR amplification of the 16S rRNA gene fragment was carried out with GeneAmp PCR System 2400 (Perkin Elmer, Foster City, CA, USA) using AmpliTaq DNA polymerase (Takara shuzo, Kyoto, Japan). The PCR amplification primers used were 16S-7 (5'-AAGAGTTTGATCATGGC-3') and 16S-1510 (5'-AGGAGGTGATCCAACCGCAG-3'), which are parts of the 16S rRNA gene of *E. coli* (EMBL/GenBank/DDBJ; AE000406) and are useful for amplifying 16S rRNA genes from various kinds of bacteria. Chromosomal DNAs, which were used as template DNA of the reaction, were prepared from the strains by using InstaGene™ Matrix (Bio-Rad, Hercules, CA, USA). For the analysis of DNA fragments, agarose gel electrophoresis was carried out under standard conditions (Sambrook et al. 1989). GeneClean kit (Bio 101, La Jolla, Calif., USA) was used to recover DNA fragments from agarose gels. The

PCR-amplified 1.5 kb fragments containing the 16S rRNA genes were ligated into the cloning vector pCR2.1 (Invitrogen, Carlsbad, Calif, USA), with the TA cloning kit (Invitrogen) and used for transformation of *E. coli* DH5$\alpha$ (Sambrook et al. 1989). The nucleotide sequence of the gene was determined with the ABI PRISM 310 genetic analyzer (Perkin Elmer). The BLASTN program (http://www.ncbi.nlm.nih.gov/BLAST/, NCBI, Bethesda, MD, USA) was used for homology searches with the standard program default. The nucleotide sequences of the 16S rRNA genes for strains SIB1, SIC1, SIS1, and CAB1 were deposited in the EMBL/GenBank/DDBJ with the accession numbers AB039737, AB039738, AB039739, and AB039736, respectively.

### 2.2.5 Phylogenetic analysis of 16S rRNA sequence

Phylogenetic trees of 16S rRNA were constructed by using the TreeView program (Page 1996) based on a multiple sequence alignment created by the CLUSTAL W program (Thompson et al. 1994).

### 2.2.6 Physiological analysis of microorganism

The isolates were physiologically characterized with the API 20NE system (bioMerieux, Marcy-I'Etoile, France). All of the methods, other than incubation temperatures (20 °C and 4 °C), were carried out according to the manufacturer's recommendations. In addition to the characteristics given in the API 20 NE kit, amylase and esterase/lipase production activities were also tested for based on the formation of clear halos around the colonies on solid medium containing 1% starch azure or 1% tributyrin, respectively (Morikawa et al. 1994).

### 2.2.7 Degradation test for alkanes, naphthalene and catechol

Since petroleum is mainly composed of aliphatic hydrocarbons and aromatic hydrocarbons, the petroleum-degrading activities of the strains were tested by using standard gas oil (a mixture of alkanes, Tokyo kasei chemical industry, Tokyo, Japan), naphthalene (an aromatic hydrocarbon, Wako pure chemicals, Osaka, Japan), and catechol (a hydroxylated aromatic hydrocarbon, Wako pure chemicals). After aerobic cultivation of the strains in the appropriate medium at 4°C for 50 h, cells were harvested by centrifugation and then washed with BM2 [5g $(NH_4)_2SO_4$, 0.5g $MgCl_2$, 1g $KH_2PO_4$, trace element mixture, and vitamin solution (Krieg et al. 1994)]. The cell pellet was resuspended in 10 ml of BM2 and either 0.1 % (v/v) of the standard gas oil, or 0.1 or 10 mM naphthalene, or catechol (final concentration). Bottles containing the cultures were sealed with rubber stoppers and aluminum crimps, and were gently shaken at 20°C or 4°C. In order to avoid adsorption of hydrocarbons to the rubber stoppers, the bottles were placed upside down in the incubator. After the appropriate cultivation period, residual substrates were analyzed by gas chromatography (GC/FID) to monitor alkane degradation (Huy et al. 1999) or by reverse-phase HPLC monitor naphthalene and catechol degradation (Seyfried et al. 1994). HPLC analysis was carried out using a COSMOSIL 5C18-AR column (4.6 × 150 mm, Nacarai tesque, Kyoto, Japan) and solvent mixtures A [10% acetonitrile/ 0.1% trifluoroacetic acid (TFA)] and B (100%

acetonitrile/ 0.1% TFA) at a flow rate of 0.5 ml/min. Adsorption, elution, and equilibration steps were carried out by changing the ratio of solvents A and B using the linear gradient mode. Substances eluted from the column were monitored by a UV detector at 260nm.

## 2.3 Results and Discussion

### 2.3.1 Isolation of cold-adapted bacteria

After aerobic incubation for a few days at 4°C, oil reservoir water samples and oil sand samples produced several colonies on solid medium; no colonies were obtained from plates incubated under anaerobic conditions. Four strains incapable of growing at 37°C were selected. The isolates from the Shibugaki petroleum gathering plant were designated as strain SIB1 (in B-medium), SIC1 (in C-medium), and SIS1 (in S-medium), and the isolate from the Canadian oil sands was designated as CAB1 (in B-medium).

### 2.3.2 Growth profiles

The growth temperatures of these strains were tested (Fig. 2-1). Strains SIB1 and SIS1 could not grow well at temperatures above 30°C, while the maximum growth rate of strains SIB1, SIS1 and CAB1 was at 20°C. However, these strains reached maximum cell densities at 4°C (SIB1 and CAB1) and 0°C (SIS1), demonstrating that they successfully adapted to the cold environment. Strains SIB1, SIS1, and CAB1 grew relatively well at 0°C, and SIS1 could grow even at -5°C. Strain SIC1 optimally grew at 30°C and did not grow at 0°C, exhibiting a growth profile similar to that of a mesophile rather than a psychrotroph.

### 2.3.3 Microscopic observations

Based on light-microscopy observations, none of the four strains were motile. The cells were also observed using a scanning electron microscope; all of the strains were rod-shaped. The sizes of the strains were as follows; SIB1 0.3 $\mu$m in diameter and 1.0-1.3 $\mu$m in length, SIC1 0.3 $\mu$m 1.3-2.0 $\mu$m, SIS1 0.3 $\mu$m 2.3-2.5 $\mu$m, and CAB1 0.6 $\mu$m by 0.8-1.5 $\mu$m (Fig. 2-2).

### 2.3.4 Phylogenetic analysis of the strains based on 16S rRNA gene sequences

In order to identify the strains, I cloned 1.5-kb DNA fragments, containing more than 95% of the 16S rRNA gene of the respective strain, and determined their complete nucleotide sequences. Phylogenetic trees of 16S rRNA gene including these bacterial strains and several related strains were constructed (Fig. 2-3). It was clearly shown that strains SIB1, SIC1 and SIS1 belong to the genus *Shewanella* and that strain CAB1 belongs to the genus *Arthrobacter*. The 16S rRNA genes of strains SIB1 and SIS1 showed, respectively, 99.9% and 99.8% identity to the 16S RNA gene of a

psychrotrophic *Shewanella* sp. AC10 (Kulakova et al. 1999). The 16S rRNA gene from strain SIC1 was 99.2% identical to that of mesophilic *S. putrefaciens* (Vogel et al. 1997), and the 16S rRNA gene of strain CAB1 showed 99.3% and 99.0% identities to those of mesophilic *Arthrobacter* sp. CF46 (ASP243243) and *Arthrobacter oxydans* (Keddie et al. 1986), respectively. *Shewanella* sp. AC10 has been isolated from Antarctic seawater and grows well at 4°C with an optimum temperature around 20°C. Genes encoding alkaline protease and alanine dehydrogenase have been cloned and studied biochemically from this strain (Kulakova et al. 1999, Galkin et al. 1999), however no hydrocarbon degradation ability has been reported. Although the growth temperature range of *A. oxydans* has not been reported, this bacteria grows well at 30°C. Rashid et al. recently reported that a new psychrotrophic isolate SN16A, which is related to *A. globiformis* (95.4% id), grew even at -5°C (Rashid et al. 1999). *Shewanella* and *Arthrobacter* seem to be popular bacterial groups in cold temperature environments of ocean, subsurface, and land surface (Loveland-Curtze et al. 1999, Bowman et al. 1997).

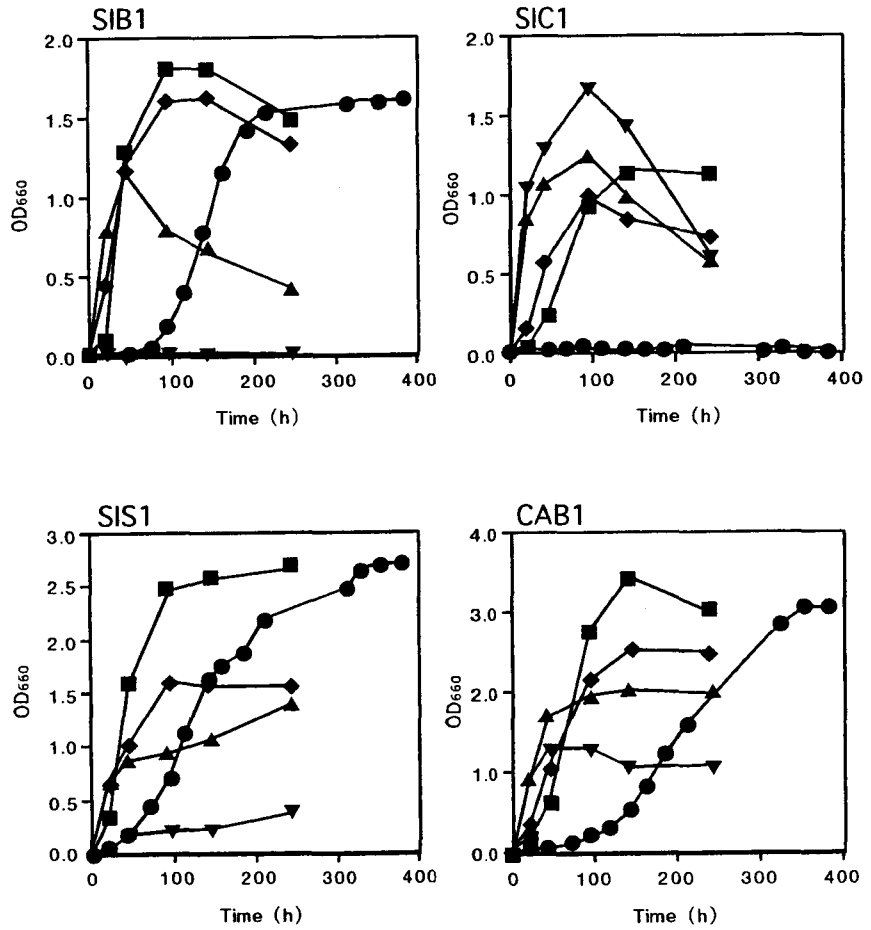


**Fig. 2-1.** Effect of temperature on the growth of strains SIB1, SIC1, SIS1 and CAB1. Cells were grown on the same medium that was used for isolation. ● 0 °C, ■ 4 °C, ◆ 10°C, ▲ 20°C, ▼ 30°C. None of the strains could grow at 37°C

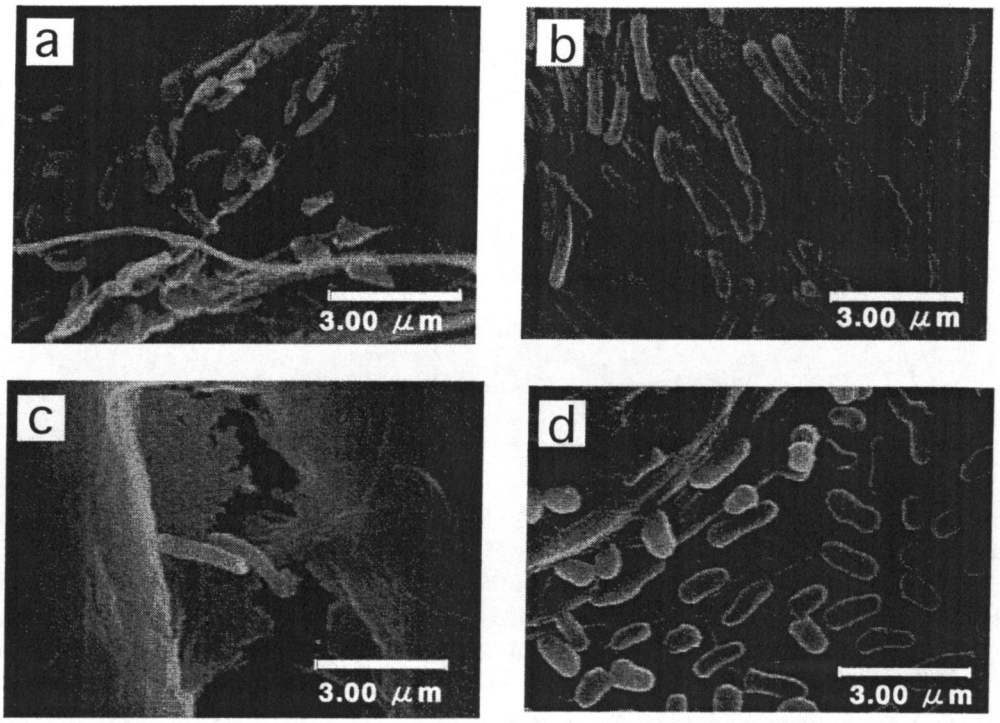


**Fig. 2-2.** Scanning electron micrographs of strains SIB1 (**a**), SIC1 (**b**), SIS1 (**c**), and CAB1 (**d**)

### 2.3.5 Physiological characterization

The API 20NE Kit was used for physiological characterization of the isolated strains (Table 2-1). Metabolic activities of strains SIB1 and SIS1 were quite similar except for the protease activity. The ability to assimilate activities of glucose, D-mannitol, *N*-acetyl-D-glucosamine, maltose and *n*-capric acid differed in strain SIC1 compared to strains SIB1 and SIS1. Besides protease, several hydrolytic activities were examined by plate tests at 4℃. All of the strains exhibited esterase (or lipase) activity. Strains SIC1 and SIB1 produced protease, and strain CAB1 showed amylase production activity. Cold adapted amylase has been reported for psychrotrophic *Alteromonas haloplanctics* (Feller et al. 1999) but not for the genus *Arthrobacter*. Gene cloning and characterization of esterase and amylase from these strains are of interest, because these studies will facilitate the understanding for cold adaptation mechanisms of industrially useful enzymes.

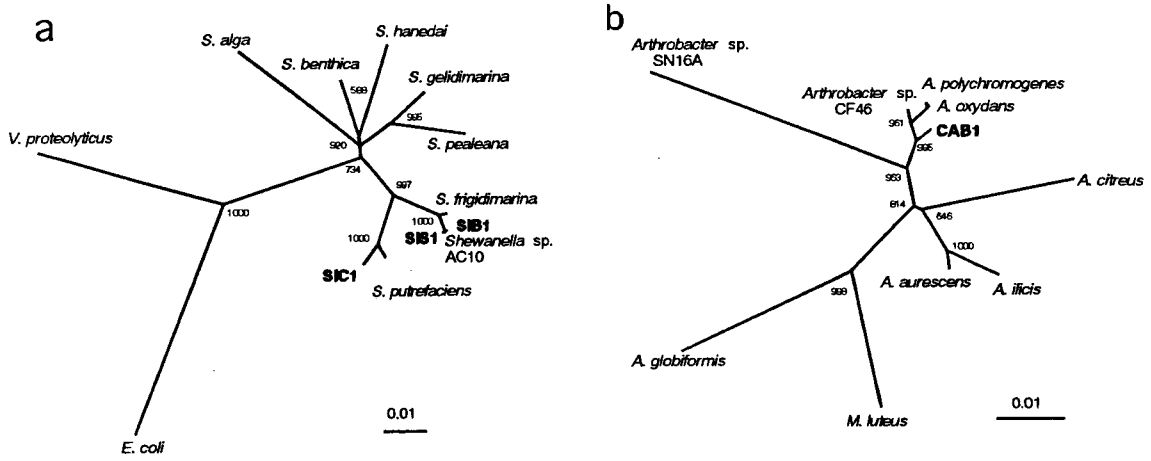


**Fig. 2-3.** Phylogenetic trees based on the 16S rRNA sequences of strains SIB1, SIC1, SIS1, and several other *Shewanella* strains (a), and based on the 16S rRNA sequences of a strain CAB1 and other *Arthrobacter* strains (b). Positions of the genera *Vibrio, Escherichia,* and *Micrococcus* are also shown in out groups. The balanced cladogram was constructed by the neighbor-joining method using the CLUSTAL W program (17). Bootstrap values were calculated from 1,000 trees. The accession numbers for each sequence are as follows; *S. alga* (AF006669), *S. benthica* (X812131), *S. hanedai* (X82132), *S. gelidimarina* (U85907), *S. pealeana* (AF011335), *S. frigidimarina* (U85903), *Shewanella* sp. AC10 (AF061557),*Shewanella* sp. CF46 (ASP243243), *S. putrefaciens* (X82133), *E. coli* (J01859), *Vibrio proteolyticus* (X74723), *A. citreus* (X80737), *A. ilicis* (X83407), *A. aurescens* (X83405), *A. globiformis* (X80746), SN16A (AB024412), *A. polychromogenes* (X80741), *A. oxydans* (X83408).

### 2.3.6 Degradation of catechol

Since strains SIB1, SIC1, SIS1, and CAB1 were isolated from petroleum-reservoir water or oil sands, their ability to degrade alkanes (aliphatic hydrocarbons), naphthalene (an aromatic hydrocarbon), and catechol (a hydroxylated aromatic hydrocarbon) were examined. Although alkanes and naphthalene were not degraded by these strains, a small but consistent amount of catechol was degraded by each of the four strains. A typical HPLC analysis, which strongly suggests that catechol was degraded by CAB1 cells upon incubation at 4°C for 128 h, is shown in Fig. 2-4a. No reduction in the amount of catechol was observed upon incubation at 20°C or 4°C for 128 h in the absence of the cells. The amounts of catechol remaining after incubation with CAB1 and SIB1 cells at 20°C or 4°C for 128 h are shown graphically in Fig. 2-4b. CAB1 degraded 63% of a 0.1 mM catechol sample upon incubation at 20°C for 128 h, and 32% upon incubation at 4°C for 128 h, while SIB1 cells degraded 41% upon incubation at 20°C for 128 h, and 17% upon incubation at 4°C for 128 h. When 10 mM catechol was used, SIB1, SIC1, SIS1, and CAB1 degraded 20%, 14%, 18%, and 19%, respectively, upon incubation at 20°C for 64 h.

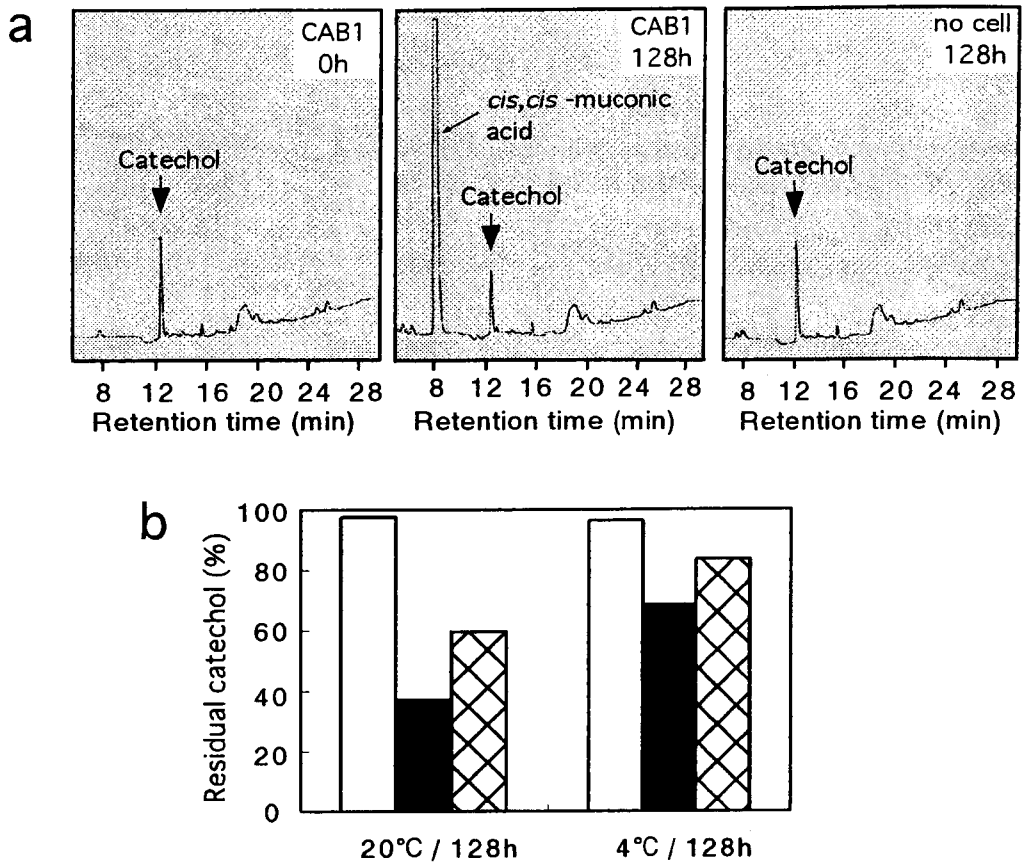


**Fig. 2-4a, b.** Degradation of catechol by representative strains *Shewanella* sp. SIB1 and *Arthrobacter* sp. CAB1. Cells were suspended in the mineral medium BM2 containing catechol at a final concentration of 0.1 mM. Bottles were gently shaken at 20 °C or 4 °C for 128 h in the presence or absence of the cells. **a** A typical HPLC analysis demonstrating that the amount of catechol was reduced upon incubation with suspensions of CAB1 at 4 °C for 128 h. No decrease in the amount of catechol was observed upon incubation at 4 °C for 128 h in the absence of cells. **b** The amounts of catechol remaining after incubation at 20 °C or 4 °C for 128 h. *Open columns* No cells, *shaded* columns CAB1, *hatched columns* SIB1.

Two pathways for the degradation of catechol have been identified to date, the catechol 1,2-dioxygenase (C12O) pathway and the catechol 2,3-dioxygenase (C23O) pathway. Catechol is transformed to *cis, cis*-muconic acid by C12O, or 2-hydroxy muconic semialdehyde by C23O (Radnoti de Lipthay et al. 1999). As shown in Fig. 2-4a, a degradation product of catechol was eluted from the HPLC column at 8.3 min, at which *cis, cis*-muconic acid eluted. A similar degradation-product peak was observed for SIB1 (data not shown). These results allowed me to conclude that psychrotrophic *Arthrobacter* and *Shewanella* strains adopted the C12O pathway in the degradation of hydroxylated aromatic hydrocarbons. The peak area of *cis, cis*-muconic acid was much larger than that of catechol reduced by degradation due to the difference in the molecular

absorption coefficients of *cis, cis*-muconic acid and catechol at 260 nm. Conversion of 0.1 $\mu$mol of catechol correspond to an increase of 5.63 absorbance units under these conditions (Ngai et al. 1990). Several psychrotrophic bacteria that can mineralize aromatic and aliphatic hydrocarbon have been reported for the genera *Pseudomonas* and *Rhodococcus*, and, *Sphingomonas* (Whyte et al. 1997, 1999, Fredrickson et al. 1995), but not for *Shewanella* and *Arthrobacter*. Further metabolic studies on these cold-adapted bacteria from petroleum-contaminated soils, sands, and water will allow the development of in situ bioremediation technologies for hazardous hydrocarons and esters.

**Table 2-1.** API20NE tests for the physiological characteristics of strains SIB1, SIC1, SIS1, and CAB1 at 20°C.

| API 20NE | | | | | |
|---|---|---|---|---|---|
| Substrates | reactions/enzymes | SIB1 | SIC1 | SIS1 | CAB1 |
| Potassium Nitrate | reduction of nitrate to nitrite | + | + | + | − |
| L-Tryptophan | Production of indole | − | − | − | − |
| L-Arginine Hydrochloride | Arginine dihydrolase | − | − | − | − |
| L-Arginine Hydrochloride | Arginine dihydrolase | − | − | − | − |
| Urea | Urease | − | − | − | − |
| Esculin | Hydrolysis ($\beta$-Glucosidase) | + | + | + | + |
| Gelatin | Hydrolysis (Protease) | + | + | − | − |
| p-Nitrophenyl $\beta$-D-Galactopyranoside | $\beta$-Galactosidase | − | − | − | + |
| Glucose | Assimilation | + | − | + | + |
| | Fermentation | − | − | − | − |
| D-Mannose | Assimilation | − | − | − | + |
| D-Mannitol | Assimilation | + | − | + | + |
| N-Acetyl-D-glucosamine | Assimilation | − | + | − | − |
| Maltose | Assimilation | + | − | + | + |
| Potassium Gluconate | Assimilation | − | − | − | + |
| n-Capric Acid | Assimilation | − | + | − | − |
| Adipic Acid | Assimilation | − | − | − | + |
| Malic Acid | Assimilation | + | + | + | + |
| Sodium Citrate | Assimilation | − | − | − | + |
| Phenyl Acetate | Assimilation | − | − | − | + |
| Tetramethyl-p-phenylenediamine | Cytochrome oxidase | + | + | + | − |

## 2.4 Summary

Four psychrotrophic strains which grew at 4°C but not at 37°C, were isolated from Japanese oil-reservoir water (strains SIB1, SIC1, SIS1) and Canadian oil sands (strain CAB1). Strains SIB1, SIS1, and CAB1 had a maximum growth rate at 20°C and grew to the highest cell densities at the cultivation temperature of 0 - 4°C. Strain SIS1 was capable of growing even at -5°C. The growth profile of strain SIC1 was rather similar to that of a mesophilic bacterium. Strains SIB1, SIC1, and SIS1 were identified as members of the genus *Shewanella*, and strain CAB1 was a member of the genus *Arthrobacter*. All these strains exhibited weak degradation activity against catechol, a hydroxylated aromatic hydrocarbon, and tributyrin. These strains are expected to be of potential use in the *in situ* bioremediation technology of hazardous hydrocarbons and esters under low-temperature conditions.

# CHAPTER 3

## Isolation and characterization of long-chain-alkane degrading *Bacillus thermoleovorans* from deep subterranean petroleum reservoirs

### 3.1 Introduction

While bacterial degradation of petroleum has been known for over 50 years, bacteria responsible have mostly been isolated from surface areas, such as soils, petroleum storage tanks, and oil spills (Zobell 1946, Britton 1984, Huy et al. 1999). Thus, biological degradation pathways for hydrocarbons have been mainly studied for mesophilic bacteria, including *Pseudomonas* sp. and *Acinetobacter* sp. (Assinder et al. 1990, Geissdorfer et al. 1999). The genetics and enzymology of the alkane metabolism have been most extensively investigated for *Pseudomonas oleovorans* (Canosa et al. 2000, Staijen et al. 2000). This alkane degradation pathway includes oxidation to alcohol [alkane monooxygenase], aldehyde [alcohol dehydrogenase], and fatty acids [aldehyde dehydrogenase]. The resulting fatty acids are converted to acyl-CoA derivatives that are then metabolized by a $\beta$-oxidation pathway. The *alk* genes, encoding proteins involved in the conversion of alkanes to the corresponding acyl-CoA derivatives have been identified and shown to form operons *alkBFGHJKL* and *alkST* on the OCT-plasmid in *P. oleovorans* (van Beilen et al. 1994). In contrast, there is limited knowledge available about alkane degradation at low or high temperatures (Whyte et al. 1997, Whyte et al. 1998, Zarilla et al. 1986, 1987, Rueter et al. 1994).

It is generally accepted that thermostable enzymes are industrially important on account of their structural stability, and many kinds of such enzymes have been purified from thermophilic bacteria and hyperthermophilic archaea (Lasa et al. 1993, Adams et al. 1996, 2000). The degradation of xenobiotics, including petroleum hydrocarbons, by thermophilic microorganisms provides crucial advantages over degradation by mesophilic or psychrophilic organisms, especially when they are applied in biotechnological processes. Limited biodegradation as a result of the low water solubility of hydrophobic contaminants may be overcome by higher water solubility at elevated temperatures. Moreover, diffusion and chemical transformation rates also increase at higher temperatures, with a consequent additional positive impact on bioavailability. These considerations prompted me to isolate thermophilic petroleum-degradaing microorganisms that degrade hydrocarbons at high temperatures and to analyze their metabolic pathways.

Deep subterranean petroleum reservoirs are expected to be a good source for the isolatation of such microorganisms, because the *in situ* temperatures are much higher than those on the Earth's surfaces. In fact, various kinds of sulfate-reducing bacteria (L'Haridon et al. 1995, Beeder et al. 1995, Rees et al. 1995, Greene et al. 1997), fermentative thermophilic bacteria, and hyperthermophilic archaea (Grassia et al. 1996, Takahata et al. 2000) have been isolated from deep petroleum reservoirs. However, none of them have been reported to utilize hydrocarbons, suggesting that they do not interact

with petroleum. The absence of oxygen would be an obstacle to microbial utilization of hydrocarbons in deep subterranean environments, although anaerobic hydrocarbon degradation has been reported for limited kinds of bacteria (Loveley et al. 1989, Evans 1991, Rueter et al. 1994, Morikawa et al. 1996, Ehrenreich et al. 2000). In addition, higher temperatures increase the membrane toxicity of hydrocarbons (Bossert et al. 1984). In fact, thermophilic *n*-alkane degrading microorganisms have thus far been isolated only from surface soils, hot springs, and sediments (Klug et al. 1967, Merkel et al. 1978, Rueter et al. 1994). Recently, it has been proposed that a naphthalene degradation pathway in *Bacillus thermoleovorans* at 60°C differs from that of mesophilic bacteria (Annweiler et al. 2000).

Here, I report the isolation of a long-chain-alkane degrading *B. thermoleovorans* from deep subterranean petroleum reservoirs in Japan. I also identifed fatty acids with corresponding chain length as the metabolites of alkane. Moreover, the gene encoding a long-chain alcohol dehydrogenase was cloned from the strain B23 and the phylogenetic position was analyzed based on the deduced amino acid sequence.

## 3.2 Material and Methods

### 3.2.1 Bacterial strains and plasmid

Strains B23 and H41 were isolated in this study. The type strain LEH-1$^{TS}$ (ATCC43513) was purchased from American Type Culture Collection as a reference strain (Zarilla et al. 1987). *Escherichia coli* DH5$\alpha$ was used as a host strain for 16S rRNA gene cloning. The cloning vector pCR2.1 (Invitrogen Corp., San Diego, CA) and pUC19 (Takara shuzo, Kyoto, Japan) were used as the cloning vectors.

### 3.2.2 Profiles of the sampling sites

Production water samples (crude oil-water emulsions) were collected from deep subterranean petroleum reservoirs in Minami-aga (Niigata, Japan) oil field, AA-5 and Yabase (Akita, Japan) oil field, S-114. Profiles of the wells were as follows; AA-5: depth, 2150 m; temperature at the bottom, 106°C; production water salinity, 1.16%; pH, 8.0. S-114: depth, 1700 m; bottom temp., 95°C; salinity, 1.87%; pH, 5.6.

### 3.2.3 Culture media

Since strain B23 and H41 grew well in a nutrient L broth, their growth profiles were determined by using L broth. L broth contained per liter: 5 g Yeast extract (Difco, Detroit, MI), 10 g Bacto-tryptone (Difco), and 5 g NaCl (pH 7.2). Freshly grown cells were inoculated (1%) into the bottles containing L-broth and kept standing under various temperature and pH conditions. LBM, a basal salts medium for strain LEH-1$^{TS}$, was used for alkane degradation experiments (Merkel et al. 1978). LBM contained per liter, 0.25 g $NaNO_3$, 0.25 g $NH_4Cl$, 0.21 g $Na_2HPO_4$, 0.20 g $MgSO_4$-$7H_2O$, 0.09 g $NaH_2PO_4$, 0.04 g KCl, 0.02 g $CaCl_2$, 1 mg $FeSO_4$, 10 ml trace mineral solution, and 1 ml (0.1%) *n*-alkane or standard gas oil. Trace mineral solution contained per liter, 7 mg $ZnSO_4$-$7H_2O$, 1 mg $H_3BO_3$, 1 mg $MoO_3$, 0.5 mg $CuSO_4$-$5H_2O$, 18$\mu$g $CoSO_4$-$7H_2O$, 7$\mu$g $MnSO_4$-

$5H_2O$. All the culture bottles were sealed with butyl rubber stoppers and aluminum crimps to avoid evaporation.

### 3.2.4 Dissolved oxygen measurement

Dissolved oxygen (DO) in the production water samples was measured by using a DO meter (Type B-505/GU-B7, Iljima electronic co. ltd., Tokyo) with a dynamic range from 0 to 20 mg/l $O_2$ and an accuracy of +/- 0.2%. The DO in the water saturated with air (1 atom, 20°C) is 9.1 mg/l (ppm).

### 3.2.5 Scanning electron microscopy

Cells washed with 0.1 M $K_2HPO_4/KH_2PO_4$ buffer (pH 7.2), cells were fixed with 2.5% glutaraldehyde in the same buffer for more than 2 h. They were then washed with the same buffer and dehydrated in acetone. After freeze-drying, specimens were coated with gold-palladium and observed with a scanning electron microscope S-4700 (Hitachi, Tokyo, Japan).

### 3.2.6 Cloning and sequencing of the 16S rRNA gene

The 16S rRNA gene was amplified by PCR in 30 cycles with a GeneAmp PCR System 2400 (Perkin Elmer, Foster City, CA, USA) using AmpliTaq DNA polymerase (Takara shuzo) as described previously (Kim et al. 1995). The PCR amplification primers used were 16S-7 (5'-AAGAGTTTGATCATGGC-3') and 16S-1510 (5'-AGGAGGTGATCCAACCGCAG-3'), which represent a 16S rRNA gene of *E. coli* (EMBL/GenBank/DDBJ accession number X80721). Chromosomal DNAs used as templates were prepared from strains B23 and H41 using InstaGene™ Matrix (Bio-Rad, Hercules, CA, USA) following manufacturer's instructions. DNA fragments were analyzed by agarose gel electrophoresis under standard conditions (Maniatis et al. 1982). A GeneClean kit (Bio 101, La Jolla, CA, USA) was used to recover DNA fragment from the agarose gels. The PCR-amplified 1.5 kb DNA fragments were ligated into the cloning vector pCR2.1, with TA cloning kit (Invitrogen Corp., Leek, The Netherland), and used for the transformation of *E. coli* DH5$\alpha$. The nucleotide sequences of the genes were determined using an ABI PRISM 310 genetic analyzer (Applied Biosystems Japan, Tokyo) with a BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems Japan). The BLASTN program (http://www.ncbi.nlm.nih.gov/BLAST/, NCBI, Bethesda, MD) was used for a gene homology search with the standard program default. The nucleotide sequences of the 16S rRNA genes for strains B23 and H41 have been deposited in the EMBL/GenBank/DDBJ databeses with accession numbers AB034836 and AB034902, respectively.

### 3.2.7 Physiological characterization test

API 20NE Kit (bioMerieux Japan Ltd., Tokyo, Japan) was used for physiological characterization of the strains. Incubation temperature was 55°C instead of 37°C.

### 3.2.8 Alkane degradation test

Cells suspensions were used to study alkane degradation. After aerobic cultivation in 10 ml L-broth at 70°C for 24 h, cells were harvested by centrifugation at 8,000 g for 10 min at 4°C and then washed with LBM. The cells were inoculated in 10 ml of LBM (*ca.* $10^{10}$ cells/ml) containing 0.1 % (v/v) filter-sterilized standard gas oil (Tokyo Kasei, Tokyo). Bottles were sealed with butyl rubber stoppers and aluminum crimps, and were incubated at 70°C without shaking. To prevent adsorption of alkanes to the rubber stoppers, the bottles were placed upside down in the incubator. Bottles with uninoculated culture were also prepared and used as negative control. After an appropriate cultivation period, residual alkanes were extracted from the cultures with 10 ml of hexane twice and directly analyzed by gas chromatography (GC/FID) and GC-mass spectrometry (GC-MS). GC/FID analyses were performed by a HP6980 instrument (Hewlett-Packard, Palo Alto, CA, USA), equipped with a 30-m non-polar capillary column (HP-1, Hewlett-Packard; internal diameter, 0.25 mm, coated with 0.25-μm film thickness cross linked methyl siloxane) and a flame ionization detector (FID). Helium was used as the carrier gas at a flow rate of 25 ml/min. The temperature program was 80°C (5 min isothermal), 80 to 300°C (10°C/min), and 350°C (10 min isothermal). The sample (1 μl) was injected by split mode at a ratio of 50:1 at 250°C. To determine the residual amounts of alkanes, fluorene was previously added to hexane at 10 mg per liter as an internal standard. GC-MS measurements were performed with a mass spectrometer (JEOL JMS-DX303, JEOL, Tokyo) at an ionization energy of 70 eV on a capillary column corresponding to HP-1.

### 3.2.9 Methanolysis of the metabolic intermediates derived from alkane

Cells were inoculated in 500 ml LBM (*ca.* $10^{10}$ cells/ml) supplemented with 0.1% of an alkane (hexadecane or heptadecane). After cultivation at 70°C for 14 d, cells were harvested by centrifugation and freeze dried. About 50 mg cells (dry wt.) were suspended with 1 ml of water and then added to 4 ml chloroform/methanol (1:2, v/v). After vigorous mixing for a few hours, the extracts were transferred to a new tube. The remaining cell pellet was extracted again with 5 ml of chloroform/methanol/water (1:2:0.8, v/v). The first and the second extracts were pooled. After evaporation, the resulting pellet was dissolved in 5 ml of 1N KOH-methanol solution, saponified at 80°C for 1h, and then acidified with an appropriate amount of 6 N HCl. Substances were extracted three times with 5 ml petroleum ether. After drying, the pellet was dissolved in 5% HCl in methanol, and hydrolyzed at 100°C for 3 to 4 h. The hydrophobic fractions containing fatty acid methyl esters and remaining intact alkanes were extracted with a *tert*-butylacetate/hexane mixture (50:50, v/v) containing 100 ppm heneicosane. The samples were analyzed by GC/FID and GC-MS as described above.

### 3.2.10 Shotgun cloning of the gene encoding an alcohol dehydrogenase

A genomic library of *B. thermoleovorans* B23 was constructed as follows. Genomic DNA of the strain B23 was prepared in a large scale by CsCl density gradient ultra-centrifugation as described previously (Imanaka et al. 1981). After complete digestion with *Eco*RI, the DNA fragments were ligated into the *Eco*RI site of pUC19. The

resultant plasmids were used to transform *E. coli* DH5$\alpha$. Cells containing recombinant plasmids were then grown on aldehyde indicator plates at 37°C.

Aldehyde indicator plates were prepared by adding 8 ml of 2.5 mg pararosaniline/ ml 1-tetradecanol and 100 mg of sodium bisulfite to 400 ml precooled L agar (Conway et al. 1987). To ensure that a positive clone exhibiting long-chain-alcohol-dependent ADH activity was isolated, 1-tetradecanol was used as a substrate in this experiment. *E. coli* cells with the enzyme activity form red colonies due to the formation of a Schiff base between aldehyde (1-tetradecanal) and pararosaniline. I did not incubate the plate at temperatures higher than 50°C because it turned red abiotically when this temperature was exceeded. Of 1,000 *E. coli* transformants examined, one colony, designated as Ec1, gave a strong red color. The plasmid isolated from this transformant contained the 2.2 kb *Eco*RI fragment that encoded a most of a putative alcohol dehydrogenase (Bt-Adh). A 2.7 kb *Cla*I fragment containing the gene encoding flanking region of Bt-Adh was amplified by inverse PCR method (Triglia et al. 1988). Finally, the entire gene (*Bt-adh*) encoding a long-chain-alcohol-dependent alcohol dehydrogenase (Bt-Adh) was cloned from strain B23. The nucleotide sequence of the *Bt-adh* was deposited in the EMBL/GenBank/DDBJ with accession number AB040809.

### 3.2.11 Phylogenetic analysis of bacterial alcohol dehydrogenases

A phylogenetic tree of representative bacterial ADHs and Bt-Adh was constructed by using the TreeView program (Page 1996). A multiple amino acid sequence alignment was created by the ClustalW program (Thompson et al. 1994).

## 3.3 Results and Discussion

### 3.3.1 Isolation of thermophilic *n*-alkane degrading bacteria

Sample were directly collected from wellheads and inoculated into various kinds of basal mineral media and nutrient media under both aerobic and anaerobic conditions. Stable bacterial growth was observed only when nutrient media were used. Direct counting of colonies on nutrient L-medium revealed that production water from the AA-5 and S-114 wells contained about 10 and 40 cells/ml, respectively. After transferring the colonies several times on L-medium or the basal medium (LBM) supplemented with standard gas oil, the cells isolated from Minami-aga (AA-5) and Yabase (S-114) were respectively designated as strains B23 and H41. Although cell growth on LBM was clearly supported by alkanes, neither strains grew on alkanes as a sole carbon source in liquid culture. It was surprising that the strains were strict aerobes. Contamination with terrestrial surface bacteria was less probable in the case of S-114 since the production water had been returned to the oil reservoir after filter-sterilization treatment and the addition of hypochlorite. In the case of AA-5, production water was never returned to the oil reservoir. A small but consistent amount of dissolved oxygen was detected under atmospheric pressure in the production water from both AA-5 (0.7 ppm) and S-114 (0.6 ppm), indicating the high probability that oxygen exists under 2000 m below the surface in these areas. Because both oil fields are located within 15 km from the shore, seawater,

which contains oxygen, may have soaked into the oil reservoir examined. Several oil bacteria have been reported to produce emulsifying reagents - biosurfactants (Kosaric et al. 1987) - which enhance the incorporation of hydrocarbons into the cells. However, an oil-displacement activity test (Morikawa et al. 1993) indicated that the amounts of biosurfactants produced by strains B23 and H41were very small.

### 3.3.2 Growth profiles

Strains B23 and H41 were able to grow at temperatures ranging from 50 to 80°C, with optimum growth at 70°C and 65°C, respectively (Fig. 3-1a). Although strain B23 grew most rapidly at 70°C, it grew at nearly the same rate at all the temperatures tested in the range from 50 to 80°C. Both strains grew optimally at pH 6.7 (Fig. 3-1b). After 24-h cultivation under the optimum conditions, the optical density at 660 nm of the B23 and H41 cultures reached to 0.15 and 0.6, respectively.

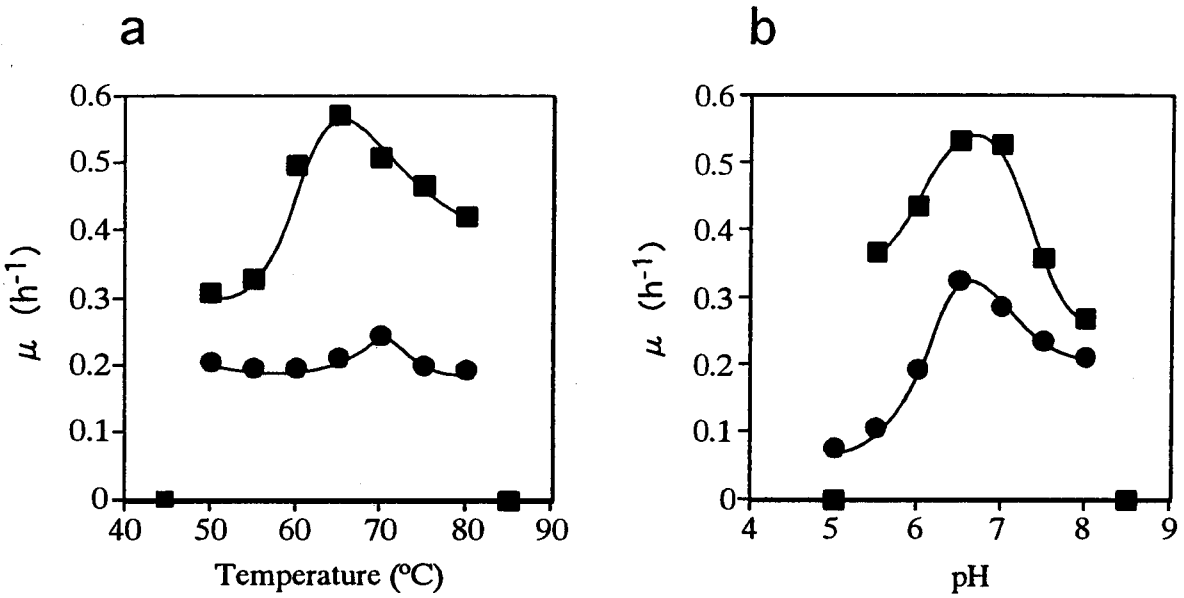


**Fig. 3-1.** Effect of temperature (a) and pH (b) on the specific growth rate, μ, of strains B23 and H41. Cells were grown on L broth at pH 7.2 (a) and at 70°C (b). Cell growth was monitored by measuring the $OD_{660}$ and μ value was determined under each condition. Symbols are; ●, B23 and ■, H41.

### 3.3.3 Microscopic observation

Cells grown in L-broth were observed under an optical microscope. They were non-motile with a rod-like shape. Scanning electron microscope images showed that strains B23 and H41 have a similar long, straight rod shape (0.3 to 0.5 μm in diameter by 2.5 μm in length, Fig. 3-2a, b).

### 3.3.4 Identification of the strains

A 1.5-kb DNA fragment from each strain carrying more than 95% of the 16S

rRNA gene, was cloned and the respective nucleotidesequences were completely determined. The gene sequences from the two strains showed the highest identity – 99.5 and 99.6%, respectively - with the sequence of *Bacillus thermoleovorans* DSM5366 (Table 3-1). The B23 and H41 genes were also 99.6% identical to each other. Sunna et al. (Sunna et al. 1997) re-examined the identification of thermophilic *Bacillus* strains by DNA-DNA hybridization method and showed that a number of thermophilic *Bacillus* species should be combined together in the species *B. thermoleovorans* despite their physiological heterogeneity. The lowest identity of the 16S rRNA sequence between two of them was 99.1%. These results strongly suggest that the strains B23 and H41 also belong to *B. thermoleovorans*. I also analyzed the physiological characteristics of strains B23 and H41 together with those of *B. thermoleovorans* type strain LEH-1$^{TS}$. The B23 and H41 16S rRNA genes did not show the highest identities to strain LEH-1$^{TS}$ (98.3% and 98.7%, respectively). However, since alkane degradation has been reported only for strain LEH-1$^{TS}$, I used this strain for comparative purposes. The results are summarized in Table 3-2. Assimilation of carbohydrates varied with the strains tested. The results obtained in the tests for arginine dihydrolase, urease, and oxidase activities for strains B23 and H41 differed from those for strain LEH-1$^{TS}$.

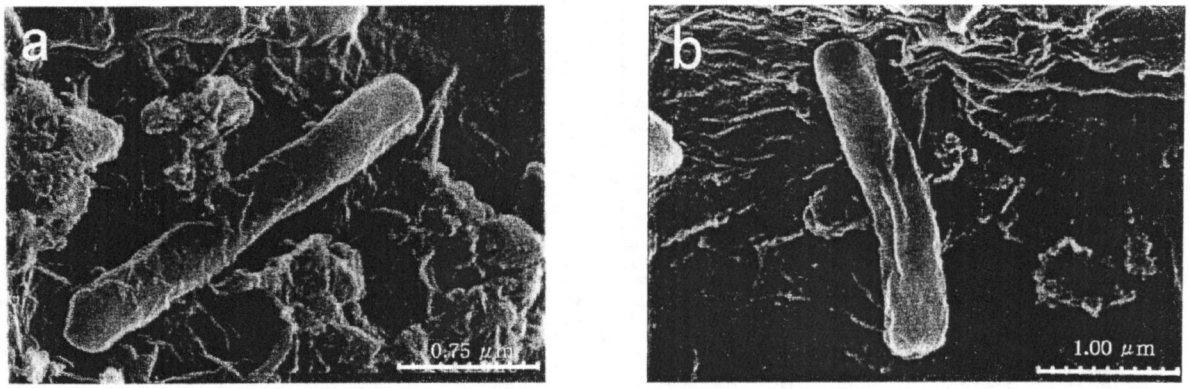


**Fig. 3-2.** Scanning electron micrographs of strains B23 (a) and H41 (b). Background of the cells is filter paper on which cells are adsorbed and fixed.

### 3.3.5 Degradation of alkane

The alkane degradation abilities of strains B23 and H41 were tested and compared with that of LEH-1$^{TS}$ at 70°C (Fig. 3-3). Although this is not the optimum growth temperature of the strains LEH-1$^{TS}$ (optimum 60°C) and H41 (optimum 65°C), because the solubility and viscosity of the substrate - which may affect susceptibility to degradation - changed at the different temperatures, alkane degradation was examined at the uniform temperature of 70°C. This was the highest temperature reported for alkane degradation by bacteria (Matels et al.

1967). In this experiment, since a large amount of the cells was inoculated into the LBM (ca. $10^{10}$ cells/ml) containing standard gas oil, no extensive cell growth was observed even in the case of strain LEH-$1^{TS}$. Alkanes in the culture bottle were reduced by 60% in the presence of the strain LEH-$1^{TS}$ or B23 in 12 d, and by 75% in the presence of the strain H41 in 20 d (Fig. 3-3). Strain LEH-$1^{TS}$ began to degrade alkanes immediately after incubation. In contrast, there is a 7-day lag in the alkane degradations by the strains B23 and H41. However, when these strains were precultured in the presence of alkanes, more than 90% of the alkanes were degraded after 20 days with no lag time. These results suggested that the enzymes responsible for the alkane degradation pathway in the strains B23 and H41 are inducible. It should be noted that the strains B23 , H41, and LEH-$1^{TS}$ did not degrade alkanes under the anoxic condition or when the cultures were shaken. The *B. thermoleovorans* strains effectively degraded alkanes only when the cultures were incubated in the presence of oxygen without shaking. Since *B. thermoloeovorans* is strictly aerobes, oxygen is probably a sole and the best electron acceptor in its dissimilating pathways, including alkane oxidation. Optical microscopic observation of strains B23 and H41 indicated that only a small number of cells attached themselves to the surface of oil droplets. Shaking during cultivation may impede attachment of the cells to the alkane phase, which could be essential for uptake of the substrate. In the uninoculated bottle, the amount of alkanes decreased to 80 and 67 % after 7 and 28 d, respectively, which was probably due to adsorption of alkane to the butyl rubber stopper. Because no decrease in the amount of alkanes was observed during the initial 7 d in the bottle containing strain B23, adsorption of alkanes to the rubber seemed to be hampered by the cells.

**Table 3-1.** Homology matrix of the nucleotide sequences of the 16S rRNA genes from the strains B23 and H41, and related thermophilic *Bacillus* species EMBL/GenBank/DDBJ data base accession numbers are; B23 (AB034836) and H41 (AB034902), *B. thermoleovorans* DSM5366 (Z26923), *B. caldodetax* (Z26922), *B. caldolyticus* (Z26924), *B. caldovelox* (Z26925), *B. kaustophilus* (X60618), *B.thermocatenulatus* (Z26926), *B. thermoleovorans* (M77488), *B. stearothermophilus* (AJ005760), *B. thermodenitrificans* (Z26928).

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Strain B23 | — | | | | | | | | | | |
| 2 | Strain H41 | 99.6 | — | | | | | | | | | |
| 3 | DSM5366 (*B. thermoleovorans*) | 99.5 | 99.6 | — | | | | | | | | |
| 4 | *B. caldotenax* | 99.3 | 99.4 | 99.7 | — | | | | | | | |
| 5 | *B. caldolyticus* | 99.1 | 99.4 | 99.4 | 99.5 | — | | | | | | |
| 6 | *B. caldovelox* | 99.0 | 99.1 | 99.1 | 99.2 | 98.9 | — | | | | | |
| 7 | *B. kaustophilus* | 98.7 | 99.0 | 99.2 | 99.0 | 98.8 | 98.5 | — | | | | |
| 8 | *B. thermocatenulatus* | 98.7 | 98.7 | 98.6 | 98.6 | 98.4 | 98.7 | 98.2 | — | | | |
| 9 | *B. thermoleovorans* LEH-1 | 98.3 | 98.7 | 98.7 | 98.5 | 98.4 | 98.1 | 98.5 | 97.8 | — | | |
| 10 | *B. stearothermophilus* | 97.6 | 97.9 | 99.7 | 97.7 | 97.4 | 97.5 | 97.1 | 97.9 | 96.5 | — | |
| 11 | *B. thermodenitrificans* | 97.2 | 97.5 | 97.5 | 97.5 | 97.3 | 97.1 | 96.8 | 97.4 | 96.3 | 98.5 | — |

**Table 3-2.** Physiological characteristics of the *B.thermoleovorans* strains B23, H41, and LEH-1.

| API 20 NE | | | | |
|---|---|---|---|---|
| Substrates | Reactions/enzymes | B23 | H41 | LEH-1 |
| Potassium Nitrate | reduction of nitrate to nitrite | + | + | + |
| L-Tryptophan | Production of indole | − | − | − |
| Glucose | Acidification | − | − | − |
| L-Arginine hydrochloride | Arginine dihydrolase | − | − | + |
| Urea | Urease | − | − | + |
| Esculin | Hydrolysis ($\beta$-glucosidase) | + | + | + |
| Gelatin | Hydrolysis (protease) | + | + | + |
| p-Nitrophenyl $\beta$-D-galactopyranoside | $\beta$-Galactosidase | + | + | + |
| Glucose | Assimilation | + | + | + |
| L-Arabinose | Assimilation | + | − | − |
| D-Mannose | Assimilation | + | − | + |
| D-Mannitol | Assimilation | − | + | + |
| N-Acetyl-D-glucosamine | Assimilation | − | − | + |
| Maltose | Assimilation | + | + | + |
| Potassium gluconate | Assimilation | + | − | + |
| n-Capric acid | Assimilation | − | − | − |
| Adipic acid | Assimilation | + | − | − |
| Malic acid | Assimilation | + | + | + |
| Sodium citrate | Assimilation | − | − | − |
| Phenyl acetate | Assimilation | − | − | + |
| Tetramethyl-*p*-phenylenediamine | Cytochrome oxidase | + | + | − |

### 3.3.6 Alkane substrate specificity of the strains

The degradation rate of alkanes with different chain lengths were determined from the decrease in the amount of individual alkanes contained in the gas oil (Fig. 3-4). Mesophilic microorganisms usually degrade alkanes with the carbon chain lengths ranging from 10 to 18 to generate energy sources for their growth (Britton 1984). Only several *Acinetobacter* strains have been reported to be able to utilize alkanes longer than C20 (Asperger et al. 1981, Sakai et al. 1994) and the corresponding fatty alcohol are dead-end products to form wax body. Strains B23 and H41 effectively degraded alkanes (>60%) longer than C12 and C15, respectively. Strain B23 degraded alkanes from C13 to C23 with nearly equal efficiencies, whereas strain H41 degraded eicosane (C20) and heneicosane (C21) most effectively. Both strains also degraded hexacosane (C26) and triacontane (C30) (data not shown). Strain LEH-1$^{TS}$ effectively degraded alkanes with carbon chain lengths of less than 18 and most effectively degraded undecane (C11). The solubility and viscosity of the hydrocarbons would respectively increase and decrease as the temperature increased. Therefore, one explanation for the unusual substrate

preferences of the strains B23 and H41 may be that they have successfully adapted themselves to high-temperature petroleum field. The difference in the substrate preferences among strains B23, H41 and LEH-$1^{TS}$ must reflect the difference in the substrate specificities of the enzymes involved in the alkane degradation pathway. Like the strain LEH-$1^{TS}$, strains B23 and H41 could not degrade aromatic hydrocarbons, such as catechol and naphtalene. On the other hand, *Bacillus* sp. A2, which was isolated from Icelandic hot spring, degrades phenol and cresols at 70°C and *B. thermoleovorans* Hamburg 2 has been shown to degrade naphtalene at 60°C (Mutzel et al. 1996, Annweiler et al. 2000).

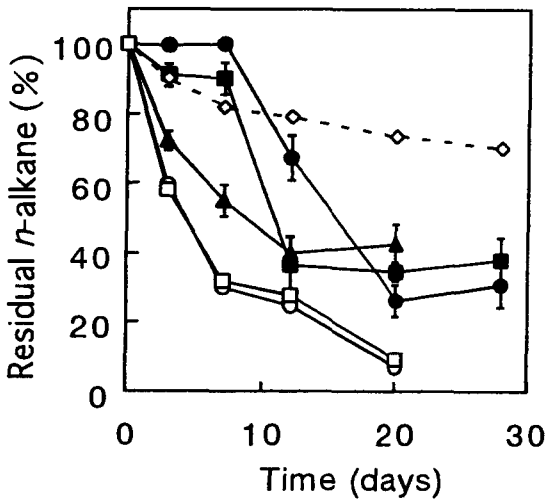


**Fig. 3-3.** Biodegradation of gas oil by the *B. thermoleovorans* strains B23, H41, and LEH-1 at 70°C. The cells were cultivated in basal salts medium LBM supplemented with 0.1 % standard gas oil. Alkanes were extracted with hexane and quantified by GC/FID analysis. Residual alkanes represent the amount of alkanes (C9-C23) extracted after incubation relative to that extracted before incubation. Experiments were performed at least twice and the error bar is also shown in the figure. Symbols are as follows; ●, B23; ■, H41; ▲, LEH-1; ◇, no cells. Cells of B23 and H41 were also harvested by centrifugation after 13 d cultivation, inoculated in LBM containing 0.1% gas oil, and incubated for another 20 d. Degradation profiles of the gas oil are shown by ○, strain B23 and □, strain H41, respectively.

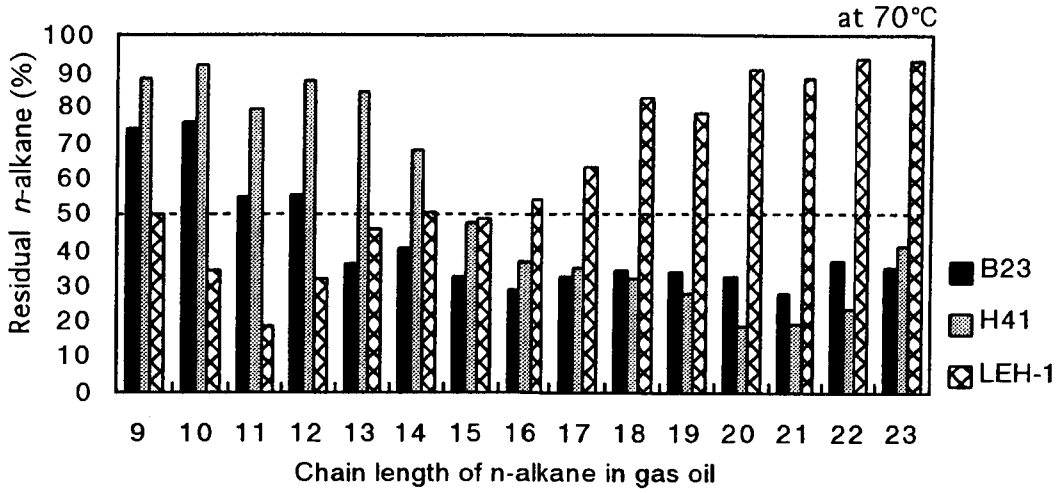


**Fig. 3-4.** Degradation activity of strains B23, H41, and LEH-1$^{TS}$ for alkanes with various carbon chain lengths. After cultivation in basal salts medium LBM supplemented with 0.1% standard gas oil for 20 d, residual *n*-alkane was extracted with hexane and quantified by GC/FID analysis. Residual *n*-alkanes represent the amount of alkanes from the bottles containing cells relative to that containing no cells.

### 3.3.7 Alkane degradation pathway of strains B23 and H41

To obtain information on the alkane degradation pathway of *B. thermoleovorans*, I identified the metabolites of alkane in the strains B23 and H41. Fatty acid derivatives of the same carbon chain length as the substrate alkane and fatty acid derivatives shorter than the substrate by two carbon units were specifically accumulated in the cells (Fig. 3-5). When the strains were grown in the presence of heptadecane, heptadecanoate and pentadecanoate was accumulated in both strains. Similarly, heptadecanoate and pentadecanoate accumulated when the strains were grown in the presence of heptadecane. These results strongly suggest that, like *Pseudomonas oleovorans* (van Beilen et al. 1994), strains B23 and H41 degrade alkanes by a terminal oxidation pathway, followed by β-oxidation pathway.

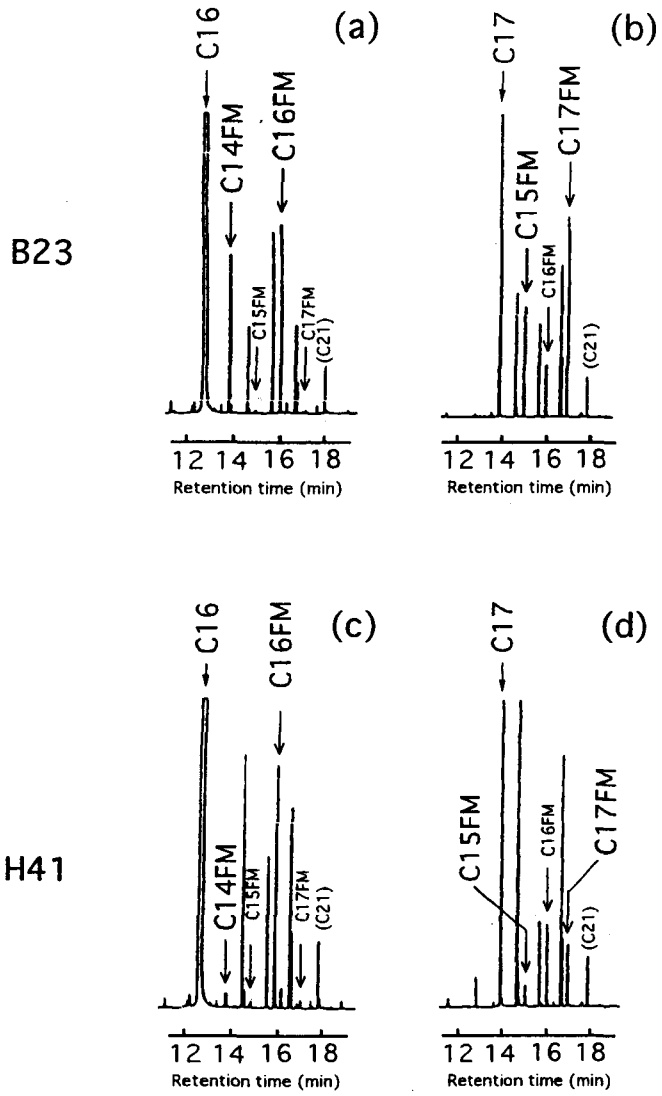


**Fig. 3-5.** GC/FID analyses of metabolic intermediates. Hexadecane (C16) or heptadecane (C17) was used as a substrate (alkane) for degradation by strains B23 and H41. The strain and the substrate *n*-alkane are B23 and C16 (a), B23 and C17 (b), H41 and C16 (c), and H41 and C17 (d), respectively. Symbols are as follows; C16, hexadecane; C17, heptadecane; C14FM, tetradecanoic acid methyl ester; C15FM, pentadecanoic acid methyl ester; C16FM, hexadecanoic acid methyl ester; C17FM, heptadecanoic acid methyl ester; (C21), internal standard heneicosane.

### 3.3.8. Cloning of an alcohol dehydrogenase gene (*bt-adh*) from the strain B23

Since a terminal oxidation pathways for *n*-alkane degradation were suggested for the strains B23 and H41, I tried to clone the gene encoding an enzyme responsible for the pathway. I tried a numbers of PCR with various kinds of oligonucleotide primers to amplify the gene homologous to alkane monooxygenase gene (*alkB*) or rubredoxin reductase gene (*alkT*) of *P. oleovorans* (EMBL/GenBank/DDBJ accession number X65936). However, no significantly amplified band was obtained. Then, I adopted a shotgun method for the cloning of an alcohol dehydrogenase gene. Finally, the entire gene

(*Bt-adh*) encoding a long-chain-alcohol dependent alcohol dehydrogenase (Bt-Adh) was cloned from strain B23. The gene *Bt-adh* encodes an open reading frame with 249 amino acid residues and its calculated molecular mass was 27,196 Da. A tyrosine residue in the active site and the glycine-rich sequence (GGXXGI/LG) constituting the probable nicotinamide adenine dinucleotide ($NAD^+$) or nicotinamide adenine dinucleotide phosphate ($NADP^+$) binding site were completely conserved in the Bt-Adh sequence at positions at 155 and 11, respectively (Neidle et al. 1992, Okibe et al. 1999). A phylogenetic tree was constructed based on the multiple alignment of the amino acid sequences of bacterial Adhs including Bt-Adh (Fig. 3-6, Table 3-3). The Adh superfamily can be divided into three groups: Group I, zinc-dependent long-chain Adhs; Gourp II, zinc-independent short chain Adhs; Group III, iron-activated Adhs (Reid et al. 1994). Group I and group II Adhs are characterized by a larger (ca. 350 residues per subunit, long chain) and smaller (ca. 250 residues per subunit, short chain) sizes, respectively. The subunit sizes of group III Adhs are about 385 residues. Bt-Adh (BTADH in Fig. 3-6) belongs to the Group II, and shows the highest sequence similarity to a hypothetical oxidoreductase (Q00593) from a hyperthermophilic strain *Thermotoga maritima* (48% identity). This protein Q00593 should be a dehydrogenase/reductase rather than an oxidoreductase on account of its potential $NAD^+/NADP^+$ binding site. Other than this protein, the T. maritima genome contains genes encoding three possible zinc-dependent type Adhs (Group I) and two possible iron-dependent ones (Group III) (Nelson et al. 1999). Although several species of *Thermotoga* have been isolated from a hot petroleum reservoir, no *n*-alkane degradation activity was reported for them (Grassia et al. 1996). Because *Bt-adh* conferred 1-tetradecanol dehydrogenase activity on *E. coli* cells, it is highly probable that *Bt*-ADH is involved in the *n*-alkane oxidation pathway of strain B23. The alcohol dehydrogenase gene (*alkJ*) in the *alk* operon of *P. oleovorans* encodes POADH which, unlike *Bt*-ADH, is a Group III enzyme. The amino acid sequences of other enzymes responsible for *n*-alkane oxidation in *B. thermoleovorans* might also be considerably different from those of *P. oleovorans*. Further experiments, such as *Bt-adh* gene disruption in the strain B23 and enzymatic characterization of Bt-ADH, are necessary to elucidate the physiological functions of Bt-ADH in the cell. Attempts to overproduce Bt-ADH in *E. coli* cells in a functional form have so far been unsuccessful, probably because recombinant Bt-ADH folds incorrectly upon overproduction or it requires an additional factor for activity. Comparison of the primary structures of long-chain alcohol dehydrogenases from *A. calcoaceticus* and *B. thermoleovorans* would be also informative for considering the common and dissimilar features that determine the substrate preference of the enzymes for long-chain alcohol.

## 3.4 Summary

Two extremely thermophilic alkane-degrading bacterial strains, B23 and H41, were respectively isolated from deep subterranean petroleum reservoirs in the Minami-aga (Niigata, Japan) and Yabase (Akita, Japan) oil fields. Both strains were able to grow at temperatures ranging from 50 to 80℃, with optimal growth at 70℃ for B23 and 65℃ for H41. From 16S rRNA gene sequence analysis and physiological characterization, both strains were identified as *Bacillus thermoleovorans* (identities of 99.5% and 99.6% to strain DSM 5366, and 98.3% and 98.7% to the type strain LEH-1 $^{TS}$, respectively). Strains B23 and H41 effectively (>60%) degraded *n*-alkanes longer than C12 and C15, respectively, at 70°C, while strain LEH-1$^{TS}$ degraded undecane (C11) most effectively. When B23 and H41 were cultivated in the presence of heptadecane, heptadecanoate and pentadecanoate were specifically accumulated in the cells. These results strongly suggest that the two strains degraded *n*-alkanes by a terminal oxidation pathway, followed by a β−oxidation pathway. In order to support above speculation a long-chain-alcohol dehydrogenase gene was cloned from the strain B23 The gene conferred 1-tetradecanol dehydrogenase activity on *Escherichia coli* cells. Bt ADH is camposed 249 amino acid residues and the calculated molecular mass is 27,196 Da. This result is in agreement with a terminal oxidation pathway.

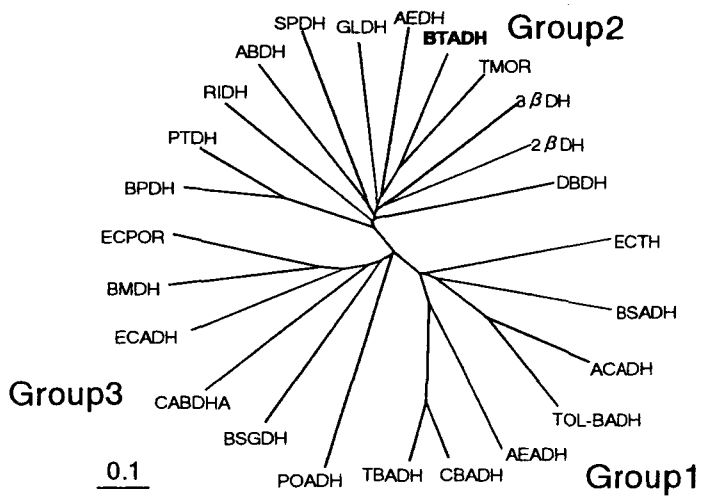


**Fig. 3-6.** A phylogenetic tree derived from amino acid sequences of several bacterial ADHs.
The sequences used for these analyses are listed in Table 3-3. The distance of the branch from a diverging point is proportional to the frequency on average of the amino acid substitution at each position. The scale bar corresponds to this frequency of 0.1.

**Table 3-3.** List of bacterial ADHs usend in the phylogenetic tree analysis.

| Group | Name | Enzyme | Organism | Accession |
|---|---|---|---|---|
| 1 | ACADH | Benzyl alcohol dehydrogenase | *Acinetobacter calcoaceticus* | U61983 |
| | AEADH | Fermentative alcohol dehydro genase | *Alcaligenes eutrophus* | J03362 |
| | BSADH | Alcohol dehydrogenase | *B. stearothermophilus* | D90421 |
| | CBADH | Alcohol dehydrogenase | *Clostridium beijerinckii* | AF157307 |
| | ECTH | L-Threonine dehydrogenase | *E. coli* | P07913 |
| | TOL-BADH | Benzyl alcohol dehydrogenase | *P. putida* (pWW0) | M94184 |
| | TBADH | Secondary alcohol dehydrogenase | *Thermoanaerobium brockii* | X64841 |
| 2 | ABDH | 1,2-Dihydroxycyclohexa-3,5-diene carboxylate dehydrogenase | *Ac. calcoaceticus* | M64747 |
| | AEDH | Acetoacetyl-CoA reductase | *Al. eutrophus* | J04987 |
| | GLDH | Glucose dehyd rogenase | *B. megaterium* | A07355 |
| | BTADH | Alcohol dehydrogenase | *B. thermoloevorans* | AB040809 |
| | DBDH | 2,3-Dihydro-2,3-dihydroxybenzoate dehydorogenase | *E. coli* | M24148 |
| | SPDH | Glucitol-6-phosphate dehydrogenase | *E. coli* | J02708 |
| | RIDH | Ribitol dehydrogenase | *Klebsiella aerogenes* | M25606 |
| | BPDH | Biphenyl dihydrodiol dehydrogenase | *P. pseudoalcaligenes* | Y07655 |
| | PTDH | *Cis*-Toluene dihydrodiol dehydro-genase | *P. putida* | J04996 |
| | 3$\beta$DH | 3$\beta$-Hydroxysteroid dehydrogenase | *P. testosteroni* | L08971 |
| | 2$\beta$DH | 20$\beta$-Hydroxysteroid dehydrogenase | *Streptomyces hydrogenans* | P19992 |
| | TMOR | Hypothetical oxidoreductase | *Thermotoga maritima* | Q56318 |
| 3 | BMDH | Methanol dehydrogenas | *B. methanolicus* | M65004 |
| | BSGDH | Glycerol dehydrogenase | *B. stearothermophilus* | M65289 |
| | CABDHA | Butanol dehydrogenase A | *C. acetobutylicum* | M96945 |
| | ECADH | Alcohol dehydrogenase | *E. coli* | M33504 |
| | ECPOR | Propanediol dehydrogenase | *E. coli* | M27177 |
| | POADH | Alcohol dehydrogenase | *P. oleovorans* | Q00593 |

# CHAPTER 4

## *Bacillus thermoleovorans* B23 shares alkane degradation pathway with eukaryotes

### 4.1 Introduction

Degradation of xenobiotics including petroleum hydrocarbons by thermophilic bacteria provides crucial advantages over that by mesophilic or psychrophilic bacteria, especially when they are applied in biotechnological processes. Limited biodegradation of hydrophobic substrates as a result of their low water solubility may be overcome at elevated temparatures because their water solubility increases. I have recently isolated an extremely thermophilic alkane-degrading bacterium, *Bacillus thermoleovorans* B23, from a deep petroleum reservoir in Japan (described in chapter 2). The strain B23 effectively degraded alkanes at 70°C with the carbon chain length of more than twelve. Since tetradecanoate and hexadecanoate or pentadecanoate and heptadecanoate were specifically accumulated as degradation products of hexadecane or heptadecane, respectively, it was indicated that the strain degraded alkanes by a terminal oxidation pathway, followed by $\beta$-oxidation pathway.

Besides their biotechnological importance, extremely thermophilic microorganisms show an interesting feature in the study on evolution of life. Microorganisms which live under extremely high temperature condition, such as hyperthermophilic archaea and hyperthermophilic bacteria, share the mechanisms for maintaining the cells with not only bacteria but also eukaryotes (Gogarten et al. 1989, Rashid et al. 1995). This is consistent with a proposal, based on a phylogenetic analysis of 16S rRNA gene, that hyperthermophiles are very primitive and the common ancestor of living organisms might be a kind of hyperthermophiles (Stetter et al. 1996). Extremely thermophilic bacteria, that survive and grow under deep subterranean environment, also might be able to provide some information on this matter because the condition at subsurface is regarded to be more stable than surface. Although alkane degradation is not a central pathway to maintain the cells, it would be informative to compare the pathway of thermophilic bacteria with those of mesophilic bacteria and eukaryotes. Since most of the studies on the alkane degradation pathway have been made on mesophilic microorganisms, such as *Pseudomonas oleovorans* (Canosa et al. 2000), *Acinetobacter* sp. (Ratajczak et al. 1998), *Candida tropicalis* (Kanai et al. 2000), and *Yarrowia lipolitica* (Wang et al. 1999), I decided to study on the alkane metabolisms of extremely thermophilic bacteria.

Here, I report that superoxide dismutase, catalase, and acyl-CoA oxidase activities were dramatically increased in the cells of *B. thermoleovorans* B23 when they were grown on alkanes. Induction of above enzymatic activities associated with alkane degradation has never been reported for bacteria but reported for yeast, such as *Candida tropicalis* (Shimizu et al. 1979, Teranishi et al. 1974). This result suggests that alkane degradation pathway is at least partly shared by eukaryotes and deep-subsurface

thermophilic bacteria.

## 4.2 Material and Methods

### 4.2.1 Cells and plasmids

An extremely thermophilic alkane-degrading bacterium, *Bacillus thermoleovorans* B23 was previously isolated from a deep petroleum reservoir in Minami-aga oil field (Niigata, Japan, described in chapter 3). *B. thermoleovorans* type strain LEH-1 (ATCC43513) was purchased from ATCC (American Type Culture Collection, Manassas, VA, Zarilla et al. 1987) and used as a comparative strain. *E. coli* DH5 $\alpha$ was used as a host strain for the gene cloning with a cloning vector pCR2.1 (Invitrogen Corp., San Diego, CA). *E. coli* strain XL1-Blue MRA (P2) was used as a host strain for construction of a phage library of B23 genome.

### 4.2.2 Culture media

Nutrient L-broth contained (per liter) 5 g of yeast extract (Difco, Detroit, MI), 10 g of Bacto-tryptone (Difco), and 5 g of NaCl (pH 7.2) was used for cultivation and storage of the strains. Cells were aerobically grown in a screw capped culture bottle containing medium without shaking at 70°C or 60°C for B23 and LEH-1, respectively. Solid medium was prepared by adding 1.5 % agar or 4% gellan gum (Wako Pure Chemicals, Osaka, Japan). Mineral salts medium, LBM (Maniatis et al. 1982), was used for alkane degradation and protein induction experiments. LBM contained per liter; 0.25 g $NaNO_3$, 0.25 g $NH_4Cl$, 0.21 g $Na_2HPO_4$, 0.20 g $MgSO_4$-$7H_2O$, 0.09 g $NaH_2PO_4$, 0.04 g KCl, 0.02 g $CaCl_2$, 1 mg $FeSO_4$, 10 ml Trace mineral solution. Trace mineral solution contained per liter; 7 mg $ZnSO_4$-$7H_2O$, 1 mg $H_3BO_4$, 1 mg $MoO_3$, 0.5 mg $CuSO_4$-$5H_2O$, 18 μg $CoSO_4$-$7H_2O$, 7 μg $MnSO_4$-$5H_2O$. Otherwise mentioned, LBM was supplemented with 1 ml single alkane or alkanes mixture (Standard gas oil, sulfur content 500 ppm level, Tokyo Chemical Industry, Tokyo, Japan). Alkanes used for cultivation substrate were filtrated (Millex-FG filter, pore size 0.2 μm, Millipore, Molsheim, France) for sterilization before use.

### 4.2.3 Scanning electron microscopy

Cells before and after growing on alkanes were washed with 0.1 M $K_2HPO_4$/$KH_2PO_4$ buffer (pH 7.2) and fixed with 2.5% glutaraldehyde in the same buffer for more than 2 h. Then, the cells were washed again with the buffer and dehydrated in acetone. After freeze-drying, specimens were coated with gold-palladium and observed with a Hitachi S-4700 scanning electron microscope (Hitachi, Tokyo, Japan).

### 4.2.4 Induction of protein productions by alkanes

After aerobic cultivation in 1L L-broth at 70°C for 24 h, B23 cells were harvested by centrifugation at 8,000 g for 10 min at 4°C and then washed with LBM. The cell pellet was suspended in 1L LBM, which contained 0.1 % (v/v) of alkane or standard gas oil. Bottles containing the suspension were closed tightly and incubated at 70°C for

appropriate periods without shaking. In order to minimize adsorption of alkanes to the teflon seal inside the cap, bottles were put upside down in the incubator when needed. Proteins induced by alkanes were analyzed by conventional SDS-12% polyacrylamide gel electrophoresis (SDS-PAGE, Laemmli et al. 1970).

### 4.2.5 Amino acid sequence determination

The N-terminal amino acid sequences of the proteins were determined with a sequencing system Procise 491 (Applied Biosystems Japan, Tokyo, Japan). Samples were prepared by electro blotting the protein band from SDS-polyacrylamide gel onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA). Electroblotting was conducted at 50mA for 1.5 h in a transfer buffer (10 mM CAPS, pH 11) containing 10 % (v/v) methanol. Proteins were stained on the PVDF membrane with 0.1 % Coomassie Brilliant Blue R in 40 % (v/v) ethanol and 1 % (v/v) acetic acid and destained for 1 min in 50 % (v/v) ethanol. A strip of the membrane containing protein band was cut into pieces and subjected to the amino acid sequence analysis.

The internal amino acid sequences were dertermind as follows. The proteins were prepared by slicing the SDS-PAGE gel and eluting out the protein by vortexing with 20 mM Tris-HCl (pH 8.0) containing 1 % SDS overnight. After digestion of the protein with lysyl endopeptidase (LEP) under standard condition (Hirano et al. 1998), each peptide fragment was fractionated by reverse phase HPLC (column: AQUAPORE RP300, 4.6×250 mm, Applied Biosystems Japan, Tokyo, Japan) and its N-terminal amino acid sequences was determined.

### 4.2.6 Construction of B23 genomic DNA library

Total genomic DNA of *B. thermoleovorans* B23 was prepared as described previously (Imanaka et al. 1981) and was partially digested with *Sau*3AI. Then the DNA fragments were ligated with phage vector Lambda EMBL3 by using Lambda EMBL3/*Bam*HI arm (Promega, Madison, WI) and packaged *in vitro* by Maxplax packaging extract kit (Epicentre Technologies, Madison, WI).

### 4.2.7 Cloning of Bt-SOD gene

Partial SOD gene was amplified by utilizing GeneAmp PCR System 2400 (Applied Biosystems Japan) with AmpliTaq DNA polymerase (Takara shuzo, Kyoto, Japan). The PCR amplification primers used were designed based on N-terminal amino acid sequence determined in this work and a sequence of consensus region (Mn dependent type SOD of *B. stearothermophilus* 193-VAKRYSEA-200, P00449), respectively. InstaGene Matrix (Bio-Rad) was used for preparation of genomic DNA .

In order to clone the entire SOD gene, inverse PCR method was adopted. Genomic DNA, which had been previously digested with *Sph*I (for subcloning of 5'-end) or *Acc*III (for subcloning of 3'-end) was self-ligated and used for template DNA. For the analysis of DNA fragments, agarose gel electrophoresis was performed under standard condition (Maniatis et al. 1982). GeneClean kit (Bio 101, La Jolla, CA) was used to recover DNA fragment from agarose gel slices. The PCR amplified gene fragment was

ligated independently into the cloning vector pCR2.1 (Invitrogen Corp.), with TA cloning kit (Invitrogen Corp.), and used for transformation of *E. coli* DH5$\alpha$. Nucleotide sequence of the gene was determined by using ABI PRISM 310 genetic analyzer (Applied Biosystems Japan).

### 4.2.8 Cloning of the P21 and P16

In order to clone the genes encoding P21 and P16, several kinds of their internal amino acid sequences were determined as follows. The proteins were prepared by slicing the SDS-PAGE gel and eluting out the protein by vortexing with 20 mM Tris-HCl (pH 8.0) containing 1 % SDS overnight. After digestion of the protein with lysyl endopeptidase (LEP) under standard condition (Hirano et al. 1998), each peptide fragment was fractionated by reverse phase HPLC (column: AQUAPORE RP300, 4.6× 250 mm, Applied Biosystems Japan, Tokyo, Japan) and its N-terminal amino acid sequences was determined. Based on these amino acid sequences, PCR primers were constructed to amplify the target gene loci. A part of the gene encoding P21 was amplified by PCR with primers designed for N-terminal amino acid sequence, AFPLSGVGGFTISADLI, and one of the internal amino acid sequences, PSLNTHYMSAGSITIPSMK (P21-37). The nucleotide sequence of this gene fragment has been submitted to EMBL/GenBank/DDBJ under accession number AB047106.

A part of the gene encoding P16 was amplified by, what I call, armed-PCR method using lambda EMBL3-B23 genomic DNA library as template DNA. The PCR amplification primers were designed for right arm of EMBL3 vector (5'-CGTCCGAGAATAACGAGTGGATC-3') and one of the internal amino acid sequences, AAQEFQTGADNITIDNGN (P16-16). The PCR amplified DNA fragments (1.8 kb) were ligated into the cloning vector pCR2.1. The complete nucleotide sequence was determined and found that the DNA fragment encodes majority of the P16 gene, including 5'-end. The nucleotide sequence of this gene fragment has been submitted to EMBL/GenBank/DDBJ under accession number AB049820.

### 4.2.9 Northern hybridization and RT-PCR

Cultures were taken from a bottle after 0, 4, and 10 days cultivation in the presence of alkanes. The cells were collected by centrifugation and frozen on dry ice. Total RNA was isolated at the same time by the method of Ausubel *et al.* (Ausubel et al. 1995). For Northern blot analysis, 20 μg each of total RNA was electrophoresed on 1 % agarose gel containing formaldehyde as a denaturant. The RNA band was blotted onto a Hybond $N^+$ membrane (Amersham Pharmacia Biotech) by using Transblot cell (Bio-Rad) under standard protocol. The PCR amplified 416 bp and 1.8 kb DNA fragments were used for detecting the mRNA of P21 or P16, respectively. Labeling the probe DNA, hybridization to the target mRNA, and detection of signals were performed by using Gene Images AlkPhos direct labeling and detection system (Amersham Pharmacia Biotech) under a standard protocol.

In order to analyze the transcription level of P16 gene, RT-PCR method was also adopted by using QIAGEN OneStep RT-PCR Kit (QIAGEN). Ten micrograms of total

RNA sample was used as the initial template for RT-PCR in each case.

### 4.2.10 Activity staining of superoxide dismutase (SOD)

Cell free extracts were prepared as follows; cells after cultivation in LBM supplemented with or without alkanes were washed and suspended with 50mM K-phosphate buffer (pH 7.8), and then disrupted by sonication in ice bath. Degree of cell disruption was monitored by microscopic observation at appropriate time interval. After a centrifugation at 15,000 g for 30 min (4°C), the resulting supernatant was subjected to gel electrophoresis using 7.5 % non-denaturing polyacrylamide gel (pH 7.5)(Laemmli 1970). Then, the SOD activity was detected by negative staining method by utilizing nitroblue tetrazolium (Beauchamp et al. 1971).

### 4.2.11 Activity staining of catalase

Cell free extracts were prepared and subjected to gel electrophoresis as mentioned above. Then, the gel was rinsed for 15 min three times with distilled water, soaked in a solution of 0.01 ml of 30% $H_2O_2$ in 100 ml water, and gently shaken for 10 min. The $H_2O_2$ solution was discarded and the gel was immediately rinsed with distilled water. A freshly prepared mixture of 30 ml each of 2 % ferric chloride and 2 % potassium ferricyanide was poured onto the gel for staining. The gel tray was gently but steadily rocked by hand over a light box. As soon as green color began to appear in the gel background, the ferricyanide mixture was rapidly removed and the gel was washed twice with water to terminate the coloring reaction (Wayne et al. 1986).

### 4.2.12 Measurement of oxidase activity

Oxidase activity was assayed by the method of Shimizu *et al.* (Shimizu et al. 1979). The reaction mixture contained in 0.4 ml of 50 mM potassium phosphate buffer (pH 7.4), 0.33 μmol 4-aminoantipyrine, 4.24 μmol phenol, 0.004 μmol $FAD^+$, 0.04 μmol substrate, 12 IU horseradish peroxidase (Sigma), and 0.1-0.2 mg cell free extract. The reaction was carried out at 70°C, and the production of $H_2O_2$ was determined by measuring the increase in absorbance at 500 nm. The molecular extinction coefficient was 6.39 /cm/μmol at pH 7.4. Tetradecane, tetradecanol, tetradecanal and tetradecanoyl-CoA were used as the substrates for assaying this enzyme activity.

## 4.3 Results and Discussion

### 4.3.1 Microscopic observations

The shape of *B. thermoleovorans* B23 cells before and after cultivation in the presence of alkanes was compared with each other by a scanning electron microscope (Fig. 4-1a, b). It was found that the cells became longer and thicker after two-week growth on alkanes. No such elongation was observed for the cells grown for 14 days in the absence of alkanes (picture not shown). This dynamic change of cell shape prompted me to analyze the cellular proteins produced in relation to alkane degradation.

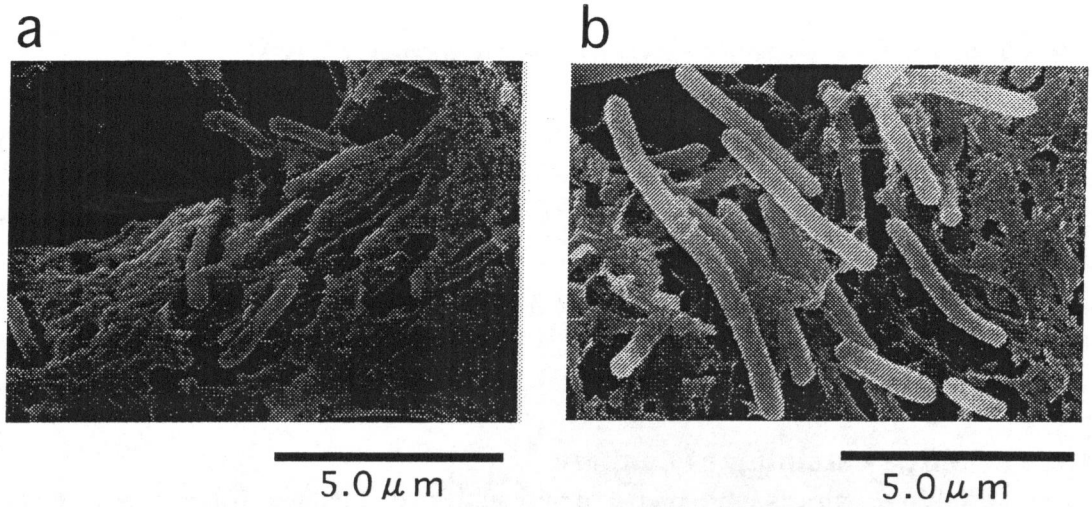


**Fig. 4-1.** Scanning electron micrographs of the strain B23 cells before (a) and after (b) cultivation on LBM supplemented with 0.1 % (v/v) alkanes. Cultivation time was 14 days. Background of the cells is cellulose fiber of filter paper on which cells are adsorbed and fixed.

### 4.3.2 Induction of protein productions by *n*-alkanes

Comparative analysis of proteins by SDS-PAGE showed that production levels of at least three kinds of proteins with relatively small size were dramatically increased after 10-day cultivation on alkanes (Fig. 4-2a). These were 24kDa, 21kDa and 16kDa proteins, which were designated as P24, P21 and P16, respectively. Although a protein band at 40kDa (P40) also seems to increase in Fig. 2a, reappearance of this phenomenon was not high (see Fig. 4-3) and then no further work was performed for P40. When alkanes were omitted from the medium, the production levels of P24, P21, and P16 remained low. It is interesting that the increase in the production level of these three proteins became significant at the time when alkane degradation was started (Fig. 4-2b). None of the production levels of the proteins were increased in the presence of palmitic acid, tributyrin, trimyristin, or dicyclopropylketone (DCPK) which is an inducer of *alk* gene expression in *P. oleovorans*.

The effect of carbon chain length of alkanes on the induction levels of the proteins was examined. It is obvious that the induction effect increases in accordance with the chain length of alkanes tested (Fig. 4-3). It has previously been shown that strain B23 effectively degrade alkanes longer than dodecane (described in chapter 2). These results strongly suggest that P24, P21, and P16 are responsible for the long-chain-alkane degradation by strain B23, otherwise the protein production was stimulated in the consequence of alkane degradation. No such induction of SOD production by alkane has ever been reported for other alkane degrading bacteria including *B. thermoleovorans* LEH-1 (Zarilla et al. 1987). Localization of the proteins in the cells was examined (Fig. 4-4). Because P24 was recovered in a soluble form after disruption of the cells, this protein is probably a cytoplasmic protein. On the other hand, P21 and P16 were recovered in an insoluble form, suggesting that they are membrane proteins.

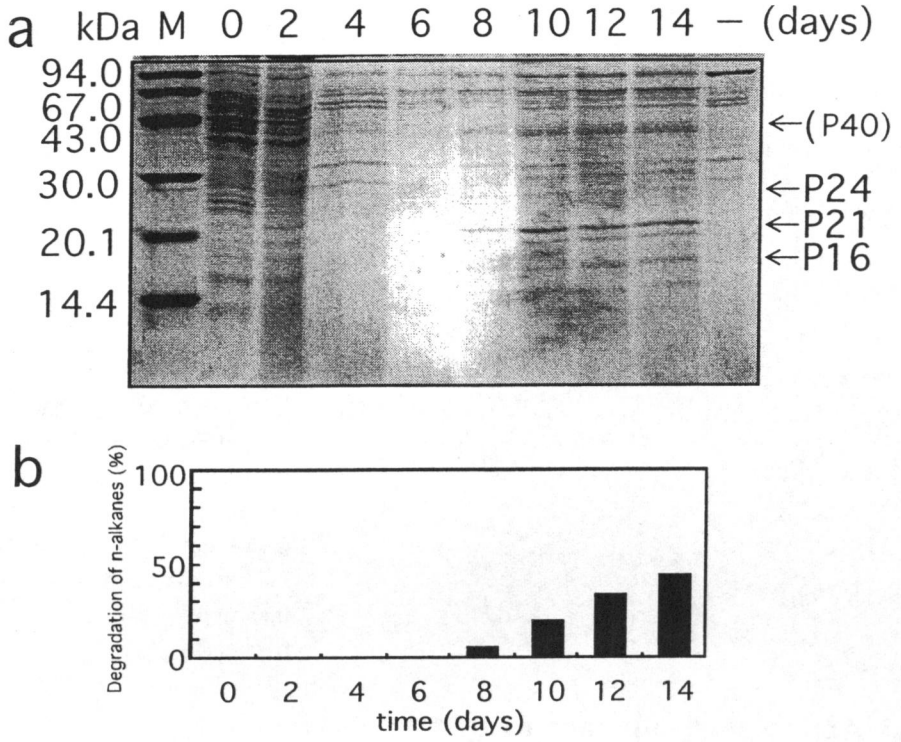


**Fig. 4-2.** a, Induction of P24, P21 and P16 productions in *B. thermoleovorans* B23. b, Degradation of *n*-alkanes by strain B23. Cells were cultivated in LBM supplemented with 0.1% alkanes (V/V) for 14 days. Total cell fractions were loaded on an SDS-12% polyacrylamide gel. Protein bands were stained with Coomassie Brilliant Blue R-250. Lanes are M, molecular size marker; 2, 2days; 4, 4 days; 6, 6 days; 8, 8 days; 10, 10 days; 12, 12 days; 14, 14 days cultivation; -, 14 days cultivation in the absence of alkanes. The degradation of alkanes was quantified by GC/FID.

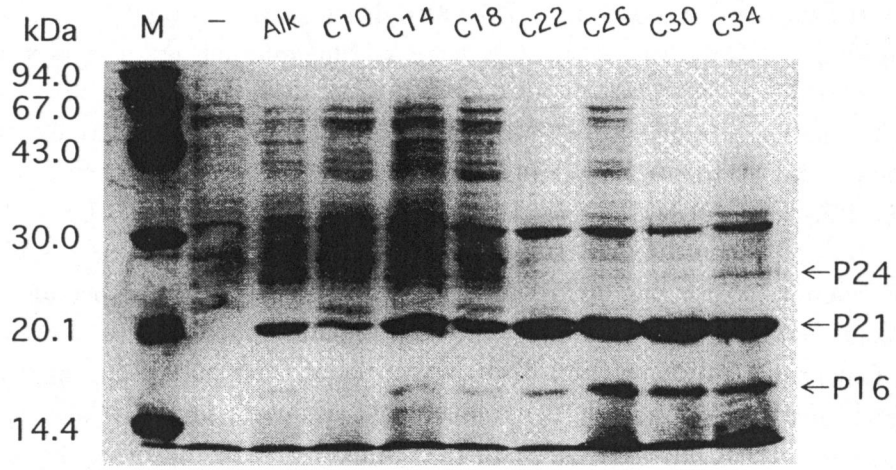


**Fig. 4-3.** Effect of single alkane with various carbon chain length on the induction of P24, P21 and P16 production. Protein was electrophoresed on an SDS-12% polyacrylamide gel and stained with Coomassie Brilliant Blue R-250. Lanes are M, molecular size marker; -, in the absence of alkanes; Alk, in the presence of alkanes; C10, tetradecane; C14, tetradecane; C18, octadecane; C22, docosane; C26, hexacosane; C30, triacontane; C34, tetracontane.

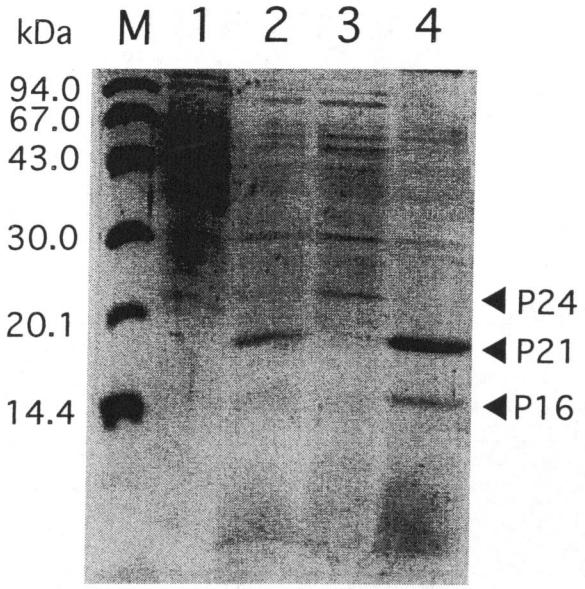


**Fig. 4-4.** Localization of P24, P21, and P16 in the cells. Lanes are M, molecular weight markers; 1, whole cell fraction cultivated in the absence of alkanes; 2, whole cell fraction cultivated in the presence of alkanes. After disrupting the cells (lane 2) by sonication, soluble (lane 3) and insoluble fractions (lane 4) were fractionated.

### 4.3.3 Amino acid sequence of P24 and P16

The amino acid sequences of P21 and P16 were determined as AFPLSGVGGFTISADLI (P21-N) and VPISGVGEFXVTFDKL (P16-N), respectively. These sequences, which are highly similar with each other, showed considerable similarity with that of cholesterol esterase from *Streptomyces lavendulae* (Fig. 4-5a, Nishimura et al. 1994). Cholesterol esterase is a secretion enzyme which hydrolyzes long-chain fatty acid esters of cholesterol mainly in mammalian tissues. In bacteria, *P. fluorescens* (Uwajima et al. 1976) and *S. lavendulae* are reported to produce this enzyme.

### 4.3.4 Cloning and analysis of P21 and P16 gene

Utilizing the information of N-terminal and internal amino acid sequences, 416 bp and 1.8 kb DNA fragments encoding a part of P21 and P16, respectively, were cloned and their nucleotide sequence was completely determined. The 416 bp fragment was confirmed to encode a part of p21 protein, because other internal amino acid sequences IDVVVTGGNVSGQNLK (P21-14) and TDNPLNVIDLSAPSLTLDK (P21-20) were found to be encoded in the gene fragment. P16 was suggested to be a secretion protein which is anchored to the cell membrane, because a possible signal peptide sequence (45 amino acid residues) was found between the initiation methionine and the N-terminal valine of mature P16 protein (Fig. 4-5b). As a result, 138 and 125 amino acid residues were determined for P21 and P16, respectively. Although cholesterol esterase from *S. lavendulae* showed relatively high sequence similarity with the N-terminal sequence of P21 and P16, the similarity score decreased when it compared to the deduced 138 and 125 amino acid residues (21.7 and 23.2%, respectively). This suggests that P21 and P16 would not be cholesterol esterase but unique membrane proteins probably responsible for the alkane uptake or degradation in *B. thermoleovorans* cells.

**a**

```
P21   1AFPLSGVGGFTISADK16
P16   1V-PISGVGEFTVQFDK15
CES   1SFSVSG-QDFKVSADK15
```

**b**

```
   1 GGAATGTATAATTGTCATAAAATTTAAACAGATTCACTAAAGCGCTTACAGCGCTGATCA        60

  61 TTCAAAAAAAGGGGGA GGAGA AACGCCATGGAAATGGCAACAAAACCGGTCATCATTGGG      120
   1                  SD     M  E  M  A  T  K  P  V  I  I  G           30

 121 GGGCGCACGGCGAAAAAGCCGCTGATCGCCGCACTGTTGGGAGGGTTCCTGCTTCTCGGA      180
  31  G  R  T  A  K  K  P  L  I  A  A  L  L  G  G  F  L  L  L  G       50

 181 GCCTTGCTGTCGATCTTCGGGCTGACAGGCGTCGCCTATGCCGTACCAATCAGCGGGGTC      240
  51  A  L  L  S  I  F  G  L  T  G  V  A  Y  A  V  P  I  S  G  V       70
                                                 P16-N
 241 GGCGAATTTACCGTTCAATTTGACAAACTGAATGGCAGCGGGTTTAAAATGTACGGCGGC      300
  71  G  E  F  T  V  Q  F  D  K  L  N  G  S  G  F  K  M  Y  G  G       90
                                                       P16-18
 301 GTGGCGGAAGCAGGCAATGCGCCGCAGACGCCGGTCTTCGTCAATGAAATGGATAAAGCG      360
  91  V  A  E  A  G  N  A  P  Q  T  P  V  F  V  N  E  M  D  K  A      110

 361 ACGATTCAAGGTCTTCGGATTTCGAAAGACTTCCCGGCCTTGGGCATTCGCGTCGTCATT      420
 111  T  I  Q  G  L  R  I  S  K  D  F  P  A  L  G  I  R  V  V  I      130
                                                          P16-23
 421 GTCGCGAGCGAGCCTGTCTCCATTGACGGCCTCGTGCAAAAAGCGACGCAAATCAACGGC      480
 131  V  A  S  E  P  V  S  I  D  G  L  V  Q  K  A  T  Q  I  N  G      150
                                                       P16-20
 481 AACATCTCCTTCGGCAGCTTGACGATGAAAGAAAACTACGTCGGCGATGTACAAGACCCG      540
 151  N  I  S  F  G  S  L  T  M  K  E  N  Y  V  G  D  V  Q  D  P      170
                                                             P16-15
 541 GTCCAAAAAGCAGCGCAAGAGTTCACCCAAGGGGCCGACAA                        583
 171  V  Q  K  A  A  Q  E  F  T  Q  G  A  D  N                       184
                  P16-16
```

**Fig. 4-5.** a, Comparison of the N-terminal amino acid sequneces of P21, P16, and cholesterol esterase from *S. lavenuae* (CES). Common amino acid residues are surrounded by square. b, Partial nucleotide sequence and deduced amino acid sequence of P16 with upstream region. SD, Shine-Dalgarno sequence. A putative signal peptide sequence was shaded. The amino acid sequences determined by peptide sequencer were underlined.

### 4.3.5 Expression level of mRNA of P21 and P16

Because P21 and P16, which are functionally unknown, were suggested to be membrane proteins, significant increase of these protein bands after 10 to 14-day cultivation on alkanes might be due to their excellent stability in the cell membrane. In order to eliminate this possibility, the induction of P21 and P16 production was examined at mRNA level. The results, which were obtained from Northern blotting experiment of P21, are shown in Fig. 4-6a. A strong signal was detected at an expected position of approximately 700 bases when RNA sample was prepared from the cells after 10-day culture. On the other hand, the probe DNA constructed for detecting mRNA of P16 hybridized with neither of RNA samples prepared. Then, RT-PCR method was adopted in this case, which is reported $10^6$ times more sensitive than Northern hybridization method (Lehrach et al. 1977). When the template RNA was prepared from 10-day culture, DNA fragment of expected size, ca. 500 bp, was clearly amplified by RT-PCR. The amplified DNA fragment was confirmed to be a part of P16 gene by determining the nucleotide sequence. No DNA amplification was observed for RNA samples prepared from 0 and 4-day culture (Fig. 4-6b).

These results strongly suggest that the productions of P21 and P16 were induced by alkanes at a transcription level. Moreover, the genes encoding P21 and P16 did not seem to form an operon and were independently transcribed. Because fatty acid, triacyl glycerol, and paraquot were no efficient inducer of P21 and P16 production, it is plausible that alkane molecules directly or indirectly affect the transcriptional regulation of P21 and P16 genes.

### 4.3.6 Amino acid sequence of P24

The N-terminal amino acid sequence of P24 was determined to be PFELPALPYPYDALEP (P24-N). According to the result obtained by the BLAST search program (http://www.ncbi.nlm.nih.gov/BLAST/, NCBI, Bethesda, MD), this sequence was completely matched with that of superoxide dismutase (SOD) from *B. stearothermophilus* and *B. caldotenax* strains. Cloning and sequencing of the entire gene encoding P24 revealed that it is a Mn-dependent type SOD of 204 amino acid residues, and showed 99.0 % identical to Mn-SOD of *B. stearothermophilus* (EMBL/GeneBank/DDBJ; P00449) or *B. caldotenax* (P28760). The amino acid residues responsible for Mn binding, 76-GGXXXHXXE-84 and 49-QD-50, were completely conserved in the sequence of P24. The nucleotide and amino acid sequence of P24, Mn-SOD of strain B23, has been deposited in the EMBL/GeneBank/DDBJ under accession number BAA95631.

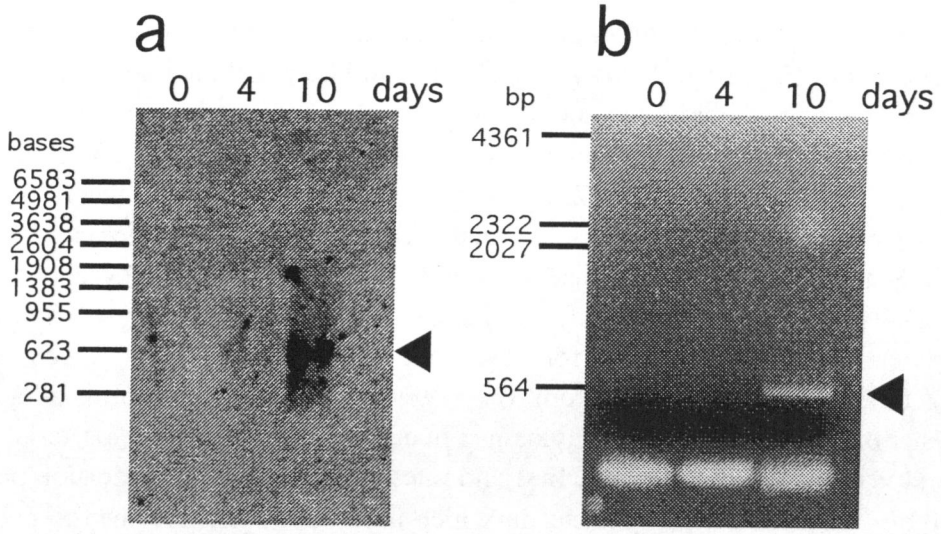


**Fig. 4-6.** Detection of mRNA for P21 and P16. Total RNA sample was prepared from *B. thermoleovorans* B23 cultures before (indicated as 0 day in the figure) or after (4 and 10 days) induction by alkanes. Positive signals were indicated by arrows. a, Northern blot analysis of mRNA for P21. AlkPhos-labeled DNA fragment gave signal at ca. 700 bases, where it hybridizes to complementary mRNA on the membrane. b, Detection of mRNA for P16 by RT-PCR analysis.

### 4.3.7 Detection of enzymatic activities responsible for erasing reactive oxygen molecules

SOD detoxifies superoxide anion to hydrogen peroxide, which in turn is generally broken down to water by catalase. The B23 cells grown in the presence or absence of alkanes were tested for SOD, catalase, and peroxidase activities by utilizing activity staining methods. The SOD activity of the B23 cells grown in the presence of alkane was higher than that of the cells grown in the absence of alkanes as expected (Fig. 4-7a). Catalase activity was detected in the B23 cells only when they were grown on alkanes (Fig. 4-7b). On the other hand, neither the SOD nor catalase was induced by alkanes in the LEH-1 cells. The reason is not clear why catalase activity was higher in the cells grown in the absence of alkanes than in the cells grown on alkanes. Although it has been reported that LEH-1 showed relatively high peroxidase activity irrespective of the presence and absence of alkane in the media (Allgood et al. 1985), this enzyme activity was not detectable level for both the B23 and H41 cells (data not shown).

### 4.3.8 Detection of acyl-CoA oxidase activity of B23

Both the SOD and catalase activities were specifically induced by alkanes at the time when strain B23 started to degrade alkanes. The SOD activity of *B thermoleovorans* B23 cells was also inducible upon addition of paraquot in the medium, which generates superoxide anion (figure not shown). It seemed most likely that the high SOD activity was required to detoxify superoxide anion, which was generated as a result of alkane degradation. So it is probable that a kind of oxidase catalyzes a step of alkane degradation pathway of *B. thermoleovorans* B23. Therefore, oxidase activity of the B23 cells was examined by using tetradecane, tetradecanal, tetradecanol, or tetradecanoyl-CoA as a substrate. Small but consistent oxidase activity was detected when tetradecanoyl-CoA was used as a substrate (Table 4-1). As far as I know, no tetradecanoyl-CoA oxidase activity has been reported for bacteria. As for acyl-CoA oxidase in bacteria, the gene encoding acyl-CoA oxidase has been cloned from *Streptomyces fradiae*, which forms a biosynthetic gene cluster of macrolide antibiotic, tylosin (Fouces et al. 1999). In the bacterial cells and mitochondria of eukaryotic cells, the first and rate-limiting step of $\beta$-oxidation pathway is catalyzed by acyl-CoA dehydrogenase, in which acyl-CoA is transformed to enoyl-CoA. This acyl-CoA dehydrogenase activity is replaced by acyl-CoA oxidase in eukaryotic peroxisome (Shultz 1991). Peroxisome is an organella which uniquely contains reactive oxygen molecules like peroxide or superoxide anions. According to the study of alkane degrading yeast *Candida*, peroxisome is highly developed in the cells grown on alkanes or fatty acids (Osumi et al. 1975). The development of peroxisomes in the cells of *C. tropicalis* grown on oleic acid was accompanied by high level expression of peroxisomal proteins, including acyl-CoA oxidase (Shimizu et al. 1979). Catalase is also a marker enzyme of peroxisome and its activity in *Candida* cells grown on hydrocarbons was much higher than that in the cells grown on lauryl alcohol, glucose or ethanol. Although acyl-CoA oxidase is reported to increase in the *Candida* cells grown on fatty acids or organic acids, too, neither palmitic acid (hexadecanoic acid) nor oleic acid (octadecenoic acid) was an effective inducer for the production of acyl-CoA oxidase in *B. thermoleovorans* B23 (Fig. 8a). I do not know whether the fatty acids could be incorporated in the B23 cells or not. The acyl-CoA oxidase activity of strain B23 showed broad substrate specificity ranging from hexanoyl-CoA to octadecanoyl-CoA (Fig. 8b). It is obvious that further experiment such as gene disruption for P24 (SOD) and acyl-CoA oxidase is necessary to conclude that these enzymes are responsible for alkane degradation pathway of the strain. However, dramatic increase in the acyl-CoA oxidase, SOD, and catalase activities in concomitant with alkane degradation strongly suggests that acyl-CoA oxidase functions at the initial step of $\beta$-oxidation and resluting superoxide anion and hydrogen peroxide would be erased by cooperative reaction of SOD and catalase (Fig. 9). This eukaryotic-type degradation mechanism of alkane in *B. thermoleovorans* cells might reflect chaotic living cell systems of common ancestor under high temperature condition of the primitive earth. I might be allowed to propose that the ancestor of *B. thermoleovorans* could be an origin of peroxisome.

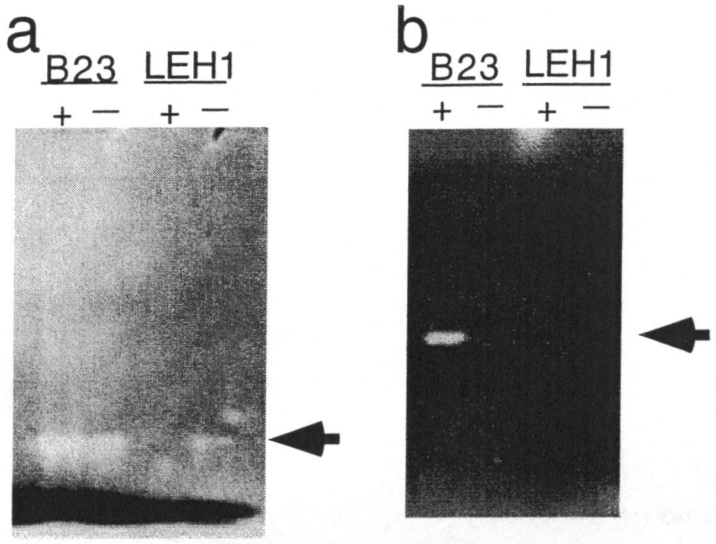


**Fig. 4-7.** Activity staining of SOD (a) and catalase (b). Crude cell extracts of *B. thermoleovorans* B23 and LEH-1 cultivated in the presence (+) or absence (-) of alkanes for 14 days were separated on 7.5% native polyacrylamide gel and stained by appropriate method. Arrows indicate respective enzyme activities.

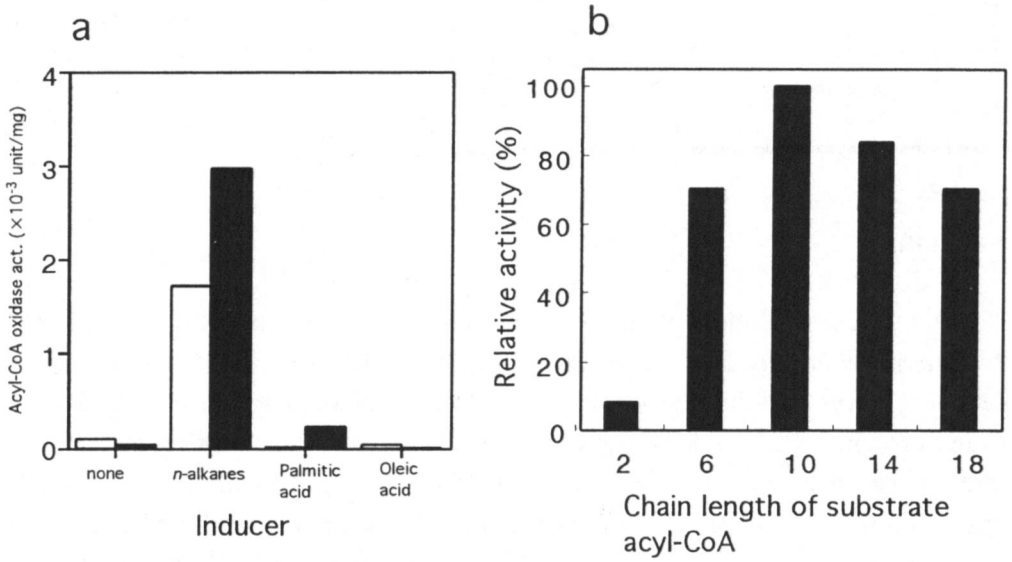


**Fig. 4-8.** Acyl-CoA oxidase activity of *B. thermoleovorans* B23. a, Induction of acyl-CoA oxidase activity by alkanes or fatty acids. *B. thermoleovorans* B23 was cultivated in the presence of alkanes or single fatty acid at 70°C for 5 days (open bar) and 10 days (closed bar). Cells grown on simple LBM were used as a negative control. Acyl-CoA oxidase activity was measured by using tetradecanoyl-CoA as a substrate. b, Substrate specificity of acyl-CoA oxidase. Enzyme activity was compared each other by using acyl-CoA with various alkyl chain length.

**Table 4-1.** Substrate specificity of oxidase activity in *B. thermoleovorans* B23 cells. Cell free extracts were prepared from the 14 days culture with 0.1 % alkanes at 70°C.

| Substrates /C14:0 | AU500 | | |
|---|---|---|---|
| | With cells (A) | No cells (B) | (A-B) |
| Tetradecane | 0.188 | 0.147 | 0.041 |
| Tetradecanal | 0.253 | 0.152 | 0.101 |
| Tetradecanol | 0.236 | 0.153 | 0.083 |
| Tetradecanoyl-CoA | 0.397 | 0.155 | 0.242 |
| None | 0.225 | 0.142 | 0.083 |

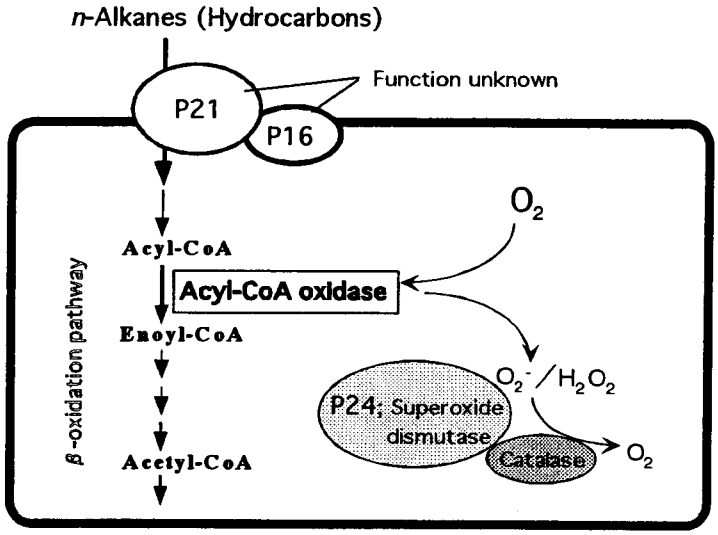


**Fig. 4-9.** A schematic diagram of alkane degradation pathway of *B. thermoleovorans* B23.

## 4.4 Summary

Alkane degradation pathway was analyzed for an extremely thermophilic *Bacillus thermoleovorans* B23, which has been recently isolated from a deep subsurface petroleum reservoir in Japan. Production levels of a cytoplasmic protein (P24) and two membrane proteins (P21 and P16) were significantly increased at the time when alkane degradation started in the cell. The induction effect was specifically observed for alkanes at a transcription level. Cloning and sequencing of the gene encoding P24 revealed that this protein is a superoxide dismutase (SOD) which consists of 204 amino acid residues (MW 22,928). Besides SOD, catalase and acyl-CoA oxidase were also increased upon alkane degradation, as far as their enzymatic activities were examined in the cell free extracts. These results strongly suggest that *B. thermoleovorans* B23 shares alkane degaradation pathway with alkane degarading yeast, *Candida tropicalis*, in which reactive oxygen molecules produced by the function of acyl-CoA oxidase are erased by cooperative function of SOD and catalase.

# CHAPTER 5

# Gene cloning and characterization of aldehyde dehydrogenase from thermophilic alkane degrading *Bacillus thermoleovorans* B23

## 5.1 Introduction

Irrespective of its chemical stability, alkane degradation ability is widely distributed among bacteria. In the group of gram-negative bacteria, *Pseudomonas* and *Acinetobacter* are best studied genera for their degradation mechanisms of alkanes (van Beilen et al. 1994, Geissdorfer et al. 1999, Ishige et al. 2000). *Pseudomonas oleovorans*, which is able to degrade C5-C12 alkanes, have been reported to possess OCT plasmid. The plasmid contained two genetic loci, *alkBFGHJKL* and *alkST*, which encode all the proteins necessary to convert alkanes to corresponding primary alcohol, aldehyde, fatty acid, and acyl-CoA (van Beilen et al. 1994). This acyl-CoA is further metabolized by TCA cycle to supply cell building blocks and reducing power. On the other hand, little is known about alkane degradation mechanism and its gene structure for gram-positive bacteria and thermophilic bacteria (Zarilla et al. 1987, 1986, Rueter et al. 1994).

I previously isolated *Bacillus thermoleovorans* B23 and H41 from deep petroleum reservoirs in Japan. They effectively degraded alkanes at 70℃ with the carbon chain length of more than 12 and 15, respectively (described in chapter 3). It has been recently reported that when the B23 cells were grown in the presence of alkanes, the production levels were significantly increased for soluble cytoplasmic proteins, such as superoxide disumutase, catalase, and acyl-CoA oxidase, and two functionally unknown membrane proteins P16 and P21 (described in chapter 4). In this report, I have analyzed the 5.2 kb DNA fragment which includes entire P21 gene. This DNA fragment contains three genes in addition to the P21 gene, and one of them was found to encode an aldehyde dehydrogenase, Bt-ALDH. The diretion of the transcription of this gene, as well as those of other two genes, is identical with that of the P21 gene. ALDH is recognized as an important enzyme for oxidation of various compounds including hydrocarbons and is widely distributed in a wide range of living organisms (Ziegler et al. 1999). Although *alkH* in *alk* operon of *P. oleovorans* has been reported to encode ALDH, the enzyme has been characterized neither enzymatically nor biochemically (van Beilen et al. 1994). In order to add knowledge on ALDH from thermophilic oil-degrading bacteria, Bt-ALDH was overproduced in *E. coli*, purified, and characterized.

## 5.2 Material and Methods

### 5.2.1 Bacterial strains and plasmid

An extremely thermophilic alkane-degrading bacterium, *B. thermoleovorans* B23 has been isolated from a deep petroleum reservoir in Minami-aga oil field (Niigata, Japan)(d). *E. coli* DH5$\alpha$ [F$^-$, $\phi$80, *lacZ* $\Delta$M15, *recA*1, *endA*1, *gyrA*96, *thi*-1,

*hsdR*17($r_k^-$ $m_k^+$), *SupE*44, *relA*1, *deoR*, Δ(*lacZYA-argF*)U169, $\lambda^-$] (Sambrook et al. 1989) and plasmid vector pCR2.1 (Invitrogen Corp., San Diego, CA) or pUC18 were used for gene cloning experiments. *E. coli* BL21(DE3) [$F^-$, *ompT*, *hsdS*$_B$($r_B^-$ $m_B^-$), *gal*( λ *cI857*, *ind1*, *Sam7*, *nin5*, *lacUV5-T7gene1*), *dcm*(DE3)] (Studier et al. 1990) and plasmid pET-25b (Novagen, Madison, WI) were used for gene expression and over production of proteins.

### 5.2.2 Culture media

Strain B23 was cultivated at 70℃ in L broth, which contained per liter 5g of yeast extract (Difco, Detroit, MI), 10 g of Bacto tryptone (Difco), 5g of NaCl, and pH was adjusted to 7.2 by 1N NaOH. All the recombinant *E. coli* cells were grown at 37℃ in L broth containing 50mg/l ampicillin. NZCYM medium containing per liter 10g of NZ amine, 5g of NaCl, 5g of yeast extract, 1g of casamino acids, and 2g of $MgSO_4$-$7H_2O$, was used for protein over production experiments.

### 5.2.3 Cloning of P21 and Bt –ALDH gene

The genomic DNA of *B. thermoleovorans* B23 was prepared as previously described (Imanaka et al. 1981). A part of the P21 gene was amplified by utilizing GeneAmp PCR System 2400 (Applied Biosystems Japan) with AmpliTaq DNA polymerase (Takara shuzo, Kyoto, Japan). The PCR amplification primers, P21-N and P21-37, were designated based on the N-terminal amino acid sequence and internal amino acid sequence of P21. The nucleotide sequences were P21-N, 5'-GCNTTYCCNAARWSNGGNGTNGGNGGNTT-3', and P21-37, 5'-CCNGCNSWCATRTARTGNGTRTT-3', where N represents A or C or G or T, Y represents C or T, R represent A or G, W represent A or T, and S represent C or G. The resulting 0.5 kb DNA fragment was used as the probe for Southern hybridization experiment to clone the entire P21 gene. P21 gene and flanking regions were cloned by inverse PCR (I-PCR) technique (Li et al. 1988, Pfeifer et al. 1989). Template DNA for I-PCR was prepared by self-ligation of the *Sph*I, *Sac*I, and *Hin*dIII digests of B23 genomic DNA. Nucleotide sequence of I-PCR amplified DNA fragment was determined by ABI Prism 310 genetic analyzer (Applied Biosystems Japan, Sanger et al. 1977).

### 5.2.4 Northern hybridization and RT-PCR

Cells were collected by centrifugation and frozen on dry ice. RNA was isolated by the method as described by Ausubel *et al.* (Ausubel et al. 1995). For Northern blot analysis, samples containing total RNA were separated on a 1% agarose gel containing formaldehyde as the denaturant (Lehrach et al. 1977). The RNA was blotted from the gel onto a Hybond $N^+$ membrane (Amersham Pharmacia Biotech) by using Transblot cell (Bio-Rad, Hercules, MI). Labelling the probe DNA, hybridization to the target mRNA, and detection of signals were performed by using Gene Images AlkPhos direct labeling and detection system (Amersham Pharmacia Biotech) under standard protocol.

In order to analyze the transcription level of the gene, RT-PCR method was also adopted. QIAGEN OneStep RT-PCR Kit (QIAGEN) was used in this case (Privitera et al. 1999).

Initial synthesis of cDNA was carried out at 50°C for 30 min by using 0.1 $\mu$g RNA as template. Temperature program for following amplification reaction was; 95°C for 15 min in the first step, and 94°C for 60 sec, 55°C for 60 sec and 72°C for 60 sec with 35 cycles, and then 72°C for 10 min in the last step. The PCR amplified products were analyzed by electrophoresis on 1% agarose gel.

### 5.2.5 Overproduction and purification of Bt-ALDH

*Bt-aldh* gene was amplified by PCR with a combination of forward (5'-TTTTTCATATGACAATGATTTCGGCG-3') and reverse (5'-TTTTTGAATTCTCCCTCCACGAGCGCCTT—3') primers. Underlines represent *Nde*I and *Eco*R sites, respectively, and the initiation codon is shown in italics. The resultant 1.5-kbp DNA fragment was digested with *Nde*I and *Eco*RI, and ligated into the *Nde*I-*Eco*RI gap of plasmid pET-25b to create an over expression plasmid pET-Bt-aldh. In this plasmid, transcription of *Bt-aldh* gene was under the control of the T7 promoter. In order to overproduce Bt-ALDH, *E. coli* BL21(DE3) cells maintaining pLysS and pET-Bt-aldh were grown at 37°C in NZCYM broth containing 50mg/l ampicillin. When optical density at 660nm of the culture reached to 0.3, isopropyl-$\beta$-D-thiogalacto-pyranoside (IPTG) was added at a final conc. 1mM. Cultivation was continued for additional 3 h. Cells were then harvested by centrifugation at 8000 × g for 10 min at 4℃ and subjected to the following purification steps.

All the purification procedures were carried out at 4℃. Cells were washed with 50mM Tris-HCl buffer (pH 7.0). After centrifugation at 8000 × g for 10 min the cell pellet was suspended with the same buffer. Cells were disrupted by a sonifier model 450 (Branson Ultrasonic Corp., Danbury, CT), and centrifuged at 15,000 × g for 30 min. The supernatant was heated at 50°C for 30 min to eliminate host proteins. The resultant supernatant was adjusted to 40% $(NH_4)_2SO_4$ saturation and centrifuged at 15,000 × g for 30 min at 4℃. The precipitate was suspended in the 50mM Tris-HCl buffer (pH 7.0) and then dialyzed against excess of the same buffer. This crude enzyme solution was applied to HiTrapQ anionic exchange column fitted to a FPLC system (Amersham Pharmacia Biotech, Piscataway, NJ). The enzyme was eluted by linearly increasing the NaCl concentration from 0 to 1.0 M in 50 mM Tris-HCl buffer (pH 7.0). Purity of the enzyme was analysed by SDS-PAGE (Laemmli et al. 1970).

### 5.2.6 Enzymatic activity

Aldehyde dehydrogenase activity was determined at 37℃ by measuring the initial rate of $NAD^+$ reduction (NADH production) which is accompanied by an increase of absorption at 340 nm (Black 1951, Okibe et al. 1999). The assay mixture (1 ml) contained 50 mM Tricine buffer (pH 8.5), 1 mM $NAD^+$, 2% Triton X-100, 1 mM substrate, and an appropriate amount of the enzyme, unless specifically stated otherwise. The enzymatic activity against all-*trans* retinal was determined in 50mM tricine buffer (pH 8.5) containing 1mM $NAD^+$, 2% Triton X-1000 and 1mM all-*trans* retinal. One unit (U) of activity is defined as the amount of enzyme catalyzing production of 1 $\mu$mol NADH/min.

To determine ALDH activity with longer aldehyde than octanal (C8) or at high temperature conditions, the resulting fatty acid was extracted with an equal volume of ethyl acetate and directly quantified by gas chromatography (GC/FID) because the reaction mixture became muddy in these cases. Aldehydes with carbon chain length longer than C14, tetradecanal, was not tested because of its commercial unavailablity.

## 5.3 Results and Discussion

### 5.3.1 DNA sequence analysis

Southern blot analysis showed that a 3.5 kb *Sph*I fragment contained P21 gene. Finally, 3.5 kb *Sph*I, 2.6 kb *Sac*I, and 1.4 kb *Hin*dIII fragments were obtained by I-PCR technique. Nucleotide sequences of these three DNA fragments were completely determined. It was found that P21 is composed of only 163 amino acid residues including a secretion signal peptide sequence (38 aa) at N-terminal end. Although P21 was migrated at a position of 21 kDa on SDS-polyacrylamide gel electrophoresis, calculated molecular weight of mature protein is 16,984. It is least probable that P21 is a modified protein, because the molecular weight of P21 recovered from SDS-polyacrylamide gel was determined ca.17,100 by ESI-mass spectrometry (LCQ, Finnigan mat, USA). Homology searches indicated that P21 has no similarity with any protein in database. P21 would be a unique membrane protein probably responsible for alkane metabolism in *B. thermoleovorans* cells (described in chapter 4). It was found that an aldehyde dehydrogenase gene (*Bt-aldh*) was located approximately 1.2 kb upstream of P21 gene (Fig. 5-1). The *Bt-aldh* gene encodes an open reading frame extending from the ATG initiation codon at position 1213 to the TAA stop codon at position 2704, with a potential Shine-Dalgarno (SD) sequence from 1201 to 1206 (Fig. 5-1). This nucleotide sequence is deposited in DDBJ with the accession number AB047016. Bt-ALDH is composed of 497 amino acid residues with a calculated moleuclar weight of 53,886 Da. Homology seaches indicated that Bt-ALDH showed high amino acid sequence identities of 49.5% to *p*-cumic aldehyde dehydrogenase from *Pseudomonas putida* (Eaton 1997), 48.5% to an aldehyde dehydrogenase, encoded by *dhaS,* from *Bacillus subtilis* (Kunst et al. 1997), and 47.3% to retinal dehydrogenase, encoded by *Raldh2,* from *Mus musculus* (Zhao et al. 1996). The amino acid sequence of Bt-ALDH was aligned with that of *p*-cumic aldehyde dehydrogenase from *Pseudomonas putida* (DDBJ/EMBL/GeneBank accession number U24215) and retinal dehydrogenase from mouse (DDBJ/EMBL/GeneBank accession number X99273) shown in Fig. 5-2. A possible consensus sequence of the $NAD^+$ binding site, G-X-T-X-X-G at 238-243 (Liu et al. 1997), and the amino acid residues demonstrated to be important for enzyme activity, such as Glu260 (Abriola et al. 1987) and Cys294 (von Bahr-Lindstrom et al. 1985) are also conserved in the Bt-ALDH sequence. Bt-ALDH shares only 27.4% of amino acid sequence identity with aldehyde dehydrogenase (AlkH) encoded in *alk* operon of *Pseudomonas oleovorans* (Kok et al. 1989), however, the hydropathy profile of the Bt-ALDH is similar to that of AlkH from *P. oleovorans* rather than that of *p*-cumic aldehyde dehydrogenase from *Pseudomonas putida*

(Fig. 5-3). Recently, gene encoding a long-chain aldehyde dehydrogenase (*ald1*) has been cloned from *Acinetobacter* sp. strain M-1 and the recombinant enzyme has been characterized (Ishige et al. 2000). Because Bt-ALDH has amino acid sequence identity of only 19.8% with Ald1, they belong to different group of ALDH. Although Ald1 showed substrate preference for long-chain alkyl aldehyde, it showed significant amino acid sequence similarity with a group of $NAD^+$ dependent low molecular weight type ALDHs including AcoD (70% identity). AcoD is an aldehyde dehydrogenase involved in acetoin and ehtanol catabolisms in *Alcaligenes eutrophus* (Priefert et al. 1992). It is also interesting that an aldehyde dehydrogenase (hd-ALDH) from alkane degrading strain HD-1 also belongs to the same group ALDH (76.1% identical to AcoD and 68.9% to Ald1) and showed ten times higher $k_{cat}/K_m$ value for decanal than acetaldehyde (Okibe et al. 1999). Substrate specificity might be governed by a very limited number of amino acid residues in this ALDH group.

The ORF-X located 196 bp upstream of the P21 gene encodes ribonucleotide reductase homologue which is 33.8% idetical with the enzyme from *Mycobacterium tuberculosis* (G70962). This protein forms class diiron-oxo protein family (Fox et al. 1994), with hydrocarbon hydroxylase and methane monooxygenase hydroxylase from *Methylococcus capsulatus* (Stainthorpe et al. 1990), toluene-4-monooxygenase from *Pseudomonas mendocina* (Yen et al. 1991), and phenol hydroxylase from *Pseudomonas* sp. CF600 (Nordlund et al. 1990). ORF-X could have monooxygenase activity against *n*-alkane in *B. thermoleovorans* B23. Another open reading frame, ORF-Y, is located at 143 bp downstream of the P21 gene. Because ORF-Y has no similarity with the proteins in database, its function is completely unknown.

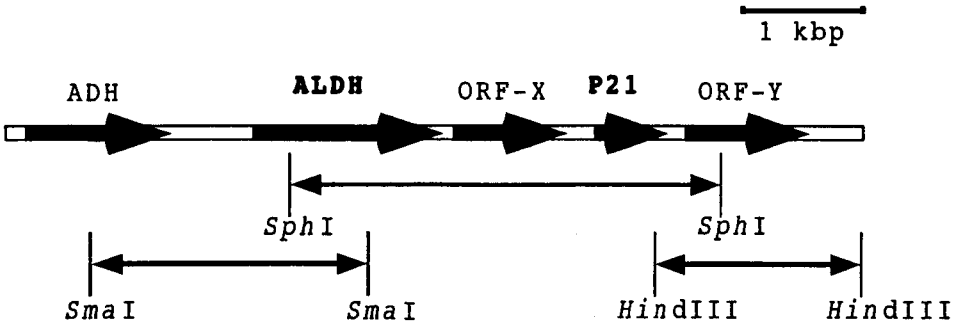


Fig. 5-1. Localization of the gene encoding Bt-ALDH, ORF-X, P21, and ORF-Y. Arrows indicate the DNA fragment which was amplified by I-PCR.

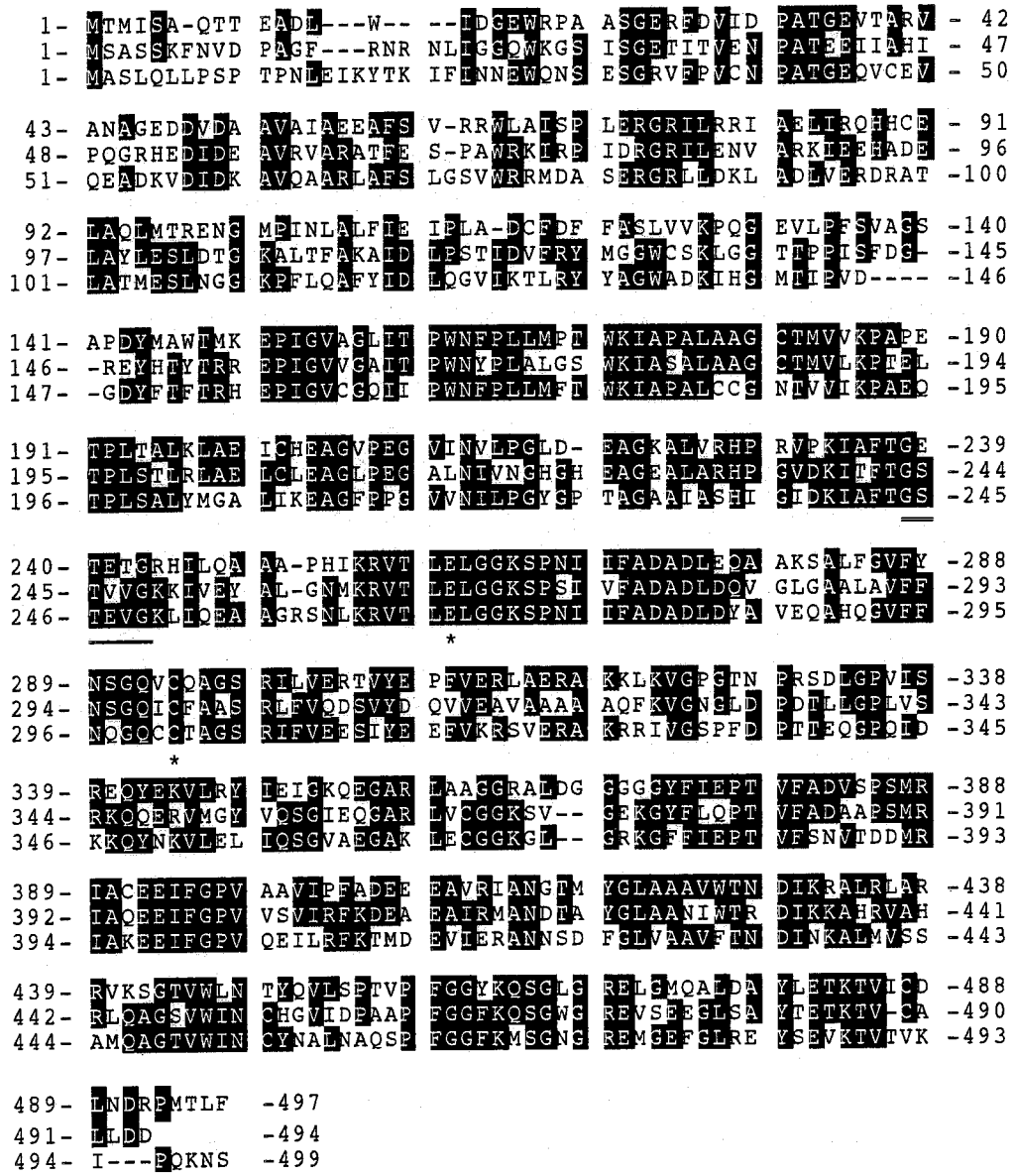

Fig. 5-2. Alignment of the amino acid sequences of ALDH. The amino acid sequence of Bt-ALDH (the upper row) is aligned with those of *p*-cumic aldehyde dehydrogenase from *Pseudomonas putida* (the middle row) and retinal dehydrogenase from mouse (the lower row). Asterisks indicate the amino acid residues which are important for enzymatic activity. A possible coenzyme-binding site (G-X-T-X-X-G) is underlined. The amino acid residues conserved in at least two different proteins are shaded.

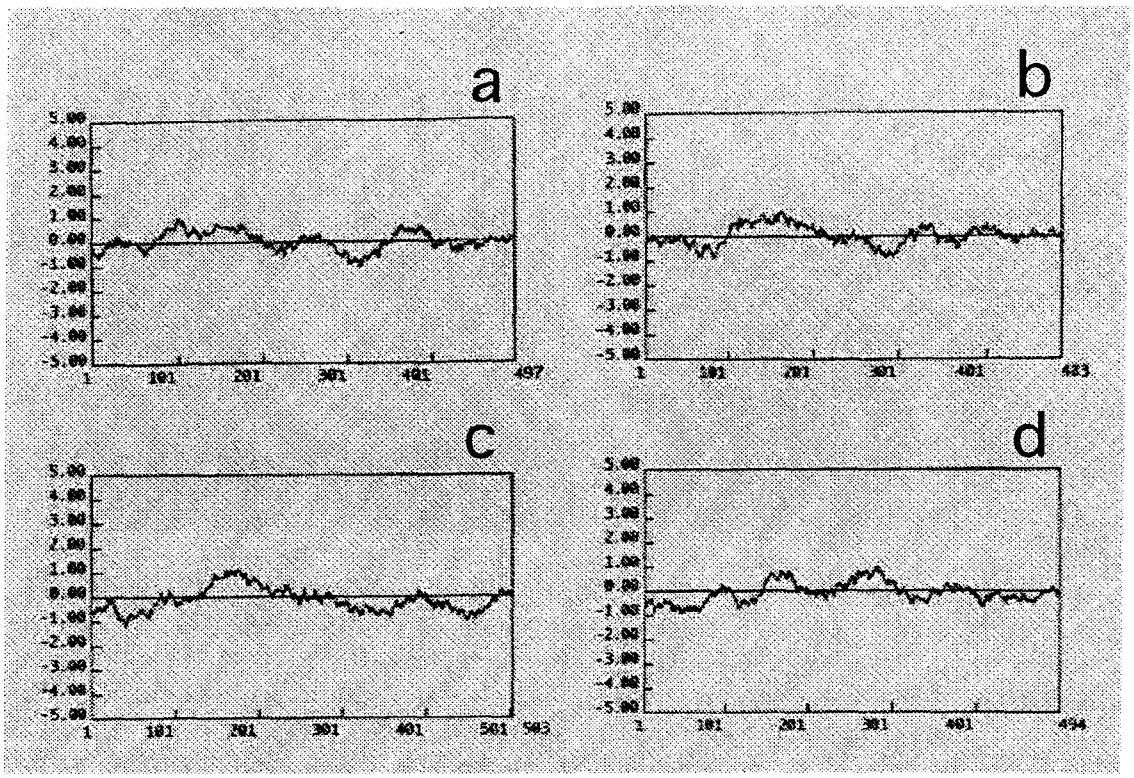


Fig. 5-3. Comparison of hydropathy profiles of ALDHs. Hydropathy profiles of Bt-ALDH (a), aldehyde dehydrogenase from *Pseudomonas oleovorans* (b), and long-chain aldehyde dehydrogenase from *Acinetobacter* sp. M-1 (c), and *p*-cumic aldehyde dehydrogenase from *Pseudomonas putida* (d) are shown. These were calculated by the algorithm of Kyte and Doolittle (Kyte et al. 1982) with a window size of 40 residues in DNASIS software (Hitachi software, Tokyo, Japan).

### 5.3.2 Induction of mRNA synthesis by *n*-alkane

Total RNA was prepared from the B23 cells grown in the presence or absence of alkane for 4 and 10 days. mRNA of the P21 gene was observed only in the B23 cells grown on alkane for 10 days (Fig. 5-4a). Alkane degradation by strain B23 was also started after 10 days cultivation on alkane. The size of mRNA of the P21 gene was approximately 700 base, suggesting that the P21 gene was transcribed independently from neighbor genes (Fig. 4a). RT-PCR analysis was also performed for B23 cells grown under the same condition as described above. Transcription levels of both the P21 and Bt-ALDH genes were similarly increased by alkane (Fig. 5-4b). These results demonstrate that the Bt-ALDH gene is expressed in response to alkane together with the expression of P21 gene.

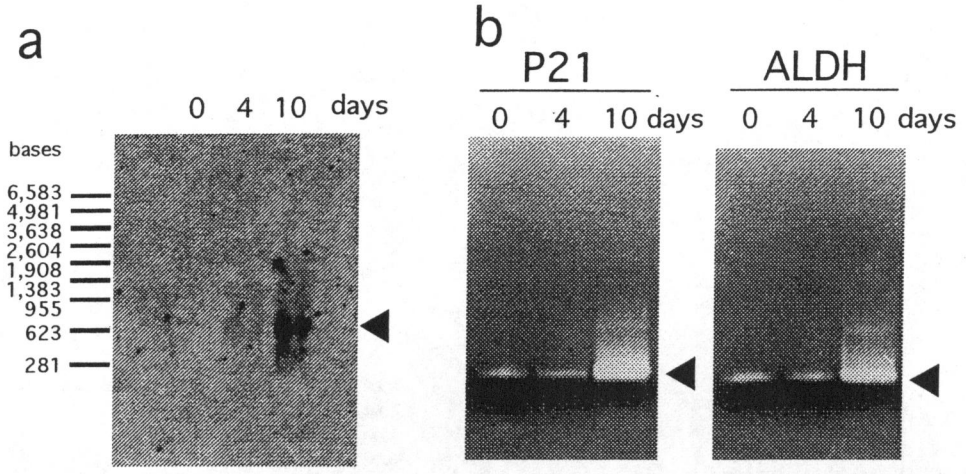


Fig. 5-4. Analyses on mRNA of P21 and *Bt-ALDH* gene. Northern blot analysis (a) and RT-PCR analysis (b) by using total RNA isolated from *B. thermoleovorans* B23 cultures before (lane 1) and after induction for 4-d (lane 2) and 10-d (lane 3) by alkanes. (a) AlkPhos-labeled *p21* DNA probes bounded with complementary mRNA were detected with a chemiluminescent substrate. 10 $\mu$g of RNA was applied for each lane. RNA molecular size marker of 6,583, 4,981, 3,638, 2,604, 1,908, 1,383, 955, 623 and 281 bp (top to bottom, respectively). (b) Comparison of transcription level of *p21* and *Bt-ALDH* by RT-PCR. RT-PCR products were analyzed on 1% agarose gel. Positive signals were detected at 511 bp position in each experiment. 0.1 $\mu$g of RNA was used as RNA template.

### 5.3.3 Overproduction and purification of Bt-ALDH

Bt-ALDH was overproduced in *E. coli* BL21(DE3) carrying expression plasmid pET-Bt-aldh and purified to homogeneity as described previously. Upon induction of Bt-ALDH gene, Bt-ALDH was produced in the cells as a major protein (Fig. 5-5; lane 2). Its production level was estimated to be roughly 10 mg/l culture, judged from the intensity of the band visualized with Coomassie brilliant blue staining following SDS-PAGE of the whole cell extract. Most of the protein accumulated in the cells was recovered in a soluble form after sonication. After purification steps, Bt-ALDH gave nearly a single band on SDS-PAGE (Fig. 5-5; lane 7). Finally 6.4 mg of Bt-ALDH was recovered from 2 l culture. The molecular weight of Bt-ALDH was estimated to be 55,000 from SDS-PAGE and 410,000 from gel permeation chromatography with Superdex-200, respectively. These results suggest that it acts as an octamer. Although it was also eluted from the column at a position for hexadecameric enzyme, its specific activity was only about 5% of octarmeric enzyme. On the other hand, retinal dehydrogenase (RALDH-2) has been reported to act as a tetramer (Zhao et al. 1996).

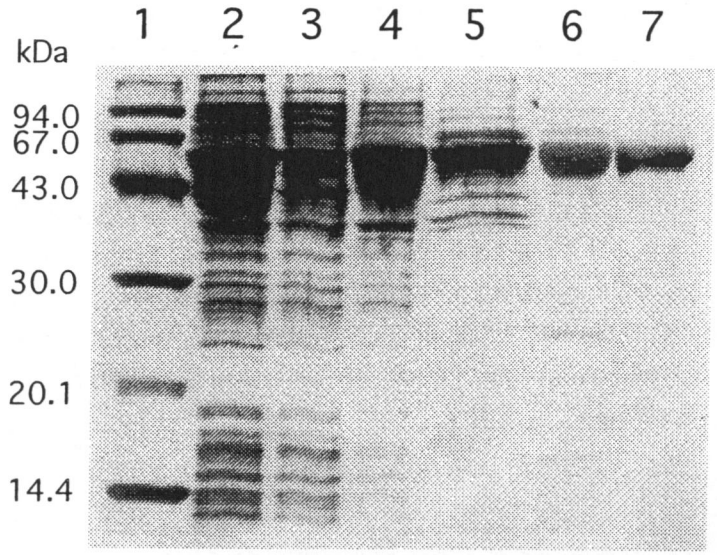


Fig. 5-5. Purification of the recombinant Bt-ALDH protein expressed in *E. coli* cells. Samples were subjected to electrophoresis on 10% polyacrylamide gel in the presence of SDS. Lane 1, molecular weight markers; phosphorylase b, 94.0 kDa; bovine serum albumin, 67.0 kDa; ovalbumin, 43.0 kDa; carbonic anhydrase, 30.0 kDa; trypsin inhibitor, 20.1kDa; and $\alpha$-lactalbumin, 14.4kDa. Lane 2, whole cell extract. Lane 3, insoluble fractions after sonication of the cells. Lane 4. soluble fractions after sonication of the cells. Lane 5. soluble fractions after heat treatment at 50°C for 30 min. Lane 6, proteins after fractionation with $(NH_4)_2SO_4$. Lane 7, Bt-ALDH purified by anionic exchange column HiTrapQ.

### 5.3.4 Properties of Bt-ALDH

The Bt-ALDH activity determined in the presence of $NAD^+$ was higher than that determined in the presence of $NADP^+$ by twelve folds by measuring the initial rate of $NAD^+$ or $NADP^+$ reduction monitoring at 340 nm. Optimum pH for the enzyme activity was around 10.0 (Fig. 5-6). Bt-ALDH specifically acted on long-chain aliphatic aldehydes (Fig. 5-7). Among various substrates tested, Bt-ALDH showed the highest activity on decanal with a specific activity of 0.44 U/mg. Retinal dehydrogenae activity of Bt-ALDH was assayed by the method described by Napoli (Napoli et al. 1990). Bt-ALDH showed no retinal dehydrogenae activity (data not shown). Although Bt-ALDH was stable up to 55℃, activity decreased to 12.7 % by heat treatment at 70°C for 30 min (Fig. 5-8). The enzymatic activity of Bt-ALDH was strongly activated by $CaCl_2$, $SrCl_2$ and $BaCl_2$, and inhibited by $MgCl_2$ and $ZnCl_2$ (Table 5-1). $SrCl_2$ was most effective co-factor for the activity. When the effect of concentration of $SrCl_2$ on the Bt-ALDH activity was tested, 2 mM was enough for the activity (data not shown). The activities of NADPH-dependent ubiquinone reductase from rat liver (Takahashi et al. 1996) and Methanol dehydrogenase from *Paracoccus denitrificans* (Harris et al. 1994) were reported to be enhanced by $Sr^{2+}$ in addition to $Mg^{2+}$ and $Ca^{2+}$, respectively. The enzymatic activity against alkyl aldehyde longer than C10 and the optimum temperature condition were determined by measuring the amount of reaction product as described previously. In the range of C2 to C14 aliphatic

aldehydes, Bt-ALDH was most active againt the C14 aldehyde (tetradecanal). Optimum temperature for the enzyme activity was determined to be 50℃. Substrate preference of Bt-ALDH were almost the same at 37°C and 65°C (data not shown).

There are several reports on aldehyde dehydrogenases, which prefer long-chain alkyl aldehydes (Okibe et al. 1999, Nagy et al. 1995, Bognar et al. 1978, Vandecasteele et al. 1982, Byers et al. 1984). Although an attempt to disrupt Bt-ALDH gene in *B. thermoleovorans* B23 has not been successful yet, substrate preference of the enzyme and transcriptional induction of the Bt-ALDH gene by alkane may allow us to propose that Bt-ALDH is an enzyme responsible for alkane degradation pathway. Anyway, further studies on Bt-ALDH would contribute to analyze the structural factors which determine the substrate specificity and thermostability.

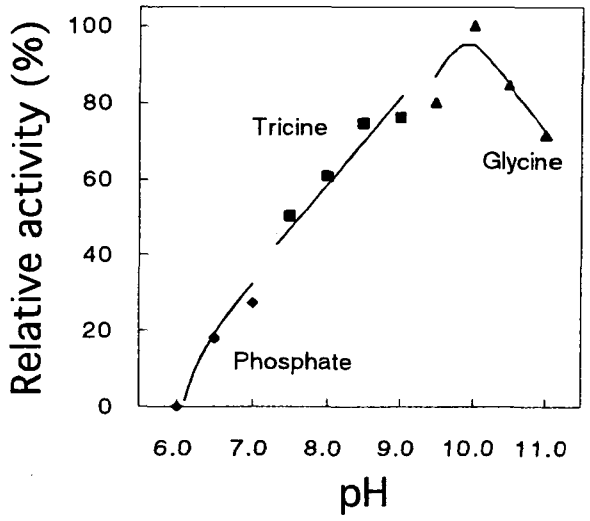


Fig. 5-6. The optimum pH for Bt-ALDH activity. The activity was assayed at 37℃ in 50mM buffer of phosphate (◆), tricine (■) or glycine (▲). Hexanal (1 mM) was adopted as a substrate for each reaction.

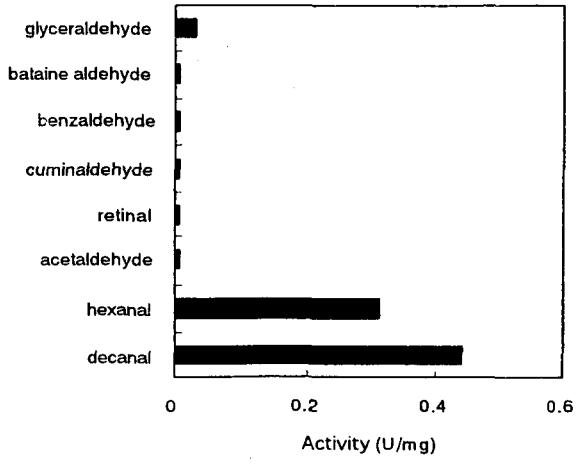


Fig. 5-7. Substrate specificity of Bt-ALDH. The enzymatic activity was determined at 37℃ in 50mM tricine buffer (pH 8.5) containing 1mM $NAD^+$ and 2% Triton X-100 by measuring the increase in the absorbance at 340 nm.

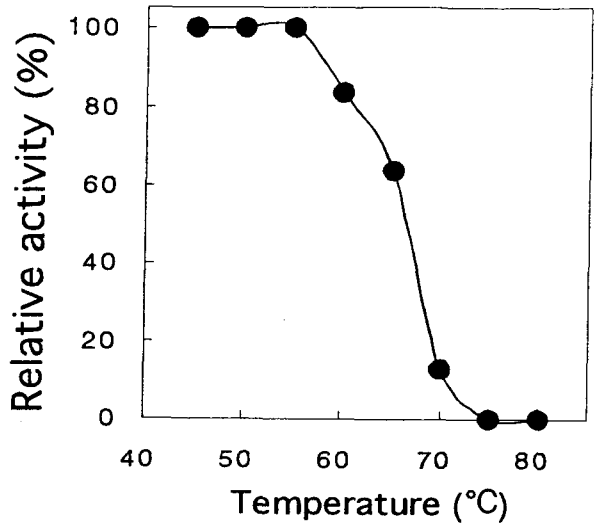


Fig. 5-8. Effect of temperature on the stability of Bt-ALDH. Thermal stability was estimated by measuring remaining activity after heated treatment at each temperature for 30 min.

| Metal ions | Relative activity |
|---|---|
| None | 1.0 |
| EDTA | 0.63 |
| $MgCl_2$ | 0.14 |
| $CaCl_2$ | 1.9 |
| $MnCl_2$ | 1.1 |
| $CoCl_2$ | 1.2 |
| $NiCl_2$ | 0.97 |
| $CuCl_2$ | 0.87 |
| $ZnCl_2$ | <0.01 |
| $SrCl_2$ | 3.4 |
| $BaCl_2$ | 2.1 |

Table 5-1. Effects of divalent metal ions and EDTA on Bt-ALDH activity. The enzyme was previously treated with 2mM EDTA and dialyzed against 50mM Tris-HCl buffer (pH 7.0) before determining its enzymatic activity. The enzymatic activity was determined at 37°C in 50mM glycine buffer (pH 10.0) containing 1mM hexanal, 1mM $NAD^+$, 2% Triton X-100, and 2mM EDTA or metal ions.

## 5.4 Summary

The gene encoding an aldehyde dehydrogenase (Bt-ALDH) from long-chain alkane degrading thermophilic *Bacillus thermoleovorans* B23 was found to be located in the upstream region of the P21 gene, whose transcription level was dramatically increased by alkanes (described in chapter 4). The Bt-ALDH gene contained an open reading frame of 1,491 bp (497 aa, MW= 53,886) and its transcription level was also increased in the cells grown in the presence of alkane like that of the P21 gene. Recombinant Bt-ALDH was overproduced in *Escherichia coli*, purified, and characterized biochemically and enzymatically. Bt-ALDH acted as an octamer, required $NAD^+$ as a coenzyme and showed high activity against aliphatic aldehyde such as tetradecanal. Optimum conditions for the enzymatic activity were 50°C and pH 10.0. The activity was elevated from two to three folds in the presence of $CaCl_2$, $BaCl_2$, and $SrCl_2$. While, $ZnCl_2$ strongly inhibited the enzymatic activity.

# CHAPTER 6

## General Conclusion

In this thesis, I described about the isolation and characterization of hydrocarbon degrading bacteria from subsurface oil fields at low and high temperature conditions. Cold-adapted bacteria from oil fields are expected to promote *in situ* bioremediation technologies for hazardous hydrocarons and esters at low temperatures. In many Arctic sites, biodegradation rates are so low that hydrocarbon contaminants remain in cold Arctic ecosystems for long periods. On the other hand, termophilic alkane degrading bacteria are expected to be applied in biotechnological processes. Limited biodegradation as a result of the low water solubility of hydrophobic contaminants may be overcome due to higher water solubility at elevated temperature. Moreover, diffusion rates and chemical transformation rates also increase at higher temperatures with an additional positive impact on bioavailability.

In Chapter 2, four catechol degrading psychrotrophic bacteria were isolated from Japanese oil reservoir water (strains SIB1, SIC1, SIS1) and Canadian oil sands (strains CAB1). The strains SIB1, SIC1 and SIS1, were identified as *Shewanella* sp., and the strain CAB1 was identified as *Arthrobacter* sp. When 0.1 mM of catechol was used for degradation test, CAB1 degraded 63% of catechol upon incubation at 20°C for 128 h and 32% upon incubation at 4°C for 128 h. On the other hand, SIB1 cells degraded 41% of catechol at 20°C for 128 h, and 17% at 4°C for 128 h. A degradation product of catechol by strains CAB1 and SIB1 was *cis, cis*-muconic acid. These results allowed me to conclude that psychrotrophic *Arthrobacter* and *Shewanella* strains adopted C1,2O pathway for hydroxylated aromatic hydrocarbon degradation pathways.

In Chapter 3, Two strains of extremely thermophilic alkane-degrading bacteria, B23 and H41, were isolated from deep petroleum reservoir in Japan. Strains B23 and H41 effectively (>60%) degraded alkanes with the carbon chain length of more than 12 and 15, respectively. According to the 16S rRNA gene sequence analysis, both strains were identified as *Bacillus thermoleovorans*, although their physiological characteristics were rather different from those of the type strain LEH-1. When these strains were grown on heptadecane, heptadecanoate and pentadecanoate accumulated in the cells. These results suggest that the strains B23 and H41 degrade *n*-alkanes by a terminal oxidation pathway, followed by a $\beta$-oxidation pathway. In order to support above speculation, a long-chain-alcohol dehydrogenase gene was cloned from the strain B23. The gene encoded an open reading frame of 249 amino acid residues and conferred 1-tetradecanol dehydrogenase activity on *Escherichia coli* cells. This result is in agreement with a terminal oxidation pathway.

In Chapter 4, superoxide emitting acyl-CoA oxidase activity was detected specifically in the cells grown on alkane. When the cells were grown in the presence of alkanes, the production levels of a soluble cytoplasmic protein (24 kDa) and two membrane proteins (16 kDa and 21 kDa) were significantly increased. Fatty acids and triacylglycerols were not effective for the productions of these proteins. Cloning and sequencing of the

gene encoding 24 kDa-protein revealed that it was a superoxide dismutase (SOD) with 204 amino acid residues. Induction of SOD synthesis by alkane has been reported for yeast but not for bacteria. Since acyl-CoA oxidase activity was also detected in the cells, *B. thermoleovorans* seems to degrade alkanes by eukaryotic-type oxidase reaction rather than bacterial-type dehydrogenase reaction.

In Chapter 5, the gene encoding aldehyde dehydrogenase (BT-ALDH) from a thermophilic alkane-degrading bacteria, *Bacillus thermoleovorans* B23, was cloned and sequenced. The gene was found in the region upsteam of the P21 gene, which was induced strongly in *B. thermoleovorans* B23 cells grown with *n*-alkanes. The gene encoding BT-ALDH had an open reading frame of 1,494 bp that encodes a polypeptide of 497 amino acid residues (molecular weight 53,886, pI 5.33). The BT-ALDH was overexpressed in *Escherichia coli*, purified, and characterized. BT-ALDH showed higher activity on aliphatic aldehyde substrate than acetoaldehyde. These results indicate that BT-ALDH highly likely concerns alkane degradation pathway of *B. thermoleovorans* B23.

Hydrocarbon degradation by microorganisms has been mainly studied for mesophilic bacteria and yeast (eukarya) which prefer moderate temperature conditions. The degradation pathway of long-chain-*n*-alkane has never been studied for thermophilic microorganisms. Based on the experimental results in this study, I could propose that an extremely thermophilic bacteria share *n*-alkane degradation mechanism with both Bacteria and Eukarya. This reminds me a chaotic metabolisms of extremely or hyper thermophilic Archaea, which are believed to inherit characteristics of common ancestor of living organisms. It is interesting from the point of view of evolutionary ecology to investigate the ecosystem in deep subsurface oil fields. Moreover, studies on thermophilic alkane degrading bacteria would expand a limit of application of microbial activity to environmental remediation and chemical processes in factories. Degradation mechanism of alkane under high temperature condition and the tolerance mechanism against hot organic solvent condition need to be clarified in the future.

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or triazone N-source. Patent EP 550,023.

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# LIST OF PUBLICATIONS

1. Kato, T., Haruki, M., Imanaka, T., Morikawa, M., and Kanaya, S. (in press) Isolation and characterization of psychrotrophic bacteria from oil reservoir water and oil sands. Appl. Microbiol. Biotechnol.

2. Kato, T., Haruki, M., Imanaka, T., Morikawa, M., and Kanaya, S. (in press) Isolation and characterization of long-chain-alkane degrading *Bacillus thermoleovorans* from deep subterranean petroleum reservoir. J. Biosci. Bioeng.

3. Kato, T., Miyanaga, A., Haruki, M., Imanaka, T., Morikawa, M., and Kanaya, S. (in press) Gene cloning of an alcohol dehydrogenase from thermophilic alkane degrading *Bacillus thermoleovorans* B23. J. Biosci. Bioeng.

4. Kato, T., Miyanaga, A., Haruki, M., Imanaka, T., Morikawa, M., and Kanaya, S. *Bacillus thermoleovorans* B23 shares alkane degradation pathway with eukaryotes. J. Bacteriol. (submitted)

5. Kato, T., Miyanaga, A., Haruki, M., Imanaka, T., Morikawa, M., and Kanaya, S. Gene cloning and characterization of aldehyde dehydrogenase from a thermophilic alkane-degrading bacteria, *Bacillus thermoleovorans* B23. (in preparation)

# PATENTS

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