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Physiological Characterization of an *n*-Butane-Utilizing Bacterium, *Alcaligenes* sp.

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An *n*-butane-utilizing bacterium was isolated from soil contaminated with petroleum and identified as *Alcaligenes* sp. by 16S-rDNA sequence homology. The bacterial isolate grew on gaseous alkane (*n*-butane), liquid alkane (*n*-hexane, *n*-octane or *n*-decane) or liquid alkane mixture (kerosene, C_{10}~C_{16}) as the sole carbon source, but did not grow on glucose, lactate or pyruvate. The specific activities of butane monooxygenase, butanol dehydrogenase, butyraldehyde dehydrogenase, lactate dehydrogenase, pyruvate dehydrogenase, isocitrate dehydrogenase and malate dehydrogenase were 6.97±0.42, 2.54±0.28, 3.78±0.31, 0.003±0.00, 0.007±0.00, 0.51±0.03 and 0.65±0.04 μM mg protein^{-1} min^{-1}, respectively. In tests of *n*-butane, *n*-hexane, *n*-octane and *n*-decane oxygenation with crude enzyme extracted from bacterial cells grown on kerosene, NADH was oxidized to NAD$^+$ coupled to production of *n*-butanol, *n*-hexanol, *n*-octanol and *n*-decanol. However, monooxygenase activity was relatively higher when the substrate for the enzyme assay was same as that used for growth than when the substrate and growth medium differed. This suggests that *Alcaligenes* sp. may produce specific monooxygenases dependent on the *n*-alkane species used as the growth substrate.

Key words : *Alcaligenes* sp., alkane oxidation, monooxygenase, *n*-butane

1. Introduction

Some bacteria are capable of aerobic growth on methane (C_1), gaseous alkane (C_2 to C_4) and liquid alkane (C_5 to C_{12}) as the sole carbon and energy source.[1,2,3,4,5,6] Methane-oxidizing bacteria can only use methane as the growth substrate, but other alkane-utilizing bacteria can catabolize gaseous alkane (C_2 to C_4), liquid alkane (C_5 to C_{12}) or both.[7-13)] The metabolic oxygenation of *n*-alkanes differs from methane oxidation catalyzed by methane monooxygenase.[14-16] However, the first reaction catalyzed by soluble butane monooxygenase, which is coupled to NADH oxidation, was reported to be similar to that of soluble methane monooxygenase.[9)] Colby *et al.*[4)] reported that the soluble methane monooxygenase of *Methylococcus capsulatus* grown on methane can catalyze oxygenation of various hydrocarbons such as *n*-alkanes, ethers and alicyclic compounds. From this report, we hypothesized that some alkane monooxygenases may be not confined to oxidation of a specific alkane, but may catalyze the oxidation of several alternative alkanes. Some gram-positive microorganisms, such as *Corynebacterium*, *Norcardia*, *Mycobacterium* and *Rhodococcus*, and gram-negative *Pseudomonas* sp. were reported to utilize short-chain alkanes other than methane.[15,17,18,19)] Also, the butane monooxygenase of *Pseudomonas butanovora* was recently reported to be induced and activated by dichloroethene isomers.[1,14,20)] However, there has been no report that crude enzyme isolated from bacteria grown on *n*-butane can catalyze monooxygenation of *n*-alkanes other than *n*-butane, or that bacteria grown on other *n*-alkanes can catalyze monooxygenation of *n*-butane. In this study, we isolated a bacterium, *Alcaligenes* sp., that utilized *n*-butane, and we characterized the alkane monooxygenase at the physiological level. We estimated and compared activity of *n*-alkane monooxygenases using crude enzyme extracted from *Alcaligenes*

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sp. grown on gaseous alkane (*n*-butane), liquid alkane (*n*-hexane, *n*-octane or *n*-decane) or a mixture of liquid alkanes (kerosene, Merck index: fractionated petroleum C_{10}~C_{16}).

2. Materials and Methods

2.1. Chemicals

n-Alkanes, kerosene and other chemicals were purchased from Sigma-Aldrich Co. (USA).

2.2. 16S-rDNA sequencing

16S ribosomal DNA from the bacterial isolate was amplified by direct PCR using the universal primers 5'-GAGTTGGATCCTGGCTCAG-3' and 5'-AAGGAGGGGATCCAGCC-3'. The reaction mixture consisted of 300 mM Tris-HCl (pH 8.8), 100 mM $(NH_4)_2SO_4$, 100 mM KCl, 20 mM $MgSO_4$, 20 pM of each primer, 20 mM of each dNTP, 2U Taq polymerase (Genenmed, USA), and 20 ng template; amplification reactions were performed in a PCR machine (T Gradient model, Biometera, German). PCR products were directly sequenced by Macrogen, Inc. (Korea) using an ABI Prism 3700 Genetic analyzer.

2.3. Selection and cultivation of bacterial isolate

To isolate *n*-butane-utilizing bacteria, soil samples were collected around local gas stations in Kanghwa-do (island). The soil samples were streaked on agar plates containing basic medium. The basic medium was composed of 50 mM KPi buffer (pH 7.0), 2 g/L NH_4Cl, 0.02 g/L Na_2SO_4, and 2 mL/L trace mineral stock solution. The trace mineral stock solution contained 0.01 g/L $MnSO_4$, 0.01 g/L $MgSO_4$, 0.01 g/L $CaCl_2$, 0.002 g/L $NiCl_2$, 0.002 g/L $CoCl_2$, 0.002 g/L $SeSO_4$, 0.002 g/L WSO_4, 0.002 g/L $ZnSO_4$, 0.002 g/L $Al_2(SO_4)_3$, 0.0001 g/L $TiCl_3$, 0.002 g/L $MoSO_4$, and 10 mM EDTA. The agar plates were incubated in a 20-L gas-tight stainless jar that was connected to a 20-L Teddler bag filled with butane-oxygen (1:1) mixture. Colonies grown on the agar plate were isolated and aerobically cultivated at 30°C in basic medium containing *n*-butane, *n*-hexane, *n*-octane, *n*-decane or kerosene as the sole carbon source. The concentration of each liquid *n*-alkane or kerosene in the basic medium was adjusted to 100 mM, except gaseous *n*-butane whose concentration was adjusted to 50% by mixing with pure oxygen (99.99%). The bacterial cells were cultivated in a 1000-mL gas-tight bottle that was sealed with butyl rubber. The medium volume was adjusted to 600 mL, and the headspace (400 mL) was filled with nitrogen-oxygen (1:1) mixture and connected to a Teddler bag (2.0 L). When butane was used as substrate, the headspace and Teddler bag were filled with butane-oxygen (1:1) mixture. Bacterial growth was measured by optical density at 660 nm. During mass culture of the isolate, the 20-L gas-tight bottle contained 2.0 L of medium. Pre-cultivated bacterial cells [5%(v/v)] were inoculated into fresh medium, which was shaken or agitated at 200 rpm during the 5-day incubation.

2.4. Isolation of cell extract

Cell extract was prepared from bacterial cells cultivated at 30°C for 48 hr in basic medium containing an *n*-alkane or kerosene. The grown cells were harvested by centrifugation at 5,000×g and 4°C for 30 min and then washed twice with 50 mM Tris-HCl buffer (pH 7.5). The washed cells were disrupted with a bead beater at 4°C for 5 min. Cell extract was obtained from disrupted cells by centrifugation at 4°C and 15,000×g for 40 min; this extract was used as crude enzyme and SDS-PAGE samples.

2.5. SDS-PAGE

Total soluble proteins in the isolates were compared by SDS-PAGE. The SDS-PAGE technique used in the present study was adapted from Laemmli.[21)] Protein concentration was determined with Bradford reagent (BioRad) as the coloring agent and bovine serum albumin as the protein standard.

2.6. Assay of NADH or NAD^+-dependent enzyme activity

The alkane oxygenation activity in the cell extract was assayed spectrophotometrically at 340 nm by measuring the oxidation of NADH to NAD^+, coupled to the oxygenation of various substrates, *n*-butane, *n*-hexane, *n*-octane and *n*-decane. The dehydrogenase activity of the cell extract was assayed spectrophotometrically at 340 nm by measuring the reduction of NAD^+ to NADH, coupled to the oxidation of specific substrates by butanol dehydrogenase (BDH), butyraldehyde dehydrogenase (BLDH), pyruvate dehydrogenase (PDH), isocitrate dehydrogenase (iCDH) and malate dehydrogenase (MDH). 2.0 mM NADH (NAD^+) and 20 mM substrates were used in all enzyme experiments; 100 μM Coenzyme A was added to the reaction mixture for the PDH assay only. The specific activity is the concentration (μM) of NAD^+ or NADH produced, coupled to the oxygenation or oxidation of substrates, per minute and mg protein.

2.7. Analysis

n-Alkanes and *n*-alkanol were analyzed by gas-liquid chromatography (GLC, Varian 3400 star, USA) equipped with a flame ionization detector and DB-1 capillary column (30 m, 0.25-μm diameter, J&W Scientific, USA). A 2-ml sample was removed from the enzyme reactor by syringe, filtered with a membrane filter (pore size 0.22 μM), and injected into the GLC injector; the injection volume was adjusted to 1.0 μL. Acetic acid was used as an internal standard. The injector and detector temperatures were 150°C and 250°C, respectively. The column temperature was increased from 50°C to 100°C at 10°C per min and then increased to 300°C at 50°C per min to clean the column. All individual experiments were repeated three to five times with less than 5.0% deviation between replicates. *n*-Alkanes and *n*-alkanols were analyzed qualitatively and quantitatively by comparing retention time and peak area with standard materials.

3. Results and Discussion

3.1. Identification of bacterial isolate

98.9% of the 16S-rDNA sequence from the isolate was identified as that of *Alcaligenes* sp. Yen AY744384. The 16S-rDNA sequence was registered in the Genbank database system (www.ncbi.nlm.nih.gov/BankIt) under accession number DQ459259.

3.2. Growth of bacterial isolate in different alkanes

In the bacterial pathway for hydrocarbon metabolism, the initial metabolic oxidation of *n*-alkane is catalyzed by monooxygenase linked to NADH oxidation and by dehydrogenase linked to NAD^+ reduction; the alkane is oxidized to fatty acid and then degraded to acetyl-CoA through the ***b***-oxidation pathway.[22] As shown in Fig. 1, the growth of the bacterial isolate tended to be proportional to the number of carbons in the *n*-alkanes, and the growth pattern on kerosene (mixture of C_{10}~C_{16}) also showed that bacterial growth is related to carbon number. This result is very reasonable, because the energy potential of alkanes is proportional to the carbon number. However, growth on *n*-hexane was relatively lower than growth on other alkanes. *n*-Hexane may badly influence the cytoplasmic membrane structure since *n*-hexane is the smallest alkane that is a liquid at ambient temperature and therefore functions as a stronger solvent for lipid than the other *n*-alkanes. According to the Merck index, hexane has been used as a solvent to extract oilseeds and non-petroleum oil.[23]

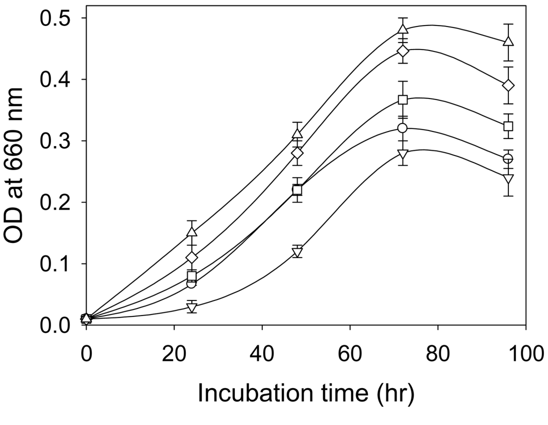

Fig. 1. Growth of the isolate on various *n*-alkanes: *n*-butane (○), *n*-hexane (▽), *n*-octane (□), *n*-decane (△) and kerosene (◇). Bacterial growth tended to be proportional to carbon number of the alkane, but growth on *n*-hexane (C_6 compounds) was lower than that on other alkanes.

3.3. Monooxygenase activity in cell extract

When crude enzyme extracted from bacterial cells grown on kerosene was used as a biocatalyst, *n*-butane, *n*-hexane, *n*-octane and *n*-decane were oxidized to *n*-butanol, *n*-hexanol, *n*-octanol and *n*-decanol, respectively, coupled to NADH oxidation to NAD$^+$, as shown in Table 1. These results indicate that the enzyme catalyzing *n*-alkane oxidation to *n*-alkanol is NADH-dependent, which has been defined as alkane monooxygenase.[5,24)] Stoichiometric imbalance between *n*-alkanol production and NADH oxidation could be caused by various factors, such as differences in the miscible ratio of *n*-alkanes with water, differences in the affinities of NADH and *n*-alkanes for the crude enzyme, and differences in the analytical methods for butanol (HPLC) and NADH (spectrophotometric) determination. The alkane monooxygenase catalyzes oxidation of the first carbon of the alkanes; we therefore supposed that the enzyme produced in bacterial cells grown on a single *n*-alkane could catalyze the oxidation of other *n*-alkanes at the same or similar level.[22)] However, as shown in Table 2, the monooxygenase activity of the cell extract was significantly higher when the substrate for the enzyme assay was the same as that used for growth than when the substrates differed. The monooxygenase activity of crude enzyme extracted from bacterial cells grown on an alkane mixture (kerosene) was obviously higher than the activity from bacterial cells grown on a single alkane, and was slightly higher when kerosene was used as a substrate for the enzyme assay than when other alkanes were used. It is very possible that the bacterial isolate produced different monooxygenases according to the alkane substrate used for growth. As shown in Fig. 2, the SDS-PAGE patterns of total soluble proteins extracted from bacterial cells grown on each single alkane or the alkane mixture differed greatly from each other, with the exception of several protein bands. This is clear evidence that alkane metabolism may be induced differently, according to the alkane species used as a growth substrate. Doughty *et al.* reported that butane monooxygenase in *P. butanovora* grown on even-chain-length alkane was repressed by the addition of

Table 1. Monooxygenase activity of crude enzyme from *Alcaligenes* sp. grown on kerosene for various *n*-alkanes. Protein concentration of cell extract was 6.8 mg/ml; NADH and substrate concentrations were adjusted to 2 mM and 20 mM, respectively. The oxygenase activity was determined by maximal NADH oxidation rates during a 60-min reaction, but butanol concentration was determined by the amount produced during a 6-hr reaction, in consideration of the GC detection limit.

Oxygenation activity	Substrates (products)			
	n-butane (*n*-butanol)	*n*-hexane (*n*-hexanol)	*n*-octane (*n*-octanol)	*n*-decane (*n*-decanol)
NADH oxidation to NAD$^+$ (mM min^{-1} mg protein^{-1})	6.97±0.42	5.45±0.23	4.85±0.18	5.15±0.21
n-Alkanols produced (mM)	1.73±0.11	0.69±0.05	1.54±0.07	1.70±0.09

Table 2. Alkane monooxygenase activity of crude enzyme extracted from *Alcaligenes* sp. grown on various alkanes. Monooxygenase activity was higher when the substrate for assay was the same as that for growth than when the substrates differed.

Growth substrate	Specific activity of monooxygenase for *n*-alkanes				
	n-Butane	*n* -Hexane	*n* -Octane	*n* -Decane	Kerosene
n-Butane	**6.97±0.42**	1.57±0.04	1.62±0.04	1.73±0.03	1.78±0.03
n -Hexane	1.52±0.05	**5.45±0.23**	1.1±0.03	1.63±0.04	1.68±0.03
n -Octane	1.33±0.04	1.27±0.02	**4.85±0.38**	1.46±0.06	1.76±0.04
n –Decane	1.00±0.02	0.54±0.01	0.96±0.03	**5.15±0.49**	1.14±0.04
Kerosene	5.6±0.37	4.74±0.32	4.76±0.39	5.00±0.12	**7.33±0.52**

Specific activity: Concentration (μM) of NADH reduced, coupled to enzymatic oxidation of alkane, per mg protein and min.

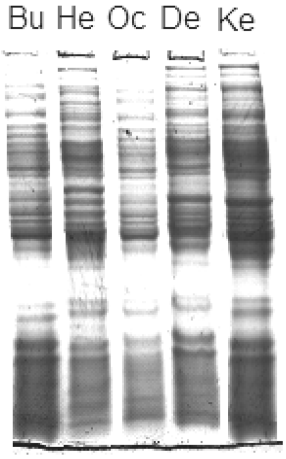

Fig. 2. SDS-PAGE patterns of total soluble protein extracted from *Alcaligenes* sp. grown in basal medium containing butane (Bu), hexane (He), octane (Oc), decane (De) and kerosene (ke) as the sole carbon source for 48 hr.

odd-chain-length alkanes such as propane, and propionate production by *P. butanovora* differed according to the substrates used for pre-cultivation and main cultivation.[11,14)] In the bacterial degradation test for 1,1-dichloroethene (DCE), 1,2-*cis* DCE and 1,2-*trans* DCE by butane-grown *P. butanovora*, the maximal degradation rate for 1,1-DCE was about seven times that for 1,2-*trans* DCE and about three times that for 1,2-*cis* DCE.[17,18,25,26)] The commonality between our results and those reported by other researchers is that the alkane monooxygenase induced by an alkane species used as a growth substrate catalyzes the oxidation of alkanes that differ from the growth substrate; however, the oxidation rates differ greatly according to the alkane species. Based on the database and experimental results, we propose that the alkane-oxidizing bacterium grown on the alkane mixture may produce different alkane monooxygenases that catalyze the oxidation of various alkanes at a higher level than the monooxygenase of bacterial cells grown on a single alkane. As shown in Table 2, more alkanol was produced by the crude enzyme extracted from bacterial cells grown on alkane mixture (Kerosene, C_{10}~C_{16}) than from those grown on single alkane.

3.4. Metabolic pathway for butane oxidation

Generally, alkane catabolism is known to depend on the pathway from alkane oxidation to alkanol, alkanol oxidation to alkanoic (lipoic acid) acid, and alkanoic acid to acetyl-CoA (b-oxidation). To design the catabolic pathway for butane, the specific activities of butane monooxygenase (BMO), butanol dehydrogenase (BDH), butylaldehyde dehydrogenase (BLDH), pyruvate dehydrogenase (PDH), lactate dehydrogenase (LDH), isocitrate dehydrogenase (iCDH) and malate dehydrogenase (MDH) were determined, as shown in Table 3. As expected, PDH and LDH activity was not detected, which is reasonable since pyruvate and lactate cannot be metabolic intermediates in the hydrocarbon oxidation pathway from *n*-alkanes to acetyl-CoA.[1,22)] The iCDH and MDH may serve as an index for metabolic pathway of *Alcaligenes* sp., since isocitrate and malate are specific metabolic intermediates in the TCA cycle. The activities of NAD^+-dependent dehydrogenases (iCDH and MDH) in the TCA cycle were lower than the activities of enzymes (BDH and BLDH) in the hydrocarbon oxidation pathway from butane to butyric acid. This difference is thought to be caused by the metabolic properties of the bacterium, which may be more dependent on the pathway from alkane to acetyl-CoA than on the TCA cycle for production of reducing power (NADH). To examine the serial metabolic pathways, growth of the isolate on glucose, pyruvate,

Table 3. Specific activities of enzymes functioning in butane oxidation pathway and TCA cycle of *Alcaligenes* sp. grown on kerosene. All enzymes are NADH- or NAD^+-dependent, which was easily assayed by spectrophotometry.

Key enzymes in butane oxidation pathway (μM mg protein^{-1} min^{-1})		Key enzymes in TCA cycle (μM mg protein^{-1} min^{-1})	
Butane monooxygenase	6.97±0.42	Pyruvate (lactate) dehydrogenase	Not detected
Butanol dehydrogenase	2.54±0.28	Isocitratedehydrogenase	0.51±0.03
Butyraldehyde dehydrogenase	3.78±0.31	Malatedehydrogenase	0.65±0.04

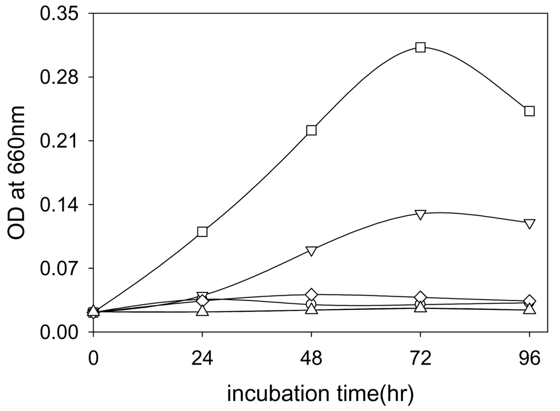

Fig. 3. Growth of *Alcaligenes* sp. on glucose (○), acetate (▽), lactate (△), *n*-butane (□) and pyruvate (◇).

lactate, acetate and *n*-butane was compared. As shown in Fig. 3, *Alcaligenes* sp. did not grow on glucose, pyruvate or lactate, but did grow on acetate and butane. We also tested the growth of *Alcaligenes* sp. on benzene, toluene and ethylbenzene, but no growth was observed (data not shown). Theoretically, acetate is not a metabolic intermediate in the pathway from alkane to acetyl-CoA, but it can be converted to acetyl-CoA by acetyl-CoA synthetase. Based on the enzyme activities and growth on hydrocarbons and carbohydrates, we proposed the catabolic pathway of alkane oxidation to CO_2 shown in Fig. 4.

4. Conclusion

Monooxygenase was produced by *Alcaligenes* sp., dependent upon the alkane used as the growth substrate; this monooxygenase catalyzed the oxidation of other alkanes at lower levels compared to the oxidation of the alkane used as the growth substrate. The alkane monooxygenase obtained from bacterial cells grown on the alkane mixture catalyzed alkane oxidation at a higher level than those grown on a single alkane. This report is a novel example of monooxygenase produced by a bacterium grown on alkane mixture having higher catalytic activity for alkane species that differ from the growth substrate than monooxygenase produced by a bacterium grown on a single alkane.

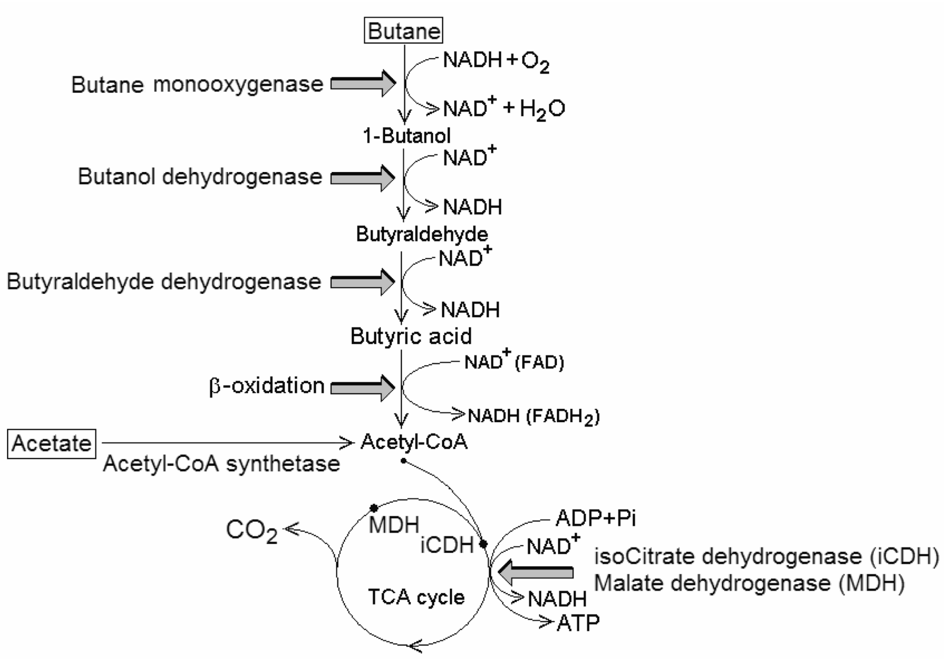

Fig. 4. Proposed metabolic pathway for butane oxidation to CO_2; this pathway was designed based on enzyme activity measurements from cell extracts and bacterial growth on *n*-butane.

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