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Microbial Oxidation of Hydrocarbons and Related Compounds by Whole-Cell Suspensions of the Methane-Oxidizing Bacterium H-2

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Previously, a thermophilic obligate methane-oxidizing bacterium, H-2 (type I), was isolated in our laboratory. H-2 is a new type of methylotroph because of the G+C content of DNA; it uses both the ribulose monophosphate pathway and the serine pathway for carbon assimilation and possesses a new quinone. In addition, we found that resting cell suspensions of H-2 had the ability to oxidize a variety of compounds different from the other methane-oxidizing bacteria as follows. (i) C_1 to C_8 *n*-alkanes are hydroxylated and further oxidized, yielding mixtures of the corresponding alcohols, aldehydes, acids, and ketones. Liquid alkanes are transformed through a different oxidative pathway from that of gaseous ones. (ii) Both gaseous (C_2 to C_4) and liquid (C_5, C_6) *n*-alkenes are oxidized to their corresponding 1,2-epoxides. (iii) Liquid monochloro and dichloro *n*-alkanes (C_5, C_6) are oxidized, yielding their corresponding acids or haloacids. (iv) Diethyl ether is oxidized to acetic acid; no ethanol and acetaldehyde are detected. (v) Cyclic and aromatic compounds are also oxidized. (vi) Secondary alcohols (C_3 to C_{10}) are oxidized to their corresponding methyl ketones.

Recently it has been pointed out by many investigators that the methane-oxidizing organisms have the ability to oxidize and transform a variety of non-growth substrates to useful products.

Methylococcus capsulatus (Bath) (type I, obligate) (3), *Methylosinus trichosporium* OB3b (type II, obligate (5), and *Methylobacterium organophilum* CRL-26 (type II, facultative) (15) oxygenate a particularly wide range of compounds. These include *n*-alkanes, *n*-alkenes, ether, alicyclic, and aromatic substrates. These oxygenative reactions are thought to be caused by methane monooxygenase (MMO). Although a variety of compounds were oxidized by whole cell suspensions of *Methylosinus trichosporium* OB3b (5), as well as by cell-free extracts (17), in the case of *Methylococcus capsulatus* (Bath) substrate specificity of whole cell suspensions was more restricted in comparison with that of cell-free extracts (18). *Methylococcus capsulatus* (Bath) differs substantially from *Methylosinus trichosporium* OB3b. The two species are representatives of the two main subdivisions of obligate methanotrophs; they differ morphologically and use fundamentally different carbon incorporation pathways.

Previously, we reported a new methane-oxidizing bacterium, H-2, isolated from a gas field (16). H-2 grows on only methane as the carbon and energy source, and the optimum temperature for growth is 50°C. Although the intracytoplasmic membrane arrangement is that of type I, H-2 uses both the ribulose monophosphate pathway and the serine pathway for carbon assimilation, and the G+C content of DNA is 58 mol%. (G.-J. Shen, Doctor's thesis, University of Tokyo, 1982). Recently a new quinone was found in H-2 (19). These facts indicate that H-2 occupies a new taxonomic position.

In this report, we describe the oxidation of a wide range of hydrocarbons by cell suspensions of H-2 and briefly summarize the properties of enzymes involved in carrying out these biotransformations.

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MATERIALS AND METHODS

Bacterial strain. A thermophilic, obligate methane-oxidizing bacterium H-2 isolated from a gas field (16) was used.

Cultivation of H-2. H-2 was grown at 50°C in 500-ml shaking flasks containing 50 ml of VCR or HP medium (2) with a gas mixture (CH_4-air-O_2-CO_2, 35:50:10:5). A large-scale culture was grown on methane (routinely 40 to 70% [vol/vol] in air) at 50°C in continuous culture at dilution rates of 0.25 to 0.30 dilutions per h in a 2-liter fermentor.

Activity assay. Cells harvested by centrifugation (5,000 × *g*, 10 min, 2°C) were washed twice with ice-cold 20 mM potassium phosphate buffer (pH 7.0) containing 5 mM $MgCl_2$. The final pellet was suspended in fresh buffer to obtain an optical density at 540 nm of 10. A 1.0-ml amount of this washed cell suspension was placed in 7-ml vials (2°C) that were sealed with rubber caps, and 0.1 ml of 40 mM sodium formate was added. In the case of gaseous substrates, 3.0 ml of the substrate was injected into vials. In the case of liquid substrates, a 2 to 50 μl of the substrate was put into vials. Vials were then incubated at 50°C on a reciprocating water bath shaker at 90 oscillations per min for 1 h. An appropriate control assay with heat-killed cells (boiled at 100°C for 30 min) was carried out. Protein was determined by the Hartree method (4).

A 2-μl sample was removed with a syringe and assayed with flame-ionized gas-liquid chromatography in a glass column (2 m by 3-mm inner diameter) packed with Chromosorb 101 (80/100 mesh) or Gaskuropack 54 (80/100 mesh), 5% polyethylene glycol 20M on 60/80 Chromosorb W AW DMCS, or 5% free fatty acid polyester on 60/80 Chromosorb G AW DMCS. The column temperature was maintained isothermally between 80 and 240°C with nitrogen carrier gas flow rates of 20 ml/min. A Model 5995A gas chromatography-mass spectrometry system (Hewlett-Packard Co.) was employed for confirmatory peak identification. A cross-linked methyl silicone capillary column (Hewlett-Packard) used for these analyses was 25 m by

TABLE 1. Oxidation of *n*-alkanes by cell suspensions of H-2

Substrate	Products (nmol formed per h per mg of protein)								
	1-ol	—CHO	—COOH	2-ol	2-on	3-ol	3-on	4-ol	4-on
Ethane	ND[a]	840	6,900						
n-Propane	ND	ND	1,200	1,500	3,000				
n-Butane	ND	ND	290	2,000	310				
n-Pentane	ND	ND	80	400	100	ND	ND		
n-Hexane	ND	ND	ND	30	20	10	ND		
n-Heptane	ND	ND	ND	10	5	3	ND	ND	ND
n-Octane	ND	ND	ND	5	ND	6	ND	2	ND

[a] ND, Not detected.

0.20-mm inner diameter. The amount of products formed was estimated from the peak area by using standard graphs which were constructed with authentic compounds.

Chemicals. All chemicals except 1,2-epoxypentane and 1,2-epoxyhexane were obtained from Tokyo Kasei Co. 1,2-Epoxypentane and 1,2-epoxyhexane were prepared from corresponding *n*-alkenes with *meta*-chloroperoxybenzoic acid. *meta*-Chloroperoxybenzoic acid (2.2 g) dissolved in 20 ml of chloroform was dropped into 10 mM of *n*-alkenes in 50 ml of chloroform with stirring at 50°C in an oil bath. After this reaction was completed, the solution was neutralized with 1 N NaOH. The chloroform layer was washed twice with ice-cold saturated $NaHCO_3$ solution and then dried by adding a little amount of anhydrous Na_2SO_4. The epoxide was separated from the substrate by silica gel chromatography.

RESULTS

Optimum temperature for the production of propylene oxide. The temperature optimum for the epoxidation of propylene by resting cell suspensions of H-2 was about 50°C (data not shown).

Oxidation of *n*-alkanes. *n*-Alkanes (C_1 to C_8 tested) were oxidized by cell suspensions of H-2. The oxidation products of methane were not identified, but the methane-dependent oxygen consumption was observed at the highest rates (11 μmol of O_2 consumed per h per mg of protein) among *n*-alkanes. Products from the oxidation of *n*-alkanes were not only alcohols but also their corresponding aldehydes, acids, and ketones (Table 1). In the case of gaseous *n*-alkanes at 50°C (C_2 to C_5), both terminal and internal oxidation was observed. However, in the case of liquid *n*-alkanes (C_6 to C_8), only the internal oxidation was observed.

Oxidation of *n*-alkenes. Cell suspensions of H-2 had broad specificity for *n*-alkenes (C_2 to C_6 tested; Table 2). Epoxides were produced not only from gaseous *n*-alkenes but also from liquid *n*-alkenes. Furthermore, about 40% as much of the epoxide was also produced as that produced at 50°C from liquid 1-pentene (boiling point, 39°C) when the reaction temperature was 30°C.

TABLE 2. Epoxidation of *n*-alkenes by cell suspensions of H-2

Substrate	Product	Activity (μmol formed per h per mg of protein)
Ethylene	Ethylene oxide	5.60
Propylene	Propylene oxide	6.00
1-Butene	1,2-Epoxybutane	3.87
cis-2-Butene	*cis*-2,3-Epoxybutane	2.35
trans-2-Butene	*trans*-2,3-Epoxybutane	1.05
Butadiene	1,2-Epoxybutene	5.13
1-Pentene	1,2-Epoxypentane	0.48
1-Hexene	1,2-Epoxyhexane	0.23

TABLE 3. Oxidation of ether, *iso*-butane, and cyclic and aromatic compounds by cell suspensions of H-2

Substrate	Products	Activity (nmol formed per h per mg of protein)
Diethyl ether	Acetic acid	5,340
iso-Butane	*iso*-Butanol	8
	iso-Butyraldehyde	23
	tert-Butanol	47
Cyclohexane	Cyclohexanol	5
	Cyclohexanone	30
Benzene	Phenol	73
Toluene	Benzyl alcohol	7
	Benzyl aldehyde	25
	Benzoic acid	8
	Cresol	5

Oxidation of ether, iso-butane, and cyclic and aromatic compounds. Cell suspensions of H-2 oxidized ethyl ether to acetic acid (Table 3). Ethanol and acetaldehyde were not detected, suggesting that H-2 has an efficient dehydrogenase system(s). H-2 also oxidized *iso*-butane, cyclohexane, benzene, and toluene. The oxidizing activities of these compounds were lower than those of *n*-alkanes.

Inhibition study. The production of propylene oxide by H-2 was inhibited by metal-binding, metal-chelating, and thiol reagents (Table 4). The effect of methane on the epoxidation of propylene and *cis*-2-butene was also studied. Methane inhibited the oxidations of propylene and *cis*-2-butene by resting cell suspensions of H-2.

Oxidation of haloalkanes. Resting cell suspensions of H-2 oxidized monochloro and dichloro C_5 and C_6 *n*-alkanes. And products from these haloalkanes were corresponding acids or haloacids, respectively (Table 5). This shows that dechlorination first occurs at a one-sided terminal.

Oxidation of secondary alcohols. Resting cell suspensions of H-2 oxidized secondary alcohols (C_3 to C_{10} tested) and produced the corresponding methyl ketones (Table 6). Among the secondary alcohols, 2-propanol was oxidized at the highest rate.

DISCUSSION

H-2 grew at a high growth rate (μ_{max} = 0.35/h) in comparison with the other obligate methane utilizers reported previously. This suggests that H-2 has an efficient methane-utilizing pathway and alcohol-oxidizing enzyme system(s), so that methane oxidants (for example, methanol) were not accumulated in the broth.

Both the system of *Pseudomonas aeruginosa* demonstrated by Van der Linden (20) and the system of *Pseudomonas oleovorans* demonstrated by Exxon group (1, 11, 12)

TABLE 4. Effect of inhbitors on the epoxidation of propylene[a]

Inhibitor	Concn (mM)	Inhibition (%)
Potassium cyanide	0.1	100
Acriflavine	1.0	95
Thiourea	1.0	58
8-Hydroxyquinoline	0.01	100
Dithiothreitol	1.0	47
2-Mercaptoethanol	1.0	97

[a] The reactions were conducted as described in the text. The products were estimated by gas chromatography after 1 h of incubation at 50°C.

epoxidized only liquid 1-alkenes from C_6 to C_{12} but not gaseous alkenes. In contrast to this, methane utilizers reported previously (obligate type I, *Methylococcus capsulatus* [Bath] and *Methylomonas methanica*; obligate type II, *Methylosinus trichosporium* OB3b; facultative type II, *Methylobacterium organophilum* CRL-26) epoxidized only gaseous *n*-alkenes (6). Recently, Hou et al. found the epoxidation of C_2 to C_6 *n*-alkenes by resting cell suspensions of propane-grown bacteria (7).

We found that the obligate methane-utilizing bacterium H-2 epoxidized not only gaseous *n*-alkenes but also liquid *n*-alkenes. In addition to this, the epoxidation of 1-pentene (boiling point, 39°C) was also observed when the reaction temperature was 30°C. This suggests that H-2 has a type of MMO that is different from that of the other methane utilizers reported previously.

With regard to the oxidation of *n*-alkanes, the oxidation pattern of *n*-alkanes that are gaseous at 50°C was different from that of *n*-alkanes that are liquid at 50°C. This suggests that the MMO of H-2 mainly oxidizes internal alkyl C-H bonds, although this MMO was thought to be both a terminal and an internal hydroxylase like that of the other methane utilizers. In the case of the terminal oxidation of *n*-alkanes, their corresponding acids were produced more than corresponding alcohols. This shows that H-2 has efficient alcohol-oxidizing enzymes.

Higgins et al. (5) reported the dechlorination of chorophenyl compounds by cell suspensions of *Methylosinus trichosporium* OB3B. Patel et al. (15) and Colby et al. (3) reported the dechlorination of chloromethane by soluble MMO from *Methylobacterium* sp. strain CRL-26 and *Methylococcus capsulatus* (Bath), respectively. But in the case of the oxidation of halobutane, products were not identified. In this paper, we reported the oxidation of monochloro and dichloro alkanes by cell suspensions of H-2 to their corresponding acids and haloacids respectively. This indicates that the dechlorination first occurs at a one-sided terminal.

The Exxon group (8, 13) found that C_1-utilizing microbes converted secondary alcohols to their corresponding methyl ketones and that the conversion was caused by the NAD-linked secondary alcohol dehydrogenase. The purified secondary alcohol dehydrogenase from *Pichia* sp. oxidized C_3 to C_9 secondary alcohols (14), but in the case of purified secondary alcohol dehydrogenase from bacteria (*Pseudomonas* sp. strain ATCC 21439) secondary alcohols longer than C_7 were not oxidized (9, 10). Both secondary alcohol dehydrogenases oxidized 2-butanol at the highest rate. In contrast to this, H-2 oxidized at least C_3 to C_{10} secondary alcohols to their corresponding methyl ketones and oxidized 2-propanol at the highest rate. Although NAD(P)H was effective as a cofactor for MMO activity of H-2 (Shen, thesis), secondary alcohols could not supply reducing power for MMO activity (H. Takigawa, T. Imai, G.-J. Shen, S. Nakagawa, T. Kodama, and Y. Minoda, manuscript in preparation). It is possible that H-2 possesses secondary alcohol-oxidizing enzyme(s) that are different from those of the other C_1-utilizing microbes, whose secondary alcohol dehydrogenase is linked to only NAD. Further information on the alcohol oxidation systems of H-2 will be published (Takigawa et al., in preparation).

TABLE 5. Oxidation of haloalkanes by cell suspensions of H-2

Substrate	Product	Activity (μmol formed per h per mg of protein)
1-Chloropentane	Pentanoic acid	3.5
1,5-Dichloropentane	5-Chloropentanoic acid	0.88
1-Chlorohexane	Hexanoic acid	3.0
1,6-Dichlorohexane	6-Chlorohexanoic acid	0.35

TABLE 6. Oxidation of secondary alcohols by cell suspensions of H-2

Substrate	Product	Activity (μmol formed per h per mg of protein)
2-propanol	Acetone	10.6
2-Butanol	2-Butanone	7.37
2-Pentanol	2-Pentanone	5.92
2-Hexanol	2-Hexanone	5.86
2-Heptanol	2-Heptanone	3.77
2-Octanol	2-Octanone	1.22
2-Nonanol	2-Nonanone	0.67
2-Decanol	2-Decanone	0.32

The thermophilic type I obligate methane-utilizing bacterium H-2, which occupies a new taxonomic position, has different oxidizing activities from the other methane-utilizing bacteria reported previously. Methane-utilizing bacteria produce not only nonspecific MMO but also other nonspecific oxidizing enzymes (for example, phenazine methosulfate (PMS)-linked methanol dehydrogenase, PMS-linked formaldehyde dehydrogenase, NAD-linked secondary alcohol dehydrogenase). It would be interesting from an industrial point of view to determine whether these nonspecific enzymes produce optically active compounds efficiently.

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