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Epoxidation of Short-Chain Alkenes by Resting-Cell Suspensions of Propane-Grown Bacteria

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Sixteen new cultures of propane-utilizing bacteria were isolated from lake water from Warinanco Park, Linden, N.J. and from lake and soil samples from Bayway Refinery, Linden, N.J. In addition, 19 known cultures obtained from culture collections were also found to be able to grow on propane as the sole carbon and energy source. In addition to their ability to oxidize *n*-alkanes, resting-cell suspensions of both new cultures and known cultures grown on propane oxidize short-chain alkenes to their corresponding 1,2-epoxides. Among the substrate alkenes, propylene was oxidized at the highest rate. In contrast to the case with methylotrophic bacteria, the product epoxides are further metabolized. Propane and other gaseous *n*-alkanes inhibit the epoxidation of propylene. The optimum conditions for in vivo epoxidation are described. Results from inhibition studies indicate that a propane monooxygenase system catalyzes both the epoxidation and hydroxylation reactions. Experiments with cell-free extracts show that both hydroxylation and epoxidation activities are located in the soluble fraction obtained after 80,000 × *g* centrifugation.

The ability of microorganisms to grow with propane as the source of carbon and energy was originally suggested by Tausz and Donath (25), in a "methanbakterium" that utilized the *n*-alkanes from methane to *n*-hexane as substrate. Since then, the widespread occurrence of propane utilizers in microbial populations has been speculated. Many propane utilizers, e.g., *Mycobacterium rhodochrous* (7, 24), *Mycobacterium convolution* (2, 4), and *Mycobacterium vaccae* (27) have been studied by Perry and his coworkers. McLee et al. (19) isolated 15 bacterial cultures that utilized ethane, propane, or *n*-butane as source of carbon and energy. These bacteria were considered to be members of two genera, *Brevibacter* and *Arthrobacter*.

The epoxidation of 1-alkenes was first demonstrated by Van der Linden (26), who detected the formation of 1,2-epoxyoctane from 1-octene by heptane-grown resting cells of *Pseudomonas aeruginosa*. Cardini and Jurtshuk (3) found that a cell extract of *Corynebacterium* sp. oxidized 1-octene to 1,2-epoxyoctane in addition to hydroxylating octane to octanol. The epoxidation of 1-octene by whole cells and a purified monooxygenase system of *Pseudomonas oleovorans* grown on *n*-octane were reported (1, 18). However, none of these epoxidation systems were found to be active on gaseous alkenes.

Previously, we described the epoxidation and hydroxylation of gaseous alkenes by resting-cell suspensions of methylotrophic microorganisms (9–13, 15–17, 20–23). Recently, we expanded our studies of gaseous hydrocarbon oxidation to include propane-utilizing microbes. Sixteen new cultures of propane-utilizing microbes were isolated. This paper describes the oxidation of short-chain alkenes to their corresponding 1,2-epoxides by resting-cell suspensions of these newly isolated cultures as well as by 19 known cultures. Optimum conditions for the epoxidation were studied in cell suspensions of propane-grown microbes, with particular reference to the epoxidation of propylene oxide. Subsequent studies revealed that the activity for both the epoxidation of propylene and the hydroxylation of propane was associated with the cell-free soluble fraction. A single enzyme system appears to be responsible for catalyzing both the hydroxylation of propane and the epoxidation of propylene.

MATERIALS AND METHODS

Bacterial strains. Propane-utilizing cultures were isolated from lake water from Warinanco Park, Linden, N.J. and from lake and soil samples from Bayway Refinery, Linden, N.J. These were classified according to *Bergey's Manual of Determinative Bacteriology*. The newly isolated cultures were deposited in the culture collection of Northern Regional Research Laboratories (Peoria, Ill.). The taxonomic characteristics of these cultures will be published elsewhere. Known cultures were obtained from the American Type Cul-

ture Collection (Rockville, Md.) and the Northern Regional Research Laboratories. These cultures, originally known to utilize liquid alkenes, were found to be able to grow on propane. The organisms were maintained on mineral salt plates in a dessicator jar under an atmosphere of propane and air (1:1, vol/vol) at 30°C. Organisms were grown at 30°C in 2.5-liter flasks containing 1 liter of mineral salt medium (8) with a propane-air mixture (1:1, vol/vol) as the sole carbon and energy source. Larger-scale cultures of propane-utilizing microbes were grown with aeration (7% propane, 93% air) at 30°C in a 30-liter fermentor (New Brunswick Scientific Co., Edison, N.J.) in a mineral salt medium (8). Growth was monitored continuously by a dissolved oxygen probe (New Brunswick Scientific Co.).

Chemicals. Gaseous alkanes, alkenes, and ethylene oxide were obtained from Matheson Gas Products (East Rutherford, N.J.). Epoxides, liquid alkanes and alkenes, and other chemicals were purchased from Matheson, Coleman and Bell, Norwood, Ohio.

Preparation of cell-free soluble fraction. Cells were harvested during exponential growth by centrifugation at 10,000 × *g* for 15 min at 4°C, washed twice with 25 mM potassium phosphate buffer (pH 7.0), and suspended in a small amount of the same buffer containing 5 mM $MgCl_2$ and DNase (0.05 mg/ml). Cell suspensions at 4°C were disintegrated by a single passage through a French pressure cell (American Instruments Co., Silver Spring, Md.) at 20,000 lb/in^2. Disintegrated cell suspensions were centrifuged at 15,000 × *g* for 15 min to remove unbroken cells. The supernatant solution was then centrifuged at 40,000 × *g* for 60 min, and the supernatant solution therefrom was again centrifuged at 80,000 × *g* for 60 min, yielding the soluble fraction.

Activity assay. When whole cells were used, the twice-washed cells were suspended in fresh 0.05 M potassium phosphate buffer to obtain an optical density at 660 nm of 0.5. A 0.5-ml portion of this washed cell suspension (containing a measured amount of dry cell mass) was placed in a 10-ml vial. The vial was sealed with a rubber cap to minimize evaporation. The gaseous phase of the vial was replaced with a gas mixture containing 50% gaseous alkene and 50% pure oxygen. In the case of liquid substrate, 5 μl of substrate was added. The reaction mixture was incubated at 30°C on a water bath rotary shaker (New Brunswick Scientific Co.) at 300 rpm. A 3-μl sample was removed with a syringe and was assayed by gas chromatography (11). The various epoxide products were identified by retention time comparisons and cochromatography with authentic standards. This identification was supplemented by observing the presence and absence of product peaks before and after bromination and hydrolysis. The amount of epoxide was determined from the peak area by using a standard curve which had been constructed with authentic epoxides. Duplicate measurements were performed for each assay. The error between these two measurements was less than 10%.

In the cell-free system, 3-ml vials contained 0.2 ml of a reaction mixture consisting of 10 μmol of potassium phosphate buffer, pH 7.0, 4 μmol of $NADH_2$, and a given amount of soluble fraction. The gaseous phase of the vial was replaced with a gas mixture of alkene and oxygen (1:1, vol/vol). In the case of a liquid oxidation substrate, 2 μl of substrate was added. The reaction was followed with gas chromatography by injecting 1- to 2-μl samples of the reaction mixture. Specific activity was expressed as μmoles of product formed per milligram of protein per 10 min (in the cell-free system) or per hour (in the whole-cell system).

RESULTS

Resting-cell suspensions of propane-grown cells of newly isolated cultures oxidized propylene to 1,2-epoxypropane, which accumulated (Table 1). After the reaction, the cell suspensions were centrifuged to remove the cells. The product propylene oxide was found totally in the supernatant fraction, indicating that the product epoxide accumulated extracellularly. Control experiments with heat-killed cells indicated that the epoxide was produced enzymatically.

The propylene oxide concentration reached a maximum after 2 h of incubation. After that, the concentration of propylene oxide decreased, indicating that a slow enzymatic oxidation of propylene oxide was occurring. The rate of

TABLE 1. Epoxidation of propylene by newly isolated propane-grown cultures

Microbe	Conversion rate (μmol/h per mg of protein) of propylene to propylene oxide
Mycobacterium sp. strain CRL51 = NRRL B-11322	0.62
Brevibacterium sp. strain CRL52 = NRRL B-11318	2.10
Pseudomonas sp. strain CRL53 = NRRL B-11329	1.20
Pseudomonas sp. strain CRL54 = NRRL B-11330	1.10
Nocardia sp. strain CRL55 = NRRL 11325	0.92
Brevibacterium sp. strain CRL56 = NRRL B-11319	2.40
Nocardia sp. strain CRL57 = NRRL 11326	2.0
Pseudomonas sp. strain CRL58 = NRRL B-11331	1.0
Arthrobacter sp. strain CRL60 = NRRL B-11315	2.5
Brevibacterium sp. strain CRL61 = NRRL B-11320	2.8
Mycobacterium sp. strain CRL62 = NRRL B-11323	0.50
Corynebacterium sp. strain CRL63 = NRRL B-11321	2.4
Nocardia sp. strain CRL64 = NRRL 11327	2.0
Pseudomonas sp. strain CRL65 = NRRL B-11332	0.4
Actinomyces sp. strain CRL66 = NRRL 11314	1.0
Acinetobacter sp. strain CRL67 = NRRL B-11313	1.2

propylene oxide production was linear for the first 60 min of incubation for all the strains tested. Therefore, epoxide production was compared within 1 h of incubation whenever the effect of a variable was tested.

Nineteen known liquid alkane-utilizing cultures selected from either ATCC or NRRL stocks were found to grow on propane as the sole carbon and energy source. Resting-cell suspensions of these cultures were also able to epoxidize propylene (Table 2).

Substrate specificity. The substrate specificity for the epoxidation of short-chain alkenes by newly isolated and known cultures of propane-grown bacteria was determined. Resting-cell suspensions of all of the strains tested epoxidized short-chain alkenes. The activity varied among strains (Table 2). Among short-chain alkenes, propylene was oxidized at higher rates.

Optimum conditions for the production of propylene oxide. (i) pH. The optimum pH for the production of propylene oxide by resting-cell suspensions of three selected strains of propane-grown microbes, *Brevibacterium* sp. strain CRL56, *Nocardia* sp. strain CRL57, and *Pseudomonas fluorescens* NRRL B-1244, was examined. Sodium phosphate buffer (0.05 M) was used for pH values from 5.5 to 8.0 and Tris buffer (0.05 M) was used for values from 8.0 to 10.0. A pH between 6.0 and 7.0 appeared to be optimum for epoxide production for all three strains tested. The initial and final pH readings in these experiments differed by less than 0.5 pH unit, and similar amounts of epoxide were produced in either buffer at pH 8.0. Authentic samples of propylene oxide (final concentration, 4 μmol/mol) were added to heat-killed cell suspensions of *Brevibacterium* sp. strain CRL56 at pH 5.5, 7.0, and 10.0 to test for nonenzymatic degradation of propylene oxide at these pH values. The propylene oxide concentration in these suspensions did not decrease during 3 h of incubation, indicating that nonenzymatic oxidation or hydrolysis of propylene oxide was negligible under these assay conditions.

(ii) Temperature. The temperature profile for the epoxidation of propylene by resting-cell suspensions of the three strains was tested in the range between 5 and 60°C. The optimum temperature found for the three strains was about 35°C. At 40°C, there was a decrease in the amount of epoxide accumulated.

(iii) Propylene and cell concentrations. Various concentrations of propylene were used to examine the production of propylene oxide by resting-cell suspensions of propane-grown *P. fluorescens* NRRL B-1244. The initial oxygen partial pressure in the gaseous phase was kept constant (50%, vol/vol). Argon gas was used to balance the rest of the gaseous phase. The amount of propylene oxide produced was assayed after 30 min of incubation. A propylene concentration, in the gaseous phase, of approximately 15% (66 μmol) supported maximum propylene oxide production. Higher propylene concentrations did not stimulate or inhibit the production of propylene oxide.

The cell concentration also influences the rate of propylene oxide production. The amount of propylene oxide accumulated after 30 min of incubation increased linearly as the cell concentration was increased up to about 12 mg/ml.

Stoichiometry of propylene oxidation. The stoichiometry of epoxidation of propylene by a cell suspension of propane-grown *P. fluorescens* NRRL B-1224 was examined. The amount of oxygen consumed during the reaction was determined polarographically with a Clark oxygen electrode. The propylene consumed and the propylene oxide formed were estimated by gas chromatography. The stoichiometry of consumption of propylene and consumption of oxygen and the production of propylene oxide at 15 min of incubation (in mole ratio) was found to be approximately 1.2:1.5:1.

Inhibition studies. The epoxidation of propylene to propylene oxide and the hydroxylation of propane to acetone by resting cell suspensions of propane-grown *P. fluorescens* NRRL B-1244 were inhibited by metal-binding and metal-chelating agents such as potassium cyanide, 1,10-phenanthroline, α,α-bipyridyl, thiourea, and imidazole (Table 3). This suggests the involvement of a metal ion(s) in both the epoxidation of propylene and the hydroxylation of propane.

The effect of propane on the epoxidation of propylene was studied to further determine whether a single enzyme system was responsible for the oxidation of both propylene and propane. The production of propylene oxide from propylene by resting-cell suspensions of propane-grown *P. fluorescens* NRRL B-1244 and *Brevibacterium* sp. strain CRL56 was assayed in the presence (gas-phase composition, propylene-propane-O_2, 25:25:50, vol/vol) or in the absence (gas-phase composition, propylene-argon-O_2, 25:25:50, vol/vol) of a given amount of propane. Argon was used to maintain the initial partial pressure of propylene. Results obtained after 15 min of incubation indicated that the epoxidation of propylene in both strains was inhibited about 55% in the presence of the hydroxylation substrate, propane. In addition, the epoxidation of propylene in both strains (about the same magnitude) was also inhibited by other gaseous *n*-alkanes: methane (46%), ethane (20%), and *n*-butane (55%).

Cell-free system. The epoxidation of 1-alkenes by propane-grown *Brevibacterium* sp. strain CRL56 was studied at the cell-free level. Cell-

TABLE 2. Epoxidation of short-chain alkenes to their corresponding 1,2-epoxides by resting-cell suspensions of various propane-grown bacteria

Strain	Epoxidation (μmol/h per mg of protein) of:					
	Ethylene to 1,2-epoxyethane	Propylene to 1,2-epoxypropane	1-Butene to 1,2-epoxybutane	1,3-Butadiene to 1,2-epoxybutene	1-Pentene to 1,2-epoxypentane	1-Hexane to 1,2-epoxyhexane
Arthrobacter petroleophagus ATCC 21494	0.41	0.32	0.36	0.59	0.10	0.06
Arthrobacter simplex ATCC 21032	0.47	0.35	0.22	0.24	0.01	0
Acinetobacter calcoaceticus ATCC 19140	0.50	0.91	0.06	0.07	0.04	0
Alcaligenes sp. strain ATCC 15525	2.60	0.57	0.15	0.11	0.04	0.02
Brevibacterium insectiphilium ATCC 15528	0.1	0.3	0.1	0.05	0.03	0
Brevibacterium sp. strain ATCC 14649	0.27	0.25	0.05	0.06	0.02	0.02
Brevibacterium fuscum ATCC 15993	0.31	1.8	0.63	0.34	0.12	0.06
Brevibacterium sp. strain CRL52 = NRRL B-11318	1.7	2.1	0.48	0.04	0.03	0
Brevibacterium sp. strain CRL56 = NRRL B-11319	0.98	2.4	0.28	0.03	0.02	0.02
Alcaligenes eutrophus ATCC 17697	0.40	0.40	0.08	0.02	0.01	0
Mycobacterium album ATCC 29676	0.36	0.36	0.16	0.08	0.05	0.01
Mycobacterium paraffinicum ATCC 12670	0.10	0.11	0.04	0.01	0.01	0
Mycobacterium rhodochrous ATCC 29670	0.30	0.20	0.15	0.08	0	0
Mycobacterium rhodochrous ATCC 26972	0.30	0.40	0.07	0.06	0.02	0.02
Mycobacterium sp. strain CRL51 = NRRL B-11322	0.52	0.62	0.25	0.07	0.03	0
Nocardia neoopaca ATCC 21499	0.07	0.37	0.68	0.70	0.03	0
Nocardia paraffinica ATCC 21198	1.46	4.30	4.0	3.20	0.11	0.03
Nocardia sp. strain CRL55 = NRRL 11325	0.20	0.92	0.68	0.42	0.02	0
Nocardia sp. strain CRL57 = NRRL 11326	0.26	2.0	1.2	0.98	0.08	0.01
Pseudomonas crucaurae NRRL B-1021	0.57	0.60	0.89	0.66	0	0
Pseudomonas fluorescens NRRL B-1244	1.70	6.10	0.82	0.33	0.07	0.03
Pseudomonas multivorans ATCC 17515	0.40	0.79	0.38	0.19	0.05	0.03
Pseudomonas putida ATCC 17453	0.44	1.90	0.68	0.77	0.05	0.01
Pseudomonas ligustri ATCC 15522	0.46	0.48	0.34	0.69	0.04	0.02
Pseudomonas sp. strain CRL53 = NRRL B-11329	1.00	1.20	0.92	0.03	0.04	
Pseudomonas sp. strain CRL54 = NRRL B-11330	0.90	1.10	0.90	0.02	0.02	
Pseudomonas sp. strain CRL58 = NRRL B-11331	0.80	1.00	0.80	0.02	0.02	

TABLE 3. Effect of inhibitors on epoxidation of propylene and hydroxylation of propane

Inhibitor (1 mM)	% Inhibition			
	Pseudomonas fluorescens NRRL B-1244		*Brevibacterium* sp. strain CRL56	
	Epoxidation	Hydroxylation	Epoxidation	Hydroxylation
Potassium cyanide	90	90	90	95
1,10-Phenanthroline	95	100	100	100
α,α-Dipyridyl	75	80	80	85
Thiourea	80	90	85	90
Imidazole	100	100	100	100

free extracts and soluble fractions were prepared according to the procedure described above. Epoxidation activity was found in the 40,000 × *g* and 80,000 × *g* soluble fractions. Results obtained from the epoxidation studies on alkenes (including straight and branched chain) and styrene by the 80,000 × *g* soluble fraction are summarized in Table 4.

Various cofactor systems were examined for the epoxidation of propylene. When $NADPH_2$ replaced $NADH_2$, about 50% of the epoxidation activity was observed. Other cofactors tested, including NAD^+, phenazine, methosulfate, potassium ferricyanide, cytochrome *c*, 2,6-dichlorindophenol, flavin-adenine dinucleotide, and ascobic acid, all failed to support the reaction.

The optimum pH for the production of propylene oxide by a cell-free soluble fraction of propane-grown *Brevibacterium* sp. strain CRL56 was also studied, using the same buffer solutions described for the resting-cell system. A pH between 6.0 and 7.0 was found to be optimum for propylene oxide production by the cell-free enzyme system.

The epoxidation-hydroxylation enzyme system of propane-grown bacteria was rather stable. The cell-free soluble fraction lost only 10% of its epoxidation activity after having been stored at 4°C for 1 week.

DISCUSSION

Resting-cell suspensions of methane-grown methylotrophic bacteria epoxidized C_2 to C_4 gaseous alkenes (11), in contrast to both the *P. aeruginosa* system of Van der Linden (26) and the *P. oleovorans* system demonstrated in our laboratory (1, 18), which epoxidized liquid 1-alkenes from C_6 to C_{12}, but not gaseous alkenes.

In this report, we describe the epoxidation of short-chain alkenes by resting-cell suspensions of propane-grown bacteria. The product 1,2-epoxides accumulated extracellularly. The nonenzymatic degradation of propylene oxide in our standard assay system was not significant even after 5 h of incubation. During the epoxidation of propylene by cell suspensions of methane-utilizing bacteria, no formation of 3-hydroxy-1-propene was detected, and the propylene oxide was not further metabolized enzymatically (11). In the case of 1,2-epoxyoctane production from 1-octene by heptane-grown cells of *Pseudomonas* sp., Van der Linden (26) stated that the epoxide was not further oxidized enzymatically. However, May and Abbott (18) reported that when 1-octene was supplied as a substrate for the ω-hydroxylation enzyme system of *P. oleovorans*, both 8-hydroxy-1-octene and 1,2-epoxyoctane were formed. In addition, Abbott and Hou (1) found that the methyl group of the latter compound was also susceptible to hydroxylation. The present results obtained from time course and stoichiometry studies indicated that propylene oxide was further metabolized enzymatically. A 1:1:1 ratio of consumption of propylene to consumption of oxygen to the production of propylene oxide was not observed, and a trace amount of 3-hydroxy-1-propene was detected, indicating that propylene was oxidized both at the double bond and at the methyl group. This is analogous to the findings of Van der Linden (26) and May and Abbott (18) in the oxidation of 1-octene, but differs from the oxidation of propylene by methylotrophic bacteria, where oxidation of the methyl group was not detected.

The octane monooxygenase system epoxidizes 1-octene and hydroxylates *n*-octane (1, 3). The methane monooxygenase system also oxidizes both methane and propylene (5, 11, 16, 23). However DeBont et al. (6) reported that the

TABLE 4. Epoxidation of alkenes and styrene by soluble fraction (80,000 × *g* centrifugation) of propane-grown *Brevibacterium* sp. strain CRL56

Substrate	Product	Rate of product formation (μmol/10 min per mg of protein)
Ethylene	Ethylene oxide	0.40
Propylene	Propylene oxide	0.52
1-Butene	1,2-Epoxybutane	0.30
1,3-Butadiene	1,2-Epoxybutene	0.44
Isobutene	Epoxyisobutene	0.46
cis-But-2-ene	*cis*-2,3-Epoxybutane	0.18
trans-But-2-ene	*trans*-2,3-Epoxybutane	0.22
1-Pentene	1,2-Epoxypentene	0.025
1-Hexene	1,2-Epoxyhexane	0.008
Styrene	Styrene oxide	0.005

ethane hydroxylation enzyme and the ethylene epoxidation enzyme were different. With the propane-grown bacteria in this study, both the epoxidation of propylene and the hydroxylation of propane were inhibited by various metal-binding and metal-chelating agents (Table 3), indicating the involvement of a metal(s)-containing enzyme system(s). The inhibition pattern and magnitude were the same for both epoxidation and hydroxylation. These results are consistent with the possibility that the propane monooxygenase system catalyzes both the hydroxylation and epoxidation reactions. The epoxidation of propylene to propylene oxide by cell suspensions of propane-grown *P. fluorescens* NRRL B-1244 and *Brevibacterium* sp. strain CRL56 was also inhibited in the presence of hydroxylation substrates (gaseous *n*-alkanes). These data further support this notion.

Although primary alcohols were not detected by our gas chromatographic assay conditions for the hydroxylation of alkanes by propane-grown bacteria, they were indeed produced and were rapidly metabolized further into aldehyde and acid (14).

The optimum conditions of pH and temperature for the in vivo epoxidation of propylene by various propane-grown bacteria are quite similar. The apparent decrease in epoxidation above 40°C may be due to both the depletion of cofactor and the volatility of the product propylene oxide (boiling point, 35°C.).

In the cell-free system, both the hydroxylation and epoxidation activities were located in the soluble fraction obtained after 80,000 × *g* centrifugation. It requires the presence of a cofactor, NADH. Compared to the methane monooxygenase system, propane monooxygenase is relatively stable. It can be stored in a freezer for several weeks without losing activity, whereas methane monooxygenase requires storage in liquid nitrogen.

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