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Production of epoxides from gaseous alkenes by resting-cell suspensions and immobilized cells of alkene-utilizing bacteria

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Summary. Newly isolated and already available strains of alkene-utilizing bacteria were able to oxidize ethene, propene or 1-butene to the respective 1,2-epoxides. Resting-cell suspensions of organisms isolated on propene and butene, when grown on these substrates converted ethene quantitatively to epoxyethane. Some, but not all ethene-utilizing strains accumulated 1,2-epoxypropane or 1,2-epoxybutane when propene or butene was supplied, although not quantitatively because the epoxides produced were partially further metabolized. Suitable epoxide producers which eventually may be employed as biocatalysts in a biotechnological process were used for immobilization in calcium alginate and *K*-carrageenan; after immobilization, 60%–100% activity for epoxide production was retained.

Introduction

The biological formation of epoxides from aliphatic alkenes has been studied in many micro-organisms often with the objective of exploring possible biotechnological applications. Most of these studies involved alkane-utilizing bacteria as, for instance, heptane-grown *Pseudomonas oleovorans* that converted 1-octene to 1,2-epoxyoctane (van der Linden 1963), or octane-grown *Pseudomonas oleovorans* cells that formed epoxides from alkenes and alkadienes (Abbott and Hou 1973; de Smet et al. 1981; de Smet et al. 1983). Epoxide formation by these organisms is due to the non-specific action of an alkane-hydroxylase that is able to form alcohols from alkanes and epoxides from alkenes. Other alkane-utilizing bacteria known to excrete epoxides from alkenes in this manner include a *Corynebacterium* sp. (Cardini and Jurtshuk 1979), propane-utilizing bacteria (Hou et al. 1983), ethane- and butane-utilizing bacteria (Patel et al. 1983) and, most remarkably, methane-oxidizing bacteria (Higgins et al. 1979; Stirling and Dalton 1979; Hou et al. 1979; Hou et al. 1983). Production of epoxides by micro-organisms capable of utilizing gaseous alkenes like ethene and propene as the sole source of carbon and energy has only been detected in a few cases to date. For instance, Furuhashi et al. (1981) found that cells of *Nocardia corallina* B-276 accumulated 1,2-epoxypropane during growth on propene while de Bont et al. (1983) reported that resting-cell suspensions of propene-grown *Mycobacterium* Py1 produced epoxyethane from ethene.

In recent years, this epoxidation of alkenes by bacteria has received considerable attention because of a possible production of certain epoxides by means of a biotechnological process. In such a process, epoxides might be obtained using either growing cells (de Smet et al. 1981; de Smet et al. 1983) or immobilized whole cells (Higgins et al. 1980) while the use of immobilized enzymes has also been considered (Parkinson 1980). In a biotechnological project aimed at producing epoxides such as epoxyethane or 1,2-epoxypropane in either a gas-solid (de Bont et al. 1983) or a multi-phase bioreactor (de Bont et al. 1981), we decided to use alkene-utilizing Mycobacteria (de Bont et al. 1980) rather than alkane-utilizing bacteria, firstly, because Mycobacteria possess a mono-oxygenase which is specific for alkenes (de Bont et al. 1979). This alkene mono-oxygenase will not hydroxylate alkanes, whereas most alkane mono-oxygenases may epoxidate as well as hydroxylate higher alkenes. Secondly, due to the composition of the cell wall (de Bont et al. 1980) the Mycobacteria are tough organisms, so it can be expected that they will be relatively stable in the presence of an organic solvent such as is employed in our multi-phase bioreactor (de Bont et al. 1981). In

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the present paper we describe the formation of epoxides from gaseous short-chain alkenes by free and immobilized cells of several alkene-utilizing Mycobacteria.

Materials and methods

Chemicals. Gaseous hydrocarbons (commercial purity) and epoxyethane were obtained from the Matheson company, East Rutherford, NY. Liquid alkanes and 1-alkenes, 1,2-epoxypropane and 1,2-epoxybutane were from Merck Schuchardt, sodium alginate (Manucol DM) and sodium carrageenan (Genugel type x-0828) from Alginate Industries, London UK and The Copenhagen Pectin Factory Ltd. Denmark, respectively. NADH, NAD^+ and CoA were purchased from Boehringer, Mannheim.

Microorganisms. The isolation and description of strains E20, 32, E44, Eu1, Eu3, and Py1 has been reported earlier (de Bont, 1976, de Bont et al. 1980), while strains Tu1, 12D, E3, E4, 2W, Py8, By1, and B were isolated by similar methods except for By1 and B where ethene or propene was replaced by 1-butene and 1,3-butadiene, respectively.

Cultivation of the organisms. Strains were cultivated in a mineral medium, supplemented with the appropriate gaseous alkene or alkane as described by Wiegant and de Bont (1980). Strains 2W, E3 and E4 used for immobilization studies were grown semi-continuously in mineral medium in a 3 dm^3 air-lift fermentor at a dilution rate of 0.015 h^{-1} at 30° C. Ethene was supplied to the culture by passing air containing 2.5% ethene at a rate of 1 dm^3/min through the vessel. The pH of the culture was maintained at 7.0. Growth on different carbon sources was determined at 30° C in 100 cm^3 Erlenmeyer flasks containing 10 cm^3 mineral medium supplemented with the appropriate carbon source. The concentration of the gaseous hydrocarbons supplied was 5% (v/v), while the concentration of liquid hydrocarbons and ordinary carbon sources was 0.2% (w/w). Hydrogen and carbon dioxide gas were supplied in a 3 : 1 ratio. Erlenmeyers without a carbon source served as controls.

Doubling times of the various strains were determined as follows: Cells were inoculated in 300 cm^3 side-arm Erlenmeyer flasks containing 30 cm^3 mineral medium. Flasks were closed with rubber stoppers with suba seals through which the appropriate gaseous alkene was injected (5% v/v). Erlenmeyers were incubated at 30° C on a rotary shaker. Growth of the strains was followed by measuring cell-density with a nephelometer. Doubling times were calculated from the growth curves obtained.

Analysis. Gaschromatographic determination of gaseous hydrocarbons and epoxides have been described previously (de Bont et al. 1979; Wiegant and de Bont 1980). The oven temperature used was 180° C.

Protein in cell suspensions and cell-free extracts was determined with the Folin-Ciocalteu reagent (Merck) as follows: 0.5 cm^3 of a cell suspension was diluted with 0.5 cm^3 1 M NaOH and boiled for 30 min. Cell-free extracts and the boiled cell suspensions were diluted appropriately with water to a final volume of 1 cm^3. Next, 2.5 cm^3 of a solution consisting of 50 cm^3 5% (w/w) Na_2CO_2, 1 cm^3 1% (w/w) $CuSO_4 \cdot 5\,H_2O$ and 1 cm^3 2% (w/w) NaKtartrate was added. After 15 min 0.5 cm^3 of the Folin-Ciocalteu (Merck) was added and after 30 min the extinction of the solutions was measured at 665 nm with a Vitatron photometer.

Oxidation of gases and epoxides by whole-cell suspensions and cell-free extracts. Preparation of washed cells and of cell-free extracts have been described previously (de Bont and Harder 1978; de Bont et al. 1979). Oxidation of hydrocarbons and epoxides and excretion of epoxides by washed cell suspensions were measured by incubating cells at 30° C in 50 mM phosphate buffer, pH 7.2 (final volume 3 cm^3) in 27 cm^3 screw cap bottles. The appropriate gas (0.5 cm^3) or 0.6 cm^3 of a 10 mM epoxide solution in 50 mM phosphate buffer was injected into the bottle. Stock solutions of epoxides in buffer were made daily prior to use. Methane (0.5 cm^3) was injected to serve as an internal standard. Samples from the gas phase were withdrawn at regular intervals for gaschromatographic analysis. Oxidation reactions with cell-free extracts were carried out as described previously (de Bont and Harder 1978; de Bont et al. 1979).

Immobilization procedures. In alginate: 10 cm^3 cell suspension (~15 mg protein/cm^3 0.9% NaCl) was thoroughly mixed with 20 cm^3 sodium alginate solution (30 g/dm^3 0.9% NaCl). The mixture was extruded through a needle into a 0.1 M $CaCl_2$ solution, resulting in the formation of calcium alginate beads. To avoid the interference of diffusional effects with the activity measurements, the size of the beads was kept small (~0.8 mm). The beads were stirred in a fresh, aerated solution of 0.1 M $CaCl_2$ for 1 h, after which the activity was determined.

In carrageenan: the procedure for immobilization in calcium alginate was also used for immobilization in potassium carrageenan but with the following modifications:

1) the cell suspension was mixed with 20 cm^3 sodium carrageenan solution (25 g/dm^3 distilled water);
2) the beads were extruded in a cold 2% (w/w) $CaCl_2 \cdot 2\,H_2O$ solution;
3) after 20 min this solution was replaced by a cold 2% (w/w) KCl solution, in which the beads were stirred for 30 min after which the activity was measured.

Oxidation of propene by immobilized cells. The propene oxidation rate and 1,2-epoxypropane accumulation rate by cells immobilized in alginate or carrageenan was measured in a recirculation reactor. A gas phase consisting of 1.5 dm^3 air (excess oxygen) and 10 cm^3 propene initially was brought into contact with the biocatalyst beads suspended in ~220 cm^3 0.05 M $CaCl_2$ solution in the case of alginate immobilization and in ~220 cm^3 0.05 M potassium phosphate buffer pH 7.0 in the case of carrageenan immobilization (total volume of the beads 30 cm^3). The reaction course was batch-wise because the gas leaving the $CaCl_2$ solution was completely recycled. Gas phase concentrations of propene and 1,2-epoxypropane were measured automatically every 30 min using a gas sampling valve. The column used was Porapak Q and the oven temperature used was 200° C. Experiments with comparable amounts of free cells suspended in ~250 cm^3 0.05 M potassium phosphate buffer pH 7.0 made it possible to establish the influence of the immobilization on the oxidation rate.

Results and discussion

Isolation, description and growth characteristics of the organisms

Bacteria able to grow on either ethene, propene or butene were isolated from various soil and water samples employing conventional enrichment and isolation techniques. Five strains were isolated on ethene (Tu1, 2W, 12D, E3, and E4), one strain on propene (Py8), one on 1-butene (By1) while another strain was isolated on 1,3-butadiene (B). Strains 2W,

Table 1. Growth of alkene-utilizing bacteria on several carbon sources

Strains tested	Substrate isolated on	Doubling time (h) on isolation substrate	Substrates tested												
			Ethene	Propene	1-butene	1-pentene	1-hexene	Epoxyethane	1,2-epoxypropane	1,2-epoxybutane	Ethane	Propane	n-butane	n-hexane	H_2/CO_2
E3	Ethene	8	+	−	−	−	−	+	−	−	−	−	−	−	−
E4	Ethene	8	+	−	−	−	−	+	−	−	−	−	−	−	−
E44	Ethene	18	+	−	−	−	−	+	−	−	−	−	−	−	−
Eu1	Ethene	19	+	−	−	−	−	+	−	−	−	−	−	−	−
Eu3	Ethene	ND	+	+	−	−	−	+	+	−	−	−	−	−	−
2W	Ethene	15	+	−	−	+	+	+	−	−	−	−	−	+	+
12D	Ethene	18	+	+	+	−	+	+	+	+	−	−	−	+	−
32	Ethene	ND	+	−	−	+	+	+	−	−	−	−	−	−	−
Tu1	Ethene	11	+	−	−	−	−	−	+	−	−	−	−	−	+
E20	Ethene	28	+	+	+	+	+	+	+	+	+	+	+	+	−
Py1	Propene	27	−	+	+	−	+	−	+	+	−	−	−	+	+
Py8	Propene	15	−	+	+	−	+	−	+	+	−	−	−	+	+
By1	1-butene	10	−	+	+	−	−	+	+	+	−	−	−	−	−
B	1,3-butadiene	14	−	−	−	−	−	+	−	−	−	−	−	−	−

All strains grew on n-decane, n-hexadecane, glucose and succinate while none grew on methane. Growth (+) or no growth (−) was determined after 4 weeks of incubation at 30° C

12D, Tu1, E3, E4, Py8, and By1 strongly resembled previously isolated alkene-utilizing Mycobacteria (de Bont et al. 1980) and were accordingly classified as species of the genus *Mycobacterium,* while strain B was tentatively classified as a species of the genus *Nocardia.* The eight newly isolated bacteria together with six previously described (de Bont 1976; de Bont et al. 1980) ethene-utilizing bacteria (E20, 32, E44, Eu1, and Eu3) and the propene-utilizing bacterium Py1 (de Bont et al. 1983) were used in the investigations described below.

The alkene-utilizing bacteria were tested for growth on saturated and unsaturated hydrocarbons as well as on other compounds (Table 1). Most ethene-isolates did not grow on propene or 1-butene. The propene and butene isolates grew on propene as well as on butene but not on ethene. The higher alkenes 1-pentene and 1-hexene were utilized by most strains while higher saturated hydrocarbons were good substrates for all strains tested. The gaseous alkanes, with the exception of strain E20, did not support growth. Growth of the bacteria on epoxides was correlated with growth on the corresponding gaseous alkenes. That is, ethene-isolated bacteria in general would grow on epoxyethane but not on 1,2-epoxypropane and 1,2-epoxybutane, whereas propene isolates and the butene isolate grew on 1,2-epoxypropane and 1,2-epoxybutane but not on epoxyethane. Ordinary carbon and energy sources like glucose and succinate supported growth of all strains. Some organisms were also able to grow autotrophically using hydrogen gas as energy source (Table 1).

Culture doubling times of various strains (Table 1) were determined while growing on the alkene upon which the bacteria were originally isolated. The ethene utilizers had doubling times ranging from 8 to 28 h, the propene utilizers doubled in 27 respectively 15 h, the butene utilizer in 10 h, while the butadiene isolate doubled in 14 h.

Excretion of epoxides by variously grown washed cell suspensions

Resting-cell suspensions of all alkene-utilizing strains studied, irrespective of the alkene utilized as growth substrate, were able to oxidize ethene, propene and butene, although at low rates varying from 6 to 50 nmoles of alkene oxidized per minute per mg protein (Table 2). These activities for ethene, propene and butene are of the same order of magnitude as reported values for alkene oxidation rates by other organisms as for instance methane and propane-utilizing bacteria (Table 3). Oxidation rates for epoxides by the alkene-utilizers were likewise low, but here for some strains the number of carbon atoms of the epoxide molecule had a pronounced effect on the

Table 2. Oxidation of alkenes and epoxides, and accumulation of epoxides by washed suspensions of alkene-grown cells

Strain	Growth substrate	Specific oxidation rate[a]						Product accumulation rate[a]		
		Ethene	Propene	1-butene	Epoxy-ethane	1,2-epoxy-propane	1,2-epoxy-butane	Epoxy-ethane	1,2-epoxy-propane	1,2-epoxy-butane
E3	Ethene	50	17	15	49	2	3	0	16	14
E4	Ethene	39	14	12	40	3	4	0	12	9
E44	Ethene	39	13	14	19	2	1	3	11	11
Eu1	Ethene	34	12	16	34	0	1	0	12	15
Eu3	Ethene	27	11	ND	27	10	ND	0	0	ND
2W	Ethene	23	6	6	14	1	3	4	5	3
12D	Ethene	37	18	23	27	13	14	3	8	13
32	Ethene	9	6	ND	8	4	ND	1	3	ND
Tu1	Ethene	23	6	6	19	2	3	3	4	3
E20	Ethene	16	7	ND	19	10	ND	0	0	ND
E20	n-butane	16	20	ND	1	3	ND	15	17	ND
Py1	Propene	15	20	17	0	19	16	15	0	0
Py8	Propene	25	30	24	0	28	21	25	0	0
By1	1-butene	19	23	26	0	17	24	19	0	0
B	1,3-butadiene	14	28	25	0	0	0	11	21	17

[a] nmoles · min^{-1} · mg $protein^{-1}$
ND = not determined

Table 3. Reported oxidation rates of gaseous alkenes by methane-utilizing, ethane-utilizing, propane-utilizing and butane-utilizing bacteria

Micro-organism	Growth substrate	Alkene oxidation rate[a]			Reference
		Ethene	Propene	1-butene	
Methylotrophic bacteria	Methane	7.5–45.8	8.3–45.8	7.5–37.5	Hou et al. (1979)
Methylosinus trichosporium OB3b	Methane	–	30–60	–	Higgins et al. (1979)
Methylococcus capsulatus (BATH)	Methane	8.0 (44)[b]	6.0 (44)	– (46)	Stirling and Dalton (1979)
Various bacteria	Ethane	8.1–17.8	15.0–28.3	2.0–4.0	Patel et al. (1983)
Various bacteria	Propane	1.7–43.3	5.3–102	0.7–15.3	Hou et al. (1983)
Various bacteria	n-butane	8.4–38.4	8.4–58.3	1.7–31.7	Patel et al. (1983)

[a] nmoles · min^{-1} · mg protein
[b] Values in parentheses refer to activities obtained after addition of 4 mM formaldehyde

oxidation rate. Several, but not all of the ethene-utilizing bacteria oxidized 1,2-epoxypropane and 1,2-epoxybutane at reduced rates compared with the oxidation rate of epoxyethane. The reverse situation existed in the propene and butene-grown bacteria in that they oxidized the C3 and C4 epoxide, but not epoxyethane.

Previous studies on alkene-utilizing bacteria have shown that ethene and propene are oxidized by a mono-oxygenase (de Bont et al. 1979), while epoxyethane may be further metabolized by a NAD^+ and CoA-dependent epoxide dehydrogenase (de Bont and Harder 1978; Wiegant and de Bont 1980). The nature of the enzyme involved in epoxypropane metabolism is not yet known (de Bont et al. 1983). Mono-oxygenase activity for ethene, propene and butene, and epoxide dehydrogenase activity for epoxyethane, 1,2-epoxypropane and 1,2-epoxybutane were tested in vitro for strains 2W, E44, E3, and Py8. The strains examined possessed a NADH-dependent mono-oxygenase that, as anticipated from the whole cell experiments, oxidized ethene as well as propene and butene (Table 4). Epoxide dehydrogenase was present in extracts of the ethene-utilizing bacteria tested and the specific activity of the enzyme strongly depended on the epoxide that was present as a substrate (Table 4). The specific activities of the mono-oxygenase and epoxide dehydrogenase in extracts are very low, also when compared with activities obtained with whole cells. Nevertheless, it may be concluded from the in vitro substrate specificities as observed for the mono-oxygenase and epoxide dehydrogenase that the behaviour of whole cells towards alkenes and epoxides is a consequence of the substrate specificities of these enzymes.

As a consequence of the differences observed in activity of whole cells for alkenes and epoxides, some strains excreted epoxides from alkenes. Figure 1

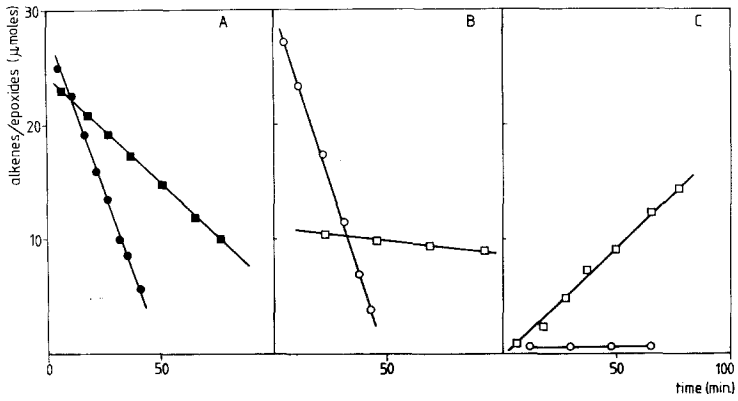

Fig. 1. Oxidation of alkenes **(A)** and epoxides **(B)**, and excretion of epoxides **(C)** by washed cell suspensions of ethene-grown cells of strain E3. Rates of oxidation and accumulation by the resting cells (all incubations 22 mg protein in 3 cm^3 50 mM phosphate buffer pH 7.2) were measured in 27 cm^3 screw-cap bottles at 30° C. ●–● ethene; ■–■ propene; ○–○ epoxyethane; □–□ 1,2-epoxypropane

Table 4. Oxidation of alkenes and epoxides by cell-free extracts of alkene-grown bacteria

Substrates[a]	Organisms[b]			
	2W	E44	E3	Py8
Ethene	0.37	1.55	0.19	2.54
Propene	0.18	1.10	0.11	2.91
1-butene	0.14	0.53	0.09	2.60
Epoxyethane	0.26	1.00	0.22	ND
1,2-epoxypropane	0.05	0.03	0.08	ND
1,2-epoxybutane	0.07	ND	0.09	ND

[a] nmoles · min^{-1} · mg protein
[b] Organisms were cultivated on ethene (2W, E3, E44) or propene (Py8)
ND = not determined

Table 5. Rates of propene oxidation and of 1,2-epoxypropane accumulation by free and immobilized ethene-utilizing bacteria

	Organisms		
	E3	E4	2W
Free cells	7.4 (6.7)[a]	7.7 (6.0)	9.0 (6.9)
Alginate	7.7 (6.5)	7.8 (7.1)	5.4 (4.1)
Carrageenan	8.4 (7.8)	5.6 (5.5)	5.3 (4.3)

[a] nmoles · min^{-1} · mg $protein^{-1}$
Values in parentheses refer to rates of 1,2-epoxypropane accumulation

shows this epoxide production for resting cells of strain E3. As expected from the oxidation rates of ethene and propene (Fig. 1a) and their corresponding epoxides (Fig. 1b) by strain E3, no epoxyethane was produced from ethene, while 1,2-epoxypropane accumulated from propene (Fig. 1c). The rates of epoxide formation for the other bacteria investigated was determined in the same manner as for E3 (Table 2). As anticipated from oxidation rates of alkenes and epoxides, epoxyethane was excreted by the propene and butene isolates while the ethene isolates would produce 1,2-epoxypropane and 1,2-epoxybutane. Strain Eu1 quantitatively produced 1,2-epoxypropane from propene. Strains E44, E3, E4, and 2W accumulated more than 80% 1,2-epoxypropane from propene while strains 12D, 32, Tu1, and B produced 1,2-epoxypropane in yields between 40% and 80%. Eu1, E44, B, E3, and E4 also produced 1,2-epoxybutane in high quantities. Production of epoxyethane by strains Py1, Py8, and By1 was stoichiometrical.

A special situation existed for strain E20. Ethene-grown cells would not produce 1,2-epoxypropane from propene because such cells oxidized 1,2-epoxypropane. However, butane-grown E20 cells did not oxidize 1,2-epoxypropane and consequently produced 1,2-epoxypropane and other epoxides almost quantitatively from the corresponding alkenes. Butane-grown E20 cells in this respect resemble methane-utilizing bacteria and other alkane-utilizers in that these organisms likewise are not able to further metabolize the epoxides.

Excretion of 1,2-epoxypropane by immobilized cells

Because of our interest in producing epoxides on a larger scale a selection of strains was made for further work. On the basis of the above results (Tables 1 and 2) strains E3, E4, 2W, and Py1 were immobilized and preliminary studies on the potential of these biocatalysts in a suitable biotechnological process were undertaken. The results obtained in a gas-solid bioreactor with the epoxyethane producing strain Py1 have been reported elsewhere (de Bont et al. 1983). Cells of the three 1,2-epoxypropane producing strains, now grown in an air-lift fermentor, were immobilized in calcium alginate and carrageenan. From the measured propene oxidation rates (Table 5) it is clear that both immobilization systems yielded active biocatalyst preparations with good retention of

activity. The rates given in Table 5 for propene oxidation and 1,2-epoxypropane accumulation are the measured values during the first 3 h of an experiment. As was the case for free cells, strains E3 and E4, when immobilized, accumulated more than 80% 1,2-epoxypropane from propene while strain 2W accumulated the epoxide to a somewhat lesser extent (75%–80%). The above observations are promising and further investigations of the possible application of immobilized alkene-utilizing Mycobacteria as potential epoxide producing biocatalysts are under way.

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