Metabolism of *trans*-2-Butene and Butane in *Nocardia* TB1

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A bacterium capable of growth on *trans*-2-butene was isolated from soil and identified as a *Nocardia* sp. The isolate also grew on propane, butane, pentane and hexane; gaseous and volatile 1-alkenes did not support growth. Intact cells grown on *trans*-2-butene or butane oxidized all *n*-alkanes, 1-alkenes, 2-alkenes and alcohols tested. Simultaneous adaptation studies, inhibitor experiments and measurements of enzyme activities in crude extracts indicated a degradative route of *trans*-2-butene via crotonic acid and of butane via butyric acid. The key enzymes in the proposed pathways were induced by growth on either *trans*-2-butene or butane. The induction of these enzymes and the substrate specificities of the enzymes suggest a relation between *trans*-2-butene and butane degradation.

INTRODUCTION

Micro-organisms attack a wide range of saturated and unsaturated hydrocarbons (Klug & Markovetz, 1971); the lower gaseous compounds are also readily degraded as originally shown by Tautz & Donath (1930). Bacteria utilizing saturated gaseous hydrocarbons mainly belong to the genera *Mycobacterium*, *Nocardia*, *Corynebacterium*, *Brevibacterium* and *Pseudomonas* (Klug & Markovetz, 1971), and most of these bacteria grow well on ethane, propane or butane (Hou *et al.*, 1983; Patel *et al.*, 1983; Philips & Perry, 1974). In general, the pathway of alkane degradation proceeds via terminal hydroxylation of the alkane, yielding the corresponding primary alcohol (Klug & Markovetz, 1971) but subterminal oxidations have also been demonstrated (Vestal & Perry, 1969).

The ability of micro-organisms to grow on gaseous alkenes has been described for ethene (de Bont, 1976; Heyer, 1976), propene and 1-butene (de Bont *et al.*, 1980; Cerniglia *et al.*, 1976; van Ginkel & de Bont, 1986), and for 1,3-butadiene (Watkinson & Sommerville, 1976). The first intermediate in the metabolism of gaseous 1-alkenes is the corresponding 1,2-epoxyalkane formed by a monooxygenase (de Bont *et al.*, 1979, 1983). The further metabolism of 1,2-epoxyethane proceeds via acetyl-CoA (de Bont & Harder, 1978), and the metabolism of 1,2-epoxypropane presumably via a C₂ + C₁ cleavage (Cerniglia *et al.*, 1976).

A major difference between alkane- and alkene-grown bacteria is the substrate specificity towards gaseous hydrocarbons of the monooxygenases responsible for the initial oxygenation. Alkane monooxygenases from alkane-utilizers oxidize alkanes and 1-alkenes (Hou *et al.*, 1983; Patel *et al.*, 1983) whereas the alkene monooxygenases are only able to epoxidate alkenes (Habets-Crützen *et al.*, 1984; van Ginkel *et al.*, 1986).

Knowledge on the metabolism of lower 2-alkenes, in contrast to the situation for 1-alkenes and alkanes, is scarce. Fujii *et al.* (1985) reported a 2-butene-utilizing *Mycobacterium* which was used in a microbial screening test. In this paper we report the isolation of a *Nocardia* sp. that can grow on *trans*-2-butene and butane, and the way in which it metabolizes these compounds.

METHODS

Chemicals. Gaseous alkenes and 1,2-epoxyethane were obtained from Hoek Loos. *trans*-2-Butene was obtained from Hicol. NADH was purchased from Boehringer. All other chemicals were obtained from Janssen Chimica.

Micro-organisms. *Nocardia* TB1 was isolated essentially as described by de Bont (1976) except that ethene was replaced by *trans*-2-butene. *Nocardia vaccinii* was a gift from Dr D. Jones, Department of Microbiology, Leicester University, UK.

Cultivation. The micro-organisms were cultivated in mineral salts medium supplemented with *trans*-2-butene or butane as described by Wiegant & de Bont (1980). Growth on different carbon sources was determined in 100 ml Erlenmeyer flasks containing 10 ml mineral salts medium supplemented with the appropriate carbon source. The concentration of gaseous hydrocarbons was set by injecting the gas in a known volume. The concentration of the gaseous substrates supplied was about 5% (v/v) while the concentration of the other carbon sources was 0.2% (w/v).

Analyses. Determination of gaseous alkenes and 1,2-epoxyalkanes was described by de Bont *et al.* (1979). CO₂ was measured as described by de Bont *et al.* (1983). Acids were measured with a Varian Aerograph series 2400 gas chromatograph fitted with a Chromosorb 101 (80 to 100 mesh) column; the column temperature was 190 °C and the carrier gas was N₂ saturated with formic acid. Total organic carbon was determined with a Dohrmann type DC organic carbon analyser. Dissolved O₂ concentrations were measured at 30 °C with a Yellow Springs model 53 monitor equipped with a polarographic sensor. Protein concentrations of washed cell suspensions and cell-free extracts were determined as described by Habets-Crützen *et al.* (1984). Mycolic acids were determined as described by Minnikin *et al.* (1975).

Determination of the doubling times. This was done by optical density measurements as described by Habets-Crützen *et al.* (1984).

Preparation of washed cell suspensions and cell-free extract. This was described by de Bont *et al.* (1979).

Oxidation of hydrocarbons and epoxyalkanes. The oxidation of hydrocarbons and the excretion of epoxyalkanes by washed cell suspensions (2.5 ml) were measured by incubating the cells in 50 mM-sodium/potassium phosphate buffer (pH 7.0) at 30 °C in Hungate tubes. The appropriate gas (0.075 ml) or 0.1 ml of a 10 mM-epoxyalkane solution were injected into the Hungate tubes. Samples from the gas phase were withdrawn at regular intervals and analysed for hydrocarbons and epoxyalkanes.

Enzyme activities. Hydrocarbon monooxygenases (EC 1.14.13.-) were measured as described by de Bont & Harder (1978) except that ethene was replaced by *trans*-2-butene or butane. For crotonic alcohol dehydrogenase (EC 1.1.1.1), crotonic aldehyde dehydrogenase (EC 1.2.1.3) and butanal dehydrogenase (EC 1.2.1.3), the reaction mixture (3 ml) contained 3 µmol alcohol or aldehyde, 3 µmol NAD and 130 µmol Tris/HCl buffer (pH 8.5); the change in A₃₄₀ due to NAD reduction was followed. Butanal reductase (EC 1.1.1.1) was assayed by following NADH oxidation. The reaction mixture (1 ml) contained 130 µmol sodium/potassium phosphate buffer (pH 7.0), 0.6 µmol NADH and the reaction was started by adding 3 µmol butanal. Thiokinases (EC 6.2.1.-) were assayed by following the formation of a hydroxamic acid complex which was measured as the increase in A₅₄₀. The reaction mixture (1 ml) contained 40 µmol Tris/HCl (pH 7), 10 µmol MgCl₂, 2.5 µmol ATP, 0.5 µmol CoA, 200 µmol hydroxylamine, 10 µmol crotonic acid or butyric acid and the reaction was stopped after 30 min by adding 1 ml of a solution of 12% (v/v) 3 M-HCl and 5% (v/v) TCA in 0.1 M-HCl. The absorption coefficient used was 641 l mol⁻¹ cm⁻¹ (Beinert *et al.*, 1953). Acetoacetyl-CoA thiolase (EC 2.3.1.9) (Senior & Dawes, 1973) and isocitrate lyase (EC 4.1.3.1) (Dixon & Kornberg, 1959) were assayed as previously described.

CO₂ production from crotonic alcohol and 2,3-epoxybutane. Washed cell suspensions of *trans*-2-butene-grown cells were incubated in 50 ml screw-cap bottles at 30 °C with 0.4% (v/v) *trans*-2-butene in the gas phase, 0.2 mM-2,3-epoxybutane or 0.2 mM-crotonic alcohol. CO₂ production was followed by measuring the CO₂ concentration in the gas phase at regular intervals.

Inhibition studies. Washed cell suspensions of *trans*-2-butene- and butane-grown *Nocardia* TB1 were incubated at 30 °C with 5 mM-arsenite or 100 mM-fluoroacetate in screw-cap bottles (50 ml). *trans*-2-Butene or butane was injected into the bottles and the change in the concentration of *trans*-2-butene or butane was measured. Any accumulation of acids was also measured by gas chromatography, using samples of the supernatant fluid collected after centrifugation. Washed cells of *trans*-2-butene-grown *Nocardia* TB1 were incubated at 30 °C in 50 ml screw-cap bottles. 2,3-Epoxybutane (2.5 µmol) was injected and the oxidation rate of the epoxide was measured. Inhibition of the oxidation rate by *trans*-2-butene was measured after 17 min by injecting various amounts of *trans*-2-butene into the screw-cap bottles.

RESULTS

Isolation and characterization

A bacterium capable of growth on *trans*-2-butene was enriched by incubating a sample of ditch water in the presence of *trans*-2-butene. From this enrichment a red-pigmented Gram-positive bacterium was isolated, which produced leathery, dry and crusty colonies when growing on a solid mineral salts medium with *trans*-2-butene in the gas phase as carbon and energy source. Aerial hyphae were not produced by this strain. The bacterium formed a branched

Table 1. Oxidation of various hydrocarbons by washed cell suspensions of *trans*-2-butene- and butane-grown *Nocardia TB1*

Substrate	Activity [nmol min ⁻¹ (mg protein) ⁻¹] after growth on:	
	<i>trans</i> -2-Butene	Butane
Methane	0	0
Ethane	0.7	1.8
Propane	1.5	3.7
Butane	2.5	4.9
<i>trans</i> -2-Butene	5.0	7.0
<i>cis</i> -2-Butene	5.7	8.0
1,3-Butadiene	3.4	7.0
Ethene	1.9	5.8
Propene	2.4	3.8
1-Butene	2.8	4.5

mycelium typical of *Nocardia* spp. On mycolic acid analysis, the methanolysate of the isolated strain showed the same pattern as that of *Nocardia vaccinii*. The bacterium was able to grow on several substrates including phenol, dextran, acetate, propionate, butyrate, ethanol, 1-propanol, 1-butanol, 2-butanol, glucose and H₂/CO₂, but not on methanol. It reduced nitrate to nitrite but did not hydrolyse gelatine or xanthine. On the basis of the above characteristics the organism was tentatively classified as a *Nocardia* sp.

Nocardia TB1 is the first bacterium isolated on *trans*-2-butene and therefore it was of interest to determine which gaseous and volatile hydrocarbons could serve as a growth substrate. This strain was able to grow on the saturated hydrocarbons propane, butane, pentane, hexane and hexadecane but not on methane and ethane. The unsaturated hydrocarbons ethene, propene, 1-butene, 1,3-butadiene, 1-pentene and 1-hexene were not growth substrates. Growth on *cis*-2-butene was very slow. Culture doubling times of the isolated strain on butane and *trans*-2-butene were 6 and 30 h, respectively.

Oxidation of hydrocarbons by trans-2-butene- and butane-grown *Nocardia TB1*

Washed cell suspensions of *trans*-2-butene-grown *Nocardia TB1* were able to oxidize all gaseous n-alkanes tested except methane (Table 1). Gaseous 1-alkenes were also oxidized, but the oxidizing activity levelled off after a short period of time when the corresponding 1,2-epoxyalkanes accumulated to a concentration of approximately 0.25 mM. From ethene and propene, the 1,2-epoxyalkanes were formed in stoichiometric amounts but from 1-butene only 50% was transformed into 1,2-epoxybutane whereas an unknown product was formed from the remainder. No epoxyalkanes accumulated from either *trans*-2-butene or *cis*-2-butene. Butane-grown cells oxidized the hydrocarbons tested twice as fast as *trans*-2-butene-grown cells but at otherwise similar relative rates (Table 1); the substrate oxidation rates also levelled off when 1,2-epoxyalkanes accumulated.

Inhibition studies with Nocardia TB1

Experiments to detect the products of butane and *trans*-2-butene oxidation were done with whole cells, using different possible product analogues of the first oxidation reactions. Alcohols and epoxides that may compete for the active site of the second enzyme in the degradative pathway of these hydrocarbons did not act as inhibitors. However, using the nonspecific enzyme inhibitor arsenite, it was possible to detect butyric acid and crotonic acid as possible intermediates of butane and *trans*-2-butene metabolism respectively (Fig. 1). Similar results were obtained using fluoroacetate as an inhibitor.

Substrate-dependent oxygen uptake

Substrate-dependent oxygen uptake was determined for *trans*-2-butene-, butane-, and succinate-grown cells (Table 2). Crotonic alcohol and 2,3-epoxybutane stimulated oxygen

Fig. 1. Effect of 5 mM-arsenite on the accumulation of intermediates by washed cell suspensions of *Nocardia* TB1 grown on either (a) butane (14.5 mg protein) or (b) *trans*-2-butene (17 mg protein). Incubations with arsenite: ●, butane; ▲, *trans*-2-butene; □, crotonic acid; ■, butyric acid. Incubations in the absence of arsenite: ○, butane; △, *trans*-2-butene.

Table 2. Increase in the rate of oxygen uptake by washed cell suspensions of *Nocardia* TB1 grown on various substrates

The endogenous rate of oxygen uptake by *trans*-2-butene-, butane- and succinate-grown cells was 13, 12 and 32 nmol min⁻¹ (mg protein)⁻¹, respectively.

Substrate	Activity [nmol min ⁻¹ (mg protein) ⁻¹] after growth on:		
	<i>trans</i> -2-Butene	Butane	Succinate
<i>trans</i> -2-Butene	64	112	0
<i>cis</i> -2-Butene	60	105	0
Crotonic alcohol	64	103	0
Crotonic aldehyde	80	102	12
Crotonic acid	63	63	19
Butane	46	53	0
1-Butanol	51	75	5
2-Butanol	19	28	0
Butanal	70	80	11
Butanone	12	38	5
Butyrate	31	52	19
3-Hydroxybutyrate	19	11	3
Acetate	57	48	32
2,3-Epoxybutane	18	16	0
Succinate	7	3	83

uptake by butane- and *trans*-2-butene-grown cells whereas the oxygen uptake by succinate-grown cells was not stimulated by these substrates. Respiration of butane-grown cells (as compared to the endogenous respiration) was increased by 1-butanol and 2-butanol. 1-Butanol and 2-butanol did not enhance or only slightly enhanced the respiration rate of succinate-grown cells. Other possible intermediates in the degradative pathways of *trans*-2-butene and butane tested enhanced the oxygen uptake of *trans*-2-butene- as well as butane- and succinate-grown cells (Table 2).

Enzyme activities

The nature of the enzyme system involved in the attack on butane or *trans*-2-butene was investigated by measuring the disappearance of butane or *trans*-2-butene using cell-free extracts. Activity was only observed when NAD(P)H and molecular oxygen were included in the reaction mixture, indicating that a monooxygenase is probably responsible for the oxidation of these hydrocarbons (Table 3).

Table 3. Specific activities of enzymes in cell-free extracts of *Nocardia TB1* grown on *trans*-2-butene, butane and succinate

Enzyme	Activity [nmol min ⁻¹ (mg protein) ⁻¹] after growth on:		
	<i>trans</i> -2-Butene	Butane	Succinate
<i>trans</i> -2-Butene monooxygenase*	2	2	0
Butane monooxygenase*	1	1	0
Crotonic alcohol dehydrogenase†	9	20	0
1-Butanol dehydrogenase‡	0	0	0
Butanal reductase*	25	12	18
Crotonic aldehyde dehydrogenase†	3	5	0
Butanal dehydrogenase†	12	76	3
Crotonic acid thiokinase‡	12	17	10
2-Butanol dehydrogenase†	5	5	4
Butyric acid thiokinase‡	15	9	10
Thiolase	154	153	150

* Requirement for NADH

† Requirement for NAD

‡ Requirement for coenzyme A

Table 4. Isocitrate lyase activity in cell-free extracts of *Nocardia TB1* after growth on various substrates

Growth substrate	Activity [nmol min ⁻¹ (mg protein) ⁻¹]
Acetate	417
Propionate	3
Butyrate	220
Succinate	1
<i>trans</i> -2-Butene	110
Butane	137
1-Butanol	171
2-Butanol	1

Crotonic alcohol dehydrogenase and crotonic aldehyde dehydrogenase were induced after growth on *trans*-2-butene or butane and a 1-butanol dehydrogenase, measured as 1-butanal reductase, was also induced in butane- and *trans*-2-butene-grown cells. 2-Butanol dehydrogenase was also present but was constitutive (Table 3). Thiokinases were induced in *trans*-2-butene-, butane- and succinate-grown cells and the requirement of the enzyme for CoA and ATP was shown by omitting these cofactors from the assay mixture. Finally, it was shown that acetoacetyl-CoA thiolase was present in *trans*-2-butene-, butane- and succinate-grown cells (Table 3).

Evidence that butane is hydroxylated at C₁ was also obtained by comparing isocitrate lyase activity in *Nocardia TB1* after growth on various carbon sources (Table 4). Growth on butane and 1-butanol resulted in the induction of isocitrate lyase, whereas 2-butanol-grown cells contained no isocitrate lyase activity.

The fate of trans-2,3-epoxybutane

trans-2,3-Epoxybutane was oxidized by butane- and *trans*-2-butene-grown *Nocardia TB1* (Table 2). The oxidation of *cis*-2,3-epoxybutane was not investigated because this compound was not available. *trans*-2,3-Epoxybutane oxidation by a washed cell suspension of *trans*-2-butene-grown cells was inhibited by *trans*-2-butene, indicating that the monooxygenase present in *Nocardia TB1* was probably responsible for the oxidation of *trans*-2,3-epoxybutane. *trans*-2,3-Epoxybutane was not oxidized to CO₂ because from this substrate hardly any additional CO₂ was formed as compared to the amount of CO₂ formed endogenously, whereas washed cell suspensions of *trans*-2-butene-grown *Nocardia TB1* produced twice the amount of CO₂ formed

during the endogenous respiration when oxidizing *trans*-2-butene or crotonic alcohol. Organic carbon derived from an unknown oxidation product accumulated in the supernatant during 2,3-epoxybutane oxidation by washed cell suspensions of *trans*-2-butene-grown *Nocardia* TB1, whereas during growth on *trans*-2-butene no organic carbon could be detected in the mineral salts medium.

DISCUSSION

Nocardia TB1 was isolated with *trans*-2-butene as carbon and energy source and also grew on several saturated, straight-chain hydrocarbons but not on 1-alkenes. Micro-organisms that grow on both saturated and unsaturated gaseous hydrocarbons have been reported i.e. *Mycobacterium* E20 (de Bont *et al.*, 1979) and a *Nocardia* sp. (Fujii *et al.*, 1985) but most hydrocarbon-utilizing bacteria cannot grow on both saturated and unsaturated hydrocarbons (Lukins & Foster, 1963; Habets-Crützen *et al.*, 1984; van Ginkel *et al.*, 1986). Both *trans*-2-butene- and butane-grown cells of *Nocardia* TB1 oxidized a wide range of hydrocarbons (Table 1), but this capacity to oxidize such a wide range of substrates is not unique. Alkane-utilizing micro-organisms are capable of hydroxylation and epoxidation reactions (Hou *et al.*, 1983; Patel *et al.*, 1983), whereas 1-alkene-utilizing bacteria can only epoxidate double bonds and do not hydroxylate saturated hydrocarbons (de Bont *et al.*, 1979; van Ginkel & de Bont, 1986). Considering the capacity of *trans*-2-butene-grown cells to oxidize saturated hydrocarbons, it would seem likely that these cells contain an alkane-type monooxygenase with broad substrate specificity, and not a 1-alkene-type monooxygenase.

It was possible to detect monooxygenase activity in extracts of *trans*-2-butene-grown cells, and the activity could theoretically result in the formation of either crotonic alcohol or 2-butanone as a result of hydroxylation activity, or in the formation of 2,3-epoxybutane as a result of epoxidation activity of the monooxygenase similar to the results of Hou *et al.* (1983).

1,2-Epoxyethane and 1,2-epoxypropane were not oxidized by *trans*-2-butene-grown cells whereas 1,2-epoxybutane and especially 2,3-epoxybutane were oxidized, which might indicate that *trans*-2-butene is metabolized via 2,3-epoxybutane. On the other hand, substrate specificity towards unsaturated and saturated hydrocarbons indicated that *trans*-2-butene is metabolized via a hydroxylation reaction. Moreover, results with washed cell suspensions of *trans*-2-butene-grown *Nocardia* TB1 showed that 2,3-epoxybutane is not oxidized to CO₂ but to an unidentified product whereas the other possible intermediate crotonic alcohol, and *trans*-2-butene itself, were both oxidized to CO₂. Indeed, it was shown that washed cells grown on *trans*-2-butene excreted a product during the oxidation of 2,3-epoxybutane and that this product probably originates from a hydroxylation reaction by the monooxygenase.

Washed cell suspensions of *trans*-2-butene-grown cells inhibited with fluoroacetate or arsenite excreted crotonic acid, which is an oxidation product of crotonic alcohol, and crotonic alcohol dehydrogenase and crotonic aldehyde dehydrogenase were induced in *trans*-2-butene-grown cells (Table 3). From these results we conclude that *trans*-2-butene is metabolized via an initial hydroxylation at the C₁ position rather than via an initial epoxidation reaction.

It was also possible to detect monooxygenase activity in butane-grown cells (Table 3). Hydroxylation of butane could result either in 1-butanol or in 2-butanol. The isocitrate lyase activities suggest that butane is hydroxylated terminally (Table 4). Further evidence for the hydroxylation of butane at the C₁ position was obtained from inhibition studies with arsenite. Butyric acid was excreted by butane-grown cells during the oxidation of butane in the presence of an inhibitor (Fig. 1). In cell-free extracts of butane-grown cells, butanal reductase activity was measured (Table 3).

The further metabolism of *trans*-2-butene and butane proceeds via β -oxidation as determined by thiokinase and acetoacetyl-CoA thiolase activities (Table 3).

Growth of *Nocardia* TB1 on *cis*-2-butene was very slow as compared to growth on *trans*-2-butene although both isomers of 2-butene were oxidized by *trans*-2-butene- and butane-grown cells at comparable rates (Tables 1 and 2). The metabolism of the *cis*-isomer was not further investigated because isomers of crotonic alcohol, crotonic aldehyde and crotonic acid were not available.

Fig. 2. Proposed degradation pathways of *trans*-2-butene and butane in *Nocardia* TB1.

On the basis of the results obtained in this study we propose pathways for the metabolism of butane and *trans*-2-butene in *Nocardia* TB1 as shown in Fig. 2. From our experiments it seems very likely that *trans*-2-butene degradation is catalysed by the same enzymes as butane degradation because substrate specificities (Tables 1 and 2) are comparable and enzymes induced in butane- and *trans*-2-butene-grown cells are able to oxidize the intermediates of both pathways.

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