Butane metabolism by butane-grown '*Pseudomonas butanovora*'

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The pathway of butane metabolism by butane-grown '*Pseudomonas butanovora*' was determined to be butane → 1-butanol → butyraldehyde → butyrate. Butane was initially oxidized at the terminal carbon to produce 1-butanol. Up to 90% of the butane consumed was accounted for as 1-butanol when cells were incubated in the presence of 5 mM 1-propanol (to block subsequent metabolism of 1-butanol). No production of the subterminal oxidation product, 2-butanol, was detected, even in the presence of 5 mM 2-pentanol (an effective inhibitor of 2-butanol consumption). Ethane, propane and pentane, but not methane, were also oxidized. Butane-grown cells consumed 1-butanol and other terminal alcohols. Secondary alcohols, including 2-butanol, were oxidized to the corresponding ketones. Butyraldehyde was further oxidized to butyrate as demonstrated by blocking butyrate metabolism with 1 mM sodium valerate. Butyrate also accumulated from butane when cells were incubated with 1 mM sodium valerate. The pathway intermediates (butane, 1-butanol, butyraldehyde and butyrate) and 2-butanol stimulated O₂ consumption by butane-grown cells. 1-Butanol, butyraldehyde and butyrate supported growth of '*P. butanovora*', as did 2-butanol and lactate.

Keywords: butane metabolism, alkane metabolism, '*Pseudomonas butanovora*', alkane oxidation

INTRODUCTION

A number of bacteria have been isolated that are capable of growth on butane. Most of these bacteria are members of the *Rhodobacter*–*Nocardia*–*Arthrobacter*–*Corynebacterium* group of Gram-positive bacteria (Ashraf *et al.*, 1994; McLee *et al.*, 1972; Perry, 1980). However, two Gram-negative bacteria which can grow on butane, '*Pseudomonas butanovora*' and *Pseudomonas* sp. strain CRL 71, have been described (Hou *et al.*, 1983; Takahashi, 1980). Butane-oxidizing bacteria can be considered as part of a larger group of bacteria which are characterized by their ability to grow on gaseous alkanes such as ethane and propane, but not methane (Ashraf *et al.*, 1994; Klug & Markovetz, 1971). This larger group is also dominated by Gram-positive bacteria, although some *Pseudomonas* spp. will grow on C₂–C₄ alkanes (Hou *et al.*, 1983). Bacteria that grow on one gaseous alkane will generally grow on other gaseous or volatile alkanes; for example, *Mycobacterium vaccae* will grow on propane (Vestal & Perry, 1969) or butane (Phillips & Perry, 1974). Bacteria that can grow on methane, methanotrophs, generally do not use other alkanes as growth substrates (Murrell, 1992) though

they can often carry out the hydroxylation of gaseous alkanes (Burrows *et al.*, 1984; Colby *et al.*, 1977).

As pointed out by Ashraf *et al.* (1994) in a recent review of the subject, the pathways for the metabolism of the light *n*-alkanes (ethane, propane and butane) have received little attention compared to those of methane and liquid *n*-alkanes. The pathway of butane metabolism has not previously been established directly for any butane-oxidizing bacterium. Butane, as with other alkanes, is generally assumed to be harvested by a monooxygenase, which results in hydroxylation of the alkane (Ashraf *et al.*, 1994; Perry, 1980). However, production of 1-butanol or 2-butanol from butane had not been directly demonstrated for any butane-grown bacterium. The subsequent metabolism of either 1-butanol, the terminal oxidation product, or 2-butanol, the subterminal oxidation product, would be expected to require different pathways. Evidence for pathways consistent with both oxidation products has been presented (Lukins & Foster, 1963; Phillips & Perry, 1974; van Ginkel *et al.*, 1987). Terminal oxidation of butane by *M. vaccae* JOB5 was proposed because cells grown on either butane or butyrate expressed isocitrate

lyase activity, which is required to assimilate the 2-carbon compounds formed as a result of further metabolism of butyrate (Phillips & Perry, 1974). In contrast, cells grown on butanone did not produce isocitrate lyase activity. Butanone was subsequently decarboxylated to propionate and further metabolized by the methylmalonate-succinate pathway (Phillips & Perry, 1974). Butane-grown *Nocardia* TB1 produced butyrate from *n*-butane while in the presence of arsenite and a pathway of butane to 1-butanol to butyraldehyde to butyrate was suggested (van Ginkel *et al.*, 1987). However, production of the first two proposed intermediates (1-butanol and butyraldehyde) was not demonstrated directly and the possibility of a concurrent pathway initiated by subterminal oxidation of butane was not eliminated. Subterminal oxidation was indicated for propane-grown *Mycobacterium smegmatis* 422, which accumulated butanone when exposed to *n*-butane (Lukins & Foster, 1963).

As with butane, the pathway of propane metabolism has received limited attention. Support for both terminal and subterminal oxidations of propane has been presented (Perry, 1980). Accumulation of acetone (from 2-propanol) supported the conclusion that propane oxidation was primarily subterminal in propane-utilizing bacteria such as *M. vaccae* JOB5 (Lukins & Foster, 1963; Perry, 1980). However, consumption of the terminal oxidation product, 1-propanol, was also demonstrated for propane-grown *M. vaccae* JOB5 (Perry, 1968). Furthermore, some propane-grown strains of *Arthrobacter* consumed 1-propanol (the terminal oxidation product of propane) but not 2-propanol, while other strains consumed both isomers (Stephens & Dalton, 1986). Both 1-propanol and 2-propanol were produced by cell-free extracts of *Arthrobacter* sp. CRL-60, *Pseudomonas fluorescens* NRRL-B-1244 and *Brevibacterium* sp. NRRL B-11319 (Patel *et al.*, 1983). Current evidence indicates that both terminal and subterminal hydroxylations of propane can occur.

'*P. butanovora*' grows on C₂-C₈ *n*-alkanes as well as on a number of alcohols and organic acids (Takahashi, 1980). However, growth on alkenes and sugars was not observed. We recently demonstrated that this bacterium, when grown on butane, could initiate the degradation of a number of chlorinated aliphatic compounds, including chloroform (Hamamura *et al.*, 1997), and this degradation appeared to be initiated by butane monooxygenase. In this work, the pathway of butane metabolism by this Gram-negative bacterium was elucidated.

METHODS

Cell growth and preparation of cell suspensions. Cells of '*Pseudomonas butanovora*' (ATCC 43655) were grown as previously described (Hamamura *et al.*, 1997). The basal medium (1 l) contained 8 g (NH₄)₂HPO₄, 1.9 g Na₂HPO₄·7H₂O, 2 g KH₂PO₄, 0.5 g MgSO₄·7H₂O, 0.06 g CaCl₂·2H₂O, 0.05 g yeast extract and 1 ml trace element solution (Wiegant & de Bont, 1980) at pH 7.1. Cells were cultured in 160 ml serum vials containing 50 ml basal medium and capped with butyl rubber stoppers and aluminium crimp seals. Each vial

received 5 ml CO₂ as an overpressure. Butane (10 ml) was added to the vial as an overpressure for growth on butane. For growth on 1-butanol, butyraldehyde or 2-butanol, appropriate volumes of each pure liquid were added directly to the sterile medium. For growth on butyrate or lactate, appropriate amounts of stock solutions (1 M) of sodium butyrate or sodium lactate were added to the medium. Cultures were shaken at 160 oscillations min⁻¹ and maintained at 30 °C during growth and harvested after 2 or 3 d. The limiting nutrient for growth was O₂; butane-grown cultures typically reached an OD₆₀₀ of 0.6 upon exhaustion of the O₂.

Cells were harvested by centrifugation (10 min at 12000 g; 10 °C) and resuspended in 1 ml buffer [8 g (NH₄)₂HPO₄, 1.9 g Na₂HPO₄·7H₂O, 2 g KH₂PO₄, 0.5 g MgSO₄·7H₂O l⁻¹, pH 7.1]. Cell suspensions were typically prepared fresh daily and used within 6 h. However, cell suspensions retained butane consumption activity for at least 30 h when stored on ice without agitation. Typical protein concentrations for the cell suspensions were 5–7 mg protein (ml suspension)⁻¹.

Measurement of cell activities. Butane consumption was measured in a 1 ml gas-tight syringe (Hamilton 1001 RN) with the needle removed. The reaction mixture consisted of 0.7 or 0.8 ml O₂-saturated buffer, 0.1 or 0.2 ml butane-saturated buffer, and addition of a cell suspension (typically 0.025 ml), other compounds (e.g. 1-propanol) as indicated, and additional buffer for a total volume of 1 ml. A glass bead in the syringe facilitated mixing of the components. Additions to the syringe were made by injection through the opening into the body of the syringe. No gas phase was present in the syringe. Movement of the plunger facilitated addition and removal of samples without introducing a gas phase. Samples of the liquid (10 µl) were removed periodically and analysed for butane content by GC as described below. In some instances, 1-butanol was also analysed. Consumption of other alkanes (0.1–0.2 µM) was measured similarly. The reactions were carried out at room temperature (20 ± 1 °C).

Consumption and accumulation of alcohols, butyraldehyde and butyrate were measured in 7 ml serum vials capped with butyl rubber stoppers and aluminium crimp seals. The reaction mixture consisted of the substrate with or without inhibitor (at the indicated concentrations), cell suspension (10–100 µl), and buffer to a total of 1 ml. Butane (1 ml) or propane (1 ml) gas, where indicated, was added as an overpressure to the headspace. Vials were shaken at 100 oscillations min⁻¹ in a 20 °C water bath during the reactions. Liquid samples (2–10 µl) were removed periodically and the concentrations of substrates and products were determined by GC.

O₂ consumption by cells in the presence of various compounds was measured with a Clark-style O₂ electrode inserted into a 1.6 ml chamber sealed with a capillary inlet through which additions were made. The contents of the electrode chamber were stirred with a magnetic stir bar. The reaction mixture consisted of air-saturated buffer with substrates and cells added as indicated. The reactions were carried out at room temperature (20 ± 1 °C).

Analytical techniques. Concentrations of alkanes, aldehydes, ketones and organic acids in the liquid phase of reaction mixtures were determined by GC with a Shimadzu GC-8A gas chromatograph equipped with a flame-ionization detector and a 30 cm long by 0.1 cm i.d. stainless steel column packed with Porapak Q. The oven temperature was varied depending on the compound analysed. The following temperatures were used for each set of compounds: 25 °C, methane and ethane; 70 °C, propane; 80 °C, butane and methanol; 100 °C, ethanol;

Fig. 1. Metabolite consumption and production by butane-grown '*P. butanovora*'. (a) Consumption of butane (●) and production of 1-butanol (■) in the presence of 5 mM 1-propanol. The reaction mixture (1 ml) contained buffer, 230 nmol butane, 915 nmol O₂, 5 μmol 1-propanol and 20 μl cell suspension (147 μg protein). (b) Consumption of 1-butanol (●) and production of butyraldehyde (■) with 1 μmol 1-butanol and 25 μl cell suspension (133 μg protein). (c) Consumption of 2-butanol (●) and production of butanone (■) with 1 μmol 2-butanol and 25 μl cell suspension (133 μg protein). (d) Production of butyrate from butane in the presence of 1 mM valerate with 6 ml air, 1 ml butane (added as an overpressure), 1 μmol sodium valerate, and either 10 μl cell suspension (47 μg protein) (■) or 40 μl cell suspension (188 μg protein) (●). For the sample with the higher cell density, the initial valerate concentration (1 mM) had decreased to 0.04 mM by 50 min. (b–d) Reactions were carried out in sealed 7 ml vials with 1 ml reaction mixture containing buffer and the indicated concentrations of substrate and cell suspension. (a–d) Liquid samples were removed at the indicated times and analysed by GC.

110 °C, pentane, butanone, acetone and butyraldehyde; 120 °C, 2-butanol, 1-propanol, 2-propanol, propionaldehyde and butanone; 130 °C, 1-butanol and butyraldehyde; 150 °C, 1-pentanol, 2-pentanol and 2-pentanone; 160 °C, butyric acid and valeric acid. Concentrations of compounds were determined by comparison of peak heights from samples to peak heights of standards of known concentration. Concentrations of gaseous compounds in standards were calculated using Henry's law and appropriate Henry's law constants (Smith & Baresi, 1989). Identities of products were determined by comparison of retention times and peak shapes to those of authentic compounds.

The protein contents of cell suspensions were determined by the Biuret assay (Gornall *et al.*, 1949) after cells were solubilized in 3 M NaOH for 30 min at 65 °C. BSA was used as a standard.

Chemicals and gases. All chemicals were of reagent grade. Acetylene was generated from CaC₂ by addition of H₂O. Propane and butane were purchased from Airgas; ethane was purchased from Matheson; methane was purchased from Airco.

RESULTS

Alkane oxidation by butane-grown '*P. butanovora*'

None of the predicted products of either terminal oxidation of butane (1-butanol or butyraldehyde) or subterminal oxidation (2-butanol or butanone) were detected in culture supernatants of butane-grown '*P. butanovora*'. Likewise, no 1- or 2-butanol was detected in resting cell suspensions during consumption of butane. Therefore, inhibitors of the subsequent metabolism of predicted products from each metabolic step were identified to cause the products to accumulate.

1-Propanol was then included in the reaction mixtures as a structural analogue of 1-butanol to compete for the enzyme(s) that further metabolized 1-butanol, thereby inhibiting 1-butanol consumption. Upon addition of 5 mM 1-propanol, 0.35 mM 1-butanol was produced after 60 min (1 ml reaction mixture, 7 ml vial, approx. 250 μg cell protein). Concentrations of 1-propanol from

Table 1. Inhibition of substrate consumption by substrate analogues or products in butane-grown '*P. butanovora*'

Substrate (mM)	Inhibitor (mM)	Inhibition (%)*
1-Butanol (1.0)	1-Propanol (5.0)	7
1-Butanol (0.1)	1-Propanol (5.0)	67
2-Butanol (0.1)	1-Propanol (5.0)	14
2-Butanol (0.2)	2-Pentanol (5.0)	79
2-Butanol (1.0)	Butanone (1.0)	88
Butanone (1.0)	2-Butanol (1.0)	100
Butyrate (0.1)	Propionate (1.0)	63
Butyrate (0.1)	Valerate (1.0)	86

* Percentage inhibition relative to rate of substrate consumption at the same concentration in the absence of inhibitor.

0.1 to 15 mM were tested; 5 mM 1-propanol led to the greatest accumulation of 1-butanol. A time course of butane consumption was inversely proportional to the time course of 1-butanol accumulation (Fig. 1a). When butane oxidation ceased (due to O₂ depletion), 1-butanol production also ceased. The ratio of 1-butanol consumption to butane production in this experiment (Fig. 1a) was 0.83. Because most of the butane consumed could be accounted for as 1-butanol, terminal oxidation of butane to 1-butanol was indicated as the predominant route of butane oxidation. The ratio of 1-butanol production to butane consumption varied with cell preparations from a low of 0.2 to a high of 0.9 with a mean value of 0.6 ± 0.2 . Butane consumption in the presence of 1-pentanol (5 mM) also led to accumulation of 1-butanol. When butane was omitted from the reactions with 5 mM 1-propanol, or when acetylene (2%, v/v), an inactivator of butane oxidation activity, was included in the reactions with butane and 5 mM 1-propanol, no 1-butanol was formed. These results indicate that 1-butanol was produced from the oxidation of butane.

The difference between the amount of 1-butanol that accumulated and the amount of butane consumed was most likely due to incomplete inhibition of the subsequent metabolism of 1-butanol. The inhibition of 1-butanol metabolism by 1-propanol was examined directly and, though substantial, was not complete, even when the 1-butanol concentration was only 2% of the 1-propanol concentration (Table 1). Therefore, some further metabolism of 1-butanol by resting cells would be expected even in the presence of 5 mM 1-propanol. The difference between the amounts of butane consumed and 1-butanol formed was not likely to be due to subterminal oxidation of a portion of the butane. No 2-butanol was detected (detection limit approx. 0.01 mM) in the reaction mixture, even in the presence of 2-pentanol (5 mM), an effective inhibitor of 2-butanol consumption (Table 1). Butane oxidation occurred primarily (if not exclusively) at the terminal carbon.

Table 2. Rates of alcohol consumption by butane-grown '*P. butanovora*'

Reactions were carried out with cell suspensions containing 133 µg protein. The initial concentration of each alcohol was 1.0 mM. The rate of consumption is measured as nmol alcohol consumed min⁻¹ (mg protein)⁻¹. ND, None detected.

Substrate	Rate of consumption	Accumulated product
Methanol	< 10	ND
Ethanol	225	ND
1-Propanol	235	Propionaldehyde
2-Propanol	158	Acetone
1-Butanol	341	Butyraldehyde
2-Butanol	150	Butanone
1-Pentanol	375	ND
2-Pentanol	254	2-Pentanone

'*P. butanovora*' grows on a variety of alkanes (Takahashi, 1980). Therefore, the consumption of several alkanes by butane-grown cells was examined. Cell suspensions (128 µg protein) consumed ethane [203 ± 16 nmol min⁻¹ (mg protein)⁻¹], propane [148 ± 23 nmol min⁻¹ (mg protein)⁻¹], butane [119 ± 8 nmol min⁻¹ (mg protein)⁻¹] and pentane [214 ± 42 nmol min⁻¹ (mg protein)⁻¹]. Methane consumption was not detected. Methane also was not a growth substrate for this bacterium (Takahashi, 1980). The products of propane oxidation in the presence of 5 mM 1-butanol (to inhibit 1-propanol consumption) were examined. Production of 1-propanol was readily observed (time course not shown). The concentration of 1-propanol produced (0.13 mM produced in 60 min by approx. 120 µg protein) was similar to the amount of 1-butanol (0.14 mM) produced by this cell suspension when inhibited with 5 mM 1-propanol. However, no 2-propanol production was detected in cells incubated with 5 mM 1-butanol or 5 mM 2-butanol (detection limit, 0.02 mM 2-propanol). As with butane, only terminal hydroxylation of propane was observed.

Alcohol consumption by butane-grown '*P. butanovora*'

Butane-grown resting cell suspensions readily consumed 1-butanol (Fig. 1b). The predicted product of an alcohol-dehydrogenase-catalysed reaction, butyraldehyde, accumulated as 1-butanol was consumed and was then subsequently consumed. The amount of butyraldehyde produced was not stoichiometric with the amount of 1-butanol consumed. Apparently some consumption of butyraldehyde took place prior to complete consumption of the 1-butanol, although the possibility that another pathway of 1-butanol consumption was functioning simultaneously has not been ruled out.

Consumption of other terminal alcohols was also examined (Table 2). The pattern paralleled that of

alkane oxidation, with all alcohols being consumed except methanol. Interestingly, oxidation of ethanol did not result in accumulation of acetaldehyde and oxidation of 1-pentanol did not result in the accumulation of valeraldehyde, while oxidation of 1-propanol did result in accumulation of propionaldehyde.

Although subterminal oxidation of butane to 2-butanol was not detected, 2-butanol was consumed by butane-grown cells (Tables 1 and 2). The consumption of 2-butanol was accompanied by the accumulation of butanone, the predicted product of the oxidation of 2-butanol (Table 2). A time course of 2-butanol consumption and butanone accumulation revealed rapid initial rates followed by a decrease in both rates as butanone accumulated (Fig. 1c). The ratio of butanone accumulated to 2-butanol consumed decreased from 0.92 (at 6 min) to 0.30 (at 102 min). In spite of the rapid initial rate of 2-butanol consumption, complete consumption of the 2-butanol was not observed even after 102 min (Fig. 1c) because the butanone that accumulated inhibited the consumption of 2-butanol (Table 1). 2-Butanol also inhibited the oxidation of butanone (Table 1). Two other secondary alcohols, 2-pentanol and 2-propanol, were also oxidized by resting cells of butane-grown '*P. butanovora*'. 2-Propanol was oxidized essentially stoichiometrically to acetone. For example, after 30 min with cells (0.3 mg protein) the concentration of 2-propanol in the reaction mixture had decreased 0.26 mM (from 1 mM) while the concentration of acetone increased to 0.27 mM. 2-Pentanol was oxidized to pentanone. For both of these secondary alcohols, the time courses of consumption were not linear. The rates decreased with time as the corresponding ketone accumulated, which is similar to the result with 2-butanol. Thus, while secondary alcohols were not produced in substantial amounts from either butane or propane, secondary alcohols were nonetheless consumed by butane-grown cells.

Aldehyde, ketone and organic acid consumption by '*P. butanovora*'

When resting cells of '*P. butanovora*' were incubated with 1 mM butyraldehyde, the butyraldehyde was readily consumed at a rate of 353 nmol min⁻¹ (mg protein)⁻¹. The consumption rate was essentially constant until all the butyraldehyde was consumed. The predicted product of the oxidation of butyraldehyde is butyrate. However, no butyrate accumulated during the oxidation of butyraldehyde. Nonetheless, butyrate consumption was readily observed. With 1 mM butyrate, consumption rates ranged from 28 to 71 nmol min⁻¹ (mg protein)⁻¹. To demonstrate that butyraldehyde was indeed oxidized to butyrate, an inhibitor of butyrate consumption was sought. Sodium valerate (1 mM) was found to be an effective inhibitor of butyrate consumption (Table 1). When cells (56 µg protein) were incubated with 1 mM butyraldehyde and 1 mM valerate, production of butyrate was observed. After 48 min, the butyrate concentration reached 0.5 mM.

The results indicated that butyraldehyde was indeed oxidized to butyrate and the cells further metabolized butyrate in the absence of a metabolic inhibitor.

When cells were incubated in the presence of butane (0.2 atm, 0.26 mM in solution) and 1 mM valerate, butyrate production was observed (Fig. 1d). The specific rate of butyrate production was 38 nmol min⁻¹ (mg protein)⁻¹. This rate is lower than typical rates of butane consumption [90–160 nmol min⁻¹ (mg protein)⁻¹], which could indicate that valerate does not completely block butyrate consumption (perhaps because of inefficient uptake), or that some intermediates other than butyrate also accumulate. With a low protein concentration (0.05 mg protein ml⁻¹), the rate of butyrate production was constant for 30 min (Fig. 1d). When the protein concentration was increased fourfold, the initial rate of butyrate production increased fourfold. However, a maximum concentration was reached at 30 min followed by complete consumption of the butyrate over the next 45 min. Butyrate consumption occurred because the cells consumed the valerate, thereby relieving the inhibition. At 50 min, the valerate concentration had decreased to 0.04 mM. No butyrate was detected when (1) butane was omitted, (2) valerate was omitted or (3) cells were pretreated with acetylene (3%, v/v, acetylene; 15 min). These results further confirm that the pathway of butane oxidation includes production of butyrate.

The consumption of ketones by butane-grown '*P. butanovora*' was examined. Because 2-butanol was consumed by butane-grown cells to produce butanone, it was of interest to determine the rate of butanone consumption. Resting cells of '*P. butanovora*' consumed butanone (1 mM) at a rate of 120 nmol min⁻¹ (mg protein)⁻¹. '*P. butanovora*' produced acetone from 2-propanol (Table 2). When acetone consumption was examined in the absence of 2-propanol, a slow rate of consumption [9 nmol min⁻¹ (mg protein)⁻¹] was observed. Recently, Ensign and coworkers (Ensign *et al.*, 1998) demonstrated that for some bacteria the first step in the metabolism of ketones involves a carboxylation reaction. For example, the consumption of acetone by resting cells of *Xanthobacter* Py2 was stimulated by addition of CO₂ (Sluis *et al.*, 1996). However, no stimulation in the rate of butanone or acetone consumption by butane-grown '*P. butanovora*' was observed upon addition of 2% CO₂ and NaHCO₃ (to 5 mM).

O₂ consumption and cell growth associated with proposed metabolites of butane

Oxidations of butane and the products of butane metabolism require the consumption of O₂ either directly by butane monooxygenase or for oxidation of the reduced electron carriers (e.g. NADH). The addition of butane or its metabolites to cells of '*P. butanovora*' is expected to stimulate O₂ consumption. However, cells of '*P. butanovora*' had a high initial rate of O₂ consumption (endogenous respiration), even without

Table 3. Rates of O₂ consumption by butane-grown '*P. butanovora*' in the presence of various metabolites

Assays were carried out with 25 µl resuspended cells (100 µg protein) in a 1.6 ml electrode chamber at 20 °C. These cells consumed butane at a rate of 160 nmol min⁻¹ (mg protein)⁻¹. O₂ consumption is measured as nmol O₂ consumed min⁻¹ (mg protein)⁻¹. The rate ± standard deviation for three replicates is indicated.

Metabolite added	Concn (mM)	O ₂ consumption	
		Initial rate	Rate after 10 min
None		163 ± 31*	52 ± 14
Butane	0.24	155 ± 11	216 ± 17
1-Butanol	1.00	378 ± 37	429 ± 19
Butyraldehyde	1.00	274 ± 35	371 ± 27
Butyrate	1.00	137 ± 5	290 ± 9
Lactate	1.00	268 ± 6	228 ± 4
2-Butanol	1.00	300 ± 6	166 ± 11

* This initial rate in the absence of added metabolite continued for 3–4 min then decayed with a half-life of about 3–4 min.

addition of substrates (Table 3). This activity was not likely to be due to residual butane because C₂H₂ was not inhibitory or due to other exogenous substrates because additional washings did not substantially diminish the activity. The endogenous respiration rate was constant for 3–5 min then decayed exponentially with a half-life of 3–4 min (data not shown). When butane was added to cell suspensions, no increase above the initial rate of endogenous respiration was observed (Table 3). However, the rate increased with time rather than decreased, which indicates that butane did support O₂ consumption. When butane was added at different time points after the endogenous respiration rate had decayed, progressively lower rates of O₂ consumption were observed. This loss of activity may indicate an inactivation of the butane monooxygenase when exposed to O₂ in the absence of an oxidizable substrate. For 1- and 2-butanol and butyraldehyde, a stimulation in the initial rate of O₂ consumption was observed. For 2-butanol, the rate of O₂ consumption decreased with time, which was expected given the time course of 2-butanol consumption (Fig. 1c). Butyrate exhibited a pattern of O₂ consumption similar to that of butane. While the time courses of O₂ consumption were complex and influenced by changes in endogenous respiration and possibly other factors (e.g. accumulation of metabolites), stimulation of O₂ consumption was evident for all the compounds tested.

'*P. butanovora*' might also be expected to grow on each of the proposed intermediates. Therefore, cell growth on 1-butanol (8.8 mM), butyraldehyde (10 and 2 mM) or butyrate (11.4 mM) was examined. Lactate (19 mM) and 2-butanol (8.8 mM) were included for comparison. All five compounds supported growth. The following

OD₆₀₀ values were reached for each substrate after 2 d growth: butane, 0.59; 1-butanol, 0.70; butyrate, 0.99; lactate, 0.65; 2-butanol, 0.54; and basal medium (containing yeast extract), 0.07. Butyraldehyde at 10 mM was toxic to the cells but supported growth at 2 mM (OD₆₀₀ = 0.21 after 1 d). While growth on 1-butanol, butyraldehyde and butyrate is consistent with the role of these compounds as intermediates in butane metabolism, growth on 2-butanol points out the shortcomings of this approach.

DISCUSSION

In this work, the pathway of butane oxidation in '*P. butanovora*' was found to be butane to 1-butanol to butyraldehyde to butyrate. For the oxidation of any *n*-alkane, a significant question is whether the oxidation occurs at the terminal or subterminal carbons, or both. The site of oxidation determines the products, which, in turn, influence the pathways required to metabolize these products. In the case of '*P. butanovora*', the terminal oxidation product of butane oxidation, 1-butanol, was readily observed (Fig. 1a), while no production of the subterminal oxidation product, 2-butanol, was detected. While a low rate of 2-butanol production cannot be ruled out (e.g. <5% of the rate of 1-butanol production), it also cannot contribute substantially to the oxidation of butane by '*P. butanovora*'. Terminal oxidation of butane was also proposed for *M. vaccae* JOB5 (Phillips & Perry, 1974) and *Nocardia* TB1 (van Ginkel *et al.*, 1987), although 1-butanol production was not demonstrated directly for either bacterium. Furthermore, the experiments did not directly eliminate the possibility that at least a portion of the butane was oxidized to 2-butanol. This distinction is important because propane-grown *M. smegmatis* 422 was demonstrated to be capable of subterminal butane oxidation (Lukins & Foster, 1963).

For propane-oxidizing bacteria, both terminal and subterminal oxidations have been proposed (Lukins & Foster, 1963; Perry, 1968, 1980; Stephens & Dalton, 1986). However, with '*P. butanovora*', again only terminal oxidation of propane was observed. Methane monooxygenases, both particulate and soluble (Burrows *et al.*, 1984; Colby *et al.*, 1977), and ammonia monooxygenase (Hyman *et al.*, 1988) oxidize both butane and propane, though these alkanes do not serve as growth substrates for either methanotrophs or ammonia-oxidizing bacteria. With methane and ammonia monooxygenases, both terminal and subterminal oxidations of these short-chain alkanes were observed. A number of bacteria can grow on longer-chain alkanes such as octane (Klug & Markovetz, 1971). The pathway of octane metabolism involves terminal oxidation of the alkane to form octanol, which is subsequently oxidized to octanal and further to octanoic acid (Baptist *et al.*, 1963). The predominantly terminal oxidation of butane and propane by '*P. butanovora*' is similar to the terminal oxidation of octane.

The results of this work support the pathway for butane

Fig. 2. Scheme of butane and 2-butanol metabolism by butane-grown '*P. butanovora*'. The upper pathway follows from the terminal oxidation of butane. The lower pathway shows the oxidation of 2-butanol, although no subterminal oxidation of butane was detected. Inhibitors of each transformation are indicated above the arrows.

oxidation depicted in Fig. 2. This pathway is supported by direct observation of the products of each step of the pathway. For conversion of butane to 1-butanol and butyraldehyde to butyrate, it was necessary to use inhibitors to slow the consumption of the product such that the product accumulated in the reaction mixture. The pathway was further confirmed by demonstration of: (1) consumption of each of the proposed intermediates, (2) enhancement of O_2 consumption by each of the proposed intermediates and (3) growth of the bacterium on each of the intermediates. Because the amount of substrate consumed was always more than the amount of product that accumulated, the possibility remains that other pathways are functioning simultaneously. However, none of the inhibitors completely blocked the subsequent oxidation of the product, which may also account for some, if not all, of the difference between the amount of substrate consumed and the amount of product that accumulated. At present, it is not known if there are specific uptake systems for alcohols such as 1- and 2-butanol or carboxylic acids such as butyrate and valerate. Inefficient uptake of inhibitors could also explain the incomplete inhibition. Whether or not alternative pathways exist, the results do indicate that the proposed pathway is the predominant pathway. Up to 90% of the butane consumed could be accounted for as 1-butanol. Substantial accumulations of butyraldehyde and butyrate are also consistent with the proposed pathway as the dominant pathway.

The proposed pathway for butane metabolism is identical to that proposed by van Ginkel *et al.* (1987) for *Nocardia* TB1. Production of butyrate from butane (when *Nocardia* TB1 cells were inhibited with arsenite) indicates a terminal oxidation of butane. However, production of 1-butanol (from butane) or butyraldehyde from either butane or 1-butanol was not demonstrated directly. The proposed pathway of butane metabolism for *Nocardia* TB1 was further supported by measurements of activities associated with the intermediates. 1-Butanol-dependent O_2 consumption was rapid in butane-grown cells and only slightly above the endogenous rate in succinate-grown *Nocardia* TB1. Cells are expected to be able to consume intermediates in a

catabolic pathway, provided the compounds are readily transported into the cells and are not toxic to cells at the concentrations used for consumption assays. Therefore, consumption of intermediates provides support for a proposed pathway. However, 2-butanol-dependent O_2 consumption was also observed in butane-grown *Nocardia* TB1 but not in succinate-grown cells. This result could indicate that subterminal oxidation of butane occurs simultaneously with terminal oxidation, that 1-butanol and 2-butanol are oxidized by the same enzyme, or that pathways for oxidation of both 1- and 2-butanol are induced by butane. In the present work, it was also demonstrated that both 1-butanol and 2-butanol stimulated O_2 consumption rates (Table 3) and both were consumed by butane-grown '*P. butanovora*' (Fig. 1b, c). Although butane-grown '*P. butanovora*' did not produce detectable levels of 2-butanol from butane, 2-butanol was nonetheless readily consumed by butane-grown cells. Therefore, the ability to consume a secondary alcohol does not ensure that a subterminal oxidation of the alkane occurs. Future work will focus on isolation and purification of the enzymes involved in butane metabolism and identification and characterization of the genes which encode these enzymes.

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