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Involvement of carbon dioxide in the aerobic biodegradation of ethylene oxide, ethene, and vinyl chloride

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Abstract

The involvement of a carboxylase in metabolism of C-2 alkenes by *Ochrobactrum* sp. strain TD and *Pseudomonas putida* strain AJ was examined. With resting cells of strain TD grown on vinyl chloride, ethene, and ethylene oxide, the maximum specific rate of ethylene oxide consumption decreased significantly in the absence of external CO_2 in comparison to cells provided with room air or added CO_2. The amount of $^{14}CO_2$ incorporated into biomass by resting cells of strain TD grown on ethylene oxide increased more than 13-fold when the assay substrate was ethylene oxide versus acetate. These results indicate that strain TD uses a carboxylase. Similar experiments were performed with strain AJ with the results suggesting that a carboxylase is not involved. In this regard, strain AJ is more similar to various *Mycobacterium* isolates that also do not appear to use a carboxylase during metabolism of vinyl chloride and ethene.

Keywords: Vinyl chloride; Ethene; Ethylene oxide; Carboxylase; Carbon dioxide; Biodegradation

1. Introduction

The involvement of coenzyme M (CoM) during aerobic metabolism of propylene has been demonstrated in *Xanthobacter* strain Py2 and *Rhodococcus rhodochrous* strain B276. The pathway involves epoxyalkane:CoM transferase, dehydrogenase, and oxidoreductase and carboxylase reactions, yielding acetoacetate as a product [1–4]. The monooxygenase and epoxide carboxylase genes for catabolism of propylene are located on a large linear plasmid (320 kb), although these genes are not clustered together [5]. Catabolism of acetone, an isomer of propylene oxide (PrO), also involves carboxylase activity under aerobic and anaerobic conditions [6].

Various bacteria have been isolated that use vinyl chloride (VC) and ethene as sole sources of carbon and energy under aerobic conditions, including several strains of *Pseudomonas* [7–9] and *Mycobacterium* [10], one strain of *Nocardioides* [10], one strain of *Ralstonia* [28], and one strain of *Ochrobactrum* [9]. As with propylene, CoM is involved in the catabolic pathway for ethene and VC in several strains of *Mycobacterium* [11,12] and in one strain each of *Pseudomonas*, *Ochrobactrum* [13], and *Nocardioides* [14]. Coleman and Spain [12] demonstrated that the product of epoxyalkane:CoM transferase from ethylene oxide (EtO) is 2-hydroxyethyl-CoM, although they did not evaluate the pathway beyond this intermediate. 2-Chloro-2-hydroxyethyl-CoM was predicted to be the product from VC-epoxide. No evidence was found for the presence of epoxide carboxylase genes near the epoxyalkane:CoM transferase gene in *Mycobacterium* strain JS60. Conversion of hydroxyethyl CoM to acetyl CoA was predicted to proceed without incorporation of CO_2 [12], as first suggested based on biochemical studies with *Mycobacterium* strain E20 [15].

Sequencing results for linear plasmid DNA from *Nocardioides* strain JS614 revealed several genes involved in alkene metabolism including an alkene monooxygenase, epoxyalkane:CoM transferase, CoA transferase, acyl-CoA synthetase, dehydrogenase, reductase, and possible CoM biosynthesis genes [14]. New evidence suggests that a carboxylase-like protein was expressed in response to VC, ethene, and ethylene oxide in *Nocardioides* strain JS614 [16]. However, no direct biochemical tests were conducted for the presence of a carboxylase in strain JS614.

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Because CoM is involved in aerobic catabolism of VC, ethene, and EtO, we hypothesized that carboxylase activity may be involved in the pathway for some of the microbes that utilize these substrates, analogous to what occurs with propylene in strains B276 and Py2. We evaluated *Ochrobactrum* sp. strain TD and *P. putida* strain AJ, which we previously isolated and characterized with respect to growth on VC, ethene and EtO [9] and the involvement of CoM in the degradation pathway [13].

2. Materials and methods

2.1. Chemicals and media

VC gas (99.5%) was purchased from Fluka (Milwaukee, WI); ethene (99.9%) from Matheson (Montgomeryville, PA); and EtO (99.5%) and PrO (99%+) from Sigma–Aldrich (Milwaukee, WI). $NaH^{14}CO_3$ was purchased from ICN Radiochemicals (50 mCi/mmol) (Irvine, CA). All other chemicals used were of reagent grade. The mineral salts medium used to grow strains AJ and TD is described in Hartmans et al. [17], except that the amount of $(NH_4)_2SO_4$ was reduced to 0.67 g l^{-1}. No vitamins or other complex growth factors were added to the mineral salts medium. The nitrate mineral salts medium used for growth of *R. rhodochrous* strain B276 is described in Whittenbury et al. [18].

2.2. Analytical methods

The total amount of VC, ethene, EtO, propylene, and PrO in culture and serum bottles was determined by gas chromatographic analysis of headspace samples, as previously described [7]. Protein was measured in the supernatants using the bicinchoninic acid–copper assay with bovine serum albumin as a standard [19]. Protein concentrations were determined by lysing cells at 65 °C for 30 min in 3 M NaOH, neutralizing with 1 M HCl, and centrifugation (10 min at 15,000 rpm; Sorvall Microspin 24S).

Glucose was measured by chemical oxygen demand using the Hach method (Hach Company, Loveland, CO; kit range = 5–150 mg l^{-1}). Acetic acid was measured by high performance liquid chromatography (Waters 717, 600E) using an Aminex HPX-87H column (300 mm × 7.8 mm) and a Micro-Guard cartridge (30 mm × 4.6 mm; BIO-RAD, Herculues, CA), a 5 mM H_2SO_4 mobile phase, and a UV–Vis detector (210 nm; Waters 490E).

2.3. Cultures and growth conditions

Cultures were grown at 30 °C in 0.725 l, 2.3 l, or 2.5 l glass bottles. The bottles that were used to grow cultures on EtO, ethene, or VC contained mineral salts medium that filled 28% of the total volume, while those used to grow cultures on propylene contained mineral salts medium that filled 20% of the total volume. The initial amount of ethene and propylene provided was 10% of the headspace volume. The initial amount of EtO and VC provided was 2%; higher percentages of VC were inhibitory. Repeated additions of EtO and VC were made as they were consumed, to provide enough substrate to reach the exponential growth phase; the amount of VC and EtO remaining was never allowed to decrease below 0.5% in the headspace. An adequate amount of oxygen was present initially to reach the exponential growth phase so that it did not have to be added during incubation. Cumulative substrate consumption was monitored as a function of time and used to calculate protein accumulation based on the observed protein yield [10]. From these curves it was apparent how much substrate had to be consumed to reach the exponential growth phase. Cells were harvested (see below) during the exponential growth phase for the maximum utilization rate and CO_2 incorporation experiments.

2.4. Effect of CO_2 on maximum substrate utilization rates

Strains AJ, TD, and B276 were grown from an initial OD_{600} of 0.04–0.085 to exponential phase (estimated OD_{600} of 0.2–0.8, based on correlation of substrate consumption and OD_{600}). Cells were centrifuged (15 min at 10,000 rpm; Servall SS-3) and washed in 50 mM phosphate buffer (KP), pH 7.2. Cells were centrifuged again, resuspended in 10–30 ml KP, and 1 ml was added to 10 ml serum bottles containing a micro stir bar. A higher dilution level (100 ml KP) was evaluated with strain AJ to determine if a difference in utilization rates could be detected at a lower cell concentration. Resting cells are defined as cultures that have been washed and suspended into KP buffer. This was done to minimize protein synthesis, based on the absence of a source of nitrogen. The treatments tested were (1) with a CO_2 trap; (2) with CO_2 and bicarbonate added; and (3) with normal room air. Preparation of CO_2 free air and nitrogen, the CO_2 trap, and the molar amounts of CO_2 and bicarbonate were the same as previously described [20]. Serum bottles were placed on a magnetic stirrer (Lab-Line Multi-Magnestir) set at 4000 rpm. Experiments were initiated by adding 2000 nmol of substrate per serum bottle. Substrate consumption was monitored by gas chromatographic analysis of headspace samples for VC, ethene, EtO, propylene, and PrO; high performance liquid chromatography for acetate; and chemical oxygen demand for glucose (see above).

2.5. Incorporation of $^{14}CO_2$ into cell suspensions

Stains AJ and TD were grown on EtO and strain B276 was grown on propylene to exponential phase. The cells were then centrifuged and resuspended in serum bottles containing 10 ml KP, as described above. Resting cells are defined as cultures that have been washed and suspended into KP buffer. This was done to minimize protein synthesis. Incorporation of $^{14}CO_2$ into biomass was determined as previously described [20]. $NaH^{14}CO_3$ was added from a stock solution to give a specific radioactivity of 59 μCi/mmol of CO_2–$NaHCO_3$. The solutions were incubated with the resting cells for 2 min followed by addition of substrate (EtO, PrO, acetate, or glucose). Assays were prepared in duplicate with one set of serum bottles containing $^{14}CO_2$ while the other set did not. When substrate consumption was complete, a 0.1 ml sample of the cell solution was removed from the bottles containing $NaH^{14}CO_3$ and passed through two filters (Whatman GF-A fiber filter placed on top of a 2.5 cm Supor filter). The filters were washed four times with 50 mM $NaHCO_3$–K_2HPO_4 (pH 8.0) and placed into liquid scintillation cocktail (Scinti Safe Plus™ 50%). Samples were counted for radioactivity with a Packard 2550 TA/RB liquid scintillation analyzer.

3. Results

3.1. Effect of CO_2 on maximum substrate utilization rates

The maximum specific rate of EtO consumption by resting cells of strain AJ grown on ethene was not affected by CO_2 removal (Fig. 1a) or addition of CO_2 (Fig. 1b), suggesting that CO_2 is not a reactant in the catabolic pathway. When only room air was available, the rate of EtO removal was similar to the treatment with CO_2 added (Fig. 1c). Different results were obtained with ethene-grown strain TD. Removal of CO_2 significantly slowed the rate of EtO consumption (Fig. 1d) in comparison to adding CO_2 (Fig. 1e) or providing only room air (Fig. 1f). These results suggest CO_2 may be involved as a reactant in the catabolic pathway of strain TD.

Maximum specific EtO utilization rates for strains AJ and TD grown on VC, ethene and EtO are summarized in Table 1. The type of growth substrate did not alter the pattern of the results described above. With strain AJ, the presence or absence of CO_2 did not have a significant affect on the rate of EtO utilization. With strain TD, removal of CO_2 by trapping in KOH reduced the rate of EtO consumption by one order of magnitude; adding CO_2 above what was present in room air did not alter the rate of EtO use.

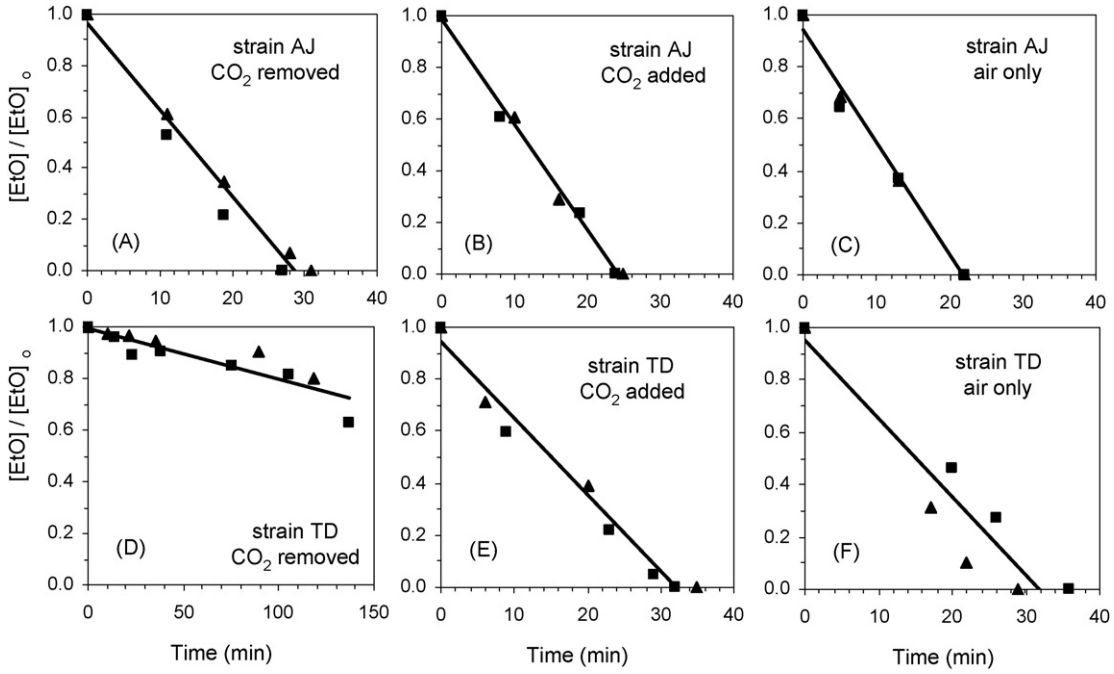

Fig. 1. Effect of CO_2 on the rate of EtO consumption by strains AJ and TD grown on ethene. Different symbols in a panel represent duplicate bottles. The ordinate axis is the ratio of EtO concentration at time t to the initial concentration.

Strain B276 was used as a positive control since previous studies showed it incorporates CO_2 during catabolism of PrO [21]. As with strain TD, removal of CO_2 significantly decreased the maximum rate of substrate utilization by strain B276, although providing an excess above what was available in room air did not stimulate the utilization rate (Table 1). A decrease in the maximum specific rate of PrO consumption during the same type of resting cell assay, when CO_2 was not available, has also been shown with propylene-grown *Xanthobacter* strain Py2 [20].

Air was present in the headspace of the serum bottles during the whole cell assays, so that oxygen was available to the cells. When air was replaced with nitrogen to minimize the availability of internally generated CO_2 (via mineralization), very little EtO consumption occurred with strains AJ and TD (data not shown).

3.2. Incorporation of $^{14}CO_2$ into cell suspensions

Incorporation of $^{14}CO_2$ into resting cells is summarized in Table 2. The amount of $^{14}CO_2$ incorporated by strain TD grown on EtO was more than 13 times higher when EtO was the substrate compared to acetate (from 0.18 to 2.47 mol CO_2 per mol of substrate), the catabolism of which does not require CO_2. With strain AJ, there was no significant difference between the amount of CO_2 incorporated when the substrate was EtO or acetate. Approximately twice as much CO_2 was incorporated by strain B276 when the assay substrate was PrO versus glucose.

Table 2 also shows the results in terms of the amount of ^{14}C incorporated into biomass as a percentage of the total $NaH^{14}CO_3$ added. For strains AJ and TD, the amount of $^{14}CO_2$ incorporated was approximately the same when no

Table 1
Effect of CO_2 on maximum specific substrate utilization rates for resting cells

Strain	Growth substrate	Assay substrate	Substrate utilization rates (nmol min^{-1} mg $protein^{-1}$)[a]		
			Air only	CO_2 added	CO_2 removed
AJ	VC	EtO	17.8 (±0.1)	17.9 (±1.9)	18.0 (±1.8)
	Ethene	EtO	24.6 (±3.6)	23.6 (±0.1)	23.6 (±0.9)
	EtO	EtO	26.1 (±0.2)	25.7 (±5.9)	25.8 (±1.3)
TD	VC	EtO	12.1 (±0.6)	13.0 (±1.2)	5.8 (±0.1)
	Ethene	EtO	42.5 (±6.7)	40.2 (±2.1)	4.3 (±2.1)
	EtO	EtO	42.3 (±5.0)	41.8 (±1.5)	5.2 (±0.1)
B276	Propylene	PrO	57.8 (±8.5)	55.6 (±1.4)	7.6 (±2.9)

[a] Average of duplicates; data ranges are in parentheses.

Table 2
CO_2 incorporation by resting cells[a]

Strain	Substrate	Mol CO_2 incorporated per mol substrate consumed	$^{14}CO_2$ incorporated into biomass (% of total ^{14}C added)
TD	EtO	2.47 (±0.16)	9.9 (±0.65)
	Acetate	0.18 (±0.04)	0.72 (±0.14)
	None	–	0.68 (±0.07)
AJ	EtO	0.35 (±0.02)	1.4 (±0.06)
	Acetate	0.38 (±0.01)	1.1 (±0.02)
	None	–	0.65 (±0.12)
B276	PrO	0.99 (±0.06)	4.0 (±0.20)
	Glucose	0.49 (±0.05)	1.5 (±0.20)
	None	–	1.4 (±0.10)

[a] Average of duplicates; data ranges are in parentheses.

substrate was present and when acetate was added. Similar results were obtained for strain B276 when no substrate was added and when glucose was added. The amount of $^{14}CO_2$ incorporated with substrates whose catabolism does not involve carboxylase activity (in this case, acetate and glucose) was not significantly different from the background amount of $^{14}CO_2$ incorporation when no substrate was present. The significantly higher percentage of ^{14}C activity incorporated into biomass during catabolism of EtO by strain TD and PrO by strain B276 is also apparent in Table 2.

4. Discussion

Strains TD and B276 are similar with respect to their use of CoM and a carboxylase during metabolism of alkenes. With strain B276, the expected stoichiometry is one mole of CO_2 incorporated per mole of PrO [3]. Our results with strain B276 are in good agreement with this expectation (Table 2), although the net amount of CO_2 incorporation is lower when subtracting out the background uptake based on glucose as a substrate. The stoichiometry of CO_2 incorporation by strain TD was approximately two moles of CO_2 per mole of EtO consumed, after subtracting out the amount of $^{14}CO_2$ incorporated when acetate was the substrate (Table 2). Based on the proposed pathway for ethene catabolism by Coleman and Spain [12], a possible pathway for EtO metabolism by strain TD would proceed by conversion to 2-hydroxyethyl CoM and oxidation to 2-ketoethyl-CoM followed by addition of one mole of CO_2 to each carbon as CoM is released. The presumptive C-4 product would be oxaloacetate, with no net gain or loss of reducing power. A similar pathway may occur when VC is converted to 2-chloro-2-hydroxyethyl CoM, although its conversion to 2-ketoethyl-CoM involves an elimination reaction rather than an oxidation, so a net input of reducing power would be required to form oxaloacetate from VC.

Considering that strain TD appears to incorporate CO_2 during EtO catabolism, it is not yet known why oxygen was required for EtO utilization during the whole cell assay even when an exogenous source of CO_2 was provided. One possibility is the need for ATP to drive EtO uptake or CO_2 incorporation. The overall conversion of 1 mole of EtO plus 2 moles of CO_2 to 1 mole of oxaloacetate is exergonic at the assay pH of 7.2 (based on free energies of formation from Lide [22]; Madigan et al. [23]; Reid et al. [24]; Voet and Voet [25] and a Henry's law constant for ethylene oxide from Reid et al. [24]). Nevertheless, one of the steps in the pathway may be endergonic and require an input of ATP. Carboxylase activity in acetone-grown strain Py2 and strain B276 is dependent on the availability of ATP and GTP, respectively [26,27]. Resting cell assays of strain Py2 and B276 were not tested in the absence of oxygen to determine if the reaction with acetone could proceed without oxygen. It is also possible that the carboxylase-like enzyme in strain TD functions differently than the one found in strain B276.

The substrate utilization assay and CO_2 incorporation results indicate that a carboxylase is not involved in the pathway for EtO catabolism used by strain AJ. When growing on VC and ethene, strain AJ appears to use a non-carboxylase pathway that may be similar to the one proposed for various *Mycobacterium* strains [12] and *Nocardioides* strain JS614 [14] via the formation of carboxymethyl-CoM. The gene organization in *Mycobacterium* strain JS60 based on sequencing results suggest the involvement of a CoA transferase and acyl-CoA synthetase [12]. These enzymes are located directly upstream from the alkene monooxygenase and CoM transferase genes. Coleman and Spain [12] suggested coenzyme A transferase and synthetase transfer coenzyme A onto 2-ketoethyl-CoM while removing CoM. Mattes et al. [14] further elucidated this pathway in VC and ethene-grown *Nocardioides* strain JS614.

In summary, the results of this study provide evidence that strain TD uses a carboxylase during catabolism of EtO (and by extension ethene and VC), while strain AJ does not. Additional studies are needed to further characterize the pathways. From an application standpoint, identification of the enzyme(s) that are unique to VC metabolism is a high priority (if indeed such enzymes exist), in order to provide a basis for distinguishing between the potential for VC and ethene biodegradation in aerobic environments.

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