Bidirectional usage of ferulate by the acetogen *Peptostreptococcus productus* U-1: CO₂ and aromatic acrylate groups as competing electron acceptors

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The influence of CO₂ on the ability of *Peptostreptococcus productus* U-1 (ATCC 35244) to use an aromatic acrylate group as an energy-conserving electron acceptor during O-methyl-dependent growth was examined. Ferulate (a methoxylated phenylacrylate), unlike hydroferulate (a methoxylated phenylpropionate), supported growth under CO₂-limited conditions. Two phases occurred during ferulate utilization in CO₂-limited cultures. In phase I (maximum growth), O-methyl-derived reductant was coupled mainly to acrylate group reduction, and acetate synthesis (CO₂ as reductant sink) was minimal. In phase II, acetate synthesis increased, but cell yields in this phase were much less than in phase I. In CO₂-enriched cultures, distinct phases were not observed; reductant was coupled equally to CO₂ and acrylate group reduction. Under CO₂-enriched conditions, O-methyl and acrylate groups were incompletely metabolized, and molar growth yields were significantly lower compared to CO₂-limited conditions. Resting cell studies indicated that O-demethylase and aromatic acrylate oxidoreductase activities were induced by ferulate. These findings demonstrated that *P. productus* U-1 can use the aromatic acrylate oxidoreductase system as a sole, energy-conserving, electron-accepting process, but is not able to prevent the simultaneous use of the bioenergetically less favourable acetyl-CoA pathway during O-methyl-dependent growth.

Keywords: acetogenic bacteria, *Peptostreptococcus productus*, phenylacrylates, O-demethylation, reductant sinks

INTRODUCTION

Acetogens utilize CO₂ as a terminal, energy-conserving electron acceptor and concomitantly synthesize acetate by the acetyl-CoA or 'Wood-Ljungdahl' pathway (Diekert & Wohlfarth, 1994; Drake, 1994; Ljungdahl, 1994; Ragsdale, 1991; Wood & Ljungdahl, 1991). Some acetogens also have the capacity to utilize aromatic acrylate groups as an alternative reductant sink (Drake *et al.*, 1994). For example, *Acetobacterium woodii* reduces aromatic acrylate groups (phenylacrylates) to phenylpropionates via the oxidation of organotrophic or lithotrophic substrates under CO₂-enriched conditions; this process conserves energy for *A. woodii* (Bache & Pfennig, 1981; Hansen *et al.*, 1988; Tschek & Pfennig, 1984). Other acetogens, including *Syntrophococcus sucromutans* (Krumholz & Bryant, 1986), TH-001 (Frazer & Young,

1985), *Clostridium (Oxobacter) pfennigii* (Krumholz & Bryant, 1985), *Clostridium acetivum* (Lux & Drake, 1992), *Clostridium formicoaceticum* (Lux & Drake, 1992) and *Peptostreptococcus productus* U-1 (Parekh *et al.*, 1992), also reduce acrylate groups under CO₂-enriched conditions; whether this is an energy-conserving process for these bacteria has not been reported.

Studies to date have shown that *P. productus* U-1 (ATCC 35244) utilizes or transforms a number of aromatic compounds (Lux *et al.*, 1990; Parekh *et al.*, 1992). Ferulate undergoes two transformations: O-demethylation and reduction of the double bond of the acrylate group (Fig. 1). Whether reduction of the aromatic acrylate group precedes O-demethylation or is regulated by environmental conditions is not known. In the present study, *P. productus* U-1 was studied to examine the influence of CO₂

Fig. 1. Bidirectional pathway for the use of ferulate by *P. productus* U-1.

on the potential of aromatic acrylate-reducing acetogens to use the aromatic acrylate oxidoreductase system as the sole, energy-conserving, terminal electron-accepting process during *O*-methyl-dependent growth.

METHODS

Organism and cultivation. *P. productus* U-1 (ATCC 35244; Lorowitz & Bryant, 1984) was cultivated anaerobically at 37 °C in a CO₂-enriched or CO₂-limited medium in butyl-rubber-stoppered, crimp-sealed tubes [18 × 150 mm, 27 ml approximate stoppered volume at 1 atm (101.33 kPa)]. The CO₂-enriched medium contained (mg l⁻¹): NaHCO₃, 7500; KH₂PO₄, 500; NaCl, 400; NH₄Cl, 400; MgCl₂·6H₂O, 50; CaCl₂·2H₂O, 10; resazurin, 1; yeast extract, 500; nicotinic acid, 0.25; cyanocobalamin, 0.25; *p*-aminobenzoic acid, 0.25; calcium D-pantothenate, 0.25; thiamin·HCl, 0.25; riboflavin, 0.25; lipoic acid, 0.25; folic acid, 0.1; biotin, 0.1; pyridoxine·HCl, 0.10; sodium nitrilotriacetate, 7.5; MnSO₄·H₂O, 2.5; FeSO₄·7H₂O, 0.5; Co(NO₃)₂·6H₂O, 0.5; ZnCl₂, 0.5; NiCl₂·6H₂O, 0.25; H₂SeO₄, 0.25; CuSO₄·5H₂O, 0.05; AlK(SO₄)₂·12H₂O, 0.05; H₃BO₃, 0.05; Na₂MoO₄·2H₂O, 0.05; and Na₂WO₄·2H₂O, 0.05. The medium was prepared by boiling and cooling the above components under 100% CO₂, adding Na₂S·9H₂O (250 mg l⁻¹) and cysteine·HCl·H₂O (250 mg l⁻¹) and dispensing under 100% CO₂ into tubes (7 ml per tube). Tubes were then crimp-sealed and autoclaved. The CO₂-limited medium was the CO₂-enriched medium with the NaHCO₃ and KH₂PO₄ replaced by Na₂HPO₄ (5.68 g l⁻¹) and NaH₂PO₄ (7.20 g l⁻¹). The CO₂-limited medium was boiled, cooled and dispensed under 100% N₂. When large quantities of cells were required, cells were grown in 1.1 l infusion bottles (Müller-Krempel; 500 ml medium per bottle). The initial pH of media was 6.6–6.8. In all experiments, growth was initiated by injecting 0.5 ml of inoculum per tube or 50 ml of inoculum per bottle, respectively. Stock solutions of substrates were prepared as previously

described (Lux *et al.*, 1990). Values for growth and substrate-product profiles are the average of duplicate experiments; the standard deviation of reported means was less than 10%.

Preparation of resting cell suspensions. Cells were harvested during mid-exponential growth by centrifugation, washed and resuspended in anoxic 50 mM sodium phosphate buffer (pH 7) to an OD₆₆₀ of 2. All cell manipulations and assay preparations (see below) were performed in an anaerobic chamber (Mecaplex; gas phase, 100% N₂).

Assay for *O*-demethylase and aromatic acrylate oxidoreductase activities. Assays were performed at 37 °C in crimp-sealed tubes containing 6 ml resuspended cells. Resting cell suspensions were pre-incubated for 20 min prior to use; CO (446 μmol) was added (via a sterile syringe) at the start of the pre-incubation period (CO being conceptualized as a source of reductant for the aromatic acrylate oxidoreductase or as a performed carbonyl moiety for acetyl-CoA synthesis via *O*-demethylase activity). Assays were initiated by injection of ferulate to a final concentration of 2.5 mM. *O*-Demethylase and aromatic acrylate oxidoreductase activities were measured by quantifying the *O*-demethylation [i.e. ferulate consumption (corrected for hydroferulate formed)] and acrylate group reduction (ferulate → hydrocaffeate or hydroferulate) of ferulate, respectively. Activities are the average of duplicate experiments and were calculated during the time interval when ferulate consumption and aromatic product formation were approximately linear (0–60 min without CO and 0–30 min with CO). One unit of activity for *O*-demethylase and aromatic acrylate oxidoreductase was equal to 1 nmol ferulate *O*-demethylated and acrylate group of ferulate reduced (min)⁻¹, respectively.

Analytical. Growth was measured at 660 nm with a Spectronic 501 spectrophotometer (Bausch & Lomb); the optical path width (inner diameter of tubes) was 1.5 cm. Because of slime production by *P. productus* U-1 during growth, the following procedure was used for cell dry weight determinations. Cells harvested by centrifugation were resuspended in buffer and transferred to pre-dried and pre-weighed Eppendorf centrifuge tubes. Cells were re-pelleted (supernatant fluid was discarded) and dried in the Eppendorf tubes at 80 °C for 48 h, cooled to room temperature in a desiccator, reweighed and dry weights calculated. An OD₆₆₀ of 1 was equal to a cell yield (mg dry wt l⁻¹) of 250 for CO₂-enriched ferulate cultures, 275 for CO₂-limited ferulate cultures and 318 for CO₂-enriched fructose cultures. The latter value was used for the calculation of cell yields from CO or pyruvate cultures. Substrates and products in culture fluids were quantified by high-performance liquid chromatography (HPLC) as previously described (Daniel *et al.*, 1990; Matthies *et al.*, 1993; Seifritz *et al.*, 1993).

RESULTS

O-Methyl-dependent growth in the presence and absence of supplemental CO₂

Both ferulate and hydroferulate supported the growth of *P. productus* U-1 under CO₂-enriched conditions (Fig. 2). However, of these two substrates, only ferulate was growth-supportive under CO₂-limited conditions, indicating that the aromatic acrylate group was essential for *O*-methyl-dependent growth during CO₂ deprivation. 3,5-Dimethoxy-4-hydroxycinnamate was also growth-supportive in the absence or presence of CO₂, while 3,4-dimethoxycinnamate did not support growth in the presence or absence of CO₂ (data not shown).

Fig. 2. Growth of *P. productus* U-1 at the expense of 5 mM ferulate (■, □) and 5 mM hydroferulate (●, ○) under CO₂-enriched (■, ●) or CO₂-limited (□, ○) conditions. Control cultures (▲, △, CO₂-enriched; △, CO₂-limited) lacked ferulate or hydroferulate.

Fig. 3. Ferulate-dependent growth and substrate-product profiles of *P. productus* U-1 under CO₂-limited (a) and CO₂-enriched (b) conditions. ●, Growth (OD₆₆₀); ■, ferulate; ○, acetate; ▲, hydroferulate; □, hydrocaffeate; and △, caffeate.

To determine if aromatic acrylate groups were essential during CO₂ limitation for growth at the expense of substrates other than *O*-methyl groups, cells were grown at the expense of H₂ under CO₂-limited conditions and in

the presence of caffeate (caffeate alone was neither growth-supportive nor metabolized). H₂, which alone was not growth-supportive under CO₂-limited conditions, supported growth when caffeate was present (data not shown).

Effects of CO₂ availability on ferulate-dependent molar growth yields and transformation patterns

Under CO₂-enriched conditions, molar growth yields [g dry wt cells formed (mol ferulate consumed)⁻¹] was approximately 3.2, compared to 6.8 for cells grown under CO₂-limited conditions. These results suggested that the presence of both CO₂ and an acrylate group as reductant sinks was detrimental to overall energy conservation.

In the absence of CO₂, two phases were observed in relation to ferulate metabolism and growth (Fig. 3a; Table 1). In phase I, ferulate was totally consumed, and hydroferulate and hydrocaffeate were formed as aromatic products, indicating initial utilization of the acrylate group as the main reductant sink; acetate formation was minimal. In phase II, hydroferulate was consumed, and acetate formation was increased. Approximately fourfold more biomass was formed per theoretical pair of reducing equivalents utilized in phase I than in phase II, demonstrating that cells were more energetically competent when growth was coupled to acrylate group reduction (phase I) than CO₂ reduction (phase II).

In CO₂-enriched cultures, distinct phases relating to ferulate utilization were not observed (Fig. 3b). Hydroferulate, hydrocaffeate, caffeate and acetate were all formed parallel to growth and, in contrast to CO₂-limited conditions, the transient accumulation and subsequent turnover of hydroferulate was not observed. Reductant derived from *O*-methyl groups was recovered nearly equally from acetate and reduced acrylate groups; similar reductant distribution patterns were observed in CO₂-limited cultures at the completion of growth (Table 1). However, compared to CO₂-limited conditions where acrylate group reduction preceded the reduction of CO₂ to acetate, the presence of supplemental CO₂ and the use of both reductive processes under CO₂-enriched conditions hindered cell energetics (Table 1). In this regard, when caffeate was added to CO₂-enriched cultures containing hydroferulate or vanillate (a growth-supportive methoxylated benzoate), the acrylate group of caffeate was reduced but *O*-methyl-dependent growth efficiencies were decreased (data not shown).

O-Demethylase and aromatic acrylate oxidoreductase activities at the cellular level

In resting cell suspensions, *O*-demethylase and aromatic acrylate oxidoreductase activities were higher in CO₂-limited, ferulate-grown cells than in CO₂-enriched, ferulate-grown cells (Table 2). With ferulate-grown cells, *O*-demethylase activities were enhanced by CO, whereas aromatic acrylate oxidoreductase activities were only slightly stimulated by the presence of CO. Pyruvate-, CO-, and fructose-grown cells displayed negligible *O*-

Table 1. Metabolism of *O*-methyl and acrylate groups of ferulate by *P. productus* U-1

Values were obtained from the time-course experiment (see Fig. 3).

Parameter assessed	Growth conditions		
	CO ₂ -limited		CO ₂ -enriched
	Phase I	Phase II*	
Substrate and product:			
Ferulate consumed (mM)	5.4	0	4.2
Cell yield (mg dry wt l ⁻¹)	31.0	11.3	10.4
<i>O</i> -Methyl group consumed (mM)	2.3	3.1	2.2
Acrylate group reduced (mM)	4.8	0.3	3.3
Acetate formed (mM)	0.4	1.4	0.6
Cell energetics:			
Sink for <i>O</i> -methyl reductant:†			
Acrylate group (%)	70.4	3.6 (31.5)	48.9
CO ₂ (%)	21.8	58.6 (44.4)	33.2
Biomass per 2[H]‡	4.6	1.2 (2.6)	1.5
Recovery:			
Reductant [H] (%)	92	62	82
Aromatic ring (%)	95	110	98

* Values in parentheses were based on overall (phase I and II) substrate-product profiles and cell yields.

† Percentage *O*-methyl reductant in the acrylate group and in CO₂ were calculated as: $[(2[H])(\text{mM acrylate group reduced})]/(6[H])(\text{mM } O\text{-methyl group consumed}) \times 100$ and $[(8[H])(\text{mM acetate formed})]/(6[H])(\text{mM } O\text{-methyl group consumed}) \times 100$, respectively.

‡ Defined as the amount (in mg) of cell biomass synthesized per theoretical pair (mmol) of reducing equivalents.

Table 2. Effects of growth conditions on *O*-demethylase and aromatic acrylate oxidoreductase activities in resting cells of *P. productus* U-1

Substrate	Growth conditions		<i>O</i> -demethylase activity [U (mg cell dry wt) ⁻¹]	Aromatic acrylate oxidoreductase activity [U (mg cell dry wt) ⁻¹]
	CO ₂	CO added to assay (446 µmol)		
Ferulate	Limited	+	44.3	49.2
		—	12.1	40.7
Ferulate	Enriched	+	20.7	34.8
		—	3.7	21.1
Pyruvate	Enriched	+	5.3	0
CO	Enriched	+	4.0	0
Fructose	Enriched	+	0	0

demethylase and aromatic acrylate oxidoreductase activities (Table 2).

DISCUSSION

Although the role and importance of CO₂ in the bioenergetics and growth of acetogens has been well-documented, the question of how acetogens respond metabolically to extracellular CO₂ remains largely un-

explored. CO₂-limited conditions have been used to assess the ability of acetogens to use alternative reductant-consuming processes for growth. This approach has revealed that some acetogens can utilize thiosulfate as an electron acceptor during methanol-dependent growth in the absence of CO₂ (Beatty & Ljungdahl, 1990), can couple H₂ oxidation to fumarate reduction when CO₂ is limited (Matthies *et al.*, 1993) or can employ ethanol fermentation during CO₂-limited, glycerol-dependent growth (Misoph

& Drake, 1996). The present findings demonstrated that *P. productus* U-1 can use an aromatic acrylate oxidoreductase system as a sole, energy-conserving, electron-accepting process for growth and that the availability of extracellular CO₂ affects the use of acrylate group reduction for the conservation of O-methyl-derived energy.

The approximate standard change in Gibb's free energy for acrylate group reduction is $-43 \text{ kJ (mol reducing equivalent)}^{-1}$ based on the H₂-dependent reduction of caffeate to hydrocaffeate, and for acetogenesis is $-13 \text{ kJ (mol reducing equivalent)}^{-1}$ based on the H₂-dependent reduction of CO₂ to acetate (Cord-Ruwisch *et al.*, 1988; Seitz *et al.*, 1990). Thus, acrylate group reduction as the principal energy-conserving process during O-methyl-dependent growth in the absence of CO₂ is thermodynamically feasible. In *A. woodii*, H₂-dependent reduction of the acrylate group is apparently coupled to energy conservation via an electron-transport-mediated process; however, the mechanism of coupling is unresolved (Hansen *et al.*, 1988).

Cell yields of *P. productus* U-1 were reduced by the simultaneous availability of acrylate groups, supplemental CO₂ and O-methyl groups. This inhibition was specific for O-methyl-grown cells since H₂- or fructose-grown cells were not inhibited by the presence of both an acrylate group (caffeate) and CO₂ (data not shown). Furthermore, when CO₂ was added to CO₂-limited medium containing ferulate, O-methyl consumption and acrylate reduction were incomplete and molar growth yields were reduced (data not shown). In the presence of CO₂, cells simultaneously routed reductant derived from O-methyl oxidation to both acrylate groups and CO₂. This simultaneous use of two reductant-consuming processes suggests that a lack of regulation in reductant flow may have resulted in the uncoupling of overall energy conservation. Only when acrylate group reduction and CO₂ reduction to acetate occurred consecutively (as observed during CO₂ deprivation) were cell energetics optimized. In contrast, *A. woodii* selectively utilizes acrylate group respiration, rather than acetogenesis, during methanol-dependent growth under CO₂-enriched conditions; however, this preference is not observed during H₂-dependent growth (Hansen *et al.*, 1988; Tschech & Pfennig, 1984).

Aromatic acrylate oxidoreductase and O-demethylase activities were induced by ferulate. Ferulate also induces both of these activities in *A. woodii*, and induction of O-demethylation activity by methoxylated aromatic compounds occurs in a number of acetogens (see Frazer, 1994, for an overview). With ferulate-grown cells of *P. productus* U-1, cells grown in the absence of CO₂ displayed higher levels of aromatic acrylate oxidoreductase and O-demethylase activities than cells grown in the presence of CO₂. Supplemental CO was not essential to aromatic acrylate oxidoreductase activities, indicating that acrylate group reduction could be coupled to the oxidation of O-methyl groups. In contrast, O-demethylase activities were significantly stimulated by the presence of CO. In resting

cell assays, the broad specificity O-demethylase of *Clostridium thermoaceticum* (*Moorella thermoacetica*) is also stimulated by CO (Wu *et al.*, 1988; Daniel *et al.*, 1991); in this organism, CO acts as a carbonyl donor (rather than as a reductive activator of the O-demethylase) for acetyl-CoA formation during O-demethylation-transfer reactions catalysed by the O-demethylase and purified enzymes (methyltransferase, corrinoid/iron-sulfur protein, and acetyl-CoA synthase) of the acetyl-CoA pathway (Kasmi *et al.*, 1994; Ragsdale, 1991).

The utilization of reductant sinks that can supplement or replace acetogenesis as an energy-conserving process might augment the competitiveness of an acetogen in its natural environment. Indeed, some acetogens prefer to utilize a reductant sink other than CO₂ even when exogenous CO₂ is abundant, suggesting that the primary function conceptualized for acetogens (i.e. the reductive synthesis of acetate from CO₂) may not always be the preferred function. For example, *C. thermoaceticum* and *Clostridium thermoautotrophicum* utilize nitrate, not CO₂, as the preferred respiratory sink during O-methyl oxidation by regulation (i.e. repression) of the acetyl-CoA pathway (Seifritz *et al.*, 1993; Fröstl & Drake, 1994). In *P. productus* U-1, the acetyl-CoA pathway is apparently not well regulated during O-methyl-coupled, acrylate group respiration, resulting in the bioenergetically unfavourable, simultaneous use of two reductant-consuming processes.

ACKNOWLEDGEMENTS

We express our appreciation to Susanna Braus-Stromeyer for review of the manuscript. This investigation was supported by the Bundesministerium für Bildung, Wissenschaft, Forschung, und Technologie (0339476A0).

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Received 13 December 1995; revised 25 March 1996; accepted 28 March 1996.