Growth inhibition and pyruvate overflow during glucose metabolism of *Eubacterium limosum* are related to a limited capacity to reassimilate CO₂ by the acetyl-CoA pathway

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(Received 18 January 1993; revised 26 February 1993; accepted 8 March 1993)

The growth characteristics of *Eubacterium limosum*, an anaerobic acetogen, have been described kinetically for fermentation of glucose. A short-lived exponential growth phase was associated with a homoacetogenic fermentation in which all catabolic CO₂ liberated was reassimilated via the acetyl-CoA pathway. During this phase no additional products were observed. A major shift in the metabolism leading to a mixed-acid fermentation pattern (acetate and butyrate) occurred coincident with the onset of the linear growth phase. Significantly diminished yields of acetate in this decelerating growth period were attributed to the limited capacity of the enzymes responsible for reductive generation of methyl donor within the acetyl-CoA pathway: CO₂ and H₂ accumulated in the gas phase. Pyruvate overflow (and to a lesser extent lactate, glycerate and carbon monoxide accumulation) were also observed, though pyruvate-ferredoxin oxidoreductase specific activity remained constant. In all experiments specific biomass formation rates were directly related to the rate of acetyl-CoA formation. The exponential growth phase was prolonged and the production of all compounds other than acetate was diminished when the medium was supplemented with tungsten, suggesting that formate dehydrogenase may be the enzyme limiting the correct functioning of the acetyl-CoA pathway.

Introduction

Eubacterium limosum is an acetogenic anaerobe having a wide spectra of metabolizable substrates and producing acetate and/or butyrate as major fermentation end-products. This micro-organism is able to develop at variable growth-rates on many multi-carbon substrates including hexoses, pentose sugars, lactate, dihydroxyacetone and also on several reduced one-carbon compounds, e.g. methanol, formate, H₂/CO₂, carbon monoxide, methoxyl-substituted aromatics (Lindley *et al.*, 1990). A high proportion of carbon substrate is converted to fermentation end-products in order to create the necessary energetic balance for efficient cell growth. The

relative proportions of the organic acids produced vary with the culture conditions and the substrate. Acidogenesis plays an essential role in energy (ATP) production via substrate-level phosphorylation, while the production of butyrate (and also H₂) can also be viewed as electron sinks to avoid the inhibitory accumulation of excess reducing equivalents. During heterotrophic growth, H₂/CO₂ can also be produced (Sharak-Genthner *et al.*, 1981), though the organism is capable of reassimilating these products to produce acetate via the acetyl-CoA pathway at low growth rates (Loubière *et al.*, 1992). This catabolic versatility makes *E. limosum* an interesting acetogenic model for the study of the regulation of carbon and energy flow in anaerobic bacteria.

The possibility for *E. limosum* to grow on one-carbon substrates is due to the acetyl-CoA pathway which enables a unicarbonotrophic synthesis of acetyl-CoA. This pathway was first described for *Clostridium thermoaceticum* by Barker & Kamen (1945) and is found in all acetogenic bacteria able to exploit one-carbon substrates and also in a similar form in some methanogenic

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bacteria (Vogels & Visser, 1983). The acetyl-CoA formed from one-carbon substrates is used for both acidogenesis and anabolic precursor synthesis via an incomplete TCA cycle and the gluconeogenic pathways (Eden & Fuchs, 1983).

During heterotrophic growth of homoacetogens like *E. limosum* on glucose, acetyl-CoA and other cell-precursors are synthesised via glycolysis. The particularity of homoacetogenic species is their capacity to fix CO₂ (via the acetyl-CoA pathway) using reduced coenzymes provided from multi-carbon substrate catabolism (Barker & Kamen, 1945; Dolfig, 1988). This autotrophic activity, which allows the synthesis of an additional acetyl-CoA per glucose, can replace H₂ and butyrate synthesis for reduced coenzyme recycling. However, for certain species (including *E. limosum*), this metabolic versatility is limited since H₂/CO₂ and butyrate are commonly produced during heterotrophic growth (Braun & Gottschalk, 1981; Lindley *et al.*, 1990; Lynd & Zeikus, 1983; Martin *et al.*, 1983).

This mixotrophic aspect of glucose consumption by *E. limosum* has received little attention in the scientific literature, although it is likely to be of prime importance in understanding the physiological growth behaviour of such mixed-acid producers. Chemostat growth experiments using glucose as carbon substrate have shown that H₂/CO₂ production is observed only at high growth rates (Loubière *et al.*, 1992). In this paper, kinetic data from batch experiments, supported by both enzyme analysis and metabolite-pool measurements, are presented which demonstrate that the growth behaviour of *E. limosum* on glucose is directly associated with CO₂ metabolism.

Methods

Organism and growth conditions. The bacterium used was a strain of *Eubacterium limosum* originally isolated and identified by INRA (Villeneuve d'Ascq, France). The organism was cultivated under strict anaerobic conditions in 10 litre glass fermenters or in 100 ml sealed bottles at 37 °C. The standard medium used was that of Pacaud *et al.* (1985) containing CO₂ (60 mM, i.e. 5 g KHCO₃ l⁻¹ under 15% CO₂), though yeast extract was omitted to enable precise determination of intracellular metabolite concentrations. In certain experiments, tungsten (tungstic acid, H₂WO₄) was added to the above medium at concentrations of 0.5 or 2 mM. The initial pH of the medium was 7.4 and for cultures in 10 litre fermenters the pH was regulated at 7.4 with NH₄OH (3 M), though in 100 ml bottles the pH was not controlled. Inocula (5%) were overnight cultures grown in the same medium.

Biomass, substrate and product analysis. Culture optical density at 660 nm was used to calculate biomass concentrations from a standard curve [correlation 0.4 g dry cell wt (OD unit)⁻¹]. A biomass formula of C₄H₇O₂N_{0.6} (Erickson, 1980) was used to convert biomass values into molar cell carbon concentrations (mmol C) for elemental balances. Glucose was analysed with an YSI-27A automatic analyser based upon a membrane-immobilized glucose oxidase reaction. Acetate and butyrate medium concentrations and the gas-phase composition were analysed using the gas-chromatographic methods described by

Loubière *et al.* (1992). Other products (polymer, lactate, glycerate and pyruvate) were identified qualitatively by HPLC analysis [separation at 40 °C with an ION-300 column (Waters) and detection with a refractometer (Waters-410)]. Elution was with H₂SO₄ (10 mM). Pyruvate was quantitatively assayed using an enzymic method coupled with fluorimetric detection of NADH. The mixture contained potassium phosphate buffer (0.2 M, pH 7), NADH (20 µM), pyruvate from diluted supernatant (0 to 15 µM) and lactate dehydrogenase (40 U).

Kinetic and stoichiometric calculations. Specific rates of growth (μ), glucose consumption (q_g) and product formation (v_p) were calculated from experimental data. Yields were calculated as the molar percentage of carbon recovered in either cells or fermentation products related to the substrate consumed (mmol C/100 mmol C substrate). The substrate-product balances were assessed for the percentage of major elements (C, H, O, N) assuming that organic acids were present as dissociated salts and that nitrogen for biomass was provided as NH₃. The low and unquantified production of polymer, lactate, glycerate and carbon monoxide during the linear growth phase were not taken into consideration.

Enzyme flux estimations. In order to compare enzyme specific activities with their *in vivo* activity, the specific carbon flux was calculated for certain reactions within the metabolic network throughout the time-course of the fermentations. These estimates were based on the manner in which specific rates of growth, substrate consumption and product accumulation evolved. The glycolytic flux was modified so as to take into account the biomass precursor requirements, assuming that a macromolecular cell composition similar to that described by Ingraham *et al.* (1983) was valid for *E. limosum* and that the relative amounts of each precursor did not vary significantly with respect to the growth rate. These same flux calculations were also used to compare the specific rate at which certain metabolites were produced or consumed. Of particular importance was the possibility to estimate the rate at which CO₂ was produced at the level of pyruvate-ferredoxin oxidoreductase, and hence compare this value with that predicted from gas phase composition and/or stoichiometric analysis, thus enabling the degree of acetate production from CO₂ to be determined.

Crude cell-free extract preparation. A gaseous atmosphere of N₂ was maintained throughout the procedure. A volume of culture corresponding to 200 mg dry wt cells was centrifuged (4 °C, 20 min at 10000 g), washed with 20 ml potassium phosphate buffer [50 mM, pH 7.4 (prepared under N₂ and containing 2 mM-dithiothreitol) to ensure an anaerobic environment] and centrifuged as before. The cell pellet was resuspended in 8 ml of the washing buffer and transferred into N₂-flushed, butyl-rubber-stoppered tubes and rapidly frozen and stored at -80 °C until extraction. No significant detrimental effect on enzyme activities was observed for frozen cells over a period of at least 5 d, compared with freshly harvested cells. Cell-free extracts were prepared in an anaerobic chamber maintained under a N₂/H₂/CO₂ gas-phase (80:10:10, by vol.). Bacteria were disrupted by sonication. The resulting crude extract was centrifuged (4 °C, 15 min at 12000 g) to remove cell debris and the supernatant was used for enzyme assays. The protein content of the extracts was determined by the method of Bradford (1976), since this procedure is not subject to interference with the thiol reagents used to maintain anaerobic conditions. Bovine serum albumin was used as the standard protein.

Determination of glycolytic enzyme activities. Enzyme activity measurements were undertaken in an anaerobic chamber using freshly prepared cell-extracts. Enzymes were assayed by coupling appropriate enzyme reactions to the spectrophotometric determination of either NADH at 340 nm or benzyl viologen at 500 nm ($\epsilon = 14.8 \text{ mm}^{-1} \text{ cm}^{-1}$). All assays were carried out at 37 °C in 2 ml cuvettes containing 1 ml of an appropriate enzyme mixture at pH 7.4. The volume of extract used

was chosen so as to ensure linearity between activity and protein concentration.

Phosphofructokinase was assayed with triethanolamine buffer (100 mM), MgCl_2 (10 mM), KCl (10 mM), NADH (0.3 mM), ATP (4 mM), 20 to 100 μl extract, fructose-bisphosphate aldolase (1 U), α -glycerophosphate dehydrogenase (2 U), triose phosphate isomerase (5 U), and the reaction was started with 20 mM-fructose 6-phosphate (4 mM).

Fructose biphosphatase was measured with potassium phosphate buffer (0.1 M), MgCl_2 (5 mM), CoCl_2 (0.4 mM), NADP (0.4 mM), 50 to 200 μl extract, glucose-6-phosphate dehydrogenase (1 U) and fructose 1,6-bisphosphate (10 mM) to initiate the reaction.

Fructose biphosphate aldolase, 3-phosphoglycerate (PGA) kinase and pyruvate kinase were measured at pH 7.4 with 50 to 200 μl extract, as described by Bergmeyer & Bernt (1974), Bergmeyer *et al.* (1974) and Hess & Wicker (1974) respectively.

Glyceraldehyde-3-phosphate dehydrogenase was measured in the gluconeogenic direction with 50 to 200 μl extract. The assay was as described by Zeikus *et al.* (1977), except that the pH was 7.4 and ATP, NADH_2 and 1,3-PGA concentrations were 5, 0.3 and 10 mM, respectively. Pyruvate-ferredoxin oxidoreductase was measured by following the pyruvate and CoA-SH dependent reduction of benzyl viologen (BV^{2+}) at 500 nm. The assay contained potassium phosphate buffer (100 mM), BV^{2+} (5 mM), 20 to 60 μl extract and pyruvate (20 mM) to initiate the reaction. NAD^+ or NADP^+ could not substitute for the viologen dye.

Phosphoenolpyruvate synthetase was measured using the method proposed by Eyzaguirre *et al.* (1982), except that pH was 7.4, and the incubation temperature was 37 °C. Maximum activity was obtained for 10–50 μl of extract.

Determination of glycolytic intermediates and cellular coenzymes. Intracellular metabolites were measured using an *in vitro* procedure based on the rapid inactivation of metabolism followed by metabolite extraction. Metabolic activity was stopped in samples of the whole culture by two different methods optimized to avoid metabolite degradation. Culture medium was pumped into a plastic beaker containing 100–200 μl of either HCl or KOH with rapid stirring, to give a final pH of 1.2 or 12.5 respectively. The acid extraction procedure was used for most metabolites and coenzymes and was achieved by incubating the HCl-treated culture (pH 1.2) at 50 °C for 8 min before neutralizing to pH 6.8 with KOH while agitating vigorously. After centrifugation at 4 °C for 5 min at 12000 g, the supernatant was used for metabolite concentration measurements. Acid-labile coenzymes [NAD(P)H] were extracted by incubating the KOH-treated culture at pH 12.5 at 50 °C for 5 min. After centrifugation at 4 °C for 5 min at 12000 g the supernatant was immediately tested for NAD(P)H without neutralizing to avoid destruction of NAD(P)H by locally high concentrations of acid. Metabolites were measured by coupling appropriate enzyme assays with fluorimetric determination of the cofactors NADH or NADPH. Emission was measured at 450 nm after excitation at 350 nm with a fluorescence spectrophotometer (Hitachi F-2000). Controls were undertaken with culture filtrate to ensure that the metabolites were exclusively localized within the cytosol.

Glucose 6-phosphate (G 6-P) and fructose 6-phosphate (F 6-P) were measured as described by Lang & Michal (1974), and fructose-1,6-bisphosphate (F-1,6-P), glyceraldehyde 3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP) concentrations were determined by following the method of Michal & Beutler (1974), except that the buffer was at pH 7 instead of 7.6. Phosphoglycerate (PGA) and 1,3-diphosphoglycerate (1,3-PGA) were measured using the following assay mixture: 200 μl triethanolamine buffer [0.5 M, pH 7.6 containing MgSO_4 (15 mM) and EDTA (4 mM), 25 μl NADH (0.5 mM), 800 μl extract, 20 μl ATP (100 mM) and 20 μl GAP dehydrogenase (500 U ml^{-1}) to initiate 1,3-PGA consumption. After the reaction

was completed, 20 μl PGA kinase (1000 U ml^{-1}) was added to measure the PGA concentration. Phosphoenolpyruvate (PEP) was determined after complete depletion of the pyruvate present in the extract using a mixture which contained: 200 μl potassium phosphate buffer [0.5 M, pH 7 containing MgCl_2 (12.5 mM)], 25 μl NADH (X mM; X was defined so as to obtain an excess of NADH after pyruvate depletion), 20 μl ADP (100 mM) and 20 μl lactate dehydrogenase (1000 U ml^{-1}). After all the pyruvate had been consumed, 20 μl pyruvate kinase (1000 U ml^{-1}) was added to initiate PEP consumption.

Oxidized nicotinamides (NAD^+ and NADP^+) were assayed as described by Klingenberg (1974). The NADH present in the alkaline extract was measured in a mixture at pH 7 containing 200 μl potassium phosphate buffer [0.5 M, pH 6 containing MgCl_2 (12.5 mM)], 800 μl alkaline extract, 20 μl pyruvate (200 mM) and 20 μl lactate dehydrogenase (1000 U ml^{-1}) to initiate the pyruvate-dependent oxidation of NADH. The NADPH was assayed at pH 7 with 200 μl triethanolamine buffer [0.5 M, pH 6 containing MgSO_4 (15 mM) and EDTA (4 mM)], 800 μl extract, 20 μl glutathione (100 mM) and 20 μl glutathione reductase (1000 U ml^{-1}) to initiate NADPH consumption.

ATP was measured as described by Jaworek *et al.* (1974a) while ADP and AMP were measured using the method of Jaworek *et al.* (1974b), except that the NADH concentration was modified so as to enable complete depletion of pyruvate from the extract before starting the reaction. The ADP concentration was assayed using the method of Gruber *et al.* (1974).

Results

Kinetic analysis of glucose fermentation

The fermentation of glucose by *E. limosum* was characterized by three phases of growth: accelerating, exponential and decelerating growth, followed by a stationary phase

Fig. 1. Batch fermentation profile of *E. limosum* grown on glucose showing substrate and product concentrations, and gas-phase partial pressures of H_2 and CO_2 . $\circ$, Biomass; $\bullet$, glucose; $\square$, acetate; $\blacksquare$, butyrate; $\blacktriangledown$, pyruvate; $\triangle$, CO_2 ; $\blacktriangle$, H_2 .

Fig. 2. Specific rates of growth, glucose consumption and product formation (a) and the respective molar yield coefficients relative to glucose consumption (b) during a batch fermentation of *E. limosum* on glucose. (a) Specific growth rate (○), specific rate of glucose consumption (●), and specific production rates of acetate (□), butyrate (■), pyruvate (▼) and CO₂ (△). (b) Biomass (○), acetate (□), butyrate (■), pyruvate (▼) and CO₂ (△).

in which some continued metabolic activity was observed (Fig. 1). During the first 4 h after inoculation, growth, glucose consumption and acetate production rates accelerated (Fig. 2). The fermentation was homoacetic, and during this period gas-phase analysis indicated that

some of the exogenous CO₂ was consumed. The stoichiometric balance representing the global situation over the 4 h accelerating phase of growth confirmed this result and also indicated a slight consumption of H₂, presumably that transferred with the inoculum.

Consumption of pyruvate that had been transferred with the inoculum also occurred within this phase. Accelerating growth was followed by a short-lived exponential growth during which the specific growth rate (μ) remained constant at a value of 0.23 h⁻¹. The specific rate at which glucose was consumed increased slightly while both the specific rate of acetate production and the acetate yield relative to glucose consumption fell significantly towards the end of the exponential growth phase. This fall in acetate could in part be explained by a shift towards a mixed acid fermentation with an increasingly important synthesis of butyrate throughout this phase of growth. However, more significantly, this diminished yield of acetate was directly related to a net production of gaseous products, H₂ and CO₂, implying that the reductive metabolism of CO₂ by the acetyl-CoA pathway was incapable of accommodating the CO₂ production from the pyruvate-ferredoxin oxidoreductase. A slight but significant production of pyruvate was also observed. The global stoichiometric balance equation for this growth period was as follows.

Throughout both the accelerating and the exponential growth phase the biomass yield remained constant despite the metabolic shifts, but fell during the prolonged period of decelerating growth which followed. The tendencies already described as regards the shift in fermentation pattern continued, and intensified, within this linear growth phase. The partial pressures of both H₂ and CO₂ increased sharply [gas-phase analysis also indicated that small amounts of CO (maximum partial pressure 1%) were also formed] and a more significant production of pyruvate took place. Trace amounts of novel fermentation products (glycerate, lactate and a viscous polymer) were produced, but their concentrations remained too low for precise quantification (i.e. < 0.25 mm) and were therefore not considered for the global stoichiometric equation of this growth phase.

Growth halted when the glucose had been depleted, though metabolic activity continued with consumption

Table 1. *Specific activities of glycolytic enzymes measured throughout the growth of E. limosum during batch fermentations on glucose*

Standard deviations represent the spread of values obtained for four samples taken throughout the active growth phase.

Enzyme	Specific activity [U (g protein) ⁻¹]
Phosphofructokinase	380 ± 15
Fructose biphosphatase	110 ± 10
Fructose biphosphate aldolase	1100 ± 50
Glyceraldehyde-3-phosphate dehydrogenase	11600 ± 200
Phosphoglycerate kinase	8800 ± 100
Pyruvate kinase	500 ± 15
Phosphoenolpyruvate synthetase	26 ± 5
Pyruvate-ferredoxin oxidoreductase	3200 ± 100
Lactate dehydrogenase	20 ± 5

Table 2. *Intracellular concentrations of glycolytic intermediates during the growth of E. limosum on glucose*

Standard deviations represent the spread of values obtained for ten samples taken throughout the active growth phase, each sample being analysed three times.

Metabolite	Intracellular concn [μmol (g dry wt) ⁻¹]
Glucose 6-phosphate	26.2 ± 0.3
Fructose 1,6-phosphate	12.3 ± 0.2
Fructose 6-phosphate	2.3 ± 0.05
Dihydroxyacetone phosphate	0.6 ± 0.03
Glyceraldehyde phosphate	0.3 ± 0.06
1,3-Diphosphoglycerate	0.8 ± 0.06
3-Phosphoglycerate	6.5 ± 0.4
Phosphoenolpyruvate	0.8 ± 0.15
NADP	0.5 ± 0.05
NADPH	< 0.1
ATP	5.8 ± 0.2
ADP	1.1 ± 0.1
AMP	0.2 ± 0.1

of pyruvate, lactate and glycerate associated uniquely with acetate production.

Biochemical analysis

During the three phases of active growth, the concentrations of all enzymes and metabolites tested remained constant (Tables 1 and 2) with the exception of phosphoglycerate, the concentration of which decreased slightly throughout the decelerating growth phase. During this same period the NAD⁺/NADH ratio dropped significantly, though this was due to a decrease in the NAD⁺ concentration without any increase in the NADH levels (Fig. 3). The cellular concentrations of the

Fig. 3. Changes in intracellular NAD(H) concentrations and energy charge during a batch fermentation of *E. limosum* on glucose. Values for other coenzymes remained constant throughout the fermentation (see Table 2). The values presented refer to the batch culture that gave the data presented in Figs 1 and 2. ○, NAD⁺; ●, NADH; ■, energy charge.

various adenosine nucleotides (energy charge = 0.9) and NADP(H) remained constant up until the stationary phase, when the energy charge of the cell decreased. In a similar manner, the intracellular concentrations of the glycolytic intermediates also fell when the glucose became depleted.

Effect of medium composition on fermentation characteristics

A number of fermentations were undertaken with media of different composition to determine whether the growth behaviour of *E. limosum* could be attributed to nutritional limitations. For these cultures, the organism was cultivated in 100 ml bottles and the pH was allowed to change freely, since control experiments had shown that no significant differences in fermentation profile could be assigned to pH variations. Macroscopic fermentation patterns were not modified when the vitamin or trace element concentrations were increased fivefold. All metabolic modifications appeared and progressed in an identical manner to those described above, indicating that the growth characteristics were not due to a deficiency in one or more components of the medium. As might have been expected from previous reports (Sharak-Genthner *et al.*, 1981), yeast extract (0.5 g l⁻¹) had a stimulatory effect on the maximum specific growth rate ($\mu = 0.3$ h⁻¹) and also reduced the duration of the accelerating growth phase. However, the progression of cultures towards prolonged linear growth phases and a mixed-acid fermentation pattern after a brief exponential growth phase was not modified.

In view of the above results, an analysis of the literature was undertaken to determine if our medium was deficient in any trace elements. The only metal

Fig. 4. Molar yield coefficients calculated throughout the growth phases and the partial pressure of H_2 for *E. limosum* on glucose in media containing tungsten with no pH regulation. ---, Changes in the same parameters in control experiments lacking tungsten. No data are presented for CO_2 because of the lack of pH regulation. $\square$, Acetate; $\blacksquare$, butyrate; $\blacktriangledown$, pyruvate; $\blacktriangle$, H_2 .

absent from our medium (and apparently from the media used by most other groups) was tungsten. Fermentation patterns for media containing tungsten (0.5 mM) were somewhat different from that of the standard medium lacking tungsten (Fig. 4). The exponential growth phase was prolonged and maximum rates of both growth and glucose consumption were improved. Moreover, as illustrated by yields of acids and the partial pressure of H_2 , the metabolic phenomena associated with the onset of linear growth were also delayed. Increasing the tungsten concentration (2 mM) did not further modify the fermentation characteristics.

Discussion

Batch cultures of *E. limosum* on glucose were typical of the kinetic behaviour of many anaerobic organisms: they were characterized by a short-lived exponential growth phase and a prolonged period of decelerating growth. This behaviour is typical of nutritional limitation or inhibition by fermentation products accumulating in the broth and occurs before the carbon and energy source becomes limiting. Since the fermentation was homoacetic during accelerating to mid-exponential growth phase, with no other catabolic products, all the CO_2 and reducing-coenzymes resulting from heterotrophic glucose metabolism were able to be reassimilated via the

acetyl-CoA pathway. During this period of the fermentation the 'autotrophic' CO_2 -fixing acetyl-CoA pathway was consequently not limited in comparison with heterotrophic metabolism. Towards the end of the exponential growth phase a number of significant modifications to the metabolism took place, which resulted in decelerating growth. Since many of these changes in the fermentation pattern became apparent at approximately the same moment, it is necessary to elucidate which of these phenomena were causative rather than merely a consequence of the modifications.

The decline in acetate production was not due to an inhibitory effect of acetate on the phosphotransferase and kinase reactions involved in the synthesis of this acid from acetyl-CoA, since supplementing the medium with acetate concentrations representative of those to be observed throughout the fermentation did not alter the culture profile. Moreover, the acetate concentration was only 5 mM when butyrate synthesis began, a value about 20 times lower than the concentration of acetate that has been shown to inhibit the growth of *E. limosum* (Pacaud *et al.*, 1986). The shift towards butyrate as co-product, while likely to diminish the production of acetate, could not account for the far more important fall in the acetate production yield. Since pyruvate accumulation is observed at approximately the same moment of the culture, one might postulate that the flux through the pyruvate-ferredoxin oxidoreductase was limiting, though the proportion of pyruvate accumulated remained extremely low compared to the flux through the enzyme. Furthermore, the appearance of CO_2 and H_2 implies that this reaction was not the cause of the fall in acetate synthesis but rather that this diminished yield of acetate was due to the incapacity of the acetyl-CoA pathway to reassimilate the CO_2 . The repercussion of this limited capacity to fix the catabolic CO_2 was that the organism was faced with a potential overproduction of reduced coenzymes necessitating alternative pathways of reducing equivalent wastage: butyrate synthesis and H_2 production. Since during the exponential growth phase, rates of CO_2 fixation were at least as high as those necessary during linear growth, it must be concluded that some nutritional element became limiting which adversely affected the organism's ability to maintain a homoacetogenic metabolism. This limitation was localized within the acetyl-CoA pathway, and in view of the positive effect of tungsten it is tempting to suggest that formate dehydrogenase was involved. Tungsten is known to have a stimulatory effect on the activity of formate dehydrogenase in two homoacetogenic species (*Clostridium formicoaceticum* and *C. thermoaceticum*) and this effect is at the structural level (Ljungdahl & Andreessen, 1975): multiple forms possessing different specific activities are thought to exist depending on the availability of certain trace elements

(i.e. iron, molybdenum, tungsten, selenium). If the formate dehydrogenase of *E. limosum* is similar to that of the clostridial species, this could explain the partial effect of tungsten supplements, since a correct proportion of the trace elements would be necessary to fully optimize the activity of this enzyme. Clearly, direct evidence of which reaction(s) within the acetyl-CoA pathway are limiting will await a detailed analysis of the enzymic activities of this pathway. Supportive evidence that the limiting enzyme lies within the reductive transformation of CO_2 to methyl donor can be seen in the manner that carbon monoxide was produced as a minor product during the linear growth phase, showing that the carbon monoxide dehydrogenase reaction was still operative.

If the incapacity of the bacterium to maintain an adequate metabolism of the CO_2 liberated during the reductive decarboxylation of pyruvate to acetyl-CoA explains the changes in the product profile, it is necessary to determine whether such modifications might themselves be the cause of the pyruvate overflow which follows shortly afterwards. Pyruvate overflow indicates that pyruvate-ferredoxin oxidoreductase could no longer support the flux arriving at pyruvate from the catabolism of glucose. The increased proportion of pyruvate accumulated throughout the linear growth period, despite the progressively diminishing rate of glucose consumption, implies that this limitation became greater throughout the fermentation. Since the specific activity of the pyruvate-ferredoxin oxidoreductase remained constant, the explanation for such a limitation must be one of biochemical inhibition rather than enzyme synthesis. It is interesting to note that when a tungsten supplement was provided and the homoacetogenic fermentation was maintained for a prolonged period, pyruvate accumulation was also delayed. In view of this observation and the existing understanding of the influence of the ferredoxin redox state on pyruvate-ferredoxin oxidoreductase activity (Raeburn & Rabonowitz, 1971), it is suggested that inhibition of the activity of this enzyme could be due to the accumulation of H_2 and resulting modifications to the proportions of oxidized to reduced coenzymes. For the moment, such a measurement of the redox state of ferredoxin has not been demonstrated, but the modified ratio of NAD:NADH observed throughout the linear growth phase does indicate that such a modified coenzyme equilibrium occurred. The complex series of oxidoreductase reactions involved in the distribution of electrons from reduced ferredoxin to other coenzymes and the eventual use of such coenzymes within dehydrogenation reactions or for H_2 synthesis via hydrogenase(s) needs further investigation.

If pyruvate overflow appears to be a direct consequence of saturation of the acetyl-CoA pathway, how do

Fig. 5. Changes in specific rates of glucose consumption and acetyl-CoA formation relative to growth rate during batch cultures of *E. limosum* on glucose. ●, Glucose consumption/ μ ; ○, acetyl-CoA formation/ μ .

these phenomena relate to the decelerating growth pattern. No evidence for energetic uncoupling as regards ATP can be seen, since the concentrations of all forms of adenosine nucleotides remained constant throughout the fermentation. The fall in the specific rate of glucose consumption which accompanied the decreased rate of growth was attributed to both a possible repression or retroinhibition of the PTS-glucose transport system by pyruvate (production of the reaction) and/or an inhibition of glyceraldehyde-3-phosphate dehydrogenase activity due to the modified NAD^+/NADH ratio. However, the overflow of other metabolites upstream of pyruvate tends to suggest that glucose consumption was sufficient to furnish adequate quantities of glycolytic intermediates. Certainly, the constant intracellular concentrations of phosphorylated intermediates do not show any signs of carbon limitation. In view of the fermentation profile, it was considered more likely that any limitations influencing the growth rate would be downstream of pyruvate. Thus, while the ratio of specific rates of consumption (q_s) to μ actually increased during growth (Fig. 5), the growth rate was directly proportional to the rate of acetyl-CoA synthesis for the entire fermentation.

It appears, therefore, that acetyl-CoA formation is potentially growth-rate-limiting, as was also the case during methylotrophic metabolism of *E. limosum* (Loubière & Lindley, 1991). This finding, implying that the major growth limitation is at the level of acetyl-CoA, perhaps explains why the use of substrate mixtures (glucose and methanol) supported growth rates significantly higher than those obtained on either substrate alone (Loubière *et al.*, 1992). In other homoacetogenic anaerobes, growth rate and yield enhancement have been observed when sugar metabolism takes place in the presence of sufficient CO_2 to permit reduced coenzymes to be used for acetate production via, presumably, the

acetyl-CoA pathway, rather than for the production of H_2 (Braun & Gottschalk, 1981; Godley *et al.*, 1990; Martin *et al.*, 1983), though these authors did not report pyruvate overflow in the absence of such carbonate supplements. The complexity of the superficially simple metabolism of the various acetogenic bacteria makes an interesting model for the study of carbon and energy flux distribution within anaerobic environments. If in this case the real cause of the growth response was most likely an inadequately formulated nutritional medium, this is a situation which the bacterium will face regularly within natural environments. In view of the niche occupied by these species in nature as part of an interactive food web, it might be imagined that their role (clearly subject to drastic changes as regards product formation) as a potentially key component of anaerobic communities is one which has been largely underestimated.

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