THE EFFECT OF FORMATE ON THE ACIDOGENIC FERMENTATION OF METHANOL BY *EUBACTERIUM LIMOSUM*

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During growth of the anaerobic acidogenic bacterium, *Eubacterium limosum*, on methanol-CO₂, formate supplements were shown to influence both rates of growth and the ratio of organic acid fermentation end-products. Formate interrupted the oxidation of methanol to CO₂ thereby lowering the yield of reducing equivalents. A consequence of this was a progressive shift towards acetic acid production at the expense of butyric acid, proportionate to the initial formate concentration. Such a finding supports the physiological model of this organism's methylotrophic metabolism, in which butyric acid production is related directly to the flux of methanol oxidation. At high formate concentrations, reversal of this catabolic pathway (i.e. assimilation of formate as methyl donor for acetyl-CoA formation) resulted in ATP-limited growth. *E. limosum* could not be acclimatized to growth on formate alone though the cells remained able to transform formate to CO₂ after several transfers.

Recently, methanol-assimilating fermentative bacteria producing high yields of organic acids (acetate and butyrate) have been isolated (1, 2). The metabolic products (particularly butyric acid) of these anaerobes are of potential economic value. So a good understanding of the energetic principles regulating the acidogenic fermentation of methanol is needed to identify the physiological bottlenecks influencing butyric acid production. Only then can a suitable fermentation strategy be devised to optimize the conversion of methanol to butyric acid.

All methylotrophic anaerobes described so far have an absolute requirement for carbon dioxide as a co-substrate for acetyl-CoA formation, despite the observed

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Fig. 1. Proposed metabolic sequences of methanol-utilizing acidogenic anaerobes. Important energy requiring (or yielding) reactions are shown, but those involving reducing equivalents are shown as XH_2 since co-enzyme specificities are not yet known.

dehydrogenation of methanol to carboxyl donor via tetrahydrofolate (THF)-intermediates (3). Further metabolism of the acetyl-CoA follows distinct pathways leading to cell biomass, acetic acid and butyric acid (Fig. 1). The metabolism is fundamentally analogous to C_2 -unit formation from $\text{CO}_2\text{-H}_2$ as described by WOOD et al. (4), though the reversed flux through the THF-sequence imposes entirely different energetic constraints. The high yield of reducing equivalents associated with methanol utilization necessitates the production of a fermentation end-product of a more reduced nature than acetate. Butyric acid is thus produced as well as acetic acid, the ratio of each being dependent on the flux through the THF-sequence (5). Only for species in which this flux is very low do homoacetic fermentations occur (6).

The possible utilization of formate as co-substrate with methanol has been reported (2, 3), but no thorough analysis of this compound's effect on growth and organic acid production has been presented. The catabolic and anabolic pathways are closely linked in methanol-utilizing anaerobes (7) and the presence of a catabolic intermediate such as formate might be expected to create energetic effects other than that of acting as an extra source of energy, as reported for certain aerobic microorganisms (8, 9). In view of this possibility, a study of the effect of formate on the methylotrophic metabolism of *E. limosum* was undertaken.

MATERIALS AND METHODS

Organism and cultivation. The fermentative bacterium *Eubacterium limosum* B2, isolated from pea-processing wastes, was cultured on the medium previously described (10) at pH 7.4 and 37°C. Methanol-CO₂ (100 mM-60 mM) were used as carbon sources throughout, except where specified in the text, with various levels of formate supplement. In order to reduce lag phases and improve growth rates sodium acetate (50 mM) was added to all cultures, though this compound does not appear to be metabolized (2). The medium was prepared under strict anaerobic conditions (11) in either sealed tubes (20 ml) or a fermenter (500 ml).

Substrate and product analysis. Methanol, acetic and butyric acids and the gas phase composition were determined by vapour chromatographic methods previously reported (10). Formate was analyzed by a colorimetric method (12).

Biomass analysis and growth rate determinations. During the growth, relative changes in culture density were measured spectrophotometrically at 660 nm and these values were used to calculate rates of growth. Absolute biomass levels were based on dry weight determinations.

Evaluation of stoichiometric balances. The substrate-product balances were assessed for the percentage recovery of the major elements (carbon, hydrogen and oxygen), assuming that organic acids were present exclusively as dissociated salts. Total reducing equivalent balances were based upon the reductive potential of each element, i.e., C=4, O=-2 and H=1 (13, 14). The reductance degree of biomass and the carbon weight fraction were taken to be 4.423 per carbon and 0.482 respectively, values shown to be common to most bacteria (15). Co-enzyme balances were calculated according to known biochemical sequences using Y_{ATP} values as quoted by STOUTHAMER (16).

RESULTS

During growth of *E. limosum* on methanol-CO₂ in the presence of 50 mM acetate, the following stoichiometric balance equation was obtained (all values are molar concentrations):

This substrate:product ratio represents a well-balanced metabolism with full recovery of all three major elements and reducing equivalents. The fermentation proceeded with a maximum specific growth rate of 0.09/hr though this exponential growth phase was short-lived, giving way to a prolonged period of decelerating growth.

The addition of formate as a third carbon source caused changes in the metabolism of *E. limosum* related to the initial formate concentration. The growth

rates were not affected at formate concentrations less than 150 mM, but thereafter progressively decreased, as did stationary phase biomass concentrations (Fig. 2). The consumption of CO_2 decreased in proportion to the initial formate to become a net production at 150 mM formate. Above that level significant quantities of both residual methanol and formate remained at the stationary phase, although the quantity of formate metabolized continued to increase. The ratio of organic acids produced shifted towards acetic acid as the formate concentration increased from 0 to 100 mM, but remained constant relative to substrate consumption at higher levels of formate. The shift in organic acid production from an approximate 50/50 molar ratio in the absence of formate to a 30/70 molar ratio in favour of acetic acid at 100 mM formate was not due principally to a fall in butyric acid production (which fell only from 23 to 19 mM), but rather to a greatly enhanced yield of acetic acid (22 to 40 mM). Thus, although butyric acid yields relative to total substrate consumption fell dramatically, this shift towards acetic acid can best be visualised as the transformation of formate (rather than methanol or CO_2) exclusively to acetic acid.

Attempts to acclimatize the bacterium to growth on formate alone were not successful though growth on methanol-formate mixtures was possible. It was observed that at certain formate concentrations (100–250 mM) the culture took on a viscous, non-homogeneous aspect which when examined microscopically was made up of bacteria entrapped in slime of a granular nature. This extracellular slime production has often been reported for *E. limosum* grown on glucose (hence the name). But in our hands the slime production has not been pronounced during growth on C_1 -compounds. Transfer of substrate from methanol-formate to formate alone was characterized by the production of acetic acid and CO_2 as major products after the first transfer, but only CO_2 production by the third transfer (Table 1). The loss of acidogenic activity was accompanied by a progressive loss of viability with no change to the biomass levels after 16 days on second transfer. The

Table 1. The effect of successive transfer of *E. limosum* into formate-containing media, on the growth and organic acid production.^a

Substrate mixture (mM)	Fermentation balance (mM)	μ_{max} (hr)	Incubation period (days)
100 CH_3OH + 60 CO_2	100 CH_3OH + 38 CO_2 $\longrightarrow$ 20 acetate + 21 butyrate + 14 cell C	0.093	3
100 CH_3OH + 60 CO_2 + 100HCOOH	100 CH_3OH + 5 CO_2 + 93HCOOH $\longrightarrow$ 61 acetate + 15 butyrate + 15 cell C	0.091	3
100 CH_3OH + 150HCOOH	83 CH_3OH + 146HCOOH $\longrightarrow$ 61 acetate + 20 butyrate + 10 cell C	— ^b	6
100 CH_3OH + 250HCOOH	67 CH_3OH + 232HCOOH $\longrightarrow$ 66 acetate + 14 butyrate + 101 CO_2 + 9 cell C	— ^b	8

^a The order in the table is representative of the transfer sequence.

^b These cultures grew in a non-homogeneous manner due to slime production and therefore accurate measurement of the growth rate was not possible.

Fig. 2.

Fig. 3.

Fig. 2. The growth, substrate consumption and organic acid production during methanol- CO_2 -formate fermentation by *E. limosum*, related to initial formate level. $\circ$: specific growth rate, $\bullet$: consumed methanol, $\square$: CO_2 consumed or produced, $\blacksquare$: consumed formate, $\blacktriangledown$: biomass, $\triangle$: butyric acid yield, $\blacktriangle$: acetic acid yield.

Fig. 3. The contribution of the various C_1 -substrates ($\square$: CO_2 , $\bullet$: methanol, $\blacksquare$: formate) to the methyl (A) and carboxyl (B) groups of acetyl-CoA, and the associated co-enzyme ($\triangle$: ATP, $\blacktriangle$: proton) balance (C). The values were based on experimental substrate consumption data assuming that the biochemical sequences described in the text occurred. The formate converted to CO_2 not incorporated into acetyl-CoA is not shown in curve B, but the reducing equivalent gain is included in the curve showing net co-enzyme yields. Further metabolism of acetyl-CoA was not taken into account for these calculations.

result of this lack of viability was progressively weaker inocula, but this alone could not explain the more general loss of metabolic activity. Similar results were obtained for fed-batch cultures in which three successive amounts of formate (100 mM) were added as the sole carbon source when the medium became depleted. Likewise, this failure to obtain sustained metabolic activity could not be attributed to the tendency of the formate-grown cultures towards basic pH values since cultures maintained at constant pH values (various) showed exactly the same inability to assimilate formate.

DISCUSSION

Since first proposed by KERBY et al. (3), the biochemical model of unicarbon acidogenesis outlined in Fig. 1 has received much support in the literature. As yet,

no alternative pathway has been postulated and it is therefore important to discuss all experimental observations with a view to testing the validity of the working model. Using the biochemical reactions shown in Fig. 1, the effect of formate supplements on the energy balance of C_2 -unit formation from various unicarbon mixtures can be calculated from substrate consumption data. With this information the likely effect of formate on growth and organic acid production can be predicted and then compared with observed experimental results. Since the vast majority of carbon is metabolized by the condensation of equimolar concentrations of methyl and carboxyl donors to give acetyl-CoA, the net contribution of each unicarbon substrate to either carbon atom of the acetyl-moiety can be calculated. Bearing in mind the various oxidoreduction steps and the phosphorylation associated with formyl-THF synthetase, the overall energy yield associated with acetyl-CoA synthesis can be estimated.

In the absence of formate, approximately one third of the consumed methanol must have been assimilated into acetyl-CoA as the carboxyl group i.e. direct fixation of CO_2 could only account for half of the carboxyl donors required. Formate progressively replaced both methanol and CO_2 as a source of carboxyl donor until the entire carboxyl group was furnished by formate (Fig. 3). At higher concentrations of formate (100 mM) the consumption data show that in addition to being oxidized to CO_2 (in excess of carboxyl donor needs) an increasing proportion of the formate must have been reduced to methyl donors to satisfy the stoichiometry of acetyl-CoA synthesis. Reduction of formate to methyl intermediate would need both reducing equivalents (supplied by CO_2 production via formate dehydrogenase) and ATP. The energy yield associated with acetyl-CoA formation would thus decrease with increasing formate consumption (Fig. 3). The net effect would thus be expected to be a shift in organic acid production in favour of acetic acid at the expense of butyric acid relative to the amount of formate metabolized. The observed shift in the organic acid production ratio (see Fig. 2) was in close agreement with this theoretical prediction, and thus further supports the proposed model.

The biomass yield was not proportional to the total substrate yield, but rather was linked directly with the concentration of the methanol consumed. It would thus seem that our strain of *E. limosum* though capable of converting formate to acetic acid, cannot derive all the requirements necessary for growth from formate. The overall co-enzyme balance of the fermentations (Fig. 4) show that even with no correction for "maintenance" requirements the ATP levels would be insufficient for good growth. This inadequate supply of ATP at formate concentrations greater than 200 mM was due presumably to the more important necessity to balance the reducing equivalent status by butyric acid production and hence a lower yield of ATP through substrate level phosphorylation reactions. The inability of this strain to adapt to growth on formate as the sole carbon source suggests that the unified model of unicarbonic acidogenesis is not yet complete. Other strains of *E. limosum* (17) and of *Butyrivibacterium methylotrophicum* (18) are able to ferment H_2 –

Fig. 4. The overall net co-enzyme (Δ : ATP, $\blacktriangle$: proton) status of the culture as calculated from stoichiometric substrate conversion data at stationary growth phase for *E. limosum* grown on methanol- CO_2 -formate. The points take into account the observed change to the pH of the medium.

CO_2 or CO with the exclusive production of acetic acid, and recently a strain of *E. limosum* able to ferment formate homo-acetically has been reported (19). These latter authors demonstrated that strains pregrown on H_2 - CO_2 were able to adapt quickly to growth on formate, whilst those grown on methanol were not always capable of growth on formate. Indeed those strains able to make the shift from methanol to formate did so only after extended periods. The conclusion reached was that the necessary changeover of metabolism was not due to enzyme induction/derepression but rather to a spontaneous mutation. Our lack of success in obtaining continued growth on formate would tend to support this theory.

The important question arising from such experiments is why some organisms containing the enzymes necessary to generate acetyl-CoA cannot use C_1 -compounds such as formate. The results presented here serve only to illustrate this problem and offer little evidence that enables a plausible answer to be formulated. However, since the major energetic problem faced by acidogens growing on formate, H_2 - CO_2 or CO is the same, i.e., ATP-limitation, the ability of some acidogens to metabolize a wide range of C_1 -compounds will almost certainly be linked with their possession of alternative ATP-generating mechanisms.

Further work is essential to characterize the fine details of metabolic versatility before the true potential of these commercially interesting bacteria can be properly assessed.

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