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Anaerobic Catabolism of Formate to Acetate and CO_2 by *Butyribacterium methylotrophicum*

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The catabolism of sodium formate to acetate and carbon dioxide by the anaerobic acetogen *Butyribacterium methylotrophicum* was analyzed by fermentation time course and ^{13}C nuclear magnetic resonance studies. Significant hydrogen production and consumption fluxes were observed during formate catabolism but not during the catabolism of formate plus CO. In the latter case, formate and CO were simultaneously consumed and label distribution studies with mixtures of ^{13}C-labeled CO and formate demonstrated their preferential incorporation into the acetate carboxyl and methyl groups, respectively. Hydrogen consumption was inhibited by CO when both were present, whereas hydrogen and formate were simultaneously consumed when CO_2 was supplied. Carbon dioxide was required for the conversion of CO to acetate, but a similar need was not observed when methanol plus CO or formate plus CO was present. These analyses indicate a bifurcated single-carbon catabolic pathway in which CO_2 is the sole single-carbon compound that directly supplies the carbonyl and methyl group synthesis pathways leading to the formation of acetyl coenzyme A, the primary reduced product. We discuss causes for the reported inability of *B. methylotrophicum* to use formate as a sole substrate.

Saccharolytic acetogens anaerobically catabolize saccharides, lower-molecular-weight alcohols, and one-carbon (C_1) compounds, coupling substrate oxidation with CO_2 reduction to acetate (and sometimes its higher-molecular-weight homologs), the primary reduced catabolic product. Current models (9, 12, 16, 21, 22, 31, 34, 35, 37) of acetogenic biochemistry include formate as one intermediate of CO_2 reduction to the acetate methyl group, and the preferential conversion of formate to the acetate methyl group by glucose-catabolizing *Clostridium thermoaceticum* cell suspensions was demonstrated in 1955 (20). Although several acetogens catabolize formate as expected (5, 6, 8, 11, 19, 27, 34), certain acetogens are reportedly unable to grow on formate itself even though other C_1 substrates or hexoses are utilized homoacetogenically (3, 32). Indeed, although we have described the acetogenic catabolism of *Butyribacterium methylotrophicum* grown on CO (24, 38), H_2 plus CO_2, glucose, CH_3OH plus CO_2 (25, 38), CH_3OH plus CO, and CH_3OH plus formate (18), we could not document its growth on formate alone (38).

We describe here the catabolism of formate by *B. methylotrophicum* and explain the discrepancies found with earlier results. We survey elsewhere the catabolic oxidoreductase enzymes required for these fermentations (manuscript in preparation).

MATERIALS AND METHODS

Gases, chemicals, and isotopes. Sodium [^{13}C]formate (99% ^{13}C enriched) was purchased from K.O.R., Inc., Cambridge, Mass. ^{13}CO (90% enriched) was purchased from MSD Isotopes (St. Louis, Mo.). High-purity gases (18) were obtained from Matheson Gas Co. (Joliet, Ill.) and, except for CO and CO_2, were passed through heated copper columns (29) to remove O_2. Chemicals and medium components were obtained from Sigma Chemical Co. (St. Louis, Mo.), Mallinckrodt (Paris, Ky.), or Difco Laboratories (Detroit, Mich.).

Organism cultivation. *B. methylotrophicum* was grown anaerobically at 37°C in a phosphate-buffered (PB), sulfide-reduced medium containing 0.05% yeast extract prepared as previously described (18). The substrate-to-product transformations and growth of *B. methylotrophicum* on various substrates were analyzed by using sealed round-bottom flasks (stoppered volume, 1.1. liter) containing 400 ml of PB medium. Medium additions included 10 ml of phosphate buffer (2.37 M, pH 7.0) and 1 ml of Na_2S (15%). Sodium formate (pH 7.0) was aseptically added to a concentration of 95 mM. Headspaces were flushed with CO to give 1.0 atm (101 kPa, 25°C) when in combination with H_2 plus CO_2 or 1.4 atm (141 kPa, 25°C) when in combination with formate. H_2 plus CO_2 (80:20) was added to 0.6 atm (61 kPa, 25°C) when combined with CO or 1.0 atm (101 kPa, 25°C) when formate was also present. Growth vessels (duplicates when CO plus CO_2 plus H_2 were substrates, triplicates in other experiments) were warmed to 37°C, inoculated with 1 to 5 ml of *B. methylotrophicum* maintained on substrates corresponding to those used in the experiment, and incubated with vigorous rotary shaking (160 rpm). The CO-adapted strain of *B. methylotrophicum* was the source inoculum for stock cultures maintained on CO plus H_2 plus CO_2, while the Marburg strain was used as the source inoculum for other substrate combinations. The derivation and characteristics of the CO-adapted strain of *B. methylotrophicum* have been reported (24).

In the pH controlled (pH 6.9) cultivation of *B. methylotrophicum* on formate, we used 2.1-liter sealed fermentation vessels (New Brunswick Sci., New Brunswick, NJ) that contained 1.2 liters of anaerobic PB medium supplemented with stock phosphate buffer (30 ml), 4 M sodium formate (30 ml), and 15% Na_2S (3 ml). Medium pH was maintained at 6.9 by the automatic addition of 3 N HCl. Warmed (37°C) medium was inoculated with 30 ml of log-phase culture growing on formate and was constantly stirred, but the mixing was much less vigorous than that in

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round-bottom flasks. This experiment was repeated three times and the averaged data are reported.

Growth on ^{13}C-enriched substrates was performed as previously described (18) in pressure tubes containing 9.25 ml of PB medium. Additions included 0.25 ml each of Na_2S (2.5%) and stock phosphate buffer and, as needed, formate (1,000 μmol when used as the sole substrate, 500 μmol when used in combination with CO) and CO (500 μmol).

Quantification of fermentation substrate-to-product transformation and growth. Liquid samples (5 ml) were removed from the growth vessel with N_2- or He-flushed syringes and partitioned as follows: 2-ml samples were immediately injected into sealed N_2-flushed small vials (5 to 8 ml) of precisely measured volume, 1.5-ml samples were frozen (−70°C) for the subsequent determination of soluble substrates and products, and the remainder was used to measure the culture growth (A_{660}, 1-cm path length cuvette) and pH. The assays of cell yield, formate, acetate, butyrate, H_2, CO, and CO_2 have been outlined previously (18). The total amount of CO_2 present in a vessel was determined in two steps: (i) headspace CO_2 was measured directly (18), and (ii) the amount present as dissolved and ionized species in the medium was determined from the amount released from the 2-ml liquid sample. Acidification of this sample to pH <3 converted the ionized species to CO_2 and, after mixing and a 15- to 30-min incubation at 25°C, the amount of gaseous CO_2 was measured. Total (gaseous and dissolved) CO_2 in the vial was calculated by using a Bunsen coefficient (1) of 0.773 and Henry's Law, and this result in turn, was multiplied by one-half the growth vessel liquid volume to give the total medium CO_2 content.

Nuclear magnetic resonance methods. Sample preparation and operation of the Nicolet NT-200 spectrometer for determination of ^{13}C-labeled substrate fractionation into acetate has been described (18). Between 84 and 2,000 accumulations were averaged per scan.

CO_2 trapping experiments. Cells were grown to a mid-log-phase A_{660} of 0.36 to 0.59 in 158-ml serum bottles containing 50 ml of PB medium plus additions and then pelleted (after pressurization with N_2 by centrifugation of the cushioned vials in a Sorvall GSA rotor at 3,000 × *g* for 60 min at 4°C). Bottles were inverted and the medium was expelled through a needle. Cells were suspended in anaerobic medium composed of the following (in grams per liter): NaCl, 1.8; $MgCl_2 \cdot 6H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.1; and 10 ml of trace mineral solution (17). This medium lacked yeast extract, vitamins, and combined nitrogen and was buffered and reduced (1.25 ml of 2.37 M phosphate buffer, pH 7; 1 ml of 2.5% cysteine-sulfide; 1 ml of 2.5% Na_2S added per 50 ml of anaerobic medium). The suspension was diluted until its acetate synthesis rate (at 37°C) from the appropriate substrate (e.g., CO if the cells were grown on CO) was ≤1 mM acetate per h. The amount of [^{14}C]formate was determined in medium supernatant samples after removal of $^{14}CO_2$ (sample acidification to pH <4, $^{12}CO_2$ sparging, sample neutralization) by fractionation on a Dowex column (1-8X, 200 mesh; Sigma) and changed to formate counter ion with 5-ml washes of distilled water (two washes), 0.05 N HCOOH (three washes), 0.1 N HCOOH (one wash), 0.4 N HCOOH (two washes), and 0.8 N HCOOH (one wash). Effluent was collected in vials containing 0.5 ml of 5 M KOH, and the radioactivity of fraction samples was determined (manuscript in preparation). Formate eluted in the 0.4 N HCOOH washes.

Carbon dioxide was trapped in pressure tubes modified by the transverse attachment of two side arms (total internal volume, 45 ml). The side arms were angled such that when their ends rested on a surface they connected to the upper side of the horizontal pressure tube slung underneath. The side arms were packed with a distal layer of absorbent cheesecloth and paper pH indicator strips and a thinner proximal layer of nonabsorbent cotton. The cheesecloth was dampened with an anaerobic solution of 2 N NaOH when CO_2 was to be trapped. Tubes were made anaerobic, flushed with CO, and rinsed with reduced resuspension medium to ensure anaerobicity. Cell suspensions (7 ml per tube), soluble substrates, and, when indicated, ^{14}CO (Amersham Corp., Arlington Heights, Ill.) were then added. Tubes were incubated horizontally at 37°C with shaking (120 oscillations per min) along the long axis of the pressure tube to maximize liquid-headspace mixing.

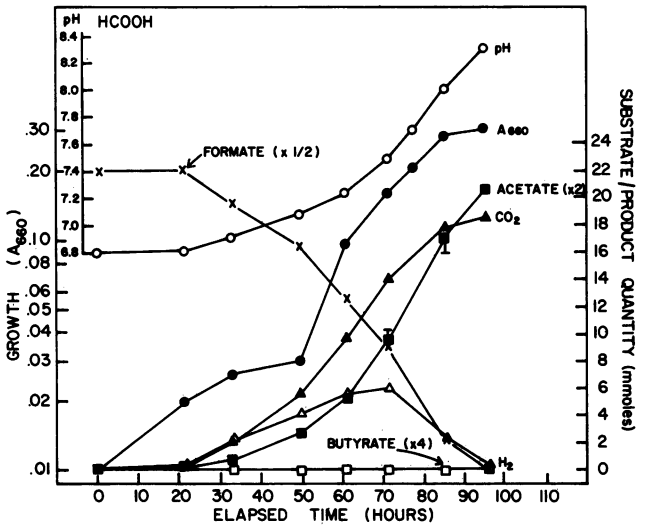

FIG. 1. Fermentation of sodium formate by *B. methylotrophicum*. Sealed flasks containing 400 ml of PB medium plus additions under an N_2 headspace were incubated at 37°C and analyzed as described in Materials and Methods. The plotted results represent averages of three growth vessels with the standard deviation of acetate production indicated by error bars where these exceed the size of the symbol. The parenthetical multipliers are factors by which the data have been multiplied to give the plotted points. For example, the formate initially present (44 mmol) has been multiplied by 0.5 to give the plotted value of 22 mmol.

RESULTS

Growth curve analyses. Acetate was the predominant reduced carbon product synthesized during the growth of *B. methylotrophicum* on oxidized substrates including sodium formate (Fig. 1), sodium formate plus H_2 plus CO_2 (Fig. 2), sodium formate plus CO (Fig. 3), and H_2 plus CO_2 plus CO (Fig. 4). However, the consumption of these substrates resulted in different H_2 levels and fluxes, cell yields, and final medium pH.

The catabolism of sodium caused an increase in medium pH (Fig. 1) despite the 59 mM buffer concentration. The initial net H_2 production (resulting in a headspace of 12% H_2 at the 72-h time point) was followed by net H_2 consumption as formate was depleted and CO_2 accumulated. Doubling times of 16 h were typical in batch cultures, with more rapid growth (ca. 10-h doubling times) observed in cultures maintained at constant pH. The cause of the decrease in growth rate at the 50-h time point is not known, but similar (though

usually less pronounced) growth variations at low growth levels were common during growth on formate. The fermentation balance of formate consumption was calculated (in micromoles per vessel) from the endpoints of Fig. 1 and a repeat experiment as follows: 41,624 formate → 9,862 acetate + 295 H_2 + 18,895 CO_2 + <18 butyrate + 1,710 cell C. Carbon and electron recoveries were 97 and 105%, respectively.

The averaged data of three formate growth experiments maintained at a constant pH demonstrated a 35% increase in cell yield (in micromoles per vessel) as follows: 103,762 formate → 24,166 acetate + 3,357 H_2 + 57,459 CO_2 + <58 butyrate + 5,748 cell C. Carbon and electron recoveries were 108 and 109%, respectively. Thus, the low yield obtained on this substrate is not a result of unfavorable pH conditions.

The hydrogen fluxes observed during growth on formate under an N_2 headspace were absent when H_2-CO_2 (80:20) replaced N_2. Under these conditions, formate and H_2 were simultaneously consumed and CO_2 synthesis was comparatively lessened (Fig. 2). The fermentation balance was (in micromoles per vessel) as follows: 35,991 formate + 23,966 H_2 → 14,900 acetate + 4,444 CO_2 + 1,781 cell C. Carbon and electron recoveries were 100 and 106%, respectively.

B. methylotrophicum simultaneously consumed formate and CO (Fig. 3), although extended lag periods (2 to 4 days) were noted with the first few transfers of a formate-grown culture into this substrate mixture. Approximately twice as much CO as formate was utilized, and, in contrast to cultures supplied only with formate, the medium pH remained constant and net H_2 production or consumption was not observed. Formate plus CO catabolism supported a higher level of growth and a higher growth rate (T_d = 6 h between 0.1 and 1.0 A_{660}) than that found on formate alone.

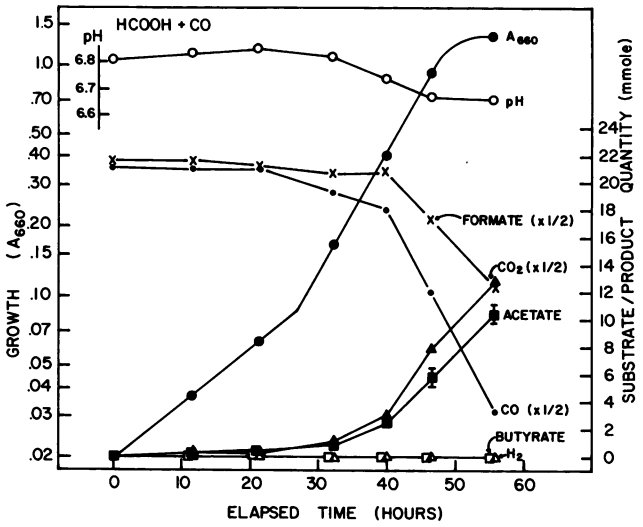

FIG. 3. Simultaneous fermentation of sodium formate plus CO by *B. methylotrophicum*. Fermentations were performed in sealed 1.1-liter flasks containing 400 ml of PB medium plus additions under a CO headspace (100%, 1.4 atm [ca. 141 kPa] at 25°C) as described in Materials and Methods. Averaged results from three growth vessels (with the standard deviations in acetate production indicated by error bars) are presented, and the parenthetial multipliers are factors by which the data have been multiplied to give the plotted points.

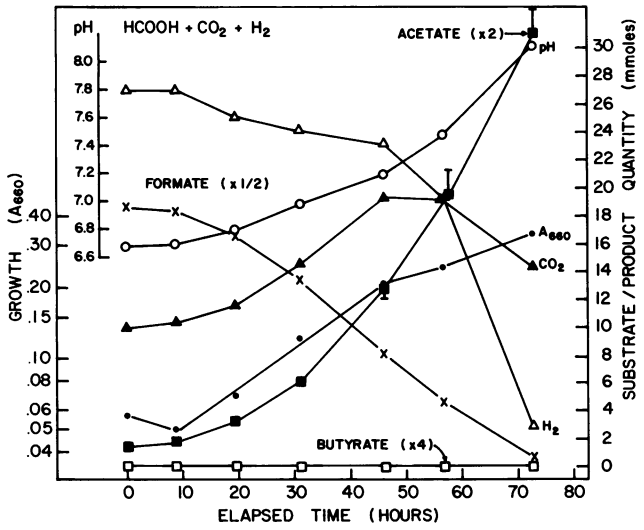

FIG. 2. Simultaneous fermentation of sodium formate plus hydrogen by *B. methylotrophicum*. The 1.1-liter flasks contained 400 ml of PB medium plus additions under a H_2-CO_2 headspace (80:20, 1 atm [ca. 101 kPa] at 25°C) and were incubated at 37°C. Analyses are as described in Materials and Methods. Average results (with the standard deviations in acetate production indicated by error bars) from three growth vessels are presented, and the parenthetical multipliers are factors by which the data have been multiplied to give the plotted points. A 34-h lag period has been omitted from the figure. Slight sulfide precipitation raised the initial A_{660} of the medium above that of the other experiments.

The fermentation balance was (in micromoles per vessel) as follows: 35,664 CO + 18,613 formate → 10,365 acetate + 25,859 CO_2 + 4,655 cell C. Carbon and electron recoveries were 94 and 95%, respectively.

The lack of net H_2 production when formate and CO were

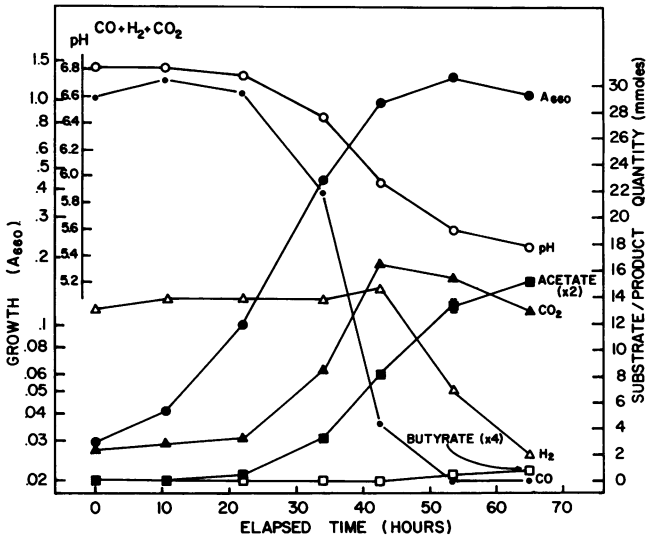

FIG. 4. Sequential fermentation of CO and hydrogen by *B. methylotrophicum*. The 1.1-liter growth vessels contained 400 ml of PB medium plus additions under a H_2-CO_2 (80:20, 0.6 atm [ca. 101 kPa] at 25°C) plus CO (1.0 atm [ca. 101 kPa] at 25°C) headspace and were incubated and analyzed as described in Materials and Methods. Averaged results from two growth vessels (with the range in acetate production indicated by error bars where these exceed the size of the symbol) are presented, and the parenthetical multipliers indicate factors by which the data have been multiplied to give the plotted points.

TABLE 1. Formate and carbon monoxide label distribution into acetate[a]

Substrate	^{13}C fractional distribution of:				
	$^{13}CH_3$-COOH	CH_3-$^{13}COOH$	$^{13}CH_3$-$^{13}COOH$	$\Sigma^{13}CH_3$	Σ-$^{13}COOH$
^{13}CO	0.04	0.08	0.88	0.48	0.52
$H^{13}COOH$	0.02	0.03	0.95	0.49	0.51
$H^{13}COOH$-^{13}CO	0.04	0.04	0.92	0.50	0.50
$H^{13}COOH$-CO	0.67	0.12	0.21	0.78	0.22
HCOOH-^{13}CO	0.10	0.61	0.29	0.24	0.76

[a] *B. methylotrophicum* was grown at 37°C with vigorous shaking in 26.4-ml pressure tubes containing 10.25 to 10.5 ml (including additions) of PB medium as described in Materials and Methods. The results present averages from at least 3 tubes per condition. Standard deviations were less than 28% of the values greater than 0.09.

supplied was assumed to reflect the observation that hydrogenase activity in extracts of *B. methylotrophicum* was inhibited by CO (24). We therefore cultivated the organism on CO plus H_2 plus CO_2 to determine whether CO also prevented H_2 consumption. *B. methylotrophicum* initially catabolized CO to acetate and CO_2 and used H_2 plus CO_2 only after the CO had been depleted (Fig. 4). The final yield and growth rate (T_d = 6 h) while CO was being consumed were similar to that of the cultures supplied with CO plus formate. If we ignore the temporal differences in H_2 and CO utilization, the fermentation balance was (in micromoles per vessel) as follows: 10,952 H_2 + 29,001 CO → 7,617 acetate + 176 butyrate + 10,811 CO_2 + 4461 cell C. Carbon and electron recoveries were 108 and 104%, respectively.

^{13}C nuclear magetic resonance analyses. Incorporation of labeled substrate into acetate indicated that the ^{13}C of formate and CO was predominantly incorporated into the methyl and carboxyl groups, respectively, of acetate (Table 1).

CO_2 trapping experiments. Methanol plus CO are simultaneously consumed (18), and when both were supplied to cell suspensions, the removal of CO_2 did not significantly affect the net amount of reduced product ("acetate") synthesized (Fig. 5). However, the ratio of acetate to butyrate was altered: more butyrate and less acetate was produced in tubes with CO_2 trapping than in those without. CO_2 removal completely inhibited acetate synthesis by cell suspensions supplied only with CO (Fig. 6).

The ^{13}C nuclear magnetic resonance experiments described above demonstrated the introduction of label from ^{13}CO into the initially unlabeled formate pool, and we decided to determine whether the transformation occurred directly or indirectly (via CO_2). CO_2 removal when CO plus formate were cosubstrates resulted in a less than 0.4-fold decrease in the rate of acetate synthesis (Fig. 7). However, the observed rate of label introduction from ^{14}CO into the large, initially unlabeled, formate pool was very dependent on the presence of CO_2 and was decreased more than 10-fold by its removal.

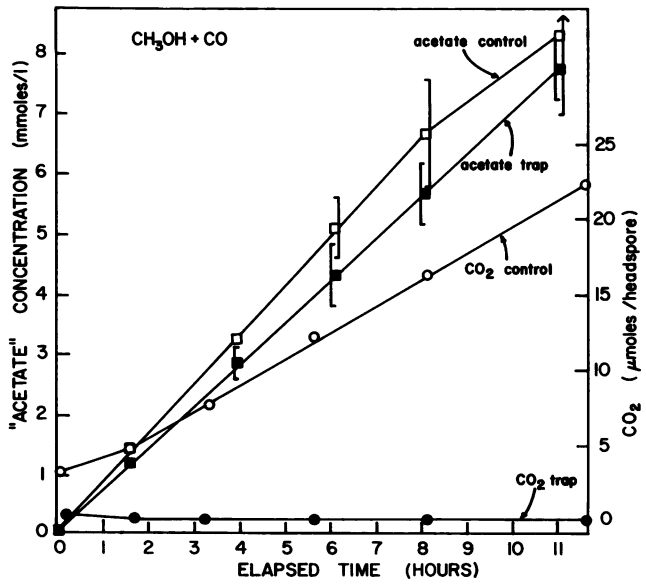

FIG. 5. "Acetate" and CO_2 production by *B. methylotrophicum* suspensions supplied with methanol (to 50 mM) and CO (1.0 atm [101 kPa], 100%). The 7-ml cell suspensions were incubated with shaking in modified pressure tubes at 37°C. CO_2 was trapped in two tubes and allowed to accumulate in two control tubes. Because CO_2 removal shifted catabolism toward greater synthesis of the more reduced butyrate, the sum of the reduced product carbon ("acetate") is reported. "Acetate" equals the combination of the acetate plus twice the butyrate concentrations present at a given time. Error bars indicate the range about the mean.

DISCUSSION

B. methylotrophicum has been shown to catabolize CO (24) and H_2 plus CO_2 (25). Here we report its growth on formate, either as the sole substrate (Fig. 1) or in combination with H_2 plus CO_2 (Fig. 2) or CO (Fig. 3).

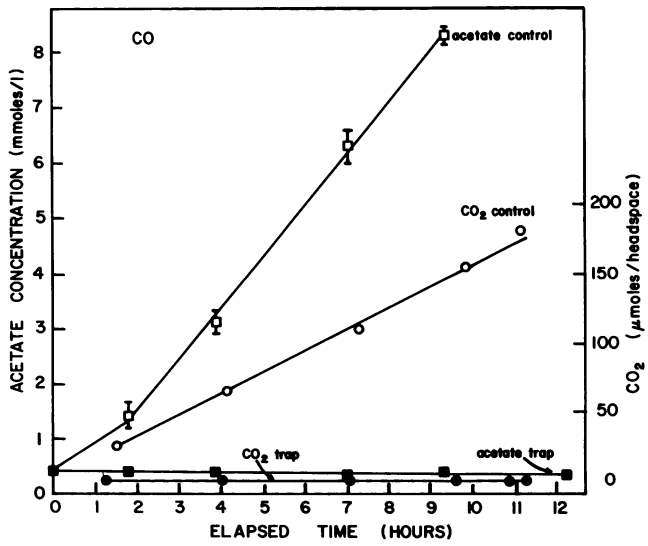

FIG. 6. Acetate and CO_2 production by *B. methylotrophicum* suspensions supplied solely with CO (1.4 atm [141.4 kPa], 100%). Error bars indicate the range about the mean from sets of duplicate tubes (± alkaline trap values).

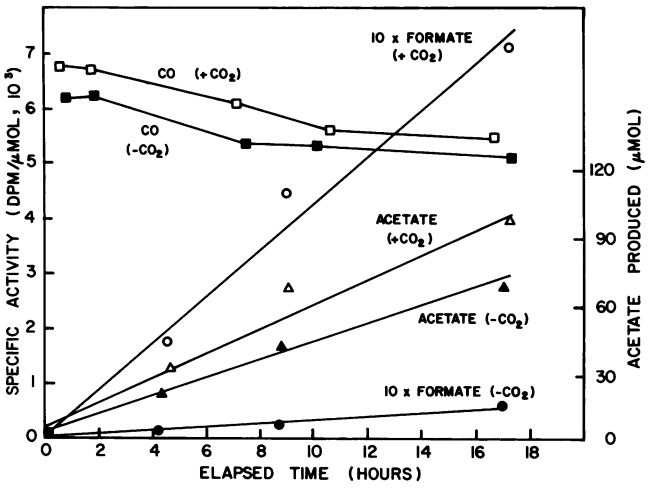

FIG. 7. Acetate production and ^{14}CO label incorporation into the formate pool by *B. methylotrophicum* grown on formate plus CO. Cell suspensions were supplied with sodium formate (ca. 200 mM) and a mixture of ^{12}CO-^{14}CO (1.2 atm [121 kPa]; 90% CO–10% He; CO specific activity, 6,500 dpm/μmol). Average levels of acetate and specific activities of CO and formate were determined in samples from six vessels, three in which CO_2 was trapped and three in which CO_2 was allowed to accumulate.

As with H_2 plus CO_2, formate was a poor substrate and significant growth required high concentrations in strongly buffered or pH-controlled fermentations. Hydrogen fluxes observed during formate consumption in the absence of CO resembled previous results with *B. methylotrophicum* supplied with either CH_3OH plus formate (18) or lactate plus formate (G. J. Shen and J. G. Zeikus, manuscript in preparation) and with *B. rettgeri* (*Eubacterium limosum* [29]) supplied with glucose plus formate (30). Probably the initial net H_2 production serves as a relief valve (15) during formate catabolism, allowing the temporary release of excess reducing equivalents while generating sufficient CO_2 for subsequent reduction to the acetate carbonyl precursor.

When CO_2 was initially present, *B. methylotrophicum* simultaneously consumed H_2 and formate (Fig. 2). H_2 consumption is also proportional to CO_2 availability during growth on multicarbon substrates (Shen and Zeikus, in preparation) and in a similar manner *Acetobacterium woodii*, which produces H_2 during growth on fructose if the initial CO_2 concentration is low, simultaneously consumes H_2 and fructose when sufficient CO_2 is supplied (7). Hydrogen production has also been reported for the thermophilic acetogen *C. thermoaceticum* growing on glucose (16, 26), with more H_2 evolved under N_2 than under CO_2 headspaces. Maximum amounts of H_2 were produced by cells catabolizing glucose under a 100% CO headspace. The exposure of *C. thermoaceticum* to CO in this experiment was much lower than the exposure of *B. methylotrophicum* to CO when formate plus CO was supplied both because the *C. thermoaceticum* cultures were not shaken and because CO is less soluble at the thermophile's higher growth temperature. Under the conditions of our experiments, detectable H_2 was neither produced (Fig. 3) nor consumed (Fig. 4) when CO was present, probably reflecting the inhibition of all hydrogenase activities, as also suggested for *E. limosum* (13).

The function of CO_2 as a necessary intermediate during CO or formate catabolism is supported by results of the CO_2 trapping experiments. CO_2 removal inhibits the consumption of CO alone (Fig. 6) but is of little consequence when a supply of acetate methyl-precursor substrate (e.g., methanol or formate) is available in addition to CO (Fig. 5 and 7). The observation (Fig. 7) that label transferral from ^{14}CO into an initially unlabeled formate pool was nevertheless dramatically reduced by CO_2 removal indicates the effectiveness of the trapping design and supports the respective positions of formate and CO on opposite forks of the catabolic pathway, with CO_2 the sole intermediate single carbon compound (37).

As demonstrated with *A. woodii* (9, 10, 18), *B. methylotrophicum* simultaneously (Fig. 3) but differentially (Table 1) catabolized formate and CO in a ratio of approximately 1:2. Formate and CO preferentially labeled the acetate methyl and carboxyl groups, respectively. Their simultaneous consumption is especially interesting considering the differences in cell yield observed when only CO or only formate is supplied. That CO, the better substrate, is not catabolized exclusively before formate consumption begins is consistent with an obligatory role for formate in C_1 substrate catabolism.

If formate is indeed an obligatory intermediate in C_1 metabolism by saccharolytic acetogens, then the question arises as to why some isolates are reported to be unable, or very weakly able, to utilize it as a substrate. Many of these results may be based on unfortunate experimental designs; either the initial formate concentration utilized was low (e.g., 10 to 20 mM; see references 14, 23, and 33) and the resulting growth minimal, or adequate formate was present but the medium was insufficiently buffered (e.g., medium containing only 11 mM phosphate buffer was used in the initial descriptive paper of *B. methylotrophicum* [38]). The availability of vitamins or trace elements could also affect formate catabolism and some examples of the effects of trace elements on formate dehydrogenase activity are known (2, 3, 4, 36). Possibly other enzymes (e.g., hydrogenase) are also required for formate catabolism.

In view of the role of free formate in the C_1 catabolic pathway, identification of saccharolytic acetogens unable to catabolize formate under suitable medium conditions would be surprising, and the biochemistry of such isolates should be investigated further. The physiology of these isolates could be comparable with methanogenic C_1 physiology in which formate and formate dehydrogenase are not believed to be intermediates in the otherwise schematically similar pathway of acetate synthesis (37).

ACKNOWLEDGMENT

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