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Carbon Monoxide Metabolism of the Methylotrophic Acidogen *Butyribacterium methylotrophicum*

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The Marburg strain of *Butyribacterium methylotrophicum* did not grow on CO alone but did consume CO during growth on a variety of substrates in the presence of a 100% CO gas phase. We selected a strain (the CO strain) that grew vigorously on CO alone. The ability of the CO strain to grow on CO was stable through multiple transfers in the absence of CO. CO dehydrogenase activity was lower in the CO strain grown on CO (13.3 μmol/min per mg of protein) than in the Marburg strain grown on methanol-acetate (47.2 μmol/min per mg of protein); thus, the levels of this enzyme did not explain the growth on CO. CO was dissimilated to acetate and CO_2 with the following stoichiometry: 4 CO → 2.17 CO_2 + 0.74 acetate. We observed a growth rate of 0.05 h^{-1}, a final optical density at 660 nm of 0.8, and a cell yield of 3.0 g of cells per mol of CO during growth of the CO strain. Growing cultures of the CO strain displayed a K_s for CO of 28 to 56 μM. The apparent thermodynamic efficiency of cell synthesis from CO was 57%. Energetic and biochemical aspects of CO metabolism are described.

The oxidation of CO by bacteria is interesting from both fundamental and applied perspectives. Fundamental studies of bacterial CO oxidation have shown that the ability to mediate this reaction is possessed by a diverse group of organisms, including both aerobes and anaerobes (2, 15). Microbial CO oxidation occurs via several biochemical mechanisms (9, 10, 16, 19) and may be either a gratuitous reaction or a reaction yielding energy for cell growth. It has been proposed that CO-oxidizing bacteria might be useful in the conversion of coal or biomass pyrolysis products into useful chemicals or fuels (33). Development of this technology has been impeded by limited fundamental research on microbial CO transformation.

CO metabolism by anaerobic bacteria has been documented for *Rhodopseudomonas gelatinosa* (5, 29), three species of clostridia (7, 12), and several methanogens (4, 17). Of these organisms, only *R. gelatinosa* and *Methanobacterium thermoautotrophicum* grow with CO as the sole energy source. The mechanism of CO oxidation in these anaerobes appears to be disproportionation of CO to CO_2 and a more reduced end product, with water donating the second oxygen atom in CO_2. Energy conservation proceeds via the following two reactions in *R. gelatinosa* and *M. thermoautotrophicum*, respectively: $CO + H_2O \rightarrow H_2 + CO_2$ ($\Delta G^{\circ\prime} = -4.8$ kcal/mol of CO) (29) and, $4CO + 2H_2O \rightarrow CH_4 + 3CO_2$ ($\Delta G^{\circ\prime} = -12.6$ kcal/mol of CO) (4).

CO oxidation by methanogens other than *M. thermoautotrophicum* and by *Clostridium* species reportedly occurs only in the presence of an additional energy source. There is no evidence to indicate that energy is conserved from CO during cosubstrate metabolism by methanogenic or *Clostridium* species. Diekert and Thauer (7) have proposed that pyruvate-dependent CO oxidation by cell suspensions of homoacetogenic clostridia is consistent with the following reaction (modified from the reaction presented by Diekert and Thauer): $4CO + 2H_2O \rightarrow 2\ CO_2 + 1CH_3\ COOH^- + 1H^+$ ($\Delta G^{\circ\prime} = -10.5$ kcal/mol of CO).

The biochemistry of CO oxidation by anaerobic bacteria has been studied most thoroughly in the homoacetogenic clostridia *Clostridium thermoaceticum* and *Clostridium formicoaceticum*. Before any investigation of CO metabolism per se, it was found that these species reduce CO_2 to acetate catabolically (18). The terminal step in this process is a corrinoid-dependent transcarboxylation reaction, with pyruvate donating the carboxyl group to methyltetrahydrofolate (24, 31). Thauer et al. (27) and Diekert and Thauer (7) reported that CO oxidation by cell extracts and suspensions of homoacetogenic clostridia and also by extracts of *Clostridium pasteurianum* was inhibited by propyl iodide, a corrinoid antagonist. These findings stimulated speculation that clostridial CO dehydrogenase might be a cobalt-containing corrinoid enzyme (7, 11, 27). However, Drake et al. (9) failed to demonstrate propyl iodide-mediated inhibition of the CO-

oxidizing activity of *C. thermoaceticum* in both purified enzyme preparations and cell extracts. Furthermore, both in vivo (6, 8) and in vitro (9) evidence has established that clostridial CO dehydrogenase is a nickel-containing enzyme. It has been shown recently (H. L. Drake et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, K42, p. 144) that CO can substitute for pyruvate as the carboxyl donor in the transcarboxylation reaction of acetyl coenzyme A synthesis from CO_2 in cell-free enzyme preparations from *C. thermoaceticum*.

Previously, we reported that the Marburg strain of *Butyribacterium methylotrophicum*, an obligate anaerobe, is capable of utilizing glucose, lactate, methanol-acetate-CO_2, and H_2-CO_2 as sources of energy for growth (36). The Marburg strain produces butyrate or acetate or both during growth on these substrates; high amounts of corrinoids (0.7% of cell dry weight) have also been found. During growth on methanol-acetate-CO_2, we observed a reaction stoichiometry of approximately 10 methanol + $2CO_2 \rightarrow$ 3 butyrate. The apparent similarities between *B. methylotrophicum* and homoacetogens (e.g., reduction of CO_2 to acetate and high levels of corrinoids) led us to examine the metabolism of CO by this recently described species.

(Preliminary results were presented at the Symposium on Trends in the Biology of Fermentations for Fuels and Chemicals, 7 to 12 December 1980. See also reference 18a.)

MATERIALS AND METHODS

Chemicals and gases. All chemicals were reagent grade. Gases were obtained from Matheson Scientific, Inc., Joliet, Ill., and had the following compositions: N_2, >99.998% pure; He, >99.995% pure; CO, >99.99% pure; N_2-CO_2, 95:5 (vol/vol), premixed; and H_2-CO_2, 80:20 (vol/vol), premixed. Enzymes and biochemicals were purchased from Sigma Chemical Co., St. Louis, Mo. ^{14}CO (40 mCi/mmol) and $^{14}CO_3^{-2}$ (1 mCi/mmol) were obtained from Amersham Searle, Arlington Heights, Ill.

Medium preparation and cultivation conditions. The anaerobic culture techniques used to grow *B. methylotrophicum* were as described previously (3). The Marburg strain was maintained on butyrogen medium, which contained the following components (per 985 ml of distilled water): KH_2PO_4, 0.3 g; $Na_2HPO_4 \cdot 7H_2O$, 2.1 g; NH_4Cl, 1.0 g; $MgCl_2 \cdot 6H_2O$, 0.2 g; $NiSO_4 \cdot 6H_2O$, 0.25 mg; trace mineral solution, 10 ml (34); vitamin solution, 5 ml (32); yeast extract (Difco Laboratories, Detroit, Mich.), 0.5 g; sodium acetate, 4.1 g; and 2% resazurin (Eastman Organic Chemicals, Rochester, N.Y.), 1.0 ml. After the medium was boiled and cooled to <50°C, 2.1 g of $NaHCO_3$ was added, the pH was adjusted to 7.2 to 7.4, and medium was dispensed anaerobically under an N_2-CO_2 atmosphere into 24-ml anaerobic culture tubes (18 by 142 mm; Bellco Glass, Inc., Vineland, N.J.) which were sealed with no. 2 black butyl stoppers (Scientific Products Div., McGaw Park, Ill.). After autoclaving, 0.25 ml of 20% methanol and 0.2 ml of 2.5% Na_2S were added to each tube aseptically with a syringe. A phosphate-buffered (PB) medium was used for all experiments. PB medium contained 0.1 g of $CaCl_2$ and the same amounts of NH_4Cl, $MgCl_2$, trace mineral solution, vitamin solution, yeast extract, and resazurin per 985 ml of distilled water as butyrogen medium. The pH was adjusted to 7.2 to 7.4 with NaOH. This medium was dispensed into culture vessels without boiling under an He or N_2 gas phase and then autoclaved. After autoclaving, 0.2 ml of phosphate buffer (15% KH_2PO_4, 29% Na_2HPO_4) and 0.2 ml of 2.5% Na_2S were added per 10 ml of medium. These and other additions to media were made with syringes. All solutions that were added after medium sterilization were autoclaved separately in sealed serum vials.

A culture headspace containing 100% CO was achieved by gassing culture vessels in a hood with a continuous stream of CO. Gas phases containing less than 100% CO were obtained by adding CO with a syringe to sealed culture vessels containing medium and an He gas phase. H_2-CO_2-CO mixtures were prepared by flushing culture vessels with CO or N_2, followed by H_2-CO_2 addition. Experiments were performed in either 158-ml serum vials (Wheaton Scientific, Millville, N.J.) or 26-ml pressure tubes (142 by 18 mm; Bellco Glass) sealed with black butyl stoppers and aluminum crimp caps (Bellco Glass). A 2% inoculum was used for cultural experiments, except when the inoculum had a low cell density, in which case a volume giving an initial optical density at 660 nm (OD_{660}) of <0.05 was used. Culture vessels were incubated vertically at 37°C on a rotary shaker.

Cells were mass-cultured with CO in a 14-liter fermentor (New Brunswick Scientific Co., Edison, N.J.) containing 8 liters of PB medium. During exponential growth it was necessary to repressurize the headspace to 1.5 atmospheres (atm) of CO every 6 to 8 h to prevent a very strong negative pressure from developing. Mass-culturing with methanol and glucose was accomplished in 18-liter glass carboys (Wheaton Scientific) which contained 15 liters of medium and were sealed with butyl rubber stoppers. Cells were harvested in late exponential phase. Cells were harvested anaerobically by centrifugation at 23,400 × *g* with a Sorvall model KSB continuous-flow system (Du Pont Institute, Bridgeport, Conn.); these cells were used directly or were frozen at −80°C.

Quantification of substrates, products, and growth. H_2, CO, and CO_2 were measured by thermal conductivity detection with a gas chromatograph, as described previously (20), except that the carrier gas flow rate was reduced to 20 ml/min at low (<10%) CO concentrations to give increased resolution of the CO peak from the trace nitrogen peak introduced by injection. The concentration of CO was determined at 25°C; CO has a solubility of 0.98 μmol of CO per ml of water per atm at 37°C (obtained by extrapolation from values at 25°C [25] and 35°C [12]), and this value was used in calculations of the K_s for CO. The concentrations of dissolved CO_2 were calculated from the gaseous CO_2 concentration and the medium pH, assuming behavior according to Henry's Law. The radioactivities of ^{14}CO and $^{14}CO_2$ were measured as described previously (20). Fatty acids, alcohols, and aldehydes were quantified by gas chromatography as described

previously (34), except that our analysis was performed at the following temperatures: column, 200°C; injection, 220°C; detector, 240°C. Formate was analyzed spectrophotometrically by standard enzymatic methods (1), using NAD-linked formate dehydrogenase. During time course experiments, gases were sampled aseptically by withdrawing 0.4 or 0.2 ml from the gas phase with a 1-ml glass syringe equipped with a mininert syringe valve (Supelco, Bellefonte, Pa.); 0.3 ml of the liquid volume was withdrawn for analyses of soluble end products.

Culture turbidities at 660 nm were determined by inserting anaerobic culture tubes into a Spectronic 20 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.). When the turbidity exceeded an OD_{660} of 0.4, we either diluted samples or used a curve of diluted versus nondiluted OD_{660} values. In serum bottle experiments, turbidities were measured by withdrawing 1-ml samples, followed by dilution. Frequently, we used a curve relating OD_{660} to dry weight. This relationship was linear with 344 mg of cells per liter at an OD_{660} of 1.0 for both methanol- and CO-grown cells. In determining the results of our cultural studies, we did not include tubes showing no growth or oxidation or both in calculations of the average values. Standard deviations are indicated as "±." Elemental analyses of glucose-, methanol-, and CO-grown cells (Galbraith Labs, Knoxville, Tenn.) gave a carbon content 45.1 ± 0.4% and a cell formula of $C_{4.86}H_{8.85}O_{2.41}N_1$ plus 7.4.% ash.

Enzyme analysis. The methods used for preparing anaerobic cell extracts and for enzyme analyses were based on modifications of previously described techniques (35). A suspension of cells in anaerobic distilled water containing 4 mM dithiothreitol was prepared and placed under continuous nitrogen gassing into a cold (4°C) French pressure cell (American Instrument Co., Inc., Silver Spring, Md.). A single disruption at 20,000 lb/in^2 resulted in 20 to 40% cell breakage. Disrupted cells were collected in an aerobic centrifuge tube sealed with a hard rubber stopper and were centrifuged for 30 min at 12,000 × *g*. The clear supernatant was transferred with a glass syringe into anaerobic vials cooled in ice. Extracts contained 9 to 27 mg of protein per ml, as determined by a Lowry assay (3). A dilution of fresh extract in anaerobic distilled water containing 4 mM dithiothreitol was prepared immediately before the assay when the enzymatic activity was too rapid to measure.

Carbon monoxide dehydrogenase, formate dehydrogenase, and hydrogenase were assayed spectrophotometrically by measuring the reduction of methyl viologen at 578 nm (ε_{578} = 9.78 mM^{-1} cm^{-1}). Assays were performed in 1.6-ml cuvettes (total volume when fitted with gray rubber stoppers) that were made anaerobic by repeatedly evacuating and flushing with N_2. The anaerobic reaction mixture (0.8 ml) contained 55 mM phosphate buffer (pH 7.2), 5 mM methyl viologen, and 5 mM sodium formate or 0.8 ml of CO, H_2, or N_2. The reaction mixture was shaken vigorously, reduced with dithionite (5 to 10 μl of a 20 mM solution) until a light blue hue was observed, and then inserted into an Eppendorf recording spectrophotometer (Brinkmann Instruments Inc., Westbury, N.Y.) with the cuvette chamber maintained at 35°C. Reactions were initiated by injecting 5- to 20-μl volumes of extract or dilution into the sealed cuvettes.

RESULTS

Selection of the CO strain. The Marburg strain of *B. methylotrophicum* grew on glucose, pyruvate, and methanol in the presence of a 100% CO gas phase; however, no growth occurred in PB medium when CO was the sole energy source.

A strain capable of growth with CO as the sole energy source was selected by the following procedure. The Marburg strain was transferred twice on butyrogen medium with a CO gas phase. A final OD_{660} of 1.0 and a strong negative pressure were observed at the end of growth under these conditions. This treatment was followed by four successive transfers at 3 to 4-week intervals on PB medium with a 100% CO gas phase but without methanol and acetate. During the first four transfers on CO, an OD_{660} of 0.1 to 0.2 was obtained after 2 to 3 weeks. The fifth transfer of this culture resulted in an OD_{660} of 0.5 and the complete elimination of CO from the gas phase in 3 days. Subsequent transfers of this culture retained the ability to grow rapidly on CO for more than 8 months through repeated cultivation on CO or on methanol in the absence of CO. The strain of *B. methylotrophicum* which was capable of growth with CO as the carbon and energy source was designated the CO strain.

We designed an experiment to examine the nature and stability of the metabolic differences between the Marburg and CO strains. Three different cultures were prepared for use as inocula. The Marburg strain inoculum was maintained on PB medium that contained methanol, acetate, and CO_2 and an N_2 gas phase; the CO strain inocula either were transferred twice (2% inoculum) on PB medium containing methanol, acetate, and CO_2 and an N_2 gas phase over a period of 1 month or were maintained on PB medium containing CO as the energy source. The experiment was initiated by transferring each of the three inocula into tubes containing PB medium, 12.5 mM $NaHCO_3$, and either methanol and acetate (N_2 gas phase), methanol, acetate, and CO, or CO alone.

Figure 1A shows that both cultures of the CO strain completed growth on CO alone in less than 6 days whether they were pregrown on CO or on methanol-acetate-N_2. The Marburg strain neither grew nor consumed CO during 22 days of incubation on CO alone. In tubes containing methanol, acetate, and CO (Fig. 1B), the Marburg strain culture and both cultures of the CO strain reached an OD_{660} of >0.8 in less than 6 days. Under these conditions, all cultures removed the CO before the onset of the stationary phase. In tubes containing methanol and acetate under an N_2 gas phase, OD_{660} values of >0.8 were obtained in less than 3 days by all cultures (data not shown). The CO strain was very stable

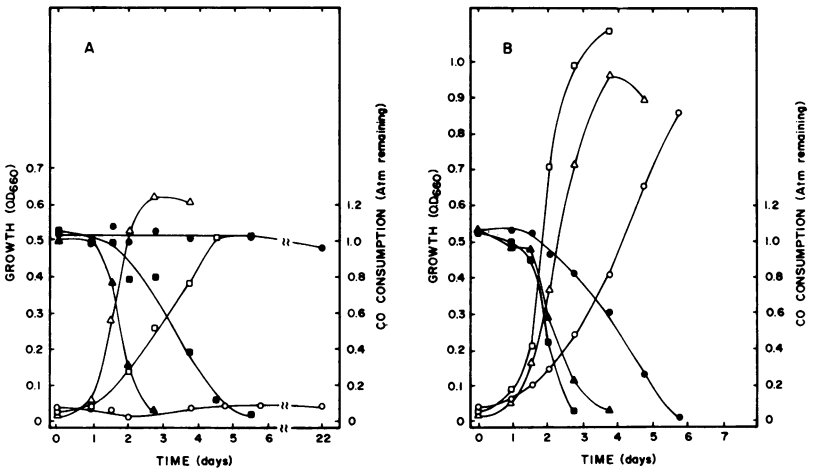

FIG. 1. CO consumption by the Marburg and CO strains of *B. methylotrophicum*. (A) Growth on CO alone. (B) Growth on CO-methanol-acetate-CO_2. Symbols: ○ and ●, Marburg strain maintained on methanol-acetate-CO_2 (N_2 gas phase); □ and ■, CO strain transferred twice on methanol-acetate-CO_2 (N_2 gas phase); △ and ▲, CO strain maintained on CO. The open symbols indicate growth, and the solid symbols indicate the CO remaining. Results are averages of duplicate to quadruplicate determinations in 27-ml pressure tubes containing 10 ml of PB medium and 1 atm of CO.

and retained the ability to grow on CO after multiple transfers on glucose or methanol-CO_2-acetate as sole growth substrates.

Growth and metabolism of the CO strain. The CO strain grew vigorously in a medium containing 1 atm of CO as the sole source of energy (Fig. 2). Inoculated controls incubated at 75°C and inoculated controls incubated in the absence of CO demonstrated that abiological oxidation of CO was insignificant and that growth was entirely dependent upon CO. Specific growth rates of approximately 0.05 h^{-1} were observed in both 28-ml tubes and 158-ml bottles containing a ratio of a headspace to medium of 2:1. Substrate consumption and production of cells were proportional throughout growth in two independent experiments. A cell yield of 3.0 ± 0.32 g of cells per mol of CO was obtained. The onset of growth on CO was inhibited 2 to 5 days by the presence of 50 mM formate. However, growth

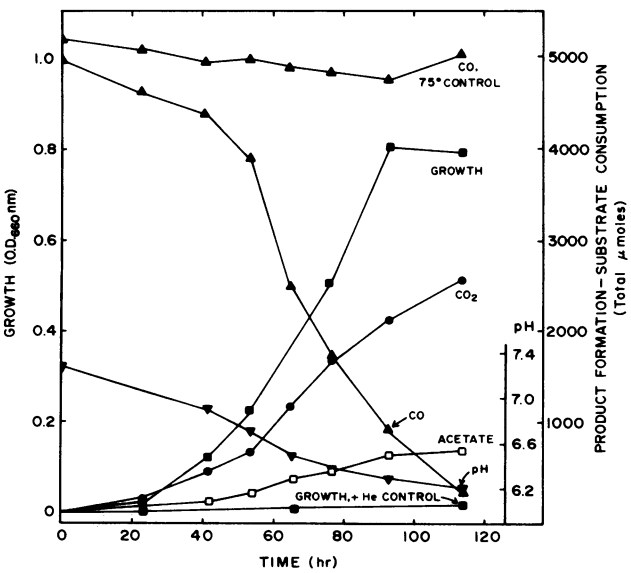

FIG. 2. CO metabolism of *B. methylotrophicum* CO strain. Results are averages of quadruplicate determinations in serum bottles containing 52 ml of PB medium and 1 atm of CO. All cultures initiated growth simultaneously; the maximum standard deviation of the OD_{660} (±0.15) was at 75 h.

of the Marburg strain on methanol-acetate-CO_2 was not altered by the same concentration of formate.

Acetate, CO_2, and protons were the only fermentation products detected after growth on CO. Endpoint analyses of cultures grown on CO revealed the following substrate-product conversion stoichiometry: $4CO \rightarrow 2.17\ CO_2 + 0.74\ CH_3COOH$ (Table 1). The production of CO_2, acetate, and cells accounted for essentially all of the carbon and electrons present in the CO consumed, with a carbon recovery of 102% and an electron recovery of 97%.

An approximate K_s value for the CO strain grown on CO was obtained by measuring the growth rate as a function of CO concentration. A plot of specific growth rate versus percent CO (Fig. 3) indicated that one-half of the maximal growth rate was observed at 4% (about 28 μM) CO. A reciprocal plot of these data (Fig. 3) gave a K_s value of 8% CO and clearly demonstrated that the growth rates at low CO concentrations were higher than would have been expected on the basis of classical Michaelis-Menten kinetics.

Biochemical activities associated with CO metabolism. To determine the effects of growth substrates on the levels of enzymes potentially involved in CO dissimilation, we compared the specific activities of CO dehydrogenase, formate dehydrogenase, and hydrogenase in cells grown on CO and in cells grown on methanol-acetate-CO (Table 2). The CO strain grown on CO displayed levels of CO dehydrogenase (13.3 μmol/min/per mg of protein) that were more than threefold lower than the levels in the Marburg strain grown on methanol (47.2 μmol/min per mg of protein). The specific activity of hydrogenase in cells of the Marburg strain grown on methanol (2.4 μmol/min per mg of protein) was higher than the specific activity of the CO strain grown on CO (0.9 μmol/min per mg of protein). CO-grown CO strain produced approximately fourfold-higher levels of formate dehydrogenase than methanol-grown Marburg strain (3.8 and 1.0 μmol/min per mg, respectively). CO strongly inhibited hydrogenase activity in extracts of cells grown on both of the substrates tested; the addition of 10 μl of CO to a gas phase containing 800 μl of H_2 resulted in 80% inhibition of hydrogenase activity.

TABLE 1. CO fermentation balance of *B. methylotrophicum* strain CO[a]

Compound	Substrate-product relationships[b]		
	Concn (μmol/bottle)		Change (total μmol)
	Initial	Final	
CO	4,744	313	4,431
CO_2	13	2,416	2,403
Acetate	<10	816	816

[a] The substrate-product conversion stoichiometry, including cells, was as follows (total moles): 4,431 CO → 2,403 CO_2 + 816 acetate + 500 cell carbon. The amount (micromoles) of cell C was calculated as follows: (3 μg of cells/micromoles of CO) × (4,431 μmol of CO used) × (0.451 μg of C/micrograms of cells) × (1 μmol of C/12 μg of C). The fermentation balance was: 4.0 CO → 2.17 CO_2 + 0.74 acetate + 0.45 cell C. The carbon recovery was: {[2,403 + 816(2) + 500]/[4,431]} × 100% = 102%. The electron recovery was: {[816(8) + 500 (4.22)]/[4,431 (2)]} × 100% = 97%. The number of electron equivalents (4.22) per micromole of C was calculated from the cell formula by the method of Harris and Adams (14).

[b] We used 158-ml serum vials that contained 55 ml of PB medium and 1 atm of CO. ^{14}CO (2.25×10^3 dpm/mol) was used to increase the precision of quantification of small amounts of CO. The results are averages of triplicate experimental vials.

DISCUSSION

The CO strain of *B. methylotrophicum* can grow on CO as the sole source of carbon and energy in a mineral medium that contains 0.05% yeast extract. The absence of growth on yeast extract alone, the linear relationship between cell synthesis and CO consumption, and the consistency of experimental and theoretical carbon and electron recoveries suggest that the contribution of the yeast extract present in the medium to the formation of cells and products is insignificant. The ability of the CO strain to grow on CO appears to be stable in the absence of selective pressure; this observation is consistent with the existence of a genetic, mutational difference between the Marburg and CO strains. Since higher levels of CO dehydrogenase were found in the Marburg strain grown on methanol-acetate-CO_2 than in the CO strain grown on CO, it is clear that more than a CO dehydrogenase activity is required for growth on carbon monoxide. The inhibition of growth on CO by formate was the only property found to be specific to either the CO strain or growth on CO alone.

Prolific growth with carbon monoxide as the only apparent source of carbon and energy qualitatively distinguishes CO utilization by *B. methylotrophicum* from CO utilization by the previously described CO-metabolizing clostridia and methanogens. Mixotrophic metabolism of CO by *B. methylotrophicum* also differs from mixotrophic metabolism of CO by clostridia and methanogens in that CO utilization contributes to cell synthesis and CO toxicity is not observed. The principal difference between the CO metabolism of *B. methylotrophicum* and the CO metabolism of *R. gelatinosa* is that acetate is the reduced catabolic product in the former, whereas the latter forms H_2 (29). Both of these species grow actively under 1 atm of carbon monoxide. In light of our findings, it will be interesting to

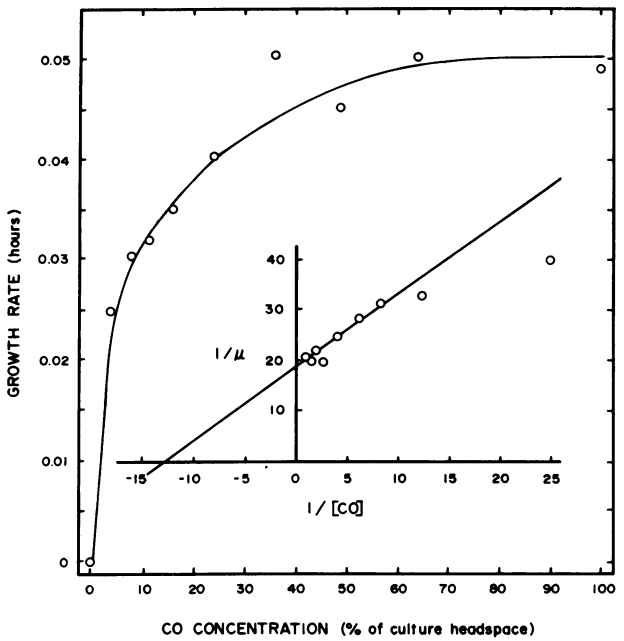

FIG. 3. Relationship between CO concentration and growth rate (μ) of *B. methylotrophicum* CO strain. Results are averages of duplicate or triplicate determinations in 27-ml pressure tubes containing 5.5 ml of PB medium. The inset shows a reciprocal plot of the same data.

determine whether other CO-utilizing anaerobes, such as *Clostridium* or *Acetobacterium* species, can be adapted to grow well on CO alone.

Our analysis of the energetics and thermodynamic efficiency of CO metabolism is as follows. illustrated below.

The apparent energetics of CO metabolism ($\Delta G^{0\prime}$) in the reaction 4 CO + 1.97 $H_2O \rightarrow$ 2.03 CO_2 + 0.76 acetate + 0.45 cell C was determined to be −38.9 kcal. The apparent thermodynamic efficiency of CO metabolism was calculated as follows:

$$\frac{\text{(moles of cells) } (\Delta G^{0\prime}{}_{ox} \text{ of cells})}{\text{(moles of substrate) } (\Delta G^{0\prime}{}_{ox} \text{ of substrate}) - \text{(moles of product) } (\Delta G^{0\prime}{}_{ox} \text{ of product})} \times 100\% =$$

$$\frac{(0.45)\ (-114.9 \text{ kcal})}{(4)\ (-61.5 \text{ kcal}) - (0.76)\ (-204.0 \text{ kcal})} \times 100\% = 57\%$$

The apparent thermodynamic efficiency of cell synthesis from CO is defined as the energy conserved as cells divided by the energy available for cell synthesis and was calculated by using an assumed value (27.3 kcal/available electron equivalent) for the free-energy oxidation ($\Delta G^{0\prime}{}_{ox}$) of cells to CO_2, H_2O, and NH_4^+ (R. F. Harris, *in Soil Nitrogen*, in press). Calculations similar to those used for determining the efficiency of *B. methylotrophicum* on CO can be performed for aerobic C_1 utilizers by using cell yield data (13) and the same thermodynamic values used above for $\Delta G^{0\prime}{}_{ox}$ cells (0.46 g of carbon per g of cells and 4.25 electron equivalents per mol of cell carbon) (14). This approach gives efficiencies of 42 to 46% for growth on methanol when the ribulose monophosphate pathway is used and 26 to 53% for growth on formate when the serine pathway is used. A thermodynamic efficiency of 57% is consistent with the range of 55 to 65% described by Payne and Williams (22, 23) for the thermal efficiency (i.e., calculated from ΔH values of composition) of heterotrophic bacteria growing on multicarbon substrates in minimal media. Our data suggest that *B. methylotrophicum* conserves energy during growth on CO with an efficiency comparable to that achieved by aerobic organisms growing on more energy-rich compounds (i.e., methanol or glucose).

The transcarboxylation reaction of *C. thermoaceticum* is thought to involve the synthesis of

TABLE 2. Relationship of energy source to dehydrogenase activities of *B. methylotrophicum*[a]

Energy source	Strain	Sp act (μmol of substrate per mg of protein per min)		
		CO dehydrogenase	Hydrogenase	Formate dehydrogenase
Methanol-acetate-CO_2	Marburg	47.2	2.4	1.0
CO	CO	13.3	0.9	3.8

[a] Cells were grown on PB medium that contained either 100 mM methanol, 25 mM sodium acetate, 12.5 mM $NaHCO_3$, and an N_2 gas phase or a CO gas phase (1.0 to 1.5 atm). Enzymes were assayed in cell-free extracts at 35°C by spectrophotometric measurement of the substrate-specific reduction of methyl viologen in anaerobic cuvettes.

acetyl coenzyme A from a carrier-bound methyl group and either CO or pyruvate (Drake et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1981). If acetate were synthesized from CO by this mechanism in *B. methylotrophicum*, 1 mol of ATP could be generated for every 4 mol of CO dissimilated via acetate kinase-mediated substrate level phosphorylation. The only other known substrate level phosphorylation which could plausibly contribute to energy conservation from CO is the hydrolysis of formyl tetrahydrofolate. This reaction is believed to be the major mode of ATP synthesis in at least one anaerobe, *Clostridium cylindrosporum* (28). The most energetically favorable electron-accepting reaction involved in the reduction of CO_2 to acetate via known pathways (33) is the reduction of a formaldehyde level intermediate. The free energy of $CO + CH_2O + H_2O \rightarrow CH_3OH + CO_2$ (−15.5 kcal) is sufficiently exergonic to be coupled to ATP synthesis via electron transport-mediated phosphorylation. The yield obtained on CO (3.0 g/mol) appears to be very consistent with the synthesis of 0.5 mol of ATP per mol of CO (2 ATP per mol of acetate produced). An ATP gain of 0.25 mol of ATP per mol of CO (1 ATP per mol of acetate) and a cell yield of 3.0 g/mol imply a Y_{ATP} of 12 g of cells per mol of ATP, a value greater than the observed and theoretical Y_{ATP} values for acetate (Y_{max}^{ATP} = 10.0 g/mol of ATP [24]), a substrate with more carbon, electrons, and energy per mole than CO.

Hypothetically, the oxidation of CO to CO_2 could proceed either directly or indirectly via a two-step process involving hydration to a formyl intermediate of some kind, followed by oxidation to CO_2. Presumably, CO dehydrogenase could catalyze direct CO oxidation; however, formate dehydrogenase could also be involved in a two-step oxidation of CO. The protein fraction that catalyzes CO oxidation in *C. thermoaceticum* is also involved in the transfer of a carboxyl group to a methyl acceptor (Drake et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1981). Thus, in vitro detection of CO dehydrogenase activity may not be correlated with an in vivo role for this enzyme in CO oxidation. The higher levels of CO dehydrogenase in cells grown on methanol than in cells grown on CO are consistent with an in vivo function of CO dehydrogenase other than CO oxidation. Hydrogenase is probably not involved in CO dissimilation because of the strongly inhibitory effect of CO on hydrogenase both in vivo and in vitro. The levels of CO dehydrogenase found in *B. methylotrophicum* are the highest levels that we know of in an anaerobe.

In previous studies on CO metabolism of clostridia (7) and methanogens (4), workers assumed that CO oxidation was coupled directly to the reduction of CO_2 to either acetate or methane. Figure 4 shows an alternative model to test for one-carbon transformations with CO conversion to acetic acid. According to this hypothetical scheme, CO is first transformed to a formyl intermediate, which may then either be oxidized to CO_2 and electrons, reduced to a methyl intermediate, or methylated to acetyl coenzyme A via transcarboxylation. Synthesis of the carboxyl group of acetate in this model is consistent with the proposed transcarboxylation function of CO dehydrogenase in *C. thermoaceticum* (Drake et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1981). Synthesis of the methyl group of acetate from CO without prior oxidation to CO_2 is proposed here because this mechanism conserves the ATP which would be expended if a carrier-bound formyl group (e.g., formyl tetrahydrofolate) had to be synthesized from free formate. Expenditure of 1 ATP per mol of acetate formed in the synthesis of a carrier-

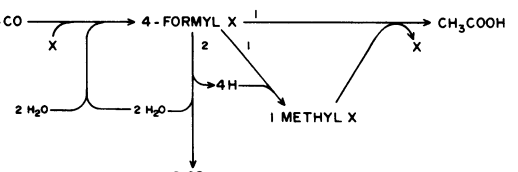

FIG. 4. Biochemical model for production of acetate and CO_2 from CO by *B. methylotrophicum*. × may represent either an enzyme active site or a carbon carrier(s).

bound formyl group does not seem likely in light of the high ATP gain observed during growth of *B. methylotrophicum* on CO. Formation of a carrier-bound formyl intermediate is associated with the favorable free energy change of converting CO plus H_2O to $HCOO^-$ plus H^+ ($\Delta G^{0\prime} = -3.9$ kcal/mol). Our demonstration of prolific growth and conversion of high concentrations of CO to acetic acid by *B. methylotrophicum* may stimulate applied investigations on anaerobic fermentation of CO and other pyrolysis products as a means to produce industrial chemicals. Current fermentation technology for acetic acid production does not compete economically with chemical processes (21). *C. thermoaceticum* glucose fermentations have been suggested as an improved route for microbial acetate production. In this regard we have used the techniques described here to obtain a strain of *C. thermoaceticum* that grows on carbon monoxide as the energy source (J. G. Zeikus and P. W. Hegge, unpublished data).

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LITERATURE CITED

1. **Bergemeyer, H. (ed.).** 1965. Methods of enzymatic analysis. Academic Press, Inc., New York.
2. **Colby, H. H., H. Dalton, and R. Whittenbury.** 1979. Biological and biochemical aspects of microbial growth on C_1 compounds. Annu. Rev. Microbiol. **33**:481–517.
3. **Cooper, T. G.** 1977. The tools of biochemistry. John Wiley & Sons, Inc., New York.
4. **Daniels, L., G. Fuchs, R. K. Thauer, and J. G. Zeikus.** 1977. Carbon monoxide oxidation by methanogenic bacteria. J. Bacteriol. **132**:118–126.
5. **Daschekvicz, M. P., and R. L. Uffen.** 1979. Identification of a CO-metabolizing bacterium as a strain of *Rhodopseudomonas gelatinosa*. Int. J. Syst. Bacteriol. **29**:145–148.
6. **Diekert, G. B., E. G. Graf, and R. K. Thauer.** 1979. Nickel requirement for CO dehydrogenase formation in *Clostridium pasteurianum*. Arch. Microbiol. **122**:117–120.
7. **Diekert, G. B., and R. K. Thauer.** 1978. Carbon monoxide oxidation by *Clostridium thermoaceticum* and *Clostridium formicoaceticum*. J. Bacteriol. **136**:597–606.
8. **Diekert, G. B., and R. K. Thauer.** 1980. The effect of nickel on CO dehydrogenase formation in *Clostridium thermoaceticum* and *Clostridium formicoaceticum*. FEMS Microbiol. Lett. **7**:187–189.
9. **Drake, H. L., S. Hu, and H. G. Wood.** 1980. Purification of carbon monoxide dehydrogenase, a nickel enzyme from *Clostridium thermoaceticum*. J. Biol. Chem. **255**:7174–7180.
10. **Ferenci, T.** 1974. Carbon monoxide-stimulated respiration in methane utilizing bacteria. FEBS Lett. **41**:94–98.
11. **Fuchs, G., G. Andress, and R. K. Thauer.** 1976. CO oxidation by anaerobic bacteria. Indications for the involvement of a vitamin B_{12} compound, p. 191–198. *In* H. Schlegel, G. Gottschalk, and N. Pfennig (ed.), Microbial production and utilization of gases. Goltze, Göttingen.
12. **Fuchs, G., U. Schnitker, and R. K. Thauer.** 1974. Carbon monoxide oxidation by growing cultures of *Clostridium pasteurianum*. Eur. J. Biochem. **49**:111–115.
13. **Goldberg, I., J. S. Roch, A. Ben-Bassat, and R. I. Mateles.** 1976. Bacterial yields on methanol, methylamine, formaldehyde, and formate. Biotechnol. Bioeng. **18**:1657–1668.
14. **Harris, R. F., and S. S. Adams.** 1979. Determination of the carbon and electron composition of microbial cells and metabolites by dichromate oxidation. Appl. Environ. Microbiol. **37**:237–243.
15. **Hegeman, G.** 1980. Oxidation of CO by bacteria. TIBS **5**:256–259.
16. **Kirkconnell, S., and G. Hegeman.** 1978. Mechanism of oxidation of carbon monoxide by bacteria. Biochem. Biophys. Res. Commun. **83**:1584–1587.
17. **Klyuver, A. J., and C. G. Schnellen.** 1947. On the fermentation of carbon monoxide by pure cultures of methane bacteria. Arch. Biochem. **14**:57–70.
18. **Ljungdahl, L. G., and H. G. Wood.** 1969. Total synthesis of acetate from CO_2 by heterotrophic bacteria. Annu. Rev. Microbiol. **23**:515–535.

18a. **Lynd, L. H., and J. G. Zeikus.** 1981. Methanol and carbon monoxide metabolism of *Butribacterium methylotrophicum*, the Marburg strain, p. 549. *In* A. Mollaender et al. (ed.), Trends in the biology of fermentations for fuels and chemicals. Plenum Press, New York.

19. **Meyer, O., and H. Schlegel.** 1980. CO:methylene blue oxidoreductase from *Pseudomonas carboxydovorans*. J. Bacteriol. **141**:74–80.
20. **Nelson, D. R., and J. G. Zeikus.** 1974. Rapid method for the radioisotopic analysis of gaseous products of anaerobic metabolism. Appl. Environ. Microbiol. **28**:258–261.
21. **Pape, M.** 1978. The competition between microbial and chemical processes for the manufacture of basic chemicals and intermediates, p. 515–530. *In* H. Schlegel and J. Barnea (ed.), Microbial energy converson. Goltze, Göttingen.
22. **Payne, W. J.** 1970. Energy yields and growth of heterotrophs. Annu. Rev. Microbiol. **24**:17–52.
23. **Payne, W. J., and M. L. Williams.** 1976. Carbon assimilation from simple and complex media by prototrophic bacteria. Biotechnol. Bioeng. **18**:1653–1655.
24. **Schulman, M., R. K. Ghambeer, L. G. Ljungdahl, and H. G. Wood.** 1973. Total synthesis of acetate from CO_2. VII. Evidence with *Clostridium thermoaceticum* that the carboxyl of acetate is derived from the carboxyl of pyruvate by transcarboxylation and not by fixation of CO_2. J. Biol. Chem. **248**:6255–6261.
25. **Stephen, H., and T. Stephen.** 1963. Solubilities of inorganic and organic compounds, vol. 1, part 1. Pergamon Press, Oxford.
26. **Stouthamer, A. H.** 1979. The search for correlation between theoretical and experimental growth yields, p. 1–48. *In* J. R. Quayle (ed.), International review of biochemistry, vol. 21. Microbial biochemistry. University Park Press, Baltimore.
27. **Thauer, R. K., G. Fuchs, B. Käfer, and B. Schnitker.** 1974. Carbon monoxide oxidation in cell-free extracts of *Clostridium pasteurianum*. Eur. J. Biochem. **45**:343–349.
28. **Thauer, R. K., K. Jungerman, and K. Decker.** 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol. Rev. **41**:100–180.
29. **Uffen, R. L.** 1976. Anaerobic growth of a *Rhodopseudomonas* species with CO as sole carbon and energy source. Proc. Natl. Acad. Sci. U.S.A. **73**:3298–3302.
30. **Wang, G., D. I. C. Wang, and R. J. Fleischaker.** 1978. A novel route to production of acetic acid by fermentation, p. 105. *In* American Institute of Chemistry Symposium Series 182, vol. 74. New York.
31. **Welty, F. K., and H. G. Wood.** 1977. Purification of the "corrinoid" enzyme involved in the synthesis of acetate by *Clostridium thermoaceticum*. J. Biol. Chem. **253**:5832–5838.
32. **Wolin, F. A., M. S. Wolin, and R. S. Wolfe.** 1963.

Formation of methane by bacterial extracts. J. Biol. Chem. 238:2882–2886.
33. **Zeikus, J. G.** 1980. Chemical and fuel production by anaerobic bacteria. Annu. Rev. Microbiol. 34:423–464.
34. **Zeikus, J. G., P. W. Hegge, and M. A. Anderson.** 1979. *Thermoanaerobium brockii*, gen. nov. and spec. nov., a new chemoorganotrophic, caldoactive, anaerobic bacterium. Arch. Microbiol. 122:41–48.
35. **Zeikus, J. G., W. Kenealy, G. Fuchs, and R. K. Thauer.** 1977. Oxidoreductases involved in cell carbon synthesis of *Methanobacterium thermoautotrophicum*. J. Bacteriol. 132:604–613.
36. **Zeikus, J. G., L. H. Lynd, T. E. Thompson, J. A. Kryzcki, P. W. Weimer, and P. W. Hegge.** 1980. Isolation and characterization of a new, methylotrophic, acidogenic anaerobe, the Marburg strain. Curr. Microbiol. 3:381–388.