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## Carbon Monoxide-Dependent Chemolithotrophic Growth of *Clostridium thermoautotrophicum*

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The acetogen *Clostridium thermoautotrophicum* was cultivated under CO-dependent chemolithotrophic conditions. CO-dependent growth profiles and energetics indicated that supplemental CO<sub>2</sub> was fundamental to efficient growth at the expense of CO. Overall product stoichiometry approximated  $6.5\text{CO} \rightarrow \text{CH}_3\text{CO}_2\text{H} + 3.5\text{CO}_2 + 0.6 \text{ cell C} + 0.5 \text{ unrecovered C}$ . Initial CO/CO<sub>2</sub> ratios of 2 to 4 yielded optimal doubling times and cell yields. Maximal  $Y_{\text{CO}}$  values approximated 2.5 g of cell dry weight per mol of CO consumed;  $Y_{\text{H}_2}$  was considerably lower than  $Y_{\text{CO}}$ . Cross-transfer growth experiments and protein profiles indicated differential expression of genes between CO and methanol cultures.

The capacity for producing acetate from two one-carbon compounds is a distinctive feature of acetogens (13, 43). During acetogenesis, CO<sub>2</sub> serves as the primary terminal electron acceptor and is reduced to the methyl level before incorporation as the methyl carbon of acetyl coenzyme A, which is sequentially converted to acetyl-phosphate and acetate. The origin of the reductant for the acetyl coenzyme A-dependent fixation of CO<sub>2</sub> differentiates heterotrophic and autotrophic acetogenesis.

Defined media have been developed for the metabolic assessment of *Clostridium thermoaceticum* and *Clostridium thermoautotrophicum* (25, 35). Although initial studies indicated that *C. thermoautotrophicum* was capable of chemolithotrophic growth at the expense of H<sub>2</sub>-CO<sub>2</sub>, growth at the sole expense of CO could not be demonstrated (35). Given the potential of this acetogen for CO-derived acetogenesis (2; J. Wiegel, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, I107, p. 112), this growth deficiency was further investigated in the present study.

### MATERIALS AND METHODS

**Cultivation.** *C. thermoautotrophicum* type strain JW701/3 (41) was cultivated at 58°C in crimp-sealed culture tubes (Bellco series 2048, 26.5-ml approximate stoppered volume at 1 atm [ca. 101.29 kPa]) as previously described (35). Culture tubes were incubated horizontally without shaking (shaking did not enhance growth). The defined medium (DM) contained the following (per liter): (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.5 g; NH<sub>4</sub>Cl, 0.5 g; 0.5 M KH<sub>2</sub>PO<sub>4</sub>, 100 ml; trace mineral solution (30), 5 ml; nicotinic acid, 2 mg; resazurin, 1.0 mg; and Na<sub>2</sub>S · 9H<sub>2</sub>O, 0.25 g. Where indicated, cysteine hydrochloride monohydrate was added as additional reducer to a final concentration of 0.25 g/liter in conjunction with 0.15 g of NaOH per liter. For each CO-CO<sub>2</sub> cultivation gas phase, the pH of the phosphate buffer solution was adjusted with 5 N KOH so that the initial pH of the medium was 6.1. Media were prepared anaerobically, and gases were passed over a copper catalyst at 450°C to remove trace levels of oxygen. Before inoculation, the gas phase composition was adjusted with filter-sterilized gases as indicated. All cultures had an initial pressure of 308 kPa (30 lb/in<sup>2</sup> over atmospheric

pressure). When necessary, N<sub>2</sub> was added as the balance gas. Growth in DM was initiated with early-log-phase inoculum from DM cultures with a headspace containing CO-CO<sub>2</sub>-N<sub>2</sub> (29:32:39) at an initial pressure of 253 kPa. The composition of undefined medium (UM) was as previously described (35), and cultures had an initial headspace containing CO-CO<sub>2</sub> (67:33). Growth in UM was initiated with early-log-phase UM cultures. In all experiments, growth was initiated by injecting 1 ml of inoculum into 10 ml of fresh medium.

For comparative growth responses with glucose, methanol, and CO-CO<sub>2</sub>, DM contained NaHCO<sub>3</sub> (1.0 g/liter). The concentrations of glucose and methanol were 10 g/liter and 10 ml/liter, respectively, and the headspace contained 100% CO<sub>2</sub> for both glucose and methanol cultures.

**Preparation of extracts and enzyme assays.** Cell extracts of *C. thermoautotrophicum* were prepared anaerobically in a Coy chamber by lysozyme digestion as previously described for *C. thermoaceticum* (25). Hydrogenase (8), carbon monoxide dehydrogenase (7, 9), and formate dehydrogenase (23) were assayed by standard procedures with methyl viologen as the electron acceptor. Enzyme activities were determined by using cell extracts prepared from mid-log-phase cultures.

**Analytical procedures.** Growth was monitored at 660 nm with a Bausch & Lomb Spectronic 88 spectrophotometer; the optical path width was 1.6 cm (inner diameter of culture tubes). Uninoculated tubes served as references, and the doubling times reported represent the minimum doubling time observed with a particular medium. Cell dry weights were determined as previously described (35); an optical density of 1.0 at 660 nm was equivalent to 453 mg of cell dry weight per liter of culture medium. For fermentation balances, the carbon content of cells was assumed to approximate 50% of the cell dry weight (16). Gases were quantitated by gas chromatography as described elsewhere (17, 22, 28, 42), and acetate was determined enzymatically by the method of Rose (34). Protein was estimated by the Bradford procedure (1) with bovine serum albumin as a standard, and slab sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis was with the Laemmli buffer system (21). The 10% polyacrylamide gels were stained with Coomassie R-250. In this paper, no distinction is made between CO<sub>2</sub> and its soluble forms.

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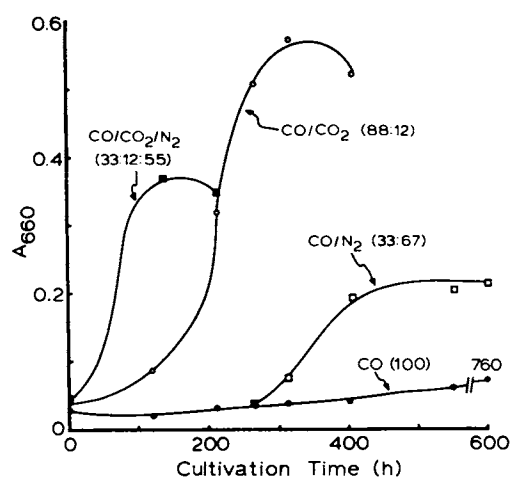


FIG. 1. CO-dependent growth profiles of *C. thermoautotrophicum*. Initial gas phases were as indicated, and the growth medium was DM with cysteine as a reducer.

## RESULTS

**CO-dependent growth of *C. thermoautotrophicum*.** Previous work demonstrated *C. thermoautotrophicum* could be cultivated in DM (which contained cysteine in the reducer) at the expense of either glucose, methanol, or  $H_2$ - $CO_2$  (67:33), but not with 100%  $CO$  (35). In subsequent experiments it was noted that when  $H_2$ - $CO_2$ -DM cultures were supplemented with  $CO$ , the resulting growth was in excess of  $H_2$ - $CO_2$  controls (data not shown). The  $H_2$ - $CO$ - $CO_2$  culture was then transferred to an environment with no  $H_2$ ;  $H_2$ -independent growth was observed, and a  $CO$ - $CO_2$  culture was established. Growth was negligible in the absence of  $CO_2$  with 100%  $CO$ , but growth with an extended lag phase was observed with a  $CO$ - $N_2$  (33:67) environment; growth in this latter environment was stimulated by  $CO_2$  (Fig. 1). Cysteine, a possible substrate for energy and biomass production, was not growth limiting since growth was not affected by its elimination, even after 25 sequential transfers in  $CO$ - $CO_2$  DM without cysteine. This unequivocally demonstrated that *C. thermoautotrophicum* was competent in  $CO$ -dependent chemolithotrophic growth.

**Product profiles and energetics.** To further assess the effects of  $CO_2$  on  $CO$ -dependent growth, the initial  $CO/CO_2$  ratio was varied and the products formed were determined upon cessation of growth. The stoichiometry of  $CO$  consumption to products formed was not constant, but approximated  $6.5CO \rightarrow CH_3CO_2H + 3.5CO_2 + 0.6 \text{ cell C} + 0.5 \text{ unrecovered C}$ . Approximately 9% of the  $CO$  consumed was accounted for in biomass carbon. In contrast, carboxydotrophic species of *Pseudomonas* and thermophilic bacilli assimilate approximately 14 and 7.5%, respectively, of  $CO$  consumed into biomass when cultivated chemolithotrophically with  $CO$  (20, 30). At moderate  $CO$  levels,  $CO$  consumption was complete regardless of the initial concentration of  $CO_2$  (Table 1, experiment A). However,  $CO$  consumption was not complete when the initial concentration of  $CO$  was high relative to the initial concentration of  $CO_2$  (Table 1, experiments B and C). High initial  $CO/CO_2$  ratios yielded high doubling times, the fastest growth being observed with initial  $CO/CO_2$  ratios of 1 to 5.

In homoacetogenesis, the ratio of acetate formed to biomass synthesized is a reflection of cell energetics, since energy production is obligately coupled to acetate formation.

TABLE 1. CO-dependent product profiles of *C. thermoautotrophicum*<sup>a</sup>

Expt	Initial gas phase			CO consumed (%)	Products formed			$t_d$ (h) <sup>c</sup>
	CO	CO <sub>2</sub>	CO/CO <sub>2</sub> ratio		Acetate	CO <sub>2</sub>	Biomass C <sup>b</sup>	
A <sup>d</sup>	676	23	29.2	664 (98)	113	291	42	33.1
	676	49	14.0	671 (99)	116	302	44	16.5
	676	131	5.2	674 (100)	108	297	48	7.6
	676	340	2.0	670 (99)	101	387	59	9.0
	676	617	1.1	669 (99)	119	359	49	10.1
B <sup>d</sup>	347	294	1.2	346 (100)	48	222	32	8.9
	710	294	2.4	708 (100)	102	405	70	9.8
	1,031	294	3.5	965 (94)	141	524	98	11.2
	1,728	294	5.9	865 (50)	132	394	67	12.0
C <sup>d</sup>	685	617	1.1	684 (100)	116	401	62	12.8
	1,090	617	1.8	1,067 (98)	163	625	106	10.1
	1,477	617	2.5	1,394 (94)	203	743	148	8.9
D <sup>e</sup>	1,475	1,024	1.4	1,337 (91)	222	705	187	7.0

<sup>a</sup> Units are micromoles per culture tube; values represent averages of triplicate or duplicate cultures. Carbon recovery ranged from 84 to 102% and averaged 94%.

<sup>b</sup> Assuming 50% carbon per unit of biomass: 1 mg of dry weight equals 41.6  $\mu$ mol of biomass carbon.

<sup>c</sup> Doubling time.

<sup>d</sup> Cultivation medium was DM.

<sup>e</sup> Cultivation medium was UM.

In  $CO$ -derived homoacetogenesis,  $CO$ -dependent growth yields ( $Y_{CO}$ ) and acetate/biomass ratios should be inversely related; this was confirmed experimentally (Fig. 2). Thus, low acetate/biomass ratios and high  $Y_{CO}$  values are indicative of cells of high energetic competence.

Figure 3 shows the relationship between  $Y_{CO}$  and the initial  $CO/CO_2$  ratio. Based on maximum  $Y_{CO}$  values observed, initial  $CO/CO_2$  ratios of 2 to 4 yielded the most efficient  $CO$ -dependent chemolithotrophic growth (i.e., based on  $Y_{CO}$ , cells experienced maximum energy gain per unit of  $CO$  consumed when the  $CO/CO_2$  ratio was between 2 and 4). When the  $CO/CO_2$  ratio was below 2 or above 4, cell energetics apparently decayed. The fastest doubling times did not necessarily correspond to the highest  $Y_{CO}$  values (or

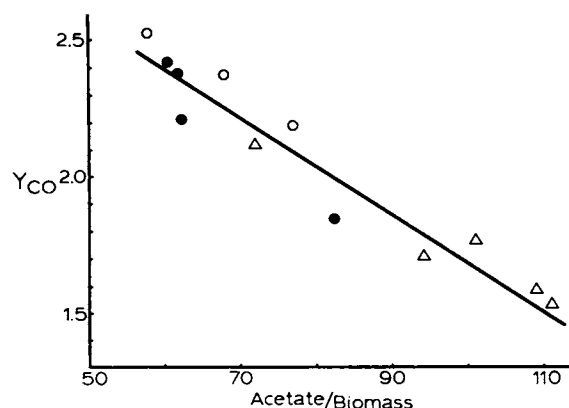


FIG. 2. Relationship between  $CO$ -dependent growth yield ( $Y_{CO}$ ) and the acetate/biomass ratio of *C. thermoautotrophicum*. Units for  $Y_{CO}$  are grams of cell dry weight per mole of  $CO$  consumed. Units for acetate/biomass ratio are micromoles of acetate formed per milligram of cell dry weight. Symbols:  $\Delta$ , experiment A;  $\bullet$ , experiment B;  $\circ$ , experiment C.

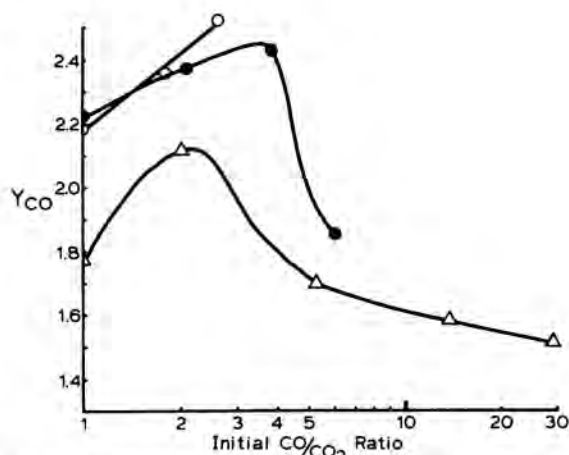


FIG. 3. Effects of initial CO/CO<sub>2</sub> ratio on CO-dependent growth yields ( $Y_{CO}$ ) of *C. thermoautotrophicum*. Units for  $Y_{CO}$  and symbols are as defined in the legend to Fig. 2.

lowest acetate/biomass ratios). In experiment A, for example, a CO/CO<sub>2</sub> ratio of approximately 5 yielded the fastest doubling time, but a CO/CO<sub>2</sub> ratio of approximately 2 yielded the most efficient conservation of CO-derived energy as determined by  $Y_{CO}$ .

Trace levels of H<sub>2</sub> and CH<sub>4</sub> were consistently produced by CO-CO<sub>2</sub> cultures of *C. thermoautotrophicum*. Cultures produced the greatest amounts of H<sub>2</sub> and CH<sub>4</sub> under energetically favorable conditions as indicated by high  $Y_{CO}$ , and amounts up to 34  $\mu$ mol of CH<sub>4</sub> per liter of culture and 427  $\mu$ mol of H<sub>2</sub> per liter of culture were evident as a result of CO-dependent chemolithotrophic growth. Significantly, cultures with partially uncoupled or impaired energetics due to inhibitory amounts of either CO or CO<sub>2</sub> did not evolve higher amounts of either H<sub>2</sub> or CH<sub>4</sub>. In contrast, *Methanosarcina barkeri* produces higher amounts of CO-derived H<sub>2</sub> when CO-dependent methanogenesis is inhibited by high CO levels (32).

**Acetogenic enzyme levels.** Hydrogenase, CO dehydrogenase, and formate dehydrogenase play vital roles in acetogenesis, and the specific activities of these enzymes were measured (Table 2). No significant differences were noted between CO and glucose cell lines. This is in contrast to the CO-dependent stimulation of hydrogenase and formate dehydrogenase levels as reported for *C. thermoacetium* (17, 18). However, the level of formate dehydrogenase in the defined medium cultures was 10-fold higher than that reported for *C. thermoautotrophicum* cultivated in an undefined medium (2).

**Cross-transfer analysis and protein profiles.** CO-DM cultures grew readily without appreciable lag when transferred into glucose DM; reciprocal transfers were also possible

TABLE 2. Enzyme levels of cell extracts of *C. thermoautotrophicum*

Medium	Sp act <sup>a</sup>		
	Hydrogenase	CO dehydrogenase	Formate dehydrogenase
DM-CO-CO <sub>2</sub> (67:33)	0.15	2.09	0.38
DM-glucose-CO <sub>2</sub>	0.13	2.57	0.73

<sup>a</sup> Micromoles of substrate converted per minute per milligram of protein.

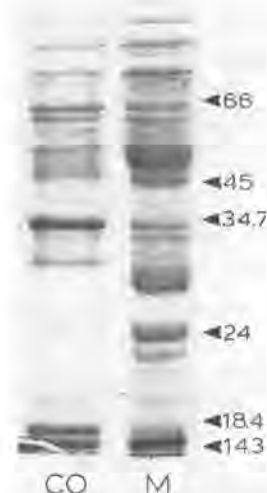


FIG. 4. Comparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis protein profiles of CO- and methanol (M)-grown cell lines. Each lane contained approximately 18  $\mu$ g of protein. Numbers indicate locations of molecular weight standards ( $M_r \cdot 10^{-3}$ ).

(data not shown). However, when CO-DM cultures were transferred into methanol-DM, growth was apparent only after an extensive lag period. In contrast, methanol-DM cultures grew readily in CO- or glucose-DM. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of cell extracts revealed dissimilar protein profiles between methanol and CO-CO<sub>2</sub> cell lines (Fig. 4), suggesting differential gene expression in methanol cultures. Glucose-DM cultures yielded protein profiles nearly identical to those of CO-DM cultures (data not shown).

## DISCUSSION

This study demonstrates that *C. thermoautotrophicum* is capable of CO-dependent chemolithotrophic acetogenesis and growth. Supplemental CO<sub>2</sub> was found to play a critical stimulatory role in this growth potential. Our findings indicate that, relative to the initial concentration of CO, there exists an optimal level of CO<sub>2</sub> which is not constant, but increases with increasing CO. Growth of *Eubacterium limosum* is inhibited by CO concentrations greater than 50% when the headspace contains 20% CO<sub>2</sub> (14); it seems likely that, as with *C. thermoautotrophicum*, this inhibition may be relieved by CO<sub>2</sub>. Supplemental CO<sub>2</sub> is also essential to prolonged cultivation of *C. thermoacetium* in glucose (100 mM)-minimal medium (25) (data not shown). In contrast, CO-dependent growth of "*Butyrivibrio methylotrophicum*" does not require supplemental CO<sub>2</sub> (31), although, as with *E. limosum* (14, 33), CO<sub>2</sub> may be stimulatory. Although *Peptostreptococcus productus* has been cultivated at the expense of CO in the presence of 20% CO<sub>2</sub>, CO<sub>2</sub> dependence was not determined (24).

The equilibrium for NADPH-dependent formate dehydrogenase from *C. thermoacetium* is far toward CO<sub>2</sub> (or HCO<sub>3</sub><sup>-</sup>) (44). A high CO<sub>2</sub> requirement for growth is consistent with this fact. Differential requirements among acetogens for CO<sub>2</sub> may, in part, be due to the type of electron donor (NADPH versus ferredoxin) used by formate dehydrogenase since this would influence the equilibrium of CO<sub>2</sub> reduction to formate.

TABLE 3. Comparative growth yields for CO- and H<sub>2</sub>-cultivated acetogens<sup>a</sup>

Acetogen	Medium	Y <sub>CO</sub>	Y <sub>H<sub>2</sub></sub>
<i>Acetobacterium woodii</i> (38)	Undefined	NR <sup>b</sup>	1.05 <sup>c</sup>
	Defined	NR	0.68
"Butyribacterium methylotrophicum" (26, 27)	Undefined	3.0	1.7
	Defined	2.53 (maximum), 1.53 (minimum)	0.82
<i>C. thermoautotrophicum</i> (35; this study)	Undefined	3.34	ND <sup>d</sup>
	Defined	2.53 (maximum), 1.53 (minimum)	0.82
<i>Eubacterium limosum</i> <sup>e</sup>	Undefined	3.38	0.84
<i>Peptostreptococcus productus</i> (24)	Undefined	1.28 <sup>f</sup>	0.39 <sup>g</sup>

<sup>a</sup> Unless otherwise indicated, units are grams of cell dry weight per mole of CO or H<sub>2</sub> consumed.

<sup>b</sup> NR, Not reported, although CO-dependent growth has recently been observed (15; M. P. Bryant and B. R. S. Genthner, personal communication).

<sup>c</sup> Original data reported as grams of cell dry weight per mole of acetate. Values shown were derived by dividing by 4 (see reaction 2); the actual Y<sub>H<sub>2</sub></sub> would likely be lower due to reductant consumed in biosynthesis.

<sup>d</sup> ND, Not determined.

<sup>e</sup> M. P. Bryant and B. R. S. Genthner, personal communication (15).

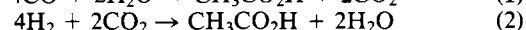
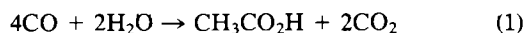
<sup>f</sup> Grams of cell protein per mole of CO consumed.

<sup>g</sup> Grams of cell protein per mole of H<sub>2</sub> consumed.

The role of CO<sub>2</sub> may be attributed to a CO<sub>2</sub> requirement in either biosynthesis or to cell energetics. CO<sub>2</sub> serves as the initial acceptor of reductant on the methyl path of acetogenesis at the level of formate dehydrogenase. In the absence of CO<sub>2</sub>, reductant flow would be impaired and normal acetogenesis would cease. However, CO-derived acetogenesis gives rise to excess CO<sub>2</sub> (see below). Thus, CO-derived CO<sub>2</sub> could theoretically provide for catabolic and anabolic CO<sub>2</sub> requirements. At low concentrations of CO, this appears to be true; but as the CO concentration increases, CO may become toxic in the absence of supplemental CO<sub>2</sub>. The rapid conversion (detoxification) of CO would therefore be dependent on non-CO-derived CO<sub>2</sub> to accept reductant from the initial oxidation of CO by CO dehydrogenase (CO + H<sub>2</sub>O → CO<sub>2</sub> + 2H<sup>+</sup> + 2e<sup>-</sup>).

The degree to which the CO concentration becomes toxic may vary among acetogenic species and the formulation of the cultivation medium. Both CO and CO<sub>2</sub> were potential inhibitors of growth and cell energetics of *C. thermoautotrophicum* (Table 1, Fig. 3). In the absence of sufficient initial CO<sub>2</sub> relative to CO, CO appears inhibitive; likewise, in the absence of sufficient CO, CO<sub>2</sub> appears inhibitive. Although inhibition of growth by CO is well documented for acetogens and methanogens (4, 14, 31, 32), this is the first report of CO<sub>2</sub>-induced inhibition of CO-dependent acetogenic growth. Although the nature of CO<sub>2</sub> inhibition is not clear, high levels of CO<sub>2</sub> relative to CO may impair the reversible oxidation of CO, thereby causing at least a partial uncoupling of CO energy conservation. The observed inhibitory effect by CO<sub>2</sub> was not due to pH or salt (due to pH adjustment) effects, since the initial pH was constant at 6.1 and salt controls were not inhibited (data not shown). The nature of the presumed CO inhibition is also not clear. CO may inhibit hydrogenase, which may in some manner be a component in the flow of acetogenic reductant.

The overall stoichiometries for CO- and H<sub>2</sub>-derived acetogenesis are



Either form of acetogenesis utilizes 8 reducing equivalents in the formation of acetate; however (Table 3), Y<sub>CO</sub> is consistently higher than Y<sub>H<sub>2</sub></sub>. This difference in growth yields may be due to (i) increased ATP synthesis per CO-derived electron pair (this assumes that Y<sub>ATP</sub> is basically the same for CO and H<sub>2</sub> cells) and (ii) reversed, energy-dependent electron flow from H<sub>2</sub> to CO on the carboxyl path of H<sub>2</sub>-CO<sub>2</sub>-dependent acetogenesis (as first postulated for methanogens [3, 11] and subsequently acetogens [5, 6]). In this case, the Y<sub>ATPS</sub> of CO and H<sub>2</sub> cell lines may differ. In support of hypothesis i, the standard changes in Gibbs free energy for the reactions shown in equations 1 and 2 (-10.5 kcal [ca. -43.9 kJ]/mol of CO and -5.6 kcal [ca. -23.4 kJ]/mol of H<sub>2</sub>, respectively [26, 37]) indicate that more energy can be obtained from CO-derived acetogenesis. Given the apparent necessity to form CO on the carboxyl path of acetogenesis, a combination of hypotheses i and ii may account for the observed differentials between CO and H<sub>2</sub> growth yields. CO-dependent growth yields of 3.7 g of cell dry weight per mol of CO have been obtained for the phototroph *Rhodospseudomonas gelatinosa* (39) cultivated in an undefined medium (Robert Uffen, personal communication). A contrastingly high Y<sub>CO</sub> of approximately 9.5 can be calculated from data reported for *M. barkeri* cultivated in defined medium (32). The Y<sub>H<sub>2</sub></sub> for methanogens is approximately half this value (40). The Y<sub>CO</sub> has not been reported for the aerobic CO-oxidizing carboxydrotrophs (Ortwin Meyer, personal communication).

Appreciable CH<sub>4</sub> formation during CO-dependent growth was never observed; thus, its formation is likely based on a minor side reaction or the expression of a vestigial pathway. The reversible formation of trace levels of CH<sub>4</sub> by some sulfate-reducing bacteria has been postulated to be a potential of an acetogenic mechanism (36). Whether acetogens are capable of oxidizing CH<sub>4</sub> remains to be seen.

Based on protein profiles, a differential expression of genes was observed with methanol-grown cells as compared with CO-grown cells. This observation, along with the fact that CO-grown cells would not grow in methanol without adaptation, indicates a potential gene-level regulatory role for methanol in methanol-dependent acetogenesis. Methanol dehydrogenase would seem a likely candidate for such regulation (10; D. W. Ivey, L. G. Ljungdahl, and J. Wiegell, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, K66, p. 182).

*C. thermoacetikum* (12) appears to be genetically very similar to *C. thermoautotrophicum* (41). Whereas *C. thermoacetikum* has been shown to obtain energy from the dissimilation of CO to acetate under heterotrophic conditions (18, 19, 29), CO-dependent chemolithotrophic growth has not been reported for this acetogen.

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