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Appl. Environ. Microbiol. 1987, 53(8):1902.

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Carbon Monoxide-Dependent Chemolithotrophic Growth of *Clostridium thermoautotrophicum*

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Received 20 January 1987/Accepted 27 May 1987

The acetogen *Clostridium thermoautotrophicum* was cultivated under CO-dependent chemolithotrophic conditions. CO-dependent growth profiles and energetics indicated that supplemental CO₂ 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/CO₂ ratios of 2 to 4 yielded optimal doubling times and cell yields. Maximal Y_{CO} values approximated 2.5 g of cell dry weight per mol of CO consumed; Y_{H_2} was considerably lower than Y_{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, CO₂ 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 CO₂ 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 H₂-CO₂, 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): (NH₄)₂SO₄, 0.5 g; NH₄Cl, 0.5 g; 0.5 M KH₂PO₄, 100 ml; trace mineral solution (30), 5 ml; nicotinic acid, 2 mg; resazurin, 1.0 mg; and Na₂S · 9H₂O, 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-CO₂ 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/in² over atmospheric

pressure). When necessary, N₂ was added as the balance gas. Growth in DM was initiated with early-log-phase inoculum from DM cultures with a headspace containing CO-CO₂-N₂ (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-CO₂ (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-CO₂, DM contained NaHCO₃ (1.0 g/liter). The concentrations of glucose and methanol were 10 g/liter and 10 ml/liter, respectively, and the headspace contained 100% CO₂ 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 CO₂ and its soluble forms.

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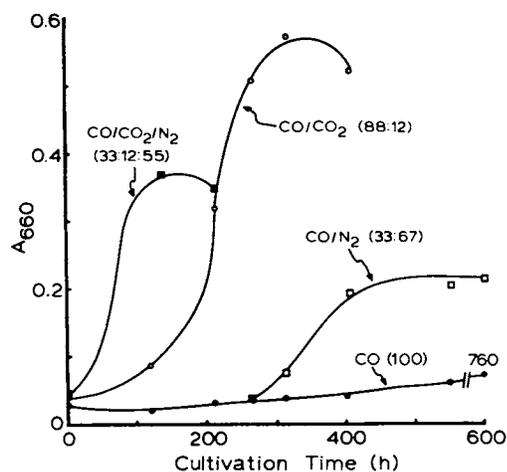


FIG. 1. CO-dependent growth profiles of *C. thermoautotrophicum*. Initial gas phases were as indicated, and the growth medium was DM with cysteine as a reducer.

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*^a

Expt	Initial gas phase			CO consumed (%)	Products formed			t_d (h) ^c
	CO	CO_2	CO/CO_2 ratio		Acetate	CO_2	Biomass C ^b	
A ^d	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 ^d	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 ^d	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 ^e	1,475	1,024	1.4	1,337 (91)	222	705	187	7.0

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

^b Assuming 50% carbon per unit of biomass: 1 mg of dry weight equals 41.6 μ mol of biomass carbon.

^c Doubling time.

^d Cultivation medium was DM.

^e 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

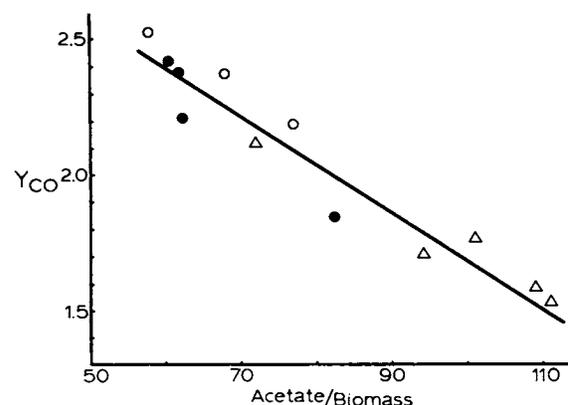


FIG. 2. Relationship between CO -dependent growth yield (Y_{CO}) and the acetate/biomass ratio of *C. thermoautotrophicum*. Units for Y_{CO} are grams of cell dry weight per mole of CO consumed. Units for acetate/biomass ratio are micromoles of acetate formed per milligram of cell dry weight. Symbols: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

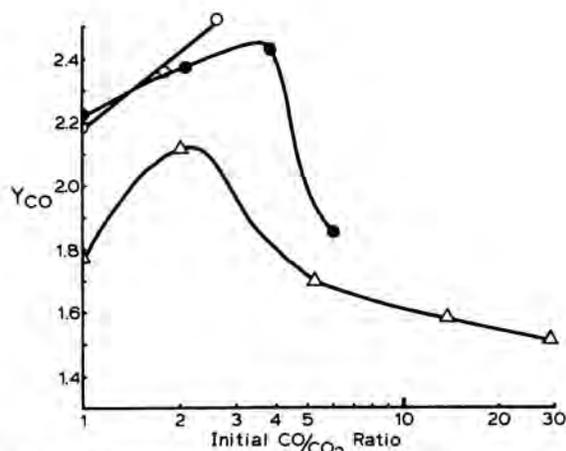


FIG. 3. Effects of initial CO/CO₂ ratio on CO-dependent growth yields (Y_{CO}) of *C. thermoautotrophicum*. Units for Y_{CO} and symbols are as defined in the legend to Fig. 2.

lowest acetate/biomass ratios). In experiment A, for example, a CO/CO₂ ratio of approximately 5 yielded the fastest doubling time, but a CO/CO₂ ratio of approximately 2 yielded the most efficient conservation of CO-derived energy as determined by Y_{CO} .

Trace levels of H₂ and CH₄ were consistently produced by CO-CO₂ cultures of *C. thermoautotrophicum*. Cultures produced the greatest amounts of H₂ and CH₄ under energetically favorable conditions as indicated by high Y_{CO} , and amounts up to 34 μ mol of CH₄ per liter of culture and 427 μ mol of H₂ 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 CO₂ did not evolve higher amounts of either H₂ or CH₄. In contrast, *Methanosarcina barkeri* produces higher amounts of CO-derived H₂ 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 ^a		
	Hydrogenase	CO dehydrogenase	Formate dehydrogenase
DM-CO-CO ₂ (67:33)	0.15	2.09	0.38
DM-glucose-CO ₂	0.13	2.57	0.73

^a Micromoles of substrate converted per minute per milligram of protein.

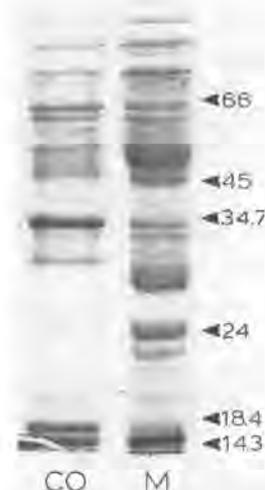


FIG. 4. Comparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis protein profiles of CO- and methanol (M)-grown cell lines. Each lane contained approximately 18 μ g of protein. Numbers indicate locations of molecular weight standards ($M_r \cdot 10^{-3}$).

(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-CO₂ 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 CO₂ 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 CO₂ 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% CO₂ (14); it seems likely that, as with *C. thermoautotrophicum*, this inhibition may be relieved by CO₂. Supplemental CO₂ 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 CO₂ (31), although, as with *E. limosum* (14, 33), CO₂ may be stimulatory. Although *Peptostreptococcus productus* has been cultivated at the expense of CO in the presence of 20% CO₂, CO₂ dependence was not determined (24).

The equilibrium for NADPH-dependent formate dehydrogenase from *C. thermoacetium* is far toward CO₂ (or HCO₃⁻) (44). A high CO₂ requirement for growth is consistent with this fact. Differential requirements among acetogens for CO₂ 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 CO₂ reduction to formate.

TABLE 3. Comparative growth yields for CO- and H₂-cultivated acetogens^a

Acetogen	Medium	Y _{CO}	Y _{H₂}
<i>Acetobacterium woodii</i> (38)	Undefined	NR ^b	1.05 ^c
	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 ^d
	Defined	2.53 (maximum), 1.53 (minimum)	0.82
<i>Eubacterium limosum</i> ^e	Undefined	3.38	0.84
<i>Peptostreptococcus productus</i> (24)	Undefined	1.28 ^f	0.39 ^g

^a Unless otherwise indicated, units are grams of cell dry weight per mole of CO or H₂ consumed.

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

^c 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_{H₂} would likely be lower due to reductant consumed in biosynthesis.

^d ND, Not determined.

^e M. P. Bryant and B. R. S. Genthner, personal communication (15).

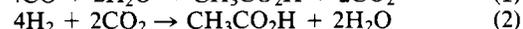
^f Grams of cell protein per mole of CO consumed.

^g Grams of cell protein per mole of H₂ consumed.

The role of CO₂ may be attributed to a CO₂ requirement in either biosynthesis or to cell energetics. CO₂ serves as the initial acceptor of reductant on the methyl path of acetogenesis at the level of formate dehydrogenase. In the absence of CO₂, reductant flow would be impaired and normal acetogenesis would cease. However, CO-derived acetogenesis gives rise to excess CO₂ (see below). Thus, CO-derived CO₂ could theoretically provide for catabolic and anabolic CO₂ 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 CO₂. The rapid conversion (detoxification) of CO would therefore be dependent on non-CO-derived CO₂ to accept reductant from the initial oxidation of CO by CO dehydrogenase (CO + H₂O → CO₂ + 2H⁺ + 2e⁻).

The degree to which the CO concentration becomes toxic may vary among acetogenic species and the formulation of the cultivation medium. Both CO and CO₂ were potential inhibitors of growth and cell energetics of *C. thermoautotrophicum* (Table 1, Fig. 3). In the absence of sufficient initial CO₂ relative to CO, CO appears inhibitive; likewise, in the absence of sufficient CO, CO₂ 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 CO₂-induced inhibition of CO-dependent acetogenic growth. Although the nature of CO₂ inhibition is not clear, high levels of CO₂ 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 CO₂ 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 H₂-derived acetogenesis are



Either form of acetogenesis utilizes 8 reducing equivalents in the formation of acetate; however (Table 3), Y_{CO} is consistently higher than Y_{H₂}. This difference in growth yields may be due to (i) increased ATP synthesis per CO-derived electron pair (this assumes that Y_{ATP} is basically the same for CO and H₂ cells) and (ii) reversed, energy-dependent electron flow from H₂ to CO on the carboxyl path of H₂-CO₂-dependent acetogenesis (as first postulated for methanogens [3, 11] and subsequently acetogens [5, 6]). In this case, the Y_{ATP}s of CO and H₂ 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 H₂, 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 H₂ 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_{CO} of approximately 9.5 can be calculated from data reported for *M. barkeri* cultivated in defined medium (32). The Y_{H₂} for methanogens is approximately half this value (40). The Y_{CO} has not been reported for the aerobic CO-oxidizing carboxydrotrophs (Ortwin Meyer, personal communication).

Appreciable CH₄ 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 CH₄ by some sulfate-reducing bacteria has been postulated to be a potential of an acetogenic mechanism (36). Whether acetogens are capable of oxidizing CH₄ 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. Wiegel, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, K66, p. 182).

C. thermoaceticum (12) appears to be genetically very similar to *C. thermoautotrophicum* (41). Whereas *C. thermoaceticum* 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.

ACKNOWLEDGMENTS

We express appreciation to J. E. Lepo for review of the manuscript.

This investigation was supported by Public Health Service grant A121852 and research career development award A100722 (to H.L.D.) from the National Institute of Allergy and Infectious Diseases.

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