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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 was fundamental to efficient growth at the expense of CO. Overall product stoichiometry approximated $6.5CO \rightarrow CH_3COOH + 3.5CO_2 + 0.6cell\;C + 0.5unrecovered\;C$. Initial CO/CO$_2$ 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, CO$_2$ 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$_2$ 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$_2$/CO$_2$, 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, 1107, p. 112), this growth deficiency was further investigated in the present study.

**MATERIALS AND METHODS**

**Cultivation.** *C. thermoautotrophicum* type strain JW7013 (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$_4$)$_2$SO$_4$, 0.5 g; NH$_4$Cl, 0.5 g; 0.5 M KH$_2$PO$_4$, 100 ml; trace mineral solution (30), 5 mL; nicotinic acid, 2 mg; resazurin, 1.0 mg; and Na$_2$S·9H$_2$O, 0.25 g. Where indicated, cysteine hydrochloride monohydrate was added as additional reductant to a final concentration of 0.25 g/L in conjunction with 0.15 g of NaOH per liter. For each CO/CO$_2$ 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$^2$ over atmospheric pressure). When necessary, N$_2$ 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$_2$-N$_2$ (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$_2$ (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$_2$, DM contained NaHCO$_3$ (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$_2$ 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$_2$ and its soluble forms.

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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$_2$ culture was then transferred to an environment with no H$_2$; H$_2$-independent growth was observed, and a CO$_2$-free medium was DM with cysteine as a reducer.

Growth was observed, and a 100% CO$_2$-dependent culture was stimulated by DM without cysteine. This unequivocally demonstrated that C. thermoautotrophicum was CO-dependent in DM (which contained cysteine in the reducer). CO$_2$-independent growth yielded high doubling times, the fastest growth being in Expt A with initial CO$_2$/CO ratio of 2 to 4 yielding the most efficient CO-dependent chemolithothrophic 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 CO$_2$ values (or

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.

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 → CH$_3$CO$_2$H + 3.5CO$_2$ + 0.6 cell C + 0.5 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, experiment A). 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.

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.

![Graph showing CO-dependent growth profiles of C. thermoautotrophicum.](http://aem.asm.org/)

**TABLE 1. CO-dependent product profiles of C. thermoautotrophicum**

<table>
<thead>
<tr>
<th>Expt</th>
<th>Initial gas phase</th>
<th>CO consumed (%)</th>
<th>Products formed</th>
<th>Yco</th>
<th>Doubling time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>CO: CO$_2$: CO/CO$_2$ ratio</td>
<td>Acetate: CO$_2$: Biomass C$^*$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A'</td>
<td>676:23:29.2</td>
<td>664:(98)</td>
<td>113:291:42</td>
<td>33.1</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>676:131:5.2</td>
<td>674:(100)</td>
<td>108:297:48</td>
<td>7.6</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>676:617:1.1</td>
<td>669:(99)</td>
<td>119:359:49</td>
<td>10.1</td>
<td></td>
</tr>
</tbody>
</table>

"Units are micromoles per culture tube; values represent averages of triplicate or duplicate cultures. Carbon recovery ranged from 84 to 102% and averaged 94%.""
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 µmol of CH₄ per liter of culture and 427 µmol 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. thermoacetica (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 (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. thermoacetica in glucose (100 mM)-minimal medium (25) (data not shown). In contrast, CO-dependent growth of Butyribacterium 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. thermoacetica 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

<table>
<thead>
<tr>
<th>Acetogen</th>
<th>Medium</th>
<th>YCO</th>
<th>YH₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetobacterium woodii</td>
<td>Undefined</td>
<td>NR</td>
<td>1.05⁰</td>
</tr>
<tr>
<td></td>
<td>Defined</td>
<td>NR</td>
<td>0.68</td>
</tr>
<tr>
<td>&quot;Butyriribacterium</td>
<td>Undefined</td>
<td>3.0</td>
<td>1.7</td>
</tr>
<tr>
<td>methylotrophicum&quot;</td>
<td>Defined</td>
<td>3.4</td>
<td>ND⁷</td>
</tr>
<tr>
<td>C. thermoautotrophicum</td>
<td>Undefined</td>
<td>3.34</td>
<td>1.53</td>
</tr>
<tr>
<td></td>
<td>Defined</td>
<td>2.53</td>
<td>(max)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(min)</td>
</tr>
<tr>
<td>Eubacterium limosum⁴</td>
<td>Undefined</td>
<td>3.38</td>
<td>0.84</td>
</tr>
<tr>
<td>Peptostreptococcus</td>
<td>Undefined</td>
<td>1.28⁸</td>
<td>0.39⁹</td>
</tr>
<tr>
<td>productus (24)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

⁵ Unless otherwise indicated, units are grams of cell dry weight per mole of CO or H₂ consumed.
⁶ NR, Not reported, although CO-dependent growth has recently been observed (15; M. P. Bryant and B. R. S. Genthner, personal communication).
⁷ 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 YH₂ would likely be lower due to reductant consumed in biosynthesis.
⁸ ND, Not determined.
⁹ M. P. Bryant and B. R. S. Genthner, personal communication (15).

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. In the absence of CO₂, reductant flow would be impaired and normal acetogenesis would cease. However, CO₂-derived acetogenesis gives rise to excess CO₂ (see below). Thus, CO₂-derived CO₂ could theoretically provide for catalytic and anabolic CO₂ requirements. At low concentrations of CO₂, this appears to be true; but as the CO₂ concentration increases, CO₂ may become toxic in the absence of supplemental CO₂. The rapid conversion (detoxification) of CO₂ would therefore be dependent on non-CO₂-derived CO₂ to accept reductant from the initial oxidation of CO by CO₂ dehydrogenase (CO + H₂O → CO₂ + 2H⁻ + 2e⁻).

The degree to which the CO₂ 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 CO₂, 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 CO₂-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 CO₂, thereby causing at least a partial uncoupling of CO energy conservation. The observed inhibition 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 CO₂ 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 CO₂- and H₂-derived acetogenesis are

\[4CO + 2H₂O \rightarrow CH₃CO₂H + 2CO₂ \] (1)
\[4H₂ + 2CO₂ \rightarrow CH₃CO₂H + 2H₂O \] (2)

Either form of acetogenesis utilizes 8 reducing equivalents in the formation of acetate; however (Table 3), YCO is consistently higher than YH₂. This difference in growth yields may be due to (i) increased ATP synthesis per CO₂-derived electron pair (this assumes that YATP is basically the same for CO₂ 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 YATPs 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 CO₂ and −5.6 kcal [ca. −23.4 kJ]/mol of H₂, respectively [26, 37]) indicate that more energy can be obtained from CO₂-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 CO₂ and H₂ growth yields. CO₂-dependent growth yields of 3.7 g of cell dry weight per mol of CO₂ have been obtained for the phototroph Rhodoseudomonas gelatinosa (39) cultivated in an undefined medium (Robert Uffen, personal communication). A contrasting high YCO of approximately 9.5 can be calculated from data reported for M. barkeri cultivated in defined medium (32). The YH₂ for methanogens is approximately half this value (40). The YCO has not been reported for the aerobic CO-oxidizing carboxydotrophs (Ortwin Meyer, personal communication).

Based on protein profiles, a differential expression of genes was observed with methanol-grown cells as compared with CO₂-grown cells. This observation, along with the fact that CO₂-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), CO₂-dependent chemolithothrophic growth has not been reported for this acetogen.

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LITERATURE CITED


