APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1993, p. 3062–3069 Vol. 59, No. 9
0099-2240/93/093062-08$02.00/0

Oxalate- and Glyoxylate-Dependent Growth and Acetogenesis by *Clostridium thermoaceticum*

STEVEN L. DANIEL AND HAROLD L. DRAKE*

Lehrstuhl für Ökologische Mikrobiologie, BITÖK, Universität Bayreuth, D-95440 Bayreuth, Germany

Received 22 February 1993/Accepted 8 July 1993

The acetogenic bacterium *Clostridium thermoaceticum* ATCC 39073 grew at the expense of the two-carbon substrates oxalate and glyoxylate. Other two-carbon substrates (acetaldehyde, acetate, ethanol, ethylene glycol, glycolaldehyde, glycolate, and glyoxal) were not growth supportive. Growth increased linearly with increasing substrate concentrations up to 45 mM oxalate and glyoxylate, and supplemental CO_2 was not required for growth. Oxalate and glyoxylate yielded 4.9 and 9.4 g, respectively, of cell biomass (dry weight) per mol of substrate utilized. Acetate was the major reduced end product recovered from oxalate and glyoxylate cultures. ^{14}C labeling studies showed that oxalate was subject to decarboxylation, and product analysis indicated that oxalate was utilized by the following reaction: $4^-OOC\text{-}COO^- + 5H_2O \rightarrow CH_3COO^- + 6HCO_3^- + OH^-$. Oxalate- and glyoxylate-dependent growth produced lower acetate concentrations per unit of cell biomass synthesized than did H_2-, CO-, methanol-, formate-, *O*-methyl-, or glucose-dependent growth. Protein profiles of oxalate-grown cells were dissimilar from protein profiles of glyoxylate-, CO-, or formate-grown cells, suggesting induction of new proteins for the utilization of oxalate. *C. thermoaceticum* DSM 2955 and *Clostridium thermoautotrophicum* JW 701/3 also grew at the expense of oxalate and glyoxylate. However, oxalate and glyoxylate did not support the growth of *C. thermoaceticum* OMD (a nonautotrophic strain) or six other species of acetogenic bacteria tested.

Acetogenic bacteria commonly occur in anoxic habitats, including soils, sediments, and the gastrointestinal tracts of humans and animals (10, 22, 35). This group of bacteria has the unique capacity to form acetate from two one-carbon compounds via an autotrophic process termed the acetyl coenzyme A (acetyl-CoA) or Wood pathway (25, 35, 43, 52, 53). Recent studies have shown that acetogenic bacteria are very versatile metabolically and can synthesize acetate from a wide range of substrates. Some of the substrates that are known to support acetogenesis include carbohydrates, alcohols, organic acids, and various one-carbon substrates (e.g., H_2-CO_2, CO, formate, and the *O*-methyl groups of aromatic compounds) (19, 22, 35, 36). In the present study, we report that *Clostridium thermoaceticum* and *Clostridium thermoautotrophicum* are capable of growth and acetogenesis at the expense of the two-carbon substrates oxalate ($^-OOC\text{-}COO^-$) and glyoxylate ($HOC\text{-}COO^-$).

MATERIALS AND METHODS

Bacterial strains. Strains and sources of strains of acetogenic bacteria used in this study included the following (cultivation temperatures are indicated in parentheses): *Acetobacterium woodii* ATCC 29683 obtained from the American Type Culture Collection (ATCC; Rockville, Md.; 35°C); *Acetogenium kivui* ATCC 33488 (55°C) obtained from the ATCC; *Clostridium aceticum* DSM 1496 (Deutsche Sammlung von Mikroorganismen [DSM], Braunschweig, Germany; 35°C) obtained from J. R. Andreesen (Institut für Mikrobiologie, Georg-August-Universität, Göttingen, Germany); *Clostridium formicoaceticum* ATCC 27076 (35°C) obtained from the ATCC; *C. thermoaceticum* OMD (55°C), DSM 2955 (55°C), and ATCC 39073 (55°C) obtained from our stock culture collection, J. R. Andreesen, and the ATCC, respectively; *C. thermoautotrophicum* JW 701/3 (55°C) obtained from J. Wiegel (Department of Microbiology, The University of Georgia, Athens); *Eubacterium limosum* RF (35°C) obtained from M. P. Bryant (Department of Dairy Science, University of Illinois, Urbana); and *Peptostreptococcus productus* ATCC 35244 (35°C) obtained from M. P. Bryant.

Culture media and growth conditions. Unless noted otherwise, strains of acetogenic bacteria were cultivated in an undefined medium in butyl-rubber-stoppered aluminum-crimp-sealed culture tubes (18 by 150 mm; Bellco series 2048; Bellco Glass Inc., Vineland, N.J.; 27.2-ml approximate stoppered volume at 1 atm [1 atm = 101.29 kPa]). The undefined medium contained the following (in milligrams per liter): $NaHCO_3$, 7,500; KH_2PO_4, 500; NaCl, 400; NH_4Cl, 400; $MgCl_2 \cdot 6H_2O$, 330; $CaCl_2 \cdot 2H_2O$, 50; resazurin, 1; yeast extract, 1,000; nicotinic acid, 0.25; cyanocobalamin, 0.25; *p*-aminobenzoic acid, 0.25; calcium D-pantothenate, 0.25; thiamine-HCl, 0.25; riboflavin, 0.25; lipoic acid, 0.30; folic acid, 0.1; biotin, 0.1; pyridoxal-HCl, 0.05; sodium nitrilotriacetate, 7.5; $MnSO_4 \cdot H_2O$, 2.5; $FeSO_4 \cdot 7H_2O$, 0.5; $Co(NO_3)_2 \cdot 6H_2O$, 0.5; $ZnCl_2$, 0.5; $NiCl_2 \cdot 6H_2O$, 0.25; H_2SeO_3, 0.25; $CuSO_4 \cdot 5H_2O$, 0.05; $AlK(SO_4)_2 \cdot 12H_2O$, 0.05; H_3BO_3, 0.05; $Na_2MoO_4 \cdot 2H_2O$, 0.05; and $Na_2WO_4 \cdot 2H_2O$, 0.05. The culture medium was prepared anaerobically by boiling and cooling the medium under 100% CO_2, adding $Na_2S \cdot 9H_2O$ (250 mg/liter) and cysteine-HCl $\cdot H_2O$ (250 mg/liter) to the medium, and dispensing the medium under 100% CO_2 into culture tubes (7 ml per tube). Tubes were subsequently crimp sealed and autoclaved. When larger quantities of cells were required, the protocol described above was scaled up to 120-ml butyl-rubber-stoppered aluminum-crimp-sealed serum bottles (50 ml of medium per bottle).

The defined medium was the undefined medium without yeast extract. The minimal medium was the defined medium with nicotinic acid as the sole vitamin and with sodium sulfide (at twice the concentration) as the sole reducing agent

* Corresponding author.

(cysteine was omitted). The basal medium was the minimal medium without nicotinic acid. The CO_2-free undefined medium was the undefined medium in which the bicarbonate and CO_2 were replaced with Na_2HPO_4 (6.4 g/liter) and NaH_2PO_4 (6.1 g/liter), and the initial gas phase was 100% N_2. The initial pH (before inoculation) of all of the media described above approximated 6.6.

C. aceticum and *C. formicoaceticum* were cultivated in the undefined medium modified by adding $NaHCO_3$ (7.5 g/liter) and $NaCO_3$ (12 g/liter) and by cooling the medium under 100% CO_2 and then dispensing the medium under 100% N_2; the initial pH (before inoculation) of this medium approximated 7.8.

Stock solutions of two-carbon substrates (acetaldehyde, acetate, ethanol, ethylene glycol, glycolaldehyde, glycolate, glyoxal, glyoxylate, and oxalate) and other substrates (fructose, glucose, methanol, vanillin, and formate) were prepared in deionized water, filter sterilized into serum bottles, and degassed by sparging and then flushing the headspace gas with sterile N_2. When necessary, free acids were neutralized with 6 N KOH. Unless noted otherwise, substrates, including gaseous substrates (CO and H_2), were added anaerobically via syringe to tubes of sterile media to the following initial concentrations (before inoculation): two-carbon substrates, 15 mM; fructose, 5 mM; glucose, 5 mM; methanol, 10 mM; vanillin, 10 mM; formate, 60 mM; CO, 413 μmol; and H_2, 619 μmol. In all experiments, growth was initiated by injecting 0.5 ml of inoculum per culture tube or 3 ml of inoculum per serum bottle; culture tubes and serum bottles were incubated horizontally (without shaking).

Distribution of ^{14}C-labeled products after [^{14}C]oxalate-dependent growth. Cells were cultivated in six tubes of the undefined medium containing 15 mM [^{14}C]oxalate (approximately 1.9×10^5 dpm/μmol; New England Nuclear Corp., Boston, Mass.). After growth of the cells, three of the tubes were used for the analysis of ^{14}C in oxalate, acetate, and biomass; the remaining three tubes were used for the analysis of ^{14}C in CO_2. For the determination of ^{14}C in oxalate and acetate, the culture medium was clarified by centrifugation and analyzed by a Beckman System Gold high-performance liquid chromatograph (HPLC; Beckman Instruments, Inc., San Ramon, Calif.) equipped with an Aminex HPX-87H column (Bio-Rad Laboratories, Inc., Richmond, Calif.; operated at room temperature with a mobile phase of 0.01 N H_2SO_4 and a flow rate of 1.0 ml/min), a diode array detector (model 168; Beckman Instruments) and a Radiomatic Flo-ONE series A-100 flow-through isotope detector (Radiomatic Instruments, Meriden, Conn.). ^{14}C activity was measured in the isotope detector with Flo-scint II scintillation cocktail (Radiomatic Instruments); the ratio between the scintillation liquid flow rate and the HPLC flow rate was 3:1. For the analysis of ^{14}C-labeled biomass, cells were harvested by filtration (pore size, 0.22 μm), washed four times with 50 mM phosphate buffer (pH 7.0), and then analyzed for ^{14}C activity by liquid scintillation in 10 ml of Ecoscint scintillation cocktail (National Diagnostics, Manville, N.J.) with a model LS-9800 liquid scintillation counter (Beckman Instruments). For the analysis of $^{14}CO_2$, gaseous CO_2 was absorbed into the culture medium by injecting 0.5 ml of 3 N NaOH into each culture tube. $^{14}CO_3^{2-}$ was measured after diffusion of $^{14}CO_2$ from an acidified reaction mixture (12) into phenethylamine (4, 13). Radioactivity trapped in the phenethylamine was counted as described above for ^{14}C-labeled biomass. Uninoculated culture medium (control) was processed in the same fashion as inoculated culture medium.

Analytical methods. Growth was monitored at 660 nm with a Spectronic 501 spectrophotometer (Bausch and Lomb, Inc., Rochester, N.Y.); the optical path width (inner diameter of culture tubes) was 1.6 cm. Uninoculated culture media served as references. Cell dry weights were determined as described previously (38). Headspace gases were measured by gas-liquid chromatography, and substrates and end products in culture fluids were quantitated by UV (210 nm) and refractive index detection in a 1090L HPLC (Hewlett-Packard Co., Palo Alto, Calif.) equipped with a fermentation monitoring column (Bio-Rad Laboratories) as described previously (14). Slab sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done with the Laemmli buffer system (33), and cell extracts for electrophoretic analysis were prepared by lysozyme digestion as described previously (37). Protein was estimated by the Pierce bicinchoninic acid protein assay (Pierce Europe B.V., Oud-Beijerland, The Netherlands). Cells were stained by the Kopeloff modification of the Gram stain (28).

Chemicals and gases. All chemicals used were of the highest purity commercially available and were purchased from Sigma Chemical Co. (St. Louis, Mo.) or Aldrich Chemical Co., Inc. (Milwaukee, Wis.). Gases (N_2, CO, and H_2) were passed over a copper catalyst at 450°C to remove trace amounts of oxygen and filter sterilized before introduction into culture tubes or serum bottles.

RESULTS

Growth of acetogenic bacteria at the expense of two-carbon substrates. The two-carbon substrates oxalate and glyoxylate supported the growth of *C. thermoaceticum* ATCC 39073 (Fig. 1). CO-cultivated cells experienced little, if any, lag when transferred to the undefined medium containing 15 mM oxalate; in contrast, cells displayed an increased lag period when transferred to the undefined medium containing 15 mM glyoxylate (data not shown). Similar oxalate- and glyoxylate-dependent growth profiles were observed when glucose-, methanol-, vanillin-, formate-, or H_2-cultivated cells were used as sources of inocula. Significantly, other two-carbon substrates (acetaldehyde, acetate, ethanol, ethylene glycol, glycolaldehyde, glycolate, and glyoxal), as well as dicarboxylates (malonate, succinate, glutarate, adipate, pimelate, malate, tartarate, fumarate, and 2-oxoglutarate), tricarboxylates (citrate, isocitrate, and aconitate), and oxamate, did not support the growth of *C. thermoaceticum* ATCC 39073 in the undefined medium.

Additional species of acetogenic bacteria, together with additional strains of *C. thermoaceticum*, were screened for growth at the expense of two-carbon substrates in undefined culture media (see Materials and Methods; data not shown). Of the two-carbon substrates tested, ethanol and ethylene glycol supported the growth of *C. aceticum*, ethanol supported the growth of *C. formicoaceticum*, and ethylene glycol supported the growth (on first transfer only) of *A. woodii*. These observations support previous findings (9, 39, 46), although the reason for our inability to maintain *A. woodii* on ethylene glycol is currently unknown. In contrast to a previous report (9), ethylene glycol was growth supportive for *C. formicoaceticum*. *C. thermoautotrophicum* and *C. thermoaceticum* DSM 2955 grew at the expense of oxalate and glyoxylate, and growth profiles of *C. thermoautotrophicum*, *C. thermoaceticum* DSM 2955, and *C. thermoaceticum* ATCC 39073 were similar when cells were cultivated at the expense of oxalate or glyoxylate. *C. thermoaceticum* OMD, *A. kivui*, *E. limosum*, and *P. productus* failed to grow at the expense of any of the two-carbon substrates tested.

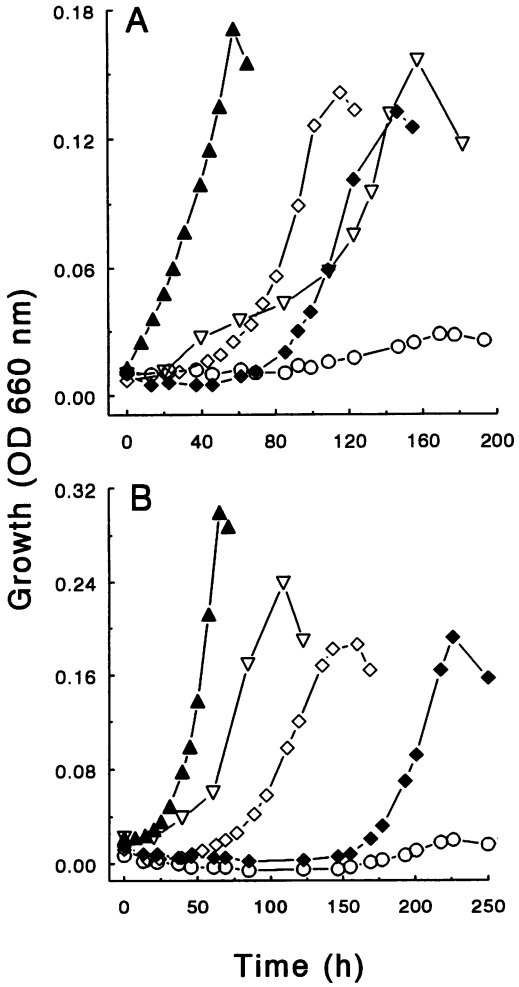

FIG. 1. Growth of *C. thermoaceticum* ATCC 39073 at the expense of 15 mM oxalate (A) and 15 mM glyoxylate (B) in the undefined (▲), CO_2-free undefined (▽), defined (◇), minimal (◆), or basal (○) culture medium. OD, optical density.

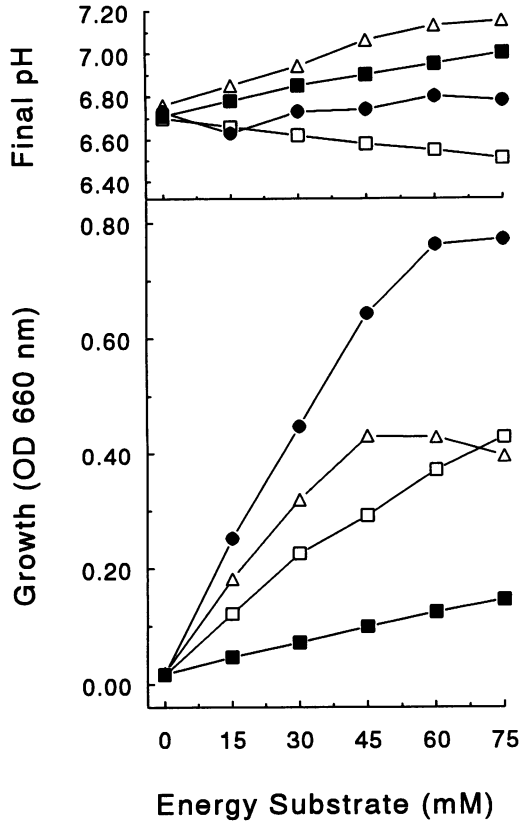

FIG. 2. Effects of increasing concentrations of oxalate (△), glyoxylate (●), formate (■), and CO (□) on the growth (measured as optical density at 660 nm [OD_{660}]) in the undefined medium and the final pH of the medium after growth. Inocula were derived from cultures maintained in undefined oxalate, glyoxylate, formate, and CO media, respectively.

Oxalate- and glyoxylate-dependent growth by *C. thermoaceticum* ATCC 39073. Undefined oxalate and glyoxylate medium cultures of *C. thermoaceticum* ATCC 39073 yielded gram-positive (strongly) rod-shaped cells when Gram stained; some cells with spores were observed. To confirm the authenticity of oxalate- and glyoxylate-dependent growth by *C. thermoaceticum* ATCC 39073, this acetogenic bacterium was reisolated from both regular and heat-shocked (100°C for 5 min) undefined glucose medium cultures on undefined glucose medium solidified with 0.75% Gelrite. Colonies were predominantly small, circular, and white in appearance. Cultures derived from 22 isolated colonies (10 and 12 from regular and heat-shocked cultures, respectively) grew at the expense of oxalate and glyoxylate. Six of the cultures derived from colonies isolated from heat-shocked cultures were also assayed for CO-dependent growth; all grew at the expense of CO. In addition, oxalate- and glyoxylate-dependent growth did not occur anaerobically at 25 or 35°C or aerobically at 25 or 55°C. These findings indicated that the *C. thermoaceticum* ATCC 39073 culture was pure.

Growth of oxalate- and glyoxylate-cultivated cells of *C. thermoaceticum* ATCC 39073 in the undefined medium increased linearly with increasing substrate concentrations up to 45 mM oxalate or glyoxylate; linear correlations were also observed between CO or formate and growth (Fig. 2). However, when compared at equimolar concentrations ranging from 15 to 45 mM, CO- and formate-dependent growth was consistently less than the growth at the expense of either oxalate or glyoxylate. As growth increased in response to increasing substrate concentrations, the pH of the culture medium decreased from 6.71 to 6.51 during CO-dependent growth, whereas the pH of the culture medium was essentially unchanged during glyoxylate-dependent growth (Fig. 2). In contrast, the pH of the culture medium increased from 6.71 to 7.00 and from 6.76 to 7.15 during formate- and oxalate-dependent growth, respectively.

Yeast extract was not required for oxalate- or glyoxylate-dependent growth by *C. thermoaceticum* ATCC 39073 (Fig. 1); the addition of yeast extract (0.1% [wt/vol]) to the defined medium did increase oxalate- and glyoxylate-dependent growth by approximately 30 and 40%, respectively (Table 1). Previous studies have shown that acetate (1 to 2 mM) is required for the synthesis of cell biomass by anaerobic oxalate-degrading bacteria (3, 17, 49). However, supplemental acetate was not required for oxalate- or glyoxylate-dependent growth by *C. thermoaceticum* ATCC 39073 (Fig. 1). Furthermore, in the defined medium, supplemental ace-

TABLE 1. Oxalate and glyoxylate product profiles of *C. thermoaceticum* ATCC 39073[a]

Energy substrate	Culture medium	Substrate consumed (mM)	Cell biomass formed (g/liter)[b]	Acetate assimilated (mM)[c]	Acetate formed (mM)	Electron recovery (%)[d]	Molar cell yield (g/mol)
Oxalate	Undefined	14.4	0.071	NA[e]	3.8	106	4.9
	Defined	14.1	0.056	1.2	2.7	111	4.0
Glyoxylate	Undefined	12.9	0.121	NA	5.3	82	9.4
	Defined	11.7	0.087	1.8	5.0	116	7.4

[a] Values are the means of duplicate or triplicate tubes. The total volume of culture tubes after inoculation was 7.7 ml.
[b] A culture optical density (at 660 nm) of 1 was equal to 0.450 g of cell biomass (dry weight) per liter.
[c] Acetate assimilation into cell biomass was calculated on the basis of the following equation (18): $17C_2H_3O_2^- + 11H_2O \rightarrow 8 < C_4H_7O_3 > + 2HCO_3^- + 150H^-$.
[d] Recovered as acetate.
[e] NA, not applicable.

tate did not influence the growth profiles of oxalate cultures over the range of concentrations examined (1.25 to 10 mM; data not shown). Growth in the basal medium containing oxalate or glyoxylate was negligible, indicating that nicotinic acid was required for growth (Fig. 1). These results agree with previous reports (14, 37) that nicotinic acid is the sole vitamin required for chemoorganotrophic (glucose) or chemolithotrophic (H_2 or CO) growth of *C. thermoaceticum*.

Oxalate- and glyoxylate-cultivated cells of *C. thermoaceticum* ATCC 39073 yielded biomass and acetate as end products (Table 1). Trace amounts of hydrogen were also detected in the headspace gas upon completion of oxalate- or glyoxylate-dependent growth; CO was not detected in such cultures. Molar cell yields (the amount of cell biomass synthesized per mole of substrate consumed) for glyoxylate-dependent growth were nearly twofold higher than those observed for oxalate-dependent growth. Overall, on the basis of substrate and end product analyses, the molar ratios (moles of substrate required to synthesize a mole of acetate) averaged 4.5 to 1 for oxalate-derived acetogenesis and 2.4 to 1 for glyoxylate-derived acetogenesis (Table 1).

In studies with [^{14}C]oxalate, $^{14}CO_2$ was the predominant ^{14}C-labeled product detected after completion of growth (Table 2); only a small percentage of the ^{14}C-labeled carbon from [^{14}C]oxalate went to acetate and biomass. Given the capacity of *C. thermoaceticum* ATCC 39073 to decarboxylate oxalate to CO_2, we reasoned that oxalate may support the growth of this acetogenic bacterium when supplemental CO_2 (required for acetogenesis) was excluded from the culture medium. Consistent with this hypothesis, oxalate, as well as glyoxylate, was growth supportive in the CO_2-free undefined medium (Fig. 1).

Energetics of growth. During growth under homoacetogenic conditions, the ratio of the amount of acetate formed per unit of cell biomass synthesized is a direct reflection of cell energetics (14, 32, 45). In this regard, the acetate-to-biomass ratios of oxalate- and glyoxylate-cultivated cells of *C. thermoaceticum* ATCC 39073 were less than those of other acetogenic substrates (Table 3). In addition, with the exception of growth at the expense of glucose, oxalate- and glyoxylate-dependent growth yielded more cell biomass per pair of reducing equivalents utilized than did the other acetogenic substrates tested (Table 3).

Protein profiles. Protein profiles of oxalate-cultivated cells of *C. thermoaceticum* ATCC 39073 were dissimilar to those of other cells, indicating oxalate-dependent, differential expression of proteins (Fig. 3). In particular, oxalate-cultivated cells contained one protein (molecular weight, approximately 33,000) that was minimally present in glyoxylate-, CO-, and formate-cultivated cells; this protein, together with another protein (molecular weight, approximately 42,000), appeared to constitute the majority of the soluble protein in oxalate-cultivated cells (Fig. 3).

DISCUSSION

The present study demonstrates that *C. thermoaceticum* (originally isolated from horse manure [23]), as well as the closely related bacterium *C. thermoautotrophicum* (originally isolated from mud [51]), has the capacity for oxalate- or glyoxylate-dependent growth and acetogenesis. This is the first report of oxalate and glyoxylate utilization by either acetogenic or thermophilic, obligately anaerobic bacteria. Of the three strains of *C. thermoaceticum* evaluated (ATCC 39073, DSM 2955, and OMD), only OMD failed to grow at the expense of oxalate or glyoxylate. Strain OMD is a nonautotroph that is not capable of H_2-, CO-, methanol-, or *O*-methyl-dependent growth (14, 15). Nevertheless, the inability of strain OMD to grow at the expense of oxalate or glyoxylate is not the result of a lack of CO dehydrogenase (acetyl-CoA synthase), since this enzyme has been detected in strain OMD (21).

TABLE 2. Distribution of ^{14}C after growth of *C. thermoaceticum* ATCC 39073 at the expense of [^{14}C]oxalate

Undefined medium[a]	Initial ^{14}C (10^5 dpm)	Distribution of ^{14}C activity (10^5 dpm)[b]			% Of initial dpm recovered
		CO_2	Biomass	Supernatant	
Uninoculated	186	0.31 (0.2)	NA[c]	183[d] (98.4)	98.6
Inoculated	188	183 (97.3)	0.54 (0.3)	1.65[e] (0.9)	98.5

[a] Contained [^{14}C]oxalate (15 mM; approximately 1.9×10^5 dpm/μmol).
[b] Values are the means of duplicate (uninoculated controls) or triplicate (inoculated) tubes. Parenthetical values represent the distribution of ^{14}C when expressed as a percentage of the initial ^{14}C activity.
[c] NA, not applicable.
[d] ^{14}C activity in oxalate.
[e] ^{14}C activity in acetate; no residual oxalate was detected.

TABLE 3. Doubling times and cell and acetate yields of *C. thermoaceticum* ATCC 39073 grown at the expense of oxalate, glyoxylate, and other acetogenic substrates[a]

Energy substrate (mM)[b]	t_d[c]	Cell biomass formed (g/liter)[d]	Molar cell yield (g/mol)	Acetate formed (mM)	Acetate/biomass ratio[e]	Biomass per 2[H][f]	Electron recovery (%)[g]
Oxalate (14.4)	14.0	0.071	4.9	3.8	54	4.9	106
Glyoxylate (12.9)	16.0	0.121	9.4	5.3	44	4.7	82
CO (53.6)	6.0	0.146	2.7	8.7	60	2.7	65
Glucose (4.7)	7.5	0.154	32.9	10.4	68	8.2	74
Methanol (9.4)	9.0	0.084	9.0	5.2	69	3.0	74
Vanillin (9.4)	9.5	0.078	8.3	6.9	88	2.8	98
Formate (56.1)	9.0	0.050	0.9	12.7	254	0.9	91
H_2 (80.5)	7.5	0.062	0.8	17.1	276	0.8	85

[a] Values are the means of triplicate tubes containing the undefined medium. The total volume of culture tubes after inoculation was 7.7 ml.
[b] Parenthetical values represent the amount (mM) of substrate consumed.
[c] t_d, doubling time in hours determined from log-based plots of exponential growth.
[d] A culture optical density (at 660 nm) of 1 was equal to 0.450 g of cell biomass (dry weight) per liter.
[e] Defined as the amount (in millimoles) of acetate formed per unit (gram) of cell biomass formed.
[f] The amount (in milligrams) of cell biomass synthesized per theoretical pair (millimole) of reducing equivalents.
[g] Recovered as acetate.

Although the metabolism of oxalate and glyoxylate by aerobic and facultatively anaerobic bacteria has been extensively investigated (27), information concerning the utilization of these substrates by obligately anaerobic bacteria is limited. To date, strains of anaerobic oxalate-degrading bacteria that have been described include the following: *Oxalobacter formigenes*, isolated from the sheep rumen (16), the ceca of swine (3) and rats (13), human feces (2), and freshwater sediments (49); and strain Ox-8, *Oxalobacter vibrioformis*, and *Clostridium oxalicum*, isolated from freshwater sediments (17, 49). Oxalate is the only substrate that supports the growth of these organisms, although *O. vibrioformis* and *C. oxalicum* can also utilize oxamate (after deamination [17]). Oxalate is a growth-supportive substrate for *Desulfovibrio vulgaris* subsp. *oxamicus*, isolated as an oxamate degrader from mud, when yeast extract and sulfate are present in the medium (42). Information on the anaerobic utilization of glyoxylate is limited to the recent isolation from marine sediments of strain PerGlx1, a strict anaerobe which converts glyoxylate to CO_2, H_2, and glycolate (24).

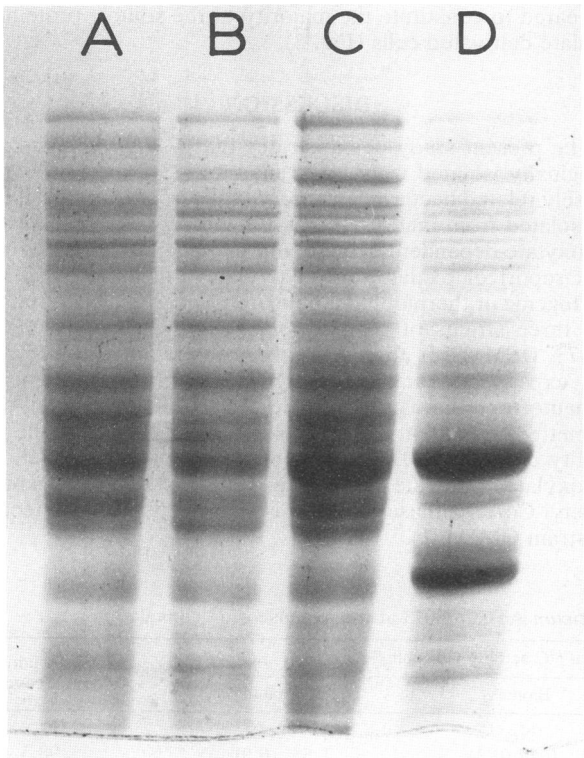

FIG. 3. Comparative SDS-PAGE protein profiles of *C. thermoaceticum* ATCC 39073. Cells were cultivated in 120-ml serum bottles containing 50 ml of the undefined medium with either CO in an initial cultivation gas phase of CO-CO_2 (50:50; total pressure of 202 kPa; A), 60 mM formate (B), 15 mM glyoxylate (C), or 15 mM oxalate (D). Each lane contained 10 μg of cell protein.

In this study, oxalate- and glyoxylate-dependent growth by *C. thermoaceticum* yielded acetate as the major reduced end product. Furthermore, in ^{14}C-labeled oxalate experiments (Table 2), oxalate was almost entirely converted (decarboxylated) by *C. thermoaceticum* to CO_2. That negligible amounts of carbon from oxalate were recovered in biomass and acetate suggests that the flow of oxalate-derived carbon (e.g., CO_2 or formate) is not tightly coupled to acetogenesis under CO_2-enriched conditions. However, this may not be the case when exogenous CO_2 is limited. Indeed, oxalate, as well as glyoxylate, supported the growth of *C. thermoaceticum* in the absence of supplemental CO_2, suggesting that the metabolism of these substrates by *C. thermoaceticum* provides the initial CO_2 equivalents (reductant sink) necessary to engage the acetyl-CoA (Wood) pathway. Growth-supportive CO_2 equivalents are also produced by *C. thermoaceticum* from the decarboxylation of carboxylated aromatic compounds (29, 30).

Fermentation balances were in agreement with the theoretical stoichiometries for oxalate- and glyoxylate-derived acetogenesis, respectively: $4^-OOC\text{-}COO^- + 5H_2O \rightarrow CH_3COO^- + 6HCO_3^- + OH^-$ (−41.4 kJ/mol of oxalate [50]) and $2HOC\text{-}COO^- + 2H_2O \rightarrow CH_3COO^- + 2HCO_3^- + H^+$ (−85.7 kJ/mol of glyoxylate [50]). From the overall findings of this study, a pathway is proposed for oxalate catabolism in *C. thermoaceticum* (Fig. 4). Oxalyl- and formyl-CoA and formate are considered potential intermediates during oxalate metabolism. The proposed role of CoA is consistent with the role of oxalyl-CoA decarboxylase and formyl-CoA transferase in the metabolism of oxalate by *O. formigenes* (3, 6–8). Whereas formate is not further metab-

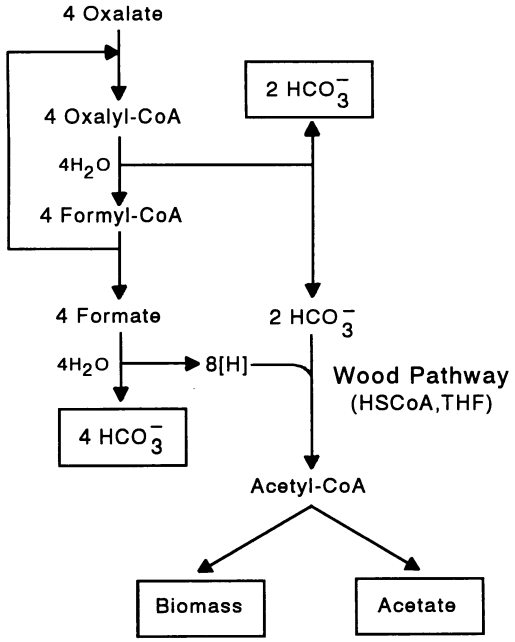

FIG. 4. Proposed pathway for oxalate-derived acetogenesis in *C. thermoaceticum* ATCC 39073. Abbreviations: HSCoA, coenzyme A; THF, tetrahydrofolate.

olized by *O. formigenes* (3, 16), with *C. thermoaceticum*, it is likely oxidized via formate dehydrogenase to CO_2 and reductant, the latter being utilized in the acetyl-CoA (Wood) pathway for energy conservation and biomass synthesis (22, 31, 35).

The molar cell yields of glyoxylate-cultivated cells were nearly double those of oxalate-cultivated cells (Table 1) and reflect the fact that, on a per mole basis, the calculated standard change in Gibbs free energy for glyoxylate-derived acetogenesis is approximately twice that of oxalate-derived acetogenesis (see equation above). Furthermore, protein profiles and physiological studies suggest that mechanisms for the metabolism of oxalate and glyoxylate are not the same in *C. thermoaceticum*. The differences observed in oxalate- and glyoxylate-dependent molar cell yields may be a result of (i) an initial decarbonylation reaction (e.g., $2HOC\text{-}COO^- \rightarrow 2HCOO^- + 2CO$) during glyoxylate catabolism which has the potential of generating a preformed carbonyl unit for acetyl-CoA synthesis or (ii) an increased capacity for energy conservation from glyoxylate-derived reductant since glyoxylate is a more reduced substrate. Significantly, when expressed on the basis of the amount of biomass synthesized per theoretical pair of reducing equivalents consumed, oxalate and glyoxylate were essentially equivalent in their capacity to support growth.

The molar cell yields of oxalate-cultivated cells of *C. thermoaceticum* were approximately three to five times greater than those of other known anaerobic oxalate-degrading bacteria (16, 17, 49). Lower molar cell yields would be anticipated given the small change in free energy for oxalate metabolism by these nonacetogenic anaerobes: $^-OOC\text{-}COO^- + H_2O \rightarrow HCOO^- + HCO_3^-$ (−26.7 kJ/mol of oxalate [50]). Furthermore, unlike *C. thermoaceticum*, these organisms do not utilize substrate level or electron transport phosphorylation for the synthesis of ATP (3, 17). Under these conditions, the energy derived from the decarboxylation of oxalate would appear to be conserved via an alternative mechanism(s). In this regard, a membrane-bound, $oxalate^{2-}$-$formate^-$ antiport protein has been described recently in *O. formigenes* (5, 40, 44); from these studies, it has been proposed that the antiport protein and a cytoplasmic decarboxylase (6, 8) work in concert to create a proton gradient (for ATP synthesis) by simultaneously coupling the electrogenic exchange of oxalate and formate across the membrane with a proton-consuming decarboxylation reaction (3). Another mechanism linking certain decarboxylation reactions to the generation of a Na^+-ion gradient via sodium-pumping decarboxylases has been proposed (20, 26, 34, 41, 47). Interestingly, the acetogenic bacteria *Sporomusa malonica* and *Sporomusa termitida* grow at the expense of succinate (decarboxylated to propionate) with essentially little, if any, acetate being produced (11, 18). Whether oxalate decarboxylation is coupled to an energy-conserving mechanism in *C. thermoaceticum* remains to be resolved.

That oxalate is subject to degradation by acetogenic bacteria may also have important implications relative to the turnover of this substrate in the environment. Oxalate is widespread in nature, occurring in soils and sediments, in many plant, algal, and fungal species, and in diets consumed by humans and animals (27), and is degraded by bacterial populations in aquatic sediments (48) and in the gastrointestinal tracts of humans and animals (1, 2, 4, 13). Since acetogenic bacteria are found in most anaerobic environments (10, 22, 35), it is possible that these bacteria participate in the flow of oxalate-derived carbon and reductant in such environments.

ACKNOWLEDGMENTS

This investigation was supported in part by the German Ministry for Research and Technology (Fördernummer 0339476AO) and by The University of Mississippi.

We express our appreciation to Milton J. Allison and Mark Rasmussen (National Animal Disease Center, Ames, Iowa) for assistance with the radioisotope studies, to Carola Matthies (Universität Bayreuth) for helpful discussions and review of the manuscript, and to Anita Gößner and Christine Wagner (Universität Bayreuth) and Cecil Hunt and Manish Parekh (University of Mississippi) for technical assistance.

REFERENCES

1. **Allison, M. J., and H. M. Cook.** 1981. Oxalate degradation by microbes of the large bowel of herbivores: the effect of dietary oxalate. Science **212**:675–676.
2. **Allison, M. J., H. M. Cook, D. B. Milne, S. Gallagher, and R. V. Clayman.** 1986. Oxalate degradation by gastrointestinal bacteria from humans. J. Nutr. **116**:455–460.
3. **Allison, M. J., K. A. Dawson, W. R. Mayberry, and J. G. Foss.** 1985. *Oxalobacter formigenes* gen. nov., sp. nov.: oxalate-degrading anaerobes that inhabit the gastrointestinal tract. Arch. Microbiol. **141**:1–7.
4. **Allison, M. J., E. T. Littledike, and L. F. James.** 1977. Changes in ruminal oxalate degradation rates associated with adaptation to oxalate ingestion. J. Anim. Sci. **45**:1173–1179.
5. **Anantharam, V., M. J. Allison, and P. C. Maloney.** 1989. Oxalate:formate exchange: the basis for energy coupling in *Oxalobacter*. J. Biol. Chem. **264**:7244–7250.
6. **Baetz, A. L., and M. J. Allison.** 1989. Purification and characterization of oxalyl-coenzyme A decarboxylase from *Oxalobacter formigenes*. J. Bacteriol. **171**:2605–2608.
7. **Baetz, A. L., and M. J. Allison.** 1990. Purification and characterization of formyl-coenzyme A transferase from *Oxalobacter formigenes*. J. Bacteriol. **172**:3537–3540.
8. **Baetz, A. L., and M. J. Allison.** 1992. Localization of oxalyl-

coenzyme A decarboxylase, and formyl-coenzyme A transferase in *Oxalobacter formigenes* cells. Syst. Appl. Microbiol. **15**:167–171.

9. **Braun, M., F. Mayer, and G. Gottschalk.** 1981. *Clostridium aceticum* (Wieringa), a microorganism producing acetic acid from molecular hydrogen and carbon dioxide. Arch. Microbiol. **128**:288–293.
10. **Breznak, J. A., and M. D. Kane.** 1990. Microbial H_2/CO_2 acetogenesis in animals guts: nature and nutritional significance. FEMS Microbiol. Rev. **87**:309–317.
11. **Breznak, J. A., J. M. Switzer, and H.-J. Seitz.** 1988. *Sporomusa termitida* sp. nov., an H_2/CO_2-utilizing acetogen isolated from termites. Arch. Microbiol. **150**:282–288.
12. **Conway, E. J.** 1962. Microdiffusion analysis and volumetric error, 5th ed. Crosby, Lockwood and Sons, Ltd., London.
13. **Daniel, S. L., P. A. Hartman, and M. J. Allison.** 1987. Microbial degradation of oxalate in the gastrointestinal tracts of rats. Appl. Environ. Microbiol. **53**:1793–1797.
14. **Daniel, S. L., T. Hsu, S. I. Dean, and H. L. Drake.** 1990. Characterization of the H_2- and CO-dependent chemolithotrophic potentials of the acetogens *Clostridium thermoaceticum* and *Acetogenium kivui*. J. Bacteriol. **172**:4464–4471.
15. **Daniel, S. L., E. S. Keith, H. Yang, Y.-S. Lin, and H. L. Drake.** 1991. Utilization of methoxylated aromatic compounds by the acetogen *Clostridium thermoaceticum*: expression and specificity of the CO-dependent O-demethylating activity. Biochem. Biophys. Res. Commun. **180**:416–422.
16. **Dawson, K. A., M. J. Allison, and P. A. Hartman.** 1980. Isolation and some characteristics of anaerobic oxalate-degrading bacteria from the rumen. Appl. Environ. Microbiol. **40**:833–839.
17. **Dehning, I., and B. Schink.** 1989. Two new species of anaerobic oxalate-fermenting bacteria, *Oxalobacter vibrioformis* sp. nov. and *Clostridium oxalicum* sp. nov., from sediment samples. Arch. Microbiol. **153**:79–84.
18. **Dehning, I., M. Stieb, and B. Schink.** 1989. *Sporomusa malonica* sp. nov., a homoacetogenic bacterium growing by decarboxylation of malonate or succinate. Arch. Microbiol. **151**:421–426.
19. **Diekert, G.** 1992. The acetogenic bacteria, p. 517–533. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The prokaryotes. A handbook on the biology of bacteria: ecophysiology, isolation, identification, applications, 2nd ed., vol. I. Springer-Verlag, Berlin.
20. **Dimroth, P.** 1987. Sodium ion transport decarboxylases and other aspects of sodium ion cycling in bacteria. Microbiol. Rev. **51**:320–340.
21. **Drake, H. L.** 1982. Occurrence of nickel in carbon monoxide dehydrogenase from *Clostridium pasteurianum* and *Clostridium thermoaceticum*. J. Bacteriol. **149**:561–566.
22. **Drake, H. L.** 1992. Acetogenesis and acetogenic bacteria, p. 1–15. *In* J. Lederberg (ed.), Encyclopedia of microbiology, vol. 1. Academic Press, Inc., San Diego, Calif.
23. **Fontaine, F. E., W. H. Peterson, E. McCoy, M. J. Johnson, and G. J. Ritter.** 1942. A new type of glucose fermentation by *Clostridium thermoaceticum* n.sp. J. Bacteriol. **43**:701–715.
24. **Friedrich, M., and B. Schink.** 1991. Fermentative degradation of glyoxylate by a new strictly anaerobic bacterium. Arch. Microbiol. **156**:392–397.
25. **Fuchs, G.** 1989. Alternative pathways of autotrophic CO_2 fixation, p. 365–382. *In* H. G. Schlegel and B. Bowien (ed.), Autotrophic bacteria. Science Tech Publications, Madison, Wis.
26. **Hilpert, W., B. Schink, and P. Dimroth.** 1984. Life by a new decarboxylation-dependent energy conservation mechanism with Na^+ as coupling ion. EMBO J. **3**:1665–1670.
27. **Hodgkinson, A.** 1977. Oxalic acid in biology and medicine. Academic Press, Inc., New York.
28. **Holdeman, L. V., E. P. Cato, and W. E. C. Moore.** 1977. Anaerobe laboratory manual, 4th ed. Virginia Polytechnic Institute and State University, Blacksburg.
29. **Hsu, T., S. L. Daniel, M. F. Lux, and H. L. Drake.** 1990. Biotransformations of carboxylated aromatic compounds by the acetogen *Clostridium thermoaceticum*: generation of growth-supportive CO_2 equivalents under CO_2-limited conditions. J. Bacteriol. **172**:212–217.
30. **Hsu, T., M. F. Lux, and H. L. Drake.** 1990. Expression of an aromatic-dependent decarboxylase which provides growth-essential CO_2 equivalents for the acetogenic (Wood) pathway of *Clostridium thermoaceticum*. J. Bacteriol. **172**:5901–5907.
31. **Hugenholtz, J., and L. G. Ljungdahl.** 1990. Metabolism and energy generation in homoacetogenic clostridia. FEMS Microbiol. Rev. **87**:383–390.
32. **Kellum, R., and H. L. Drake.** 1986. Effects of carbon monoxide on one-carbon enzymes and energetics of *Clostridium thermoaceticum*. FEMS Microbiol. Lett. **34**:41–45.
33. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
34. **Laubinger, W., and P. Dimroth.** 1988. Characterization of the ATP synthase of *Propionigenium modestum* as a primary sodium pump. Biochemistry **27**:7531–7537.
35. **Ljungdahl, L. G.** 1986. The autotrophic pathway of acetate synthesis in acetogenic bacteria. Annu. Rev. Microbiol. **40**:415–450.
36. **Ljungdahl, L. G., J. Hugenholtz, and J. Wiegel.** 1989. Acetogenic and acid-producing clostridia, p. 145–191. *In* N. P. Minton and D. J. Clarke (ed.), Clostridia. Biotechnology handbooks, vol. 3. Plenum Publishing Corp., New York.
37. **Lundie, L. L., Jr., and H. L. Drake.** 1984. Development of a minimally defined medium for the acetogen *Clostridium thermoaceticum*. J. Bacteriol. **159**:700–703.
38. **Lundie, L. L., Jr., H. Yang, J. K. Heinonen, S. I. Dean, and H. L. Drake.** 1988. Energy-dependent, high-affinity transport of nickel by the acetogen *Clostridium thermoaceticum*. J. Bacteriol. **170**:5705–5708.
39. **Lux, M. F., and H. L. Drake.** 1992. Reexamination of the metabolic potentials of the acetogens *Clostridium aceticum* and *Clostridium formicoaceticum*: chemolithoautotrophic and aromatic-dependent growth. FEMS Microbiol. Lett. **95**:49–56.
40. **Maloney, P. C., V. Anantharam, and M. J. Allison.** 1992. Measurement of the substrate dissociation constant of a solubilized membrane carrier. J. Biol. Chem. **267**:10531–10536.
41. **Matthies, C., and B. Schink.** 1992. Energy conservation in fermentative glutarate degradation by the bacterial strain WoGl3. FEMS Microbiol. Lett. **100**:221–226.
42. **Postgate, J. R.** 1963. A strain of *Desulfovibrio* able to use oxamate. Arch. Mikrobiol. **46**:287–295.
43. **Ragsdale, S. W.** 1991. Enzymology of the acetyl-CoA pathway of CO_2 fixation. Crit. Rev. Biochem. Mol. Biol. **26**:261–300.
44. **Ruan, Z.-S., V. Anantharam, I. T. Crawford, S. V. Ambudkar, S. Y. Rhee, M. J. Allison, and P. C. Maloney.** 1992. Identification, purification, and reconstitution of OxlT, the oxalate: formate antiport protein of *Oxalobacter formigenes*. J. Biol. Chem. **267**:10537–10543.
45. **Savage, M. D., and H. L. Drake.** 1986. Adaptation of the acetogen *Clostridium thermoautotrophicum* to minimal medium. J. Bacteriol. **165**:315–318.
46. **Schink, B., and M. Bomar.** 1992. The genera *Acetobacterium*, *Acetogenium*, *Acetoanaerobium* and *Acetitomaculum*, p. 1925–1936. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The prokaryotes. A handbook on the biology of bacteria: ecophysiology, isolation, identification, applications, 2nd ed., vol. 2. Springer-Verlag, Berlin.
47. **Schink, B., and N. Pfenning.** 1982. *Propionigenium modestum* gen. nov. sp. nov. a new strictly anaerobic, nonsporing bacterium growing on succinate. Arch. Microbiol. **133**:209–216.
48. **Smith, R. L., and R. S. Oremland.** 1983. Anaerobic oxalate degradation: widespread natural occurrence in aquatic sediments. Appl. Environ. Microbiol. **46**:106–113.
49. **Smith, R. L., F. E. Strohmaier, and R. S. Oremland.** 1985. Isolation of anaerobic oxalate-degrading bacteria from freshwater lake sediments. Arch. Microbiol. **141**:8–13.
50. **Thauer, R. K., K. Jungermann, and K. Decker.** 1977. Energy

conservation in chemotrophic anaerobic bacteria. Bacteriol. Rev. **41**:100–180.

51. **Wiegel, J., M. Braun, and G. Gottschalk.** 1981. *Clostridium thermoautotrophicum* species novum, a thermophile producing acetate from molecular hydrogen and carbon monoxide. Curr. Microbiol. **5**:255–260.
52. **Wood, H. G.** 1991. Life with CO or CO_2 and H_2 as a source of carbon and energy. FASEB J. **5**:156–163.
53. **Wood, H. G., and L. G. Ljungdahl.** 1991. Autotrophic character of the acetogenic bacteria, p. 201–250. *In* J. M. Shively and L. L. Barton (ed.), Variations in autotrophic life. Academic Press, Inc., New York.