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Effect of Nitrate on the Autotrophic Metabolism of the Acetogens *Clostridium thermoautotrophicum* and *Clostridium thermoaceticum*

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Although nitrate stimulated the capacity of *Clostridium thermoautotrophicum* and *Clostridium thermoaceticum* to oxidize (utilize) substrates under heterotrophic conditions, it inhibited autotrophic H_2-CO_2-dependent growth. Under basal medium conditions, nitrate was also inhibitory to the use of one-carbon substrates (i.e., CO, formate, methanol, or the O-methyl groups of vanillate or syringate) as sole carbon energy sources. This inhibitory effect of nitrate was bypassed when both O-methyl groups and CO were provided concomitantly; H_2-CO_2 did not replace CO. These results indicated that nitrate blocked the reduction of CO_2 to the methyl and carbonyl levels. On the basis of the inability of acetogenic cells (i.e., cells cultivated without nitrate) to consume or reduce nitrate in resting-cell assays, the capacity to dissimilate nitrate was not constitutive. Nitrate had no appreciable effect on the specific activities of enzymes central to the acetyl-coenzyme A (CoA) pathway. However, membranes obtained from cells cultivated under nitrate-dissimilating conditions were deficient in the *b*-type cytochrome that was typical of membranes from acetogenic cells, i.e., cells dependent upon the synthesis of acetate for the conservation of energy. Collectively, these findings indicated that (i) *C. thermoautotrophicum* and *C. thermoaceticum* cannot engage the carbon-fixing capacities of the acetyl-CoA pathway in the presence of nitrate and (ii) the nitrate block on the acetyl-CoA pathway occurs via an alteration in electron transport.

Most investigations of the CO_2-fixing acetyl-coenzyme A (CoA) pathway of acetogenic bacteria have focused on the dissimilatory and energy-conserving features of the pathway (13, 16, 61). However, the anabolic and catabolic functions of the pathway are conceived to be integrated (Fig. 1). The concept that acetogens use the acetyl-CoA pathway for the autotrophic assimilation of carbon is based on a relatively limited number of observations, mainly the absence of other autotrophic CO_2-assimilating enzymes in *Acetobacterium woodii* and carbon-labeling patterns obtained with *Clostridium thermoaceticum* (22, 37). Under heterotrophic conditions (i.e., conditions which do not require obligatory use of the acetyl-CoA pathway for the assimilation of carbon), acetogens may not be strictly dependent on this process, since a number of alternative energy-conserving electron acceptors can be utilized, including fumarate (14, 42), aromatic acrylates (1, 43, 57), inorganic sulfur compounds (2, 3, 27), pyruvate (44), or nitrate (52). Because the use of alternative electron acceptors by acetogens has been examined primarily under heterotrophic conditions, the potential impact of competing reductant sinks on the autotrophic potentials of acetogens is largely unknown. The acetogens *C. thermoaceticum* (20) and *Clostridium thermoautotrophicum* (60) are phylogenically very closely related (55) and dissimilate nitrate preferentially to the reduction of CO_2 under heterotrophic conditions (21, 52). The main objective of the present study was to resolve the impact of nitrate on the one-carbon metabolism and autotrophic capacities of these two acetogens.

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MATERIALS AND METHODS

Culture media and growth conditions. *C. thermoautotrophicum* (ATCC 33924) and *C. thermoaceticum* (ATCC 39073) were cultivated at 55°C in butyl rubber-stoppered, aluminum crimp-sealed tubes (18 by 150 mm; approximately 27-ml stoppered volume at atmospheric pressure; 7 ml of medium per tube). The basal medium contained the following (in milligrams per liter): $NaHCO_3$, 7,500; KH_2PO_4, 500; NaCl, 400; NH_4Cl, 160; $MgCl_2 \cdot 6H_2O$, 50; $CaCl_2 \cdot 2H_2O$, 10; nicotinic acid, 2; cyanocobalamin, 0.25; *p*-aminobenzoic acid, 0.25; calcium D-pantothenate, 0.25; thiamine · HCl, 0.25; riboflavin, 0.25; lipoic acid, 0.25; folic acid, 0.1; biotin, 0.1; pyridoxal · HCl, 0.1; 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; $Na_2WO_4 \cdot 2H_2O$, 0.05; $Na_2S \cdot 9H_2O$ (reducer), 500; and resazurin (redox indicator), 1. Undefined medium was basal medium that lacked NH_4Cl but was supplemented with yeast extract (1 g/liter). Unless otherwise indicated, the cultivation gas phase was 100% CO_2; the pH of both basal and undefined media approximated 6.8. Supplemental substrates were as indicated elsewhere. Growth was initiated with 0.5 ml of inoculum. When larger quantities of cells were required for assays of resting cells, cell extracts, and membranes, cultivation was done in 1.2-liter infusion flasks (Müller-Krempel, Bülach, Switzerland) containing 500 ml of medium. Unless otherwise indicated, culture tubes and bottles were incubated horizontally without shaking.

Acetogenium kivui (ATCC 33488), *A. woodii* (DSM 1030), and *Peptostreptococcus productus* (ATCC 35244) were cultivated at 55, 30, and 37°C, respectively, in the undefined medium described above. *Clostridium aceticum* (DSM 1496) and *Clostridium formicoaceticum* (DSM 92) were cultivated in a similar undefined medium at pH 8 and 37°C (42).

Preparation of resting-cell assay. Cells (cultivated in undefined medium [5 g of yeast extract per liter] with 60 mM methanol and 10 mM nitrate as indicated) were harvested during mid-growth (an optical density at 660 nm of approximately 0.25) by centrifugation and washed with 50 mM potassium phosphate buffer (pH 7) under a gas atmosphere of 100% N_2. The cells were resuspended to a final optical density at 660 nm of 7 in 100 mM potassium phosphate buffer (pH 7) containing 10 mM $MgCl_2$ and 0.05% Na_2S (to maintain reduced conditions). Five-milliliter cell suspensions were preincubated for 5 min at 55°C in closed, N_2-flushed tubes (28 ml); reactions were initiated by injecting CO_2, methanol, and nitrate (final concentrations of 20, 60, and 5 mM, respectively). Aliquots were removed for analysis; the reaction was terminated by icing and aerobically vortexing the collected sample.

Preparation of cell extracts and enzyme activity assays. Cells were harvested anaerobically and digested with lysozyme as outlined previously (40). All enzyme activity assays were carried out under anaerobic conditions (100% N_2) at 55°C. Cells used for the assessment of acetyl-CoA synthase activity were obtained from cultures grown in defined medium supplemented with pyruvate, ethanol, and

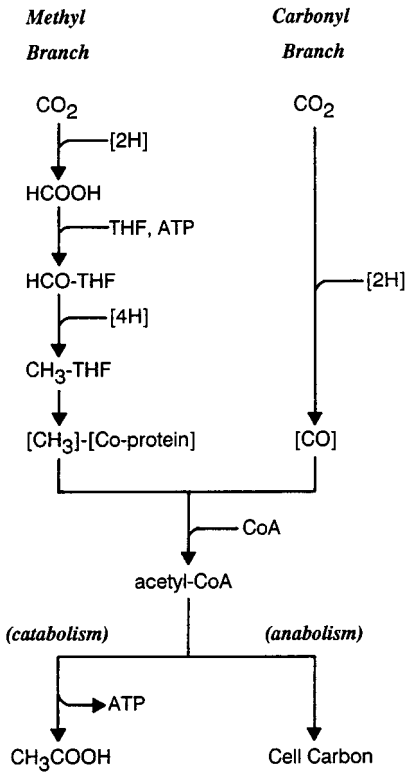

FIG. 1. Pathway for the autotrophic synthesis of acetate and biomass via the acetyl-CoA pathway (modified from reference 16). Abbreviations: THF, tetrahydrofolate; [Co-protein], corrinoid protein.

vanillate (10 mM each); this combination of substrates yielded maximal growth under defined conditions. Cells used for the assessment of all other enzyme activities were obtained from cultures grown in methanol (60 mM)-supplemented undefined medium (modified with 5 g of yeast extract and 0.4 g of NH_4Cl per liter). These media were supplemented with 10 mM nitrate as indicated.

Reactions were initiated by the addition of cell extract (containing 20 to 30 mg of protein per ml). Hydrogenase, CO dehydrogenase, and formate dehydrogenase activities were determined by following the H_2-dependent, CO-dependent, or formate-dependent reduction of benzyl viologen at 578 nm, respectively (15). The assay buffer was 50 mM Tris HCl (pH 8.5) plus 5 mM benzyl viologen; assay tubes containing 5 ml of assay buffer were supplemented with 125 μmol of H_2, 125 μmol of CO, or 50 μmol of formate for the respective enzyme assay. Formyltetrahydrofolate synthetase (47, 49), methylenetetrahydrofolate dehydrogenase (59), and methylenetetrahydrofolate reductase (7) activities were assayed spectrophotometrically according to published protocols. For hydrogenase, CO dehydrogenase, formate dehydrogenase, methylenetetrahydrofolate reductase, and methylenetetrahydrofolate dehydrogenase, 1 U of activity was defined as 1 μmol of reductant pair equivalent consumed per min. For formyltetrahydrofolate synthetase, 1 U of activity was defined as 1 μmol of formyltetrahydrofolate synthesized per min.

Acetyl-CoA synthase activity (note that this enzyme is the same as CO dehydrogenase and catalyzes both the synthesis of acetyl-CoA and the reversible oxidation of CO [50]) was assayed as previously described (18, 48), except the assessment of acetate (used to assess acetyl-CoA synthesis) was not dependent on the use of ^{14}C-labeled methyltetrahydrofolate. The assay was performed in stoppered reaction vials (12 ml) containing 520 μl of the following mixture (in millimolar except as specified otherwise): sodium phosphate (pH 6.0), 80; ADP, 5; $MgCl_2$, 10; CoA, 2; dithiothreitol, 10; 5-methyltetrahydrofolate, 2; 2-mercaptoethanol, 9 (present in the 5-methyltetrahydrofolate stock solution); and CO, 19 (10 μmol). The reaction was initiated by injecting 50 μl of cell extract (containing approximately 1 mg of protein) and terminated at 15 min with 0.2 ml of 2 M $HClO_4$. Acetate was analyzed by high-performance liquid chromatography. Control reactions that were terminated immediately or control reaction mixtures that lacked either 5-methyltetrahydrofolate or CO yielded negligible or nondetectable amounts of acetate. For acetyl-CoA synthase, 1 U of activity was defined as 1 nmol of acetyl-CoA synthesized per min.

Difference spectra of membranes. Cells were cultivated in undefined medium that contained either methanol (60 mM) or CO (60 mM [these cultures were shaken on a rotary shaker at 90 rpm]) and that was supplemented with nitrate (10 mM) as indicated. Cells were disrupted under anaerobic conditions with a French press, and membranes were prepared and washed by ultracentrifugation as previously described (17, 30). Washed membranes were resuspended to a final concentration of 1 mg of protein per ml in 50 mM potassium phosphate buffer (pH 7) containing 10 mM $MgSO_4$ and 10% glycerol. Membranes were reduced with sodium dithionite, and the reduced-minus-oxidized spectra were obtained with a Uvikon 930 (Kontron Instruments, Milan, Italy) double-beam recording spectrophotometer at room temperature.

Analytical methods. Growth was monitored at 660 nm with a Spectronic 501 photometer (Bausch and Lomb, Inc., Rochester, N.Y.). Cell dry weights were calculated as previously described (51). Protein was estimated by the method of Bradford (5). Analysis of culture liquid and gas phases was facilitated by high-performance liquid chromatography and gas chromatography, respectively, as previously described (9, 10). Gas solubilities were calculated from standard solubility tables (58), and the amounts of gas consumed or produced were calculated by taking into account both gas and liquid phases. Millimolar concentrations of a specific gas were calculated by dividing the total amount of said gas present in the culture tube by the volume of the liquid phase. Ammonium (19, 24), nitrite (26), and nitrate (6) were determined spectrophotometrically by standard methods. All chemicals used were of the highest purity available and were purchased from Sigma Chemie (Deisenhofen, Germany) or Aldrich Chemie (Steinheim, Germany). In this study, no distinctions between (i) carboxylic acids and their salt forms, (ii) CO_2 and its carbonate forms, and (iii) ammonia and its salt form, ammonium, were made. All results and values are representative of replicate experiments.

RESULTS

Capacity of acetogens to dissimilate nitrate. After *A. kivui*, *A. woodii*, *C. aceticum*, *C. formicoaceticum*, and *P. productus* were cultivated in undefined media with a variety of substrates, their growth rates and cell yields were found not to be appreciably affected by nitrate; in each case, nitrate consumption was minimal or not apparent (data not shown). These results were in marked contrast with results attesting to the capacities of *C. thermoautotrophicum* and *C. thermoaceticum* to dissimilate nitrate.

Effect of nitrate on one-carbon metabolism and substrate turnover under heterotrophic conditions. In the presence of nitrate under yeast extract-enriched conditions (i.e., conditions that provide the cell with utilizable carbon for biosynthesis from yeast extract), *C. thermoautotrophicum* grew with one-carbon substrates. This growth was coupled to the reduction of nitrate, and the acetyl-CoA pathway was not engaged, as was indicated by the cell's inability to synthesize acetate from H_2-CO_2, CO, and formate (Table 1). Similar results were obtained with *C. thermoaceticum* (data not shown). The flow of reductant was shifted towards the reduction of nitrate; nitrite and ammonium were the main reduced products detected. Formate was formed by H_2 (and to a lesser extent CO) cultures supplemented with nitrate. The amounts of acetate formed by glucose and pyruvate cultures were reduced under nitrate-supplemented basal conditions (data not shown); this result was consistent with the loss of the function of the acetyl-CoA pathway relative to the reduction of CO_2.

With undefined medium, the amount of biomass formed per unit of reductant consumed by *C. thermoautotrophicum* and *C. thermoaceticum* was consistently greater under nitrate-supplemented conditions (Table 1 and data not shown). For example, at low H_2 levels (15 mM), nitrate-coupled growth was eightfold greater than acetogenic growth (on the basis of the amount of biomass formed per unit of reductant consumed). In addition, although ethanol and *n*-propanol were only marginally growth supportive in the absence of nitrate, these alcohols yielded high amounts of biomass per unit of reductant consumed under nitrate-dissimilating conditions (Table 1). These results demonstrated that certain substrates not readily growth supportive under acetogenic conditions can nonetheless serve as growth-supportive sources of reductant via nitrate dissimilation. Reductant recovery in nitrate-dissimilating, undefined, and basal medium cultures approximated only 60% (Table 1

TABLE 1. Effect of nitrate on the growth and product profiles of *C. thermoautotrophicum* cultivated in undefined medium

Substrate (mM)	Concn of substrate consumed (mM)	Biomass per 2[H] consumed (mg/mmol)[a]	Concn of nitrate consumed (mM)	Concn of product (mM)[b]							Recovery (%)	
				Nitrite	Ammonium	Acetate	Formate	Propionate	Protocatechuate	Catechol	N	[H]
None (control)	NA[c]	NA (7)	0	0	0.1	0	0				NA	NA
NO_3^- (5) (control)[d]	NA	NA (7)	0.2	0.2	0.1	0	0				95	NA
H_2 (15)[e]	13.0	0.6 (14)	ND[f]	ND	ND	3.5	0				NA	108
H_2 (15) + NO_3^-[g]	8.7	4.8 (49)	3.4	2.9	0.6	0	0				102	56
H_2 (60)	53.4	0.9 (54)	ND	ND	ND	12.5	0				NA	94
H_2 (60) + NO_3^-	16.2	2.4 (46)	3.8	2.6	1.1	0	3.2				96	61
CO (60)	29.4	2.7 (86)	ND	ND	ND	6.4	0				NA	87
CO (60) + NO_3^-	18.6	6.8 (133)	4.1	0.7	2.6	0	0.6				81	62
Formate (15)	14.6	0.9 (20)	0	0	0.1	3.6	0				NA	99
Formate (15) + NO_3^-	15.7	3.1 (56)	5.0	3.2	1.6	0	0				98	61
Vanillate (5)	5.6	2.6 (15)	ND	ND	ND	3.4	0		3.4	0.8	NA	82
Vanillate (5) + NO_3^-	3.3	5.3 (33)	2.9	1.0	0.8	0	0		0.9	1.3	71	41
Ethanol (10)	4.8	0.5 (12)	ND	ND	ND	6.2	0				NA	86
Ethanol (10) + NO_3^-	4.3	6.5 (64)	3.4	2.2	0.8	4.0	0				86	58
n-Propanol (10)	10.0	0.4 (15)	0	0	0.2	4.9	0	10.8			NA	97
n-Propanol (10) + NO_3^-	2.4	5.6 (33)	2.3	2.2	0.5	0	0	2.6			116	80

[a] Values in parentheses are milligrams (dry weight) of cells per liter.
[b] The values reported were corrected for amounts carried over with inoculum. Inocula were derived from cultures maintained under the growth conditions indicated.
[c] NA, not applicable.
[d] When nitrate was added, it was added at 5 mM throughout.
[e] Inoculum was derived from maintained H_2 (60 mM) culture (undefined medium).
[f] ND, not determined.
[g] Inoculum was derived from maintained H_2 (60 mM) plus NO_3^- (5 mM) culture (undefined medium).

and data not shown); with basal medium cultures, reductant recovery was 80 to 110% after the amounts of reductant theoretically in the biomass were taken into account (data not shown). Because essentially identical results were obtained to this point with both *C. thermoautotrophicum* and *C. thermoaceticum*, *C. thermoautotrophicum* was selected as the primary model for subsequent evaluations.

Turnover of nitrate during growth and by resting cells. Time course studies with glucose (Fig. 2) and pyruvate (data not shown) demonstrated that the reduction of nitrate to nitrite was correlated with growth. Although these results suggested that energy was conserved in the reduction of nitrate to nitrite, it remains unknown if further reduction of nitrite to ammonium was energy conserving.

To determine if the cell's capacity to dissimilate nitrate was constitutive, cells that had been cultivated at the expense of methanol in undefined medium with or without nitrate were resuspended in methanol-supplemented buffer and challenged with nitrate. Cells cultivated in the absence of nitrate displayed no capacity either to consume or to reduce nitrate over a 60-min assay period. In contrast, cells cultivated with nitrate readily reduced nitrate to nitrite, with the amount of nitrite formed accounting for approximately 75% of the nitrate consumed. Rates of nitrate consumption by resting nitrate-cultivated cells approximated 0.1 μmol of nitrate consumed per min per mg (dry weight) of cells, which was about one-fourth of the nitrate consumption rate observed during growth.

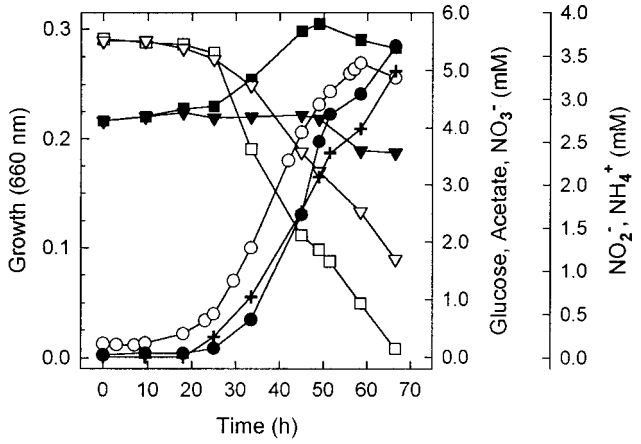

FIG. 2. Effect of time on the substrate and product profiles of *C. thermoautotrophicum* cultivated with glucose in nitrate-supplemented basal medium. Symbols: ○, growth; ▽, glucose; +, acetate; □, NO_3^-; ●, NO_2^-; ▼, NH_4^+; ■, NH_4^+ corrected for the theoretical nitrate-derived N in the biomass (46).

Inhibition of chemolithoautotrophic growth by nitrate. Nitrate inhibited the H_2-CO_2 chemolithoautotrophic growth of *C. thermoautotrophicum*, i.e., cultures could not be maintained beyond the first transfer in H_2-CO_2 basal medium supplemented with nitrate (Table 2). This result suggested that the loss of the capacity to synthesize acetate via the acetyl-CoA pathway was coincident with the loss of the cell's capacity to assimilate CO_2-derived carbon. Although growth could not be maintained in H_2-CO_2-nitrate basal medium, initial transfers of cells into this medium resulted in the formation of large amounts of formate (Table 2), suggesting that the flow of reductant and carbon towards acetyl-CoA was blocked beyond the formate dehydrogenase step in the acetyl-CoA pathway. Certain acetogens have the specialized capacity to use reverse acetogenesis for the assimilation of acetate (62). However, supplemental acetate did not serve as a carbon source (Table 2) and, thus, did not provide the cell with an anabolic bypass for the apparent block imposed by nitrate on the acetyl-CoA pathway.

Effect of nitrate on growth at the expense of one-carbon substrates under basal medium conditions. Under nitrate-supplemented basal conditions, cultures of *C. thermoautotrophicum* could not be maintained with CO, formate, methanol, vanillate, or syringate after these one-carbon acetogenic substrates were added singly as medium supplements; in contrast,

TABLE 2. Effect of nitrate on the H_2-dependent growth and product profiles of initial transfers of *C. thermoautotrophicum* in basal medium[a]

Substrate	Biomass formed (mg [dry wt]/liter)[b]	Concn of product (mM)	
		Acetate	Formate
None (control)	3	0.1	0
NO_3^- (control)	1	0	0
H_2	51 (44)	14.7	0
H_2 + NO_3^-	33 (2)	0	8.1
Acetate	2	0.1	0
Acetate + NO_3^-	1	0.1	0
H_2 + acetate	34 (38)	13.4	0
H_2 + acetate + NO_3^-	19 (0)	0.1	10.6

[a] Inoculum was derived from a culture maintained in H_2-supplemented (60 mM) undefined medium. The initial substrate concentrations (millimolar) were as follows: nitrate, 5; H_2, 60; and acetate, 5. Values are the maximum values observed during growth.
[b] The values in parentheses are second-transfer values.

these substrates were growth supportive when nitrate was omitted from the basal medium (data not shown). (Vanillate and syringate are referred to as one-carbon substrates because *C. thermoaceticum* and *C. thermoautotrophicum* only make use of one-carbon substituent groups of these aromatic compounds [references 11 and 28 and data not shown].) After either vanillate or syringate was combined with CO in basal medium, maintainable growth was obtained in the presence of nitrate (Table 3 and data not shown). Thus, the apparent nitrate-dependent blockage of the assimilatory function of the acetyl-CoA pathway was bypassed when both preformed methyl- and carbonyl-level precursors were available for growth, indicating that the acetyl-CoA formed under these conditions was used exclusively for the anabolic assimilation of carbon rather than the synthesis of acetate. H_2 may enhance the capacity of certain acetogens to use aromatic O-methyl groups (35, 36). However, growth was not obtained in the presence of nitrate with vanillate- or syringate-supplemented basal medium after CO was replaced with H_2, indicating that H_2-dependent reduction of CO_2 to the carbonyl level did not occur in the presence of nitrate. Experiments with *C. thermoaceticum* yielded essentially identical results (data not shown).

Effect of nitrate on enzyme activities in cell extracts. Enzyme activities central to the acetyl-CoA pathway were present in cell extracts of *C. thermoautotrophicum* obtained from cultures grown in the presence of nitrate (Table 4). The difference in the specific activities of cultures cultivated with and without nitrate was minimal; activity levels in both cases were similar to those previously observed with *C. thermoautotrophicum* (8, 30). Although they were not measured directly, the methyl transferase (18) and corrinoid protein (29) that collectively facilitate the transmethylation of acetyl-CoA synthase from methyltetrahydrofolate (16, 50, 61) appeared also to be present in an active form in nitrate-grown cells (since both enzymes were required for the synthesis of acetyl-CoA from methyltetrahydrofolate and CO). The addition of nitrate or nitrite in enzyme assays had no appreciable effect on CO dehydrogenase and formyltetrahydrofolate synthetase activities in cell extracts (data not shown). The specific activity of hydrogenase decreased in cells cultivated under nitrate-dissimilating conditions (Table 4).

Effect of nitrate on the occurrence of cytochrome *b* in cell membranes. The difference spectrum of membranes obtained from *C. thermoautotrophicum* cultivated with either CO or methanol under nitrate-dissimilating conditions indicated that membranes from such cells were dissimilar to those obtained from cells cultivated in the absence of nitrate (Fig. 3). In particular, cytochrome *b* (previously reported with glucose- and methanol-cultivated cells) (25, 30) with absorption peaks at approximately 430 and 560 nm decreased to nondetectable levels. Similar results were obtained with *C. thermoaceticum*, i.e., membranous cytochrome *b* was present in cells cultivated without nitrate but was not detected in the membranes of nitrate-cultivated cells.

DISCUSSION

The acetyl-CoA pathway is conceived to have both dissimilatory and assimilatory functions relative to carbon and energy flow (Fig. 1). The collective findings of the present study provide confirmatory evidence (22, 37) that the acetyl-CoA pathway is indeed central to the autotrophic assimilation of carbon for *C. thermoautotrophicum* and *C. thermoaceticum*. Furthermore, because a shift in the flow of reductant towards the dissimilation of nitrate altered the routing of CO_2 to both acetate and biomass, the findings also provide evidence that the primary dissimilatory and assimilatory reductive functions of the acetyl-CoA pathway of these two acetogens are not regulated separately during nitrate dissimilation. Both the dissimilatory and assimilatory CO_2-reducing functions of the pathway appear to have been short circuited by nitrate, and the cell consequently lost its capacity to grow autotrophically.

Despite this negative effect of nitrate on the in situ functionality of the acetyl-CoA pathway, cell yields were enhanced during the dissimilation of nitrate under heterotrophic growth conditions (i.e., when the cell could obtain carbon for biomass synthesis from reduced carbon precursors [e.g., from yeast

TABLE 3. Effect of nitrate on the growth and product profiles of *C. thermoautotrophicum* cultivated in basal medium supplemented with vanillate plus CO[a]

Substrate	Biomass formed (mg [dry wt]/liter)	Concn of substrate consumed (mM)			Biomass per 2[H] consumed (mg/mmol)	Concn of product (mM)						Recovery (%)	
		Vanillate	CO	Nitrate		Nitrite	Ammonium	Acetate	Protocatechuate	Catechol	H_2	N	[H]
None (control)	6.0	NA[b]	NA	ND[c]	NA	ND	−0.2	0			ND	NA	NA
NO_3^- (control)	5.5	NA	NA	0	NA	0	0	0			ND	NA	NA
Vanillate + CO	68.0	4.6	16.0	0	2.1	0	−1.0	6.7	2.4	1.2	0.1	NA	90
Vanillate + CO + NO_3^-	89.8	3.6	13.1	3.4	3.5	1.2	2.0[d]	0	0.2	3.6	0.8	92	40

[a] The initial substrate concentrations (millimolar) were as follows: nitrate, 5; vanillate, 5; and CO, 15.
[b] NA, not applicable.
[c] ND, not determined.
[d] Values were corrected by including the theoretical amount of ammonium assimilated in the biomass. For 100 mg (dry weight) of biomass, biomass ammonium equivalents were assumed to approximate 1 mmol (on the basis of a cell N content of 14% [46]).

TABLE 4. Effect of nitrate on enzyme activities in cell extracts of *C. thermoautotrophicum*

Enzyme activity	Sp act (U/mg)	
	Cultures without nitrate	Cultures with nitrate
Formate dehydrogenase	6.8	3.6
Formyltetrahydrofolate synthetase	1.1	2.0
Methylenetetrahydrofolate dehydrogenase	2.8	0.8
Methylenetetrahydrofolate reductase	3.8	2.3
CO dehydrogenase	7.0	7.1
Acetyl-CoA synthase	0.5	0.3
Hydrogenase	5.4	0.4

extract]). Nitrate dissimilation to ammonium is energetically more favorable than acetogenesis (for $NO_3^- + 2H^+ + 4H_2 \rightarrow NH_4^+ + 3H_2O$, $\Delta G^{\circ\prime} = -600$ kJ per reaction [56], whereas for $2CO_2 + 4H_2 \rightarrow CH_3COO^- + H^+ + 2H_2O$, $\Delta G^{\circ\prime} = -95$ kJ per reaction [22]). In addition, the higher redox potential of nitrate compared with that of CO_2 (note that the redox potential of the nitrate-nitrite half-cell is +0.42 V [39] while that of the CO_2-acetate half-cell is −0.34 V [23]) may account for the ability of these acetogens to utilize propanol and ethanol under nitrate-dissimilating conditions. Relative to the in situ environmental roles of acetogens, it is noteworthy that an acetogen can engage such a high-redox-potential terminal electron acceptor. In this regard, of the acetogens evaluated, only *C. thermoautotrophicum* and *C. thermoaceticum* dissimilated nitrate. Although this capacity appeared to be restricted to these two acetogens, other acetogens engage alternative electron-accepting processes, such as the dissimilation of fumarate, aromatic acrylates, or pyruvate; indeed, many, if not most, acetogens are not strictly dependent on the acetyl-CoA pathway for the conservation of energy (1–3, 14, 42–44, 57).

Although the specific electron carriers engaged in the dissimilation of nitrate are not resolved, such high-redox-potential electron carriers as menaquinone and rubredoxin have been previously identified in the membranes of *C. thermoautotrophicum* (31, 38). The metabolic roles of these carriers are less than certain from existing data (38). The nitrate-specific electron transport system would conceivably alter the capacity of the cell to reduce methylenetetrahydrofolate to methyltetrahydrofolate (catalyzed by methylenetetrahydrofolate reductase), the terminal ion-translocating step in acetogenic electron transport-mediated phosphorylation (12, 38, 45).

On the basis of resting-cell studies, the capacity of cells to dissimilate nitrate was not constitutive, suggesting that either nitrate-dissimilating enzymes or nitrate-specific electron carriers were induced or strongly regulated at the protein level. Different respiratory components are expressed in response to electron acceptor availability in denitrifying (e.g., *Paracoccus denitrificans*) and nitrate-dissimilating (e.g., *Escherichia coli*) bacteria (53). Both the type and levels of cytochromes in the membranes of the acetogen *Sporomusa ovata* are regulated by the substrate (i.e., origin of reductant) used during growth (32).

Nitrate dissimilation resulted in a complete loss of the autotrophic and most C_1-assimilating potentials of *C. thermoautotrophicum* and *C. thermoaceticum*. The formation of significant amounts of formate by initial transfers into nitrate-supplemented, H_2-CO_2 basal medium indicated that the acetyl-CoA pathway was blocked directly or indirectly by nitrate subsequent to the reduction of CO_2 to formate. Simultaneous availability of CO and O-methyl groups alleviated the nitrate-dependent inhibition of autotrophic growth, indicating that preformed carbonyl- and methyl-level C_1 units bypassed the nitrate-dependent block of the acetyl-CoA pathway. Methyl groups from phenylmethylethers enter the acetyl-CoA pathway via methyltetrahydrofolate (4, 33, 54), and CO can enter the pathway directly at the carbonyl level (12, 50). That preformed carbonyl- and methyl-level C_1 units bypassed the nitrate block of carbon assimilation provided additional evidence that acetyl-CoA synthase was indeed present and active under nitrate-dissimilating basal conditions. These results also provided evidence that inducible, broad-specificity *O*-demethylase (11, 33) was functional in nitrate-dissimilating, O-methyl group-cultivated cultures.

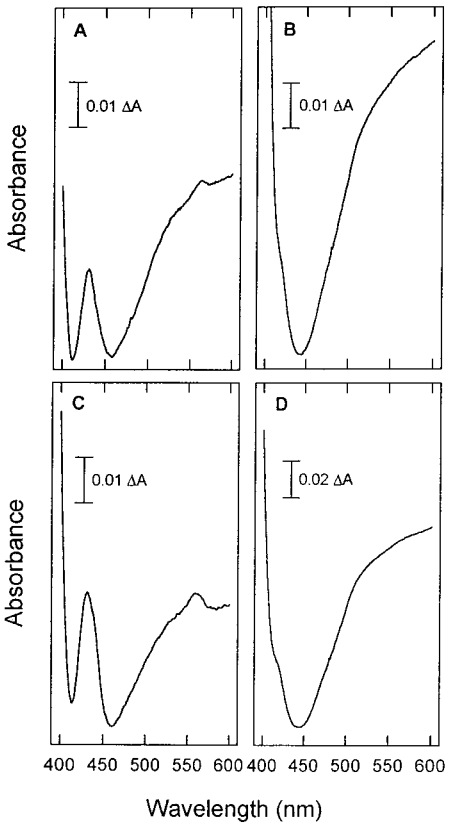

FIG. 3. Redox difference spectra of *C. thermoautotrophicum* membranes obtained from cells cultivated in undefined medium with methanol (A and B) or CO (C and D). Cultivation was in the absence (A and C) or presence (B and D) of nitrate.

Cytochrome *b* was essentially absent in membranes from nitrate-dissimilating cells. This change in otherwise acetogenic cells was coincident with the loss of the cell's capacity to reductively synthesize acetate from CO_2. Numerous studies indicate that many of the catalysts and electron acceptors thought to be central to acetogenesis are localized or associated with the membrane (30, 31, 38). Membranous cytochrome *b* is conceptualized as facilitating the electron flow of methylenetetrahydrofolate reductase (which catalyzes the reductive conversion of methylenetetrahydrofolate to methyltetrahydrofolate [38]), and we hypothesize that the loss of membranous cytochrome *b* alters this step in the acetyl-CoA pathway. However, additional steps that may or may not be affected by the loss of cytochrome *b* also appear to be altered. In particular,

nitrate-dissimilating cells appeared to be unable to reduce CO_2 to the carbonyl level; exogenous CO bypassed this block. Hydrogenase specific activity was reduced 10-fold under nitrate-dissimilating conditions; this factor might have accounted for the apparent inability of cells to reduce CO_2 to the CO level. In addition, the electron transport system for hydrogenase and CO dehydrogenase (cytochrome *b*?) might have also been disrupted by nitrate, or the more favorable energetics of nitrate reduction to nitrite (0.42 V [39]) by H_2 may have disallowed the reduction of CO_2 to CO (-0.52 V [23]). Although the H_2 uptake role of hydrogenase during H_2-dependent autotrophic growth is obvious, the role of hydrogenase during heterotrophic acetogenesis is less than certain (10, 18, 34, 41). An open question is whether hydrogenase (i.e., the enzyme that is assayed as hydrogenase) is involved in the flow of reductant in the formation of the carbonyl carbon under heterotrophic conditions.

The reduction of CO_2-level carbon to the carbonyl level is conceived to be dependent upon reverse electron transport (12). Although enzymes of the acetyl-CoA pathway were present in cell extracts of nitrate-dissimilating cells, it is not known how nitrate potentially influences the membranous association of these catalysts. If their primary in situ function in acetate synthesis is dependent upon their being membrane associated, alteration of this association would clearly influence their functionality in carbon flow. In this regard, it is noteworthy that the acetyl-CoA theoretically formed by CO plus vanillate in nitrate-dissimilating basal medium cultures did not proceed towards acetate but rather was used exclusively for biomass synthesis. In contrast, the acetyl-CoA theoretically formed from glucose under nitrate-dissimilating basal medium conditions (presumably via decarboxylation of pyruvate [16] rather than the reduction of CO_2) yielded acetate (Fig. 2). These results indicate that the cell is able to selectively control the flow of carbon from acetyl-CoA towards either assimilatory or dissimilatory channels.

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REFERENCES

1. **Bache, R., and N. Pfennig.** 1981. Selective isolation of *Acetobacterium woodii* on methoxylated aromatic acids and determination of growth yields. Arch. Microbiol. **130:**255–261.
2. **Beaty, P. S., and L. G. Ljungdahl.** 1990. Thiosulfate reduction by *Clostridium thermoaceticum* and *Clostridium thermoautotrophicum* during growth, abstr. I-7, p. 199. *In* Abstracts of the 90th Annual Meeting of the American Society for Microbiology 1990. American Society for Microbiology, Washington, D.C.
3. **Beaty, P. S., and L. G. Ljungdahl.** 1991. Growth of *Clostridium thermoaceticum* on methanol, ethanol, propanol and butanol in medium containing either thiosulfate or dimethylsulfoxide, abstr. K-131, p. 236. *In* Abstracts of the 91st General Meeting of the American Society for Microbiology 1991. American Society for Microbiology, Washington, D.C.
4. **Berman, M. H., and A. C. Frazer.** 1992. Importance of tetrahydrofolate and ATP in the anaerobic O-demethylation reaction for phenylmethylethers. Appl. Environ. Microbiol. **58:**925–931.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
6. **Cataldo, D. A., M. Haroon, L. E. Schrader, and V. L. Young.** 1975. Rapid colorimetric determination of nitrate in plant tissue by titration of salicyclic acid. Commun. Soil Sci. Plant Anal. **6:**81–90.
7. **Clark, J. E., and L. G. Ljungdahl.** 1984. Purification and properties of 5,10-methylenetetrahydrofolate reductase, an iron-sulfur flavoprotein from *Clostridium formicoaceticum*. J. Biol. Chem. **259:**10845–10849.
8. **Clark, J. E., S. W. Ragsdale, L. G. Ljungdahl, and J. Wiegel.** 1982. Levels of enzymes involved in the synthesis of acetate from CO_2 in *Clostridium thermoautotrophicum*. J. Bacteriol. **151:**507–509.
9. **Daniel, S. L., and H. L. Drake.** 1993. Oxalate- and glyoxylate-dependent growth and acetogenesis by *Clostridium thermoaceticum*. Appl. Environ. Microbiol. **59:**3062–3069.
10. **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.
11. **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.
12. **Diekert, G., and G. Wohlfarth.** 1994. Energetics of acetogenesis from C_1 units, p. 157–179. *In* H. L. Drake (ed.), Acetogenesis. Chapman and Hall, Inc., New York.
13. **Diekert, G., and G. Wohlfarth.** 1994. Metabolism of homoacetogens. Antonie Leeuwenhoek **66:**209–221.
14. **Dorn, M., J. R. Andreesen, and G. Gottschalk.** 1978. Fermentation of fumarate and L-malate by *Clostridium formicoaceticum*. J. Bacteriol. **133:**26–32.
15. **Drake, H. L.** 1982. Demonstration of hydrogenase in extracts of the homoacetate-fermenting bacterium *Clostridium thermoaceticum*. J. Bacteriol. **150:**702–709.
16. **Drake, H. L.** 1994. Acetogenesis, acetogenic bacteria, and the acetyl-CoA "Wood/Ljungdahl" pathway: past and current perspectives, p. 3–60. *In* H. L. Drake (ed.), Acetogenesis. Chapman and Hall, Inc., New York.
17. **Drake, H. L., S.-I. Hu, and H. G. Wood.** 1980. Purification of carbon monoxide dehydrogenase, a nickel enzyme from *Clostridium thermoaceticum*. J. Biol. Chem. **255:**7174–7180.
18. **Drake, H. L., S.-I. Hu, and H. G. Wood.** 1981. Purification of five components from *Clostridium thermoaceticum* which catalyze synthesis of acetate from pyruvate and methyltetrahydrofolate. Properties of phosphotransacetylase. J. Biol. Chem. **256:**11137–11144.
19. **Fawcett, J. K., and J. E. Scott.** 1960. A rapid and precise method for the determination of urea. J. Clin. Pathol. **13:**156–159.
20. **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.
21. **Fröstl, J. M., and H. L. Drake.** 1994. Dissimilatory reduction of nitrate by the acetogen *Clostridium thermoautotrophicum*, abstr. K-22, p. 279. *In* Abstracts of the 94th General Meeting of the American Society for Microbiology 1994. American Society for Microbiology, Washington, D.C.
22. **Fuchs, G.** 1986. CO_2 fixation in acetogenic bacteria: variations on a theme. FEMS Microbiol. Rev. **39:**181–213.
23. **Fuchs, G.** 1994. Variations on the acetyl-CoA pathway in diversely related microorganisms that are not acetogens, p. 507–520. *In* H. L. Drake (ed.), Acetogenesis. Chapman and Hall, Inc., New York.
24. **Gadkari, D.** 1984. Influence of herbicides Goltix and Sencor on nitrification. Zentrabl. Mikrobiol. **139:**623–631.
25. **Gottwald, M., J. R. Andreesen, J. LeGall, and L. G. Ljungdahl.** 1975. Presence of cytochrome and menaquinone in *Clostridium formicoaceticum* and *Clostridium thermoaceticum*. J. Bacteriol. **122:**325–328.
26. **Harrigan, W. F., and M. E. McCance.** 1966. Laboratory methods in microbiology. Academic Press, London.
27. **Heijthuijsen, J. H. F. G., and T. A. Hansen.** 1989. Selection of sulphur sources for the growth of *Butyribacterium methylotrophicum* and *Acetobacterium woodii*. Appl. Microbiol. Biotechnol. **32:**186–192.
28. **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.
29. **Hu, S.-I., E. Pezacka, and H. G. Wood.** 1984. Acetate synthesis from carbon monoxide by *Clostridium thermoaceticum*: purification of the corrinoid protein. J. Biol. Chem. **259:**8892–8897.
30. **Hugenholtz, J., D. M. Ivey, and L. G. Ljungdahl.** 1987. Carbon monoxide-driven electron transport in *Clostridium thermoautotrophicum*. J. Bacteriol. **169:**5845–5847.
31. **Hugenholtz, J., and L. G. Ljungdahl.** 1989. Electron transport and electrochemical proton gradient in membrane vesicles of *Clostridium thermoautotrophicum*. J. Bacteriol. **171:**2873–2875.
32. **Kamlage, B., A. Boelter, and M. Blaut.** 1993. Spectroscopic and potentiometric characterization of cytochromes in two *Sporomusa* species and their expression during growth on selected substrates. Arch. Microbiol. **159:**189–196.
33. **Kasmi, A. E., S. Rajasekharan, and S. W. Ragsdale.** 1994. Anaerobic pathway for conversion of the methyl group of aromatic methyl ethers to acetic acid by *Clostridium thermoaceticum*. Biochemistry **33:**11217–11224.
34. **Kellum, R., and H. L. Drake.** 1984. Effects of cultivation gas phase on hydrogenase of the acetogen *Clostridum thermoaceticum*. J. Bacteriol. **160:**466–469.
35. **Liu, S., and J. M. Suflita.** 1993. H_2-CO_2-dependent anaerobic O-demethylation activity in subsurface sediments and by an isolated bacterium. Appl.

Environ. Microbiol. **59:**1325–1331.
36. **Liu, S., and J. M. Suflita.** 1995. H_2 as an energy source for mixotrophic acetogenesis from the reduction of CO_2 and syringate by *Acetobacterium woodii* and *Eubacterium limosum*. Curr. Microbiol. **31:**245–250.
37. **Ljungdahl, L. G.** 1986. The autotrophic pathway of acetate synthesis in acetogenic bacteria. Annu. Rev. Microbiol. **40:**415–450.
38. **Ljungdahl, L. G.** 1994. The acetyl-CoA pathway and the chemiosmotic generation of ATP during acetogenesis, p. 63–87. *In* H. L. Drake (ed.), Acetogenesis. Chapman and Hall, Inc., New York.
39. **Loach, P. A.** 1976. Oxidation-reduction potentials, absorbance bands and molar absorbance of compounds used in biochemical studies, p. 122–130. *In* G. D. Fasman (ed.), Handbook of biochemistry and molecular biology, 3rd ed., vol. 1. Physical and chemical data. CRC Press, Boca Raton, Fla.
40. **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.
41. **Martin, D. R., L. L. Lundie, Jr., R. Kellum, and H. L. Drake.** 1983. Carbon monoxide-dependent evolution of hydrogen by the homoacetogen-fermenting bacterium *Clostridium thermoaceticum*. Curr. Microbiol. **8:**337–340.
42. **Matthies, C., A. Freiberger, and H. L. Drake.** 1993. Fumarate dissimilation and differential reductant flow by *Clostridium formicoaceticum* and *Clostridium aceticum*. Arch. Microbiol. **160:**273–278.
43. **Misoph, M., S. L. Daniel, and H. L. Drake.** Bidirectional usage of ferulate by the acetogen *Peptostreptococcus productus* U-1: CO_2 and aromatic acrylate groups as competing electron acceptors. Microbiology, in press.
44. **Misoph, M., and H. L. Drake.** 1996. Effect of CO_2 on the fermentation capacities of the acetogen *Peptostreptococcus productus* U-1. J. Bacteriol. **178:**3140–3145.
45. **Müller, V., and G. Gottschalk.** 1994. The sodium ion cycle in acetogenic and methanogenic bacteria: generation and utilization of a primary electrochemical sodium ion gradient, p. 127–156. *In* H. L. Drake (ed.), Acetogenesis. Chapman and Hall, Inc., New York.
46. **Neidhardt, F. C., J. L. Ingraham, and M. Schaechter.** 1990. Physiology of the bacterial cell: a molecular approach, p. 1–29. Sinauer Associates, Inc., Sunderland, Mass.
47. **O'Brien, W. E., and L. G. Ljungdahl.** 1972. Fermentation of fructose and synthesis of acetate from carbon dioxide by *Clostridium formicoaceticum*. J. Bacteriol. **109:**626–632.
48. **Pezacka, E., and H. G. Wood.** 1984. Role of carbon monoxide dehydrogenase in the autotrophic pathway used by acetogenic bacteria. Proc. Natl. Acad. Sci. USA **81:**6261–6265.
49. **Rabinowitz, J. C., and W. E. Pricer, Jr.** 1962. Formyltetrahydrofolate synthetase. J. Biol. Chem. **237:**2898–2902.
50. **Ragsdale, S. W.** 1994. CO dehydrogenase and the central role of this enzyme in the fixation of carbon dioxide by anaerobic bacteria, p. 88–126. *In* H. L. Drake (ed.), Acetogenesis. Chapman and Hall, Inc., New York.
51. **Savage, M. D., and H. L. Drake.** 1986. Adaptation of the acetogen *Clostridium thermoautotrophicum* to minimal medium. J. Bacteriol. **165:**315–318.
52. **Seifritz, C., S. L. Daniel, A. Gößner, and H. L. Drake.** 1993. Nitrate as a preferred electron sink for the acetogen *Clostridium thermoaceticum*. J. Bacteriol. **24:**8008–8013.
53. **Stouthammer, A. H.** 1988. Dissimilatory reduction of oxidized nitrogen compounds, p. 245–303. *In* A. J. B. Zehnder (ed.), Biology of anaerobic microorganisms. Wiley Interscience, New York.
54. **Stupperich, E., and R. Konle.** 1993. Corrinoid-dependent methyl transfer reactions are involved in methanol and 3,4,-dimethoxybenzoate metabolism by *Sporomusa ovata*. Appl. Environ. Microbiol. **59:**3110–3116.
55. **Tanner, R. S., and C. R. Woese.** 1994. A phylogenic assessment of the acetogens, p. 254–269. *In* H. L. Drake (ed.), Acetogenesis. Chapman and Hall, Inc., New York.
56. **Thauer, R. K., K. Jungermann, and K. Decker.** 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol. Rev. **41:**100–180.
57. **Tschech, A., and N. Pfennig.** 1984. Growth yield increase linked to caffeate reduction in *Acetobacterium woodii*. Arch. Microbiol. **137:**163–167.
58. **Umbreit, W. H., R. H. Burris, and J. F. Stauffer.** 1972. Manometric and biochemical techniques. Burgess Publishing Co., Minneapolis.
59. **Uyeda, K., and J. C. Rabinowitz.** 1967. Enzymes of clostridial purine fermentation. Methylenetetrahydrofolate dehydrogenase. J. Biol. Chem. **242:**4378–4385.
60. **Wiegel, J., M. Braun, and G. Gottschalk.** 1981. *Clostridium thermoautotrophicum* species novum, a thermophile producing acetate from molecular hydrogen and carbon dioxide. Curr. Microbiol. **5:**255–260.
61. **Wood, H. G., and L. G. Ljungdahl.** 1991. Autotrophic character of acetogenic bacteria, p. 201–250. *In* J. M. Shively and L. L. Barton (ed.), Variations in autotrophic life. Academic Press, San Diego, Calif.
62. **Zinder, S. H.** 1994. Syntrophic acetate oxidation and "reversible acetogenesis," p. 386–415. *In* H. L. Drake (ed.), Acetogenesis. Chapman and Hall, Inc., New York.