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Ethanol Production by Thermophilic Bacteria: Metabolic Control of End Product Formation in *Thermoanaerobium brockii*

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Specific changes in the chemical and microbial composition of *Thermoanaerobium brockii* fermentations were compared and related to alterations of process rates, end product yields, and growth parameters. Fermentation of starch as compared with glucose was associated with significant decreases in growth rate and intracellular fructose-1,6-bisphosphate concentration and with a dramatic increase in the ethanol/lactate product ratio. Glucose or pyruvate fermentation in the presence of acetone was correlated with increased substrate consumption, growth (both rate and yield), acetate yield, and quantitative reduction of acetone to isopropanol in lieu of normal reduced fermentation products (i.e., H_2, ethanol, lactate). Acetone altered pyruvate phosphoroclastic activity of cell extracts in that H_2, lactate, and ethanol levels decreased, whereas the acetate concentration increased. Glucose fermentation in the presence of exogenous hydrogen was associated with inhibition of endogenous H_2 production and either increased ethanol/acetate product ratios and decreased growth at less than 0.5 atm (51 kPa) of H_2 or total growth inhibition at 1.0 atm (102 kPa). The effects of exogenous hydrogen on glucose fermentation were totally reversed by the addition of acetone. Glucose fermentation in coculture with *Methanobacterium thermoautotrophicum* correlated with increased growth (both rate and yield), acetate yield, and the formation of methane in lieu of monoculture reduced products. In coculture, but not monoculture, *T. brockii* grew on ethanol as the energy source, and acetate and methane were the end products as a direct consequence of hydrogen consumption by the methanogen.

Biotechnological exploitation of anaerobic bacteria for chemical and fuel production necessitates a knowledge of metabolic features that control the rates, yields, and kinds of fermentation products formed by both pure and mixed cultures. Regulation of end product formation in pure cultures of anaerobic bacteria (i.e., intraspecies control) and mixed cultures (i.e., interspecies control) is poorly understood and appears complex, with multiple factors interacting to control the rates and yields of specific product formation. This includes parameters influencing the flow of carbon and electrons in a given metabolic path as well as species tolerance to the fermentation products formed.

The parameters which govern the catabolic electron flux in anaerobic bacteria are determined by thermodynamic considerations and the specific activities of the catabolic enzymes as a unit (7, 8, 10, 12). Thermophilic ethanologenic bacteria examined to date (10, 19) employ a branched pathway and form multiple fermentation products. Ethanol production is greatly influenced by regulation of electron flow at the NAD^+:ferredoxin oxidoreductase level, as exemplified in recent studies of *Clostridium thermocellum* and *Thermoanaerobium brockii* (10). Fermentation of cellobiose by *T. brockii* yields a reduced product ratio of 224 ethanol/20 H_2/352 lactate, whereas for *C. thermocellum* strain LQRI, the ratio is 157 ethanol/286 H_2/24 lactate. In *T. brockii*, ethanol yield is higher as a consequence of electron flow from pyruvate to ethanol via pyruvate:ferredoxin reductase, ferredoxin:NAD (and NADP) reductase, and NADH (NADPH):acetaldehyde reductase. In *C. thermocellum* strain LQRI, ferredoxin:NAD reductase and NADPH:acetaldehyde reductase are not detectable during cellobiose fermentation. In *C. thermocellum*, H_2 yield is higher because of higher hydrogenase activity and the absence of electron flow from reduced ferredoxin or NADPH to lactate or ethanol. Higher lactate yields in *T. brockii* corresponded with higher intracellular levels of FDP, the allosteric activator of lactate dehydrogenase (10), and electron

flow from pyruvate to NADH via ferredoxin: NAD reductase.

General characterization of the catabolic enzyme activities of *T. brockii* suggested that electron flow from pyruvate to fermentation products was interconnected via several different oxidoreductase activities (9, 10). In addition, the reversible NADP-linked alcohol dehydrogenase of *T. brockii* was purified and shown to have a broad substrate specificity that included acetone and other ketones, aldehydes, and secondary alcohols (R. Lamed and J. G. Zeikus, Biochem. J., in press). Control of the metabolism of *T. brockii* will be documented here by showing how different energy sources and exogenous electron donors and acceptors influence in vivo expression of catabolic enzyme activity (i.e., as measured by a change in fermentation products and growth parameters).

MATERIALS AND METHODS

Chemicals. All chemicals were reagent grade. Enzymes and coenzymes were obtained from Sigma Chemical Co., St. Louis, Mo.; N_2, H_2, N_2-CO_2 (95:5), and H_2-CO_2 (80:20) were purchased from Matheson Scientific, Inc., Joliet, Ill., and were passed through heated (310°C) copper filings to remove traces of O_2.

Organisms and cultivation procedures. *T. brockii* neotype strain HTD4 (21) and *Methanobacterium thermoautotrophicum* strain YTB (20) were used in this study. Both strains were isolated from thermal features in Yellowstone National Park and were cultured as previously described (21, 22).

Minimal medium (LPBB medium) contained (per liter of distilled water): NH_4Cl, 1.0 g; $MgCl_2 \cdot 6H_2O$, 0.2 g; KH_2PO_4, 0.30 g; $Na_2HPO_4 \cdot 7H_2O$, 2.0 g; trace mineral solution, 10 ml; 2.5% $FeSO_4$, 0.03 ml; 0.2% resazurin, 1 ml; and vitamin solution, 5 ml (21); 20 ml of 2.5% Na_2S, was added after sterilization. The trace mineral solution contained (grams per liter of distilled water): nitrilotriacetic acid neutralized to pH 6.5 with KOH, 12.8; $FeSO_4 \cdot 7H_2O$, 0.1; $MnCl_2 \cdot 4H_2O$, 0.1; $CoCl_2 \cdot 6H_2O$, 0.17; $CaCl_2 \cdot 2H_2O$, 0.1; $ZnCl_2$, 0.1; $CuCl_2$, 0.02; H_3BO_3, 0.01; $NaMoO_4 \cdot 2H_2O$, 0.01; NaCl, 1.0; and Na_2SeO_3, 0.02. The culture medium gas phase contained N_2. The pH of the medium was 7.2 after autoclaving. Minimal medium was supplemented with 0.1% yeast extract (Difco Laboratories, Detroit, Mich.) and 0.5% glucose unless specified in the text. Complex medium (TYEG) contained LPBB medium supplemented with 1% tryptone (Difco), 0.3% yeast extract, and 0.5% glucose, which was autoclaved separately before addition.

Most growth and metabolic studies were performed in 24-ml anaerobic culture tubes (18 by 142 mm) from Bellco (Bellco Glass, Inc., Vineland, N.J.) that contained 10 ml of medium and were sealed with no. 1 neoprene stoppers. Experiments that employed a H_2 gas phase or methanogens were performed in anaerobic pressure tubes (18 by 142 mm) from Bellco that contained 10 ml of medium and were sealed with a pressure bung and a metal serum stopper crimp.

Metabolic measurements. All growth and metabolic experiments employed duplicate or more anaerobic culture tubes, and individual experiments were duplicated or triplicated. Growth was determined by measuring the increase in turbidity at 540 or 660 nm. Optical density was quantified directly by insertion of the anaerobic culture tubes into a Spectronic 20 (Bausch & Lomb, Inc., Rochester, N.Y.) spectrophotometer. Cell dry weight was determined by filtration of the fermentation broth through a 0.45-μm membrane filter (Millipore Corp., Bedford, Mass.) filter followed by drying overnight at 65°C to a constant weight. Glucose was determined with Statzyme reagent (Worthington Diagnostics, Chicago, Ill.). Fermentation products formed during growth were quantified by gas chromatography and by enzymatic analysis. Gases were analyzed, using the procedures described by Nelson and Zeikus (11). Organic alcohols and acids were determined as described by Zeikus et al. (21). L-Lactic acid was determined, using a standard enzyme assay (1). Preparation and metabolic activity analysis of cell extracts were as described by Lamed and Zeikus (9). Intracellular FDP concentration was determined as described previously (10).

RESULTS

Control of fermentation by energy source(s). The amount of growth and specific fermentation product yields of *T. brockii* in complex medium varied with respect to the kind of energy source(s) fermented (Table 1). Lactate was the major fermentation product with glucose as the energy source, whereas ethanol was the major end product of starch fermentation. The growth rate on starch (5-h doubling time) was approximately one-third of the rate on glucose; starch fermentation was associated with lower intracellular FDP concentrations (4% of glucose alone value) and a threefold-higher ethanol/lactate ratio. Growth of *T. brockii* on Trypticase (BBL Microbiology Systems)-yeast extract alone was slight, but it was significant in the presence of added acetone. Growth was also significant (absorbance of 0.35 at 540 nm [A_{540}]) in acetone-minimal medium supplemented with 0.6% yeast extract but not with Casamino Acids or Trypticase soy broth alone. The specific substrates in yeast extract serving as an electron donor(s) for energy metabolism under these conditions have not been identified. The addition of acetone to glucose complex medium dramatically increased growth and acetate yield but significantly decreased the intracellular FDP concentration (40% of glucose alone value) and the H_2, lactate, and ethanol yields.

Control of fermentation by chemical electron acceptors and donors. Acetone was shown to affect glucose metabolism of *T. brockii* by functioning as a catabolic electron acceptor. The addition of acetone to glucose minimal me-

dium significantly increased growth (both yield and rate), glucose consumption, and acetate yield and resulted in quantitative reduction of acetone to isopropanol (Table 2; Fig. 1). The effect of acetone on end product formation and growth was concentration dependent, and increasing acetone concentrations correlated with higher total end product formation and growth yields. Acetone addition decreased the amount of normal reduced end products (i.e., H_2, ethanol, and lactate) formed in favor of isopropanol production. With 200 mM acetone, hydrogen decreased more significantly than other reduced products. The addition of acetone, regardless of specific concentration, decreased the observed product ratio of lactate formed/total products formed. For example, this ratio was 0.58, 0.39, and 0.18 during glucose fermentation alone or with either 200 or 600 mM acetone, respectively. In separate experiments, the addition of 30 to 50 mM 2-butanone or 2-methylcyclohexanone altered glucose metabolism of *T. brockii* in a manner similar to acetone, except these ketones were reduced to their corresponding alcohols.

Table 3 compares the effect of acetone on pyruvate metabolism in growing cells and in cell extracts. The effect of acetone on pyruvate-dependent growth was the same as observed above for glucose growth, except lactate was not a significant end product. Acetone addition significantly decreased the amounts of H_2, ethanol, and lactate formed during growth and increased the acetate yield. Likewise, acetone altered the flow of electrons in an identical manner when cell extracts decomposed pyruvate.

Experiments were initiated to examine whether hydrogen, at a high partial pressure, could reduce both endogenous and exogenous electron acceptors during saccharide fermentation by *T. brockii*. Figure 1 and Table 4 show the relation of H_2 to glucose metabolism in the presence and absence of acetone. Increasing the partial pressure of H_2 during glucose fermentation resulted in a decrease in the growth rate and yield. Complete growth inhibition was achieved at 1 atm (101 kPa) of hydrogen. Acetone addition reversed the growth inhibition caused by H_2 and increased the specific growth rate. The effect of exogenous H_2 on fermentation product formation also displayed a pronounced

TABLE 1. *Relation of energy sources to growth, [FDP], and fermentation product yields of T. brockii in complex medium*[a]

Addition	Growth (A_{540})	[FDP] (nmol/mg of cells)	End products formed (total μmol per tube)				Ethanol/lactate ratio
			H_2	Ethanol	Acetate	Lactate	
None	0.15		8	33	11	16	2.0
Glucose	1.3	52	11	110	15	162	0.70
Starch	1.0	2	20	215	29	81	2.65
Acetone	0.40		3	3	90	15	0.2
Glucose + acetone	2.3	21	4	15	262	20	0.75

[a] Culture tubes contained TYE medium and 0.5% glucose (250 μmol) or starch (263 μmol of hexose) and 100 mM acetone (1 mmol) where indicated. The experiments were initiated with 0.2 ml of culture grown on TYEG medium and incubated for 12 to 48 h at 65°C.

TABLE 2. *Effect of acetone on glucose metabolism by T. brockii in minimal medium*[a]

Addition (μmol)	Growth (A_{540})	End products formed (total μmol per tube)					Glucose fermented (total μmol)	Acetone consumed (total μmol)	Carbon recovery
		H_2	Ethanol	Acetate	L-Lactate	Isopropanol			
None	0.09	9	6	0	9	0		0	
Acetone (500)	0.15	0	0	25	4	40		40	
Glucose	0.48	21	39	6	93	0	70	0	0.99
Glucose + acetone (200)	0.72	9	30	71	128	94	98	94	1.17
Glucose + acetone (600)	0.85	6	16	139	92	269	123	269	1.0

[a] Culture tubes contained LPBB medium, 0.1% yeast extract, and additions indicated. The glucose concentration was 0.5% (250 μmol). The tubes were inoculated with 0.2 ml of a culture grown on TYEG medium and incubated at 65°C for 20 to 24 h. Carbon recovery represents the ratio of carbon in glucose consumed to carbon in products formed. The amount of CO_2 produced was estimated (i.e., 1 mol of CO_2 per mol of ethanol or acetate).

concentration dependence. An H_2 concentration of 0.3 to 0.5 atm (34 to 51 kPa) in glucose fermentation most noticeably decreased net H_2 formations and increased the ethanol/acetate ratio, whereas at 1.0 atm of pressure, H_2 production was not detectable, and all product yields decreased dramatically. In the presence of acetone, hydrogen consumption occurred that was dependent on the initial H_2 partial pressure. This effect correlated with increased isopropanol formation at higher H_2 partial pressures without a noticeable change in final growth yield.

Control of fermentation by methanogens. In nature and anaerobic digestors, the terminal electron acceptors of microbial fermentative processes are often methanogenic bacteria which scavenge H_2 and form CH_4 as a consequence of their energy-yielding metabolism. Recently, *T. brockii* and *M. thermoautotrophicum* were shown to coexist in the same niche as part of a thermophilic microbial carbon cycle formed in association with volcanic activity (20). Therefore, experiments were initiated to examine the effect of methanogenic activity on the metabolism of *T. brockii*. It is important to note that *M. thermoautotrophicum* has a very limited range (i.e., H_2-CO_2 or CO) of energy sources for growth (5, 22). Figure 2 demonstrates the effect of *M. thermoautotrophicum* on glucose metabolism of *T. brockii*. In coculture, the dramatic increase in growth rate and yield correlated with increased acetate yield and decreased ethanol, lactate, and hydrogen production. Methane was formed in lieu of the normal amounts of reduced monoculture end products (i.e., H_2, ethanol, and lactate).

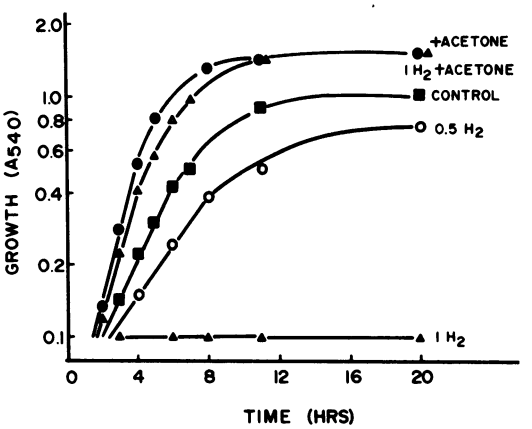

FIG. 1. *Effect of H_2 on T. brockii growth. Pressure tubes contained LPBB medium, 0.1% yeast extract, 0.5% glucose (250 μmol), and an N_2 gas phase. Acetone (700 μmol) or H_2 (0.5 or 1 atm) or both were added as indicated. The tubes were inoculated with 0.2 ml of culture grown on TYEG medium.*

Most interesting, *T. brockii* was able to catabolize substrates in the presence of *M. thermoautotrophicum* that did not serve as energy sources for growth in monoculture. Growth of the coculture on ethanol involved the coupled metabolism of ethanol to acetate and H_2 by *T. brockii* and the conversion of H_2-CO_2 to CH_4 by *M. thermoautotrophicum* (Table 5). The coculture also grew and converted yeast extract alone to acetate and CH_4. Cocultures did not utilize lactic acid as a carbon source for growth, nor did *T. brockii* monocultures grow on ethanol in the presence of chemical electron acceptors, such as acetone.

DISCUSSION

The addition of exogenous electron donors or acceptors or both (e.g., H_2 or acetone) to *T.*

TABLE 3. *Effect of acetone on pyruvate metabolism by T. brockii cultures and cell extracts*

Conditions	Growth (ΔA_{540})	Pyruvate consumed (μmol)	Products formed (total μmol per tube)						Carbon recovery
			H_2	Ethanol	Acetate	Lactate	Isopropanol	CO_2	
Cultures[a]									
Pyruvate									
7 h	0.03	34	1	8	34	<0.5		44	1.23
24 h	0.16	175	7	49	127	11		176	1.07
Pyruvate + acetone									
7 h	0.16	81	<0.5	<0.5	89	<0.5	85	89	1.1
24 h	0.36	251	<0.5	<0.5	285	<0.5	267	285	1.13
Cell extracts[b]									
Pyruvate		ND	1	6	20	12	0	18	
Pyruvate + acetone		ND	<0.1	1	29	1	26	26	

[a] Culture tubes contained LPBB medium, 0.1% yeast extract, pyruvate (570 μmol), and acetone (700 μmol) as indicated. Tubes were inoculated at 65°C and analyzed at 7 and 24 h as indicated.

[b] Enzyme reaction mixtures (1 ml) contained: 0.05 M potassium phosphate (pH 7.1), 400 μmol of pyruvate, 0.1 μmol of coenzyme A, 5 mg of extract protein (0.2 ml), and 500 μmol of acetone as indicated. The products were measured after a reaction time of 1 min at 37°C. ND, Not determined.

TABLE 4. *Effect of H_2 on glucose fermentation products of T. brockii*[a]

Addition	H_2 produced (+) or consumed (−) (μmol)	Total products per tube (μmol)			
		Isopropanol	L-Lactate	Ethanol	Acetate
None	+44		235	166	38
0.3 atm of H_2	+11		205	174	26
0.5 atm of H_2	ND		230	150	16
1.0 atm of H_2	ND		50	20	19
Acetone	+6	495	112	23	316
0.3 atm of H_2 + acetone	−77	508	135	21	300
0.5 atm of H_2 + acetone	−118	552	116	20	287
1 atm of H_2 + acetone	−153	615	164	18	198

[a] Pressure tubes contained LPBB medium, 0.1% yeast extract, 0.5% glucose (250 μmol), and an N_2 gas phase (1 atm). Acetone (700 μmol) or H_2 (0.3 to 1 atm) or both were added as indicated. The tubes were inoculated with 0.2 ml of cells grown on TYEG medium and incubated for 20 to 24 h at 65°C. ND, Not detectable.

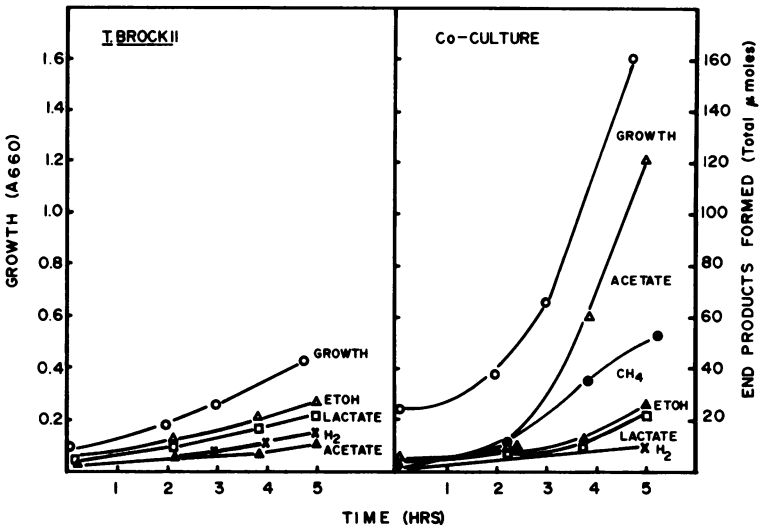

FIG. 2. *Comparison of T. brockii glucose catabolism in mono- and coculture with M. thermoautotrophicum. T. brockii moncultures were grown in pressure tubes that contained 5 ml of TYEG medium and an N_2 gas phase. Coculture experiments were performed as follows: the methanogen was grown to an optical density of 0.18 at 540 nm in pressure tubes that contained 5 ml of TYE medium and 3 atm (XX kPa) of H_2-CO_2 (80:20), the gas phase was replaced with N_2, and 0.5 ml of inoculum of T. brockii and glucose (125 μmol) was added. All experimental tubes were incubated at 65°C without shaking. ETOH, Ethanol.*

brockii saccharide fermentations drastically altered metabolism by control of intraspecies electron flow. The in vivo physiological data presented here support the conclusions of in vitro enzymological studies (10), in which the flow of electrons from energy sources to end products of *T. brockii* is mediated by interconnected oxidoreductases and common electron carriers [i.e., NAD(P) and ferredoxin]. These data (i.e., growth on ethanol) also suggest that the ferredoxin-NAD(P) oxidoreductase activities in *T. brockii* are reversible ince the in vitro analysis of enzymes only demonstrated electron flow from ferredoxin to ethanol, lactate, and H_2 (10). Biochemical relationships that account for the effect of acetone, hydrogen, and *M. thermoautotrophicum* on the metabolism of *T. brockii* are shown in Fig. 3. The addition of hydrogen or chemical and microbial electron acceptors influenced specific enzymatic reactions, and this altered carbon and electron flow. Acetone was reduced by the NADP-linked alcohol dehydrogenase (reaction 5). Electron removal via acetone reduction to isopropanol decreased the amount of normal reduced end products (i.e., H_2, ethanol, and lactate) and increased the metabolic rate and acetate yield. The acetone effect was concentration dependent and could drastically decrease the yield of normal reduced products, probably as a consequence of the higher affinity and activity of the organism's novel pyridine nucleotide-linked alcohol-aldehyde/ke-

tone oxidoreductase towards acetone rather than acetaldehyde (Lamed and Zeikus, in press). In essence, acetone appears to alter the thermodynamics of electron flow during fermentation because of its recognition by the organism's catabolic enzymes. Acetone, at a low concentration, decreased the amount of hydrogen more significantly than it decreased that of lactate or ethanol, probably as a consequence of more favorable thermodynamics associated with the flow of electrons from reduced ferredoxin rather than from NADH to isopropanol. In the presence of acetone, lactate was a less significant product of pyruvate than glucose fermentation because there is less NADH generation and more thermodynamically favorable electron flow.

Exogenous hydrogen was oxidized by hydrogenase (reaction 2), and increasing the H_2 partial pressure led to complete inhibition of H_2 production, altered reduced product yields via reactions 4 to 9, and decreased metabolic rates. Complete growth inhibition by 1 atm of H_2 in the absence but not in the presence of acetone suggests that reduction of common electron carriers [i.e., NAD(P) and ferredoxin] by H_2 is the basis for H_2 inhibition. The observed relationship between H_2 oxidation and acetone reduction to isopropanol during glucose catabolism demonstrated the reversibility of electron flow from H_2 to alcohol. Glucose fermentation in coculture with *M. thermoautotrophicum* indicated that interspecies hydrogen metabolism (reactions 2 and 3) altered electron flow, such that H_2 was formed by electrons generated from reactions 10 and 9, in addition to altering pyru-

TABLE 5. *Fermentation of ethanol by T. brockii and M. thermoautotrophicum*

Culture[a]	Growth (OD_{660})[b]	Ethanol consumed (μmol)	Products formed (total μmol per tube)		
			Acetate	H_2	CH_4
T. brockii	0.07	0	10	9	0
M. thermoautotrophicum	0.01	0	0	0	0
T. brockii + *M. thermoautotrophicum*	0.3	187	200	1.6	167
T. brockii + *M. thermoautotrophicum* (control; no ethanol)	0.1		20	1.8	55

[a] Pressure tubes contained LPBB medium, 0.1% yeast extract, and 0.2% ethanol (435 μmol/tube) as indicated. The gas phase was CO_2-N_2 (5:95) at 1 atm. The coculture experiments were inoculated with 1 ml of coculture grown in the same medium. Monoculture experiments were inoculated with 0.2 ml of a *T. brockii* culture grown on LPBB medium, 0.1% yeast extract, and glucose (0.5%; 250 μmol) or with 0.5 ml of a *M. thermoautotrophicum* culture grown on LPBB medium and 3 atm of H_2-CO_2 (80:20). All experimental tubes were incubated at 65°C for 48 h.

[b] OD_{660}, Optical density at 660 nm.

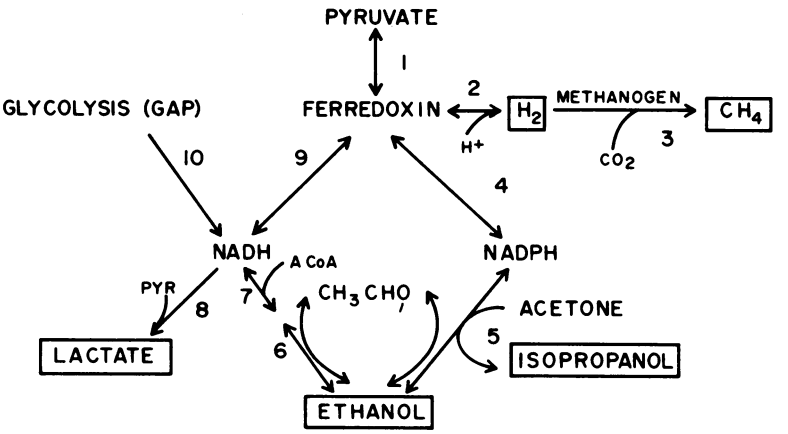

FIG. 3. *Metabolic control of catabolic electron flow by reversible oxidoreductases in T. brockii. The numbers refer to the following enzyme activities: 1, pyruvate ferredoxin oxidoreductase; 2, hydrogenase; 3, "methanogenases"; 4, ferredoxin-NADP oxidoreductase; 5, ethanol-NADP oxidoreductase; 6, ethanol-NAD oxidoreductase; 7, NADH-acetyl coenzyme A oxidoreductase; 8, NADH-pyruvate reductase; 9, ferredoxin-NAD oxidoreductase; and 10, glyceraldehyde-3-phosphate dehydrogenase. ACoA, Acetyl coenzyme A; GAP, glyceraldehyde 3-phosphate. Note that the specific product of enzyme 5 depends on the substrate (i.e., acetaldehyde, ethanol, or acetone).*

vate-ferredoxin reductase activity (reaction 1). Growth of the coculture on ethanol to H_2 via reactions 3, 4 to 7, and 9 occurred, provided that reduced electron carriers were oxidized via methanogen H_2 consumption. This finding is similar to that of studies of the "*Methanobacillus omelianskii*" mixed culture (2, 16) which concluded that growth of the "S-organism" on ethanol was thermodynamically feasible only at the low partial pressures of H_2 maintained by the methanogen. Notwithstanding, the physiological properties and substrate range of *T. brockii* are quite different from those of the S-organism.

The data indicate that regulation of lactate yield in *T. brockii* is complex and that it is controlled by both electron flow and lactate dehydrogenase activity. Lactate yield on starch was lower than that on glucose as a consequence of a lower [FDP] level caused by slower metabolic activity. On the other hand, lactate yield was lower on glucose-acetone, probably as a consequence of a lower [FDP] level caused by increased metabolic activity and removal of pyruvate or NADH required for lactate dehydrogenase activity or both. The results discussed above and the inability of *T. brockii-M thermoautotrophicum* cocultures to grow on lactate support the irreversible character of the lactate dehydrogenase of *T. brockii* (10) and the described regulatory properties for this enzyme in lactic acid bacteria (16, 17).

Ultimately, the catabolic enzyme activities of a bacterial species are probably determined by the ecological niche in which the organism evolved and by phylogenetic diversity. In this regard, the metabolic interrelationships demonstrated for *T. brockii* and *M. thermoautotrophicum* are worth mentioning. These species are found associated with anaerobic decomposition of the algal-bacterial biomass formed in thermal features (20). The data support some of the suggested biological role(s) for methanogens in nature (3, 6, 13, 16, 18). Namely, by regulation of interspecies electron flow via H_2 consumption, methanogens can prevent formation of toxic levels of intermediary metabolites (i.e., ethanol, lactic acid, and hydrogen) and increase the rate of organic matter decomposition. These data clearly show that a methanogen can alter electron flow in fermentative bacteria that form lactic acid in high yield, provided that the organism also produces hydrogen via pyruvate dehydrogenase. Also worth mentioning is that methanogen metabolism also increases the substrate range of fermentative bacteria, probably as a consequence of providing a thermodynamically favorable electron-accepting reaction. Thus, in the presence of *M. thermoautotrophicum*, *T. brockii* fermented yeast extract and ethanol.

Knowledge gained from an understanding of catabolic electron flow in *T. brockii* may help explain metabolic phenomena in different anaerobic bacterial species. H_2-dependent growth inhibition of fermentative bacteria that form H_2 is a strain-specific character (10). For example, *Clostridium cellobioparum* (4), *Clostridium thermohydrosulfuricum* (15), and *T. brockii* (10) strains are inhibited but *C. thermocellum* (10, 14) and *Clostridium pasteurianum* (7, 8) strains are not. This may be related to specific differences in the ferredoxin-NAD(P) oxidoreductase activities, as demonstrated in *T. brockii* and *C. thermocellum* strains (10). In this regard, we observed the same general physiological findings on electron control in *C. thermohydrosulfuricum* strain 39E as those reported here for *T. brockii*. The data also suggest that anaerobic bacterial fermentations of readily soluble substrates (e.g., glucose) may be limited by the rate at which reduced intracellular electron carriers (i.e., NADH, NADPH, and ferredoxin) are oxidized. This speculation is based on the findings that the metabolic rate of *T. brockii* was enhanced by the addition of acetone or a methanogen and was inhibited by hydrogen in the absence of an exogenous electron acceptor.

These results may have practical significance for commercial anaerobic bacterial fermentations. Manipulation of fermentation parameters (e.g., energy source and exogenous electron donors-acceptors) that control intraspecies electron flow in ethanologens that form multiple reduced end products can increase ethanol yields in lieu of producing metabolic mutants that lack hydrogenase or lactate dehydrogenase. In general, analysis of metabolic control of intra- and interspecies electron flow in *T. brockii* fermentations suggests that ethanolic saccharide fermentations possess potentially higher product yields (i.e., gram of product formed per gram of substrate fermented) than do methanogenic fermentations but have lower process rates. The major process limitations of bioethanol production with wild-type thermophilic bacterial strains are low ethanol tolerance and final solvent concentrations of $\leq 1\%$ in the fermentation liquor. In methanogenic fermentations, end product tolerance is not a problem, but control of metabolic interactions between methanogenic and non-methanogenic species is often process limiting.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. **Bergmeyer, H. U.** 1965. Methods of enzymatic analysis, p. 266–284. Academic Press, Inc., New York.
2. **Bryant, M. P., E. A. Wolin, and R. S. Wolfe.** 1967. *Methanobacillus omelianskii*, a symbiotic association of two species of bacteria. Arch. Mikrobiol. **59**:20–31.
3. **Chen, M., and M. J. Wolin.** 1977. Influence of CH_4 production by *Methanobacterium ruminantium* on the fermentation of glucose and lactate by *Selenomonas ruminantium*. Appl. Environ. Microbiol. **34**:756–759.
4. **Chung, K.-T.** 1976. Inhibitory effects of H_2 on growth of *Clostridium cellobioparum*. Appl. Microbiol. **31**:342–348.
5. **Daniels, L. D., G. Fuchs, R. K. Thauer, and J. G. Zeikus.** 1977. Carbon monoxide oxidation by methanogenic bacteria. J. Bacteriol. **132**:118–126.
6. **Iannoti, E. L., D. Kafkewitz, M. J. Wolin, and M. P. Bryant.** 1973. Glucose fermentation products of *Ruminococcus albus* grown in continuous culutre with *Vibrio succinogenes*: changes caused by interspecies transfer of H_2. J. Bacteriol. **114**:1231–1240.
7. **Jungermann, K., M. Kern, V. Riebeling, and R. K. Thauer.** 1976. Function and regulation of ferredoxin reduction with NADH in *Clostridia*, p. 85–96. *In* H. Schlegel, G. Gottschalk, N. Pfennig (ed.), Microbial production and utilization of gases. Erich Goltz K.G., Gottingen.
8. **Jungermann, K., R. K. Thauer, G. Leimenstoll, and K. Decker.** 1973. Function of reduced pyridine nucleotide-ferredoxin oxidoreductases in saccharolytic clostridia. Biochim. Biophys. Acta **305**:268–280.
9. **Lamed, R., and J. G. Zeikus.** 1980. Glucose fermentation pathway of *Thermoanaerobium brockii*. J. Bacteriol. **141**:1251–1257.
10. **Lamed, R., and J. G. Zeikus.** 1980. Ethanol production by thermophilic bacteria: relationship between fermentation product yields and catabolic enzyme activities in *Clostridium thermocellum* and *Thermoanaerobium brockii*. J. Bacteriol. **144**:569–578.
11. **Nelson, R. D., and J. G. Zeikus.** 1974. Rapid method for the radioisotopic analysis of gaseous products of anaerobic metabolism. Appl. Environ. Microbiol. **28**:258–261.
12. **Petitdemange, H., C. Cherrier, G. Raval, and R. Gay.** 1976. Regulation of the NADH and NADPH-ferredoxin oxidoreductases in clostridia of the butyric group. Biochim. Biophys. Acta **421**:334–347.
13. **Scheiffinger, C. C., B. Linehan, and M. J. Wolin.** 1975. H_2 production by *Selenomonas ruminantium* in the absence and presence of methanogenic bacteria. Appl. Microbiol. **19**:480–483.
14. **Weimer, P. J., and J. G. Zeikus.** 1977. Fermentation of cellulose and cellobiose by *Clostridium thermocellum* in the absence and presence of *Methanobacterium thermoautotrophicum*. Appl. Environ. Microbiol. **33**:289–297.
15. **Wiegel, J., L. G. Ljungdahl, and J. R. Rawson.** 1979. Isolation from soil and properties of the extreme thermophile *Clostridium thermohydrosulfuricum*. J. Bacteriol. **139**:800–810.
16. **Wolin, M. J.** 1976. Interactions between H_2-producing and methane-producing species, p. 141–150. *In* H. Schlegel, G. Gottschalk, N. Pfennig (ed.), Microbial production and utilization of gases. Erich Goltze K. G., Gottingen.
17. **Yamada, T., and J. Carlsson.** 1975. Regulation of lactate dehydrogenase and change of fermentation products in streptococci. J. Bacteriol. **124**:55–61.
18. **Zeikus, J. G.** 1977. The biology of methanogenic bacteria. Bacteriol. Rev. **41**:514–541.
19. **Zeikus, J. G.** 1980. Thermophilic bacteria: ecology, physiology and technology. Enzyme Microb. Technol. **1**: 243–252.
20. **Zeikus, J. G., A. Ben-Bassat, and P. W. Hegge.** 1980. Microbiology of methanogenesis in thermal, volcanic environments. J. Bacteriol. **143**:432–440.
21. **Zeikus, J. G., P. W. Hegge, and M. A. Anderson.** 1979. *Thermoanaerobium brockii* gen. nov. spec. nov. A new caldoactive anaerobic bacterium. Arch. Microbiol. **122**: 41–47.
22. **Zeikus, J. G., and R. S. Wolfe.** 1972. *Methanobacterium thermoautotrophicus* sp. n., an anaerobic, autotrophic, extreme thermophile. J. Bacteriol. **109**:707–713.