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Metabolism of H_2-CO_2, Methanol, and Glucose by *Butyribacterium methylotrophicum*

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The fermentative metabolism of *Butyribacterium methylotrophicum* grown on either H_2-CO_2, methanol, glucose, or CO is described. The following reaction stoichiometries were obtained: 1.00 H_2 + 0.52 CO_2 → 0.22 acetate + 0.06 cell C; 1 methanol + 0.18 CO_2 + 0.01 acetate → 0.24 butyrate + 0.29 cell C; and 1.00 glucose → 0.31 CO_2 + 1.59 acetate + 0.21 butyrate + 0.13 H_2 + 1.58 cell C. Cell yields of 1.7 g (dry weight) per mol of H_2, 8.2 g (dry weight) per mol of methanol, 42.7 g (dry weight) per mol of glucose, and 3.0 g (dry weight) per mol of CO were obtained from linear plots of cell synthesis and substrate consumption. Doubling times of 9.0, 9.0, and 3 to 4 h were observed during batch growth on H_2-CO_2, methanol, and glucose, respectively. Indicative of a growth factor limitation, glucose fermentation in defined medium displayed a lower cell synthesis efficiency than when yeast extract (0.05%) was present. *B. methylotrophicum* fermentation displayed atypically high substrate/cell carbon synthesis conversion ratios for an anaerobe, as greater than 24% of the carbon was assimilated into cells during growth on methanol or glucose. The data indicate that *B. methylotrophicum* conserves carbon-bound electrons during growth on single-carbon or multicarbon substrates.

The catabolic reduction of CO_2 to acetic acid was first reported by K. T. Wieringa in *Clostridium aceticum* (40). During the next 10 years, acetate formation from CO_2 was described in *Clostridium acidi-urici* (8), *Butyribacterium rettgeri* (7), and *Clostridium thermoaceticum* (5). Two additional species of CO_2-reducing, acetate-producing bacteria were characterized in the 1970s: *Clostridium formicoaceticum* (1) and *Acetobacterium woodii* (3, 4). *C. aceticum*, *C. thermoaceticum*, *C. formicoaceticum*, and *A. woodii* can form acetate as the sole fermentation end product and are thus referred to as homoacetogens. The biochemistry of acetate synthesis from CO_2 by anaerobic bacteria has been the subject of general investigation for 40 years (2, 6, 15, 22, 23, 26–28, 30–32, 35, 39). As a result, the homoacetogenic fermentation is to date the most thoroughly described one-carbon transformation pathway operative in anaerobic bacteria.

We recently began studying the physiology of *Butyribacterium methylotrophicum*, a novel anaerobic, acid-producing bacterium that ferments one carbon compounds (24, 43). *B. methylotrophicum* grows on a wide range of substrates including H_2-CO_2, methanol, lactate, pyruvate, glucose, and CO. Butyrate or acetate (or both) was produced from all of these substrates, and CO_2 reduction accompanied acetate production on H_2-CO_2 and methanol (43). *B. methylotrophicum* also grows on a variety of energy sources in the presence of CO, and an adapted strain was selected to grow vigorously on high partial pressures of CO alone (24). The general metabolic features of *B. methylotrophicum* appear similar to those of *Eubacterium limosum* (13, 14, 25, 34).

The present investigation was undertaken to elucidate the stoichiometries, yields, and energetics of H_2-CO_2, methanol, and glucose metabolism. In addition, the efficiency of cell synthesis during growth of *B. methylotrophicum* on heterotrophic and unicarbonotrophic substrates is compared.

MATERIALS AND METHODS

Chemicals and gases. All chemicals were reagent grade. Gases (N_2-CO_2, 95:5 [vol/vol] premixed and H_2-CO_2, 80:20 [vol/vol]) were obtained from Matheson (Joliet, Ill.). Methanol (Nanograde) was purchased from Mallinckrodt (St. Louis, Mo.).

Media preparation and cultivation techniques. Anaerobic cultivation and media preparation techniques were as described previously (24). PB medium, a phosphate-buffered mineral medium with 0.05% yeast extract (24), was routinely used for both maintenance of stock cultures and experiments. Media were prepared with a gas phase of either N_2-CO_2 (95:5 [vol/vol]) or H_2-CO_2 (80:20 [vol/vol]); media prepared with an H_2-CO_2 headspace were supplemented with 1.5 ml

of 0.5 M $NaHCO_3$ after autoclaving. A defined medium (PBD) contained the following (per 990 ml of distilled water): NH_4Cl, 1.0 g; $MgCl_2 \cdot 6H_2O$, 0.2 g; $CaCl_2$, 0.1 g; $NiSO_4 \cdot 6H_2O$, 26 mg; trace mineral solution (44), 10 ml; resazurin (Eastman, 0.2%), 1.0 ml. After autoclaving the medium, a sterile solution containing phosphate buffer (2.4 mol/liter, either pH 7.0 or 7.5) and filter-sterilized pantothenic acid and biotin (10 and 4 mg/liter, respectively) was added (0.1 ml/10 ml of medium) by syringe. Initial fermentation substrate concentrations were as follows: glucose, 10 to 20 mM; methanol–acetate–CO_2, 50 mM, 50 mM, 700 μmol/158-ml vial, respectively; H_2-CO_2, 0.8 atm (ca. 81.04 kPa) of H_2–0.4 atm (ca. 40.52 kPa; total) of CO_2. Conditions for growth of the adapted strain on CO and CO fermentation analyses were as described previously (24).

All experiments were conducted in 158-ml serum vials (Wheaton Scientific, Millville, N.J.) sealed with black butyl stoppers and aluminum crimp caps (Bellco Glass, Inc., Vineland, N.J.). Experimental vials contained approximately 50 ml of medium (including additions) and were incubated vertically at 37°C on a rotary shaker. A 1% inoculum was used to initiate experiments.

Quantification of substrates, products, and growth. Fatty acids, alcohols, lactate, CO_2, and H_2 were analyzed by gas chromatography as described previously (24). Glucose was quantified spectrophotometrically with a hexokinase–NADP-linked glucose 6-phosphate dehydrogenase mixture (Sigma Chemical Co., St. Louis, Mo.) as described previously (9). Formate was assayed spectrophotometrically with cell-free extracts of the CO strain of *B. methylotrophicum* serving as the source of formate dehydrogenase (24).

Culture turbidity at 660 nm (optical density at 660 nm [OD_{660}]) was measured in either a Spectronic 20 spectrophotometer for $OD_{660} > 0.3$ (1.8-cm path length; Bausch & Lomb, Inc., Rochester, N.Y.) or a Gilford 240 spectrophotometer for $OD_{660} < 0.3$ (1-cm path length, Gilford Instruments, Oberlin, Ohio). Samples were diluted to $OD_{660} < 0.5$ before measurement. Dry weight was determined by reference to a standard curve that related OD_{660} to dry weight and which was based on over 20 independent measurements. This curve displayed a linear relationship from 0 to 2.7 OD_{660} with a slope of 344/ml per OD_{660} unit, and it was generated from culture samples taken from glucose-, methanol-, and CO-grown cells. The symbol ± represents standard deviation. Dry weights were determined by collecting cells on membrane filters (0.45 μm; Millipore Corp., Bedford, Mass.) and drying at 60°C until the weight remained constant. Carbon and electron recoveries in metabolites and cells were calculated as previously described (24).

Fermentation balance analysis. Liquid samples (1 ml) were aseptically removed (i.e., to prevent contamination) from experimental vials at the beginning and at the end of experiments; 0.2 ml was used for analysis of soluble compounds, and the remaining volume was used to measure OD_{660}. Gases were sampled via withdrawal of 0.4 cm^3 from the headspace with a syringe equipped with a mininert valve (Supelco, Bellefonte, Pa.). Initial concentrations of H_2 were measured by aseptically sampling the experimental vials. Initial concentrations of CO_2 were determined from control vials which were acidified with 0.25 ml of 12 M HCl, shaken vigorously, and incubated for >15 min before sampling. Final concentrations of gases in experimental vials were determined after acidification, shaking, and incubation. Fermentation balance experiments were designed to be substrate limited to ensure that yields and efficiencies determined at the end of growth were not distorted by uncoupled substrate utilization. Experiments were terminated as soon as the growth substrate was essentially exhausted (~24 h for glucose and less than 3 days for the other substrates).

Fermentation time-course analysis. Quantifications of gases, soluble metabolites, and growth were with 1-ml liquid samples and 0.4-cm^3 gas samples. Vials were held at 37°C in a water bath during sampling of the liquid and gaseous phases. The optical density of cultures used in overall fermentation balances was ~1.3 for glucose, 1.0 for methanol, and 0.3 for H_2-CO_2. In these experiments total CO_2 was calculated after acidification when <0.1% of the CO_2 was dissolved. The total amount of CO_2 present during a fermentation time course was estimated by methods previously cited (39) and according to the fraction of CO_2 dissolved at a given pH and temperature, the Bunsen absorption coefficient for CO_2, and Henry's law. This calculation was not a good absolute measurement for CO_2 during H_2-CO_2 fermentation time-course experiments because total amounts of CO_2 up to 60% lower were obtained when samples were acidified before analysis. H_2-CO_2 vials were pressurized to 1.5 atm (ca. 152.0 kPa) with N_2 when a negative pressure was observed during liquid sampling. CO_2 in the text indicates a mixture of gaseous CO_2 and dissolved CO_2, HCO_3^-, and CO_3^{2-}, whose relative composition is pH dependent.

RESULTS

Fermentation of H_2-CO_2. *B. methylotrophicum* produced acetate and cells during growth on H_2-CO_2 (Fig. 1). H_2 and CO_2 were used in a ratio of approximately 2:1 during growth. A doubling time of 9.0 h was observed during logarithmic growth. Cultures reached an OD_{660} of approximately 0.3 on 0.8 atm of H_2–0.4 atm of CO_2, and growth ceased because of substrate depletion. The medium was buffered with both phosphate and bicarbonate; thus, the final pH (7.2) was only slightly lower than the initial pH (7.4). The final acetate concentration was 16 mM. An $OD_{660} > 0.4$ was obtained when vials were repressurized with H_2-CO_2, and substrate consumption and acetate formation continued even when growth ceased during longer incubations.

Table 1 shows a representative fermentation balance for growth on H_2-CO_2. Trace amounts of butyrate (<0.5 mM) were occasionally detected after growth on H_2-CO_2. A stoichiometry (in micromoles) of 3,260 H_2 + 620 CO_2 → 693 acetate + 18 butyrate + 174 cell C was observed. This reaction displayed a balanced carbon and available electron recovery.

Fermentation of methanol. *B. methylotrophicum* consumed methanol and CO_2 in the pres-

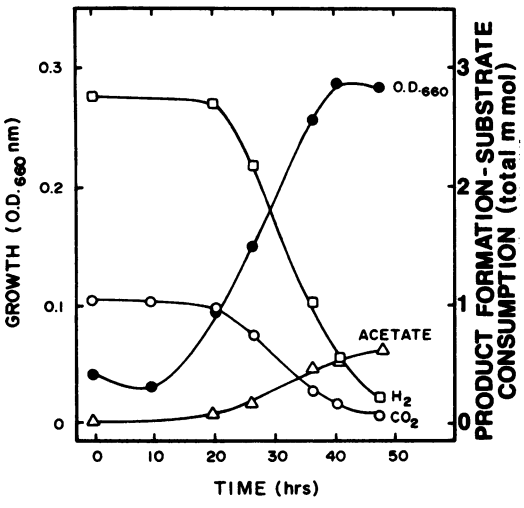

FIG. 1. H_2-CO_2 metabolism time course of *B. methylotrophicum*. The results are averages of quadruplicate 158-ml vials initially containing 51 ml of PB medium, 0.64 atm (ca. 64.9 kPa) of H_2, and 0.3 atm (ca. 30.4 kPa) of CO_2.

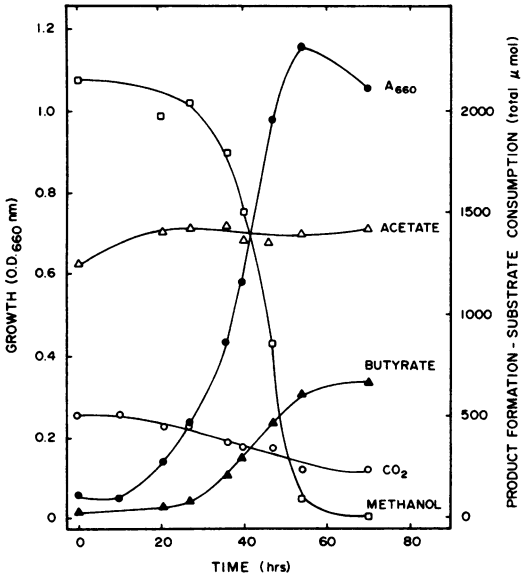

FIG. 2. Methanol metabolism time course of *B. methylotrophicum*. The results are averages of quadruplicate 158-ml vials initially containing 51 ml of PB medium, 48 mM methanol, 56 mM acetate, and 672 μmol of CO_2.

ence of acetate and produced butyrate as an end product (Fig. 2). Cultures grown on methanol-acetate-CO_2 exhibited a doubling time of 9.0 h during the logarithmic phase. The pH changed from an initial value of 7.4 to a final value of 7.0; the final concentration of butyrate was 15 mM.

The change in the acetate concentration during the fermentation of methanol by *B. methylotrophicum* was variable and dependent upon the concentrations of CO_2 and acetate present at the onset of growth. When 50 mM acetate and excess CO_2 (e.g., >700 μmol/158-ml vial) were present initially, the acetate concentration remained relatively constant. Net acetate production (20 to 30 mM) was observed when the initial concentration of acetate was 10 mM with 700 μmol of CO_2 per 158-ml vial. Net acetate consumption (10 to 20 mM) occurred when a low concentration of CO_2 was present initially (220 μmol/158-ml vial) with an initial acetate concentration of 50 mM. Growth was not observed after a 7-day incubation when amounts of CO_2 of ≤150 μmol/158-ml vial were present. *B. methylotrophicum* could be transferred on methanol–CO_2 in the absence of added acetate; however, a lag time of 3 to 5 days preceded the onset of growth.

Table 2 shows a representative fermentation balance for growth on methanol. A stoichiometry (in micromoles) of 2,742 methanol + 544 CO_2 + 197 acetate → 735 butyrate + 823 cell carbon was obtained. This reaction displayed a

TABLE 1. H_2-CO_2 fermentation balance for *B. methylotrophicum*[a]

Concn	H_2 (μmol)	CO_2 (μmol)	Acetate (μmol)	Butyrate (μmol)	Cells (mg)
Initial	3,260	1,665	<10	<5	0.05
Final	<2	45	693	18	4.68
Δ	3,260	1,620	693	18	4.63

[a] Results are averages of triplicate 158-ml vials containing 50 ml of PB medium. The pH dropped from 7.4 to 7.2 over the course of the experiment. Values representing < are equal to the limits of detection for the respective substrates. Carbon recovery: {[693(2) + 174 + 18(4)]/1,620} × 100 = 100%. Available electron recovery: {[693(8) + 174(4.22) + 18(20)]/3,260(2)} × 100 = 101%.

TABLE 2. Methanol fermentation balance for *B. methylotrophicum*[a]

Concn	Methanol (μmol)	Acetate (μmol)	Butyrate (μmol)	CO_2 (μmol)	Cells (mg)
Initial	2,769	2,891	<5	839	0.4
Final	27	2,694	735	295	22.3
Δ	2,742	197	735	544	21.9

[a] Results are averages of quadruplicate 158-ml vials containing 53 ml of PB medium. The pH dropped from 7.2 to 6.9 over the course of the experiment. Carbon recovery: {[735(4) + 823]/[2,742 + 548 + 197(2)]} × 100 = 102%. Available electron recovery: {[823(4.22) + 735(20)]/[2,742(6) + 197(8)]} × 100 = 101%.

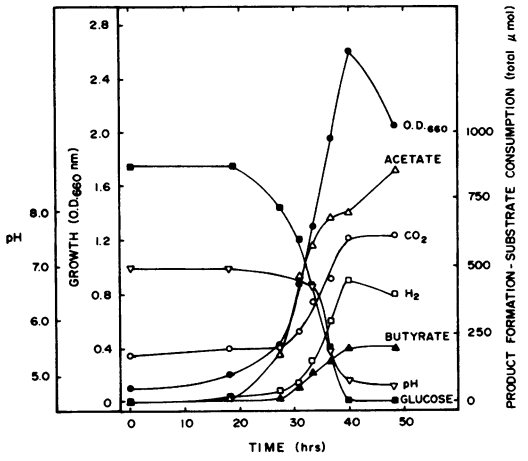

FIG. 3. Glucose metabolism time course of *B. methylotrophicum*. The results are averages of quadruplicate 158-ml vials containing 50 ml of PB medium and 17.2 mM glucose.

balanced carbon and available electron recovery.

Fermentation of glucose. Glucose was metabolized to acetate, butyrate, and H_2-CO_2. Trace amounts (<0.25 mM) of lactate were observed occasionally. Figure 3 shows a glucose fermentation time course. Notably, acetate was the first metabolite to increase; then CO_2, H_2, and butyrate formation paralleled acetate. The pH decreased during the time course from 7.0 to 4.8. Final concentrations of acetate and butyrate were 22 and 5 mM, respectively. Exponentially growing cells exhibited a doubling time of 3 to 4 h. Growth stopped after 40 h, but H_2 consumption and acetate production continued.

Table 3 shows a representative fermentation balance for growth on glucose. A stoichiometry (in micromoles) of 495 glucose → 754 acetate + 112 butyrate + 117 H_2 + 135 CO_2 + 808 cell C was obtained. The low CO_2 and hydrogen yields (<0.3 mol/mol of glucose) are indicative of extensive conservation of the electrons and carboxyl of intermediary pyruvate.

Yields and energetics of *B. methylotrophicum* fermentations. Figure 4 shows a comparison of the cell yields of *B. methylotrophicum* during exponential growth on H_2-CO_2, methanol, glucose, and CO. Cell yields were determined by plots of micrograms (dry weight) of cells produced per micromole of substrate consumed during fermentation time courses. Values obtained by this procedure were 42.7 ± 1.7 g/mol of glucose, 8.2 ± 0.3 g/mol of methanol, 3.0 ± 0.3 g/mol of CO, and 1.7 ± 0.15 g/mol of hydrogen. The data presented in Fig. 4 indicate that substrate consumption and growth were related by a constant factor throughout logarithmic growth. These relationships also suggest balanced growth (i.e., minor growth phase-dependent accumulation or utilization of storage compounds).

The results of multiple trials of fermentation balance experiments with growth of *B. methylotrophicum* on H_2-CO_2, CO, methanol, and glucose in both complex and defined media are presented in Table 4. The mean cell yields determined by endpoint analysis are in close agreement with the cell yields obtained from measurements of cell production and substrate consumption over the course of growth (Fig. 4). With the exception of glucose in defined medium, the mean carbon and electron recoveries obtained were all within 5% of 100% recovery. Notably, growth on glucose in defined medium resulted in reduced acetate production and increased production of butyrate, H_2, and CO_2. The mean yields on glucose in defined and complex medium were 38.0 and 42.0 g/mol, respectively.

Cells were a very significant product in the *B. methylotrophicum* fermentations (Table 4). Greater than 12% of substrate electrons or carbon was assimilated into cells from either H_2-CO_2, CO, glucose, or methanol-acetate-CO_2. The high efficiency of incorporation of substrate carbon and electrons into cells prompted us to look at the extent of conservation of available substrate energy in cells. Payne and co-workers (17, 29) proposed that efficiencies of anaerobic fermentations can be represented by "Y_{kcal}," which is equal to the ratio between the cell yield (grams of cells per mole of substrate) and the energy removed from the medium. It was further

TABLE 3. Glucose fermentation balance for *B. methylotrophicum*[a]

Concn	Glucose (μmol)	Acetate (μmol)	Butyrate (μmol)	H_2 (μmol)	CO_2 (μmol)	Cells (mg)
Initial	495	<10	<5	<2	229	0.9
Final	<5	754	112	117	365	22.4
Δ	495	754	112	117	136	21.5

[a] Results are averages of quadruplicate 158-ml vials containing 50 ml of PB medium. The pH dropped from 7.3 to 6.4 over the course of the experiment. Carbon recovery: {[754(2) + 112(4) + 136 + 808]/[495(6)]} × 100 = 98%. Available electron recovery: {[808(4.22) + 754(8) + 117(2) + 112(20)]/[495(24)]} × 100 = 100%

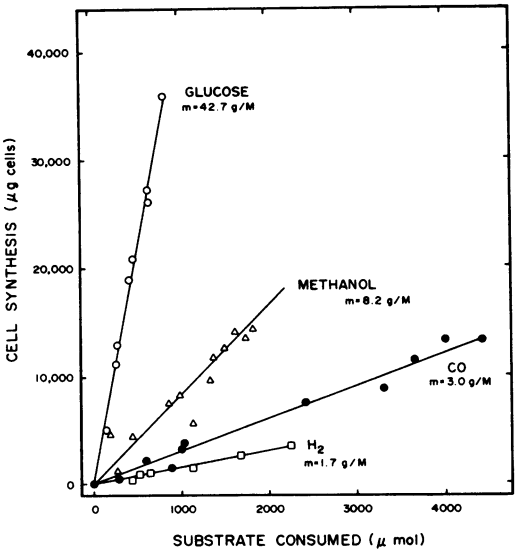

FIG. 4. Relation of cell yield to energy source consumption during logarithmic-phase growth of *B. methylotrophicum*. The results for each substrate are from two separate experiments for each substrate in PB medium performed in triplicate or quadruplicate vials.

suggested by Payne that the energy removed from the medium is equal to the difference between the stoichiometrically weighted heats of combustion of the substrates and products.

The actual thermodynamic driving force in the growth of microorganisms is a function of not only enthalpy effects but also of entropy, including the effect of substrate and end product concentration. Because of the decrease in the product/reactant ratio during growth in batch culture, the free-energy change of converting substrate to product decreases during the time course. In Table 4 the actual free energy change after 50% substrate utilization (ΔG) associated with batch culture was compared with the standard-state free energy change at pH 7 ($\Delta G^{0\prime}$) and the enthalpy change at pH 7.0 ($\Delta H'$). For these fermentations, $\Delta G^{0\prime}$ more nearly approximates the actual thermodynamic driving force than does $\Delta H'$. This is largely because the entropy changes in fermentations involving production or consumption of gas molecules is accounted for in calculating $\Delta G^{0\prime}$, but not $\Delta H'$. Thus, we chose to define an efficiency index in terms of $\Delta G^{0\prime}$ instead of enthalpy.

The standard state thermodynamic efficiencies of cell synthesis from H_2-CO_2, CO, methanol, and glucose are presented in Table 4. These values represent the fraction of free energy conserved as cells divided by the energy available for cell synthesis (i.e., the energy removed from the medium). The mean values observed for the different fermentations varied from 58 to 76; in some cases, the differences in growth efficiency appear to be significant (e.g., CH_3OH versus CO). In defined medium, the efficiency of cell synthesis from glucose was lower, implying a growth factor limitation.

DISCUSSION

Cells were a significant product of *B. methylotrophicum* fermentations. The percentage of substrate carbon and electrons incorporated into cells was always greater than 12% and was more than 24% for CH_3OH or glucose. We expect that cells are probably a significant product in anaerobic fermentations of other organisms, and that standard indexes such as carbon recovery and oxidation-reduction index, often calculated without including cells, would be more accurate if cell production were considered. During growth on one-carbon compounds, the cell yield was correlated with the degree of reduction of the carbon substrate; e.g., the cell yield on methanol (6 available electrons per mol of C) was higher than on CO (2 available electrons per mol of C) which was higher than on H_2-CO_2 (0 available electrons per mol of C). The higher yield on CO than on H_2-CO_2 supports the suggestion that the organism does not necessarily have to oxidize CO to H_2 plus CO_2 before its assimilation into an acetate (i.e., carbon-bound electrons appear conserved). This interpretation is in line with the studies of Hu et al. (18), who demonstrated the direct assimilation of CO into acetyl coenzyme A in cell extracts of *Clostridium thermoaceticum*. The difference in cell yields on CO and H_2-CO_2 could also be due to coupling ATP synthesis to CO oxidation. The higher growth yield of *B. methylotrophicum* on methanol than on CO, together with the similar free energy changes for the fermentation of these substrates, suggests that *B. methylotrophicum* fermentations conserve the carbon electrons in methanol, and an energetic price may have to be paid for the synthesis of reduced carbon compounds. The high cell yield during growth on methanol may be due to direct assimilation of substrate into cell carbon, as has recently been demonstrated in *Methanosarcina barkeri* (19).

The values calculated for the standard state thermodynamic efficiencies of cell synthesis are fairly consistent with the range of 55 to 65% reported for the enthalpic efficiency (i.e., calculated from heats of combustion) of aerobic heterotrophic bacteria grown on multicarbon compounds in minimal media (17, 29). The relative similarity of anabolic efficiency of *B. methylotrophicum* on one-carbon and multicarbon compounds is interesting in that it suggests that the number of carbon-carbon bonds in the substrate

TABLE 4. Stoichiometric, synthetic, and energetic relationships of *B. methylotrophicum* fermentations[a]

Energy source	Substrate-product stoichiometry[b]	Carbon recovery (%)	Electron recovery (%)	Yield (per mole of substrate)				Cell synthesis efficiency[c] (%)	Substrate/cell synthesis conversion ratio (%)	
				Y_s (g of cells)	$\Delta G^{0'}$[d] (kcal)	ΔH[e] (kcal)	ΔG[f] (kcal)		Carbon	Electrons
H_2-CO_2	1.00 H_2 + 0.517 CO_2 + 0.013 $NH_4^+ \rightarrow$ 0.216 CH_3COO^- + 0.002 $CH_3(CH_2)_2COO^-$ + 0.063 $[C_1H_{1.82}O_{0.50}N_{0.21}]$ + 0.231 H^+	97 ± 4	102 ± 2	1.6 ± 0.2	−4.2	−15.0	−3.9	63 ± 9	12	13
CO[g]	1.000 CO + 0.027 $NH_4^+ \rightarrow$ 0.172 CH_3COO^- + 0.001 $CH_3(CH_2)_2COO^-$ + 0.129 $[C_1H_{1.82}O_{0.50}N_{0.21}]$ + 0.497 CO_2 + 0.200 H^+	97 ± 5	97 ± 0	3.5 ± 0.5	−10.8	−16.4	−11.0	58 ± 4	13	27
Methanol	1.000 CH_3OH + 0.183 CO_2 + 0.060 $NH_4^+ \rightarrow$ 0.016 CH_3COO^- + 0.234 $CH_3(CH_2)_2COO^-$ + 0.284$[C_1H_{1.82}O_{0.50}N_{0.21}]$ + 0.31 H^+	105 ± 3	100 ± 1	7.5 ± 0.5	−10.4	−14.2	−8.7	76 ± 3	24	20
Glucose (complex medium)	1.000 $C_6H_{12}O_6$ + 0.332 $NH_4^+ \rightarrow$ 1.593 CH_3COO^- + 0.707 $CH_3(CH_2)_2COO^-$ + 0.155 H_2 + 0.313 CO_2 + 2.132 H^+ + 1.580 $[C_1H_{1.82}O_{0.50}N_{0.21}]$	99 ± 8	100 ± 6	43.0 ± 0.5	−64.9	−41.2	−65.3	74 ± 17[h]	26	28
Glucose (defined medium)	1.000 $C_6H_{12}O_6$ + 0.303 $NH_4^+ \rightarrow$ 1.136 CH_3COO^- + 0.291 $CH_3(CH_2)_2COO^-$ + 0.439 H_2 + 0.593 CO_2 + 1.73 H^+ + 1.44 $[C_1H_{1.82}O_{0.50}N_{0.21}]$	91 ± 5	91 ± 3	38.0 ± 4				59 ± 6		

[a] All values are averages of two to four trials, each consisting of triplicate or quadruplicate vials. The change in pH was approximately 7.4 to 7.2 for H_2-CO_2, 7.2 to 6.9 for methanol, 7.2 to 6.3 for glucose (in both complex [PB] and defined [PBD] medium), and 7.0 to 6.2 for CO.

[b] The cell formula was calculated from the elemental analysis. The quantity of NH_4^+ used was calculated from the quantity of cells made and cell N content. The third decimal place is not significant in all cases.

[c] Standard state thermodynamic efficiency was calculated as follows. For a bacterium growing on substrate A, making reduced products B, C, $D \ldots N$, and cell material Z, growth may be represented by: $aA \rightarrow bB + cC + dD \ldots nN + zZ$, where lowercase letters define the stoichiometric amounts of each component. Efficiency can be calculated by:

$$100 \times \frac{(Z) \times (\Delta G^{0'}_{ox,cells})}{[(a) \times (\Delta G^{0'}_{ox,A})] - [(b) \times (\Delta G^{0'}_{ox,B}) + \ldots (n) \times (\Delta G^{0'}_{ox,N})]}$$

$\Delta G^{0'}_{ox}$ cells = (27.314 kcal/electron equivalent) [(4.22 electron equivalents)/mol of cell C] = 115.26 kcal/mol of cell C. This definition assumes that N remains in the fully reduced state and that logarithmic growth can be represented by a single stoichiometry.

[d] ΔG^0 values are calculated by using standard state free energies of oxidation (ΔG^0) of reaction components to CO_2, H_2O, and NH_4^+ at pH 7 and 25°C. A value of −27.314 kcal per available electron equivalent was used for the ΔG^0 of cells. This value and the $\Delta G^{0'}_{ox}$ of reaction components were provided by R. H. Harris (16) and from unpublished calculations.

[e] $\Delta H'$ values are calculated as is $\Delta G^{0'}$, except that the $\Delta H'_{ox}$ of cells and reaction components was used instead of $\Delta G^{0'}_{ox}$.

[f] $\Delta G = \Delta G^0 + RT \ln (P/R) - 1.36$ (pH 7.0) (H^+ produced per mole reaction); where R is the universal gas constant; T is 298°K; and P/R is the ratio of the concentration of products and reactants, each raised to the power of its stoichiometric coefficient, where the concentrations are those when 50% of the substrate is converted to products.

[g] Results are from reference 24 and a repeat experiment.

[h] Values from individual trials for the efficiency of growth on glucose in defined medium were 64, 65, 75, and 100%; the last value was calculated for a fermentation balance with carbon and electron recoveries of 110%.

has a minor effect on the efficiency of cell synthesis. Although the efficiency of cell synthesis was calculated for complex anaerobic fermentations from relatively few trials, the standard deviation obtained was comparable to that for more conventional growth indexes such as cell yield and carbon recovery. Some of the difference in efficiencies observed for different fermentations may be due in part to different values in carbon recoveries, since higher carbon recovery leads to a higher calculated growth efficiency. Anabolic growth efficiency has received considerable interest in aerobic bacteria (12, 13, 17, 21, 36). We hope that the limited results presented above with *B. methylotrophicum* will generate more interest in the anaerobic cell synthesis efficiency as well.

The fermentation of methanol by *B. methylotrophicum* is unique among described methylotrophic microorganisms in that intermolecular redox reactions involving methanol- and carbon-containing electron acceptors are required for growth. This requirement exists because methanol (6 available electrons per mol of C) is more reduced than either cells (4.21 available electrons per ml of C), or butyrate (5 available electrons per mol of C). The cell yield and doubling time of *B. methylotrophicum* (8.2 g/mol, 9 h) are considerably higher than those reported for *M. barkeri* (3.9 g/mol, 18 h), a methylotrophic methanogen (20, 38). The fermentation of both methanol and glucose appeared more dynamic than fermentation of H_2-CO_2 because multiple electron acceptors (i.e., acetate, CO_2, H^+) can be generated from the oxidation of these substrates. In this regard, the actual ratios of fermentation products formed from both methanol and glucose were related to the levels of different electron acceptors. Thus, acetate was either produced or reduced (i.e., consumed) during growth on methanol, depending on the initial acetate and CO_2 concentrations. Further studies on the path of carbon and the specific enzymes used are required before speculations on the physiological mechanisms that account for the high growth yield of *B. methylotrophicum* on methanol can be stated.

The growth yield of *B. methylotrophicum* on H_2-CO_2 was 1.7 g/mol of H_2; to our knowledge, growth yield data have not been reported for other homoacetogens grown on this substrate (10, 35). The carbon and electron recoveries for fermentation of H_2-CO_2 by *B. methylotrophicum* are consistent with an autotrophic growth mode. Autotrophy has been further substantiated by the development of a defined medium for growth of *B. methylotrophicum* on hydrogen with CO_2 as the sole carbon source (T. Moench and J. G. Zeikus, submitted for publication).

The cell yield observed during the fermenta-

tion of glucose in PB medium (42.7 g/mol of glucose) is consistent with the value of 40 to 50 g/mol of glucose obtained by Andreesen et al. (2) for *C. thermoaceticum* in complex medium. The cell yield observed by Andreesen and co-workers caused them to hypothesize a higher ATP gain per mole of glucose than would be expected based on the pathways of glucose dissimilation and acetate formation known at the time. The apparently high ATP gain of *B. methylotrophicum* on glucose may be due to electron transport-mediated phosphorylation as suggested by Andreesen et al. (1, 2) for *C. thermoaceticum*. However, the recent finding that the transcarboxylation reaction operative in *C. thermoaceticum* produces 2 mol of acetyl coenzyme A from pyruvate and a carrier-bound methyl group (11, 18) invited speculation (24) that this reaction could increase ATP synthesis from glucose via a novel substrate-level phosphorylation. Assuming that this mechanism of substrate-level phosphorylation occurs, we calculate an ATP gain of 2.73 mol of ATP per mol of glucose and a Y_{ATP} value of 15.6 g of cells per mol of ATP, assuming no energy conservation by electron transport-mediated phosphorylation. This value is consistent with the range reviewed by Stouthamer (33) for energy conservation from glucose in complex medium via substrate-level phosphorylation. Thus, the observed yields of *B. methylotrophicum* on glucose alone do not necessarily lead one to hypothesize electron transport-mediated phosphorylation.

Anaerobic bacteria have drawn considerable attention recently as agents of chemical and fuel production from renewable or inexpensive (or both) substrates (41). Wang et al. (37) have considered the potential of *C. thermoaceticum* for use in acetic acid production from glucose. The acetate yield of *B. methylotrophicum* (0.53 g of acetate per g of glucose) is significantly lower than that of *C. thermoaceticum* (0.85 g of acetate per g of glucose). However, butyrate may also be a desirable industrial chemical, and because both acids are derived from the same precursor, production of either can undoubtedly be enhanced by metabolic control or mutation. The maximum doubling time of *C. thermoaceticum* with no pH control (5.8 h) is nearly twice that observed for *B. methylotrophicum*. In addition, the growth rate of *C. thermoaceticum* is reduced to 15% of its maximal value when the pH is lowered from the optimum of 7.1 to 6.9 (37). *B. methylotrophicum* grows logarithmically at its maximal rate on glucose in medium with a pH of $\leq$6.0. The fermentation of methanol and CO_2 to butyrate and H_2-CO_2 or CO to acetate is also interesting from an applied perspective because of the availability of these substrates from pyrolysis of biomass or coal (41).

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