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Differential Metabolism of Cellobiose and Glucose by *Clostridium thermocellum* and *Clostridium thermohydrosulfuricum*

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Clostridium thermohydrosulfuricum consumed glucose in preference to cellobiose as an energy source for growth. The rates of substrate uptake in glucose- and cellobiose-grown cell suspensions were 45 and 24 nmol/min per mg (dry weight), respectively, at 65°C. The molar growth yields (i.e., grams of cells per mole of glucose equivalents) were similar on cellobiose and glucose (19 and 16, respectively). Both glucose- and cellobiose-grown cells contained a glucose permease activity and high levels of hexokinase (>0.34 μmol/min per mg of protein at 40°C). Growth on cellobiose was associated with induction of a cellobiose permease activity. In contrast, *Clostridium thermocellum* metabolized cellobiose in preference to glucose as an energy source and displayed lower growth rates on both substrates. The substrate uptake rates in cellobiose- and glucose-grown cell suspensions were 18 and 17 nmol/min per mg (dry weight), respectively. The molar yields were 38 on cellobiose and 20 on glucose. Extracts of glucose- and cellobiose- grown cells both contained cellobiose phosphorylase and phosphoglucomutase activities, whereas only glucose-grown cells contained detectable levels of glucose permease and hexokinase activities. The general catalytic and kinetic properties of the glucose- and cellobiose-catabolizing enzymes in the two species are described, and a model is proposed to distinguish differential saccharide metabolism by these thermophilic ethanologens.

The basic repeating unit of cellulose, lichenan, laminaran, and other related polysaccharides is cellobiose, a β-1,4-linked glucose dimer (4-*O*-β-D-glucopyranosyl-β-D-glucopyranose). Cellobiose is also the principal hydrolysis product formed by the action of cellobiohydrolase activity present in microbial cellulases (EC 3.2.1.4) (13). In most cellulolytic microorganisms examined, cellobiose is cleaved into glucose by cellobiase (13). However, in cellulolytic bacteria, which include *Cellvibrio gilvus* (34), *Ruminococcus flavefaciens* (5, 6), and *Clostridium thermocellum* (2, 31), cellobiose is converted into glucose 1-phosphate and glucose by cellobiose phosphorylase (EC 2.4.1.20). This enzyme is novel and results in conservation of the β-1,4 bond energy during activation of glucose with inorganic phosphate. Cellulolytic species that contain cellobiose phosphorylase prefer cellobiose to glucose as an energy source for growth (11, 14, 15, 16, 21, 24, 32; R. F. Gomez and P. Hernandez, Abst. Int. Ferment. Symp., 6th, and Int. Symp. Yeasts, 5th, 1980, p. 91). Notably, *C. gilvus* differentially metabolizes the glucose molecules in cellobiose in that the reducing moiety is preferentially converted to CO_2 (14, 15, 34).

Renewed interest in thermophilic saccharide fermentation has been generated because of the potential technological development of active and stable saccharidases and production of chemicals or fuels by direct fermentation of biomass components (37, 38). *C. thermocellum* actively ferments cellulose via extracellular cellulase (24, 25, 30) and forms stable metabolic associations with other anaerobes, including methanogens (36) and ethanologens (23). Recently, endoglucanases (EC 3.2.1.4) which hydrolyze carboxymethyl cellulose or Avicel microcrystalline cellulose have been purified from *C. thermocellum* (26, 29). Also, a cell-bound β-glucosidase that is active on oligodextrins and cellobiose has been purified from *C. thermocellum* (1; N. Creuzet, personal communication).

Previous reports on the ability of *C. thermocellum* to ferment glucose are somewhat contradictory because this metabolic feature appears as a variable result (21, 24, 27, 28, 35; Gomez and Hernandez, Abstr. 6th Int. Ferment. Symp. and 5th Int. Symp. Yeasts, p. 91). We previously described (19) the general enzymatic activities associated with the production of fermentation products by strains of *C. thermocellum* that grow on glucose, cellulose, or cellobiose. Recently, these strains were shown to form stable

cocultures with *Clostridium thermohydrosulfuricum*, a noncellulolytic, saccharolytic species that improves ethanol yields during cellulose fermentation (23). Detailed studies on the saccharolytic metabolism of *C. thermohydrosulfuricum* have not been presented. The present report is in part an attempt to understand the metabolic basis for the stable *C. thermocellum*-*C. thermohydrosulfuricum* coculture and to compare growth and enzyme activities associated with conversion of cellobiose and glucose into phosphorylated hexoses in these two thermophilic, saccharolytic species.

MATERIALS AND METHODS

Chemicals. Analytical coupling enzymes, coenzymes, sodium phosphoenolpyruvate (PEP), NADPH, α-methyl-D-glucose, 2-deoxy-D-glucose, and carbonyl cyanide-*m*-chlorophenyl hydrazone were obtained from Sigma Chemical Co., St. Louis, Mo. D-[*U*-^{14}C] glucose (323 mCi/mmol) was obtained from New England Nuclear Corp., Boston, Mass. 4-*O*-β-D-[*U*-^{14}C] glucopyranosyl-D-glucopyranose (2.7 mCi/mmol) and 4-*O*-β-D-glucopyranosyl-D-[*U*-^{14}C]glucopyranose (0.85 mCi/mmol) were enzymatically synthesized from glucose 1-phosphate and glucose according to the procedure of Swisher et al. (34) as modified by T. K. Ng (Ph.D. thesis, University of Wisconsin, Madison, 1981). *N,N'*-Dicyclohexylcarbodiimide was kindly provided by Jack L. Pate, Department of Bacteriology, University of Wisconsin, Madison. All other chemicals were obtained commercially and used as analytical reagent grade.

Organisms and cultivation conditions. *C. thermocellum* wild-type strain LQRI (23) and *C. thermohydrosulfuricum* wild-type strain 39E (39) were obtained and maintained by the anaerobic culture techniques described previously (23). Experimental cultures were grown without shaking in N_2-gassed 125-ml Wheaton serum bottles or in anaerobic pressure tubes (Bellco Glass, Inc., Vineland, N.J.) that were sealed with butyl rubber bungs and aluminum crimps. Large-scale cultivations of cells for enzymatic and growth yield studies were grown in 14-liter fermentors (New Brunswick Scientific Co., New Brunswick, N.J.) that contained 12 liters of medium each. *C. thermocellum* and *C. thermohydrosulfuricum* cultures were incubated at 60 and 65°C, respectively. GS medium, described previously (23) for growth of *C. thermocellum*, was used throughout this study. Stock cultures of *C. thermocellum* and *C. thermohydrosulfuricum* were maintained on cellulose and xylose, respectively. Stock cultures were grown for one transfer in cellobiose or glucose before initiation of growth experiments with these substrates. Cells were harvested at the late-exponential phase of growth by centrifugation at 12,000 × *g* for 30 min in a Sorvall RC-5 centrifuge (Du Pont Co., Newton, Conn.) equipped with a KSB continuous-flow system.

Growth and fermentation analysis. Growth was determined by measuring the optical density of the culture tubes at 660 nm with a Spectronic 20 colorimeter (Bausch & Lomb, Inc., Rochester, N.Y.). Dissolved and evolved gases, including $^{14}CO_2$, were quantified by the gas chromatography-gas proportional counting system described by Nelson and Zeikus (22). Soluble organic fermentation products were determined by the procedures described by Zeikus et al. (41). L-Lactate was determined enzymatically with lactate dehydrogenase. Glucose was quantified enzymatically (25) by Glucostat (Worthington Diagnostics, Freehold, N.J.); cellobiose was determined by hydrolysis to glucose by almond β-glucosidase (Sigma) and glucose determination by Glucostat.

Cell yield studies were conducted in 12 liters of GS medium with 1% sugar. The pH was maintained at 6.8 by continuous addition of 6 M NaOH. Samples (400 ml of culture) were periodically withdrawn from the fermentor during the exponential phase of growth and were centrifuged at 10,000 × *g* for 15 min. The supernatants were collected for quantification of sugars. The cell pellets were washed in 200 ml of 100 mM potassium phosphate (pH 6.8) and recentrifuged at 10,000 × *g* for 15 min. They were then dried at 100°C and weighed until constant weights were obtained. The cell yield was then determined from the ratio of the rate of sugar consumption to the rate of cell mass production.

Enzymatic analysis. Cell extracts were prepared by suspending 3 g of wet cell paste in 10 ml of 100 mM Tris-hydrochloride buffer (pH 7.6) with 3 mM dithiothreitol and 10 mM $MgCl_2$. This suspension was disrupted by passage through a French pressure cell at 68 MPa. The supernatant was collected from the cell lysate by centrifugation at 30,000 × *g* for 30 min at 4°C. Protein concentration was determined by the method of Lowry et al. (20).

All enzyme activities except that of cellobiose phosphorylase were assayed spectrophotometrically at 40°C by modified standard procedures (8) in 1-ml quartz cuvettes with 1-cm light paths. The formation of cellobiose plus P_i from glucose 1-phosphate and glucose by cellobiose phosphorylase activity was determined as described by Alexander (3). Inorganic phosphate was quantified by the phosphomolybdate-ascorbic acid procedure (17). The composition of the reaction mixtures (1 ml total) used for the following enzymes were: ATP:D-glucose-6-phosphotransferase (hexokinase, EC 2.7.1.1), 100 mM Tris-hydrochloride (pH 7.6), 10 mM $MgCl_2$, 10 μM EDTA, 10 mM glucose, 10 mM ATP, 1 mM NADP, 3 U of glucose 6-phosphate dehydrogenase, and 2 mM dithiothreitol; phosphoglucomutase (EC 2.7.5.1), same as for hexokinase, except ATP was omitted and glucose was replaced with 10 mM glucose 1-phosphate and 30 μM glucose 1,6-bisphosphate; cellobiose phosphorylase, same as for hexokinase, except glucose was replaced with 30 mM cellobiose, 20 mM sodium phosphate (pH 7.6), 3 U of phosphoglucomutase, 3 U of hexokinase, and 30 μM glucose 1,6-bisphosphate. β-D-glucosidase (EC 3.2.1.21) and cellobiase (EC 3.2.1.21) were assayed according to the procedures of Ng and Zeikus (25).

PEP-glucose phosphotransferase activity was assayed by two methods: (i) in vitro PEP-dependent phosphorylation of glucose to glucose 6-phosphate was assayed with the same reaction mixture as above for hexokinase, except ATP was replaced by 10 mM PEP (6); (ii) in vivo PEP-glucose phosphotransferase activity was assayed in toluene-treated cells by the direct assay of Kornberg and Reeves (18). Resting

cells were prepared by growing the culture in serum bottles until the mid-exponential growth phase and harvesting by centrifugation at 3,000 × *g* for 30 min at room temperature with an IEC model K centrifuge (International Equipment Co., Div. of Damon Corp., Needham Heights, Mass.) equipped with a horizontal rotor. The harvested cells inside the culture vessels were washed once with prereduced 50 mM sodium phosphate buffer (pH 7.2) containing 10 mM $MgCl_2$, recentrifuged at 3,000 × *g* for 30 min, and resuspended in the same buffer. The whole process was performed under anaerobic conditions by maintenance of an N_2 headspace inside the culture vessels and addition and removal of reduced fluids via a syringe. Toluene-treated cells were prepared immediately before the assay by syringe injection of 1 ml of cell suspension into a sealed, anaerobic test tube, addition of 10 μl of a toluene-ethanol mixture (1:9, vol/vol), and vigorous blending in a Vortex mixer for 1 min at 4°C. The assay was initiated by the addition of 100 μl of toluenized cells (~100 μg of cells, dry weight) to 0.9 ml of a reaction mixture that contained 100 mM Tris-hydrochloride (pH 7.2), 10 mM $MgCl_2$, 10 mM PEP, 10 mM NADH, 4 U of lactate dehydrogenase, 10 mM glucose, and 2 mM dithiothreitol. Pyruvate was measured spectrophotometrically by lactate dehydrogenase-dependent NADH reduction (8).

Pyridine nucleotide reduction or oxidation reactions were measured spectrophotometrically at 334 nm (extinction coefficient at 334 nm = 6.10 $mM^{-1}cm^{-1}$) with an Eppendorf recording spectrophotometer. Enzyme activities were determined under conditions at which they were linear with time and protein concentration. One unit of enzyme activity is expressed as 1 μmol of substrate converted per min per mg of protein. The apparent affinity for substrate $[S]_{0.5v}$ and enzyme turnover (v_{max}) of individual enzymes were calculated from Lineweaver-Burk plots. Temperature coefficient (Q_{10}) and activation energy (E_a) values were determined from Arrhenius plots. K_i values were obtained from Dixon-Webb plots of $1/v$ verus inhibitor concentration [I] with fixed concentrations of substrate.

Sugar uptake. Resting cells were prepared as described above for the in vivo PEP-glucose phosphotransferase assay except that the cells were suspended the second time in GS medium without a carbon source or toluene treatment. Cell suspensions (5 ml each) were transferred anaerobically to 15-ml vials with 100% N_2 gas phases. The vials were incubated at 60°C (for *C. thermocellum*) and 65°C (for *C. thermohydrosulfuricum*) for 30 min before uptake measurement. The reaction was initiated by the addition of [^{14}C]glucose or 4-*O*-β-D-[^{14}C]glucopyranosyl-β-D-glucopyranose and the inhibitors indicated below. Samples (1 ml) were withdrawn periodically with a 1-ml syringe that was prerinsed in 5% hydrogen peroxide. The sample was rapidly filtered through a Millipore HAWP membrane filter (0.45-μm) and washed twice with 5 ml of 50 mM potassium phosphate buffer (pH 7.2) containing 50 mM LiCl. The filters were placed in scintillation vials with 15 ml of Filter Count scintillation fluid (Packard Instrument Co., Inc., Downers Grove, Ill.) and counted in a Packard model PLD Tri-Carb liquid scintillation spectrometer. The protein content of the cell suspension was determined by incubating 1 ml of the cell suspension with 1 ml of 1 M NaOH at 100°C for 15 min. After neutralization with 1 ml of 1 M HCl, the protein content was determined by the method of Lowry et al. (20).

Incorporation of ^{14}C-labeled compounds. The experiments performed were similar to that described above for uptake studies. Samples (2 ml) were withdrawn every 30 s and filtered through Millipore HAWP membrane filters (0.45-μm). The filters were extracted first with 3 ml of 50% ethanol for 60 min and then twice with 3 ml of 20% ethanol for 30 min each. The fractions were pooled, evaporated to dryness on a Buchi model EL rotary evaporator (Brinkmann Instruments, Chicago, Ill.) at 30°C, suspended in a minimal volume of water, centrifuged for 10 min with an Eppendorf microcentrifuge (Brinkmann), and then stored at −20°C until used.

Separation and autoradiography of ^{14}C-labeled intermediates. Sugars and sugar phosphates were separated and identified by standard thin-layer chromatographic procedures on Ecteola cellulose plates (20 cm by 20 cm by 0.1 mm; EM Reagent, Newhaven, Conn.) impregnated with 0.1 M ammonium tetraborate (pH 9.0). Chromatograms were developed at 25°C in 95% ethanol–0.1 M ammonium tetraborate (pH 9.0) (60:40, vol/vol). The sugar phosphates were made visible with molybdate (ammonium)-perchloric acid spray (33). Autoradiography was performed as described by Daniels and Zeikus (10). Thin-layer plates were dried at 40°C overnight, placed in X-ray exposure holders (36 by 43 cm), and exposed to Kodak SB-5 X-ray film. The films were developed and fixed after being exposed for 14 days. The locations of the unknown sugar phosphates were determined by authentic sugar phosphate standards. The cellulose was then carefully scraped from the radioactive spots on the chromatogram, placed in 5-ml plastic scintillation vials with 5 ml of Filter Count scintillation fluid, and counted as described above.

RESULTS

General metabolic features. The growth properties of *C. thermocellum* and *C. thermohydrosulfuricum* cultured on glucose or cellobiose as an energy source are compared in Table 1. *C. thermohydrosulfuricum* displayed an identical lag period on both substrates, regardless of whether the cell inoculum was grown on cellobiose or glucose. On the other hand, *C. thermocellum* always displayed a much longer lag period when grown on glucose. The molar growth yield on either substrate was nearly equivalent for *C. thermohydrosulfuricum*; however, the growth yield of *C. thermocellum* on cellobiose was nearly double that of *C. thermohydrosulfuricum* or *C. thermocellum* grown on glucose. The growth rate of *C. thermohydrosulfuricum* on either saccharide was faster than that of *C. thermocellum*. Also, growth of *C. thermocellum* on cellobiose was always associated with greater amounts of a yellow soluble pigment that was not formed by *C. thermohydrosulfuricum*.

To understand the basis for the long lag on glucose displayed by *C. thermocellum*, experiments were performed to compare the growth

TABLE 1. General growth parameters of thermophilic anaerobes on glucose or cellobiose

Organism and growth substrate	Lag period (h)	Molar growth yield[a]	Theoretical ATP yield[b]	Cell yield[c]	Growth rate (h^{-1})
C. thermocellum					
Glucose	12–24	20	2.0	0.4	0.3
Cellobiose	3–5	38	3.8	0.5	0.4
C. thermohydrosulfuricum					
Glucose	3–5	16	1.6	0.3	0.7
Cellobiose	3–5	19	1.9	0.3	0.5

[a] Determined as grams of cells (dry weight) per mole of glucose equivalent metabolized.

[b] Calculated on the basis that 1 mol of ATP is equivalent to 10 g of cells (dry weight).

[c] Measured as milligrams of cells (dry weight) per milliliter divided by absorbance at 660 nm.

substrate preferences of the two species in medium that contained both cellobiose and glucose. Regardless of the inoculum energy source, *C. thermohydrosulfuricum* preferentially utilized glucose over cellobiose (Fig. 1). The preference for glucose was confirmed by the more rapid depletion of glucose during growth and the lack of significant $^{14}CO_2$ production from [^{14}C]cellobiose until glucose was nearly exhausted from the medium. *C. thermocellum* displayed an absolute preference for cellobiose when the inoculum energy source was cellobiose but consumed both saccharides simultaneously when the inoculum energy source was glucose (Fig. 2).

C. thermocellum produced glucose during growth in medium with high cellobiose concentration (i.e., ≥2%). At the end of growth, approximately 7% of the total cellobiose consumed accumulated as glucose (Fig. 3). In these experiments, growth paralleled $^{12}CO_2$ production; [^{14}C]glucose was added at 4 h to demonstrate that the glucose inside and outside the cell existed in dynamic equilibrium. (That is, $^{14}CO_2$ produced from [^{14}C]glucose was a result of isotopic exchange, not net metabolism of extracellular glucose.)

Experiments were performed to determine whether *C. thermocellum* differentially metabolized the glucose moieties in cellobiose, as does *C. gilvus*. *C. thermocellum* was grown in cellobiose medium that contained either 4-*O*-β-D-[*U*-^{14}C]glucopyranosyl-β-D-glucopyranose (specific activity of 2460.8 dpm/μmol) or 4-*O*-β-D-glucopyranosyl-D-[*U*-^{14}C]glucopyranose (specific activity of 3034.8 dpm/μmol). Notably, the specific activities of the carbon dioxide produced (average of 208 dpm/μmol) during the fermentation of cellobiose labeled at the nonreducing glucose moiety were similar to that of the carbon in cellobiose (specific activity of 205 dpm/μmol). The specific activities of the carbon dioxide evolved (average of 262 dpm/μmol) from the reducing end of cellobiose were also comparable to that of the substrate (specific activity of 253 dpm/μmol).

Glucose and cellobiose activation enzymes. To identify a biochemical basis for the differential substrate metabolism described above, we examined the enzymatic activities associated with

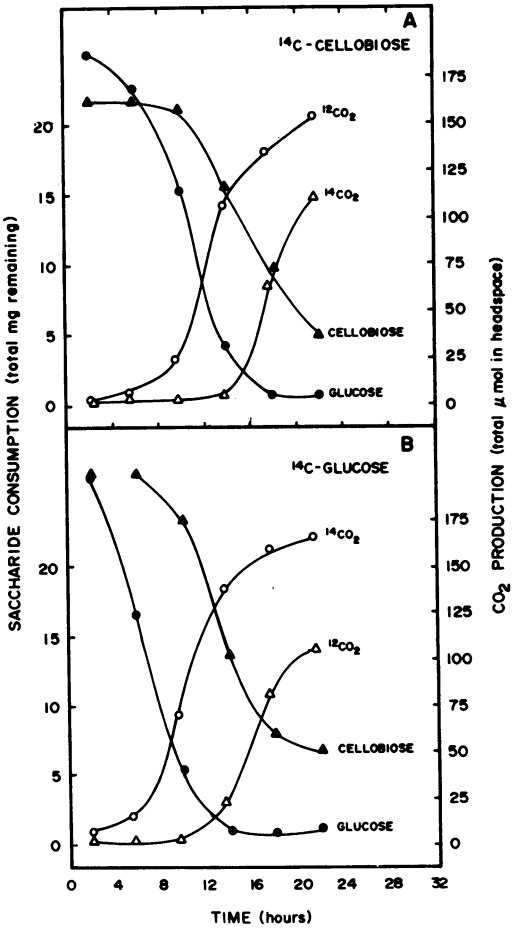

FIG. 1. Combined-substrate metabolism of *C. thermohydrosulfuricum* on glucose and cellobiose. Anaerobic culture tubes contained 10 ml of GS medium with 0.2% glucose and 0.2% cellobiose and were incubated at 65°C without shaking. (A) Tubes contained 1 × 10^6 dpm of [^{14}C]cellobiose labeled at the reducing end and were inoculated with cells grown on glucose. (B) Tubes contained 3 × 10^6 dpm of [*U*-^{14}C]glucose and were inoculated with cells grown on cellobiose. $^{14}CO_2$ was calculated from specific activity measurements and represents the total amount of CO_2 derived from the unlabeled and ^{14}C-labeled substrate indicated. $^{12}CO_2$ represents the total amount of CO_2 produced from the unlabeled substrate.

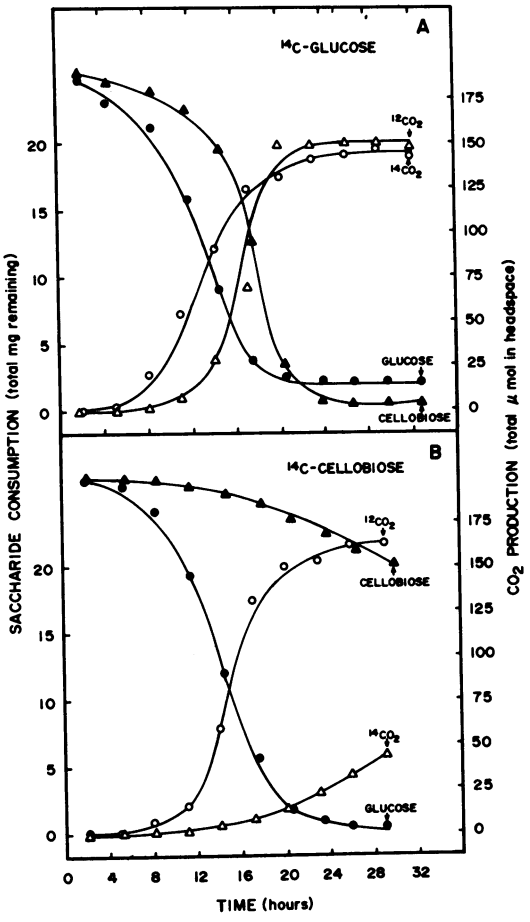

FIG. 2. Combined-substrate metabolism of *C. thermocellum* on glucose and cellobiose. Anaerobic culture tubes contained 10 ml of GS medium with 0.2% glucose and 0.2% cellobiose and were incubated at 60°C without shaking. (A) Tubes contained 1×10^6 dpm of [^{14}C]cellobiose labeled at the reducing end and were inoculated with cells grown on glucose. (B) Tubes contained 3×10^6 dpm of [*U*-^{14}C]glucose and were inoculated with cells grown on cellobiose.

conversion of saccharides to glucose 6-phosphate in both species grown on either glucose or cellobiose (Table 2). This analysis revealed that *C. thermohydrosulfuricum* differed from *C. thermocellum* in that it contained hexokinase and cellobiase when grown on either substrate but lacked cellobiose phosphorylase. Notably, in *C. thermocellum* extracts, hexokinase was not detectable in cellobiose-grown cells, and low levels of β-glucosidase activity but not cellobiase were detectable. The forward reaction of cellobiose phosphorylase (i.e., cellobiose + $P_i \rightarrow$ glucose 1-phosphate + glucose) was eight times slower than the backward reaction, which had a rate of 0.82 μmol/min per mg of protein.

TABLE 2. Enzyme activities involved in conversion of saccharides to glucose 6-phosphate by thermophilic anaerobes[a]

Enzyme	Sp act (μmol/min per mg of protein at 40°C)			
	C. thermocellum		*C. thermohydrosulfuricum*	
	Glucose	Cellobiose	Glucose	Cellobiose
Hexokinase	0.17	<0.01	0.59	0.34
Cellobiase	<0.001	<0.001	0.1	0.1
β-Glucosidase	0.01	0.06	0.3	0.3
Cellobiose phosphorylase	0.04	0.10	<0.001	<0.001
Phosphoglucomutase	0.24	0.66	0.15	0.52

[a] The enzyme activities were determined in extracts of glucose- or cellobiose-grown cells as described in the text. Cellobiase was assayed as glucose released from cellobiose; β-glucosidase was measured as liberation of *p*-nitrophenyl-β-D-glucoside. The data represent the average of four independent determinations from different cell extracts.

To understand the mechanism of cellobiose metabolism in *C. thermocellum*, we examined the kinetic properties of cellobiose phosphorylase, hexokinase, and phosphoglucomutase (Table 3). Cellobiose phosphorylase activity was strongly influenced by the presence of hexokinase. In the absence of externally added hexokinase, the rate of cellobiose phosphorolysis was

TABLE 3. Enzyme kinetic properties for conversion of cellobiose to glucose 6-phosphate in *C. thermocellum*[a]

Enzyme	E_a (kJ)	Q_{10}	(ν_{max} μmol/min per mg of protein)	$[S]_{0.5v}$[b] (mM)	K_i (mM)	K_{act} (mM)
Cellobiose phosphorylase	66.5	2.47	0.25	0.26	0.31[c]	5.17[d]
Hexokinase	27.2	1.57	0.29	0.33	ND	ND
Phosphoglucomutase	ND	ND	0.66	0.27	ND	0.012[e]

[a] Enzyme activities were determined at 40°C in cell extracts of *C. thermocellum* grown on cellobiose, except for hexokinase which was determined in glucose-grown cells. E_a, Activation energy; Q_{10}, temperature coefficient; ν_{max}, enzyme turnover; K_i, inhibitor constant; K_{act}, activator constant; ND, not determined.

[b] Cellobiose, glucose, and glucose 1-phosphate were the respective substrates for cellobiose phosphorylase, hexokinase, and phosphoglucomutase.

[c] Arsenate was the inhibitor.

[d] Phosphate was the activator.

[e] Glucose 1,6-bisphosphate was the activator.

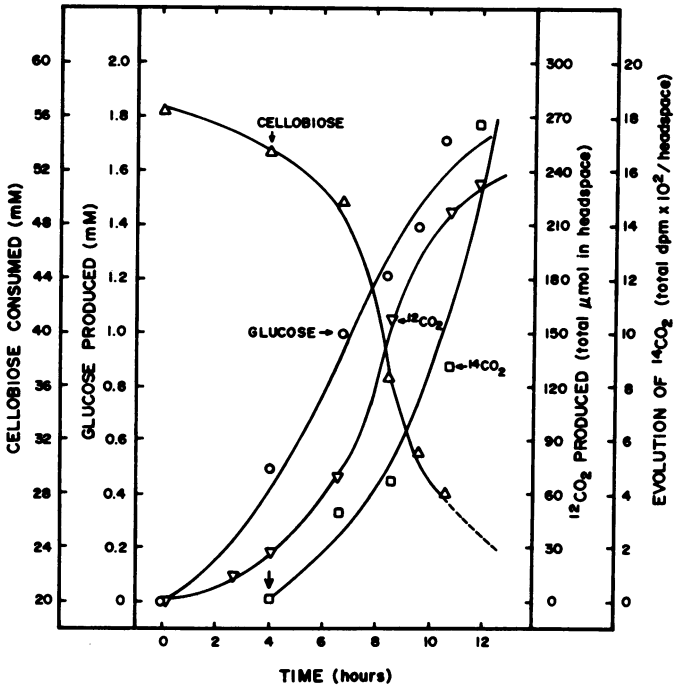

FIG. 3. Single-substrate metabolism of *C. thermocellum* on excess cellobiose. Anaerobic culture tubes contained 10 ml of GS medium with 2% cellobiose. Experiments were performed with cells grown on cellobiose and incubated at 60°C without shaking. Arrow indicates the time at which 1×10^6 dpm of [*U*-^{14}C]glucose (specific activity of 323 mCi/mmol) was added.

0.01 μmol/min per mg of protein. The forward reaction of cellobiose phosphorylase (cellobiose + $P_i \rightarrow$ glucose 1-phosphate + glucose) was totally dependent on the phosphate concentration. Activity was totally inhibited by 10 μM glucose in the absence of added hexokinase. Since the K_i for arsenate, an analog of phosphate, was 17 times lower than the $[S]_{0.5v}$ for phosphate, arsenate was an effective, competitive inhibitor of the enzyme. Linear relationships were observed between 40 and 60°C for cellobiose phosphorylase and hexokinase activities in Arrhenius plots. Glucose 1,6-bisphosphate served as a high-affinity activator for phosphoglucomutase activity.

To analyze the mechanism of cellobiose-derived glucose metabolism, cell suspensions were incubated for 0.5 to 8 min with either [^{14}C]glucose or [^{14}C]cellobiose differentially labeled in the glucose moieties, and then the distribution of label in sugar phosphates was determined. Glucose 6-phosphate, glucose 1-phosphate, and fructose 6-phosphate were significantly (>11% in each) labeled with either [^{14}C]glucose or [^{14}C]cellobiose, whereas fructose 1,6-bisphosphate constituted only a small proportion (<5%) of the total radioactivity at 0.5 min. With [^{14}C]glucose as the substrate, glucose 6-phosphate constituted the highest percentage of total labeled compounds at all incubation times. The cellular intermediates formed were differentially labeled by the two glucose moieties of cellobiose. Most notably, [^{14}C]glucose was not detected from 4-*O*-β-D-[^{14}C]glucopyranosyl-β-D-glucopyranose, indicating the absence of cellobiase, and [^{14}C]glucose 6-phosphate accounted for 21% of the label recovered after 0.5 min of incubation.

Sugar uptake systems. In view of the different sugar activation enzymes expressed during differential substrate metabolism by the two species, experiments were performed to compare the uptake of cellobiose and glucose by cell suspensions (Table 4). Only cell suspensions of glucose-grown *C. thermocellum* incorporated glucose, whereas cellobiose was incorporated by cells grown on either substrate. On the other hand, the glucose uptake system in *C. thermohydrosulfuricum* was present in both glucose- and cellobiose-grown cells and was three to four times more rapid than that of *C. thermocellum*. The ability to transport cellobiose was present in cellobiose-grown cells of *C. thermohydrosulfuricum* but was not detectable in glucose-grown cells.

The effects of energy uncouplers and inhibitors on the initial rate of sugar uptake is shown in Table 5. α-Methyl-β-D-glucose at 3.3 mM did

TABLE 4. Kinetic properties of glucose and cellobiose uptake in *C. thermocellum* and *C. thermohydrosulfuricum*[a]

Organism and growth substrate	v_{max} (nmol/min per mg [dry wt])		K_m (μM)	
	Glucose uptake	Cellobiose uptake	Glucose uptake	Cellobiose uptake
C. thermocellum				
Glucose	17	*	990	*
Cellobiose	ND	18	ND	136
C. thermohydrosulfuricum				
Glucose	45	ND	644	ND
Cellobiose	47	24	731	292

[a] Uptake studies were initiated by the addition of appropriate labeled substrate to 5 ml of cell suspension (1 μg of cells [dry weight] per μl). Samples were withdrawn periodically, and the incorporation of radioactivity was determined. ND, Not detectable (i.e., ≤0.01 nmol/min per mg of cells); *, too low (i.e., <3.3 nmol/min per mg of cells) to measure kinetic properties.

not affect the uptake rate of sugars in either thermophile, but 2-deoxyglucose, notably, inhibited the cellobiose uptake rate more than the glucose uptake rate in *C. thermocellum*. Oxygen at 1% saturation level exerted a complete inhibition on the sugar uptake rates in both microorganisms. In contrast, carbonyl cyanide-*m*-chlorophenyl hydrazone, an uncoupler for oxidative phosphorylation, was not an inhibitor. *N,N'*-Dicylohexylcarbodiimide, an inhibitor for membrane-bound ATPase, significantly reduced the sugar uptake rates in both organisms, but sodium azide and sodium cyanide at 164 μM had no effect. Sodium fluoride, which inhibits certain glycolytic enzymes of the Embden-Meyerhof-Parnas pathway, decreased the sugar uptake rates in both microorganisms by approximately 50%. Sodium arsenate, an analog of phosphate, completely inhibited cellobiose but not glucose uptake by *C. thermocellum*, whereas it had less effect on sugar transport in *C. thermohydrosulfuricum*.

DISCUSSION

A proposed model to explain the differential metabolism of glucose and cellobiose by the two thermophilic anaerobes is shown in Fig. 4. Glucose metabolism is constitutive in *C. thermohydrosulfuricum*, whereas cellobiose metabolism is constitutive in *C. thermocellum*. Thus, *C. thermocellum* appears to be novel, because glucose is a general catabolite repressor in most other microorganisms examined. The two species also significantly differ in the enzyme activities responsible for cellobiose metabolism in that *C. thermohydrosulfuricum* utilizes cellobiase and hexokinase, but *C. thermocellum* utilizes cellobiose phosphorylase and phosphoglucomutase. Although hexokinase was not measured by conventional methods in cellobiose-grown cell extracts of *C. thermocellum*, evidence of a hexokinase-type activity was provided by the detection of [^{14}C]glucose 6-phosphate from [^{14}C]cellobiose. This activity may be a key regulatory enzyme(s) that couples the phosphorolytic cleavage of cellobiose with glucose phosphorylation in the cell membrane and is required for cellobiose phosphorylase activity, because the reaction equilibrium of cellobiose phosphorylase strongly favors cellobiose formation (2, 3). The conversion of either glucose moiety in cellobiose to glucose 6-phosphate is followed by further degradation via the Embden-Meyerhof-Parnas pathway (40). It is worth noting here that *C. thermocellum* differs from *C. gilvus*, which differentially metabolizes the different glucose moieties of cellobiose to CO_2 (34).

The data suggest that glucose is transported by both species in a similar fashion but that the mechanism of cellobiose uptake differs. The exact nature of the saccharide transport mechanism was not delineated by this study; however, the experimental results with transport inhibitors and uncouplers suggest that it involves

TABLE 5. Effect of inhibitors, uncoupling agents, or glucose analogs on the initial rate of [^{14}C]cellobiose or [^{14}C]glucose uptake by cell suspensions of thermophilic anaerobes[a]

Compound	Concn (μM)	Inhibition of uptake rate (%)			
		C. thermocellum		*C. thermohydrosulfuricum*	
		Glucose	Cellobiose	Glucose	Cellobiose
α-Methyl-D-glucose	3,300	0	0	0	0
2-Deoxyglucose	3,000	46	73	35	32
Oxygen	1%	100	100	100	100
CCCP[b]	100	0	0	0	0
DCCD[c]	10	87	55	54	46
Sodium fluoride	164	59	48	41	52
Sodium arsenate	32	53	100	23	36
Sodium cyanide	164	0	0	0	0
Sodium azide	164	0	0	0	0

[a] The reaction mixture (5 ml total) consisted of 5 mg of cells (dry weight) in 50 mM sodium phosphate buffer (pH 7.2) with 10 mM $MgCl_2$, plus substrate and inhibitor. [^{14}C]glucose uptake was measured in glucose-grown cells; [^{14}C]cellobiose uptake was measured in cellobiose-grown cells.

[b] CCCP, Carbonyl cyanide-*m*-chlorophenyl hydrazone.

[c] DCCD, *N,N'*-Dicyclohexylcarbodiimide.

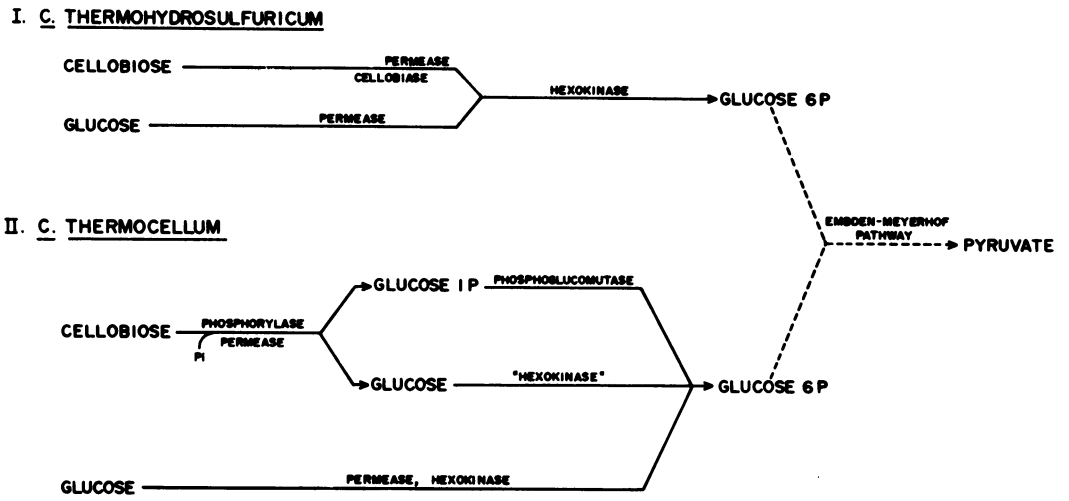

FIG. 4. Proposed model to summarize the relationship between growth saccharide and enzymatic activities associated with formation of glucose 6-phosphate in thermophilic anaerobes. The cellobiose-metabolizing enzymes were constitutive in *C. thermocellum* whereas the glucose-metabolizing enzymes were constitutive in *C. thermohydrosulfuricum*.

glucose permease and ATP rather than a PEP-sugar phosphotransferase or a respiration-linked transport system (9). *C. thermocellum* grown on cellobiose was more strongly inhibited by 2-deoxyglucose than was *C. thermohydrosulfuricum*, because cellobiose phosphorylase also catalyzes the formation of 4-*O*-β-D-glucopyranosyl-D-deoxyglucose plus phosphate from 2-deoxyglucose plus glucose 1-phosphate (3). Arsenate has a much higher affinity for this enzyme than does phosphate (2) and thus also effectively blocked the uptake of cellobiose.

The present study supports a recent report (Gomez and Hernandez, Abstr. 6th Int. Ferment. Symp. and 5th Int. Symp. Yeasts, p. 91) that glucose fermentation requires a long lag period when *C. thermocellum* is transferred from cellulose to a glucose medium. The data detailed here indicate that this is due to the requirement for induction or derepression of glucose-metabolizing enzyme activities. Thus, the ability of *C. thermocellum* strains to ferment glucose appears to be multifaceted in that, in addition to strains that require induction (the present study), other strains are apparently constitutive or lack the genetic information (12, 19, 22, 24, 27, 28, 30; Gomez and Hernandez, Abstr. 6th Int. Ferment. Symp. and 5th Int. Symp. Yeasts, p. 91).

The cell yields of *C. thermocellum* on glucose and *C. thermohydrosulfuricum* on glucose or cellobiose were nearly identical to that reported for *Thermobacteroides acetoethylicus* (7), which employs the same general glycolytic pathway (40). The cell yield of *C. thermocellum* on cellobiose was much higher, probably as a consequence of cellobiose phosphorylase, which, unlike cellobiase, conserves the energy of hydrolysis of the β-1,4 bond with the formation of glucose 1-phosphate. Therefore, *C. thermocellum* has a higher degree of metabolic efficiency than *C. thermohydrosulfuricum* on cellobiose because it conserves 1 mol more of ATP per mol of substrate metabolized. *C. thermocellum* also contains a cellodextrin phosphorylase (2), and in view of the present findings, the growth yield of the organism should be even higher on cellodextrins. However, a metabolic consequence appears to be related to the possession of cellobiose phosphorylase; namely, glucose utilization becomes a rate-limiting step in cellobiose metabolism. This suggestion is supported by both the accumulation of glucose during growth on medium containing high concentrations of cellobiose and the enhancement of cellobiose phosphorylase activity in cell extracts by hexokinase addition.

These data also help, in part, to explain the metabolic basis for stable cocultures between *C. thermocellum* and *C. thermohydrosulfuricum* during growth on cellulose (23). Glucose and cellobiose are hydrolytic end products of the cellulase of *C. thermocellum* (25), and glucose is also an accumulable end product of cellobiose phosphorylase. However, glucose metabolism is constitutive to *C. thermohydrosulfuricum*, not to *C. thermocellum*. In addition, the in vivo growth and cellobiose consumption rates of *C. thermohydrosulfuricum* (120-min doubling time, 131 μmol/min per ml of culture) were more rapid than those of *C. thermocellum* (150-min doubling time, 98 μmol/min per ml of culture).

Hence, these findings indicate that *C. thermohydrosulfuricum* can successfully compete for both cellobiose and glucose formed during cellulose fermentation by *C. thermocellum*.

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