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Jilai Zhou
Dartmouth College

Daniel G. Olson
Dartmouth College

D. Aaron Argyros
Mascoma Corporation

Yu Deng
Dartmouth College

Walter M. van Gulik
Delft University of Technology

See next page for additional authors

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Jilai Zhou,[a,b] Daniel G. Olson,[a,b] D. Aaron Argyros,[c] Yu Deng,[a,b] Walter M. van Gulik,[d] Johannes P. van Dijken,[d] Lee R. Lynd[a,b,c]

Thayer School of Engineering, Dartmouth College, Hanover, New Hampshire, USA[a]; BioEnergy Science Center, Oak Ridge, Tennessee, USA[b]; Mascoma Corporation, Lebanon, New Hampshire, USA[c]; Delft University of Technology, Delft, the Netherlands[d]

Cofactor specificities of glycolytic enzymes in *Clostridium thermocellum* were studied with cellobiose-grown cells from batch cultures. Intracellular glucose was phosphorylated by glucokinase using GTP rather than ATP. Although phosphofructokinase typically uses ATP as a phosphoryl donor, we found only pyrophosphate (PP_i)-linked activity. Phosphoglycerate kinase used both GDP and ADP as phosphoryl acceptors. In agreement with the absence of a pyruvate kinase sequence in the *C. thermocellum* genome, no activity of this enzyme could be detected. Also, the annotated pyruvate phosphate dikinase (*ppdk*) is not crucial for the generation of pyruvate from phosphoenolpyruvate (PEP), as deletion of the *ppdk* gene did not substantially change cellobiose fermentation. Instead pyruvate formation is likely to proceed via a malate shunt with GDP-linked PEP carboxykinase, NADH-linked malate dehydrogenase, and NADP-linked malic enzyme. High activities of these enzymes were detected in extracts of cellobiose-grown cells. Our results thus show that GTP is consumed while both GTP and ATP are produced in glycolysis of *C. thermocellum*. The requirement for PP_i in this pathway can be satisfied only to a small extent by biosynthetic reactions, in contrast to what is generally assumed for a PP_i-dependent glycolysis in anaerobic heterotrophs. Metabolic network analysis showed that most of the required PP_i must be generated via ATP or GTP hydrolysis exclusive of that which happens during biosynthesis. Experimental proof for the necessity of an alternative mechanism of PP_i generation was obtained by studying the glycolysis in washed-cell suspensions in which biosynthesis was absent. Under these conditions, cells still fermented cellobiose to ethanol.

The ultimate low-cost configuration for conversion of cellulose to biofuels is via consolidated bioprocessing (CBP) (1). *Clostridium thermocellum* is a candidate for CBP because of its ability to rapidly solubilize cellulose and produce ethanol (2). Currently its commercial potential is limited by low alcohol yields. Although tools for genetic modification in *C. thermocellum* have been developed (3, 4) and applied to the problem of increasing ethanol yield by eliminating organic acid production (5–7), further improvement in yield is desired (6). In order to develop rational metabolic engineering strategies for improving ethanol yield, it is important to understand the basic metabolic pathways of *C. thermocellum*. Unlike those of model organisms such as *Escherichia coli* and *Saccharomyces cerevisiae*, the central metabolic pathways of *C. thermocellum* have been less well studied (8, 9).

Some features of cellobiose fermentation in *C. thermocellum* are generally agreed upon. Cellobiose transport in *C. thermocellum* is mediated by an ATP-binding cassette transporter instead of the phosphotransferase system (10, 11). Cellobiose is then phosphorylated to glucose-1-phosphate and glucose by cellobiose phosphorylase (11). There is a broad consensus that glycolysis is the predominant pathway for conversion of intracellular glucose and glucose-1-phosphate to pyruvate. Based on analysis of the genome, all of the genes in the glycolysis pathway except pyruvate kinase have been reported to exist in *C. thermocellum* (12, 13). A variety of key glycolytic enzymes have been detected in cell extracts (8, 11). Furthermore, ^{14}C-glucose tracer experiments have demonstrated high flux through glycolysis and low or no flux through the pentose phosphate pathway or the Entner-Doudoroff pathway (8). Despite this, there are conflicting claims about a number of enzyme activities in glycolysis.

Glucokinase mediates phosphorylation of glucose to glucose-6-phosphate. Gene *clo1313_0489* has been annotated as a glucokinase and shown to be both transcribed (14) and translated (15). There have been several reports showing the absence or very low activity of ATP-dependent glucokinase in *C. thermocellum* (10, 11, 16) and one account showing high activity of glucokinase (9). Ng and Zeikus showed that glucokinase activity is induced during growth on glucose but was not detected during growth on cellobiose (11). They were able to detect the production of glucose-6-phosphate from cellobiose using ^{14}C labeling, although they did not discover the enzyme responsible. Thus, it is not clear at this time what enzyme is responsible for conversion of glucose to glucose-6-phosphate in cellobiose-grown cells.

Phosphofructokinase (PFK) mediates the phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate. Two genes have been annotated as potential phosphofructokinases, *clo1313_0997* and *clo1313_1876* (12). Both genes have been shown to be transcribed (14) and translated (15). There have been two reports describing ATP-linked phosphofructokinase activity (9, 17). Rydzak et al. (15) annotated different cofactor specificities for the two genes, with *cthe_1261* (homologous to *clo1313_0997*) being ATP linked (EC 2.7.1.11) and *cthe_0347* (homologous to *clo1313_1876*) being PP_i linked (EC 2.1.7.90). They found higher expression of *cthe_0347* compared with *cthe_1261* (15). The presence of two types of PFK enzyme has also been reported for *Entamoeba histolytica* (18) and *Propionibacterium shermanii* (19). It is not known whether this is the case for *C. thermocellum* as well.

For conversion of phosphoenolpyruvate (PEP) to pyruvate, three possibilities exist: pyruvate kinase (PK) (EC 2.7.1.40), phosphoenolpyruvate synthase (PEPS) (EC 2.7.9.2), and pyruvate

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Address correspondence to Lee R. Lynd, Lee.Lynd@Dartmouth.edu.

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phosphate dikinase (PPDK) (EC 2.7.9.1). Of these three, the evidence for pyruvate kinase is the weakest. Although no standard pyruvate kinase has been found (15, 20), in one study, where the presence of a pyruvate kinase gene was presumed, a nonstandard pyruvate kinase was identified (13). PPDK has been shown to allow PEP-to-pyruvate conversion in *Thermoproteus tenax* (21) and *E. histolytica* (22). *C. thermocellum* has a gene annotated as *ppdk* (*clo1313_0949*), and it has been shown to be expressed at high levels in both transcriptomic and proteomic data (14, 15). The gene thought to confer PEPS activity (*clo1313_1005*) shows a low expression level and thus seems unlikely to play a major role in glycolysis (14, 15).

Thus, the following questions remain about central metabolism in *C. thermocellum*. (i) How does glucose enter glycolysis? (ii) Since two genes are annotated for PFK in the genome, what is the nature of the phosphofructokinase? (iii) How is PEP converted to pyruvate?

Here, we attempted to answer these questions using enzyme assays and targeted gene deletion. In the course of pursuing these questions, we discovered that glycolysis was different from the standard model with respect to cofactors of several key enzymes.

MATERIALS AND METHODS

Organism, medium, and growth. *Clostridium thermocellum* DSM1313 was obtained from the DSMZ culture collection. *Thermoanaerobacterium saccharolyticum* JW/SL-YS485 (23) was kindly provided by Juergen Wiegel. Strains were grown anaerobically at 55°C in modified MTC medium (24, 25) in 125-ml glass bottles with a working volume of 50 ml with shaking at 250 rpm. Fermentations were allowed to proceed for 72 h, at which point the cells had reached stationary phase and samples were collected for analysis.

Medium composition and preparation. Modified MTC medium contained 5 g/liter cellobiose, 9.25 g/liter MOPS (morpholinepropanesulfonic acid) sodium salt, 2 g/liter urea, 2 g/liter potassium citrate monohydrate, 1.25 g/liter citric acid monohydrate, 1 g/liter Na_2SO_4, 1 g/liter KH_2PO_4, 2.5 g/liter $NaHCO_3$, 2 g/liter urea, 1 g/liter $MgCl_2 \cdot 6H_2O$, 0.2 g/liter $CaCl_2 \cdot H_2O$, 0.1 g/liter $FeCl_2 \cdot 6H_2O$,1 g/liter L-cysteine HCl monohydrate, 0.02 g/liter pyridoxamine HCl, 0.004 g/liter *p*-aminobenzoic acid (PABA), 0.004 g/liter D-biotin, 0.002 g/liter vitamin B_{12}, 0.005 g/liter $MnCl_2 \cdot 4H_2O$, 0.005 g/liter $CoCl_2 \cdot 6H_2O$, 0.002 g/liter $ZnCl_2$, 0.001 g/liter $CuCl_2 \cdot 2H_2O$, 0.001 g/liter H_3BO_3, 0.001 g/liter $Na_2MoO_4 \cdot 2H_2O$, and 0.001 g/liter $NiCl_2 \cdot 6H_2O$. It was prepared by combining six sterile solutions under a nitrogen atmosphere. All of the six solutions were sterilized through a 0.22-μm-pore-size filter (product number 430517; Corning). A solution, concentrated 2.5-fold, contained cellobiose, MOPS sodium salt, and distilled water. B solution, concentrated 25-fold, contained potassium citrate monohydrate, citric acid monohydrate, Na_2SO_4, KH_2PO_4, $NaHCO_3$, and distilled water. C solution, concentrated 50-fold, contained urea and distilled water. D solution, concentrated 50-fold, contained $MgCl_2 \cdot 6H_2O$, $CaCl_2 \cdot H_2O$, $FeCl_2 \cdot 6H_2O$, L-cysteine HCl monohydrate, and distilled water. E solution, concentrated 50-fold, contained pyridoxamine HCl, *p*-aminobenzoic acid (PABA), D-biotin, vitamin B_{12}, and distilled water. F solution, concentrated 1,000-fold, contained $MnCl_2 \cdot 4H_2O$, $CoCl_2 \cdot 6H_2O$, $ZnCl_2$, $CuCl_2 \cdot 2H_2O$, H_3BO_3, $Na_2MoO_4 \cdot 2H_2O$, $NiCl_2 \cdot 6H_2O$, and distilled water. All chemicals mentioned above were reagent grade and obtained from Sigma.

Plate reader growth experiments. Growth rates were determined as previously described (26). Briefly, strains were grown anaerobically at 55°C in 200 μl modified MTC medium in a 96-well plate. At 3-min intervals the plate was shaken for 30 s, followed by measuring the optical density at 600 nm (OD_{600}).

Deletion of pyruvate phosphate dikinase (*ppdk*) gene. Strain M1631 (Δ*hpt* Δ*ppdk*) was derived from strain M1354 (6) by deletion of the pyruvate phosphate dikinase (*ppdk*) gene (*clo1313_0949*) using plasmid pMU2051 (GenBank accession number KC146550). This plasmid has three regions of homology to the *ppdk* gene region corresponding to a 522-bp region upstream of *ppdk* amplified by the following two primers: 5′-CGGCCGCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGAAGCCATGCAACCTGGACGT-3′ and 5′-TAGTGAAGGCAATGCfATCAATGAGAGACCTGCTTGGAGGAGTATCATGCTCTCCGTTCCG-3′, a 520-bp region downstream of *ppdk* amplified by the following primers: 5′-CAAGCCTTGCAATCGGCACACGGAACGGAGAGCATGATACTCCTCCAAGCAGGTCTCTCA-3′ and 5′-CTATATTGCTATAAAGAATGAGGAGGGAACTAGTTGAAGCTGCTTGCGATCCTAAGTCTT-3′, and a 549-bp region internal to *ppdk* amplified by the following primers: 5′-ACTCTTTAGAATCTTTTCCTCTCTTTCGGAAAAGAAATACACTCCATATCCTGCATATCT-3′ and 5′-AGCAAGGTGTAGAAAGTGCCATGAAGTCCCGCGGACTTAATCAGCGATGTTGTCATGGAG-3′.

Washed-cell experiments. Cells for washed-cell experiments were harvested at exponential phase and centrifuged at 12,000 relative centrifugal force (RCF) units for 2 min. The supernatant was discarded, and cells were resuspended in 50 ml of medium (MTC medium without cellobiose and without urea—i.e., both carbon and nitrogen limited). This washing procedure was repeated one additional time. Washing was performed in a COY anaerobic chamber (COY Laboratory Products, MI) with an atmosphere of 85% nitrogen, 10% carbon dioxide, 5% hydrogen, and <5 ppm oxygen. Cells were then resuspended in one of three modified MTC medium recipes (10 ml each)—MTC medium, MTC medium without cellobiose and urea, or MTC medium without urea—and incubated anaerobically at 55°C. Over the course of 72 h, several 1-ml aliquots were removed for further analysis. At each time point, the optical density of the culture at 600 nm was measured and after centrifugation (5 min, 12,000 RCF units), the supernatant was analyzed by high-pressure liquid chromatography (HPLC) (described below).

Analytical techniques. Fermentation products (cellobiose, glucose, acetate, lactate, and ethanol) were analyzed by a Waters HPLC system with an Aminex HPX-87H column operated at 60°C. Sample collection and processing were as reported previously (27).

Preparation of cell extracts for enzyme assays. All manipulations were performed under anaerobic conditions in a COY anaerobic chamber. Cells used for enzymatic activities were harvested at the exponential phase of growth and washed twice with a buffer containing 50 mM Tris-HCl (pH 8.0) and 5 mM dithiothreitol (DTT). To prepare cell extracts, cells were centrifuged and suspended in 50 mM potassium phosphate buffer (pH 7.0 at 55°C) containing 5 mM dithiothreitol. The cells were disrupted by sonic oscillation (Misonex) for 2 min (30 s for disrupting and 30 s for cooling per cycle, 4 cycles total) at 4°C. The cell extracts were obtained after centrifugation (5 min, 12,000 RCF units). The total amount of protein in the extracts was determined by Bradford assay, using bovine serum albumin as the standard (28). In all cases, the final protein concentration was between 2.5 and 5.4 mg/ml. Cell extracts were stored at −20°C and used within 1 week of preparation.

Assays of enzyme activities. Enzymes were assayed by measuring changes in absorbance at 340 nm with reactions coupled to NADP reduction or NADH oxidation. An Agilent 8453 spectrophotometer was used for these measurements with a Peltier temperature control module (part number 89090A) to maintain a temperature of 55°C during the assay. The reaction volume was 1 ml, in reduced-volume quartz cuvettes (part number 29MES10; Precision Cells Inc., NY) with a 1.0-cm path length. Assays were performed in a COY anaerobic chamber. All enzyme activities are expressed as $\mu mol\ of\ product \cdot min^{-1} \cdot (mg\ of\ cell\ extract\ protein)^{-1}$. For each enzyme assay, at least two concentrations of cell extract were used to confirm that specific activity was proportional to the amount of extract added.

All biochemicals except for ADP-glucose were obtained from Sigma. ADP-glucose was obtained from Santa Cruz Biotechnology. All chemicals and coupling enzymes were prepared fresh monthly and stored in −80°C in water except NADH, which was prepared in 1 M Tris buffer (pH 9.0). The following coupling enzymes (also from Sigma, with catalog numbers) were used: glucose-6-phosphate dehydrogenase (recombinant, expressed

in *E. coli* from *Leuconostoc mesenteroides*, G2921), phosphoglucomutase (from rabbit muscle, P3397), aldolase (from rabbit muscle, A2714), α-glycerophosphate dehydrogenase (from rabbit muscle, G6880), triosephosphate isomerase (from *S. cerevisiae*, T2507), glyceraldehyde-3-phosphate dehydrogenase (from *S. cerevisiae*, G5537), pyruvate kinase (from *Bacillus stearothermophilus*, P1903), and lactate dehydrogenase (recombinant from *E. coli*, 59747).

Glucokinase (EC 2.7.1.2) was assayed based on the formation of glucose-6-phosphate (29).The assay mix contained 5 mM $MgCl_2$, 2 mM glucose, 2 mM NADP, 2 U of glucose-6-phosphate dehydrogenase (yeast) per ml, and 20 μl cell extract in 50 mM Tris-HCl buffer (pH 7.0). To achieve the maximum activity, 60 mM KCl was added into the reaction mix. The reactions were started by the addition of 2 mM phosphoryl donor, ATP, GTP, and PP_i, respectively.

Phosphoglucomutase (EC 5.4.2.2) was assayed by the method of Ye et al. (30) with minor modifications. The assay mixture contained 50 mM Tris-HCl (pH 7.0), 5 mM $MgCl_2$, 2 mM NADP, 2 mM glucose-1-phosphate, 2 U of glucose-6-phosphate dehydrogenase (yeast) per ml, and 20 μl cell extract. The reaction was started by adding glucose-1-phosphate.

Phosphofructokinase (EC 2.7.1.11 or EC 2.7.1.90) was assayed by the method of de Jong-Gubbels et al. (31) with minor modifications. The assay mixture contained 50 mM Tris-HCl (pH 7.0), 5 mM $MgCl_2$, 0.15 mM NADH, 1 mM fructose-6-phosphate, 4 U/ml aldolase, 4 U/ml α-glycerophosphate dehydrogenase, 4 U/ml triosephosphate isomerase, and 20 μl cell extract. The assay was started with 2 mM phosphoryl donor, ATP, or PP_i.

Phosphoglycerate kinase (EC 2.7.2.3) was assayed from the formation of 1,3-diphosphoglycerate according to the method of van Hoek et al. (32) with minor modifications. The assay mixture contained 50 mM Tris-HCl (pH 7.0), 5 mM $MgCl_2$, 3-phosphoglycerate 2 mM, 2 mM EDTA, 0.15 mM NADH, and 2 U/ml glyceraldehyde-3-phosphate dehydrogenase. The assay was started by the addition of phosphoryl donors.

Pyruvate kinase (EC 2.7.1.40) in extracts from both *C. thermocellum* and *T. saccharolyticum* was assayed by coupling pyruvate production to NADH oxidation using the lactate dehydrogenase enzyme according to the protocol of de Jong-Gubbels et al. (31) with minor modifications. The assay mixture contained 50 mM Tris-HCl (pH 7.0), 2 mM PEP, 0.15 mM NADH, 5 mM $MgCl_2$, 4 U/ml lactate dehydrogenase, and 50 μl cell extract. The reaction was started by the addition of 2 mM ADP, 1 mM fructose-1,6-biphosphate. Ribose-5-phosphate and glucose-6-phosphate were added into the mixture and tested as potential activators of pyruvate kinase activity.

Pyruvate phosphate dikinase (EC 2.7.9.1) was assayed in the same way as pyruvate kinase. However, the reaction was started by adding 2 mM AMP and 2 mM PP_i instead of ADP.

PEP synthase (EC 2.7.9.2) was assayed in the same way as pyruvate kinase. However, the mixture contained 50 mM potassium phosphate buffer (pH 7.0) instead of Tris-HCl buffer. The reaction was started by adding 2 mM AMP instead of ADP.

PEP carboxykinase (EC 4.1.1.32 or EC 4.1.1.38 or EC 4.1.1.49, depending on cofactor specificity) was assayed by the formation of oxaloacetate (OAA) from PEP, following the protocol from Jabalquinto et al. (33) with minor modifications. The assay mix contained 50 mM Tris-HCl (pH 7.0), 50 mM $NaHCO_3$, 5 mM $MgCl_2$, 2 mM PEP, 0.15 mM NADH, 6 U/ml malate dehydrogenase, and 20 μl cell extract. The reaction was initiated by adding 2 mM GDP or 20 mM P_i.

Malate dehydrogenase (EC 1.1.1.37) was assayed by measuring the decrease of NADH with conversion of OAA to malate following the protocol of Rokosh et al. (34) with minor modifications. The assay mix contained 50 mM Tris-HCl (pH 7.0), 0.15 mM NADH, 5 mM DTT, and 20 μl cell extract. The assay was started by adding 2 mM OAA.

Malic enzyme (EC 1.1.1.40) was assayed by measuring the reduction of NADP following the protocol of Lamed and Zeikus (35) with minor modifications. The assay mix contained 50 mM Tris-HCl (pH 7.0), 5 mM DTT, 5 mM NH_4Cl, 0.15 mM $NADP^+$, and 20 μl cell extract. The assay was initiated by adding 2 mM malate.

TABLE 1 Activity of phosphorylating enzymes in glycolysis of *Clostridium thermocellum*

Enzyme	EC number	Cofactor	Sp act (U/mg of protein)
Glucokinase	EC 2.7.1.2	ATP	0.03 ± 0.01
		GTP	2.16 ± 0.14
		PP_i	<0.01
Phosphoglucomutase	EC 5.4.2.2	N/A[a]	0.42 ± 0.01
Phosphofructokinase	EC 2.7.1.11	ATP	<0.01
	EC 2.7.1.90	PP_i	0.98 ± 0.04
Phosphoglycerate kinase	EC 2.7.2.3	ADP	6.52 ± 0.06
	EC 2.7.2.10	GDP	6.35 ± 0.01
		P_i	<0.01
Pyruvate kinase	EC 2.7.1.40	ADP	<0.01
PEP carboxykinase	EC 4.1.1.49	ADP	0.31 ± 0.08
	EC 4.1.1.32	GDP	4.14 ± 0.43[b]
	EC 4.1.1.38	P_i	<0.01
Malate dehydrogenase	EC 1.1.1.37	NADH	1.67 ± 0.11[b]
Malic Enzyme	EC 1.1.1.40	NADP	1.97 ± 0.25[b]

[a] N/A, not applicable.
[b] Data are from Deng et al. (7).

Calculation of PP_i. The calculation of the amount of pyrophosphate generated in the biosynthesis of cell components was performed with the metabolic network model of Taymaz-Nikerel et al. (36).

RESULTS

Activities of glycolytic enzymes. Enzymes in the glycolysis pathway were tested for cofactor specificity (Table 1, Fig. 1). Glucokinase was found to be GTP linked, with activity 50-fold higher using GTP compared to ATP. Phosphofructokinase was found to be PP_i linked. Phosphoglycerate kinase was found to be equally active with either ADP or GDP. No activity was found for pyruvate kinase, pyruvate phosphate dikinase, or PEP synthase. Activity of PEP carboxykinase was detected with the presence of GDP and was 15-fold higher than that with ADP. No activity was found when GDP was replaced by inorganic phosphate (P_i), indicating that PEP carboxytransphosphorylase (PEP + $P_i \Leftrightarrow$ OAA + PP_i) (EC 4.1.1.38) is absent. Cell extracts also contained high activities of NADH-linked malate dehydrogenase and NADP-linked malic enzyme. Therefore, the sequence of reactions catalyzed by PEP carboxykinase, malate dehydrogenase, and malic enzyme can compensate for the absence of pyruvate kinase in the generation of pyruvate from PEP (Fig. 1).

Deletion of *ppdk* gene. As mentioned in the previous paragraph, both pyruvate kinase and *ppdk* were not detectable in cellobiose-grown cells of *C. thermocellum*. Pyruvate kinase was, however, readily detectable in extracts of *T. saccharolyticum* (data not shown), but we did not possess a suitable reference assay for the *ppdk* reaction. To further investigate a possible role of this enzyme in the generation of pyruvate from PEP in *C. thermocellum*, we studied the properties of a *ppdk* deletion mutant and compared it to the wild type and the parent strain (strain M1354, which has only the *hpt* deletion). All three strains grew readily on cellobiose. There was a slight decrease in growth rate between the wild type and the parent strain (0.47 versus 0.38), which is likely due to the effect of the *hpt* deletion. There was no significant difference between the growth rates of the parent (M1354) and *ppdk* deletion (M1631) strains. Furthermore, all strains showed similar final culture densities (as measured by absorbance at 600 nm) (Table 2).

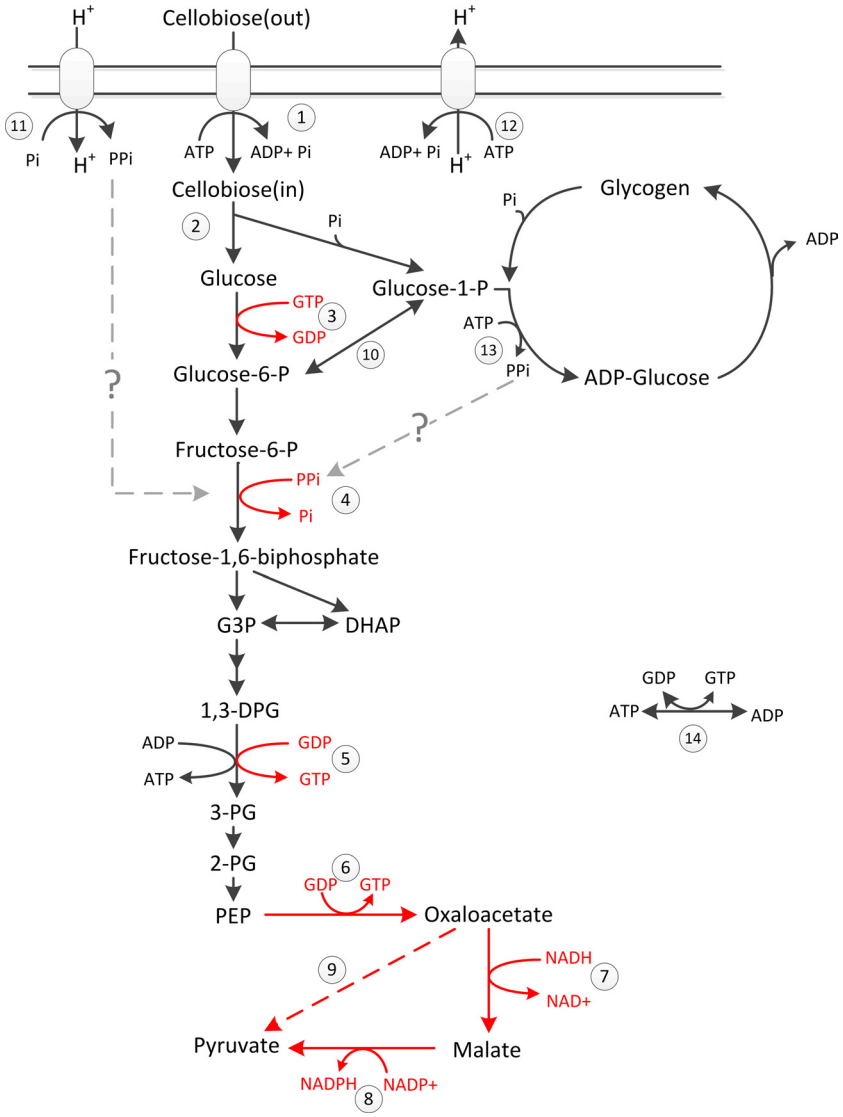

FIG 1 Aberrant glycolysis and glycogen cycle in *C. thermocellum*. Numbers in circles indicate enzymes as follows: 1, ABC transporter; 2, cellobiose phosphorylase (EC 2.4.1.20); 3, glucokinase (EC 2.7.1.2); 4, phosphofructokinase (EC 2.7.1.90); 5, phosphoglycerate kinase (EC 2.7.2.3); 6, PEP carboxykinase (EC 4.1.1.32); 7, malate dehydrogenase (EC 1.1.1.37); 8, malic enzyme (EC 1.1.1.40); 9, OAA decarboxylase (EC 4.1.1.3); 10, phosphoglucomutase (EC 5.4.2.2); 11, membrane-bound pyrophosphatase (EC 3.6.1.1); 12, ATP-energized proton pump; 13, ADP-glucose synthase (EC 2.7.7.27); 14, NDP-kinase (EC 2.7.4.6). The abbreviations represent metabolites as follows: G1P, glucose 1-phosphate; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; G3P, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate; 1,3 DPG, 1,3-biphosphoglycerate; 3 PG, 3-phosphoglycerate; 2 PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate. Gray question marks represent possible sources for pyrophosphate in glycolysis.

The final concentrations and ratio of the main fermentation products, acetate and ethanol, were similar. There were slight differences in lactate and glucose production (Fig. 2).

Stoichiometry of pyrophosphate turnover in cellobiose metabolism. In the literature on PP_i-dependent glycolysis, it is usually assumed that biosynthetic reactions are the exclusive source of pyrophosphate (37–39). Indeed, metabolic network analysis reveals that substantial amounts of PP_i are generated in the biosynthesis of protein, RNA and DNA, lipids, and glycogen (Table 3). However, this amount is by far not enough to satisfy the PP_i requirement in glycolysis. Using a representative bacterial biomass composition presented in Table 3, the elemental composition corresponding to this biomass composition, and a biomass yield of 0.1 g cells/g glucose, a simple flux diagram can be constructed (Fig. 3). Approximately 11 mmol PP_i is generated during the formation of 1 g biomass (Table 3). Sufficient PP_i is generated in the production of cellular polymers to provide for the PP_i used in the associated assimilation processes; however, there is not much left over for dissimilation reactions. In order to reach a biomass yield of 100

TABLE 2 Growth rate and maximum optical density of strains[a]

Strain	Maximum specific growth rate (h^{-1})	Maximum OD_{600} (AU)
Wild type	0. 47 ± 0.02	1.17 ± 0.02
M1354 (Δ*hpt*)	0.38 ± 0.02	1.08 ± 0.04
M1631(Δ*hpt* Δ*ppdk*)	0.39 ± 0.01	1.14 ± 0.04

[a] Error is one standard deviation; $n = 3$.

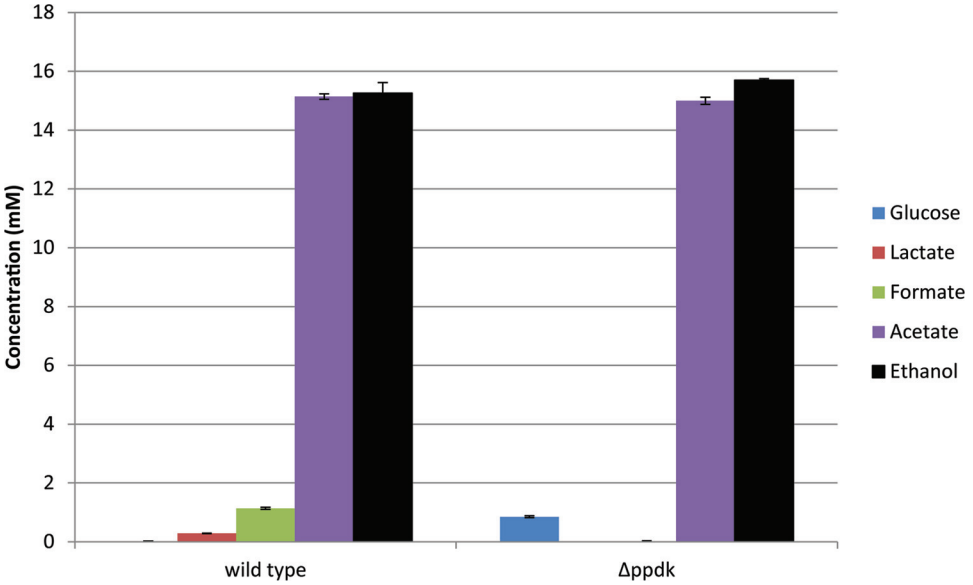

FIG 2 Analysis of fermentation products of the wild-type and Δ*ppdk* strains. Error bars represent one standard deviation of duplicate fermentations.

g cells/1,000 g glucose (= 5,555 mmol glucose), the total amount of PP_i required is 5,555 mmol PP_i, assuming that all glucose fluxes via the PFK reaction. Thus, in growing cells, biosynthetic reactions can only provide approximately 1,097/5,555 ≈ 20% of the pyrophosphate required to sustain the total glycolytic flux. Expressed relative to the dissimilative part of the glycolytic flux, the contribution is only 364/4,822 ≈ 8% (Fig. 3). It can therefore be concluded that anaerobic bacteria that possess a PP_i-dependent glycolysis must possess alternative mechanisms to generate PP_i. Note that the amount of additional pyrophosphate generated by biosynthesis is an order of magnitude lower than what would be required if biosynthesis were the sole source of pyrophosphate, and thus this conclusion is robust even when somewhat different values of biomass yield and composition are used.

Cellobiose fermentation by washed-cell suspensions. In addition to metabolic network analysis, it can be shown experimentally that biosynthetic processes cannot be the sole source of PP_i. If biosynthesis of cellular polymers were the exclusive source of PP_i, fermentation of cellobiose by nongrowing cells would be impossible. To determine the extent to which biosynthesis is required for metabolism, washed cells were reinoculated into three variants of MTC medium: unmodified, MTC lacking urea (nitrogen source), and MTC lacking both cellobiose (carbon source) and urea (nitrogen source). Cells inoculated into the unmodified medium (Fig. 4A) showed an increase in OD_{600} from 0.68 to 2.85 and then a gradual decrease to 1.44. All of the 15.5 mM cellobiose initially

TABLE 3 Amount of pyrophosphate produced in the synthesis of cell components

Component	Pyrophosphate liberated (mmol/g cells)			
	% of biomass[a]	Precursor biosynthesis	Polymerization	Total
Protein	65.4	1.259	6.409	7.668
RNA and DNA	15.1	0.659	0.502	1.160
Lipids and lipopolysaccharide	11.9	2.110		2.110
Glycogen	0.5	0.032		0.032
Other	0.7			
Ash	6.4			
Total	100			10.970

[a] Data are from Taymaz-Nikerel et al., Table 4, for *E. coli* cells growing anaerobically at a dilution of 0.3 h^{-1} (36).

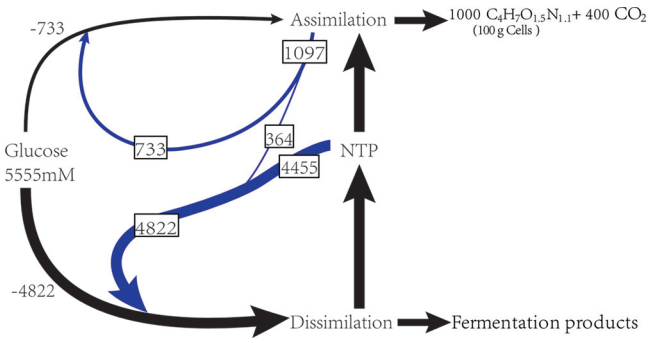

FIG 3 Example of calculation of the fluxes of pyrophosphate in microbial anaerobic heterotrophic sugar metabolism with a PP_i-dependent phosphofructokinase. Blue arrows represent the pyrophosphate flux in *C. thermocellum*. For this calculation the following biomass compositions (as weight percentages) were used: protein, 65.4; RNA, 13.1; DNA, 1.98; lipids, 11.2; lipopolysaccharides, 0.65; polysaccharide (glycogen), 0.48; peptidoglycan (murein), 0.48; putrescine, 0.22; spermidine, 0.07; ash, 6.43 (36). Biomass with this composition can be represented as $CH_{1.64}N_{0.27}O_{0.37}S_{0.007}P_{0.012}$. This is based on measurement of *E. coli* grown anaerobically. The evidence would still be robust even if the composition were to change. A biomass yield of 0.1 g/g hexose is assumed (represented in mmol). The molecular weight of the biomass is 100 including ash. Biomass formation is associated with a net carbon loss of approximately 10% resulting from decarboxylation reactions. For simplicity it is assumed that all carbon required for the biosynthesis of cells (733 mmol) passes the PFK reaction. Although the biosynthesis of biomass is self-supporting with respect to PP_i supply, a large amount of pyrophosphate is required to sustain the catabolic flux. The additional PP_i required for dissimilation (4,458 mmol) must be obtained via ATP hydrolysis with a membrane-bound pyrophosphatase or glycogen cycling (Fig. 1).

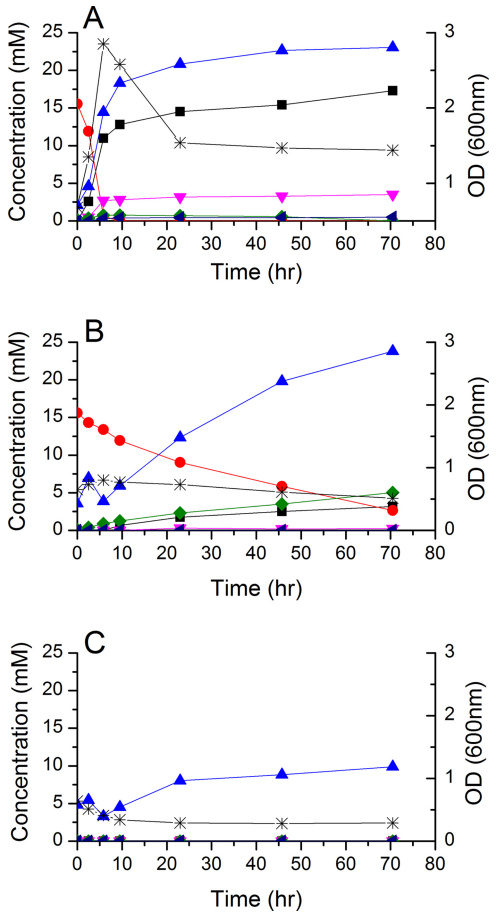

FIG 4 Results of washed-cell experiments in MTC medium (A), MTC medium without urea (B), and MTC medium without cellobiose and without urea (C). Concentrations of cellobiose (red circle), glucose (green diamond), acetate (black square), ethanol (blue triangle), lactate (pink triangle), and formate (dark blue triangle) and the OD (*) were determined as indicated in Materials and Methods.

present was consumed, and 23.1 mM ethanol and 17.3 mM acetate were produced. Cells were inoculated into MTC lacking urea but containing cellobiose (Fig. 4B). The optical density of cells in this medium remained relatively constant, showing a slight increase from 0.65 to 0.80 and then a decrease to 0.51. Of the 15.6 mM cellobiose that was originally present, 13.0 mM was consumed, and 23.8 mM ethanol, 3.2 mM acetate, and 5.0 mM glucose were produced. Cells were also inoculated into MTC without cellobiose or urea (Fig. 4C). Cells under this condition showed a decrease in OD_{600} from 0.64 to 0.29, and 9.9 mM ethanol was produced. This small amount of ethanol probably arises from reserve carbohydrates, such as glycogen, that are present in the cells. Surprisingly, in the absence of nitrogen, the ethanol-to-acetate ratio increased to 7.5:1 from the typical value of 1:1.

The data presented in Fig. 4 thus show that cellobiose fermentation in nongrowing cells, albeit slower than in growing cells, is not obligately dependent on biosynthesis of cell material.

DISCUSSION

Early literature on the biochemistry of glycolysis in *C. thermocellum*. Although the enzyme assay data from Patni and Alexander (9) are frequently cited in the literature, this work is at the heart of a number of issues concerning the biochemistry of glycolysis in *C. thermocellum*. They report high activity of ATP-linked glucokinase on cellobiose-grown cells, but we found glucokinase activity to be mainly GTP linked in cellobiose-grown cells (Table 1). Furthermore, Ng and Zeikus specifically note that ATP-linked glucokinase activity was absent in cellobiose-grown cells (11). Patni and Alexander report activities for glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, but this is the only report of these activities in *C. thermocellum*, and genes coding for these activities are conspicuously absent from the genome annotation (12, 15). The presence of these activities would suggest a complete oxidative pentose phosphate pathway, which is contradicted by ^{14}C tracer experiment results presented by Lamed and Zeikus (8). Patni and Alexander reported ATP-linked phosphofructokinase activity, which contradicts our data that phosphofructokinase is primarily PP_i linked with small amounts of GTP-linked activity. Finally, Patni and Alexander reported pyruvate kinase activity, which again contradicts our results. One possible explanation for these disparities is that the activities they found are specific to strain 651. This strain was originally isolated by McBee (40) and is distinct from strain DSM 1313, ATCC 27405, or any other strain of *C. thermocellum* currently available in commercial culture collections. In fact, as of 1986 the strain was reported to have been lost (16). Therefore, the results of Patni and Alexander do not seem representative of the enzyme activities of the strains of *C. thermocellum* currently in use. In this respect, the purity of the strain used by Patni and Alexander also must be considered. *C. thermocellum* strains are generally isolated from nature in a mixture with saccharolytic organisms such as *Thermoanaerobacter* spp., which are known to contain the enzyme activities associated with the issues discussed here. Culture purity checks are especially required in research with *C. thermocellum* strains (41).

Absence of pyruvate kinase. We were unable to detect pyruvate kinase (Table 1), though the enzyme was readily detectable in extracts of *T. saccharolyticum*. This is at variance with the proposed annotation of *clo1313_2626* by Roberts et al. (13) as a possible pyruvate kinase. Roberts et al. (13) found the pyruvate kinase based on their reverse blast search against all the proteins annotated by UniProt as pyruvate kinase, and they selected a candidate gene that is most likely to have the function of pyruvate kinase. However, they did not further confirm the function of this gene experimentally, and it does not have the PK or PK_C Pfam domains typically found in pyruvate kinase enzymes (42). We therefore conclude that *C. thermocellum* strain DSM1313 does not possess a pyruvate kinase, and we see no evidence that pyruvate kinase is present in other strains of *C. thermocellum*.

Options for PEP-to-pyruvate conversion. In the absence of PK, there are three apparent options for the conversion of PEP to pyruvate: pyruvate phosphate dikinase, PEP synthase, and carboxylation of PEP to oxaloacetate (Fig. 1). As *ppdk* deletion strain M1631 proceeded with only a slightly diminished rate, we conclude that this enzyme is not important for glycolysis in *C. thermocellum*. PEP synthase seems to be an unlikely candidate for PEP-to-pyruvate conversion due to its low expression at the transcript (14) and protein level (15). The remaining option is conversion via PEPCK, which was present at a high level of activity, and we suspect that the majority of PEP is converted to OAA via this enzyme (7).

OAA can be converted directly to pyruvate via the OAA decar-

boxylase activity of malic enzyme (EC 1.1.1.40) (Fig. 1). It was not reported by Lamed and Zeikus (35) whether OAA can also serve as a substrate for the unusual *C. thermocellum* malic enzyme, as described for some other malic enzymes. OAA can also be converted directly to pyruvate by the membrane-bound, proton-translocating OAA decarboxylase (EC 4.1.1.3) (*clo1313_1523*); however, its activity was not assayed because of the high rate of (nonenzymatic) chemical decomposition of OAA. It can be converted to pyruvate indirectly via malate with the activities of malate dehydrogenase and malic enzyme, both of which have high activity *in vitro*. This so-called malate shunt is believed to function in *Entamoeba histolytica* (37) and *Tritrichomonas foetus* (43), which also lack pyruvate kinase in glycolysis.

Stoichiometry of pyrophosphate turnover. The calculations presented in Table 3 and Fig. 3 show that the amount of PP_i generated in biosynthetic reactions is only a fraction of the total amount of PP_i required for the anaerobic metabolism of sugars via PP_i-linked PFK. Although this calculation was performed for only one specific cell composition, it is evident that the shortage of PP_i holds for any cell composition, even when precursors for cell polymers are provided in the growth medium as yeast extract. This consequence of a PP_i-dependent glycolysis is entirely neglected in the literature. It is even frequently stated that the use of a PP_i-dependent PFK increases the ATP yield from glycolysis (37, 39, 44). However, our findings are confirmed by other authors. Heinonen (38), in his book on the biological role of inorganic pyrophosphate, reported that the amount of PP_i is only a fraction of the total amount of ATP generated. Klemme (45) calculated that the amount of PP_i produced in biosynthesis is 10 mmol/g biomass, comparable to our value of 11. It must be stressed that the value of 10 to 11 mmol PP_i applies only to calculations for glycolysis with an ATP-linked PFK. The results of our metabolic network analysis presented in Fig. 3 show that the net formation of PP_i in the overall synthesis of biomass from sugars is much smaller than 10 mmol/g cells when cell constituents are synthesized via a PP_i-dependent PFK. In this case, only a very small fraction of the PP_i generated in biosynthesis remains available for dissimilation of sugars to fermentation products (Fig. 3). Therefore, an increased ATP yield from glycolysis does not appear to be operative in *C. thermocellum* when the PFK reaction is PP_i dependent (37, 39, 44). Most of the PP_i required does arise as a by-product from biosynthesis but must be synthesized from ATP or GTP by a separate mechanism.

The assumption that biosynthesis can be a sole source of pyrophosphate is also falsified by the results of washed-cell experiments. Nongrowing cells are still capable of carrying out a PP_i-dependent glycolysis (Fig. 4). The reduced rate of glycolysis in such cells is likely due to a limitation by the rate of ATP dissipation. ATP cannot anymore be used for biosynthesis, and the ATP (or GTP) generated in glycolysis must be dissipated in futile cycles.

Alternative sources of pyrophosphate in metabolism. As biosynthetic reactions yield insufficient PP_i for sugar catabolism in a PP_i-dependent glycolysis, other mechanisms must exist to supply the PP_i needed for glycolysis in *C. thermocellum*. Various possibilities exist in this respect.

(i) ATP-pyrophosphatase (ATP $\Leftrightarrow$ AMP + PP_i) (EC 3.6.1.8) as present in *Spirochaeta thermophila* (46).

(ii) PP_i yielding reactions in the conversion of pyruvate to fermentation products such as PP_i-dependent acetate kinase (acetylphosphate + P_i $\Leftrightarrow$ acetate + PP_i) (EC 2.7.2.12) as occurs in *E. histolytica* (47) or PP_i-linked PEP carboxykinase (PEP + P_i + CO_2 $\Leftrightarrow$ OAA + PP_i) (EC 4.1.1.38) as occurs in *P. shermanii* (48) and *E. histolytica* (49). In *C. thermocellum*, however, acetate kinase has been shown to be ATP dependent (50) and P_i could not replace GDP in the PEP carboxykinase reaction (Table 1).

(iii) Reversed reaction of a membrane-bound ion-pumping inorganic pyrophosphatase (2 P_i $\Leftrightarrow$ PP_i + H_2O). It is well known that this enzyme can generate PP_i in *Rhodospirillum rubrum* and plants (38, 51). In the *C. thermocellum* genome, a gene encoding a potential proton pumping PP_i-ase, *clo1313_0823*, is present, and it is highly expressed at the transcription level (52) but not at the translation level (15).

(iv) ADP-glucose synthase (ATP + glucose-1-P $\Leftrightarrow$ ADP-glucose + PP_i). Simultaneous formation and degradation of glycogen has been observed in various cellulolytic bacteria such as *Fibrobacter succinogenes* (53), *Ruminococcus albus* (54) and *Clostridium cellulolyticum* (55). A key enzyme in this process is ADP-glucose synthase (glucose-1-phosphate + ATP $\Leftrightarrow$ ADP-glucose + PP_i). This enzyme is annotated in the genomes of these bacteria and is also present in the *C. thermocellum* genome. In saccharolytic thermophiles, such as *Thermoanaerobacter* spp., polysaccharide synthesis proceeds via UDP-glucose but not ADP-glucose (12).

The mechanisms for PP_i generation in *C. thermocellum* metabolism remain to be investigated. Glycogen cycling deserves special attention in this respect, as a variety of cellulolytic bacteria such as *C. cellulolyticum*, *F. succinogenes*, and *R. albus* exhibit this peculiar phenomenon (53–55). ADP-glucose synthase activity was present in cellobiose-grown *C. thermocellum* (Table 1) and the *C. thermocellum* genome also contains the other genes required for cycling of glycogen. It may well be that the primary function of glycogen cycling in bacteria is the supply of pyrophosphate for glycolysis, as has been suggested for the PP_i-dependent glycolysis in the noncellulolytic *Actinomyces naeslundii* (29).

ATP yield of glycolysis in *C. thermocellum*. Our study shows that published schemes on pyrophosphate-dependent glycolysis in anaerobic heterotrophic bacteria are not valid. This holds in particular for *C. thermocellum*. Not only ATP but also GTP is generated, and inorganic pyrophosphate is consumed (Table 1 and Fig. 1). Entrance of cellobiose into the cell by an ABC transporter may require between one and two ATP (56). It has, however, been calculated from biomass yields that oligosaccharide transport by this mechanism probably requires one ATP equivalent in both *C. thermocellum* (57) and *E. coli* (58). The relative amounts of GTP and ATP are dependent on the *in vivo* cofactor specificities of an ABC transporter for ATP or GTP (59) and of the phosphoglycerate kinase reaction (Table 1). Pyrophosphate must be generated directly or indirectly via ATP or GTP hydrolysis (Fig. 3). Assuming that both cellobiose transport and pyrophosphate synthesis require one ATP equivalent, and in the (unlikely) event that pyruvate formation proceeds exclusively via the malate shunt, without direct decarboxylation of OAA, the equation for glycolysis in cellobiose-grown *C. thermocellum* can be written as 1 cellobiose + 4 NADP + 4 NDP $\Leftrightarrow$ 4 pyruvate + 4 NADPH + 4 NTP.

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REFERENCES

1. **Lynd L, van Zyl W, McBride J, Laser M.** 2005. Consolidated bioprocessing of cellulosic biomass: an update. Curr. Opin. Biotechnol. **16**:577–583.
2. **Demain AL, Newcomb M, Wu JHD.** 2005. Cellulase, clostridia, and ethanol. Microbiol. Mol. Biol. Rev. **69**:124–154.
3. **Olson D, Tripathi S, Giannone R, Lo J, Caiazza N, Hogsett D, Hettich R, Guss A, Dubrovsky G, Lynd L.** 2010. Deletion of the Cel48S cellulase from *Clostridium thermocellum.* Proc. Natl. Acad. Sci. U. S. A. **107**:17727–17732.
4. **Tyurin M, Desai S, Lynd L.** 2004. Electrotransformation of *Clostridium thermocellum.* Appl. Environ. Microbiol. **70**:883–890.
5. **Tripathi SA, Olson DG, Argyros DA, Miller BB, Barrett TF, Murphy DM, McCool JD, Warner AK, Rajgarhia VB, Lynd LR, Hogsett DA, Caiazza NC.** 2010. Development of *pyrF*-based genetic system for targeted gene deletion in *Clostridium thermocellum* and creation of a *pta* mutant. Appl. Environ. Microbiol. **76**:6591–6599.
6. **Argyros D, Tripathi S, Barrett T, Rogers S, Feinberg L, Olson D, Foden J, Miller B, Lynd L, Hogsett D, Caiazza N.** 2011. High ethanol titers from cellulose by using metabolically engineered thermophilic, anaerobic microbes. Appl. Environ. Microbiol. **77**:8288–8294.
7. **Deng Y, Olson DG, Zhou J, Herring CD, Joe Shaw A, Lynd LR.** 2013. Redirecting carbon flux through exogenous pyruvate kinase to achieve high ethanol yields in *Clostridium thermocellum.* Metab. Eng. **15**:151–158.
8. **Lamed R, Zeikus J.** 1980. Ethanol production by thermophilic bacteria: relationship between fermentation product yields of and catabolic enzyme activities in *Clostridium thermocellum* and *Thermoanaerobium brockii.* J. Bacteriol. **144**:569–578.
9. **Patni N, Alexander J.** 1971. Utilization of glucose by *Clostridium thermocellum*: presence of glucokinase and other glycolytic enzymes in cell extracts. J. Bacteriol. **105**:220–225.
10. **Nochur SV, Jacobson GR, Roberts MF, Demain AL.** 1992. Mode of sugar phosphorylation in *Clostridium thermocellum*. Appl. Biochem. Biotechnol. **33**:33–41.
11. **Ng T, Zeikus J.** 1982. Differential metabolism of cellobiose and glucose by *Clostridium thermocellum* and *Clostridium thermohydrosulfuricum.* J. Bacteriol. **150**:1391–1399.
12. **Kanehisa M, Goto S, Sato Y, Furumichi M, Tanabe M.** 2012. KEGG for integration and interpretation of large-scale molecular data sets. Nucleic Acids Res. **40**:D109–D114.
13. **Roberts S, Gowen C, Brooks J, Fong S.** 2010. Genome-scale metabolic analysis of Clostridium thermocellum for bioethanol production. BMC Syst. Biol. **4**:31.
14. **Riederer A, Takasuka T, Makino S-I, Stevenson D, Bukhman Y, Elsen N, Fox B.** 2011. Global gene expression patterns in *Clostridium thermocellum* as determined by microarray analysis of chemostat cultures on cellulose or cellobiose. Appl. Environ. Microbiol. **77**:1243–1253.
15. **Rydzak T, McQueen P, Krokhin O, Spicer V, Ezzati P, Dwivedi R, Shamshurin D, Levin D, Wilkins J, Sparling R.** 2012. Proteomic analysis of *Clostridium thermocellum* core metabolism: relative protein expression profiles and growth phase-dependent changes in protein expression. BMC Microbiol. **12**:214.
16. **Golovchenko NP, Chuvilskaya NA, Akimenko VK.** 1986. Regulation of biosynthesis of cellylolytic enzymes and enzymes catalyzing initial catabolism of glucose and cellobiose in *Clostridium thermocellum.* Microbiology **55**:23–25.
17. **Cooney CL, Demain AL, Sinskey AJ, Wang DIC.** 1987. Degradation of lignocellulosic biomass and its subsequent utilization for the production of liquid fuels. Technical Report SERI/STR-231-3180. Solar Energy Research Institute, Golden, CO.
18. **Chi A, Deng Z, Albach R, Kemp R.** 2001. The two phosphofructokinase gene products of *Entamoeba histolytica.* J. Biol. Chem. **276**:19974–19981.
19. **Meurice G, Deborde C, Jacob D, Falentin H, Boyaval P, Dimova D.** 2004. In silico exploration of the fructose-6-phosphate phosphorylation step in glycolysis: genomic evidence of the coexistence of an atypical ATP-dependent along with a PPi-dependent phosphofructokinase in *Propionibacterium freudenreichii subsp. shermanii*. In Silico Biol. **4**:517–528.
20. **Burton E, Martin V.** 2012. Proteomic analysis of *Clostridium thermocellum* ATCC 27405 reveals the up-regulation of an alternative transhydrogenase-malate pathway and nitrogen assimilation in cells grown on cellulose. Can. J. Microbiol. **58**:1378–1388.
21. **Tjaden B, Plagens A, Dörr C, Siebers B, Hensel R.** 2006. Phosphoenolpyruvate synthetase and pyruvate, phosphate dikinase of *Thermoproteus tenax*: key pieces in the puzzle of archaeal carbohydrate metabolism. Mol. Microbiol. **60**:287–298.
22. **Reeves RE.** 1968. A new enzyme with the glycolytic function of pyruvate kinase. J. Biol. Chem. **243**:3202–3204.
23. **Mai V, Lorenz WW, Wiegel J.** 1997. Transformation of *Thermoanaerobacterium* sp. strain JW/SL-YS485 with plasmid pIKM1 conferring kanamycin resistance. FEMS Microbiol. Lett. **148**:163–167.
24. **Hogsett DAL.** 1995. Cellulose hydrolysis and fermentation by *Clostridium thermocellum* for the production of ethanol. Dartmouth College, Hanover, NH.
25. **Baskaran S, Ahn HJ, Lynd LR.** 1995. Investigation of the ethanol tolerance of *Clostridium thermosaccharolyticum* in continuous culture. Biotechnol. Prog. **11**:276–281.
26. **Olson D, Lynd L.** 2012. Computational design and characterization of a temperature-sensitive plasmid replicon for gram positive thermophiles. J. Biol. Eng. **6**:5.
27. **Zhang Y-HP, Lynd L.** 2005. Regulation of cellulase synthesis in batch and continuous cultures of *Clostridium thermocellum.* J. Bacteriol. **187**:99–106.
28. **Kruger N.** 1994. The Bradford method for protein quantitation, p 9–15. *In* Walker J (ed), Basic protein and peptide protocols, **vol 32**. Humana Press, Clifton, NJ.
29. **Takahashi N, Kalfas S, Yamada T.** 1995. Phosphorylating enzymes involved in glucose fermentation of *Actinomyces naeslundii.* J. Bacteriol. **177**:5806–5811.
30. **Ye RW, Zielinski NA, Chakrabarty AM.** 1994. Purification and characterization of phosphomannomutase/phosphoglucomutase from *Pseudomonas aeruginosa* involved in biosynthesis of both alginate and lipopolysaccharide. J. Bacteriol. **176**:4851–4857.
31. **de Jong-Gubbels P, Vanrolleghem P, Heijnen S, van Dijken JP, Pronk JT.** 1995. Regulation of carbon metabolism in chemostat cultures of *Saccharomyces cerevisiae* grown on mixtures of glucose and ethanol. Yeast **11**:407–418.
32. **Van Hoek P, Van Dijken J, Pronk J.** 1998. Effect of specific growth rate on fermentative capacity of baker's yeast. Appl. Environ. Microbiol. **64**:4226–4233.
33. **Jabalquinto A, Laivenieks M, Zeikus J, Cardemil E.** 1999. Characterization of the oxaloacetate decarboxylase and pyruvate kinase-like activities of *Saccharomyces cerevisiae* and *Anaerobiospirillum succiniciproducens* phosphoenolpyruvate carboxykinases. J. Protein Chem. **18**:659–664.
34. **Rokosh DA, Kurz WGW, LaRue TA.** 1973. A modification of isocitrate and malate dehydrogenase assays for use in crude cell free extracts. Anal. Biochem. **54**:477–483.
35. **Lamed R, Zeikus J.** 1981. Thermostable, ammonium-activated malic enzyme of *Clostridium thermocellum.* Biochim. Biophys. Acta **660**:251–255.
36. **Taymaz-Nikerel H, Borujeni A, Verheijen P, Heijnen J, van Gulik W.** 2010. Genome-derived minimal metabolic models for *Escherichia coli* MG1655 with estimated in vivo respiratory ATP stoichiometry. Biotechnol. Bioeng. **107**:369–381.
37. **Mertens E.** 1993. ATP versus pyrophosphate: glycolysis revisited in parasitic protists. Parasitol. Today **9**:122–126.
38. **Heinonen JK.** 2001. Biological role of inorganic pyrophosphate. Kluwer Academic Publishers, Boston, MA.
39. **Bielen A, Willquist K, Engman J, van der Oost J, van Niel E, Kengen S.** 2010. Pyrophosphate as a central energy carrier in the hydrogen-producing extremely thermophilic *Caldicellulosiruptor saccharolyticus.* FEMS Microbiol. Lett. **307**:48–54.
40. **McBee RH.** 1948. The culture and physiology of a thermophilic cellulose-fermenting bacterium. J. Bacteriol. **56**:653–663.
41. **Erbeznik M, Jones C, Dawson K, Strobel H.** 1997. *Clostridium thermocellum* JW20 (ATCC 31549) is a coculture with *Thermoanaerobacter ethanolicus.* Appl. Environ. Microbiol. **63**:2949–2951.
42. **Finn RD, Mistry J, Tate J, Coggill P, Heger A, Pollington JE, Gavin OL, Gunasekaran P, Ceric G, Forslund K, Holm L, Sonnhammer ELL, Eddy SR, Bateman A.** 2010. The Pfam protein families database. Nucleic Acids Res. **38**:D211–D222.
43. **Hrdý I, Mertens E, Van Schaftingen E.** 1993. Identification, purification

and separation of different isozymes of NADP-specific malic enzyme from *Tritrichomonas foetus.* Mol. Biochem. Parasitol. **57**:253–260.
44. **Chastain C, Failing C, Manandhar L, Zimmerman M, Lakner M, Nguyen T.** 2011. Functional evolution of C(4) pyruvate, orthophosphate dikinase. J. Exp. Bot. **62**:3083–3091.
45. **Klemme JH.** 1976. Regulation of intracellular pyrophosphatase activity and conservation of phosphoanhydride energy of inorganic pyrophosphate in microbial metabolism. Z. Naturforsch. C **31**:544–550.
46. **Janssen P, Morgan H.** 1992. Glucose catabolism by *Spirochaeta thermophila* RI 19.B1. J. Bacteriol. **174**:2449–2453.
47. **Fowler M, Ingram-Smith C, Smith K.** 2012. Novel pyrophosphate-forming acetate kinase from the protist *Entamoeba histolytica.* Eukaryot. Cell **11**:1249–1256.
48. **Lochmüller H, Wood HG, Davis JJ.** 1966. Phosphoenolpyruvate carboxytransphosphorylase. II. Crystallization and properties. J. Biol. Chem. **241**:5678–5691.
49. **Reeves RE.** 1970. Phosphopyruvate carboxylase from *Entamoeba histolytica*. Biochim. Biophys. Acta **220**:346–349.
50. **Lin WR, Peng Y, Lew S, Lee CC, Hsu JJ, Hamel J-F, Demain AL.** 1998. Purification and characterization of acetate kinase from *Clostridium thermocellum.* Tetrahedron **54**:15915–15925.
51. **Baltscheffsky M, Schultz A, Baltscheffsky H.** 1999. H^+-PPases: a tightly membrane-bound family. FEBS Lett. **457**:527–533.
52. **Raman B, McKeown C, Rodriguez M, Brown S, Mielenz J.** 2011. Transcriptomic analysis of *Clostridium thermocellum* ATCC 27405 cellulose fermentation. BMC Microbiol. **11**:134.
53. **Gaudet G, Forano E, Dauphin G, Delort A.** 1992. Futile cycling of glycogen in *Fibrobacter succinogenes* as shown by in situ 1H-NMR and 13C-NMR investigation. Eur. J. Biochem. **207**:155–162.
54. **Hungate RE.** 1963. Polysaccharide storage and growth efficiency in *Ruminococcus albus.* J. Bacteriol. **86**:848–854.
55. **Guedon E, Desvaux M, Petitdemange H.** 2000. Kinetic analysis of *Clostridium cellulolyticum* carbohydrate metabolism: importance of glucose 1-phosphate and glucose 6-phosphate branch points for distribution of carbon fluxes inside and outside cells as revealed by steady-state continuous culture. J. Bacteriol. **182**:2010–2017.
56. **Mimmack M, Gallagher M, Pearce S, Hyde S, Booth I, Higgins C.** 1989. Energy coupling to periplasmic binding protein-dependent transport systems: stoichiometry of ATP hydrolysis during transport in vivo. Proc. Natl. Acad. Sci. U. S. A. **86**:8257–8261.
57. **Strobel H, Caldwell F, Dawson K.** 1995. Carbohydrate transport by the anaerobic thermophile *Clostridium thermocellum* LQRI. Appl. Environ. Microbiol. **61**:4012–4015.
58. **Muir M, Williams L, Ferenci T.** 1985. Influence of transport energization on the growth yield of *Escherichia coli.* J. Bacteriol. **163**:1237–1242.
59. **Zhong X, Tai PC.** 1998. When an ATPase is not an ATPase: at low temperatures the C-terminal domain of the ABC transporter CvaB is a GTPase. J. Bacteriol. **180**:1347–1353.