Glycolysis without pyruvate kinase in *Clostridium thermocellum*

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Abstract

The metabolism of *Clostridium thermocellum* is notable in that it assimilates sugar via the EMP pathway but does not possess a pyruvate kinase enzyme. In the wild type organism, there are three proposed pathways for conversion of phosphoenolpyruvate (PEP) to pyruvate, which differ in their cofactor usage. One path uses pyruvate phosphate dikinase (PPDK), another pathway uses the combined activities of PEP carboxykinase (PEPCK) and oxaloacetate decarboxylase (ODC). Yet another pathway, the malate shunt, uses the combined activities of PEPCK, malate dehydrogenase and malic enzyme. First we showed that there is no flux through the ODC pathway by enzyme assay. Flux through the remaining two pathways (PPDK and malate shunt) was determined by dynamic ¹³C labeling. In the wild-type strain, the malate shunt accounts for about 33±2% of the flux to pyruvate, with the remainder via the PPDK pathway. Deletion of the *ppdk* gene resulted in a redirection of all pyruvate flux through the malate shunt. This provides the first direct evidence of the in-vivo function of the malate shunt.

Key words

¹³C flux analysis; pyruvate kinase; malate shunt; malic enzyme; malate dehydrogenase; oxaloacetate decarboxylase

Background

Clostridium thermocellum is a bacterium used to produce ethanol from cellulose as a renewable biofuel. Ethanol yields have been successfully increased (Argyros et al., 2011; Biswas et al., 2015, 2014; Deng et al., 2013; Papanek et al., 2015; Tian et al., 2016; Tripathi et al., 2010), but incomplete understanding of metabolic pathways hampers rapid progress. A number of interesting features of the metabolic network of *C. thermocellum* have been discovered. One of the most notable of these is the lack of pyruvate kinase (Rydzak et al., 2012; Zhou et al., 2013). Pyruvate is a key metabolic intermediate for most organisms. In organisms that use the Embden-Meyerhof-Parnas (EMP) pathway, pyruvate is

typically produced from phosphoenolpyruvate (PEP) either by the pyruvate kinase enzyme (EC 2.7.1.40) or by the phosphotransferase system (PTS) during sugar import. *C. thermocellum* uses the EMP pathway for conversion of sugars to pyruvate (Lamed and Zeikus, 1980), but it does not use the PTS system (Deng et al., 2013)(Nochur et al., 1992), and does not have a pyruvate kinase enzyme (Zhou et al., 2013).

Figure 1. Pathways for conversion of phosphoenolpyruvate to pyruvate in *C. thermocellum*. Engineered strains of *C. thermocellum* have been developed that contain a heterologous pyruvate kinase enzyme from *T. saccharolyticum*, which is indicated by a green arrow. Note that PEPS is generally thought to function only in the direction of PEP production from pyruvate (i.e. opposite to what is shown here) based on thermodynamic considerations, but it has been included here in the PEP to pyruvate direction since we are enumerating all of the possible routes for flux from PEP to pyruvate.

There are a number of potential pathways for conversion of PEP to pyruvate (Figure 1). Wild type *C. thermocellum* has two enzymes that could directly convert PEP to pyruvate: phosphoenolpyruvate synthetase (PEPS, EC 2.7.9.2) and pyruvate phosphate dikinase (PPDK, EC 2.7.9.1). While both of these enzymes are typically thought to be involved in gluconeogenesis (Tjaden et al., 2006), PPDK has been shown to be reversible in several organisms (Varela-Gomez et al., 2004)(South and Reeves, 1975) and is expressed at high levels in *C. thermocellum*(Taillefer et al., 2015). Since there are no known growth conditions in *C. thermocellum* under which one might expect gluconeogenesis to occur, it could participate in glycolysis. However deletion in *C. thermocellum* did not affect the production of acetate or ethanol (Zhou et al., 2013). PEPS is expressed at very low levels in *C. thermocellum*(Riederer et al., 2011).

Another possible pathway involves the conversion of PEP to oxaloacetate (OAA), malate, and finally to pyruvate, via three enzymes, phosphoenolpyruvate carboxykinase (PEPCK, EC 4.1.1.32), malate

dehydrogenase (MDH, 1.1.1.37) and malic enzyme (ME, EC 1.1.1.39/1.1.1.40). This pathway was first proposed by Lamed and Zeikus in 1980 (Lamed and Zeikus, 1980) and is known as the malate shunt. All of the required enzymes are present and highly active in *C. thermocellum* (Deng et al., 2013) (Lamed and Zeikus, 1981) (Taillefer et al., 2015), however its *in-vivo* function has not been conclusively demonstrated.

In yet another pathway, OAA can be converted to pyruvate by decarboxylation. This conversion can be mediated by a variety of enzymes, but does not appear to be present in *C. thermocellum*.

Although pyruvate kinase (PYK, EC 2.7.1.40) is not present in wild-type *C. thermocellum*, a heterologous pyruvate kinase from *Thermoanaerobacterium saccharolyticum* has been expressed in *C. thermocellum* (Deng et al., 2013). Disruption of the malate shunt was only achieved in strains with a heterologous pyruvate kinase (Deng et al., 2013). The malate shunt was disrupted by insertion of an antibiotic resistance cassette into the malic enzyme gene which increased ethanol production. This change in fermentation products suggested an important role for malic enzyme, but it could not be determined which pathways were actually being used for conversion of PEP to pyruvate.

In order to produce ethanol at high yield and titer, the pathways for nicotinamide cofactors (NADH and NADPH) need to be balanced. Since the choice of pathway for PEP to pyruvate conversion affects this balance, we set out to characterize this node of metabolism via gene deletions, enzyme assays and ¹³C flux analysis.

Materials and methods

Growth medium

C. thermocellum DSM1313 was obtained from the DSMZ culture collection. Strains were grown anaerobically at 55°C in AN7 medium. This medium is similar to previously described minimal medium (Amador-Noguez et al., 2010). Per liter, it contained 1.02 g ammonium sulfate ((NH₄)₂SO₄), 0.1 g magnesium chloride hexahydrate (MgCl₂ • 6 H₂O), 0.02 g calcium chloride dihydrate (CaCl₂ • 2 H₂O), 1.0 g L-cysteine HCl monohydrate, 0.02 g iron sulfate heptahydrate (FeSO₄ • 7 H₂O), 2.5 g cellobiose, 40 ml vitamin solution (see below), 1 ml trace mineral solution (see below) and 5.71 g potassium phosphate dibasic trihydrate (K₂HPO₄ • 3 H₂O). Components were added in the order listed. To prevent precipitation, the potassium phosphate was dissolved in water before being added. The final pH of the medium was usually 7.2, although when necessary it was adjusted with HCl. The medium was sterilized by passing through a 0.22 µm filter. Usually this filter sterilization step was performed in the anaerobic chamber (see enzyme assay section for details of anaerobic chamber).

The vitamin solution was made at 25x concentration and contained, per liter, 25 mg d-biotin, 38 mg nicotinic acid (vitamin B3), 25 mg cyanocobalamin (vitamin B12), 63 mg p-aminobenzoic acid, 63 mg thiamine-HCl, 63 mg pantothenic acid hemicalcium salt (vitamin B5), 80 mg riboflavin phosphate (vitamin B2), 38 mg pyridoxamine dihydrochloride. This solution was filter sterilized and stored at 4°C for up to a year.

The trace mineral solution was made at 1000x concentration and contained, per liter, 5000 mg iron (II) sulfate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$), 1440 mg zinc sulfate heptahydrate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$), 800 mg manganese (II) chloride tetrahydrate ($\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$), 250 mg copper (II) sulfate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$), 200 mg sodium tetraborate ($\text{Na}_2\text{B}_4\text{O}_7$), 1000 mg ammonium molybdate ($(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$), 40 mg nickel chloride hexahydrate ($\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$), 37 mg cobalt chloride hexahydrate ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$), 30 mg boric acid (H_3BO_3), 20 mg sodium selenite (Na_2SeO_3) and 10.2 ml of 12.2N hydrochloric acid. This solution was filter sterilized and stored at 4°C for up to a year.

In previous work we have used either CTFUD rich medium (Olson and Lynd, 2012) or MTC defined medium (Zhou et al., 2013). For these experiments we chose not to use CTFUD medium because the yeast extract would have interfered with our quantification of extracellular fluxes and also interfered with LC/MS analysis. We used MTC in some of our preliminary experiments, but found that the MOPS and citrate in this medium also interfered with LC/MS analysis. Because the buffering capacity in AN7 medium is lower than that of MTC, we found that wild type *C. thermocellum* cultures could only consume 2.5 g/l cellobiose before becoming limited by low pH, so that was the maximum concentration of cellobiose we used with this medium.

Strain construction

Strains of *C. thermocellum* were constructed by transformation with plasmid DNA followed by successive rounds of positive and negative selection as previously described (Olson and Lynd, 2012). The genotypes of strains used in this work are listed in Table 1. Plasmids pDGO134 and pZJ08 used in this work are based on plasmid pDGO-68, which is the standard plasmid backbone we use for making gene deletion plasmids. Plasmid pDGO-68 is based on plasmid pAMG258 (Olson and Lynd, 2012). Plasmid pAMG258 was designed to be used with yeast-mediated ligation cloning (Shanks et al., 2006). When we switched to homology-based cloning (i.e. Gibson Assembly) (Gibson, 2011), we found that we needed to add a 30 bp stretch of random DNA on either side of the PvuII cloning site, ATGCTTTAATACATACCTGGCCAGTAGTT and CTAATAGTAGTGAAAAAATCAGGATAATG. We suspect this may have disrupted secondary structure that was previously causing problems with cloning. Plasmid pMU2051 was a gift from the Mascoma Corporation.

Name	Genotype	Notes	Accession number*	Source
pDGO-68		Plasmid backbone used for making deletion plasmids, based on plasmid pAMG258 (Olson and Lynd, 2012)	KU682275	This work
pMU2051		Plasmid for deletion of the <i>ppdk</i> gene	KC146550	(Zhou et al., 2013)
pDGO134		Plasmid used to delete heterologous pyruvate kinase	KU682277	This work
pZJ08		Plasmid for deletion of the <i>peps</i> gene	KU682276	This work
pYD14		Plasmid used for disruption of malate shunt	KF806700	(Deng et al.,

				2013)
pDGO75		Plasmid used for attempted deletions of Clo1313_1523 (putative <i>odc</i> gene)	KU695468	This work
LL345	Δhpt	Derived from wild type <i>C. thermocellum</i> , DSMZ 1313 by deletion of <i>hpt</i> gene. The <i>hpt</i> gene is not thought to affect pyruvate metabolism. Also known as M1354.		(Argyros et al., 2011)
LL1010	$\Delta hpt \Delta ldh::P_{eno}-pyk(Tsc)$	Also known as strain DS8, derived from strain LL345 by insertion of pyruvate kinase from <i>T. saccharolyticum</i> at <i>ldh</i> locus.	SRP073622	(Deng et al., 2013)
LL1113	$\Delta hpt \Delta ldh::P_{eno}-pyk(Tsc) \Delta me::cat-hpt$	Also known as strain YD02, derived from strain LL1010 using plasmid pYD14.		(Deng et al., 2013)
LL1138	$\Delta hpt \Delta ppdk$	Also known as strain M1631, derived from strain LL345 by deletion of <i>ppdk</i> gene using plasmid pMU2051.		(Zhou et al., 2013)
LL1163	$\Delta hpt \Delta ldh::P_{eno}-pyk(Tsc) \Delta me-mdh$	Derived from strain LL1113 by resolving the merodiploid with 8AZH selection against the <i>hpt</i> marker.		This work
LL1213	$\Delta hpt \Delta ldh::P_{eno}-pyk(Tsc) \Delta me-mdh \Delta ppdk$	Derived from strain LL1163 by deletion of <i>ppdk</i> gene using plasmid pMU2051.		This work
LL1227	$\Delta hpt \Delta ldh$	Derived from strain LL1010, the heterologous pyruvate kinase enzyme was deleted using plasmid pDGO134		This work
LL1251	$\Delta hpt \Delta ldh::P_{eno}-pyk(Tsc) \Delta me-mdh \Delta ppdk \Delta peps$	Derived from strain LL1213		This work

* Note, accession numbers for strains refer to raw resequencing data from the JGI Sequence Read Archive. Accession numbers for plasmids refer to GenBank <http://www.ncbi.nlm.nih.gov/genbank/>.

Table 1. Strains and plasmids used in this work

Whole genome resequencing

Whole genome resequencing was used to verify strain construction and check for secondary mutations. It was performed as previously described (Zhou et al., 2015). Raw data is available from the JGI Sequence Read Archive. Briefly, genomic DNA was submitted to the Joint Genome Institute (JGI) for sequencing with an Illumina MiSeq instrument. Unamplified libraries were generated using a modified version of Illumina's standard protocol. 100 ng of DNA was sheared to 500 bp using a focused ultrasonicator (Covaris). The sheared DNA fragments were size-selected using SPRI beads (Beckman Coulter). The selected fragments were then end-repaired, A-tailed, and ligated to Illumina compatible adapters (IDT, Inc.) using KAPA- Illumina library creation kit (KAPA biosystems). Libraries were quantified using KAPA Biosystems' next-generation sequencing library qPCR kit and run on a Roche LightCycler 480 real-time PCR instrument. The quantified libraries were then multiplexed into pools for sequencing. The pools were loaded and sequenced on the Illumina MiSeq sequencing platform utilizing a MiSeq Reagent Kit v2 (300 cycle) following a 2 × 150 indexed run recipe. Paired-end reads were generated, with an

average read length of 150 bp and paired distance of 500 bp. Data was analyzed with CLC Genomics Workbench v8 (Qiagen).

Enzyme assays

Preparation of cell-free extract

Cells were grown in AN7 medium to mid-log phase ($OD_{600} = 0.2 - 0.6$), separated by centrifugation at $7000 \times g$ for 6 minutes at room temperature ($\sim 25^{\circ}\text{C}$). The supernatant was discarded and the cell pellet was washed two times with 1 ml of a buffer containing 100 mM Tris-HCl (pH 7.5 at 25°C) and 2 mM dithiothreitol (DTT). The cell pellet was resuspended in a final volume of 200 – 1000 μl of the wash buffer. 10 μl (4000 – 8000U) of a 1:50 dilution of Ready-Lyse Lysozyme enzyme (Epicentre) was added and cells were incubated at room temperature for 30 minutes or until an increase in viscosity (due to DNA) was observed. Then 5 μl DNase I (Thermo Fisher) was added to reduce the viscosity of the solution and it was incubated for an additional 10 minutes. The resulting solution was centrifuged at $10,000 \times g$ for 3 minutes at room temperature and the supernatant was used as cell-free extract (CFE) for enzyme assays. CFE was used immediately or stored at 4°C for up to one week.

The initial centrifugation step was performed aerobically, although the tubes were kept tightly capped when outside of the anaerobic chamber. The subsequent washes and all other steps were performed in a COY anaerobic chamber (COY labs, Grass Lake, MI). The anaerobic atmosphere was 10% CO_2 , 1-3% hydrogen and the balance nitrogen. Anaerobic conditions were maintained (less than 5 ppm oxygen) with a palladium catalyst.

Enzyme assays

Enzyme activity was measured in an anaerobic chamber. Continuous assays were performed by spectrophotometer. Quantification of discontinuous assays was performed by HPLC.

Spectrophotometer-based assays were performed using an Agilent 8453 UV-vis spectrophotometer with a water bath used to maintain assay temperature. Reactions were measured at 55°C unless otherwise noted. The reaction volume was 1 ml, in reduced-volume quartz cuvettes (part number 29MES10; Fireflysci, NY) with a 1.0 cm path length. The ODC assay was performed in a cuvette or in a 96-well microplate using a Powerwave XS reader (Biotek, VT). For assays at 265 nm, a Corning UV-transparent microplate was used. Since the pathlength depended on the assay volume, standards of known concentration were included in each plate.

The units for all enzyme activities are expressed as $\mu\text{mol of product} \cdot \text{min}^{-1} \cdot (\text{mg of cell extract protein})^{-1}$. 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 chemicals and coupling enzymes were purchased from Sigma. For assays involving NADH or NADPH, an extinction coefficient of $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ was used.

Pyruvate kinase (PYK, EC 2.7.1.40)

PYK was measured by coupled assay with lactate dehydrogenase (LDH), similar to the assay described by Zhou et al. (Zhou et al., 2013). The assay buffer contained 50 mM Tris-HCl, pH 7.5 (at 55°C), 5 mM

dithiothreitol (DTT), 10 mM KCl, 12 mM MgCl₂, 10 mM ADP, 0.1 mM 3-phosphoglyceric acid (3PG), 5 mM PEP, 12 U LDH enzyme (Sigma 59747) and 0.3 mM NADH. Water was added to a final volume of 1.0-1.3 ml. PEP was converted to pyruvate, which was converted to lactate by LDH with the concomitant reduction of NADH to NAD⁺. The decrease in NADH concentration over time was measured at 340 nm by spectrophotometer. The reaction could be started with either ADP or PEP. For routine assays it was started with PEP. The assay is sensitive to MgCl₂ levels and did not work when MgCl₂ was lower than 2 mM. In some cases, NADH reduction was observed in the absence of LDH. This background activity (likely from the combined activity of PEPCK and MDH) was subtracted from the reported value.

Pyruvate-phosphate dikinase activity (PPDK, EC 2.7.9.1)

PPDK was measured by coupled assay with LDH, similar to the assay described by Zhou et al. (Zhou et al., 2013). The assay buffer contained 50 mM Tris-HCl, pH 7.5 (at 55°C), 5 mM DTT, 0.3 mM NADH, 5 mM MgCl₂, 20 mM NH₄Cl, 2 mM PEP, 12 U LDH enzyme (Sigma 59747), 2 mM AMP and 1 mM pyrophosphate (PP_i). Unless otherwise noted, the reaction was started with the addition of PP_i, although it could also be started by adding AMP or PEP. The decrease in NADH concentration over time was measured at 340 nm by spectrophotometer.

The inclusion of NH₄Cl is based on the assay of Saavedra-Lira (Saavedra-Lira et al., 1998), and may explain why previous reports failed to detect PPDK activity in *C. thermocellum* (Zhou et al., 2013). At 55°C, MgCl₂ and PP_i form a precipitate. The combination of 5 mM MgCl₂ and 1 mM PP_i was stable at 55°C for 30 minutes, but formed a precipitate after 1 hour. This was slow enough that it did not interfere with the assay, but slight increases in the concentration of either compound resulted in rapid precipitation.

Phosphoenolpyruvate synthase (PEPS, EC 2.7.9.2)

PEPS was measured by two different methods. The first method is similar to the PYK assay, but potassium phosphate buffer (pH 7.0 at 55°C) was used in place of Tris-HCl and AMP was used in place of ADP. This assay measured conversion of PEP to pyruvate by coupling with the LDH enzyme. The assay buffer contained 50 mM potassium phosphate buffer (pH 7.0 at 55°C), 5 mM dithiothreitol (DTT), 10 mM KCl, 20 mM MgCl₂, 10 mM AMP, 5 mM PEP, 12 U LDH enzyme (Sigma 59747) and 0.3 mM NADH. Water was added to a final volume of 1.0-1.3 ml. The decrease in NADH concentration over time was measured at 340 nm by spectrophotometer.

The second method measured conversion of pyruvate to PEP. The reaction volume was 1 ml and contained 50 mM Tris-HCl, pH 7.5 (at 55°C), 5 mM DTT, 10 mM KCl, 20 mM MgCl₂, 10 mM ATP and 5 mM pyruvate. At ~5 minute intervals, an 80 µl sample was taken, diluted 1:5 by adding 320 µl water and acidified with 20 µl of 10% sulfuric acid (H₂SO₄) to stop the reaction. These samples were stored at 4°C until they could be analyzed by HPLC. Components were separated on an Aminex HPX-87H column (BioRad Laboratories). PEP concentrations were measured with the absorbance (UV) detector at 225 nm and could be quantified in the range of 0.01 to 10 mM. Pyruvate concentrations were measured with the refractive index (RI) detector and absorbance (UV) detector at 225 nm, and could be quantified in the range of 0.2 mM to 20 mM. See HPLC section for additional details.

Oxaloacetate decarboxylase (ODC, EC 4.1.1.3)

ODC activity was measured by several methods. The first method is based on assay described by Dimroth et al. (Dimroth, 1981). The disappearance of oxaloacetate (OAA) was determined directly by measuring the decrease in absorbance at 265 nm. The final reaction volume was 1.0-1.3 ml and contained 50 mM Tris-HCl buffer, pH 7.5 (at 55°C), 5 mM DTT, 20 mM NaCl or 2 mM MgCl₂ or 2 mM MnCl₂ and 1.6 mM OAA. After the OAA was added, the absorbance at 265 nm increased for a short time as the keto-enol equilibrium was reached. At 25°C this took 5-15 seconds. At 55°C this took less than 5 seconds. The reaction was then started by adding cell-free-extract, then the rate of decrease in absorbance at 265 nm was measured. An extinction coefficient of $\epsilon_{265} = 0.88 \text{ mM}^{-1} \text{ cm}^{-1}$ was determined based on a standard curve. The cuvette pathlength was 1 cm. This assay was also performed in a Powerwave XS microplate reader (Biotek, VT). When this device was used, the reaction volume was 100 μl , the pathlength was 0.27 cm and an OAA concentration of 10 mM was used. This assay was performed at both 25°C and 55°C.

The second method is also based on an assay described by Dimroth et al. (Dimroth, 1981). The pyruvate produced from OAA was converted to lactate by lactate dehydrogenase (LDH) and coupled to the concomitant reduction of NADH to NAD⁺, which was measured by a decrease in absorption at 340 nm. This assay could not be used in the presence of large amounts of malate dehydrogenase activity (which converts NADH and OAA to NAD⁺ and malate), so it was only used with strains LL1163 and LL1251. The final reaction volume was 1.0-1.3 ml and contained 50 mM Tris-HCl buffer, pH 7.5 (at 55°C), 5 mM DTT, 20 mM NaCl or 2 mM MgCl₂ or 2 mM MnCl₂, 2.5 mM OAA, 12 U of LDH enzyme (Sigma 59747) and 0.3 mM NADH.

The third method is based on an assay described by Hsu and Lardy (Hsu and Lardy, 1966). The final reaction volume was 1 ml and contained 50 mM Tris-HCl, pH 7.5 (at 55°C), 5 mM DTT, 10 mM NaCl, 10 mM MgCl₂ and 10 mM OAA. The reaction was started by adding cell-free extract. At ~5 minute intervals, an 80 μl sample was taken, diluted 1:5 by adding 320 μl water and acidified with 20 μl of 10% sulfuric acid (H₂SO₄) to stop the reaction. These samples were stored at 4°C until they could be analyzed by HPLC. Components were separated on an Aminex HPX-87H column (BioRad Laboratories). OAA concentrations were measured with the refractive index (RI) detector and absorbance (UV) detector at 225 and 265 nm, and could be quantified in the range of 0.2 mM to 20 mM. Pyruvate concentrations were measured with the refractive index (RI) detector and absorbance (UV) detector at 225 nm, and could be quantified in the range of 0.2 mM to 20 mM. See HPLC section for additional details.

Due to the spontaneous decarboxylation of OAA to pyruvate, 0.5 M stock solutions of OAA in water were prepared fresh daily. The spontaneous non-enzymatic decarboxylation of OAA was always measured with appropriate controls. This reaction was exacerbated at high temperature and in the presence of divalent cations (Mg²⁺ or Mn²⁺) (Ivanishev and Kurganov, 1992). At 25°C without divalent cations, the rate was 0.02 mM/min. At 55°C with 6 mM MgCl₂, the rate was 0.5 mM/min. For all assays, a commercial preparation of ODC (Sigma O4878) was used as a positive control. This enzyme requires divalent cations for maximum activity, although showed at least some activity under all conditions tested.

Phosphoenolpyruvate carboxykinase (PEPCK, EC 4.1.1.32)

PEPCK activity was measured by coupled assay with malate dehydrogenase (MDH), similar to the method described by Zhou et al. (Zhou et al., 2013). The reaction volume was 1.0-1.3 ml and contained 50 mM Tris-HCl, pH 7.5 (at 55°C), 5 mM DTT 0.3 mM NADH, 50 mM NaHCO₃, 2 mM MgCl₂, 2 mM PEP, 12 U MDH enzyme (Sigma M1567) and 2 mM GDP. The reaction was started with GDP. The decrease in NADH concentration over time was measured at 340 nm by spectrophotometer.

Malate dehydrogenase (MDH, EC 1.1.1.37)

MDH activity was measured with a method similar to that described by Zhou et al. (Zhou et al., 2013). The reaction volume was 1.0-1.3 ml and contained 50 mM Tris-HCl, pH 7.5 (at 55°C), 5 mM DTT 0.3 mM NADH and 10 mM OAA. The reaction was started with OAA. The decrease in NADH concentration over time was measured at 340 nm by spectrophotometer.

Malic enzyme (ME, EC 1.1.1.39/1.1.1.40)

ME activity was measured with a method similar to that described by Zhou et al. (Zhou et al., 2013). The reaction volume was 1.0-1.3 ml and contained 50 mM Tris-HCl, pH 7.5 (at 55°C), 5 mM DTT, 2 mM NADP⁺, 5 mM NH₄Cl and 2.5 mM malate. The reaction was started with malate. The increase in NADPH concentration over time was measured at 340 nm by spectrophotometer.

Note that the Enzyme Commission (EC) has numbers for three types of malic enzyme. EC 1.1.1.38 is NADH-linked and has oxaloacetate decarboxylase activity. EC 1.1.1.39 is NADH-linked and does not have oxaloacetate decarboxylase activity. EC 1.1.1.40 is NADPH-linked and has oxaloacetate decarboxylase activity. There is currently no EC number for an enzyme that is NADPH-linked and does not have oxaloacetate decarboxylase activity.

Fumarase (fumarate hydratase, FH, EC 4.2.1.2)

FH activity was measured by the method described by Sridhar et al. (Sridhar et al., 2000). The reaction volume was 1.0-1.3 ml and contained 50 mM Tris-HCl, pH 7.5 (at 55°C), 10 mM DTT and 50 mM malate. The production of fumarate was determined by measuring increases in absorbance at 240 nm. An extinction coefficient of $\epsilon_{240} = 2.27 \text{ mM}^{-1} \text{ cm}^{-1}$ was determined based on a standard curve. Fumarase enzyme F1506 (Sigma) was used as a positive control. The absorbance of cell-free-extract at 240 nm limited the amount that could be added to about 20 μl (30 μg protein).

¹³C labeling

Dynamic ¹³C labeling experiments were performed using the filter technique described by Hörl et al. (Hörl et al., 2013). The extraction solution was a 2:2:1 ratio of acetonitrile:methanol:water with 5 μM 2-keto-4-(methyl-d₃)pentanoic acid (used as an internal standard, Sigma, USA, part number 616621) and 1.6 mM phenylhydrazine-HCl for derivitization of keto-acids such as pyruvate and oxaloacetate (Zimmermann et al., 2014). Best derivitization results were obtained when the phenylhydrazine was added to the extraction immediately before use.

Cells were pre-cultured to mid-log phase (OD₆₀₀ = 0.2 – 0.5) on AN7 medium with 2.5 g/l naturally-labeled cellobiose. 5-15 ml of this culture was transferred to a membrane filter, vacuum-filtered,

followed by a 10 s washing phase with preheated AN7 medium with 0.1 mM naturally labeled cellobiose. ¹³C-labeling was initiated by changing the washing solution to 0.1 mM uniformly ¹³C-labeled cellobiose (Omicron Biochemicals, IN, USA).

The duration of ¹³C-labeling ranged from 0 to 120 seconds. The labeling experiment was performed within a COY anaerobic chamber and the whole chamber was incubated to 55°C (50-52°C when measured right at the filter). At the end of the ¹³C-labeling wash, the filter was removed from the vacuum apparatus and placed culture-side-down in a plastic petri dish with 1.6 ml cold extraction solution to quench metabolism and start extracting the metabolites. The extraction solution was kept cold by being placed in contact with a 4-inch-thick aluminum block pre-chilled to -80°C. Labeling experiments were performed in triplicate for each strain with cells from different pre-cultures.

The petri dishes with extraction solution and filter were placed at -80°C for 1 to 24 hours to allow a pause in the protocol. They were then thawed, and the cells were washed off of the filter by pipetting (using gentle scraping as needed). The cells and extraction solution were transferred to a 1.5 ml microcentrifuge tube and centrifuged at 15,000 x g for 1 minute. The supernatant was collected and placed in a rotary evaporator (Labconco Centrivap Concentrator) at a vacuum of 25-30 mm Hg and temperature of 25-30°C until dry, which usually took 4-6 hours. For steady-state experiments, the cell pellet was used to obtain protein-bound amino acids (Zamboni et al., 2009).

Samples were re-suspended in 100 µl of MilliQ water, distributed in sealed 96-well microtiter plates, and injected into a Waters Acquity UPLC (Waters Corp., Milford, MA) with a Waters Acquity T3 column (150 mm x 2.1 mm x 1.8 µm, Waters Corp.) coupled to a Thermo TSQ Quantum Ultra triple quadrupole instrument (Thermo Fisher Scientific Inc., Waltham, MA) with electrospray ionization (Rühl et al., 2012). For determination of amino acid ¹³C-labeling patterns by GC-MS, bacterial pellets were washed twice with 1 ml of 9 g l⁻¹ NaCl, hydrolyzed in 1 ml of 6 M HCl for 24 h at 110°C, and desiccated overnight at 85°C under a constant air stream. The hydrolyzate was dissolved in 50 µl of 99.8% (wt/vol) dimethyl formamide and subsequently transferred into a new tube. For derivatization, 30 µl of *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide, was added to the hydrolyzate and incubated at 85°C for 60 min. The ¹³C-labeling patterns of proteinogenic amino acids were determined on a 6890N Network GC system with a 5975 inert XL mass selective detector (Agilent Technologies Inc., Santa Clara, CA) as described previously (Nanchen et al., 2007).

Due to the expense of labeled ¹³C cellobiose, we first determined the minimum cellobiose concentration. Concentrations of 0.001, 0.01, 0.1 and 1 mM were tested. Full labeling of these metabolites was observed for 1 and 0.1 mM concentrations, so 0.1 mM was used for subsequent experiments (Supplementary Figure S1).

Fermentation conditions

For extracellular flux measurements, cultures were grown in AN7 medium with 2.5 g/l cellobiose in glass bottles sealed with rubber stoppers. The working volume was 50 ml and the headspace volume was 100 ml and was composed of 100% N₂. The inoculum size was 5% (v/v). Bottles were incubated at 55°C

without shaking. At several points during growth, samples of the liquid culture and headspace gas were taken for analysis.

Analytical techniques

Cellobiose, glucose, pyruvate, formate, acetate, ethanol, succinate and lactate were measured by HPLC using an Aminex HPX-87H column (BioRad, CA, USA) with refractive index and ultraviolet detectors as described previously (Zhou et al., 2015). Carbon and nitrogen from cell pellets was determined by elemental analysis with a TPC-V CPH and TNM-1 analyzer (Shimadzu, Kyoto, Japan) as described previously (Zhou et al., 2015). Dry cell weight (DCW) was calculated from pellet nitrogen using a conversion factor of 0.048 mg DCW per mmol pellet N, as described previously (Holwerda et al., 2013). Hydrogen gas was measured by gas chromatograph using a thermal conductivity detector and nitrogen as the carrier gas, as described previously (Zhou et al., 2015).

To determine concentrations of glucose-6-phosphate (G6P), glucose-1-phosphate (G1P), fructose-6-phosphate (F6P), and malate, samples were diluted 1:20 and measured by ion-pairing LC-MS/MS. 5 μ l were injected into a Waters Acquity UPLC (Waters Corp., Milford, MA) with a Waters Acquity T3 column (150 mm $\times$ 2.1 mm $\times$ 1.8 μ m, Waters Corp.) coupled to a Thermo TSQ Quantum Ultra triple quadrupole instrument (Thermo Fisher Scientific Inc., Waltham, MA) with electrospray ionization (Buescher et al., 2010). For quantification, a dilution series of a defined mixture of standards was used.

To determine amino acids including glycine, alanine, serine, proline, valine, asparagine, glutamine, glutamate, leucine, methionine and phenylalanine, samples were diluted 1:20 and measured by HILIC LC-MS/MS. 5 μ l were injected on an Agilent HILIC Plus RRHD column (100 $\times$ 2.1 mm $\times$ 1.8 μ m; Agilent, Santa Clara, CA) coupled to a 5500 QTRAP triple quadrupole mass spectrometer in positive mode and MRM scan type (AB SCIEX, Foster City, CA) (Link et al., 2015). Calibration curves were obtained with an amino acid standard solution (AA-S-18, Sigma-Aldrich) supplemented with L-glutamine, L-tryptophan and L-asparagine.

Determining extracellular fluxes

For each fermentation, the exponential phase of growth was determined by log-transforming the biomass (gDCW), plotting the increase over time, and choosing at least 3 points in the linear region. The slope of this line was used to determine the specific growth rate μ . The same set of points was used to determine the extracellular fluxes for each metabolite by plotting the metabolite concentration (mM) against the cell mass (gDCW) and multiplying by specific growth rate. The units for these fluxes were mM gDCW⁻¹ h⁻¹. In cases where the R² value was less than 0.8, that flux measurement was ignored. This eliminated about 10% of our measurements.

Flux fitting

The mass distribution vectors of the free intracellular metabolites were corrected for the natural abundance of all stable isotopes using MatLab (The Mathworks Inc., Natick, MA). To determine the ratio

between the PPDK¹ and malate shunt pathways, the M+0 isotopomers of PEP, malate and pyruvate were fitted to a simple dynamic model consisting of the following reactions:

The set of differential equations corresponding to this model was constructed as described by Nöh et al. (Nöh and Wiechert, 2011). The v1 and v2 fluxes, and pool sizes for PEP, malate and pyruvate were allowed to vary. The fit was optimized by minimizing the sum of squares difference between predicted and observed data for all 7 time points using the GRG Nonlinear solver (Microsoft Excel). Data from each replicate was fitted separately, and the results were averaged. The flux ratio error is the standard deviation, n=3. For strain LL1138, v1 was set to 0. For strains LL1163 and LL1251, v1 was set to 1.

The determined linear system of mass-balances, flux ratios, quantitative physiology data, and biomass requirements was then solved with the *fmincon* function from MatLab using the Netto module from FiatFlux (Zamboni et al., 2005) to obtain the net metabolic fluxes as described previously (Fischer et al., 2004). Confidence intervals for each calculated flux are estimated as a function of the experimental errors from the Jacobian matrix of the output function.

Results and Discussion

Flux distribution in the wild type strain

One goal of this work was to understand how PEP is converted to pyruvate in the wild type strain (LL345). First we measured the enzyme activities of the enzymes in the various pathways. Activities for the PPDK and malate shunt pathways were present (Table 2). Activities of ODC, PEPS and FH were below the limit of detection for all strains (< 0.05 U).

Specific Activity (U/mg CFE)	LL345 (wt)		LL1138		LL1163		LL1251	
Genotype	Δhpt		$\Delta hpt \Delta pdk$		$\Delta hpt \Delta Idh::P_{eno}^-$ $pyk(Tsc) \Delta me-mdh$		$\Delta hpt \Delta Idh::P_{eno}^-$ $pyk(Tsc) \Delta me-mdh$ $\Delta pdk \Delta peps$	
	avg	error	avg	error	avg	error	avg	error
PEPCK	1.10	± 0.60	1.02	± 0.34	1.94	± 0.89	2.36	± 0.96
PPDK	0.12	± 0.09	< 0.05		0.12	± 0.03	< 0.05	

¹ Note that since PPDK and PYK only differ in their cofactor usage, they are considered as a single reaction

PYK	< 0.05		< 0.05		0.37	± 0.12	0.40	± 0.17
MDH-NADH	2.19	± 0.70	1.93	± 0.64	0.12	± 0.06	0.15	± 0.09
ME-NADP ⁺	1.69	± 1.00	1.40	± 0.67	< 0.05		< 0.05	

Table 2. Enzyme activity in cell-free extract of various strains of *C. thermocellum*. Data presented is the mean of at least 3 biological replicates. Error represents one standard deviation. The limit of detection was < 0.05 U. One unit of activity (U) is equal to conversion of one μ mol of substrate per minute. Specific activity is activity per mg cell-free extract (CFE) protein. Metabolic flux data is presented in units of mmol/gDCW/hr. Assuming that 1 g DCW is equivalent to 0.5 g CFE protein, enzyme activity here can be multiplied by a factor of 30 to convert to flux units. The complete set of raw enzyme assay measurements is presented in Supplementary Table S1

Since there are some reports in the literature suggesting the presence of ODC in *C. thermocellum* (Rydzak et al., 2012; Zhou et al., 2013), and our results consist of negative data (the absence of ODC enzyme activity) we reviewed the ODC literature to identify the range of assay conditions that have been used. There are three types of oxaloacetate decarboxylase (ODC) enzymes in the KEGG database. One type is a side-activity of malic enzyme. This type of enzyme was first observed in pigeon liver, is soluble, and requires divalent cations, such as $MnCl_2$ (Hsu and Lardy, 1966). Malic enzymes with oxaloacetate decarboxylating side-activity are given the EC number 1.1.1.38 if they are NAD-linked and 1.1.1.40 if they are NADP-linked. The malic enzyme from *C. thermocellum* was recently purified and found not to have ODC activity and to be NADP⁺-linked (Taillefer et al., 2015). This shares features with EC 1.1.1.39 (does not decarboxylate OAA) and 1.1.1.40 (NADPH-linked), but does not have its own EC number at present. A second type of ODC enzyme (EC 4.1.1.3) is soluble and requires divalent cations. It has been found in a number of organisms, including *Acetobacter xylinum* (Benziman et al., 1978), *Pseudomonas stutzeri* (Labrou and Clonis, 1999), *Corynebacterium glutamicum* (Klafl and Eikmanns, 2010), *Pseudomonas ovalis* (Horton and Kornberg, 1964), *Pseudomonas citronellolis* (O'Brien et al., 1977), *Veillonella parvula* (Ng et al., 1982), *Azotobacter vinelandii* (Plaut and Lardy, 1949) and *Enterococcus faecalis* (Repizo et al., 2013). A third type of ODC enzyme (EC 4.1.1.3) is membrane-bound, does not require divalent cations, but does require sodium ions, which it pumps across the cell membrane in a coupled reaction with ODC activity. This enzyme has been studied extensively in *Klebsiella pneumoniae* by Peter Dimroth (Dimroth, 1981). *C. thermocellum* has two genes that have been annotated as ODC enzymes (EC 4.1.1.3) in the KEGG database (Kanehisa et al., 2016), Clo1313_1523 and Clo1313_1712, and both are expressed at moderate levels (Riederer et al., 2011; Rydzak et al., 2012). Since the first type of ODC activity was ruled out by Taillefer et al., we focused on the second (requires divalent cation) and third (requires sodium ion) types and assayed each by three different methods (see materials and methods). In all cases, we found no evidence of ODC activity. Thus, two putative paths for PEP to pyruvate conversion remain.

To distinguish between the two remaining pathways: PPDK and malate shunt, we performed a dynamic ¹³C flux experiment with 100% uniformly ¹³C-labeled (UL-¹³C) cellobiose. As the ¹³C-labeled atoms propagated through the metabolic network, a decrease in unlabeled (M+0) isotopomer was observed (Figure 2). As expected when feeding labeled sugars, the compounds in glycolysis were labeled first in all strains (G6P as fast as FBP and before 2/3-PG and DHAP and PEP), followed by compounds in the TCA cycle (Mal before AKG and Glu and Suc). Aspartate, which is presumably derived from oxaloacetate, was rapidly labeled after PEP, suggesting that oxaloacetate was also rapidly labeled. Pyruvate is labeled

more slowly than PEP, but substantially faster than malate (Supplementary Figure S2), suggesting that flux through both PPDK and the malate shunt contribute to pyruvate formation.

Figure 2. Decrease dynamics of unlabeled metabolites (M+0 fragment) after addition of uniformly-labeled ^{13}C cellobiose. Time $t = 0$ is when the feed was switched from naturally-labeled (i.e. unlabeled) to uniformly-labeled cellobiose. The following abbreviations are used: G6P, glucose-6-phosphate; FBP, fructose-1,6 bisphosphate; DHAP, dihydroxyacetonephosphate; xPG, 3-phosphoglycerate and 2-phosphoglycerate; PEP, phosphoenolpyruvate; Asp, aspartate; Mal, malate; Pyr, pyruvate; AKG, alpha ketoglutarate; Glu, glutamate; Suc, succinate. Error bars represent one standard deviation, $n = 3$. The complete set of ^{13}C labeling data is presented in Supplementary Table S2

To quantify these observations, the flux split ratio between the PPDK and malate shunt pathways was calculated by fitting the isotopomer data from PEP, malate and pyruvate to a dynamic flux model as described in the materials and methods. For the wild type strain, the malate shunt pathway accounted for $33 \pm 2\%$ of the pyruvate flux and with the balance accounted for by the PPDK pathway. This is the first in-vivo demonstration of the malate shunt in *C. thermocellum*. Furthermore, it was previously thought that the PPDK pathway was unimportant (Zhou et al., 2013), but here we show that it supports a substantial fraction of glycolytic flux. Flux fit data for other strains and comparison of flux fit vs experimental data can be found in the supplement (Supplementary Table S3 and Supplementary Figure S3).

Diverting flux towards the malate shunt pathway

Having demonstrated that PPDK and the malate shunt are the two main pathways for PEP to pyruvate conversion in the wild type strain, we investigated the extent to which either of those pathways could serve as the sole route for flux to facilitate further metabolic engineering. We attempted to generate

strains where the malate shunt was disrupted and also where the malate shunt was the sole route for PEP to pyruvate conversion (Figure 3, Supplementary Figure S4).

Figure 3. Metabolic pathways for conversion of phosphoenolpyruvate to pyruvate in engineered strains of *C. thermocellum*. Pathways thought to be present in the wild type organism are indicated with black arrows. Engineered strains of *C. thermocellum* have been developed that contain a heterologous pyruvate kinase enzyme from *T. saccharolyticum*, which is indicated by a green arrow. Grey arrows indicate pathways that are present, but have low activity based on enzyme assay data. The wild type strain (LL345) can use either the malate shunt or PPDK pathways. The *ppdk* deletion strain (LL1138) can only use the malate shunt. Strain LL1163 can use either the PPDK or heterologous PYK pathways. Strain LL1251 can only use the heterologous PYK pathway.

In strain LL1138, the *ppdk* gene is deleted, and thus all of the flux from PEP to pyruvate should occur via the malate shunt. The elimination of PPDK activity was confirmed by enzyme assay (Table 2). In this strain pyruvate was labeled more slowly compared with the wild type strain (Figure 2). Furthermore, since pyruvate was labeled at the same rate as malate, it suggests that all of the flux was diverted through the malate shunt.

Diverting flux away from the malate shunt pathway

Previous work demonstrated that the malate shunt could be disrupted by deletion of malic enzyme, but only in the presence of a heterologous pyruvate kinase (Deng et al., 2013). Starting with the previously-created strain with a malic enzyme disruption (LL1113, $\Delta hpt \Delta ldh::P_{eno-pyk}(Tsc) \Delta me::cat-hpt$), we used 8AZH counter-selection to remove the *cat-hpt* cassette by homologous recombination. This had the effect of deleting 38 bp from the 3' end of the malate dehydrogenase gene (*mdh*) as well as causing an R305Q mutation, which may have partially obstructed the catalytic cavity (Supplementary Figure S5). The resulting strain was named LL1163 ($\Delta hpt \Delta ldh::P_{eno-pyk}(Tsc) \Delta me-mdh$). It had a complete deletion

of malic enzyme and a partial deletion of malate dehydrogenase, leaving PPDk and PYK as paths for conversion of PEP to pyruvate. The presence of PYK activity and the absence of ME activity were confirmed by enzyme assay (Table 2). The activity of MDH was reduced by ~90%, which may be a result of the R305Q mutation. We observed an increase in PEPCK activity relative to the wild type strain. We reviewed the resequencing data (see SRA accession numbers in Table 1) however we did not find any mutations in the vicinity of the *pepck* gene (Clo1313_0415) that could explain the change in activity.

In the heterologous *pyk* gene, we observed a single nucleotide polymorphism (SNP) mutation in strain LL1113 at a frequency of about 10%. In the course of engineering this strain to create strain LL1163, the mutation frequency increased to almost 100% (Supplementary Table S5). The presence of this SNP results in an I480K mutation in the Pyk protein. To determine the effect of the I480K point mutation in the heterologous Pyk protein from *T. saccharolyticum*, we also measured the PYK activity in strains LL1010 (wild type *T. saccharolyticum* *pyk* gene). The activity was 0.63 ± 0.16 , which is not significantly different ($p > 0.01$) from the value measured for strains LL1163 and LL1251 (mutant *pyk* gene, Table 2). This suggests that the I480K mutation does not affect specific activity, however it is possible that it affects in vivo flux in some other way, such as by altering allosteric regulation.

Although strain LL1163 had blocked all of the flux through the malate shunt pathway by the deletion of malic enzyme, there were still several pathways for the direct conversion of PEP to pyruvate. These include: PPDk (encoded by Clo1313_0949), PEPS (encoded by Clo1313_1005) and the heterologous PYK. First, we attempted to delete the *ppdk* gene from strain LL1163, using plasmid pMU2051. This was successful and resulted in strain LL1213 ($\Delta hpt \Delta ldh::P_{eno}\text{-}pyk(Tsc) \Delta me\text{-}mdh \Delta ppdk$). Although we did not expect that the PEPS pathway carried any flux from PEP to pyruvate based on enzyme assay data and thermodynamic considerations, we nevertheless deleted the *peps* gene from strain LL1213, resulting in strain LL1251 ($\Delta hpt \Delta ldh::P_{eno}\text{-}pyk(Tsc) \Delta me\text{-}mdh \Delta ppdk \Delta peps$), where the heterologous *pyk* gene from *T. saccharolyticum* is the only known pathway for conversion of PEP to pyruvate (Figure 3).

For strains LL1163 and LL1251, the ^{13}C -labeling data shows more rapid labeling of the glycolytic metabolites upstream of PEP (Figure 2). This may be a result of the heterologous *pyk* gene from *T. saccharolyticum* that is expressed in these strains. An anomaly in the labeling data of these strains was the increase in unlabeled pyruvate after 40 seconds. Although reproducibly observed in all replicates, it suggests metabolism of a carbon source other than cellobiose. We suspect this may be an experimental artifact due to the presence of unlabeled ethanol in the atmosphere of the anaerobic chamber.

As an alternative strategy to constrain the metabolic pathways of strain LL1163, we attempted to delete the heterologous *pyk* gene from *T. saccharolyticum*. Despite several attempts we did not succeed and therefore were unable to create a strain in which PPDk was the sole route for PEP conversion to pyruvate. To confirm that the *pyk* deletion plasmid (pDGO134) was functional, we used it to delete the heterologous *pyk* gene from strain LL1010 ($\Delta hpt \Delta ldh::P_{eno}\text{-}pyk(Tsc)$). This was successful and resulted in strain LL1227 ($\Delta hpt \Delta ldh$) (Supplementary Figure S4). Thus it appears that the heterologous PYK enzyme is capable of serving as the sole route for PEP to pyruvate conversion, however, the PPDk enzyme is not.

Impact of flux redirection on redox balance

To determine the impact of flux redirection on redox balance, we measured extracellular metabolic fluxes for the 4 key strains (wild type, LL1138, LL1163 and LL1251) (Table 3).

Metabolite	Metabolic fluxes (mmol/gDCW/hr)							
	LL345 (wt)		LL1138		LL1163		LL1251	
cellobiose	-4.52	±0.33	-5.47	±0.36	-4.65	±0.41	-4.22	±0.17
glucose	-0.05	±0.03	0.27	±0.03	0.18	±0.22	-0.04	±0.00
pyruvate	0.37	±0.02	0.34	±0.03	0.16	±0.06	0.11	±0.01
formate	2.57	±0.07	1.80	±0.19	3.49	±0.38	3.97	±0.17
acetate	3.76	±0.19	3.33	±0.37	2.14	±0.16	2.10	±0.10
ethanol	2.24	±0.21	2.87	±0.07	6.52	±0.56	6.48	±0.28
succinate								
lactate	0.10	±0.02	0.22	±0.03	0.00	±0.01		
G6P	0.00	±0.00	0.01	±0.00	0.04	±0.04	0.00	±0.00
F6P					0.02	±0.03		
G1P	0.08	±0.02	0.65	±0.07	0.22	±0.14	0.05	±0.01
malate	0.03	±0.00	0.11	±0.03	0.24	±0.02	0.22	±0.01
Gly	0.01	±0.00	0.02	±0.00	0.00	±0.01		
Ala	0.05	±0.01	0.06	±0.01	0.03	±0.01	0.03	±0.00
Pro	0.00	±0.00	0.00	±0.00	0.00	±0.00	0.00	±0.00
Val	1.00	±0.02	1.47	±0.15	0.29	±0.14	0.31	±0.05
Asn	0.04	±0.00	0.03	±0.00	0.02	±0.00	0.02	±0.00
Gln	0.00	±0.00	0.00	±0.00	0.00	±0.00	0.00	±0.00
Leu	0.04	±0.00	0.04	±0.00	0.02	±0.00	0.02	±0.00
Glu	0.05	±0.01	0.40	±0.13	0.06	±0.00	0.02	±0.01
Met	0.03	±0.01	0.03	±0.00	0.04	±0.01	0.04	±0.01
Phe	0.01	±0.00	0.01	±0.00	0.00	±0.00	0.01	±0.00
Pellet N	3.14	±0.30	2.84	±0.15	4.00	±0.35	3.77	±0.28
H ₂	11.64	±1.06	18.70	±4.03	5.19	±1.03	4.26	±0.10
CO ₂ (estimated)	5.70	±0.32	7.97	±1.01	6.64	±0.42	5.99	±0.36
Carbon balance	77%		79%		92%		93%	
	Specific growth rate (1/hr)							
	LL345 (wt)		LL1138		LL1163		LL1251	
	0.39	±0.04	0.35	±0.02	0.49	±0.05	0.47	±0.03

Table 3. Metabolic fluxes and growth rates. For each strain, metabolic fluxes were calculated by the slope of linear regression of metabolite concentration vs. gDCW. Metabolite concentration data is presented in Supplementary Table S5. DCW was calculated from Pellet N (nitrogen) data. Missing values indicate that the flux value was not included because the R² value was less than 0.8 for all samples. For each strain, fluxes were calculated individually for each replicate and then averaged. Error is one standard deviation, n = 2 or 3 (depending on whether samples were removed due to low R² values).

In general, the deletion strains exhibit two pattern of extracellular fluxes. The first pattern, exhibited by the wild type and LL1138 strain (*ppdk* deletion) involved high levels of hydrogen production and slightly more acetate than ethanol (Table 3). The second fermentation pattern, exhibited by strains LL1163 and

515 LL1251, involved reduced hydrogen production and ethanol to acetate ratios of about 3:1. In addition,
516 this second set of strains showed a roughly 30% higher growth rate. Interestingly, this second set of
517 strains indicates that one can engineer *C. thermocellum* to simultaneously have high ethanol yield and a
518 faster growth rate.

519 We combined the extracellular flux data with the pyruvate flux ratio from the ^{13}C labeling data (note
520 that for strains other than the wild type, the metabolic network was fully determined by gene deletions)
521 to constrain flux balance analysis. The metabolic reaction network was adapted from a recently
522 published model (Thompson et al., 2015) and is given in Supplementary Table S6. Although the oxidative
523 pentose phosphate pathway (PPP) is a canonical pathway for NADPH generation, it was shown to be
524 absent in *C. thermocellum* based on isotope labeling data (Lamed and Zeikus, 1980), which was
525 confirmed by our analysis (i.e. fitted flux of zero when we included it in the model) and thus we did not
526 include the PPP in the final metabolic model.

527 Based on our enzyme assay and labeling results, we eliminated the ODC reaction from the model. We
528 assumed that hydrogen production proceeds solely via the bifurcating hydrogenase based on the
529 following observations:

- 530 1. More H_2 is produced than pyruvate is oxidized (in the wild type strain)
- 531 2. It is thermodynamically difficult to produce H_2 using NAD(P)H
- 532 3. The homolog of the *Ruminococcus albus* bifurcating hydrogenase (Zheng et al., 2014) is highly
533 expressed in *C. thermocellum* (Gowen and Fong, 2010; Raman et al., 2011; Riederer et al., 2011).

534 The genes for the NADH dependent reduced ferredoxin:NADP $^+$ oxidoreductase (NFN) reaction ($\text{NADH} +$
535 $\text{Fd}_{\text{red}} + 2 \text{NADP}^+ \leftrightarrow \text{NAD}^+ + \text{Fd}_{\text{ox}} + 2 \text{NADPH}$) and proton-transporting ferredoxin:NAD $^+$ oxidoreductase
536 (RNF) reaction ($\text{NAD}^+ + \text{H}_{\text{int}}^+ + \text{Fd}_{\text{red}} \leftrightarrow \text{NADH} + \text{H}_{\text{out}}^+ + \text{Fd}_{\text{ox}}$) are both present in *C. thermocellum*. Since
537 both reactions are thought to be reversible, they can be combined to allow for unconstrained
538 transhydrogenation ($\text{NADH} + \text{NADP}^+ \leftrightarrow \text{NAD}^+ + \text{NADPH}$) or NADPH-FNOR activity ($\text{Fd}_{\text{red}} + \text{NADP}^+ + \frac{1}{2} \text{H}_{\text{int}}^+$
539 $\leftrightarrow \text{Fd}_{\text{ox}} + \text{NADPH} + \frac{1}{2} \text{H}_{\text{out}}^+$). For obtaining determined systems to calculate fluxes we constrained the
540 RNF reaction to allow only the forward (i.e. NADH-generating) direction. It is possible that other
541 reactions remain for the transfer of electrons between NADH, NADPH, Ferredoxin and H_2 , but they were
542 not included in this simplified model. Furthermore, we did not include ion pumping reactions. We also
543 lumped the PYK and PPDK reactions, since they only differ in their phosphate cofactors ($\text{Pi} + \text{ADP} \rightarrow \text{ATP}$
544 vs $\text{PPi} + \text{AMP} \rightarrow \text{ATP}$), which we have already lumped together. The resulting fluxes were plotted to show
545 metabolic flux distributions for each strain (Figure 4).

calculated flux and estimated as a function of the experimental errors of the ratios and physiological constrains. Fdred is an abbreviation for reduced ferredoxin. The complete set of fitted fluxes is presented in Supplementary Table S6.

In the wild type strain (LL345), 33 ±2% of pyruvate is generated from the malate shunt (based on ¹³C labeling data, see previous section). This means that the malate shunt is able to supply most of the NADPH needed for biosynthesis (Figure 5). Consequently, the primary electron transfer reaction is the bifurcating hydrogenase (Bifur-Hyd), and flux through the other electron transfer reactions (RNF and NFN) is only 25% of the Bifur-Hyd (Inset of Figure 4). Indeed, a system for regulation of malate shunt activity based on changing relative levels of PP_i and NH₄⁺ has recently been proposed (Taillefer et al., 2015). In this system, malic enzyme is strongly activated by NH₄⁺ and strongly inhibited by PP_i, which is a substrate for the PPK reaction, suggesting that PP_i levels play a role in the flux split between PPK and the malate shunt. Our results are consistent with this hypothesis.

Figure 5. NADPH production and consumption based on fitted net fluxes (mmol g⁻¹ h⁻¹) for all 4 strains, LL345 (wt), LL1138, LL1163 and LL1251. Abbreviations are biomass, bm; isocitrate dehydrogenase, icd; malic enzyme, me; NADH dependent reduced ferredoxin:NADP⁺ oxidoreductase, nfn. The amino acids category includes valine, alanine, cysteine, asparagine, leucine, methionine, glutamate and phenylalanine. In all cases, valine was the predominant amino acid. The NADPH

production and consumption is based on the respective fitted net fluxes. Error from the fitted fluxes was linearly propagated to generate a combined error for the sum of NADPH production or consumption.

In strain LL1138 (*ppdk* deletion), all of the pyruvate is generated via the malate shunt. This results in a catabolic oversupply of NADPH (Figure 5). This excess NADPH is presumably balanced by running the NFN reaction in the reverse direction (Inset Figure 4). Additional flux through Bifur-Hyd and RNF are needed to balance the ferredoxin and nicotinamide cofactor pools. A predicted consequence of the reverse NFN reaction is increased H₂ production, and in fact, H₂ production increases by 60% in strain LL1138, compared with the wild type (LL345) (Table 3).

In strains LL1163 and LL1251, all of the pyruvate is generated by PPDK or PYK (and none by the malate shunt) resulting in an under-supply of NADPH (Figure 5). This is balanced by running the NFN reaction in the forward direction.

Based on our current understanding, *C. thermocellum* has two primary mechanisms for generating the NADPH needed for biosynthesis: the malate shunt and the NFN reaction. In the wild type strain, it appears to primarily use the malate shunt; however, when this pathway is disrupted, the NFN reaction is used instead. One puzzle that remains is the question of why strains LL1163 and LL1251 show increased ethanol production. Previously it was suggested that disruption of the malate shunt increased the supply of NADH, which resulted in increased ethanol production (Deng et al., 2013). If this were the main explanation, we would expect a decrease in ethanol production in strain LL1138 (*ppdk* deletion), but ethanol levels remain unchanged. Previously it has been shown that disrupting H₂ production in *C. thermocellum* leads to increased ethanol production (Biswas et al., 2015). It is possible that the expression of PYK and disruption of the malate shunt in strains LL1163 and LL1251 somehow inhibited the Bifur-Hyd reaction. Alternatively, NADH availability could still be the explanation, and strain LL1138 may balance electron flux differently than the wild type. Electron metabolism in *C. thermocellum* is an interesting area for future work.

Conclusions

In this work, we have adapted the method for use in *C. thermocellum* to unravel a longstanding question about the role of the malate shunt. We have shown that it does, in fact, operate in-vivo, based on gene deletion experiments and the relative rate of ¹³C malate and pyruvate labeling in the *ppdk* deletion strain (LL1138). In the wild type strain, the malate shunt carries about a third of the glycolytic flux and is capable of supplying most of the NADPH needed for biosynthesis.

Although there has been some speculation that ODC is present in *C. thermocellum*, we have provided evidence that this is not the case.

Previous work suggested that the PPDK reaction did not play a large role in metabolism due to the lack of change in fermentation products when it was deleted (Zhou et al., 2013). Here we have shown that PPDK activity is present, that it contributes substantially to the production of pyruvate from PEP, and that it may play an important role in regulating NADPH production. It does not appear, however, to be able to function as the sole means for converting PEP to pyruvate, since disruption of the malate shunt seems to require the presence of a heterologous pyruvate kinase.

The improved understanding of *C. thermocellum* metabolism gained from these experiments should facilitate subsequent efforts to engineer this organism for improved ethanol production.

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