**CO$_2$-fixing one-carbon metabolism in a cellulose-degrading bacterium *Clostridium thermocellum***

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*Clostridium thermocellum* can ferment cellulosic biomass to formate and other end products, including CO$_2$. This organism lacks formate dehydrogenase (Fdh), which catalyzes the reduction of CO$_2$ to formate. However, feeding the bacterium 13C-bicarbonate and cellubiose followed by NMR analysis showed the production of 13C-formate in *C. thermocellum* culture, indicating the presence of an uncharacterized pathway capable of converting CO$_2$ to formate. Combining genomic and experimental data, we demonstrated that the conversion of CO$_2$ to formate serves as a CO$_2$ entry point into the reductive one-carbon (C1) metabolism, and internalizes CO$_2$ via two biochemical reactions: the reversed pyruvate:ferredoxin oxidoreductase (rPFOR), which incorporates CO$_2$ using acetyl-CoA as a substrate and generates pyruvate, and pyruvate:formate lyase (PFL) converting pyruvate to formate and acetyl-CoA. We analyzed the labeling patterns of proteinogenic amino acids in individual deletions of all five putative PFOR mutants and in a PFL deletion mutant. We identified two enzymes acting as rPFOR, confirmed the dual activities of rPFOR and PFL crucial for CO$_2$ uptake, and provided physical evidence of a distinct in vivo “rPFOR-PFL shunt” to reduce CO$_2$ to formate while circumventing the lack of Fdh. Such a pathway precedes CO$_2$ fixation via the reductive C1 metabolic pathway in *C. thermocellum*. These findings demonstrated the metabolic versatility of *C. thermocellum*, which is thought of as primarily a cellulosic heterotroph but is shown here to be endowed with the ability to fix CO$_2$ as well.

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**Significance**

High carbon yield in the bioengineering of heterotrophic bacteria is hindered by carbon loss to CO$_2$ production. We provide evidence showing *Clostridium thermocellum*, a cellulose-degrading bacterium and a model consolidated bioprocessing organism, can fix CO$_2$ while growing predominantly on cellubiose, a cellulose-derived disaccharide. By adding 13C-bicarbonate to the bacterial culture and tracking 13C-labeled metabolites, we discovered active reductive one-carbon (C1) metabolism in this bacterium. We further identified critical enzymes responsible for fixing CO$_2$ and channeling the fixed carbon to the C1 metabolic pathway. Our findings pave the way to future engineering of this bacterium to use cellulose and CO$_2$ simultaneously as a means to improve microbial carbon efficiency that is constrained by theoretical limitation and to reduce CO$_2$ in the environment.

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The authors declare no conflict of interest.

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measure the isomomers of fermentation products and proteinogenic amino acids using NMR and GC/MS, and uncover a route C. thermocellum employs to fix CO₂ when grown in a primarily heterotrophic mode supplemented with sodium bicarbonate.

**Results**

**Effects of Bicarbonate on C. thermocellum Growth and Metabolism.** To examine C. thermocellum’s ability to use inorganic carbon, we compared cell growth and metabolic product formation during cellulose fermentation with and without bicarbonate supplementation (20 mM). To eliminate any potential buffering effect exerted by bicarbonate, fermentation was performed in batch mode with pH controlled at 7.0 throughout. Upon consumption of all cellulose, cultures with added bicarbonate yielded ~40% more cell biomass than in its absence (Table 1). Without accounting for the excreted amino acids that have been observed in overflow metabolism (3), this result increased the possibility that extra total carbon output (i.e., cell biomass, lactate) could emerge from incorporation of the inorganic carbon (bicarbonate) as suggested by the calculated apparent carbon efficiency (65.7% without bicarbonate and 75.5% with bicarbonate; Table 1). We also observed higher apparent carbon efficiency in bicarbonate-fed cultures in non-pH-controlled fermentation (Table S1) buffered with 50 mM (N-morpholino)propanesulfonic acid (MOPS).

**Reduction of Bicarbonate to Formate as Revealed by NMR Analysis Using 13C-Tracer.** To track how bicarbonate could be metabolized by the bacterium, we fed C. thermocellum 13C-bicarbonate (20 mM) and unlabeled cellulose (14.6 mM) and analyzed the labeled fermentation products that were excreted into the medium with 13C-NMR. Data from Fig. L4 show 1D 13C spectra of the culture-spend medium after cells were grown to late log phase. In the medium, we detected small peaks at 160.3 and 128.0 ppm (Fig. L4, double asterisks), which correspond to residual bicarbonate and CO₂, respectively (Fig. L4, Top and Middle), and a signature formate carbonyl peak with a chemical shift at 171.1 ppm (Fig. L4, Middle and Bottom). To confirm the formation of 13C-formate further, common fermentation products were also assessed for the assignment of the new peak at 171.1 ppm. Organic acids, further, common fermentation products were also assessed for the assignment of the new peak at 171.1 ppm. Organic acids, which have a carboxylic observed peak. One class of doubtable compounds is alpha-keto acids (i.e., pyruvate, 2-oxoglutarate), which do not have directly bound1H on their hydrogen directly bound to the carbonyl carbon. Pyruvate and 2-oxoglutarate, however, do not have directly bound1H on their respective carbonyl carbons, leading to the conclusion that the new peak at 171.1 ppm is indeed derived from formate. In addition to 13C-formate, two small peaks at 182.5 and 174.4 ppm (Fig. L4, marked with asterisks) correspond to other fermentation products that may also be derived from 13C-bicarbonate labeling but in very small amounts. Other peaks from 5 to 65 ppm likely arose from natural abundance components in the defined media and did not change in relative intensity from sample to sample.

**Putative Reductive C1 Metabolism Based on Genome Annotation.** The above labeling experiments provided evidence of a significant CO₂-to-formate reduction pathway that remains to be characterized. To identify genes that are putatively responsible for this conversion, we reconstructed the C1 metabolic pathway de novo, based on the genomic information of C. thermocellum DSM1313 strain (20). Its genome sequence suggests that C. thermocellum is capable of converting formate to methionine, serine, and pyruvate via the methyl branch of the Wood–Ljungdahl pathway (Fig. 2). However, it lacks Fdh, the enzyme microbes typically use to convert CO₂ to formate (Fig. 2).

Lacking the fdh gene, the only other gene encoding formate production found in C. thermocellum genome is pyruvate-formate lyase (PFOR), catalyzing the conversion of pyruvate and CoA to acetyl-CoA and formate (21). We therefore hypothesize that a bypass consisting of two reactions catalyzed by pyruvate:ferredoxin oxidoreductase (PFOR) in the reversed direction and Fd may realize the CO₂-to-formate conversion. Presumably, the reversed PFOR activity fixes CO₂ by catalyzing the formation of pyruvate using CO₂ and acetyl-CoA as substrates. The fixed carbon atom from CO₂ forms the carbonyl group of pyruvate, which was then split to formate by Fd (Fig. 2), with the remaining two carbons in pyruvate making up the acetyl group in acetyl-CoA. Both pfLA and pfB genes are annotated in the C. thermocellum genome. Genes for pyruvate formate lyase (pfLA, clo1313_1717) and its activating enzyme (pfLB, clo1313_1718) are adjacent to each other on the genome. Several putative genes encoding for PFOR (clo1313_0673, clo1313_0200-0023, clo1313_0382-0385, and clo1313_l353-1356) were also annotated, hence making our hypothesis genetically feasible.

**13C-Tracer Data Demonstrated the Reversed PFOR Activity.** To investigate this hypothesized pathway, we first test if a reversed PFOR (rPFOR) activity exists in vivo. We set up a steady-state tracer experiment by feeding 13C-bicarbonate (20 mM) along with unlabeled cellulose (14.6 mM) to the cells and detecting the labeling pattern of proteinogenic amino acids by GC/MS. The isotopomer fingerprint of pyruvate can be traced by the isotopomer fingerprint of alanine, which is a direct transaminated product of pyruvate. As shown in Fig. 3A, the topomer fingerprint of pyruvate can be traced by the isotopomer fingerprint of alanine, which is a direct transaminated product of pyruvate. As shown in Fig. 34, the topomer fingerprint of pyruvate can be traced by the isotopomer fingerprint of alanine, which is a direct transaminated product of pyruvate. As shown in Fig. 34, the topomer fingerprint of pyruvate can be traced by the isotopomer fingerprint of alanine, which is a direct transaminated product of pyruvate. As shown in Fig. 34, the topomer fingerprint of pyruvate can be traced by the isotopomer fingerprint of alanine, which is a direct transaminated product of pyruvate. As shown in Fig. 34, the topomer fingerprint of pyruvate can be traced by the isotopomer fingerprint of alanine, which is a direct transaminated product of pyruvate. As shown in Fig. 34, the topomer fingerprint of pyruvate can be traced by the isotopomer fingerprint of alanine, which is a direct transaminated product of pyruvate. As shown in Fig. 34, the topomer fingerprint of pyruvate can be traced by the isotopomer fingerprint of alanine, which is a direct transaminated product of pyruvate.
CO2 in malate shunt (7, 14) which is known to assimilate and release 13C atoms among all possible candidate pathways, including the pyruvate at the carboxylic carbon, we examined the fate of carbon catalyzed by PEPCK, resulting in 13C-labeling at position 4 of malate via malate dehydrogenase and is subsequently lost as double asterisks indicate a contaminant in the 13C-bicarbonate. The signals single asterisk indicates other products of bicarbonate metabolism. The within the braces indicate natural abundance of aliphatic and methyl carbons from the medium or metabolites. (Δ) 13C-lactate with a labeling pattern consistent with the labeling pattern of pyruvate (Figs. 2 and 3), presumably propagated from the labeled pyruvate (position 1), and not to the label exchanged PFL. The question mark indicates a missing reaction in C. thermocellum. RuBisCO was ruled out. Another possible carboxylase enzyme that could presumably result from the medium or metabolites. (Δ) JHC dipolar coupling of 195 Hz further confirmed the identity of the peak at 171 ppm as formate.

To confirm rPFOR activity as the main mechanism contributing to the labeled pyruvate (position 1), and not to the exchange reaction, we fed C. thermocellum [2,13C]acetate (40 mM) supplemented with unlabeled bicarbonate (20 mM) when cells were grown primarily in clocebose (5 g/L). Results showed labeled carbons at the expected position in pyruvate (position 3) and acetyl-CoA (position 2) in both wild type (Δhyp; Fig. 3C) and PFL-inactivated mutant (ΔhypΔpflA; Fig. S1). For confirmation and characterization of the ΔhypΔpflA mutant, see Fig. 5 and Figs. S2 and S5A. These results also ruled out the possibility of labeled pyruvate transiting from acetyl-CoA via reversed PFL activity. Results are described in greater detail in SI Results. The observed labeled carbon at position 3 of pyruvate is independent of the label exchange specific to the carboxyl group, unlike previously reported in Clostridium acetobutylicum (22).

To determine which pathway(s) may also lead to 13C-labeled pyruvate at the carboxylic carbon, we examined the fate of carbon atoms among all possible candidate pathways, including the malate shunt (7, 14) which is known to assimilate and release CO2 in C. thermocellum. As shown in Fig. 3B, CO2 fixation is catalyzed by PEPCK, resulting in 13C-labeling at position 4 of oxaloacetate. This labeled carbon is propagated to position 4 of malate via malate dehydrogenase and is subsequently lost as 13CO2 by malic enzyme. Therefore, malate shunt, per se, does not contribute to the 13C-labeling at the carboxylic group of pyruvate. Another possible carboxylase enzyme that could presumably result in the labeling at the carboxylic group of pyruvate is ribulose bisphosphate carboxylase/oxygenase (RuBisCO). However, genes encoding for the highly conserved RuBisCO enzyme are not present in the C. thermocellum genome or in other sequenced Clostridium species. Thus, RuBisCO was ruled out.

We also considered the possibility of a reversible conversion between malate and fumarate catalyzed by malate hydrolyase (Clo1313_0640). The symmetry of the fumarate molecule can interconvert labeling on carbon atoms in positions 1 and 4; hence, it could contribute to the resulting 13C-carboxylic group in pyruvate. Although the alanine labeling pattern (Fig. 3A) cannot rule out the malate hydrolyase reaction, data from genetic KO experiments (Fig. 4) presented below are consistent with rPFOR activity and not malate hydrolyase activity.

We also investigated the isotopomer pattern for serine, which could be derived from phosphoglycerate, a triose phosphate interconverted with pyruvate in the glycolytic pathway (Fig. 2). Assuming that serine is exclusively derived from glycolysis and shares an identical synthetic route with alanine, the labeling pattern of serine and alanine should be very similar. However, in contrast to mostly unlabeled Pyr2–3, we observed a nonnegligible m1 peak of Ser2–3 with 12% enrichment (Fig. 3A). One carbon labeled at Ser2–3 implies a distinct serine pathway from pyruvate, consistent with the metabolic pathway in which a 13C-methylene-THF from reductive C1 metabolism is combined with glycine by glycine hydroxymethyltransferase (glyA, clo1313_1155) and forms the hydroxymethylene group of serine at position 3

Fig. 1. 13C-NMR analysis of supernatant from C. thermocellum (Ct) culture fed with 20 mM 13C-bicarbonate and 14.6 mM unlabeled clocebose. (A) 13C-NMR signal at 171 ppm is consistent with a formate carbonyl carbon that is observed in the spent medium of C. thermocellum (red spectrum). Defined medium supplemented with 20 mM 13C-bicarbonate (green spectrum) and 20 mM 13C-formate (black spectrum) was analyzed as the references. The single asterisk indicates other products of bicarbonate metabolism. The double asterisks indicate a contaminant in the 13C-bicarbonate. The signals within the braces indicate natural abundance of aliphatic and methyl carbons from the medium or metabolites. (B) JHC dipolar coupling of 195 Hz further confirms the identity of the peak at 171 ppm as formate.

Fig. 2. Schematic of reductive C1 metabolism initiated from rPFOR and reversed PFL. The question mark indicates a missing reaction in C. thermocellum DSM1313 genome annotations. Pink-shaded pathways illustrate the reduction of CO2 to formate and CO2 to pyruvate. Yellow-shaded pathways include steps of serine and methionine biosynthesis. Fhdh is not annotated in C. thermocellum genome. Fhs, formyl-tetrahydrofolate synthase (clo1313_0030); FolD, methyl-tetrahydrofolate dehydrogenase/cyclohydrolase (clo1313_1120); GlyA, glycine hydroxymethyltransferase (clo1313_1155); Metf, methyl-tetrahydrofolate reductase (clo1313_2132); MS, methionine synthase (clo1313_1587-1598, clo1313_1581-1582, clo1313_0372). Dotted lines indicate multistep catalysis. Carbons in red indicate 13C-isotopes propagated from 13C-bicarbonate or 13C-formate in the tracer experiments.
(Fig. 2). These results implied that $^{13}$C isotope propagated to pyruvate was able to flow into the C1 pathway via formate, contributing to the biosynthesis of serine in C. thermocellum.

Knocking Out PFOR Genes Impairs Pyruvate Synthase Activity for CO$_2$ Fixation. The CO$_2$-fixing C1 pathway initiated by PFOR in the reductive direction was further examined through the deletion of putative PFOR genes and the analysis of altered labeling patterns in the KO mutants. Multiple genes or gene clusters are annotated as PFOR (clo1313_0020-0023, 1353-1356, 0673, and 0382-0385) or pyruvate ferredoxin oxidoreductase (clo1313_1615-1616) in the C. thermocellum genome. These genes were respectively deleted via homologous recombination following the schemes shown in Fig. S2A, and validation of the genotype via colony PCR is shown in Fig. S3 and Table S2. All PCR products were further confirmed by commercial sequencing of the genome. These results implied that rPFOR and the malate shunt (gray box). OAADC, oxaloacetate decarboxylase complex; MDH, malate dehydrogenase; ME, malic enzyme. The labeled ($^{13}$C) and unlabeled ($^{12}$C) carbon atoms are represented by closed and open circles, respectively. (A) Isotopomer analysis of proteinogenic alanine and leucine in Δhpt mutants, with reduced labeling at the carboxylic group in mutants ΔhptΔclo1313_0020-0023 and ΔhptΔclo1313_1353-1356, were fed with 20 mM $^{13}$C-bicarbonate and 14.6 mM unlabeled cellobiose for isotope tracer experiments. The relative abundance of m0 and m1 shown for Pyr1–3 (M_ala_057; Fig. 4) and for Pyr2–3 (M_ala_085; Fig. S4) indicates that the carbon is labeled predominantly on the carboxylic group in pyruvate in both wild type (Δhpt) and mutants, but the relative abundance of m1 is significantly lower in mutants ΔhptΔclo1313_0020-0023 and ΔhptΔclo1313_1353-1356 (Fig. 4). By taking the ratio of pyruvate labeled at the carboxylic group (m1) to unlabeled ones (m0), data showed that m1/m0 ratios are not significantly different in individual PFOR deletion mutants ΔhptΔclo1313_1615-1616 and ΔhptΔclo1313_0673, or exhibited little difference in mutant ΔhptΔclo1313_0382-0385. However, the m1/m0 ratios are significantly lower in ΔhptΔclo1313_0020-0023 and ΔhptΔclo1313_1353-1356, with reduced labeling at the carboxylic group. The results identified Clo1313_0020-0023 and Clo1313_1353-1356 functioning as a pyruvate synthase, catalyzing the incorporation of CO$_2$ originating from $^{13}$C-bicarbonate to form pyruvate. Based on the relative ratios of m1 to m0, Clo1313_0020-0023 and Clo1313_1353-1356 account for ~50% and ~30% (Fig. 4) of pyruvate synthase activity, respectively, for carbon fixation. The differential m1/m0 ratios in these mutants also suggest that rPFOR contributes to the $^{13}$C-carboxylic carbon labeling in pyruvate, which cannot originate from the interconversion between malate and fumarate alone. However, potential interplay between the putative PFORs and the interconversion between malate and fumarate is beyond the scope of this study, and hence is not addressed here.

PFL KO Mutant Is Deficient in Reductive C1 Metabolism. To assess the involvement of PFL in reductive C1 metabolism, we inactivated PFL by deleting the pflA gene as illustrated in Fig. S2B, adapting a scheme developed previously (23). Deletion of the pflA gene was confirmed by PCR analysis (Fig. S5A) and commercial sequencing of the PCR products. Mutant ΔhptΔpflA grew slower than the wild type in defined medium, consistent with previous observations (12). We also measured the batch fermentation products when all substrates were consumed (Fig. S5B). As expected, although the wild type produces 2.3 mM formate, formate is no longer detected in the ΔhptΔpflA culture-spent medium (Fig. S5B), confirming complete inactivation of PFL and the fact that PFL is the only enzyme responsible for formate production from pyruvate.

To investigate how PFL deficiency affects carbon flux toward C1 metabolism, we compared the labeling pattern of isotopomers in both ΔhptΔpflA and wild type by feeding $^{12}$C-formate (20 mM) and $^{13}$C-bicarbonate (20 mM), respectively (Fig. 5). In either case, unlabeled cellobiose (14.6 mM) was added to the cell culture. We collected isotopomer data for proteinogenic amino acids that may be synthesized from this pathway, including alanine, serine, and methionine. With the addition of $^{13}$C-formate, an m1 peak with 16% enrichment in Pyr1–3 was observed for wild type, whereas the corresponding m1 ratio was merely 4% in ΔhptΔpflA. In the isotopomer of Pyr2–3, we observed no 50% and 30% (Fig. 4) of PFL by deleting the pflA gene as illustrated in Fig. S2B, adapting a scheme developed previously (23). Deletion of the pflA gene was confirmed by PCR analysis (Fig. S5A) and commercial sequencing of the PCR products. Mutant ΔhptΔpflA grew slower than the wild type in defined medium, consistent with previous observations (12). We also measured the batch fermentation products when all substrates were consumed (Fig. S5B). As expected, although the wild type produces 2.3 mM formate, formate is no longer detected in the ΔhptΔpflA culture-spent medium (Fig. S5B), confirming complete inactivation of PFL and the fact that PFL is the only enzyme responsible for formate production from pyruvate.

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significant difference in labeling pattern between the strains. Taken together, the results supported that the 13C-carbon atom in formate was internalized by the cells and became the carboxylic carbon of pyruvate, which was catalyzed by the reversed PFL activity. We next analyzed the Ser and Met isotopomers that could be synthesized from the C1 pathway downstream of 13C-formate. Based on the labeling patterns of Ser1-3, Ser2-3, and Ser1-2 (Fig. 5A), we deduced that the 13C-carbon propagated from 13C-formate is mainly at position 3 of Ser (hydroxymethylene group), consistent with our constructed de novo C1 metabolic pathway toward serine biosynthesis (Fig. 2). As for the isotopomer of Met shown in Fig. 5A, both ∆hptΔpfIA and wild type indeed demonstrated a dominant peak at m1 upon 13C-formate addition, validating the incorporation of the methyl group propagated from 13C-formate into Met. Detailed descriptions of Ser and Met isotopomer labeling analyses are provided in SI Results.

We also analyzed the isotopomers of proteinogenic amino acids for 13C-bicarbonate labeling experiments in ∆hptΔpfIA. Based on our hypothesized "rPFOR-PFL shunt," incorporation of the 13C-carbon from bicarbonate into the C1 metabolism is mediated by PFL, which could be validated by comparing 13C-influx into the C1 metabolism in the presence and absence of an active PFL. Similar pyruvate labeling patterns were observed for wild-type and ∆hptΔpfIA strains, both of which showed m0 and m1 dominant peaks for Pyr1-3 and m0 signal for Pyr2-3 fragment. Results suggest that PFL deletion does not affect CO2 incorporation catalyzed by the pyruvate synthase activity. As for serine labeling, both Ser1-3 and Ser2-3 fragments containing the hydroxyl methylene group had a lower labeling ratio in ∆hptΔpfIA mutant than in wild type. For Ser2-3, mutant ∆hptΔpfIA had a much lower m1 peak (2.6%) than the control (12.7%). Correspondingly, the unlabeled m0 peak of Ser2-3 for ∆hptΔpfIA strain is 10.1% higher than in the parental strain (97.4% vs. 87.3%). Collectively, reduced isotope enrichment in the mutant strongly supports that C1 metabolism originating from 13C-bicarbonate is disrupted by knocking out pfIA. It is also evident that PFL activity is essential in internalizing the 13C-carbon in bicarbonate in C. thermocellum, and our hypothesis that PFL coupled with the rPFOR activity initiates the reductive C1 pathway in C. thermocellum was corroborated. We observed a lower extent of fractional labeling of methionine in the ∆hptΔpfIA mutant than in wild type, which is described in greater detail in SI Results.

Discussion

This study reveals that during cellobiose metabolism, C. thermocellum also assimilates CO2 via a reductive C1 metabolic pathway that is initiated by the dual actions of reversed PFO and PFL. PFO has been greatly considered in the forward direction in C. thermocellum, catalyzing the oxidative decarboxylation of pyruvate to acetyl-CoA and CO2 and generating reduced ferredoxin (Fdred). The PFOR activity in C. thermocellum has been associated with the oxidative decarboxylation of pyruvate to acetyl-CoA. Uyeda and Rabinowitz (24, 25) and Raeburn and Rabinowitz (26) demonstrated in vitro that low potential electron donors, such as Fdred, can drive PFOR to catalyze the reductive carboxylation of acetyl-CoA as a pyruvate synthase. The rPFOR serves as a key enzyme for autotrophic CO2 fixation via the Wood–Ljungdahl pathway in acetogens (27). In this work, the in vivo isotopomer data measured postfeeding C. thermocellum 13C-bicarbonate and [2-13C]-acetate double-confirmed rPFOR activity and revealed that PFOR activity accounted for over 40% of the labeled carbon in pyruvate. Measurements in individual PFOR mutants further identified rPFOR activity predominantly catalyzed by Clo1313_0020-0023 and Clo1313_1353-1356. Consistent with previous global expression studies, Clo1313_0020-0023 is the most highly expressed PFOR based on proteomics (28) and transcriptomics (29) analyses. Clo1313_1353-1356 is the third most highly expressed PFOR based on proteomics data, but it has a low transcript level based on microarray data, and the difference could be due to different growth conditions in these studies.

By feeding 13C-bicarbonate to the PFL-inactivated mutant and the wild type, we confirmed the incorporation of these tracers into formate (Fig. 1), lactate (Fig. 3A), and the downstream amino acids linked to C1 metabolism (Figs. 3 and 5). We provided multiple lines of evidence showing that the fixed CO2 was channeled to the C1 metabolism through PFL, which could operate reversibly (21, 30) and contribute to the biosynthesis of essential amino acids, including serine and methionine. In addition, the reductive C1 pathway mediated by PFL may play a critical role in nucleotide biosynthesis (reconstructed purine biosynthesis pathway in C. thermocellum shown in Fig. S6). The impaired growth observed in PFL-inactivated mutant is consistent with a previous finding (12) and underscores PFL’s importance. A transcriptome study reported that folate-dependent enzymes are in the most highly expressed category, which also suggests an active C1 metabolism in vivo (31).

Carbon loss in the form of CO2 from the conversion of pyruvate to acetyl-CoA across a wide range of bacterial heterotrophs reduces the theoretical maximum yield of a carbon product of interest per carbon consumed (carbon efficiency) downstream of acetyl-CoA. A synthetic nonoxidative glycolysis has been shown to bypass the loss of CO2 in the conversion of pyruvate to acetyl-CoA (32). The rPFOR activity shown here allows some of the lost CO2 to be recaptured into pyruvate, a critical branch point and precursor to numerous natural and nonnatural compounds. Based on our data, CO2 utilization has increased the titers of organic carbon compounds (Table 1) by incorporating CO2 into the biomass.
formula, as detected in proteinogenic serine and methionine, and, as such, supplementing cell growth.

It is interesting that C. thermocellum uses the rPFOR-PFL shunt for CO2 uptake in the absence of Fdh. We speculate that gain insights into how widespread the rPFOR-PFL shunt could be via the malate shunt, with GTP produced via PEPCK promoting pathway, the rPFOR-PFL route for CO2 uptake may provide added metabolism and a key node connecting glycolysis and the C1 metabolic pathway for generating Fdred to yield reduced biofuels and PFOR functioning in the oxidative direction serves as a major trade-off of the flux toward electron generation. We speculate chemicals. Thus, the metabolic flux toward biosynthesis may be a formula, as detected in proteinogenic serine and methionine, and, other major redox pathways (i.e., H2 production) to enhance the carbon economy without causing redox imbalance. To satisfy our curiosity, we tested C. thermocellum autotrophic growth by feeding either bicarbonate plus 100% H2 or an 80% H2/20% (vol/vol) CO2 gas mixture, but detected no growth (Fig. S8). Nevertheless, the rPFOR-PFL shunt uncovered in this study may have important ramifications in the metabolic plasticity of a cellulose degrader and in other heterotrophs, and hence warrants more in-depth investigations.

Materials and Methods
A more complete discussion is provided elsewhere (16, 23, 33–35), as well as in SI Methods.

Unless otherwise noted, C. thermocellum DSM1313-derived strains, Δhpt (hpt encoding for hypoxanthine phosphoribosyl transferase is used for counterselection), and other mutants were routinely grown anaerobically at 55 °C on 5 g/L cellobiose. The hpt gene deleted strain is used as the wild type for the studies herein. To test the inorganic carbon utilization, 20 mM sodium bicarbonate was added to cell cultures also fed with cellobiose. We used the DSM122-defined medium referred to as CTFUD medium (36). Full details on strain construction, fermentation conditions, and 13C-tracer experiments are provided in SI Methods.

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14. Taillefer M, Rydzak T, Levin DB, Oresnik IJ, Sparling R (2015) Reassessment of the Feeding CO2 may also enhance the transient CO2 fixation pathway requires Fdred as an electron donor. On the other hand, substrate feeding may not be readily available, the presence of these species have Fdh, whereas 51 species have both PFOR and PFL (Fig. S7). Although the in vivo reversibility of PFOR can be organism- and/or growth condition-specific, and information as to whether these bacteria can use inorganic carbon during organic substrate feeding may not be readily available, the presence of these genes renders it genetically feasible.

It should be noted that carbon fixation via the rPFOR-PFL shunt requires Fdred as an electron donor. On the other hand, PFOR functioning in the oxidative direction serves as a major pathway for generating Fdred to yield reduced biofuels and chemicals. Thus, the metabolic flux toward biosynthesis may be a trade-off of the flux toward electron generation. We speculate that CO2 enhances growth by regenerating oxidized Fd, which, in turn, provides a driving force for faster cellobiose consumption. Feeding CO2 may also enhance the “transient” CO2 fixation via the malate shunt, with GTP produced via PEPCK promoting C. thermocellum growth. The biosynthesis vs. energy requirement at the pyruvate node needs to be evaluated comprehensively with 19. Schuchmann K, Müller V (2014) Autotrophy at the thermodynamic limit of life: A model for energy conservation in acetogenic bacteria. Nat Rev Microbiol 12(12):809–821.


