Physiological roles of pyruvate ferredoxin oxidoreductase and pyruvate formate-lyase in *Thermoanaerobacterium saccharolyticum* JW/SL-YS485

Jilai Zhou\(^1,3\), Daniel G Olson\(^1,3\), Anthony A Lanahan\(^1,3\), Liang Tian\(^1,3\), Sean Jean-Loup Murphy\(^1,3\), Jonathan Lo\(^2,3\) and Lee R Lynd\(^1,2,3^*\)

**Abstract**

**Background:** *Thermoanaerobacterium saccharolyticum* is a thermophilic microorganism that has been engineered to produce ethanol at high titer (30–70 g/L) and greater than 90 % theoretical yield. However, few genes involved in pyruvate to ethanol production pathway have been unambiguously identified. In *T. saccharolyticum*, the products of six putative *pfor* gene clusters and one *pfl* gene may be responsible for the conversion of pyruvate to acetyl-CoA. To gain insights into the physiological roles of PFOR and PFL, we studied the effect of deletions of several genes thought to encode these activities.

**Results:** It was found that pyruvate ferredoxin oxidoreductase enzyme (PFOR) is encoded by the *pforA* gene and plays a key role in pyruvate dissimilation. We further demonstrated that pyruvate formate-lyase activity (PFL) is encoded by the *pfl* gene. Although the *pfl* gene is normally expressed at low levels, it is crucial for biosynthesis in *T. saccharolyticum*. In *pforA* deletion strains, *pfl* expression increased and was able to partially compensate for the loss of PFOR activity. Deletion of both *pforA* and *pfl* resulted in a strain that required acetate and formate for growth and produced lactate as the primary fermentation product, achieving 88 % theoretical lactate yield.

**Conclusion:** PFOR encoded by *Tsac_0046* and PFL encoded by *Tsac_0628* are only two routes for converting pyruvate to acetyl-CoA in *T. saccharolyticum*. The physiological role of PFOR is pyruvate dissimilation, whereas that of PFL is supplying C1 units for biosynthesis.

**Keywords:** *Thermoanaerobacter saccharolyticum*, Pyruvate metabolism, Pyruvate ferredoxin oxidoreductase, Pyruvate formate-lyase, C1 metabolism
To achieve high-yield ethanol production in fermentative microbes with catabolism featuring pyruvate conversion to acetyl-CoA, the electrons from this oxidation must end up in ethanol, presumably via nicotinamide cofactors. In the case of PFOR, this means that electrons from reduced ferredoxin need to be transferred to NAD$^+$ or NADP$^+$. In the case of PFL, this means that electrons from formate must be transferred to NAD$^+$ or NADP$^+$. Shaw et al. [9] have detected ferredoxin-NAD(P)H activity, corresponding to reaction B in Table 2, in cell extracts. A $\text{pfor}$ gene (Tsac_2085) has also been identified [9]. A recent study has confirmed that the electron-bifurcating enzyme complex TfnAB, encoded by Tsac_2085 and Tsac_2086, plays a key role for generating NADPH from reduced ferredoxin in $T$. saccharolyticum [14]. Formate dehydrogenase (FDH) is another possible route for electron transfer to ethanol. However FDH (equation D in Table 2) has not been found in $T$. saccharolyticum, either by sequence homology or enzyme assay [9–11].

The conversion of pyruvate to acetyl-CoA is thought to proceed by the PFOR reaction in $T$. saccharolyticum; however, few of the specific genes responsible for ethanol formation from pyruvate in $T$. saccharolyticum have been unambiguously identified. For example, in the closely related species, C. thermocellum, despite the presence of a complete genome sequence, gene deletions and enzyme assays were required to determine a number of key aspects of central metabolism [15]. Following this, we decided to closely examine pyruvate metabolism in $T$. saccharolyticum. In particular, we wished to confirm whether PFOR is responsible for pyruvate dissimilation, identify which of the many PFOR enzymes are most important, gain insight into the function of PFL and examine the physiological consequences of deleting these genes individually and in combination.

**Results**

**Deletion of $\text{pfor}$**

There are six gene clusters in the $T$. saccharolyticum genome annotated as pyruvate ferredoxin/flavodoxin oxidoreductases according to KEGG [10, 11] (Table 1). In our first round of deletions, we succeeded in deleting four of the six clusters: $\text{pforA}$, $\text{pforB}$, $\text{pforD}$ and $\text{pforF}$ separately in the wild-type strain (LL1025). Deletion of $\text{pforA}$ resulted in the elimination of PFOR enzyme activity. The other deletions did not affect PFOR activity (Fig. 2). As expected from the enzyme assay data, only the $\text{pforA}$ deletion resulted in a change in products of fermentation (Table 3).

Fermentation profiles for individual colonies of the $\text{pforA}$ deletion strain revealed two different phenotypes with respect to lactate production. These strains were named LL1139 and LL1140. Strain LL1140 had less...
lactate production than LL1139 (Table 3). Both LL1139 and LL1140 showed elevated formate production compared to the wild-type strain. They were not able to consume more than 10 % of the 5 g/L cellobiose initially present in the medium (Table 3). The maximum ODs of these two strains in MTC-6 medium were reduced by 60 % in the presence of yeast extract (Additional file 1: Figure S1) and over 90 % in the absence of yeast extract (Fig. 3). Growth rates and lag phases of these two strains were similar to wild type with the presence of yeast extract (Additional file 1: Figure S1), but they did not grow without yeast extract in the MTC-6 medium over the course of 20 h (Fig. 3). Of the eight colonies analyzed, seven had the LL1139 phenotype and only one had the LL1140 phenotype.

To improve strain fitness, we adapted both LL1139 and LL1140 in MTC-6 medium for 20 transfers (approximately 140 generations) until no additional changes in growth rate were observed. Adapted cultures of strains LL1139 and LL1140 were named LL1141 and LL1142, respectively. Both strains produced more formate compared with their unadapted parent strains. Strain LL1141 produced more lactate and less pyruvate than LL1142, but otherwise their fermentation profiles were similar. Both strains were able to consume about half of the 5 g/L cellobiose initially present in the medium (Table 3), and the maximum cell density and growth rate were greater than the unadapted parent strains in the defined medium but did not recover to wild-type level (Fig. 3).

In all pfor deletion strains, the expression levels of pyruvate formate-lyase genes were increased about sixfold compared with the parent strain (Fig. 4). Transcriptional analysis also indicated that pfl had higher expression level in LL1142 than LL1141, which corresponds to higher formate production in LL1142.

We also deleted pforA in the high ethanol-producing strain of T. saccharolyticum, LL1049, previously developed by Mascoma [4]. The resulting strain was named LL1159. This strain grew slower than LL1139 or LL1140.

### Table 1 Clusters of pfor and pfl genes

| Gene cluster | Gene  | Annotated gene productsa |
|--------------|-------|--------------------------|
| pforA        | Tsac_0046 | Pyruvate ferredoxin/flavodoxin oxidoreductase |
| pforB        | Tsac_0380 | 2-Oxooacid acceptor oxidoreductase subunit alpha |
|              | Tsac_0381 | Pyruvate ferredoxin/flavodoxin oxidoreductase subunit beta |
| pforC        | Tsac_0915 | Pyruvate ferredoxin/flavodoxin oxidoreductase |
|              | Tsac_1064 | 4Fe–4S ferredoxin |
| pforD        | Tsac_1065 | Pyruvate flavodoxin/ferredoxin oxidoreductase domain-containing protein |
|              | Tsac_1066 | Thiamine pyrophosphate TPP-binding domain-containing protein |
|              | Tsac_1067 | Pyruvate/ketoisovalerate oxidoreductase |
|              | Tsac_2160 | Pyruvate/ketoisovalerate oxidoreductase |
| pforE        | Tsac_2161 | Thiamine pyrophosphate TPP-binding domain-containing protein |
|              | Tsac_2162 | Pyruvate flavodoxin/ferredoxin oxidoreductase domain-containing protein |
|              | Tsac_2163 | 4Fe–4S ferredoxin |
|              | Tsac_2177 | Pyruvate/ketoisovalerate oxidoreductase subunit gamma |
| pforF        | Tsac_2178 | Thiamine pyrophosphate TPP-binding domain-containing protein |
|              | Tsac_2179 | Pyruvate flavodoxin/ferredoxin oxidoreductase domain-containing protein |
|              | Tsac_2180 | 4Fe–4S ferredoxin |
| pfl          | Tsac_0628 | Pyruvate formate-lyase |
|              | Tsac_0629 | Pyruvate formate-lyase-activating enzyme |

a The gene product annotations were based on NCBI genome project (NC_017992.1).

### Table 2 Potential reactions related to pyruvate dissimilation in T. saccharolyticum

| Reaction ID | Enzyme name | Reaction catalyzed by the enzyme |
|-------------|-------------|---------------------------------|
| A           | Pyruvate ferredoxin oxidoreductase (PFOR) | Pyruvate + CoA + ferredoxin (ox) → acetyl-CoA + ferredoxin (red) + CO2 |
| B           | Ferredoxin/NAD(P)H oxidoreductase (FNOR) | Ferredoxin (red) + NAD(P)+ + H+ → ferredoxin (ox) + NAD(P)H |
| C           | Pyruvate formate-lyase (PFL) | Pyruvate + CoA → acetyl-CoA + formate |
| D           | Formate dehydrogenase (FDH) | Formate + NAD(P)+ → CO2 + NAD(P)H |
in MTC-6 medium and it was unable to consume more than 10 % of 5 g/L cellobiose (Table 3).

Deletion of pfl
To investigate the physiological role of PFL in T. saccharolyticum, we deleted the pfl gene cluster in the wild type (LL1025). The pfl deletion in strain LL1025 gave two different phenotypes (high lactate and low lactate), which were stored as strain LL1164 and LL1170. Of eight colonies picked, two had the LL1164 phenotype and six had the LL1170 phenotype. Strain LL1170 consumed more cellobiose, produced more acetate and ethanol and less lactate than strain LL1164 (Table 3).

Both pfl deletion strains grew more poorly in MTC-6 medium than in CTFUD medium. The biggest difference between CTFUD and MTC-6 medium is the presence of yeast extract. The addition of yeast extract could restore the growth of pfl deletion strains in the MTC-6 medium (Additional file 2: Figure S2). The growth of both strains was stimulated by addition of formate, serine or lipoic acid (Fig. 5). In cases where formate was added, a small amount was consumed by all three strains (less than 1 mM, which is equivalent to 0.05 mmol in 50 mL culture as shown in Table 3).

Double deletion of pfor and pfl
In the adapted pforA deletion strains (LL1141 and LL1142), formate production was significantly increased, and carbon flux toward acetate and ethanol formation was presumptively via the PFL reaction. To show that PFOR encoded by pforA and PFL encoded by pfl were the only two routes for the conversion of pyruvate to ethanol in T. saccharolyticum, we deleted pfl in strain LL1141 (which already contained the pforA deletion). To create this deletion, it was necessary to supplement the medium with 4 mM sodium acetate.

The resulting pfor/pfl double deletion strain (LL1178) consumed about 70 % of the 5 g/L cellobiose initially present, which was about the same as its parent strain (LL1141). It required sodium acetate for growth, even in the presence of yeast extract. Lactate became the main product of fermentation, with 3.5 mol of lactate produced for each mole of cellobiose consumed (or 88 % of the theoretical maximum yield) (Table 3).

Genomic sequence of mutants
Comparing resequencing results for the pfor deletion strains (LL1139 and LL1140) (Additional file 3: Table S1), we found a mutation in lactate dehydrogenase gene of LL1140, which was maintained during the adaptation process and also found in strain LL1142 (adapted version of LL1140).

As described before, we isolated two different phenotypes (high and low lactate) when we deleted pfl in T. saccharolyticum. They were named as LL1164 and LL1170, respectively. Strain LL1164 could not consume all 5 g/L cellobiose initially present in the medium and produced lactate as the main product of fermentation. After comparing the genome resequencing data of LL1164 and LL1170 (two phenotypes of pfl deletion strains from wild-type T. saccharolyticum), we found two mutations that were present in LL1164, but not in LL1170. One mutation is a synonymous mutation in Tsac_1304, which is annotated as uncharacterized protein, the other one is found in Tsac_1553, which is annotated as ferredoxin hydrogenase.

Discussion
The major route for pyruvate dissimilation
Wild-type T. saccharolyticum produces 2.7 mol of C2 products (ethanol and acetate) for each mole of cellobiose consumed (since the theoretical maximum is 4, this is 68 % of the theoretical maximum yield). Deletion of the primary pfor gene, pforA, resulted in a dramatic decrease in growth, indicating the importance of pforA in pyruvate dissimilation. Since ethanol was still produced, it was hypothesized that pfl partially compensated for the deletion of pfor. Creation of a double deletion strain (LL1178),

![Fig. 2 Enzymatic activity of pyruvate ferredoxin oxidoreductase from cell-free extract of T. saccharolyticum mutants. Error bars represent the standard deviation of three replicates. ND (not detected), the specific activities were below detection limit 0.005 U/mg.](image)
Table 3  Fermentation profiles of T. saccharolyticum knockout strains

| Strains         | Additions to medium | Fermentation profile<sup>a</sup> | Consumption of cellulose | Residual cellulose | Formate | Lactate | Acetate | Ethanol | Succinate | Pyruvate | Malate | Pellet carbon | Hydrogen | Carbon recovery (%) | Electron recovery (%) |
|-----------------|---------------------|---------------------------------|--------------------------|--------------------|---------|---------|---------|---------|-----------|----------|--------|-------------------|----------|-------------------|------------------------|
| LL1025 Wild type| None                | Consumed cellulose: 0.70 mmol  | Residual cellulose: 0.00 | Formate: 0.01     | Lactate: 0.28 | Acetate: 0.71 | Ethanol: 1.18 | Succinate: 0.01 | Pyruvate: 0.00 | Malate: 0.00 | Pellet carbon: 0.80 | Hydrogen: 1.68 | Carbon recovery: 90 | Electron recovery: 94 |
| LL1049 Ethanologenic strain| None                | Consumed cellulose: 0.70 mmol  | Residual cellulose: 0.00 | Formate: 0.22     | Lactate: 0.00 | Acetate: 0.04 | Ethanol: 2.10 | Succinate: 0.00 | Pyruvate: 0.00 | Malate: 0.00 | Pellet carbon: 0.72 | Hydrogen: 0.22 | Carbon recovery: 86 | Electron recovery: 90 |
| LL1139 LL1025 ΔpforA-1| None                | Consumed cellulose: 0.07 mmol  | Residual cellulose: 0.63 | Formate: 0.15     | Lactate: 0.04 | Acetate: 0.05 | Ethanol: 0.09 | Succinate: 0.00 | Pyruvate: 0.00 | Malate: 0.00 | Pellet carbon: 0.18 | Hydrogen: 0.01 | Carbon recovery: 98 | Electron recovery: 99 |
| LL1140 LL1025 ΔpforA-2| None                | Consumed cellulose: 0.02 mmol  | Residual cellulose: 0.68 | Formate: 0.01     | Lactate: 0.00 | Acetate: 0.00 | Ethanol: 0.00 | Succinate: 0.00 | Pyruvate: 0.00 | Malate: 0.00 | Pellet carbon: 0.19 | Hydrogen: 0.10 | Carbon recovery: 100 | Electron recovery: 101 |
| LL1141 Adapted from LL1139| None                | Consumed cellulose: 0.37 mmol  | Residual cellulose: 0.34 | Formate: 0.28     | Lactate: 0.67 | Acetate: 0.06 | Ethanol: 0.29 | Succinate: 0.00 | Pyruvate: 0.06 | Malate: 0.27 | Pellet carbon: 0.03 | Hydrogen: 0.02 | Carbon recovery: 91 | Electron recovery: 92 |
| LL1142 Adapted from LL1140| None                | Consumed cellulose: 0.34 mmol  | Residual cellulose: 0.36 | Formate: 0.38     | Lactate: 0.26 | Acetate: 0.03 | Ethanol: 0.42 | Succinate: 0.02 | Pyruvate: 0.22 | Malate: 0.21 | Pellet carbon: 0.04 | Hydrogen: 0.04 | Carbon recovery: 89 | Electron recovery: 90 |
| LL1155 LL1025 ΔpforD| None                | Consumed cellulose: 0.70 mmol  | Residual cellulose: 0.00 | Formate: 0.02     | Lactate: 0.39 | Acetate: 0.72 | Ethanol: 0.01 | Succinate: 0.00 | Pyruvate: 0.00 | Malate: 0.72 | Pellet carbon: 1.65 | Hydrogen: 1.65 | Carbon recovery: 91 | Electron recovery: 94 |
| LL1156 LL1025 ΔpforF| None                | Consumed cellulose: 0.70 mmol  | Residual cellulose: 0.00 | Formate: 0.02     | Lactate: 0.15 | Acetate: 0.80 | Ethanol: 1.24 | Succinate: 0.01 | Pyruvate: 0.00 | Malate: 0.89 | Pellet carbon: 1.74 | Hydrogen: 1.74 | Carbon recovery: 92 | Electron recovery: 94 |
| LL1157 LL1025 ΔpforC| None                | Consumed cellulose: 0.70 mmol  | Residual cellulose: 0.00 | Formate: 0.00     | Lactate: 0.00 | Acetate: 0.38 | Ethanol: 0.76 | Succinate: 1.19 | Pyruvate: 0.00 | Malate: 0.73 | Pellet carbon: 1.64 | Hydrogen: 1.64 | Carbon recovery: 91 | Electron recovery: 94 |
| LL1159 LL1049 ΔpforA| None                | Consumed cellulose: 0.07 mmol  | Residual cellulose: 0.63 | Formate: 0.01     | Lactate: 0.00 | Acetate: 0.01 | Ethanol: 0.00 | Succinate: 0.00 | Pyruvate: 0.00 | Malate: 0.23 | Pellet carbon: 0.05 | Hydrogen: 0.05 | Carbon recovery: 92 | Electron recovery: 92 |
| LL1164 LL1025 Δpfl-1| Formate: 0.48      | Consumed cellulose: 0.21 mmol  | Residual cellulose: -0.02 | Formate: 1.13     | Lactate: 0.19 | Acetate: 0.33 | Ethanol: 0.00 | Succinate: 0.00 | Pyruvate: 0.47 | Malate: 0.00 | Pellet carbon: 0.48 | Hydrogen: 0.48 | Carbon recovery: 96 | Electron recovery: 98 |
| LL1170 LL1025 Δpfl-2| Formate: 0.69      | Consumed cellulose: 0.00 mmol  | Residual cellulose: -0.03 | Formate: 0.24     | Lactate: 0.81 | Acetate: 1.22 | Ethanol: 0.00 | Succinate: 0.00 | Pyruvate: 0.92 | Malate: 0.00 | Pellet carbon: 0.69 | Hydrogen: 0.69 | Carbon recovery: 92 | Electron recovery: 95 |
| LL1178 LL1025 ΔpforA; Δpfl| Formate and Acetate: 0.48 | Consumed cellulose: 0.25 mmol  | Residual cellulose: -0.01 | Formate: 1.69     | Lactate: 0.12 | Acetate: 0.08 | Ethanol: 0.00 | Succinate: 0.00 | Pyruvate: 0.00 | Malate: 0.31 | Pellet carbon: 0.00 | Hydrogen: 0.00 | Carbon recovery: 94 | Electron recovery: 96 |

<sup>a</sup> The amount of fermentation end products are reported in millimoles in a volume of 50 mL serum bottle. The amounts of initial cellulose were 0.70 mmol for all fermentations. Cultures were incubated for 72 h at 55 °C with an initial pH of 6.2 in MTC-6 medium.  
<sup>b</sup> To improve the growth of LL1164, LL1170, 0.20 mmol formate was added into 50 mL MTC-6 medium. Negative values represent that a certain amount of sodium formate was consumed during fermentation. 
<sup>c</sup> LL1178 requires supplementation of both formate and acetate to grow in MTC-6 medium. 0.20 mmol sodium formate and 0.20 mmol sodium acetate were added into 50 mL MTC-6 medium. Negative values represent that a certain amount of sodium formate and sodium acetate was consumed during fermentation.
with both pf or and pf l deleted, produced almost no C2 products and carbon flux was redirected to lactate production. The C2 yield in this strain is $-0.08$ mol per mole of cellobiose consumed (the slight negative value is due to the consumption of sodium acetate), whereas the C3 (i.e., lactate) yield is 3.52 (88 % of theoretical).

The recA gene is Tsac_1846, annotated as a DNA recombination and repair protein, which usually has a consistent expression level across many strains and environmental conditions.

**Fig. 3** Growth curves of Δpf or strains in MTC-6 medium. Black plus signs represent wild-type strain (LL1025), cyan circles represent Δpf or-1, magenta crosses represent Δpf or-2, blue diamonds represent adapted Δpf or-1, red stars represent adapted Δpf or-2.

**Fig. 4** Relative mRNA level of Tsac_0046, Tsac_0628 and Tsac_0629 in adapted pf orA deletion strains. Tsac_0046 encodes pyruvate ferredoxin oxidoreductase, Tsac_0628 encodes pyruvate formate-lyase and Tsac_0629 encodes pyruvate formate-lyase activating enzyme. The recA gene is Tsac_1846, annotated as a DNA recombination and repair protein, which usually has a consistent expression level across many strains and environmental conditions.

**Fig. 5** Growth of pf l deletion strains in MTC-6 medium (black), and with 4 mM formate (red), with 4 mM glycine (yellow), MTC with 4 mM serine (blue), MTC with 10 mg/L lipoic acid (green).
via the PFL pathway, formate production and C2 yield should be equivalent on a molar basis. For strain LL1141, formate production can account for about 80 % of the C2 products. For strain LL1142, formate production can account for about 84 % of the C2 products (Table 3). One possible explanation for the residual C2 production is consumption of formate for biosynthesis. Another possible explanation is PFOR activity from a gene cluster other than pforA. Although PFOR activity was eliminated after deletion of pforA, adaptation resulted in the appearance of very low levels of PFOR activity (less than 1/100th) that could be from one of the other annotated pfor genes (Fig. 2).

The gene encoding PFOR

Based on data from enzyme assay and gene deletions, it appears that pforA is the gene encoding the primary PFOR enzyme in T. saccharolyticum, which is different from the gene cluster, pforB, as suggested by Shaw et al. [9]. Single deletion of the pforA cluster in wild-type T. saccharolyticum completely eliminated the PFOR activity, while deletions of other pfor gene clusters had no effect under tested conditions (Fig. 2). This result is also consistent with proteomic data for T. saccharolyticum, in which PFOR encoded by pforA is the most abundant protein among all PFOR enzymes [13]. Enzymes encoded by other pfor gene clusters are expressed at a much lower level, at least ten times lower than that encoded by pforA [13]. The role of these other gene clusters remains unknown.

Pyruvate dehydrogenase and pyruvate decarboxylase activity

The genes for PDC and PDH were absent in the genome of T. saccharolyticum [9–11]. Shaw et al. also did not detect PDH or PDC activities by enzyme assay (which we have confirmed). There are reports that PFOR can decarboxylate pyruvate directly to acetaldehyde, functioning as pyruvate decarboxylase (PDC) in Pyrococcus furiosus [16] and Thermococcus guaymasensis [17]. Although in both cases, the acetyl-CoA production rates are higher than acetaldehyde production rates (roughly 5:1 in both organisms [17]), the PDC side activity of PFOR is still thought to be one of the options for acetaldehyde production in hyperthermophiles [17]. Another possibility is through aldehyde ferredoxin oxidoreductase (AOR), which can convert acetate to acetaldehyde [18, 19]. According to this ratio of PFOR activity versus PDC activity, the PDC activity should be in the order of 0.1–1 U/mg if the PFOR in T. saccharolyticum has this side activity. However, we did not detect PDC activity in cell extracts (<0.005 U/mg), so this activity (if it exists) does not likely play a significant physiological role.

We also examined the existence of PDH in several other species that are closely related to T. saccharolyticum (Table 4). In some Thermoaerobacter species, they possess all genes required to encode the PDH complex, but their function and physiological roles remain to be determined experimentally.

Role of pfl and C1 metabolism

Pyruvate formate-lyase was only expressed at low levels and was not the major route for pyruvate dissimilation in the wild-type strain. It was, however, required for growth of T. saccharolyticum grown in MTC-6 medium. The consumption of added formate and restoration of stronger growth upon addition of formate by all pfl deletions strains (Table 3) supports the hypothesis that PFL is required for biosynthesis.

It has been previously reported that PFL has an anabolic function in Clostridium species and furnishes cells with C1 units [20, 21]. The results presented here suggest that this might also be the case in T. saccharolyticum, which belongs to class Clostridia. In Clostridium acetobutylicum, 13C labeling experiments showed that over 90 % of C1 units in biosynthetic pathways come from the carboxylic group of pyruvate and are likely to be derived from the PFL reaction [22]. Due to the impaired growth of pfl deletion strains, we think this is likely the case in T. saccharolyticum also. In the case of C. acetobutylicum, Amador-Noguez et al. [22] found that glycine is not formed from serine, and thus that the methyl group from serine is not transferred to tetrahydrofolate (THF) in this organism. However, in the case of T. saccharolyticum, the growth of pfl deletion strains was restored by the addition of serine, suggesting that C1 units are transferred from serine to THF.

Although additional glycine did not stimulate the growth of T. saccharolyticum, additional lipoic acid did improve growth (Fig. 5). In fact, T. saccharolyticum has all of the genes required for the glycine cleavage system and the lipoic acid salvage system. Since it does not have lipoic acid biosynthesis pathways, it required additional lipoic acid for H protein formation, which is essential for the glycine cleavage system [23]. The proposed one carbon metabolism in T. saccharolyticum is shown in Fig. 6 based on the generic C1 metabolism network from KEGG [10].

Among other species that we have examined, most of the Thermoaerobacter species have the glycine cleavage system and either the lipoic acid biosynthesis or the lipoic acid salvage system for H protein formation (Table 4). However, Caldicellulosiruptor species do not have either PFL or glycine cleavage system. Therefore, we think they may use serine aldolase (EC 2.1.2.1) for the supply of C1 units.
Mutations found in genomic resequencing

In one pfor deletion strain lineage (lineage 2, Additional file 3: Table S1), which includes ΔpforA-2 (strain LL1140) and its adapted descendant (strain LL1142), we found an SNP in lactate dehydrogenase (Tsac_0179). This SNP causes an amino acid change from asparagine to serine. According to the protein structure of LDH from Bacillus stearothermophilus [24], which shares 48% identity with that from T. saccharolyticum, we found this mutation was near the catalytic site. We suspect that this SNP may explain the decrease in lactate production in strains LL1140 and LL1142.

In one pfl deletion strain (LL1164) but not another (strain LL1170, a different colony from the pfl deletion experiment, see previous description), an SNP was found in the ferredoxin hydrogenase, subunit B (hfsB, Tsac_1153). A non-functional hfs gene could inhibit the PFOR reaction by preventing the oxidation of reduced ferredoxin. Shaw et al. [25] found that deletion of the entire hfs operon resulted in a decrease in hydrogen and acetate production and increase in lactate production. We see similar trends for hydrogen, acetate and lactate. Shaw et al. found a slight decrease in ethanol production (22%), whereas we see a much larger decrease (73%). The similarities in the patterns of fermentation data between our hfsB mutant and the hfs deletion from Shaw et al. suggest that the hfs mutation may in fact be responsible for the change in distribution of products of fermentation between strains LL1164 and LL1170.

Conclusion

In this study, we have identified genes and enzymes responsible for pyruvate ferredoxin oxidoreductase and pyruvate formate-lyase activities in T. saccharolyticum. The primary physiological role of PFOR appears to be pyruvate dissimilation, while the role of PFL appears to be supplying C1 units in biosynthesis. PFOR encoded by Tsac_0046 and PFL encoded by Tsac_0628 are only two routes for converting pyruvate to acetyl-CoA in T. saccharolyticum. The combination deletion of these two

---

Table 4 Comparison of genes involved in pyruvate metabolism and C1 metabolism between T. saccharolyticum and its relative species

| Organisms                                      | Enzymes | Glycine cleavage system | Lipoic acid synthesis | Lipoic salvage system |
|------------------------------------------------|---------|-------------------------|-----------------------|-----------------------|
| Clostridium thermocellum DSM1313               | +       | +                       | _                      | _                      |
| Clostridium clariflavum DSM 19732             | +       | +                       | _                      | _                      |
| Clostridium stercorarium subsp. stercorarium DSM 8532 | +       | +                       | _                      | _                      |
| Thermoanaerobacter saccharolyticum JW/SL-Y5485 | +       | +                       | _                      | _                      |
| Thermoanaerobacter tengcongensis MB4(T)       | +       | +                       | _                      | _                      |
| Thermoanaerobacter sp. X514                   | +       | +                       | _                      | _                      |
| Thermoanaerobacter pseudethanolicus ATCC 33223 | +       | +                       | _                      | _                      |
| Thermoanaerobacter italicus Ab9               | +       | +                       | _                      | _                      |
| Thermoanaerobacter mathani subsp. mathanii A3  | +       | +                       | _                      | _                      |
| Thermoanaerobacter brockii subsp. finnii Ako-1 | +       | +                       | _                      | _                      |
| Thermoanaerobacter wiegelii RbB1              | +       | +                       | _                      | _                      |
| Thermoanaerobacter kivui DSM 2030             | +       | +                       | _                      | _                      |
| Thermoanaerobacterium thermosaccharolyticum DSM571 | +       | +                       | _                      | _                      |
| Clostridium thermocellum DSM1313               | +       | +                       | _                      | _                      |
| Caldicellulosiruptor saccharolyticus DSM 8903 | +       | +                       | _                      | _                      |
| Caldicellulosiruptor bescii DSM 6725          | +       | +                       | _                      | _                      |
| Caldicellulosiruptor obsidians DSM 847        | +       | +                       | _                      | _                      |
| Caldicellulosiruptor hydrothermaliis 108      | +       | +                       | _                      | _                      |
| Caldicellulosiruptor owensensis OL            | +       | +                       | _                      | _                      |
| Caldicellulosiruptor kristjanssonii 177R1B    | +       | +                       | _                      | _                      |
| Caldicellulosiruptor kronoksakensis 2002      | +       | +                       | _                      | _                      |
| Caldicellulosiruptor lactoacetici DSM 6A      | +       | +                       | _                      | _                      |

a T. thermosaccharolyticum DSM571 has pfl annotated, whereas T. thermosaccharolyticum M0795 does not have it. It is also confirmed with protein blast using PFL protein sequence from T. saccharolyticum.

b No information about lipoic acid metabolism of C. thermocellum DSM1313, C. clariflavum DSM 19732, C. kristjanssonii 177R1B and C. lactoaceticus 6A in KEGG. The existence of lipoic acid biosynthesis and lipoic salvage system are confirmed by protein blast using lipoate synthase from C. bescii and lipoate protein ligase from T. saccharolyticum.
genes virtually eliminated pyruvate flux to acetyl-CoA, which can be seen by the shift of carbon flux to lactate production at high yield (88% of theoretical).

**Methods**

**Strains and plasmids**

*T. saccharolyticum* JW/SL-YS485 (aka LL1025, DSM8691) was kindly provided by Juergen Wiegel (University of Georgia, Athens, GA, USA) and stored in the laboratory strain collection. Strain LL1049 (aka M1442) was a gift from the Mascoma Corporation [4]. All other strains were from commercial sources or developed in our laboratory (Table 5). Plasmids are described in Table 5.

**Media and growth conditions**

Genetic modifications of *T. saccharolyticum* were performed in CTFUD medium, containing 1.3 g/L (NH₄)₂SO₄, 1.5 g/L KH₂PO₄, 0.13 g/L CaCl₂·2H₂O, 2.6 g/L MgCl₂·6H₂O, 0.001 g/L FeSO₄·7H₂O, 4.5 g/L yeast extract, 5 g/L cellobiose, 3 g/L sodium citrate tribasic dihydrate, 0.5 g/L l-cysteine-HCl monohydrate, 0.002 g/L resazurin and 10 g/L agarose (for solid media only). The pH was adjusted to 6.7 for selection with kanamycin (200 μg/mL), or adjusted to 6.1 for selection with erythromycin (25 μg/mL). Measurement of fermentation products and growth of *T. saccharolyticum* were performed in MTC-6 medium [26], including 5 g/L cellobiose, 9.25 g/L MOPS (morpolineproanesulfonic acid) sodium salt, 2 g/L ammonium chloride, 2 g/L potassium citrate monohydrate, 1.25 g/L citric acid monohydrate, 1 g/L Na₂SO₄, 1 g/L KH₂PO₄, 2.5 g/L NaHCO₃, 2 g/L urea, 1 g/L MgCl₂·6H₂O, 0.2 g/L CaCl₂·2H₂O, 0.1 g/L FeCl₃·6H₂O, 1 g/L l-cysteine HCl, 0.002 g/L pyridoxamine HCl, 0.004 g/L p-aminobenzoic acid (PABA), 0.004 g/L D-biotin, 0.002 g/L vitamin B12, 0.04 g/L thiamine, 0.005 g/L MnCl₂·4H₂O, 0.005 g/L CoCl₂·6H₂O, 0.002 g/L ZnCl₂, 0.001 g/L CuCl₂·2H₂O, 0.001 g/L H₂BO₃, 0.001 g/L Na₂MoO₄·2H₂O and 0.001 g/L NiCl₂·6H₂O. It was prepared by combining six sterile solutions (A–F) with minor modification under nitrogen atmosphere as described before [15]. All six solutions were sterilized through a 0.22 μm filter (Corning, #430517). Solution A, concentrated 2.5-fold, contained cellobiose, MOPS sodium salt and distilled water. Solution B, concentrated 5-fold, contained potassium citrate monohydrate, citric acid monohydrate, Na₂SO₄, KH₂PO₄, NaHCO₃ and distilled water. Solution C, concentrated 50-fold, contained ammonium chloride and distilled water. Solution D, concentrated 50-fold, contained MgCl₂·6H₂O, CaCl₂·2H₂O, FeCl₃·6H₂O and l-cysteine HCl monohydrate. Solution E, concentrated 50-fold, contained thiamine, pyridoxamine HCl, p-aminobenzoic acid (PABA), D-biotin and vitamin B12. Solution F, concentrated 1000-fold, contained MnCl₂·4H₂O, CoCl₂·6H₂O, ZnCl₂, CuCl₂·2H₂O, H₂BO₃, Na₂MoO₄·2H₂O and NiCl₂·6H₂O. Some fermentations required supplementation with additional components. These were added after the first six solutions were combined. The final pH was adjusted to 6.1. Fermentations of *T. saccharolyticum* were done in 125-mL glass bottles at 55 °C under a nitrogen atmosphere. The working volume was 50 mL with shaking at 250 rpm. Fermentations were allowed to proceed for 72 h, at which point samples were collected for analysis.

---

**Fig. 6** Proposed one carbon metabolic pathway in *T. saccharolyticum*. Green arrows indicate the pathway for 10-formyl-THF production. Note that this pathway requires formate, which is presumably generated by FPL in *T. saccharolyticum*. Blue arrows indicate the active pathways of pfl deletion strains grown in MTC-6 supplemented with additional serine. Orange arrows indicate active pathways in pfl deletion strains grown in MTC-6 supplemented with additional lipoic acid. EC numbers represent enzymes responsible for catalyzing that reaction. In *T. saccharolyticum*, formate tetrahydrofolate ligase (EC 6.3.4.3) is encoded by Tsac_0941.
OD measurements were performed in a 96-well plate incubated at 55 °C in the absence of oxygen as previously described [27]. Each well contained 200 μL MTC-6 medium. The plate was shaken for 30 s every 3 min, followed by measuring the optical density at 600 nm.

*Escherichia coli* strains used for cloning were grown aerobically at 37 °C in Lysogeny Broth (LB) [28] medium with either kanamycin (200 μg/mL) or erythromycin (25 μg/mL). For cultivation on solid medium, 15 g/L agarose was added.

All reagents used were from Sigma-Aldrich unless otherwise noted. All solutions were made with water purified using a MilliQ system (Millipore, Billerica, MA, USA).

**Plasmid construction**

Plasmids for gene deletion were designed as previously described [29] with either kanamycin or erythromycin resistance cassettes from plasmids pMU433 or pZJ23 flanked by 1.0- to 0.5-kb regions homologous to the 5′ and 3′ regions of the deletion target of interest. Plasmids pZJ13, pZJ15, pZJ16, pZJ17 and pZJ20 were created based on pMU433. The backbone and kanamycin cassettes from plasmid pMU433 were amplified by the primers shown in Table 6. Homologous regions of deletion targets of interest were amplified by primers shown in Table 6. Homologous regions of deletion targets of interest were amplified with the primers shown in Table 6. Homologous regions of deletion targets of interest were amplified.

All primers used were from Gibson Assembly Master Mix (New England Biolabs, Ipswich, MA). The assembled circular plasmids were transformed into *E. coli* DH5α chemical competent cells (New England Biolabs, Ipswich, MA) for propagation. Plasmids were purified by a Qiagen miniprep kit (Qiagen Inc., Germantown, MD, USA).

### Table 5 Strains and plasmids

| Strain or plasmid | Description | Accession number | References |
|-------------------|-------------|-----------------|------------|
| Strains           |             |                 |            |
| *E. coli* DH5α    | *E. coli* cloning strains | N/A            | New England Biolabs |
| *T. saccharolyticum* |            |                 |            |
| LL1025            | Wild-type strain | SRA234880       | [32]       |
| LL1040 (aka ALK2) | High ethanol-producing strain, Kanr, Ermr | N/A            | [2]        |
| LL1049 (aka M1442) | High ethanol-producing strain | SRA233073       | [4]        |
| LL1139            | LL1025 ΔpforA : Kanr, colony 1 | SRA234882       | This study |
| LL1140            | LL1025 ΔpforA : Kanr, colony 2 | SRA233066       | This study |
| LL1141            | Adapted LL1139 | SRA234883       | This study |
| LL1142            | Adapted LL1140 | SRA234884       | This study |
| LL1155            | LL1025 ΔpforD : Kanr, | N/A            | This study |
| LL1156            | LL1025 ΔpforB : Kanr, | N/A            | This study |
| LL1157            | LL1025 ΔpforF : Kanr, | N/A            | This study |
| LL1159            | LL1049 ΔpforA : Kanr, | N/A            | This study |
| LL1164            | LL1025 Δpfl : Kanr, colony 1 | SRA233080       | This study |
| LL1170            | LL1025 Δpfl : Kanr, colony 2 | SRA233074       | This study |
| LL1178            | LL1141 Δpfl : Ermr | SRA234885       | This study |
| Plasmids          |             |                 |            |
| pMU433            | Cloning vector pta/ack, Kanr | KP057684        | This study |
| pZJ13             | pforA knockout, Kanr, pta/ack | KP057685        | This study |
| pZJ15             | pforA knockout, Kanr, pta/ack | KP057686        | This study |
| pZJ16             | pforD knockout, Kanr, pta/ack | KP057687        | This study |
| pZJ17             | pforF knockout, Kanr, pta/ack | KP057688        | This study |
| pZJ20             | pfl knockout, Kanr, pta/ack | KP057689        | This study |
| pZJ23             | Cloning vector Ermr, Ampr | KP057690        | This study |
| pZJ25             | pfl knockout, Ermr, Ampr | KP057691        | This study |

**Note.** Accession numbers for strains refer to raw resequencing data from the JGI Sequence Read Archive. Accession numbers for plasmids refer to the Genbank database.

Kanr kanamycin resistant, Ermr erythromycin resistant, Ampr ampicillin resistant, pta/ack is a negative selective marker.

---

Zhou *et al.* Biotechnol Biofuels (2015) 8:138
Table 6 Oligonucleotides used in this study

| Primer   | Target gene       | Sequence (5’–3’)              |
|----------|-------------------|--------------------------------|
| JP75     | Kanamycin cassette from pMU43 | TAAACGGCTAAAGGCATGA            |
| JP76     | pMU43 backbone    | CTATCTGATGCTGTTTTTC            |
| JP77     | pMU433 back bone  | AGTATGAGATTTGAGCGA             |
| JP78     | AAAGAGGGGATACAGGAA |
| JP209    | Erythromycin cassette from ALK2 | TGAGCTGAGATACAGGAA            |
| JP210    | ALK2              | GAATCCCTTCTTAGAACGTGA          |
| JP211    | Replication region from pUC19 | CATTAATGAAATCccccacacac             |
| JP212    |                   | CTACCTATATGGCTGTGTCCTTTTTTTTTT |
| JP143    | external to pforA cluster | GCTGTCGCAACCTAAACAAA            |
| JP144    |                   | CTCATATACATACGGCTGCTCTTTTT     |
| JP167    | external to pforB cluster | GTGTTGTTTTGCTGGTAGG             |
| JP168    |                   | AGCTTTCTATTCACTAGTACG           |
| JP169    | external to pforD cluster | CGTGCCTTTTGACCCTTCC            |
| JP170    |                   | CTGCATGATGCTGTTTACCTTTT        |
| JP171    | external to pforF cluster | CCAATTACCACCCAGCCA             |
| JP172    |                   | GAATTTGAAAAACCCCGCA            |
| JP181    | external to pfl cluster | ATCCCTTGTGCTTCTTATAC           |
| JP182    |                   | TGTTTGGGTTGGTGGTTATG           |
| recA-F   | qPCR for Tsac_1846 (recA) | GAAGGCCTTGTGAGGGAAATG          |
| recA-R   |                   | GAAAGCTCAACATGCGATCGG           |
| pfor-F   | qPCR for Tsac_0046 | ATCCAGGCTTGGAGGTGTTG           |
| pfor-R   |                   | CGTGTGGAGCGCTTGGTGC            |
| pfl-F    | qPCR for Tsac_0628 | CTATAGCATGCGCTGCTTGG           |
| pfl-R    |                   | TGGATAACCGCGCTTATATAC          |
| pfl_oe-F | qPCR for Tsac_0629 | ATGGCGCATAACCGCTGACCA          |
| pfl_oe-R |                   | TAGGCTCTCCATACGGCTACCG         |

Transformation of *T. saccharolyticum*

Plasmids were transformed into naturally competent *T. saccharolyticum* as described before [25, 30]. Mutants were grown and selected on solid medium with kanamycin (200 μg/mL) at 55 °C or with erythromycin (20 μg/mL) at 48 °C in an anaerobic chamber (COY Labs, Grass Lake, MI, USA). Mutant colonies appeared on selection plates after about 3 days. Target gene deletions, with chromosomal integration at both homology regions, were confirmed by PCR with primers external to the target genes (Table 6).

Preparation of cell-free extracts

*Thermoanaerobacterium saccharolyticum* cells were grown in CTFUD medium in an anaerobic chamber (COY labs, Grass Lake, MI, USA), and harvested in the exponential phase of growth at OD between 0.6 and 0.8. To prepare cell-free extracts, cells were collected by centrifugation at 6,000×g for 15 min and washed twice under similar conditions with a deoxygenated buffer containing 100 mM Tris–HCl (pH 7.5) and 5 mM dithiothreitol (DTT). Cells from 50 mL culture were resuspended in 3 mL of the washing buffer. Resuspended cells were lysed by adding 10 μL of 1:100 diluted Ready-Lyse lysozyme solution (Epicentre, Madison, WI, USA) and 2 μL of DNase I solution (Thermo scientific, Waltham, MA, USA) and then incubated at room temperature for 20 min. The concentration of Ready-Lyse lysozyme solution varies from 20 to 40 KU/μL and the DNase I solution is 25 U/μL. The crude lysate was centrifuged at 12,000×g for 5 min and the supernatant was collected as cell-free extract. The total amount of protein in the extract was determined by Bradford assay [31], using bovine serum albumin as the standard.

Enzymes assays

Enzyme activity was assayed in an anaerobic chamber (COY labs, Grass Lake, MI, USA) using an Agilent 8453 spectrophotometer with Peltier temperature control module (part number 89090A) to maintain assay temperature. The reaction volume was 1 mL, in reduced-volume quartz cuvettes (part number 29MES10; Precision Cells Inc., NY, USA) with a 1.0 cm path length. The units for all enzyme activities are expressed as μmol of product · min⁻¹ (mg of cell extract protein)⁻¹. For each enzyme assay, at least two concentrations of cell extract were used to confirm that the specific activity was proportional to the amount of extract added.

All chemicals and coupling enzymes were purchased from Sigma except for coenzyme A, which was purchased from EMD Millipore (Billerica, MA, USA). All chemical solutions were prepared fresh weekly.

Pyruvate ferredoxin oxidoreductase was assayed by the reduction of methyl viologen, which was monitored at 578 nm, at 55 °C with minor modifications as described before [32]. An extinction coefficient of ξ₅₇₈ = 9.7/mM/cm was used for calculating the activity. The assay mixture contained 100 mM Tris–HCl (pH = 7.5), 5 mM DTT, 2 mM MgCl₂, 0.4 mM coenzyme A, 0.4 mM thiamine pyrophosphate, 1 mM methyl viologen, cell extract and approximately 0.25 mM sodium dithionite (added until faint blue, A₅₇₈ = 0.05–0.15). The reaction was started by adding 10 mM sodium pyruvate. Activities were expressed as acetyl-CoA production rate.

Adaptation experiment

Inside the anaerobic chamber, strains were inoculated into polystyrene tubes (Corning, Tewksbury, MA, USA), containing 10 mL MTC-6 medium. The growth of cells in culture was determined by measuring OD₆₀₀. 200 μL of cultures was transferred into tubes with 10 mL fresh medium at the exponential phase of growth as indicated by OD₆₀₀mm = 0.3.
RNA isolation, RT-PCR and qPCR for determining transcriptional expression level

3 mL of bacterial culture was pelleted and lysed by digestion with lysozyme (15 mg/mL) and proteinase K (20 mg/mL). RNA was isolated with an RNeasy minikit (Qiagen Inc., Germantown, MD, USA) and digested with TURBO DNase (Life Technologies, Grand Island, NY, USA) to remove contaminating DNA. cDNA was synthesized from 500 ng of RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Quantitative PCR (qPCR) was performed using cDNA with SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA) at an annealing temperature of 55 °C to determine expression levels of Tsac_0046, Tsac_0628 and Tsac_0629. In each case, expression was normalized to recA RNA levels. To confirm removal of contaminating DNA from RNA samples, cDNA was synthesized in the presence and absence of reverse transcriptase followed by qPCR using recA primers to ensure only background levels were detected in the samples lacking reverse transcriptase. Standard curves were generated using a synthetic DNA template (gBlock, IDT, Coralville, IA, USA) containing the amplicons. Primers used for qPCR are listed in Table 6.

Genomic sequencing

Genomic DNA was submitted to the Joint Genome Institute (JGI) for sequencing with an Illumina MiSeq instrument. Paired-end reads were generated, with an average read length of 150 bp and paired distance of 500 bp. Raw data were analyzed using CLC Genomics Workbench, version 7.5 (Qiagen, USA). First reads were mapped to the reference genome (NC_017992). Mapping was improved by two rounds of local realignment. The CLC Probabilistic Variant Detection algorithm was used to determine small mutations (single and multiple nucleotide polymorphisms, short insertions and short deletions). Variants occurring in less than 90 % of the reads and variants that were identical to those of the wild-type strain (i.e., due to errors in the reference sequence) were filtered out. The fraction of the reads containing the mutation is presented in Additional file 3: Table S1.

To determine larger mutations, the CLC InDel and Structural Variant algorithm was run. This tool analyzes unaligned ends of reads and annotates regions where a structural variation may have occurred, which are called breakpoints. Since the read length averaged 150 bp and the minimum mapping fraction was 0.5, a breakpoint can have up to 75 bp of sequence data. The resulting breakpoints were filtered to eliminate those with fewer than ten reads or less than 20 % “not perfectly matched.” The breakpoint sequence was searched with the Basic Local Alignment Search Tool (BLAST) algorithm [33] for similarity to known sequences. Pairs of matching left and right breakpoints were considered evidence for structural variations such as transposon insertions and gene deletions. The fraction of the reads supporting the mutation (left and right breakpoints averaged) is presented in Additional file 3: Table S1.

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 Biosystem’s 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.

Analytical techniques

Fermentation products: cellobiose, glucose, acetate, lactate, formate, pyruvate, succinate, malate and ethanol were analyzed by a Waters (Milford, MA) high-pressure liquid chromatography (HPLC) system with an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA). The column was eluted at 60 °C with 0.25 g/L H2SO4 at a flow rate of 0.6 mL/min. Cellobiose, glucose, acetate, lactate, formate, succinate, malate and ethanol were detected by a Waters 410 refractive-index detector and nitrogen was detected by a Waters 2487 UV detector. Sample collection and processing were as reported previously [34].

Carbon from cell pellets was determined by elemental analysis with a TOC-V CPH and TNM-I analyzer (Shimadzu, Kyoto, Japan) operated by TOC-Control V software. Fermentation samples were prepared as described with small modifications [35]. A 1 mL sample was centrifuged to remove the supernatant at 21,130g for 5 min at room temperature. The cell pellet was washed twice with MilliQ water. After washing, the pellet was resuspended in a TOCN 25 mL glass vial containing 19.5 mL MilliQ water. The vials were then analyzed by the TOC-V CPH and TNM-I analyzer.

Hydrogen was determined by gas chromatography using a Model 310 SRI Instruments (Torrence, CA, USA) gas chromatograph with a HayeSep D packed column using a thermal conductivity detector and nitrogen carrier gas. The nitrogen flow rate was 8.2 mL/min.

Carbon balances were determined according to the following equations, accounting for carbon dioxide and
formate through the stoichiometric relationship of its production to levels of acetate, ethanol, malate and succinate [25]. The overall carbon balance is as follows:

\[
C_t = 12\text{CB} + 6\text{G} + 3\text{L} + 3\text{A} + 3\text{E} + 3\text{P} + 3\text{M} + 3\text{S} + 1\text{Pe},
\]

where \(C_t\) is the total carbon, CB the cellobiose, G the glucose, L the lactate, E the ethanol, P the pyruvate, M the malate, S the succinate, Pe the pellet and

\[
C_R = \frac{C_{tf}}{C_{t0}} \times 100\%,
\]

where \(C_R\) is the carbon recovery, \(C_{t0}\) the total carbon at the initial time, and \(C_{tf}\) the total carbon at the final time. Electron recoveries were calculated in a similar way, with the following numbers of available electrons per mole of compound: per mole 48 for cellobiose, 24 for glucose, 8 for acetate, 12 for ethanol, 12 for lactate, 14 for succinate, 10 for pyruvate, 12 for malate, 2 for hydrogen and 2 for formate. The electrons contained in the cell pellet was estimated with a general empirical formula for cell composition (CH\(_2\)\(_N\)O\(_{2.5}\)O\(_{0.3}\)); therefore, the available electrons per mole cell carbon was assumed to be 4.75 per mole. The calculation follows the equations below:

\[
E_t = 48\text{CB} + 24\text{G} + 12\text{L} + 8\text{A} + 12\text{E} + 14\text{S} + 10\text{P} + 12\text{M} + 2\text{H} + 2\text{F} + 4.75\text{Pe},
\]

\[
E_R = \frac{E_{tf}}{E_{t0}} \times 100\%,
\]

where \(E_t\) is the total electrons, \(E_R\) the electron recovery, F the formate and H the hydrogen; other abbreviations are the same as shown above.

**Additional files**

**Additional file 1:** Figure S1. Growth curves of \(\Delta\)pfor strains in MTC-6 medium with 4.5 g/L yeast extract. Black plus represent wild type strain (LL1025), black cyan circle represent \(\Delta\)pfor-1, green magenta cross represent \(\Delta\)pfor-2, blue diamond represent \(\Delta\)pfor-1, red star represent adapted \(\Delta\)pfor-2.

**Additional file 2:** Figure S2. Growth curves of \(\Delta\)pfl strains in MTC-6 medium with 4.5 g/L yeast extract. Circles represent growth curves of wild type (black), \(\Delta\)pfl-1 (red), \(\Delta\)pfl-2 (blue) in MTC-6 medium with 4.5 g/L yeast extract. Circles represent growth curves of wild type (black), \(\Delta\)pfl-1 (red), \(\Delta\)pfl-2 (blue) in MTC-6 medium without yeast extract.

**Additional file 3:** Table S1. Mutations found in genomic analysis.

**Abbreviations**

CBP: consolidated bioprocessing; PFOR: pyruvate ferredoxin oxidoreductase; PFL: pyruvate formate-lyase; PDH: pyruvate dehydrogenase; PDC: pyruvate decarboxylase; THF: tetrahydrofolic acid.

**Authors’ contributions**

JZ, DGO and LRL conceived the study; AAL performed the qPCR experiment; LT performed the PDH enzyme assay; JZ and JL performed the PFOR enzyme assay; SL-JM performed the fermentation experiments; JZ, DGO and LT performed the genomic sequence analysis; JZ carried out all the molecular genetic studies and all other analyses. JZ drafted the manuscript together with DGO, AAL and LRL, who also supervised the work. All authors read and approved the final manuscript.

**Author details**

1 Thayer School of Engineering, Hanover, NH 03755, USA. 2 Department of Biological Sciences at Dartmouth College, Hanover, NH 03755, USA. 3 BioEnergy Science Center, Oak Ridge, TN 37830, USA.

**Acknowledgements**

We would like to thank the Mascoma Corporation for giving us the strain LL1049 (aka M1442) as well as preliminary sequencing data of that strain. We thank Marybeth I. Maloney for preparing genomic DNA for resequencing and Dr. Johannes P. van Dijken for providing valuable suggestions and comments on the manuscript. The BioEnergy Science Center is a U.S. Department of Energy Bioenergy Research Center supported by the Office of Biological and Environmental Research in the DOE Office of Science. The genomic resequencing work conducted by the U.S. Department of Energy Joint Genome Institute, a DOE Office of Science User Facility, is supported by the Office of Science of the U.S. Department of Energy under Contract No. DE-AC02-05CH11231. Notice: This manuscript has been authored by Dartmouth College under Subcontract No. 4000115284 and Contract No. DE-AC05-00OR22725 with U.S. Department of Energy. The US Government retains and the publisher, by accepting the article for publication, acknowledges that the US Government retains a non-exclusive, paid-up, irrevocable worldwide license to publish or reproduce the published form of this manuscript or allow others to do so, for US Government purposes. (End of Notice)

**Compliance with ethical guidelines**

**Competing interests**

LRL is affiliated with Mascoma Corporation, which partly funded this research.

Received: 9 April 2015   Accepted: 3 August 2015

Published online: 15 September 2015

**References**

1. Lee Y-E, Jain MK, Lee C, Zelus JG (1993) Taxonomic distinction of saccharolytic thermophilic anaerobes: description of Thermoanaerobacterium xylointolyticum gen. nov, sp. nov, and Thermoanaerobacterium saccharolyticum gen. nov, sp. nov; reclassification of Thermoanaerobium brockii, Clostridium thermosulfurogenes, and Clostridium thermohydrosulfuricum E100-69 as Thermoanaerobacter brockii comb. nov, Thermoanaerobacter thermosulfurogenes comb. nov, and Thermoanaerobacter thermohydrosulfuricus comb. nov, respectively; and Transfer of Clostridium thermohydrosulfuricum 39E to Thermoanaerobacter ethanolicus. Int J Syst Bacteriol 43:41–51
2. Shaw AJ, Podkaminer K, Desai S, Bardstyle J, Rogers S, Thorne P et al (2008) Metabolic engineering of a thermophilic bacterium to produce ethanol at high yield. Proc Natl Acad Sci USA 105:13769–13774
3. Shaw AJ, Covalla SF, Miller BB, Frielik BT, Hoggett DA,Henning CD (2012) Urease expression in a Thermoanaerobacteriacum saccharolyticum ethanologen allows high titer ethanol production. Metab Eng 14:528–532
4. Henning CD, Kenealy WR, Shaw AJ, Raman B, Tschaplinski TJ, Brown SD et al (2012) Final report on development of Thermoanaerobacterium saccharolyticum for the conversion of lignocellulose to ethanol. Golden, CO (United States)
5. Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS (2002) Microbial cellulose utilization: fundamentals and biotechnology. Microbiol Mol Biol Rev 66:506–577
6. Lynd LR, van Zyl WH, McBride JE, Laser M (2005) Consolidated bioprocessing of cellulosic biomass: an update. Curr Opin Biotechnol 16:577–583
7. Olson DG, Sparling R, Lynd LR (2015) Ethanol production by engineered thermophiles. Curr Opin Biotechnol 33:103–141
8. Lo J, Zheng T, Hon S, Olson DG, Lynd LR (2015) The bifunctional alcohol and aldehyde dehydrogenase gene, addH, is necessary for ethanol production in Clostridium thermocellum and Thermoanaerobacterium saccharolyticum. J Bacteriol 197:J.B.02450–14
