The pyruvate:ferredoxin oxidoreductase of the thermophilic acetogen, *Thermoanaerobacter kivui*

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Pyruvate:ferredoxin oxidoreductase (PFOR) is a key enzyme in bacterial anaerobic metabolism. Since a low-potential ferredoxin (Fd2−) is used as electron carrier, PFOR allows for hydrogen evolution during heterotrophic growth as well as pyruvate synthesis during lithoautotrophic growth. The thermophilic acetogenic model bacterium *Thermoanaerobacter kivui* can use both modes of lifestyle, but the nature of the PFOR in this organism was previously unestablished. Here, we have isolated PFOR to apparent homogeneity from cells grown on glucose. Peptide mass fingerprinting revealed that it is encoded by *pfor1*. PFOR uses pyruvate as an electron donor and methylene blue (1.8 U/mg) and ferredoxin (Fd; 27.2 U/mg) as electron acceptors, and the reaction is dependent on thiamine pyrophosphate, pyruvate, coenzyme A, and Fd. The pH and temperature optima were 7.5 and 66°C, respectively. We detected 13.6 mol of iron/mol of protein, consistent with the presence of three predicted [4Fe–4S] clusters. The ability to provide reduced Fd makes PFOR an interesting auxiliary enzyme for enzyme assays. To simplify and speed up the purification procedure, we established a protocol for homologous protein production in *T. kivui*. Therefore, *pfor1* was cloned and expressed in *T. kivui* and the encoded protein containing a genetically engineered His-tag was purified in only two steps to apparent homogeneity. The homologously produced PFOR1 had the same properties as the enzyme from *T. kivui*. The enzyme can be used as auxiliary enzyme in enzymatic assays that require reduced Fd as electron donor, such as electron-bifurcating enzymes, to keep a constant level of reduced Fd.

The Wood–Ljungdahl pathway (WLP) was probably the first CO2 fixation pathway on Earth [1] and is still employed by strictly anaerobic microorganisms: acetogenic bacteria [2], methanogenic archaea [3], and sulfate-reducing bacteria and archaea [4]. In the WLP, 2 moles of CO2 are converted to the central metabolite acetyl-coenzyme A (acetyl-CoA) and acetogens metabolize this further to acetate [5,6]. Most acetogens can sustain a chemolithoautotrophic lifestyle by using molecular H2 and/or carbon monoxide (CO) as electron donors for fixing CO2 in the WLP to make acetate [7]. The net ATP gain of the WLP is zero; thus,

**Abbreviations**

[4Fe-4S], iron-sulfur cluster; CoA, coenzyme A; CODH/ACS, carbon monoxide dehydrogenase/acetyl-CoA synthase; Ech, energy-converting hydrogenase; Fd, ferredoxin; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; MB, methylene blue; PFOR, pyruvate:ferredoxin oxidoreductase; P-loop, phosphate-binding loop; Rnf, Rhodobacter nitrogen fixation; SEM, standard error of the mean; TCA, tricarboxylic acid; TPP, thiamine pyrophosphate; VIT, vacuolar iron transporter; WLP, Wood–Ljungdahl pathway.
they depend on a chemiosmotic gradient for energy conservation to sustain cellular homeostasis and fuel anaabolic processes [8]. The central switchpoint between catabolic and anabolic processes is acetyl-CoA. The two enzymes that can make this key metabolite are the bifunctional carbon monoxide dehydrogenase/acyetyl-CoA synthase (CODH/ACS), which units both branches of the WLP by fusing a methyl-group with enzyme-bound CO [9–13], and the pyruvate:ferredoxin oxidoreductase (PFOR), which can reduce and carboxylate acetyl-CoA to pyruvate [14]. The redox potentials of the CO/CO₂ and pyruvate/acyetyl-CoA redox pairs are, however, very low (E₀[pyruvate/acyetyl-CoA]/[CO/CO₂] = −500/−520 mV) [15,16], requiring an electron donor with an even lower potential. In both cases, a ferredoxin (Fd) takes on this role, requiring an electron donor with an even lower potential. In both cases, a ferredoxin (Fd) takes on this role, whose redox potential can be as low as ∼−450 to −500 mV [16]. The produced pyruvate is then further converted via the incomplete reductive tricarboxylic acid (TCA) cycle to give rise to many different building blocks [17–19].

Most acetogens can also sustain a heterotrophic lifestyle using sugars, organic acids, or alcohols as growth substrates [20], and under these circumstances, the PFOR’s role is to provide acetyl-CoA and low-potential Fd²⁻ from pyruvate. The Fd²⁻ can then be used by energy-converting hydrogenases (Ech) [21] or the Rnf complex to establish the chemiosmotic gradient [22], or provide electrons for a range of different soluble enzymes including Fe-hydrogenases [23] or electron-bifurcating enzyme complexes such as the lactate dehydrogenase (LDH/Etf) [24], the caffeyl-CoA reductase (CarCDE) [25], the NADH-dependent Fd²⁻:NADP⁺ oxidoreductase (Nfn, Stn) [26,27], several electron-bifurcating hydrogenases [28], and the butyryl-CoA-dehydrogenase (Bcd-EtfAB) [26].

The ability to provide reducing equivalents in the form of reduced Fd makes the PFOR an invaluable enzyme during in vitro studies of ferredoxin-dependent enzymes. So far, most enzymatic assays requiring Fd in the reduced state rely on strong chemical reducing agents such as sodium dithionite or titanium (III) citrate. However, these reducing agents often interfere with the physiological reactions by, for example, reducing the enzyme directly. An improvement to chemical reductants was achieved when a purification protocol was established for the CODH/ACS from Acetobacterium woodii, which can reduce Fd with CO as reductant [29]. However, CO is a potent inhibitor of many enzymes including most hydrogenases [30], and the enzyme is highly O₂-sensitive [11]. Therefore, we decided to identify, purify, and characterize a PFOR that can be used to provide Fd²⁻ in a physiological and nontoxic manner.

**Results**

**Identification of potential PFOR-encoding genes in Thermoanaerobacter kivui**

We used the acetogenic bacterium *T. kivui* as a model organism, a thermophilic organism that is able to sustain a lithotrophic and heterotrophic lifestyle [31]. Therefore, it must possess at least one PFOR, which functions reversibly and the enzyme should be thermostable, which is very advantageous for its potential application and storage. First, inspection of genomic data had indicated that a PFOR is encoded by the TKV_c19260-19290 cluster [31] which would translate to proteins with molecular masses of 20.2, 27.3, 39.1, or 7.7 kDa, respectively (Fig. 1A,B). These four genes are usually fused in bacteria [32] but may retain as four separate genes in ancient complexes still found in archaea [14,33] or hyperthermophilic bacteria [34,35].

A closer examination of the amino acid sequence revealed that the described cluster might correspond either to the four subunits of a 2-oxoglutarate:ferredoxin oxidoreductase (δβγ), that catalyzes the interconversion of 2-oxyglutarate and succinyl-CoA in the incomplete reductive TCA cycle [1] or indeed catalyze the oxidation of pyruvate to acetyl-CoA like in Thermotoga maritima [34,35]. Oxoglutarate:ferredoxin oxidoreductase has not been demonstrated in *T. kivui*, neither has the synthesis of oxoglutarate been studied. Searching for other PFOR-encoding genes revealed two candidate genes, *pfor1* (TKV_c04340) and *pfor2* (TKV_c21450; Fig. 1A). The amino acid sequences share 73% sequence identity with one another and the resulting protein products have predicted molecular masses of 129.8 (PFOR1) or 130.4 kDa (PFOR2; Fig. 1B).

Upstream of *pfor1* lies a small gene that encodes a vacuolar iron transporter (VIT) family protein with three transmembrane helices that shows similarities to ruberythrin. This protein is involved in an oxidative stress protection system in many anaerobes like the sulfate-reducing bacterium Desulfovibrio vulgaris [36,37]. Downstream of *pfor1* lies a putative phosphohydrolase (HDIG domain-containing protein). Upstream of *pfor2* is a small gene encoding a protein of unknown function and downstream is a gene encoding a putative threonine 3-dehydrogenase. Since the bioinformatic analyses did not allow a clear designation which of the two genes encode the PFOR, we attempted a purification of the PFOR based on its activity.
Identification of pyruvate:ferredoxin oxidoreductase activity in *T. kivui* and purification of the corresponding PFOR

To investigate whether *T. kivui* has PFOR activity, initially, an enzyme assay had to be established to monitor this activity. Besides ferredoxin (Fd), isolated from *Clostridium pasteurianum* [56], methylene blue (MB) proved to be a suitable electron acceptor for the reaction. This artificial one-electron acceptor has a $E^{0}$ of $+11 \text{ mV}$ and turns from blue in the oxidized form to colorless in the reduced state [38]. A pyruvate:MB oxidoreductase activity in an assay containing cell-free extract of glucose-grown cells could be observed with $20 \pm 3.2 \text{ mU} \cdot \text{mg}^{-1}$ (using 50 $\mu\text{M}$ MB), while PFOR activity was $553 \pm 26.9 \text{ mU} \cdot \text{mg}^{-1}$ with 30 $\mu\text{M}$ Fd as electron acceptor. Thus, the pyruvate:MB activity assay was used to screen for the presence of the PFOR, but further determination of the purification success and characterization of the enzyme were carried out using the physiological PFOR activity. To purify the PFOR, cell-free extract of *T. kivui*, grown on glucose to the late exponential growth phase, was prepared. The cell-free extract was separated into membranes and cytoplasm and the PFOR was purified from the cytoplasm by ion exchange chromatography on Q-Sepharose, hydrophobic interaction chromatography on Phenyl-Sepharose followed by a size exclusion chromatography on Superdex 200. Using this procedure, the enzyme was purified 50-fold to apparent homogeneity with an average specific PFOR activity of $27.2 \pm 4.1 \text{ U} \cdot \text{mg}^{-1}$ or pyruvate:MB oxidoreductase activity of $1.8 \pm 0.3 \text{ U} \cdot \text{mg}^{-1}$ and a yield of 0.8 mg (Table 1).

Analyses of the purified enzyme separated on a 12% SDS/PAGE revealed one protein with an apparent molecular mass of 130 kDa (Fig. 2). Using peptide mass fingerprinting, this protein could be identified as the gene product of *pfcr1* (TKV_c04340). Analytical size exclusion chromatography revealed a molecular mass of 245 kDa for the purified complex, which is consistent with PFOR1 being a homodimer.

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*Fig. 1.* Genetic organization, architecture, and cofactors of possible PFORs of *Thermoanaerobacter kivui*. The genome of *T. kivui* encodes for three different *pfcr* clusters (A). Regardless of gene arrangements, PFORs maintain a basic composition of domains I, II, III, and VI, with domain V also present in most PFORs. Domain IV is present only in dimeric PFORs. Cartoons of the domain arrangement for the catalytic units of possible PFORs in *T. kivui* are shown (B). Domains are indicated with colored boxes, with their respective domain numbers inside. Black bars connecting domains indicate that the domains are found on the same polypeptide chain. The domains that bind TPP and [4Fe–4S] cluster are indicated at the top of each domain. VIT; vacuolar iron transporter.
Biochemical characterization of PFOR1

First, we assessed key biochemical properties of the purified PFOR1, including temperature and pH stability, substrate affinities, and cofactor dependence. To ensure an ideal reflection of the physiological conditions, we exclusively used the PFOR assay. The purified PFOR1 reduced Fd with pyruvate as reductant with an average specific activity of $27.2 \pm 4.1$ U$\cdot$mg$^{-1}$ (Fig. 3). PFOR1 was active at temperatures ranging from 22 to 80°C with a maximal activity of $24.3 \pm 1.1$ U$\cdot$mg$^{-1}$ at the optimal growth temperature of $T$. kivui (66°C) (Fig. 4A). The PFOR1 was not only active at mesophilic and thermophilic conditions but also extremely stable, with 70% activity remaining after 172 days of storage at 4°C. The pH range was relatively narrow with only 20% activity at pH 6 and 8 and an optimal activity of $26.9 \pm 0.4$ at pH 7.5 (Fig. 4B). All further analyses were subsequently carried out at pH 7.5 and 66°C to ensure optimal enzyme activity.

Next, we assessed the $K_m$ values for all reaction partners of the PFOR1. Since the enzyme was purified from cells grown on glucose, the physiological direction of the enzyme is to oxidize pyruvate to acetyl-CoA, which is then further converted to acetate at the gain of ATP from substrate-level phosphorylation. As mentioned, this PFOR activity of PFOR1 was $27.2 \pm 4.1$ U$\cdot$mg$^{-1}$. The dependence of the reaction on Fd, pyruvate, and CoA was hyperbolic with saturation at 10 mM pyruvate, 50 µM Fd, and 200 µM CoA (Fig. S1). The $K_m$ values for pyruvate, Fd, and CoA were $0.11 \pm 0.02$ mM, $19.1 \pm 3.4$ µM, and $25.4 \pm 4.7$ µM, respectively (Fig. S1A–C). Unsurprisingly, the absence of any reaction partner led to a complete loss of activity.

Cofactor determination of PFOR1

From bioinformatic analyses, it was inferred that PFOR1 should contain three iron–sulfur clusters.

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**Table 1.** Purification of PFOR1 from *Thermoanaerobacter kivui*.

| Purification step  | Protein (mg) | PFOR activitya (U) | PFOR activity (U$\cdot$mL$^{-1}$) | PFOR activity (U$\cdot$mg$^{-1}$) | Purification (-fold) | Yield (%) |
|-------------------|--------------|--------------------|----------------------------------|---------------------------------|---------------------|----------|
| Cell-free extract | 1152         | 645                | 13.4                             | 0.54                            | 1                   | 100      |
| Cytoplasm         | 1036         | 577                | 12.8                             | 0.56                            | 1                   | 89.5     |
| Q-Sepharose       | 99           | 212                | 8.5                              | 2.1                             | 3.9                 | 32.9     |
| Phenyl-Sepharose  | 6.4          | 49                 | 4                                | 7.6                             | 14.1                | 7.6      |
| Superdex 200      | 0.8          | 23                 | 45.8                             | 27.2                            | 50.4                | 3.6      |

*aPFOR activity was measured with pyruvate as electron donor and ferredoxin as electron acceptor*
used by several PFOR enzymes [33,40], because TPP acts as a potent nucleophile that forms an adduct with pyruvate and enzyme-bound [4Fe–4S] clusters deliver electrons into or out of the active site [41]. A binding site for TPP was also detected in our in silico analyses for PFOR1 (Fig. 1B). And indeed, purified PFOR1 exhibited only a residual activity of 1.1 ± 0.2 U·mg⁻¹ when TPP was omitted from the enzyme assay as opposed to 27.2 ± 4.1 U·mg⁻¹ with 200 µM TPP in the assay. The dependence of the reaction on TPP was hyperbolic, reaching a saturation at 200 µM TPP, and the K_m value was 287.2 ± 0.1 nm (Fig. S1D).

Rapid and simple production of genetically modified PFOR1 in *T. kivui*

To increase the yield and simplify the purification of PFOR1, we took advantage of a plasmid, pMU131, which is replicating in *T. kivui* [42]. The plasmid has already been used for gene expression in *T. kivui* to complement growth phenotypes. Among others, *T. kivui* phosphofructokinase *fruK* in a ΔfruK background proving the involvement of *fruK* in fructose metabolism [42]. Here, we aimed for overproduction of PFOR1. Therefore, we cloned the *pfor1* gene (TKV_c04340) together with a gene sequence coding for a 10x histidine-tag into a plasmid containing the S-layer promoter of *T. kivui* (Fig. 5). Naturally competent cells of *T. kivui* were transformed with the verified plasmid (Fig. S2) and cell-free extract of the genetically modified *T. kivui* strain was prepared as

![Absorbance vs Time](image)

**Fig. 3.** Pyruvate-oxidizing activity of the purified PFOR1. Enzymatic activity was measured in 1.8-mL anoxic cuvettes containing an overall liquid volume of 1 mL. The assay contained 5 µg PFOR, 200 µM CoA, and 50 µM TPP in buffer (50 mM Tris(HCl), 10 mM NaCl, 2 mM DTE, 4 µM resazurin, pH 7.5) under a 100% N₂ atmosphere at 66 °C. 30 µM Fd served as electron acceptor. Reduction of Fd was measured at 430 nm.

![Temperature vs pH](image)

**Fig. 4.** pH optimum and temperature profile of purified PFOR1. Temperature (A) or pH (B) dependence of the pyruvate-dependent Fd reduction was measured in 1.8-mL anoxic cuvettes containing an overall liquid volume of 1 mL under a 100% N₂ atmosphere at 20–80 °C (A) or 66 °C (B). The assay contained 1 mL of buffer A (50 mM Tris/HCl, 10 mM NaCl, 2 mM DTE, 4 µM resazurin, pH 7.5) or buffer B (50 mM Tris, 50 mM MES, 50 mM CHES, 50 mM CAPS, 50 mM Bis/Tris, 10 mM NaCl, 2 mM DTE, 4 µM Resazurin, pH 5–10), 5 µg PFOR, 200 µM CoA, 50 µM TPP, 30 µM Fd and 10 mM pyruvate. Shown is the average of two measurements from one representative experiment out of two independent replicates. Error bars represent the SEM.
described for the wild type. Undisrupted cells were removed by centrifugation and the His-tagged PFOR1 was purified from the cell-free extract on Ni²⁺-NTA-Sepharose followed by a size exclusion chromatography on Superdex 200. This procedure yielded an apparently homogeneous preparation with just two purification steps (Fig. 6). 5.4 mg of the enzyme was purified 36-fold to apparent homogeneity out of a 1 l culture (Table 2). The purified tagged version exhibited almost similar average specific activity of 21.8 ± 2.3 U·mg⁻¹ as the untagged version. Therefore, the tag did not interfere with PFOR1 activity. Using this protocol, it was possible to obtain fully functional PFOR1 with a 135 times higher yield in one rather than 4 days.

**Discussion**

In this work, we discovered, purified, and characterized a very stable PFOR from the anaerobic acetogenic bacterium *T. kivui*. Moreover, we developed an improved and simple purification protocol for PFOR, based on homologous overproduction in a strict anaerobe, a method that may be transferred to other strictly oxygen-sensitive proteins.

PFOR1 is the enzyme in *T. kivui* that provides acetyl-CoA and reduced Fd from pyruvate during heterotrophic growth. Under heterotrophic conditions, the two electrons that are generated as a result of pyruvate decarboxylation are used in the reduction of low-potential ferredoxins (Fd²⁻), which are used to fuel the chemiosmotic gradient by the respiratory Ech-complex [21] and the reduction of CO₂ to protein-bound CO by the CODH/ACS [43] in the WLP.
Cell-free extract responsible for CoA binding [41]. The same P-loop for (MtOOR) revealed that residues of domain without TPP supplementation. in assays containing enriched PFOR1 from explains why activity was still present (although lower) cules [32]. The burial of TPP in the dimerized PFOR tramic core that binds two thiamine diphosphate mole-
pyrophosphate-binding module. Two of a kind can assemble, giving rise to another protein subunit. In the mid-
le of the protein is the domain III (gamma domain). Recently, a crystal structure of a PFOR from M. ther-
moacetica (MtOOR) [41] or Desulvobrio africanus (DaOOR) [46], respectively. The N-terminal region comprises a pyruvate flavodoxin/ferredoxin oxidoredu-
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lowed by a second domain II, which might be involved in interaction with another protein subunit. In the mid-
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moacetica (MtOOR) revealed that residues of domain III form a phosphate-binding loop (P-loop) that is responsi-
ble for CoA binding [41]. The same P-loop for CoA binding is conserved in PFOR1 of T. kivui. Next, a 33-residue-long conserved domain containing an EKR sequence motif (domain IV) follows. It has a hitherto unknown function and links the PFOR1 domain to a following Fe-S-cluster binding domain (domain V) with a C-X2-C-X2-C-X3-C binding motif. Finally, at the C terminus is a TPP- and pyruvate-binding domain (do-
main VI). In PFOR1, the N- and C-terminal region con-
tains a thiamine diphosphate-binding fold, comprising two functional modules: the pyridine-binding and pyrophosphate-binding module. Two of a kind can assemble, giving rise to a homodimer with a heterote-
tramic core that binds two thiamine diphosphate mole-
cules [32]. The burial of TPP in the dimerized PFOR explains why activity was still present (although lower) in assays containing enriched PFOR1 from T. kivui without TPP suplementation.

Besides PFOR1, the genome of T. kivui encodes for a 73% identical second PFOR with an exact similar six-
domain arrangement (PFOR2; Fig. 1B). Surprisingly, after the purification from glucose-grown cells, there were no indications that PFOR2 was copurified. This could be explained by an absence of the protein due to no or very low gene expression, or a different activity of this enzyme. Indeed, the occurrence of multiple PFOR-
encoding genes is also found in several other organisms like Thermoanaerobacter saccharolyticum [47], M. thermoacetica [41,48], or Pyrococcus furiosus [33,49]. While pfor1 in T. kivui encodes the catalytic PFOR1, the other presumably encodes a similar enzyme with another substrate specificity (possibly to another α-ketoacid). Alternatively, it might also convert pyruvate, but use an alternate electron acceptor. Good candidates for this could be flavodoxin, rubredoxin, or thioredoxin [50], which may substitute the role of Fd under iron deprivation. After all, not much is known about these electron carriers in acetogens.

In silico analysis using ‘InterPro’ [44] revealed that PFOR1 belongs to the 2-oxoacid:ferredoxin oxidore-
ductase (OFOR) superfamily and has a typical six-do-
main arrangement (Fig. 1B) [45]. PFOR1 is 65% or 61% identical to well-studied PFORs of Moorella ther-
moacetica (MtOOR) [41] or Desulvobrio africanus (DaOOR) [46], respectively. The N-terminal region comprises a pyruvate flavodoxin/ferredoxin oxidoreductase (domain I) that is involved in TPP-binding, fol-
lowed by a second domain II, which might be involved in interaction with another protein subunit. In the mid-
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encoding genes is also found in several other organisms like Thermoanaerobacter saccharolyticum [47], M. thermoacetica [41,48], or Pyrococcus furiosus [33,49]. While pfor1 in T. kivui encodes the catalytic PFOR1, the other presumably encodes a similar enzyme with another substrate specificity (possibly to another α-ketoacid). Alternatively, it might also convert pyruvate, but use an alternate electron acceptor. Good candidates for this could be flavodoxin, rubredoxin, or thioredoxin [50], which may substitute the role of Fd under iron deprivation. After all, not much is known about these electron carriers in acetogens.

In this work, we established a simple and rapid purification method for PFOR1 from T. kivui that can be used as ‘supporting-enzyme’ for biochemical analy-
sis in the future. The ability to reduce Fd (even from C. pasteurianum) at moderate pH values, and mesophilic and thermophilic conditions makes the PFOR1 of T. kivui an invaluable enzyme during in vitro studies. Moreover, the enzyme is stable for a long time. Reduced Fd is not only an electron donor for many electron-bifurcating enzymes [51], but is also required for the acetylogenic respiratory enzymes, like Ech complexes [21] or Rnf complexes [22]. PFOR1 of T. kivui catalyzes the oxidation of pyruvate to acetyl-CoA and CO2 (E0' = -500 mV) [15,16] coupled to the reduction of Fd (E0[Fd2+/Fd] = -450 to -500 mV) [16] as electron donor. Since this system does not involve strong chemical reducing agents such as sodium dithionite or titanium (III) citrate, which often interfere with the physiological reaction of several enzymes [21,22,52], it is an ideal way to provide reduced Fd for biochemical assays in a physiological and nontoxic manner.

### Methods

#### Growth of cells and purification of the PFOR

*T. kivui* (DSM 2030) was grown at 66 °C under anoxic conditions in 20-L bottles (Glaserätebau Ochs, Bovenden-Lenglern, Germany) using 28 mM d-glucose as substrate. The medium and all buffers were prepared using the anaerobic techniques described previously [31,53,54]. All buffers used for preparation of cell extracts and purification contained 2 mM DTE, 4 μM resazurin, and 20% (v/v) glycerol. All purification steps were performed under strictly anaerobic conditions at room temperature in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI, USA) filled with 95–98% N2 and 2–5% H2. Cells of *T. kivui* were har-
vested and washed twice in buffer A (50 mM Tris/HCl, 2 mM DTE, 4 μM resazurin, 20% (v/v) glycerol, pH 8.0). The cells were resuspended in 50 mL buffer A including 0.5 mM

| Purification | Protein yield | PFOR activity | PFOR activity | Purification fold | Yield (%) |
|--------------|---------------|---------------|---------------|------------------|-----------|
| Cell-free extract | 193 (mg) | 120.2 (U) | 0.59 (U·mg⁻¹) | 1 | 100 |
| Ni²⁺-NTA | 6.5 | 114 | 17.5 | 30 | 94.8 |
| Superdex 200 | 5.4 | 112.9 | 21.1 | 35.8 | 93.9 |

*PFOR activity was measured with pyruvate as electron donor and ferredoxin as electron acceptor.*
Chalfont, UK) equilibrated with buffer E (50 mM Tris/HCl, 1 M NaCl, 20 mM MgSO₄, 2 mM DTE, 4 µM resazurin, 20% (v/v) glycerol, pH 8.0). PFOR activity eluted at around 3–13 mM NaCl or conductivity of 3.8–8.4 mS⋅cm⁻¹. Ammonium sulfate (1 M) was added to the pooled fractions and these were loaded onto a Phenyl-Sepharose high-performance column (1.6 cm × 10 cm, 27 mL Phenyl-Sepharose) equilibrated with buffer C (50 mM Tris/His, 20 mM MgSO₄, 1 M (NH₄)₂SO₄, 2 mM DTE, 4 µM resazurin, 20% (v/v) glycerol, pH 7.5). Protein was eluted with a linear gradient of 170 mL from 1 to 0 M (NH₄)₂SO₄ in buffer D (50 mM Tris/His, 20 mM MgSO₄, 2 mM DTE, 4 µM resazurin, 20% (v/v) glycerol, pH 7.5). PFOR activity eluted in a peak around 0.84–0.68 M (NH₄)₂SO₄ or conductivity of 64–55 mS⋅cm⁻¹. Pooled fractions were concentrated using ultrafiltration in 50-kDa VIVASPIN tubes (Sartorius Stedim Biotech GmbH, Germany). The sample was loaded on a Superdex 200 increase 10/300 GL (GE Healthcare Life Sciences, Little Chalfont, UK) equilibrated with buffer E (50 mM Tris/His, 150 mM NaCl, 20 mM MgSO₄, 2 mM DTE, 4 µM resazurin, 20% (v/v) glycerol, pH 7.5) and eluted at a flow rate of 0.5 mL⋅min⁻¹. PFOR activity eluted in a single peak with a maximum at 12.5 mL elution volume. Fractions corresponding to this peak were pooled and stored in buffer E at 4 °C.

Cloning of pMU131_pfor1-His

Plasmid pMU131_pfor1-His was used for the expression of pfor1 (TKV_e04340). The plasmid is based on plasmid pMU131 [55] which is replicating in T. kivui and confers resistance to kanamycin [42]. The insert was amplified by using the primers PFOR1_His_for (5′-CAAGGGAGAGTGACTGTAAGTAACTAAGG-3′) and PFOR1_His_rev (5′-TCC TGGATAATATTAAAAAATATTAAATGATGATTGATTTG-3′). The backbone pMU131 was amplified by using the primers pMU131_for (5′-TTTATTAAATTTATCATGATAAAAGAGAAGAC-3′) and pMU131_rev (5′-ACA GTCTAATCCCTCGTG-3′), followed by the fusion of the PCR products via Gibson Assembly. T. kivui (DSM 2030) was transformed with the generated plasmid pMU131_pfor1-His, taking advantage of its natural competence for DNA uptake [42]. Following the transformation protocol of Basen et al. [42], cells were plated on agar medium using 28 mM glucose as carbon source and 200 µg⋅mL⁻¹ kanamycin as selection marker. To verify the transformation, colonies were picked and the transformed plasmids were checked by using primer pairs seq1_for (5′-TCTAACAGACATTATCATTAAGTAGTGATA-3′)/seq2_rev (5′-AGTATTGTCTAATACTTGAAAGGCA-3′) binding on the pMU131 backbone and amplifying the complete pfor1 locus.

Production and purification of His-tagged PFOR1 in T. kivui

For the purification of the His-tagged PFOR1, T. kivui pMU131_pfor1-His cells were grown in the presence of 28 mM glucose and 200 µg⋅mL⁻¹ kanamycin. The preparation of cell-free extract was carried out as described previously, using a modified buffer A (50 mM Tris/HCl, 100 mM NaCl, 20 mM MgSO₄, 10 mM imidazole, 0.5 mM DTE, 4 µM resazurin, 20% (v/v) glycerol, pH 7.5). Protein purification was carried out on a nickel nitrioltriacetic acid (Ni²⁺-NTA) resin (Qiagen, Hilden, Germany) using a gravity flow column under anaerobic conditions. Cell-free extract was incubated with 1 mL resin for 10 min at room temperature. Afterward, the resin was washed with buffer F (50 mM Tris/His, 150 mM NaCl, 20 mM MgSO₄, 30 mM imidazole, 0.5 mM DTE, 4 µM resazurin, 20% (v/v) glycerol, pH 7.5) to remove loosely bound proteins from the resin. Subsequently, specifically bound proteins were eluted by adding 400 mM imidazole-containing elution buffer G (50 mM Tris/His, 150 mM NaCl, 20 mM MgSO₄, 400 mM imidazole, 0.5 mM DTE, 4 µM resazurin, 20% (v/v) glycerol, pH 7.5). One milliliter fractions were collected, pooled, concentrated, using 50-kDa VIVASPIN tubes, and separated on a Superdex 200 increase 10/300 GL (GE Healthcare Life Sciences) as described above. Fractions containing PFOR1-His were pooled and stored at 4 °C.

Measurement of PFOR enzyme activity

Enzyme assays were routinely performed at 66 °C in 1.8-mL anaerobic cuvettes (Glasgeratebau Ohcs, Bovenden-Lenngern, Germany) sealed by rubber stoppers in a N₂ atmosphere with buffer H (50 mM Tris/His, 10 mM NaCl, 4 mM DTE, 4 µM resazurin, pH 7.5) at an overall liquid volume of 1 mL. PFOR activity was measured with MB or Fd as electron acceptor and measured at 665 nm (ε = 53.1 mM⁻¹⋅cm⁻¹) or 430 nm (ε = 13.1 mM⁻¹⋅cm⁻¹), respectively. Fd was purified from C. pasterianum as described previously [56]. The assay was supplemented with cell-free extract, cytoplasm or enriched PFOR preparations, 50 µM MB or 30 µM Fd, 200 µM CoA, and 100 µM TPP. The reaction was started by addition of 10 mM sodium pyruvate. For Kₘ determination, the CoA, pyruvate, TPP and Fd concentrations ranged between 0–200 µM, 0–10 mM, 0–20 µM, and 0–50 µM, respectively. For the determination of the pH and temperature profile, the assay and protein were preincubated for 10 min at the pH or
temperature indicated. The buffer used for the pH optima determination was 50 mM MES, 50 mM CHES, 50 mM CAPS, 50 mM Bis/Tris, 50 mM Tris, 10 mM NaCl, 4 mM DTE, 4 μM resazurin at pH 5–10.

Analytical methods
The concentration of proteins was measured according to Bradford [57]. Proteins were separated in 12% SDS/PAGE and stained with Coomassie Brilliant Blue G250. The molecular mass of the purified PFOR was determined using a calibrated Superdex 200 column, buffer E, and defined size standards (ovalbumin: 43 kDa; albumin: 158 kDa; catalase: 232 kDa; ferritin: 440 kDa). The isolated PFOR was identified by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) analysis. Peptide mass fingerprinting by MALDI-TOF analysis was performed by the ‘Functional Genomics Center Zürich’ at the ETH Zurich, Switzerland, and results were analyzed using the Scaffold-Proteome Software version 4.10.0 (Proteome Software Inc., Portland, OR, USA). The iron content of the purified enzyme was determined by colorimetric methods [39]. Flavin determination was performed by TLC as described before [25].

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Conflict of interest
The authors declare no conflict of interest.

Data accessibility
The data that support the findings of this study are available from the corresponding author (vmueller@bio.uni-frankfurt.de) upon reasonable request.

Author contributions
VM, AK, MCS, and MB designed the experiments. AK and MCS performed the experiments. AK, MCS, MB, and VM wrote the paper.

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**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1.** Ferredoxin, pyruvate, CoA and TTP dependence on PFOR1 activity. PFOR activity was measured in 1.8-mL anoxic cuvettes containing an overall liquid volume of 1 mL under a 100% N2 atmosphere at 66 °C. The assay contained 1 mL of buffer A (50 mM Tris/HCl, 10 mM NaCl, 2 mM DTE, 4 µM resazurin, pH 7.5), 5 µl PFOR, different amounts of pyruvate (A), Fd (B), CoA (C), or TPP (D). Shown is the average of two measurements from one representative experiment out of two independent replicates. Error bars represent the SEM.

**Fig. S2.** Verification of the *pMU131_pfor1-His* construct transformed in *Thermoanaerobacter kivui*. To verify the nature of the plasmid *pMU131_pfor1-His* after propagation, *T. kivui* colonies were picked and the plasmid was checked by using primer pairs seq1-for (5′)/seq2_rev (6) binding on the *pMU131* backbone and amplifying the complete *pfor1-His* (A). The resulting size was 4054 (B). M, Gene Ruler 1 kb DNA ladder.