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Pyruvate Formate-Lyase Enables Efficient Growth of *Escherichia coli* on Acetate and Formate

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Supporting Information

ABSTRACT: Pyruvate formate-lyase (PFL) is a ubiquitous enzyme that supports increased ATP yield during sugar fermentation. While the PFL reaction is known to be reversible *in vitro*, the ability of PFL to support microbial growth by condensing acetyl-CoA and formate *in vivo* has never been directly tested. Here, we employ *Escherichia coli* mutant strains that cannot assimilate acetate via the glyoxylate shunt and use carbon labeling experiments to unequivocally demonstrate PFL-dependent co-assimilation of acetate and formate. Moreover, PFL-dependent growth is faster than growth on acetate using the glyoxylate shunt. Hence, growth via the reverse activity of PFL could have substantial ecological and biotechnological significance.

The reversibility of metabolic reactions can be a complex issue. While some reactions are clearly reversible within the cellular context, and others are clearly irreversible, there are reactions whose *in vivo* reversibility is not entirely resolved. In some cases, there is a direct physiological significance to a reaction's reversibility, as it might be able to support novel growth strategies. One such interesting case is the reversibility of pyruvate formate-lyase (PFL). This ubiquitous, oxygen-sensitive enzyme plays a central role in many organisms by supporting the production of an extra ATP molecule (three ATP molecules instead of only two) during glucose fermentation.[1] As the reverse activity of PFL could sustain novel strategies for acetate assimilation and/or formate fixation, it may have a substantial metabolic and ecological significance.

According to its standard Gibbs energy, $\Delta_rG'^{\circ} \approx -21$ kJ/mol ($6 \leq$ pH ≤ 8, $0 \leq I \leq 0.25$ M),[2] and considering that cellular metabolite concentrations mostly vary between 1 μM and 10 mM,[3,4] the PFL reaction could fluctuate in a range of -70 kJ/mol $\leq \Delta_rG'^{\circ} \leq 25$ kJ/mol and hence is expected to be reversible. Indeed, the enzyme was demonstrated to be fully reversible *in vitro*.[5−7] However, while a few previous studies suggested that PFL might support the condensation of acetyl-CoA and formate *in vivo*,[8−10] validation and characterization of this activity have never been provided. Notably, three of PFL's reactants—pyruvate, CoA, and acetyl-CoA—are central metabolites with very high connectivity within the metabolic networks of all organisms. Therefore, their cellular concentrations might be substantially more constrained than those of other metabolic intermediates, which could potentially prevent the reverse activity of PFL under physiological conditions.

Here, we aim to resolve the question of PFL's *in vivo* reversibility by directly selecting for its capacity to condense acetyl-CoA and formate within the model bacterium *Escherichia coli*. To this aim, we employed an *E. coli* strain in which the *aceA* gene, encoding isocitrate lyase, has been deleted. This strain cannot assimilate acetate via the glyoxylate shunt, the activity of which is dependent on isocitrate lyase. We further utilized a double-mutant strain Δ*aceA* Δ*pflB* in which the gene encoding the endogenous PFL enzyme has also been deleted. We transformed the wild type (WT) and both deletion strains with a plasmid that contains the native *pflB* and *pflA* genes (the latter encoding for a PFL-activating enzyme) and monitored the anaerobic growth of these strains on M9 medium supplemented with 50 mM nitrate (as an electron acceptor) and 15 mM acetate with or without 15 mM formate.

As shown in Figure 1A, the overexpression of *pflB* and *pflA* substantially burdens the WT strain's growth on acetate (dashed lines). However, while the addition of formate only slightly contributed to the growth of the WT strain, it dramatically increased the growth rate of PFL-overexpressing cells (solid lines). The Δ*aceA* strain cannot grow on acetate alone (dashed lines in Figure 1B), as it cannot operate the glyoxylate shunt. Supplemented with formate, the strain overexpressing PFL shows considerably better growth recovery than the Δ*aceA* strain, which possesses only endogenous PFL activity (solid lines in Figure 1B). This suggests that without an active glyoxylate shunt, the native expression level of PFL is not sufficient for optimal growth on acetate and formate. Finally, as expected, the Δ*aceA* Δ*pflB* strain can grow on acetate only if *pflB* is expressed from a plasmid and the medium is supplemented with formate (Figure 1C).

These results confirm that PFL can catalyze the condensation of acetyl-CoA and formate *in vivo* and serve as a sole route for microbial growth on acetate and formate. The PFL-dependent growth is quite efficient, supporting a doubling time of 4.7 ± 0.1 h (green solid lines in Figure 1B,C), shorter than the doubling time observed for the WT strain growing on acetate alone, 7.9 ± 0.2 h (dashed black line in Figure 1A). Still, unsurprisingly, growth of the WT strain on 15 mM pyruvate is faster, having a doubling time of 2.4 ± 0.1 h, and also results in higher biomass yield, achieving an OD of ∼0.5 (data not

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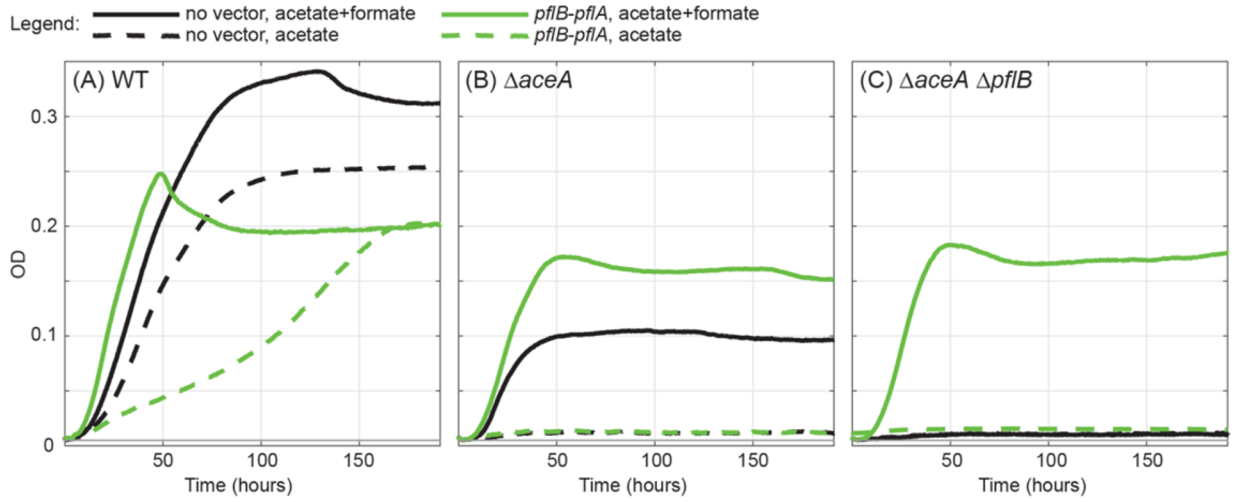

Figure 1. Growth on acetate and formate of WT (BW 25113) and *E. coli* deletion strains. Growth in an anaerobic chamber was tested on M9 medium supplemented with 50 mM nitrate and 15 mM acetate with (solid lines) or without (dashed lines) 15 mM formate. WT, Δ*aceA*, and Δ*aceA* Δ*pflB* strains are shown in panels A–C, respectively. Each strain was tested without a plasmid (black lines) and also transformed with a plasmid that harbors the native *pflB* and *pflA* genes (encoding PFL and PFL-activating enzymes, respectively, green lines). OD measurements were taken every ~17 min within a 96-plate reader and were corrected to represent OD values in standard cuvette tubes (Supporting Information). No growth was observed without nitrate. OD at inoculation was 0.005 (shown by a thin gray line). Measurements were taken in triplicate, whose averages are shown; error bars are not presented because the deviations between all triplicates, at all time points, were <5%.

shown). This indicates that the assimilation of acetate and formate to pyruvate limits cellular growth under the condition tested, which could probably be attributed to the slow rate of acetate activation (acetyl-CoA synthetase has a low k_{cat} of ~1 s^{-1},[11]) rather than the reverse activity of PFL that, at least *in vitro*, was found to be efficient.[5−7]

To prove that the formate-dependent acetate assimilation of the strain lacking isocitrate lyase is mediated through PFL's reverse activity, we performed labeling experiments. We fed the Δ*aceA* strain overexpressing *pflB* and *pflA* with labeled [^{13}C]formate and/or [1-^{13}C]acetate and compared the labeling pattern of proteogenic alanine and valine to that of a WT strain that was fed with unlabeled acetate or [1-^{13}C]acetate. Alanine and valine were chosen as they are derived solely from pyruvate. While alanine's carbons directly mirror those of pyruvate, valine biosynthesis involves condensation of two pyruvate molecules, in which one carboxylic acid is lost (Figure 2A). Hence, a single labeling in a non-carboxyl carbon of pyruvate results in a twice-labeled valine, while a labeled carboxyl carbon of pyruvate gives rise to a singly labeled valine (Figure 2B).

As shown in Figure 3, the labeling of alanine and valine in the WT strain growing on [1-^{13}C]acetate is consistent with the activity of the glyoxylate shunt: as approximately half of the alanine and valine molecules are singly labeled, we can deduce that approximately half of the carboxyl groups of pyruvate are labeled, and almost all of its non-carboxyl carbons are not labeled. We note that only half of the pyruvate molecules are labeled because the activity of the TCA cycle reshuffles the carbons of acetate in such a way that only half of the carboxyls of the TCA cycle's C4 intermediates are labeled (and none of the non-carboxyl carbons are labeled).

The labeling of alanine and valine in the Δ*aceA* strain overexpressing *pflB* and *pflA* and growing on [1-^{13}C]acetate and formate fits the hypothesis of PFL-dependent synthesis of pyruvate: as alanine is labeled once and valine is labeled twice (Figure 3), it is evident that pyruvate is labeled once on a non-carboxyl carbon that is derived from the carboxyl group of

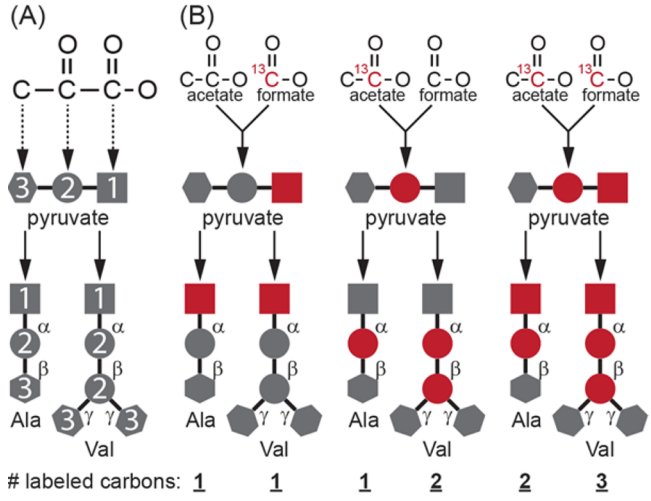

Figure 2. Expected effect of the carbon labeling in pyruvate on those in alanine ("Ala") and valine ("Val"). (A) Schematic correspondence between pyruvate's carbons and those of alanine and valine. (B) Expected numbers of labeled carbons (marked in red) in alanine and valine as dictated by the labeling position(s) in pyruvate, which could be derived from PFL-dependent assimilation of [^{13}C]formate and/or [1-^{13}C]acetate.

acetate. When the cells are fed with acetate and [^{13}C]formate, both alanine and valine are labeled once (Figure 3), which is consistent with formate being converted to the carboxyl carbon of pyruvate. Finally, the labeling of alanine and valine upon feeding with both [1-^{13}C]acetate and [^{13}C]formate follows the expected pattern of having pyruvate's carboxyl carbon and one non-carboxyl carbon labeled (Figure 3).

Although, by itself, the labeling of pyruvate's carboxyl could result from an exchange reaction with labeled formate, rather than net formate-dependent pyruvate production, the combined pattern presented in Figure 3 makes it clear that the entire cellular pool of pyruvate is derived from acetate and formate in a manner supporting the activity of PFL.

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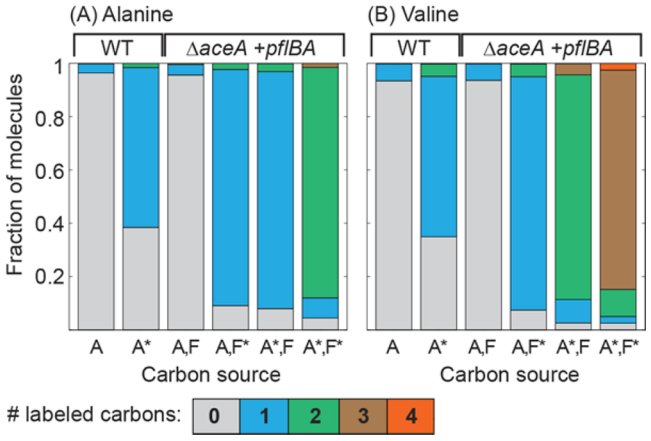

Figure 3. Labeling pattern within proteogenic alanine and valine upon feeding the WT strain and the Δ*aceA* strain overexpressing *pflB* and *pflA* with labeled and unlabeled acetate and formate. A corresponds to unlabeled acetate, A* to [1-^{13}C]acetate, F to unlabeled formate, and F* to [^{13}C]formate. Labeling patterns were detected using LC−MS/MS (Supporting Information).

Altogether, we demonstrate that (i) growth of a Δ*aceA* strain is dependent on the presence of formate in the medium and on the presence of PFL and (ii) labeling patterns of alanine and valine in a Δ*aceA* strain are inconsistent with acetate assimilation via the glyoxylate shunt and pyruvate formation via a cataplerotic reaction and instead are completely in line with pyruvate synthesis via the condensation of acetyl-CoA with formate. These results unequivocally confirm that PFL supports growth on acetate and formate.

The capacity of PFL to support growth via the condensation and assimilation of acetate and formate is expected to be ecologically significant in environments where the two compounds are available. As both compounds are fermentation products of multiple organisms, their combined presence in confined environments is quite prevalent.[12,13] Without an external electron acceptor, these compounds can serve as substrates for methanogenesis.[14] However, if an electron acceptor becomes available, respiration of acetate and formate could easily outcompete methanogenesis and microorganisms that harbor PFL can use the enzyme for efficient assimilation of these compounds into biomass. Note, we do not claim that all organisms that have an active PFL utilize it for growth on acetate and formate, but rather that such PFL-dependent growth is a possibility that some microbes might use under the right conditions.

As acetate can be converted to formate, e.g., via the reductive acetyl-CoA pathway operating in the oxidative direction,[15] PFL might be able to support growth on acetate as a sole carbon source. Similarly, as formate can be converted to acetate, e.g., via the reductive acetyl-CoA pathway operating in the reductive direction or via the acetogenic glycine synthase-reductase pathway of purine-fermenting anaerobes,[16,17] PFL could support growth on formate as a sole carbon source.

Interestingly, as formate is an intermediate of the reductive acetyl-CoA pathway,[15] it is possible that a variant of the pathway exists in which acetyl-CoA assimilation to pyruvate proceeds via PFL rather than pyruvate synthase (i.e., pyruvate ferredoxin:oxidoreductase[18]). In environments relatively rich with formate, this pathway variant might have an advantage over its ubiquitous counterpart as it is less dependent, thermodynamically and kinetically, on the external CO_2 concentration. In support of this hypothesis, the genomes of at least five sequenced acetogenic bacteria harbor at least one gene predicted to be a PFL ortholog: *Acetobacterium woodii*, *Clostridium ljungdahlii*, *Clostridium aceticum*, *Clostridium autoethanogenum*, and *Clostridium ultunense*.

Because formate can be efficiently produced by electrochemical reduction of CO_2,[19,20] microbial growth on formate is currently pursued as a means to establish electricity-dependent microbial cultivation and production of value-added chemicals.[21,22] PFL-dependent formate assimilation could significantly contribute to these efforts: via identification of metabolic routes that recycle acetyl-CoA from pyruvate, it should be possible to create a cycle that supports the net assimilation of formate into biomass. As PFL is not strictly anaerobic but can rather operate under microaerobic conditions,[23,24] it should be possible for PFL-dependent growth to use cheap and available oxygen as an electron acceptor. In support of such a biotechnological perspective, it was previously demonstrated that establishing an active PFL in a foreign host is quite straightforward and does not represent a substantial challenge.[25,26] Further characterization of microbial growth via the reverse activity of PFL could therefore have a direct biotechnological as well as ecological significance and could contribute to our understanding of the enzymology of one-carbon metabolism.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.6b00184.

A detailed account of the experimental methods used in this work (PDF)

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Notes

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