Phosphoketolase pathway engineering for carbon-efficient biocatalysis

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Abstract

Recent advances in metabolic engineering have facilitated the development of microbial biocatalysts capable of producing an array of bio-products, ranging from fuels to drug molecules. These bio-products are commonly generated through an acetyl-CoA intermediate, which serves as a key precursor in the biological conversion of carbon substrates. Conventional biocatalytic upgrading strategies proceeding through this route are limited by low carbon efficiencies, in large part due to carbon losses associated with pyruvate decarboxylation to acetyl-CoA. Bypass of pyruvate decarboxylation offers a means to dramatically enhance carbon yields and, in turn, bioprocess economics. Herein, we discuss recent advances and prospects for employing the phosphoketolase pathway for direct biosynthesis of acetyl-CoA from carbon substrates, and phosphoketolase-based metabolic engineering strategies for carbon efficient biocatalysis.

Introduction

Progress in metabolic engineering and synthetic biology has enabled a rapid expansion of industrial microbiology, wherein cellular metabolism is rewired to target production of tailored molecules. This has facilitated the design of microbial biocatalysts capable of converting renewable feedstocks, including non-food biomass, agricultural and municipal waste, and atmospheric CH₄ and CO₂, into an array of petroleum replacement products, which mitigates greenhouse gas emissions associated with petroleum-based products. Strain engineering strategies targeting production of fuels, industrial platform chemicals, pharmaceuticals, and biomaterials commonly exploit central metabolic pathways in which sugars and/or sugar phosphates are converted to pyruvate through the Embden-Meyerhof-Parnas (EMP) pathway, and subsequently decarboxylated to generate acetyl coenzyme A (acetyl-CoA), a key precursor to a myriad of bioproducts. Given the resultant loss of carbon dioxide, the carbon efficiency of such biological conversion processes is inherently limited. Though re-fixing of CO₂ offers a means to maximize carbon yield, this comes at an energetic expense (2 NADPH and 3 ATP per CO₂ fixed in photoautotrophic processes), or requires extensive metabolic engineering for anaerobic CO₂ fixation pathways. Thus, strategies targeting bypass of pyruvate decarboxylation offer a direct route to dramatically enhance carbon yields and process economics.

Phosphoketolases

Phosphoketolases (Pkts) catalyze the irreversible phosphorylytic cleavage of xylulose-5-phosphate and/or fructose-6-phosphate to acetyl-phosphate (acetyl-P) and glyceraldehyde-3-phosphate or erythrose-4-phosphate, respectively [1]. These enzymes are thiamine pyrophosphate (TPP)-dependent and require divalent cations for their activity [2]. To date, two distinct Pkt activities have been described based on substrate utilization: fructose-6-phosphate Pkt (FPkt, equation 1, E.C. 4.1.2.22) and xylulose-5-phosphate Pkt (XPkt, equation 2, E.C. 4.1.2.9). Many of the Pkts that have been characterized exhibit dual substrate specificity, but these Pkt variants typically have higher activity toward xylulose-5-phosphate [3,4].

Pkts enable a catabolic variant to the canonical pentose phosphate pathway (PPP), and are integral for sugar-phosphate metabolism in obligate and heterofermentative bacteria, as well as in some species of microalgae, cyanobacteria, and fungi [5]. FPkt is the key enzyme of the bifid-shunt, a pathway used by *Bifidobacterium* species to convert hexoses into acetic and lactic acids [6]. XPkt action bypasses the non-oxidative branch of the PPP by directly splitting

the xylulose-5-phosphate formed in the nonreversible reactions of the PPP oxidative branch (oxPPP) into glyceraldehyde-3-phosphate and acetyl-P [7]. The acetyl-P produced by Pkts serves as an easily assimilated energy source.

In lactic acid bacteria and bifidobacteria, Pkt operates with either acetate kinase (Ack) to generate acetate and ATP, or phosphotransacetylase (Pta) to generate acetyl-CoA and inorganic phosphate. In bacteria, *pkt* and *pta* or *ack* genes are usually positioned in an operon on the chromosome, which supports their physiological role in production of acetate and/or acetyl-CoA. More recently, Pkts have also been discovered in eukaryotic and basidiomycete fungi [5,8,9]. In fungi, Pkt is proposed to operate with Ack and acetyl-CoA synthetase (Acs), since all Ack-expressing fungi encode at least one, and in some cases two, Pkts, but do not express Pta. Although Pkt genes are often found in close proximity to genes encoding Ack or Pta in bacteria and fungi, some individual *pkt* open reading frames exist, particularly in species that encode two Pkts. These remote *pkt* genes suggest that independent Pkt functionality and regulation may exist in certain organisms, especially those that also encode a *pkt-pta/ack* operon.

Phosphoketolase pathway engineering

Increasing flux through the Pkt pathway can be an effective tool for metabolic engineering (Figure 1). Recent metabolic engineering efforts have shown that this pathway can be used with both pentose and hexose sugars to improve the yields of target molecules (Table 1). These improved yields are accomplished by increasing the pools of NADPH and acetyl-CoA, as well as by reducing the loss of carbon as CO₂. In addition to enhancing sugar-based biocatalytic processes, the Pkt pathway also offers the potential for deployment in conjunction with C1 assimilation pathways for increased carbon conversion efficiency (CCE).

I. Biocatalysis of sugars to fuel and chemical precursors

One of the first demonstrations of the benefit of increasing flux through the Pkt pathway was carried out in *Corynebacterium glutamicum*. Overexpression of the *Bifidobacterium animalis xfp* gene (encoding a Pkt with dual substrate specificity) in a *C. glutamicum* strain that was previously engineered to overproduce L-glutamic acid (L-Glu) resulted in a 14% increase in the L-Glu yield from glucose [10]. By introducing other mutations into the Pkt-producing strain, engineered *C. glutamicum* was able to produce acetyl-CoA from glucose via the Pkt pathway, while also limiting CO₂ loss [10], presumably by bypassing the CO₂-producing pyruvate dehydrogenase reaction. Thus, the loss of CO₂ increased the maximum theoretical yield of L-Glu from 81.7% to 98.0% (mol/mol L-Glu produced/glucose consumed).

Saccharomyces cerevisiae serves as a microbial cell factory for the production of a variety of bio-based fuels, platform chemicals, and pharmaceuticals [11]. Recent advances in strain engineering leveraging the Pkt pathway have

increased the yield of a variety of products in this organism, highlighting the potential of the Pkt pathway in biocatalysis. Papini *et al.* heterologously expressed XPkt and Ack from *Aspergillus nidulans* for acetate production in *S. cerevisiae*. ¹³C-labeling experiments showed flux through the engineered Pkt pathway from glucose to acetate, representing the first demonstration of an active synthetic Pkt pathway in *S. cerevisiae* [12]. Other engineered *S. cerevisiae* strains with synthetic Pkt pathways for increased flux of glucose to acetyl-CoA, either by the conversion of acetate to acetyl-CoA via Acs or the conversion of acetyl phosphate to acetyl-CoA via Pta, indicate that the Pkt pathway can increase production of polyhydroxybutyrate (PHB) [13], fatty acid ethyl ester (FAEE) [14], and isoprenoids [15,16].

In all of the above approaches, glucose was used as a feedstock. However, the Pkt pathway can also be employed to target efficient biocatalysis of other sugars to increase yields of valuable compounds. For example, a number of yeast, including *S. cerevisiae*, have native Pkts for xylose fermentation [17,18]. By using the native Pkt and heterologously expressing genes downstream in the Pkt pathway (acetaldehyde dehydrogenase and *pta*) as a way to drive flux through the pathway, ethanol yields from xylose have been increased by 25% [19]. Interestingly, heterologous overexpression of the dual substrate Pkt from *Bifidobacterium lactis* did not increase the ethanol yield, although this strain exhibited a 10-fold increase in Pkt activity compared to wild-type *S. cerevisiae* [19]. In *Aspergillus nidulans*, overexpression of the native Pkt has been used to increase the acetyl-CoA pool available for production of 6-methylsalicylic acid (6-MSA) [20]. Interestingly, the 6-MSA yield produced by an *A. nidulans* strain expressing Pkt decreased compared to wild-type when cells were grown on glucose, but increased when cells were grown on glycerol. The highest yields of 6-MSA were achieved when cells were grown on xylose with glyceraldehyde-3-phosphate dehydrogenase inhibited [20], which likely redirects carbon flux through the heterologous Pkt pathway.

A variation of the Pkt pathway, termed non-oxidative glycolysis (NOG) [21], was recently developed and tested both *in vitro* and *in vivo* in *E. coli*. Unique to NOG compared to a native Pkt pathway, fructose 1,6-bisphosphatase (Fbp) functions in coordination with Pkt and the non-oxidative branch of the PPP to drive a cyclic pathway. Heterologous expression of F/XPkt from *B. adolescentis* and overexpression of the native Fbp in an *E. coli* strain with competing fermentative pathways disrupted produced near the theoretical yield of acetate from xylose, supporting the use of NOG *in vivo* [21].

The Pkt pathway has been used successfully to increase yields of multiple valuable products by increasing CCE and providing a larger pool of acetyl-CoA. Despite these benefits, there are cases in which the Pkt pathway is detrimental to product yield. For example, both lactic acid and mannitol, which can be produced via carbon flux through the Pkt pathway, have higher yields if the Pkt pathway is disrupted and flux is directed toward the PPP or EMP pathways [22,23]. In both

cases, the Pkt pathway drives flux towards competing products (primarily acetate), resulting in decreased yield of desired products.

II. Biocatalysis of C1 substrates to fuel and chemical precursors

The use of a synthetic Pkt pathway in C1 metabolism has been proposed [21,24,25], and is an attractive strategy to enhance bioprocess economics. A Calvin-Benson-Bassham (CBB)-Pkt pathway addresses one of the major obstacles in photosynthetic biocatalysis: inefficient carbon fixation by the ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) enzyme [26]. Indeed, for each acetyl-CoA produced in a CBB-Pkt pathway, only two CO₂ molecules need to be fixed by RuBisCO, whereas three CO₂ are needed in a CBB-EMP pathway (Figure 1). Thus, a CBB-Pkt pathway would require less energy input in the form of ATP to synthesize one molecule of acetyl-CoA. This increased efficiency could dramatically enhance process economics of acetyl-CoA-derived products in photoautotrophic platform organisms, including cyanobacteria, microalgae, and terrestrial feedstocks.

Obligate methanotrophic bacteria use methane or methanol as their sole carbon and energy source, making them attractive for use in methane valorization and greenhouse gas mitigation. However, to date, methane biocatalysis has been limited by low-productivity cultures attributed to the high energy input required for methane activation and poor CCE [27,28]. In conjunction with the ribulose monophosphate (RuMP) pathway of gammaproteobacterial methanotrophs [29], engineering of an obligate Pkt pathway would bypass the pyruvate dehydrogenase-mediated decarboxylation step in the EMP pathway and increase CCE in methanotrophic biocatalysis (Figure 1). However, several questions exist with regard to carbon flux in methanotroph intermediary metabolic pathways and must be considered when engineering a heterologous Pkt pathway in these organisms. The genomes of gammaproteobacterial methanotrophs indicate that these organisms encode the oxidative branch of the PPP [30-32], but it is unclear whether ribulose-5-phosphate is preferentially formed through oxPPP or from a riboneogenic pathway initiating from downstream EMP pathway substrates. This is an important question that needs to be answered to facilitate rational strain engineering efforts related to native or heterologous Pkt overexpression. If carbon flux primarily occurs through the oxPPP (Figure 2A), one carbon equivalent will be lost in the 6-phosphogluconate dehydrogenase-mediated reaction prior to xylulose-5-phosphate formation, limiting CCE to acetyl-CoA but conserving the production of reducing equivalents. If carbon flux occurs through a riboneogenic cycle arrangement (Figure 2B) an obligate RuMP-Pkt pathway would be energy and redox neutral, potentially inhibiting vital cellular processes. Flux through either metabolic arrangement is unlikely to be mutually exclusive in methanotrophic bacteria. However, in either case, elevated flux into the non-oxidative branch of the PPP by Pkt could potentially increase the intracellular ribulose-5-phosphate (Ru5P) concentration, thereby fueling the RuMP pathway

and carbon fixation while concurrently bypassing pyruvate dehydrogenase-dependent carbon loss.

Concluding Remarks

Rapid enhancements in metabolic engineering, metabolic modeling, and synthetic biology have opened the door for an array of targeted strain-engineering approaches, aimed at the development of petroleum replacement products from renewable carbon substrates. These biocatalytic approaches offer great promise for the future development of green, microbial cell factories. However, such strategies are inherently limited by the poor carbon efficiency of native metabolic machinery. Pyruvate decarboxylation, in particular, is a key limiting cost driver in biological carbon upgrading processes targeting products derived from an acetyl-CoA intermediate.

Here we have reviewed recent pathway engineering advances in a high-potential alternative route, the phosphoketolase pathway, which facilitates bypass of pyruvate decarboxylation and enables complete carbon conservation in bioprocesses targeting PPP and/or acetyl-CoA-derived products. This pathway and variants thereof have been successfully deployed for targeted production of an array of bioproducts from sugar substrates. Biocatalytic approaches employing C1 substrates, including CH₄ and CO₂, also present promising opportunities for deployment of the Pkt pathway. Further, inherent to bypass of pyruvate decarboxylation is enhanced flux to PPP, which opens the door for targeted diversion of carbon flux to a series of PPP-derived products.

Importantly, metabolic, energetic, and redox considerations must be taken into account for effective deployment of obligate Pkt pathway engineering strategies. Hexoses are catabolized via the EMP pathway and the oxPPP in many organisms. Since EMP and oxPPP pathways result in the loss of a carbon equivalent, interruption of both would be required to force flux from C6 substrates through a native or heterologous Pkt pathway (Figure 1). Conversely, disruption of EMP alone would effectively shunt C5s and C1s through the Pkt pathway, as arabinose and xylose catabolism bypasses the CO₂ lost in the oxPPP, and these sugars are naturally metabolized through the Pkt pathway in several organisms. Notably, an obligate Pkt pathway that bypasses the oxPPP and downstream glycolytic pathway is redox and energy neutral, which may alter anabolic processes. Indeed, increased production of target compounds through the Pkt pathway has resulted in growth defects and decreases in biomass yields [12-14, 19]. This suggests that Pkt engineering strategies may be best suited for continuous and/or immobilized culture systems, wherein energy and carbon needed to maintain biomass accumulation is negligible. However, the loss of NAD(P)H could prove to be problematic if product synthesis is reducing equivalent-demanding, e.g. fatty acid biosynthesis.

Lastly, a largely unexplored function for Pkts is their possible role in cell signaling and transcriptional regulation. At high intracellular concentrations, acetyl-P can act as a phospho-donor and activate response regulator transcription factors of two-component systems in prokaryotes [33-35]. In eukaryotes, acetyl-CoA serves as the acetyl-donor by which histone acetyltransferase acetylates DNA, promoting the establishment of transcriptionally active euchromatin [36]. Thus, flux through the Pkt pathway could regulate key metabolites used for transcriptional activation in prokaryotic and eukaryotic organisms. These hypotheses warrant further investigation, as the potential for global regulatory side effects must be considered when deregulating Pkt in host organisms.

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Table 1. Metabolic engineering efforts using the phosphoketolase pathway.

Host organism	Substrate	Product (yield increase)	Phosphoketolase	Other host modifications	Reference
<i>S. cerevisiae</i>	Xylose	Ethanol (27%)	native <i>S. cerevisiae</i> OR <i>xfp</i> from <i>B. lactis</i>	<i>pta</i> (<i>B. subtilis</i>), <i>EhADH2</i> (<i>E. histolytica</i>)	Sonderegger 2004
<i>S. cerevisiae</i>	Glucose	Acetate (19%)	<i>xpkA</i> from <i>A. nidulans</i>	<i>ack</i> (<i>A. nidulans</i>), <i>sth</i> (<i>A. vinelandii</i>), <i>PCK1</i> (<i>S. cerevisiae</i>)	Papini <i>et al.</i> 2012
<i>S. cerevisiae</i>	Glucose	Polyhydroxybutarate (50%)	<i>xpkA</i> from <i>A. nidulans</i>	<i>ack</i> (<i>A. nidulans</i>), <i>Acs</i> ^{L461P} (<i>S. enterica</i>), heterologous PHB pathway	Kocharin <i>et al.</i> 2013
<i>S. cerevisiae</i>	Glucose	Fatty acid ethyl esters (70%)	<i>xpkA</i> from <i>A. nidulans</i>	<i>ack</i> (<i>A. nidulans</i>) OR <i>pta</i> (<i>B. subtilis</i>), <i>ws2</i> (<i>M. hydrocarbonoclasticus</i>)	de Jong <i>et al.</i> 2014
<i>S. cerevisiae</i>	Glucose	Isoprenoids (not reported)	various	<i>pta</i> , <i>ACSI</i> , <i>ACS2</i> , <i>ALD6</i> , heterologous MEV pathway	Garner <i>et al.</i> 2013
<i>C. glutamicum</i>	Glucose	L-glutamic acid (14%)	<i>xfp</i> from <i>B. animalis</i>	<i>odhA</i>	Chinen <i>et al.</i> 2007
<i>A. nidulans</i>	Xylose	6-methylsalicylic acid synthase (62%)	<i>xpkA</i> from <i>A. nidulans</i>	<i>msaS</i> (<i>P. griseofulvum</i>); GAPDH inhibited by iodoacetate	Panagiotou 2009
<i>E. coli</i>	Xylose	Acetate (500%)	<i>xfp</i> from <i>B. adolescentis</i>	<i>fbp</i> (<i>E. coli</i>), <i>ldhA</i> , <i>adhE</i> , <i>frdBC</i> , <i>pflB</i>	Bogorad <i>et al.</i> 2013

Figure 1. Phosphoketolase Pathway Engineering. Simplified schematic of engineering strategies for glucose (Glu), xylose (Xyl), methane (CH₄), and carbon dioxide (CO₂) biocatalysis through an obligate phosphoketolase (Pkt) pathway (purple) using a fructose-6-phosphate (F6P)-dependent Pkt (FPkt) or a xylulose-5-phosphate (X5P)-dependent Pkt (XPkt). Acetyl-CoA (AcCoA) or acetate is formed by phosphotrasacetylase (Pta) or acetate kinase (Ack) from Pkt-derived acetyl-phosphate (AcP), respectively. Acetate can also be converted to AcCoA by AcCoA synthetase (Acs). Disruption of the downstream Embden-Meyerhof-Parnas (EMP) pathway (green) and oxidative branch of the pentose phosphate pathway (PPP) will increase flux through the synthetic Pkt pathway, but at the expense of NAD(P)H and ATP production. CH₄ and CO₂ are assimilated through the ribulose monophosphate (RuMP) pathway and Calvin-Benson-Bassham (CBB) cycle in methanotrophic and photoautotrophic organisms, respectively. G6P, glucose-6-phosphate; G3P, glyceraldehyde-3-phosphate

Figure 2. Two potential routes for carbon flux through the ribulose monophosphate (RuMP) pathway of gammaproteobacterial methanotrophs.

Methane or methanol is assimilated via the RuMP pathway by formaldehyde (CH_2O) condensation with ribulose-5-phosphate (Ru5P) to ultimately form fructose-6-phosphate (F6P). F6P can enter the oxidative branch of the pentose phosphate pathway (oxPPP) (blue, arrangement A), or the Embden-Meyerhof-Parnas (EMP) pathway (orange, arrangement B). Both metabolic arrangements can regenerate the Ru5P needed for CH_2O fixation but with different production of ATP and NAD(P)H. Engineering a phosphoketolase-mediated bypass of the downstream EMP pathway after carbon flux through oxPPP will decrease NAD(P)H and block ATP production, while bypass of both the oxPPP and EMP pathways will be redox and energy neutral. G6P, glucose-6-phosphate; 6PGL, 6-phosphogluconolactone; 6PG, 6-phosphogluconate; X5P, xylulose-5-phosphate; R5P, ribose-5-phosphate; S7P, sedoheptulose-7-phosphate; G3P, glyceraldehyde-3-phosphate; E4P, erythrose-4-phosphate; AcCoA, acetyl-CoA.

Figure 1

Figure 2