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Exploring and Engineering Cyanobacterial Metabolism for Biofuel and Sugar Productions

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Exploring and Engineering Cyanobacterial Metabolism for Biofuel and Sugar Productions

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Chemical Engineering

by

Shih-Wei Chuang

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ABSTRACT OF THE DISSERTATION

Exploring and Engineering Cyanobacterial Metabolism for Biofuel and Sugar Productions

by

Shih-Wei Chuang

Doctor of Philosophy in Chemical Engineering

University of California, Los Angeles, 2020

Professor James Liao, Chair

Direct conversion of CO_2 into chemical production through cyanobacteria is a desirable solution to utilizing the excess amount of greenhouse gases released by burning fossil fuel. Several important chemical products including iso-butanol, n-butanol and 3-hydroxypropionate have been demonstrated in our and other laboratories. However, several issues remain unsolved before practical deployment of such technologies. These problems require both scientific and engineering investigations. In this thesis, we start by reporting an effort on improving n-butanol production from *Synechococcus elongatus* PCC7942 by determining the bottleneck in a production strain. Then, we focus on determining the physiological role of phosphoketolase (Xpk), which is an enzyme that can be used in the synthetic pathway, non-oxidative glycolysis. Although this enzyme is prevalent in cyanobacteria, its physiological role or biochemical properties are still unknown. Therefore, the second part of my thesis describes the characterization of the biochemical and physiological roles of Xpk in cyanobacteria.

To improve n-butanol production, pathway optimization for heterologous expression is one of the important strategies to enhance the carbon flux toward the targeted metabolite. Here, butyryl-CoA is identified as the bottlenecks in the original n-butanol producer through heterologous expression of *Clostridial* CoA dependent butanol production pathway in *Synechococcus elongatus* PCC7942. The combined strategy of enhancing the alcohol dehydrogenase (PduP) activity and overexpression of the single unit acetyl-CoA carboxylase from *Yarrowia lipolytica,* increases n-butanol production from 285mg/L in the original producer to 355mg/L in the strain DC11 after induction 10 days.

To further improve metabolite production derived from acetyl-CoA, Xpk expression has been suggested to form the non-oxidative glycolysis pathway that conserves carbon. Xpk is an enzyme that links several primary metabolic pathways in cyanobacteria, including Calvin-Benson-Bassham (CBB) pathway, oxidative pentose phosphate (OPP) pathway, Embden–Meyerhof–Parnas (EMP) pathway and the tricarboxylic acid (TCA) cycle. Putative sequences annotated as Xpk are widespread in most well-studied cyanobacteria strains. However, little is known about its physiological functions. Here, we show that Xpk from *S. elongatus* PCC7942 is inhibited by ATP. Further surveying Xpk's from different organisms indicates that ATP inhibition is conserved among cyanobacteria strains, suggesting its potential roles under low ATP conditions. Moreover, Xpk in *S. elongatus* PCC7942 and *Cyanothece* ATCC51142 show that they are dusk-peaked genes in day-night (12hr/12hr) cycle grown cultures, suggesting their roles in dark conditions. Finally, we find that knocking out *xpk* in *S. elongatus* PCC7942 abolished acetate production under dark and anaerobic condition. Interestingly, under this condition, Xpk pathway is particularly important in response to osmotic shock such as $NaHCO_3$, $KHCO_3$ and NaCl and its deletion causes viability decrease and glucose secretion as part of the incomplete glycogen degradation.

The dissertation of Shih-Wei Chuang is approved.

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- Lan E. I., Chuang D. S., Shen C. R., Lee A. M., Ro S. Y., Liao J. C. Metabolic engineering of cyanobacteria for photosynthetic 3-hydroxypropionic acid production from CO_2 using Synechococcus elongatus PCC 7942. *Metab Eng*. 31, 163-170(2015).
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Conference Abstract

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- Wannien fellowship at UCLA from 2011-2014

1. Introduction

The growing environmental concern has stimulated the interests in biofuel production. Current renewable bioprocesses focus on using microorganisms to convert glucose [1], cellulose [2], protein waste [3], methanol [4] and CO_2 [5] into valuable chemical feedstocks. To date, the feasibility of using various microorganisms to produce a wild spectrum of chemicals has demonstrated the potential to replace the petroleum-based chemical production process [6]. Among all the microbial platforms, photosynthetic organisms such as cyanobacteria, algae and plants are very promising as they can harvest sunlight and water to drive Calvin-Benson-Bassham (CBB) pathway (Fig.1.1) for carbon fixation. Net reaction: $3CO_2 +6NADPH+6H^+ +9ATP \rightarrow 6G3P+6NADP^+ +9ADP+3H_2O+8Pi$. Especially, cyanobacteria are more genetically malleable than algae and have a shorter growth cycle than plant cultivation. In this study, we engineer *S. elongatus* PCC7942 (thereafter PCC7942) for improvement of n-butanol production and explore PCC7942 metabolism with a focus on phosphoketolase (Xpk). Xpk is an enzyme widespread in most common cyanobacteria strains and links primary metabolic pathways together. However, little is known about its physiological function. Here, we find that Xpk is essential for survival under dark and anaerobic condition. We also find that glucose secretion can be harvested in Δ*xpk* strain under the same condition. Compared to industrial glucose production, our finding can skip many complicated processes and is relatively easy to scale-up.

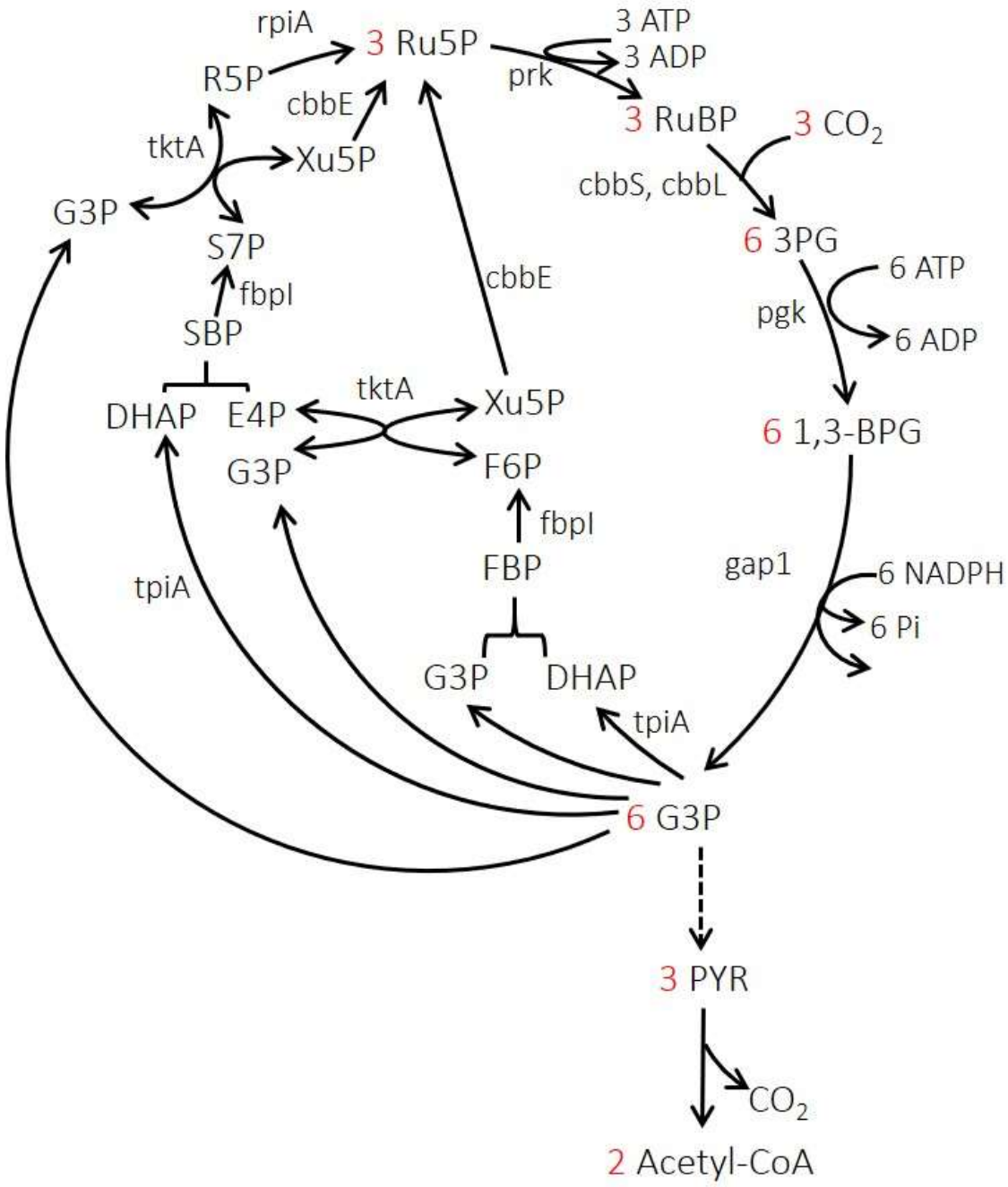

Fig.1.1 CBB cycle in cyanobacteria. Gene symbols: *cbbL,* rubisco large subunit; *cbbS*, rubisco small subunit; *pgk*, phosphoglycerate kinase; *gap1*, glyceraldehyde-3-phosphate dehydrogenase; *tpiA*, triosephosphate isomerase; *fbpl*, fructose 1,6-bisphosphatase; *tktA*, transketolase; *cbbE*, ribulose-phosphate 3-epimerase; *rpiA*, ribose 5-phosphate isomerase; *prk*, phosphoribulokinase

1.1 Cyanobacterial physiology and metabolism

The flexibility of cyanobacteria metabolism determines the organism's abilities to live in different habitats ranging from dry soils, freshwater and marine environment. Some species can be found in extreme conditions such as high temperature or high saline. In general, all cyanobacteria can perform oxygenic photosynthesis, in which water is used as the electron donors and the reducing powers generated by photosystem are used to drive CBB cycle for carbon fixation and growth. Some cyanobacteria can live photoheterotrophic conditions under low light conditions. In most cases, autotrophy is the major mode of growth and light availability is the key factor to power their photosynthesis.

Photosynthetic cyanobacteria are sensitive to intracellular energy level under dark or any stressed conditions as they are required to maintain a certain energy level waiting for the conditions favorable to initiate photosynthesis again. When light is insufficient or in the dark condition, cyanobacteria start to consume storage glycogen to generate energy for survival. Glycogen is one of the major carbon sinks in the light condition. Knocking out glucose-1-phosphate adenylyltransferase (*glgC*) in PCC7942 impairs growth. The defected growth phenotype can be rescued by introducing iso-butanol production pathway as the alternative carbon sink [7]. Glycogen also plays an important role in awakening chlorotic cells, which are suffering from nitrogen starvation in light condition in non-nitrogen fixing cyanobacteria such as PCC7942. Degradation of glycogen via oxidative pentose phosphate (OPP) pathway and glycolysis provides the initial energy and reducing power required for resuscitation from chlorosis. Knocking out glycogen phosphorylation (*glgP2*) in *Synechocystis* sp. PCC6803 impairs the ability to exit dormancy [8].

Cyanobacteria are the only prokaryotes known to possess a robust circadian clock [9]. It is a self-bolstering oscillation, which enables them to adapt a specific physiological event to the correct time. For example [10], under day and night cycle (12hr/12hr) condition, the transcriptions of the main carbon fixation enzyme, ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) and other CBB cycle involving enzymes such as phosphoribulokinase (*prk*) and NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase (*gap*) reaches peak at the beginning of the day cycle and gradually decrease in the day time. On the other hand, the transcription of glucose-6-phosphate 1-dehydrogenase (*zwf*) and 6-phosphogluconolactonase (*pgl*) and 6-phosphogluconate dehydrogenase (*gnd*) reaches peak at the beginning of dark cycle, showing that OPP pathway might be important for dark survival. The circadian rhythm is one of the key strategies for cyanobacteria to adapt to the dynamic light change following the rise and fall of sunlight.

Diazotrophic cyanobacteria can fix atmospheric nitrogen into ammonium. They also play an important role in the global nitrogen cycle and are the only prokaryotes capable to perform both oxygenic photosynthesis and nitrogen fixation [11]. Nitrogenase is an oxygen-sensitive enzyme. On the other hand, photosynthesis splits water to generate oxygen and electrons. Cyanobacteria have developed two different strategies to accommodate these two incompatible processes. One strategy is temporal separation [12]. For example, in the unicellular *Cyanothece* ATCC51142, the expression of nitrogenase reaches peak in the beginning of night while RubisCO reaches peak in the beginning of daytime. The other strategy is spatial separation [13]. For example, when Anabaena undergo nitrogen starvation, vegetative cells start to differentiate into specialized cells called heterocyst, which cannot perform photosystem II, and therefor provide low oxygen environment to allow nitrogenase to be active. Nitrogen fixation is triggered by nitrogen starvation regardless of light availability and it requires 16ATP for N_2 being fixed as $2NH_3$. Therefore, fixing

nitrogen under low light or dark condition needs stringent energy regulation. For example, carbohydrates in the form of sugars in the vegetative cells move to heterocyst and degradation of sugars provides additional energy for nitrogen fixation. In return, ammonium and amino acid are transported to vegetative cells from heterocyst.

Recent research that focuses on primary metabolism includes improving carbon fixation efficiency or bypassing pyruvate dehydrogenase complex for acetyl-coA synthesis. These approaches allow the increase of the carbon yield of key metabolites and circumvent the problem of the RubisCO limitation, as the slow rate of RubisCO ultimately limits growth, biomass and all downstream production. It is reported that introducing an malyl-coA glycerate pathway enhanced acetyl-coA pool by 2 folds [14].

1.2 Cyanobacterial biofuel and sugar productions

The first ethanol production in cyanobacteria was achieved by introducing *Zymomonas mobilis* ethanol production pathway including pyruvate decarboxylase and a primary alcohol dehydrogenase [15]. This heterologous pathway directs carbon flux from pyruvate to acetaldehyde and then ethanol. However, ethanol is not the ideal candidate to meet the need of current infrastructure due to relatively low energy density (24MJ/L) and high hygroscopicity. On the other hand, long chain alcohol such as n-butanol and iso-butanol are the potential alternatives for gasoline as both have higher energy densities and low hygroscopicities. Additionally, various chemical and sugar (Table 1.1 and Fig. 1.2) productions through heterologous pathway expression in cyanobacteria have demonstrated the feasibility of photosynthetic platforms to replace petroleum-based chemical production. To efficiently direct carbon flux toward the targeted products, several important strategies have been identified and employed such as cofactor

preference, designing a synthetic driving-force, reducing product toxicity and removing side-product formation.

Table 1.1 Cyanobacterial chemical and sugar productions

Compound	Organism	Titers	Reference
acetone	*Synechocystis* sp. PCC6803	36mg/L	Zhou et al.(2012)[16]
2-propanol	*S.elongatus* sp. PCC7942	26.5mg/L	Kusakabe et al.(2013)[17]
2,3-butanediol	*S.elongatus* sp. PCC7942	2.4g/L	Oliver et al.(2013)[18]
1-butanol	*S.elongatus* sp. PCC7942	300mg/L in flask	Lan et al.(2013)[19]
ethylene	*Synechocystis* sp. PCC6803	718±19μl $l^{-1}h^{-1}$ perA_{730nm}	Xiong et al.(2015)[20]
1,2-propanediol	*S.elongatus* sp. PCC7942	150mg/L	Li and Liao(2013)[21]
3-hydroxybutyrate	*Synechocystis* sp. PCC6803	533.4 mg/L	Wang et al.(2013)[22]
3-hydroxypropionate	*S.elongatus* sp. PCC7942	665 mg/L	Lan et al.(2015)[23]
	Synechocystis sp. PCC6803	837 mg/L	Wang et al.(2015)[24]
isobutanol	*S.elongatus* sp. PCC7942	450mg/L	Atsumi et al.(2009)[5]
isobutyraldehyde	*S.elongatus* sp. PCC7942	1.1 g/L	Atsumi et al.(2009)[5]
2-methyl-1-butanol	*S.elongatus* sp. PCC7942	200mg/L	Shen and Liao (2012)[25]
fatty acid	*Synechocystis* sp. PCC6803	197 mg/L	Liu et al.(2011)[26]
ethanol	*Synechocystis* sp. PCC6803	5.5 g/L*	Gao et al.(2012)[27]
1,3-propanediol	*S.elongatus* sp. PCC7942	288mg/L	Hirokawa et al(2016)[28]
sucrose	*S.elongatus* sp. PCC7942	10mM	Ducat et al(2012)[29]
erythritol	*Synechocystis* sp. PCC6803	256mg/L	van der Woude et al(2016)[30]
mannitol	*S.elongatus* sp. PCC7002	1.1g/L	Jacobsen and Frigaard(2014)[31]

*by photobioreactor

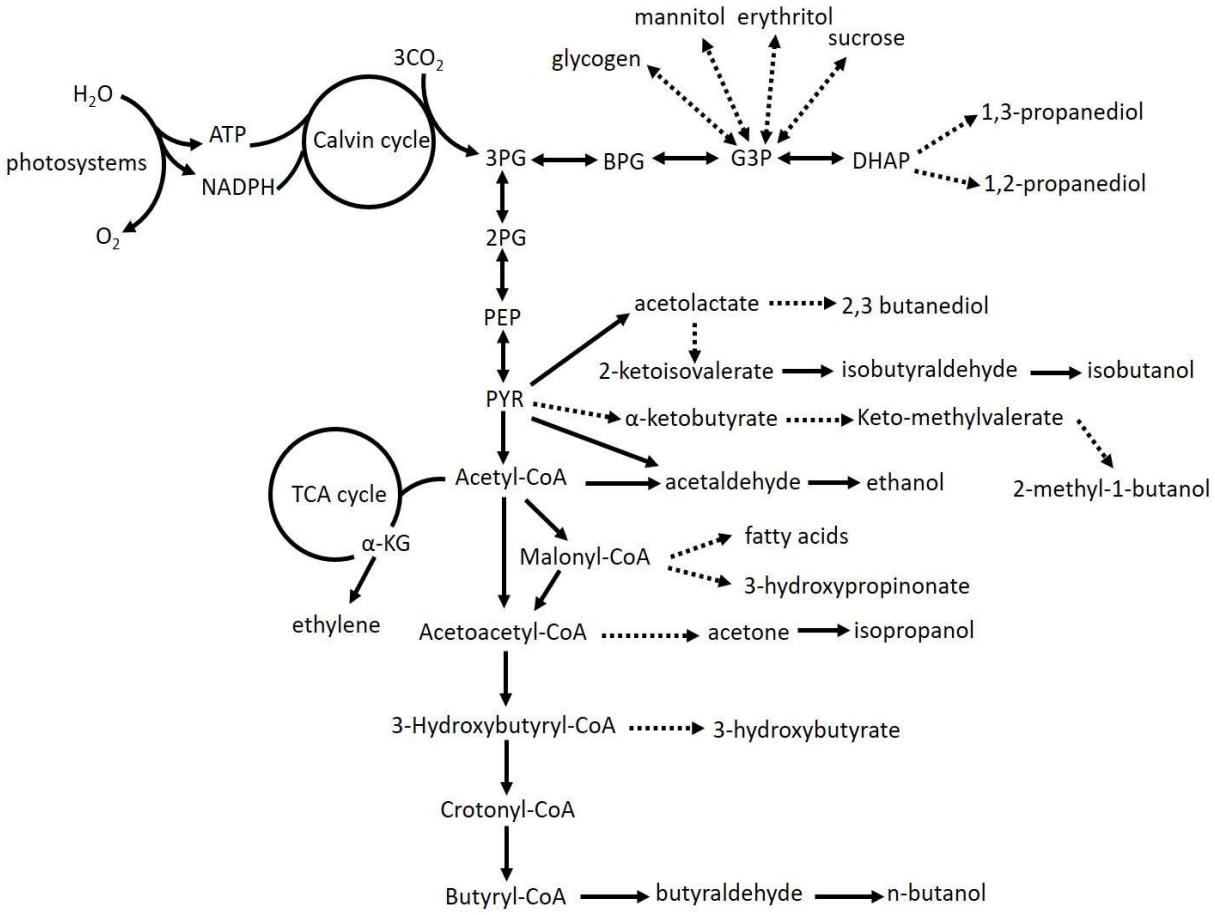

Fig. 1.2 Overview of pathways for biofuels and sugar productions from cyanobacteria

There are many acetyl-coA derived chemical and alcohols including n-butanol, acetone, 2-propanol, 3-hydropropinoate and 3-hysroxybutyrate. One of the bottlenecks in 2-propanol production is the reaction catalyzed by acetate/3-ketoacid CoA transferase. This enzyme needs acetate as a substrate in order to push the reaction toward acetoacetate biosynthesis. However, cyanobacteria do not produce acetate under autotrophic condition, and the acetate production pathway remains unknown. Feeding external acetate cannot increase 2-propanol production, perhaps due to regulation of acetate uptake.

3-Hydroxypropionate (3HP) is an important compound as it is the precursor for several industrially relevant chemicals such as acrylic acid, acrylamide, and 1,3-propanediol. Moreover, biodegradable and biocompatible poly-3HP can be made by using 3HP as the monomer. Three pathways have been proposed for 3HP production by microbial platforms (Fig. 1.3). The glycerol dependent pathway needs a coenzyme B12-dependent glycerol dehydratase. However, feeding external coenzyme B12 increases additional cost. Moreover, B12-independent glycerol dehydratase is an oxygen-sensitive enzyme which is not ideal for application in oxygenic cyanobacteria. Therefore, direct transfer of glycerol-dependent pathway into cyanobacteria for 3HP production would become difficult. On the other hand, 3HP can be produced from malonyl-CoA, a precursor for fatty acid synthesis which is common to most microorganisms. By introducing different malonyl-CoA reductases and malonate semialdehyde reductases, 3HP productions achieve titers between 124 and 659 mg/1 in *S. elongatus* PCC 7942 (ref). Another pathway is through β-alanine dependent pathway, which is able to redirect carbon fluxes from phosphoenolpyruvate to 3HP. Expression of both β-alanine-dependent pathway and the malonyl-CoA-dependent pathway can achieve highest 3HP production, which can outperform only the individual pathways. However, this enhancement is potentially restricted by 3HP toxicity to cyanobacteria. Previous report also mentioned that knocking out acetyl-CoA synthase can enhance tolerance to 3HP and other organic acids in *Synechococcus* sp. PCC 7002.

Sugar productions such as sucrose and trehalose are part of the response to salt stresses particularly in fresh-water cyanobacteria. When treating cyanobacteria with high concentrations of NaCl, growth rate decreases and cells start to secret non-ionic solutes such as sucrose and trehalose against outside osmotic pressure. Productivity of sucrose secretion can be significantly enhanced through introduction of sucrose permease (*cscB*) from *E. coli*. The final production reaches up to

10mM in 160 hours, which is considered as a competitive amount relative to sugarcanes from plants.

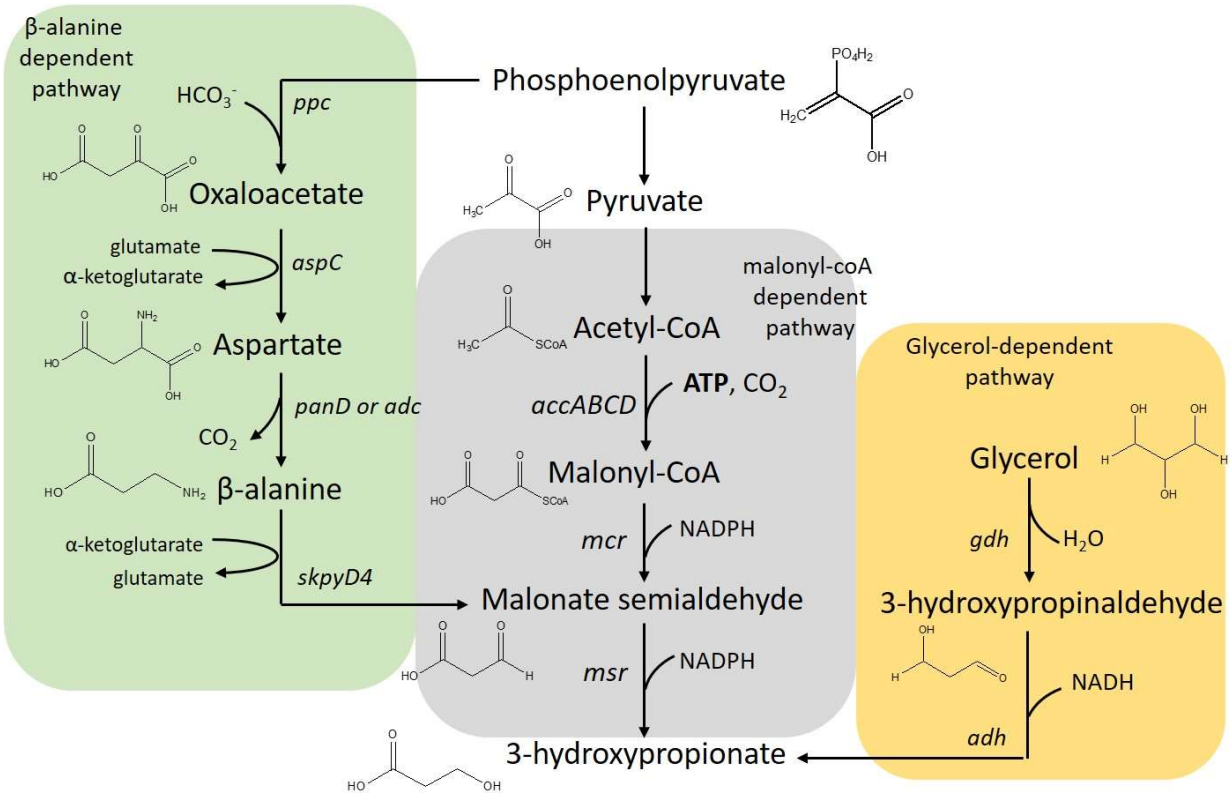

Fig.1.3 3-Hydroxypropionate(3HP) synthesis via glycerol-dependent, malonyl-CoA-dependent, and β-alanine-dependent pathways. Gene symbols are: *ppc*, phosphoenolpyruvate carboxylase; *aspC*,aspartate transaminase; *panD* or *adc*: aspartate decarboxylase; *skpyD4*, β-alanineaminotransferase; *mcr*, malonyl-coA reductase; *msr*, malonate semialdehyde reductase; *gdh*, glycerol dehydratase; *adh*, alcohol dehydrogenase.

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2. Improvement of n-butanol production in *Synechococcus elongatus* PCC7942

Ethanol is naturally produced in *Saccharomyces cerevisiae* and *Zymomonas mobilis,* and it has attracted much attention for application as an alternative fuel. However, it has a few unfavorable properties such as miscibility in water, relatively low energy density and relatively high vapor pressure. When compared to ethanol, middle chain (C4-C6) and long chain alcohols have properties closer to gasoline (table 2.1), which are more favorable for the application as fuels. Moreover, butanol can be used as an additive to increase octane number in gasoline.

Table 2.1 Comparison of chemical properties

Fuels	ethanol (C2)	n-butanol (C4)	gasoline (mixtures of alkanes)
energy density (MJ/L)	24	29.2	34.2
vapor pressure (kPa)[$]	5.95	0.8	0.68-206
octane number (RON)[*]	108.6	92	96-98[#]
hygroscopicity	high	low	low
compatibility with current infrastructure	no	yes	yes

[$] 25°C
*RON: research octane number
premium gasoline in USA

2.1. Challenge: heterologous expression of *clostridial* CoA dependent n-buOH pathway

The CoA-dependent pathway is naturally used by *clostridium* for n-butanol production as part of the acetone-butanol-ethanol (ABE) fermentation under anaerobic condition. Direct transfer of this oxygen-sensitive pathway into other user-friendly organisms such as *E. coli* and cyanobacteria also presents challenges and engineering strategies that are needed for high titer n-butanol productions.

When transferring native clostridial n-butanol pathway into *E. coli*, n-butanol titer is much lower than the original host. This result indicates the difficulty of transferring a pathway into a nonnative host. One potential bottleneck is that butyryl-coA dehydrogenase electron transferring flavoprotein

(Bcd/Etf) complex is poorly expressed in recombinant *E. coli*. The poor expression problem can be solved by utilizing trans-2-enyol-coA reductase (Ter) for the reduction of crotonyl-coA. Enzyme assay indicates that Ter can be readily expressed in recombinant *E. coli* and use NADH as a cofactor. Moreover, FAD-mediated Bcd/Etf catalyzes a reversible reaction while Ter is an irreversible step favorable for butyryl-coA formation. This strategy effectively enhances n-butanol productivity from 150mg/L/d to 800-1600mg/L/d in recombinant *E. coli* [1]. Nevertheless, increased n-butanol titers cannot compete with the original host. On the other hand, decarboxylation can be found in ethanol, isopropanol, and isobutanol pathways and is considered as a driving force in which one carbon dioxide irreversibly escapes from the cell. To construct a synthetic driving force for high titer n-butanol production in recombinant *E. coli*, NADH pool is built up by knocking out other NADH-consuming pathways including *ldhA*, *adhE* and *frdBC* [1]. Moreover, overexpression of formate dehydrogenase further increases the NADH pool. Knocking out acetate consumption pathway helps to increase acetyl-coA pool. The combined efforts of increasing NADH and acetyl-coA pools significantly direct carbon flux to n-butanol production with a titer up to 15g/L in *E. coli*.

When Ter-dependent n-butanol pathway is transferred into cyanobacteria, n-butanol titer is much lower than in *E. coli*. While knocking out competitive pathways to accumulate NADH and acetyl-coA pools is an effective strategy in recombinant *E. coli*, most cyanobacteria do not have mixed acid fermentation pathways and reducing power is replenished by photosynthesis. One of the challenges for n-butanol production is expressing a pathway from a strict anaerobe in oxygen-evolving cyanobacteria as enzymes involved may be sensitive to oxygen. By inhibiting the oxygen-evolving capability or growing under anoxic and dark condition, trace amounts of n-butanol is produced from cyanobacteria [2].

Acetyl-coA pool in cyanobacteria is assumed insufficient as they do not have fermentation pathway and tricarboxylic acid cycle (TCA) is unlikely to be active under autotrophic condition when compared to other fermentative bacteria such as *E. coli*. One of the hypotheses is that acetyl-coA is not enough to drive the thermodynamic unfavorable reaction catalyzed by acetyl-coA acetyltransferases. This problem can be solved by taking alternative pathway which involves a native acetyl-coA carboxylase (ACCase) and a heterologous acetoacetyl-coA synthase [3]. In cyanobacteria, native ACCase is an ATP dependent enzyme responsible for the biosynthesis of malonyl-coA, a precursor for fatty acid production. Under light condition, ATP production is generated by photosynthesis and create a driving force toward malonyl-coA biosynthesis. Acetoacetyl-coA synthase irreversibly catalyzes condensation of acetyl-coA and malonyl-coA to form acetoacetyl-coA with simultaneously release of one carbon dioxide as a driving force. This alternative pathway successfully increases n-butanol titers from about 1 mg/L to 29.9mg/L. Furthermore, n-butanol production significantly improves up to 300mg/L by replacing oxygen sensitivity butyraldehyde dehydrogenase (bldh) with an oxygen tolerant alcohol dehydrogenase (pduP) from *Salmonella enterica*, which indicates that oxygen sensitivity of coA-acylating aldehyde dehydrogenase seems to be the key step for alcohol production from cyanobacteria [4]. The butanol pathway in strain BUOHSE is shown in Fig. 2.1.

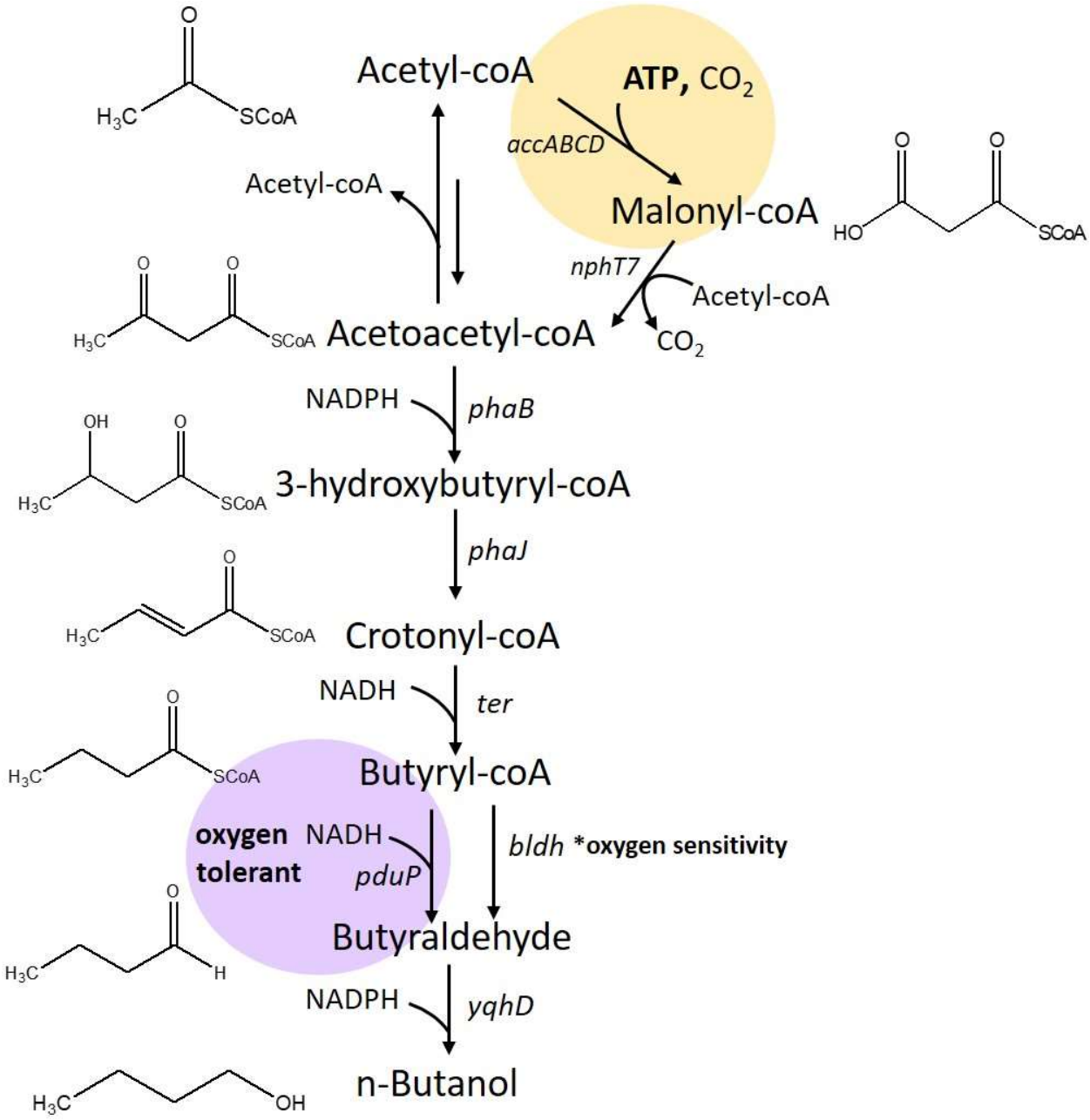

Fig. 2.1 n-butanol production via CoA-dependent pathway implemented in *S. elongatus* PCC7942. Gene symbols are: *accABCD*, acetyl-coA carboxylase; *nphT7,* acetoacetyl-coA synthase; *phaB*, acetoacetyl-coA reductase; *phaJ*, R-specific enoyl-coA hydratase; *ter*, trans enoyl-coA reductase; *pduP*: CoA-acylating propionaldehyde dehydrogenase; *bldh*: butyraldehyde dehydrogenase; *yqhD*: NADPH-dependent alcohol dehydrogenase.

2.2 Identification of bottlenecks in n-buOH producer BHOHSE

To identify potential rate-limiting steps in the butanol producer BUOHSE, our collaborators in Osaka University used metabolomics analysis to compare the relative metabolite pool sizes in three different butanol producers, EL14, EL22 and BUOHSE strains and found out butyryl-coA (Fig. 2.2) was the potential bottlenecks in the BUOHSE strain [5]. One hypothesis was that the improvement of butyryl-coA flux to butanol might facilitate the release of free coA, and therefore help to increase acetyl-coA synthesis.

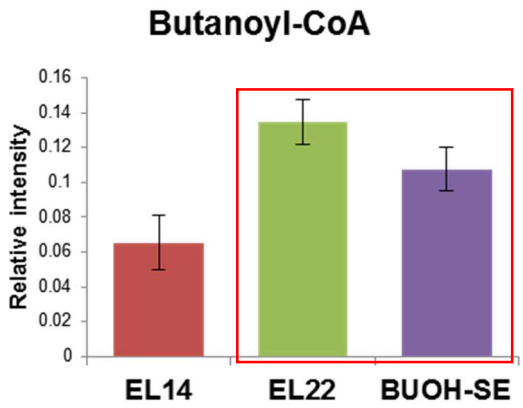

Fig.2.2 Relative butyryl-coA pool size in strains EL14, EL22 and BUOH-SE [5]

2.3 Design of different ribosome binding sites (RBS) for improvement of alcohol dehydrogenase activity

There are few approaches that can be engineered to potentially increase *pduP* expression including codon optimization, or introduction of additional copy of *pduP* or, replacement with a strong promoter or replacement with a strong RBS. Here I chose to design different RBS because it can save time and recombination site for segregation. Also, changing RBS is presumably better than changing a strong promotor because it has less unpredicted effect on the heterologous butanol pathway operon (Fig. 2.3).

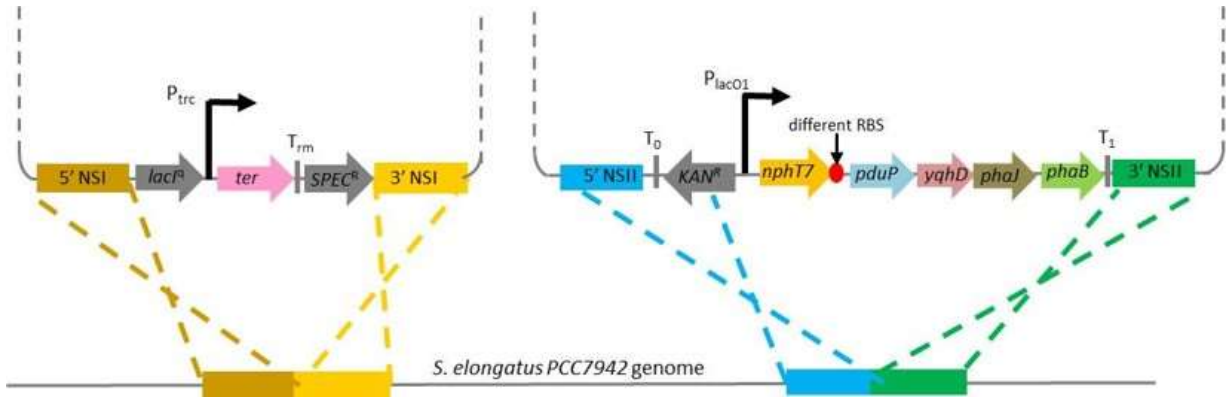

Fig.2.3 Heterologous butanol pathway in BUOHSE. Red dot indicates the RBS in front of *pduP*.

RBS sequence is also known as the Shine-Dalgarno Sequence in bacteria. The length is usually less than 20 base pairs. The location is upstream of the start codon (UTP) of an mRNA transcript about 8-10 base pairs or sometimes without any spacer between start codon and the RBS sequence. The consensus sequence is an AG rich area. For example, AGGAGG or GAGG are commonly found in *E. coli*. RBS is responsible for recruiting ribosomes in the process of protein translation from mRNA. Different RBS have different binding efficiencies with ribosome and therefore affect translational initiation frequency and protein expression. Recently, RBS calculator developed by Salis [6] is considered as a useful tool to generate synthetic RBS variants. To improve alcohol dehydrogenase(*pduP*) activities in BUOHSE strain, I collected 6 different RBS sequences (Table 2.2): three are synthetic RBS which are generated by Salis calculator [7] and the other three are native RBS in front of essential and important genes including RubisCo large subunit(*rbcL*), acetyl-coA carboxylase subunit(*accB*) and malonyl-coA-acyl carrier protein transacylase(*fabD*). These RBS sequences are used to replace the original RBS in the strain BUOHSE. Among six newly constructed strains, *pduP* expression with native RBS produce less butanol than the original producer while expression with synthetic RBS all produce more than the original producer. The highest producer is the one with the synthetic RBS-3 yielding a final titer 369mg/L in 12 days,

compared with 323mg/L in the original producer (Fig. 2.4). The increase is about 14%. Our results indicate that RBS calculator is a useful tool to generate RBS variance and it helps *pduP* expression (Fig. 2.5) by 45%. The enhanced pduP activity contributes to more butanol production.

Table 2.2 List of RBS sequences in front of *pduP*

origin	RBS sequence
original RBS for *pduP* in BUOHSE	GCATGCAGGAGAAAGGTACC
RubisCo large subunit	AGGGAGAGACGAC
malonyl-coA-acyl carrier protein transacylase	GAACTGAGAAGGGAGTATCACT
acetyl-coA carboxylase subunit	AGAGAGGAGTTGAGCGATCGCT
synthetic RBS-1	CAGACCGCCTACAAGAAGGAGGTTAATA
synthetic RBS-2	TGGTCGCACAGACCCCCAACAAAAGGCAGGTACCC
synthetic RBS-3	TCACAAAATACTTACCAACAAAGGAGGATCCC

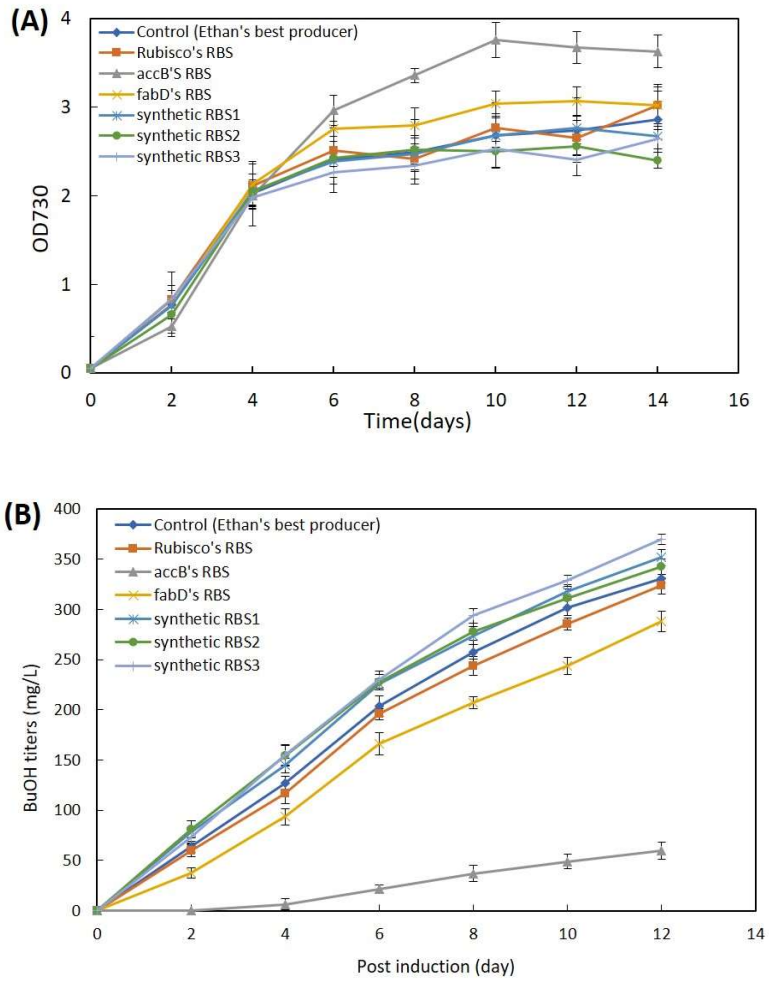

Fig.2.4 (A) growth curve (B) butanol production with different RBS in front of *pduP*

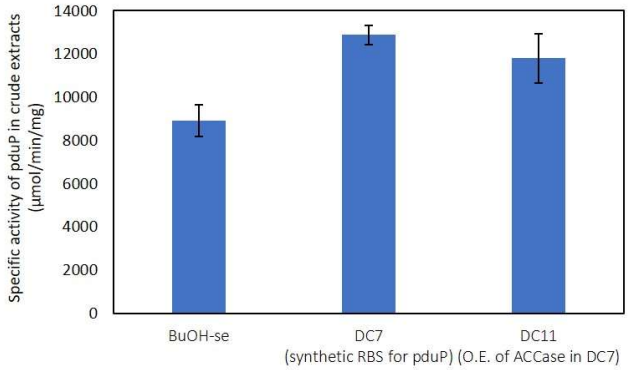

Fig.2.5 *pduP* activity in crude extracts from strains BUOHSE, DC7 and DC11. Strain DC7 has pduP with synthetic RBS-3. Strain DC11 has ACCase from *Yarrowia lypolitica* on top of DC7.

2.4 Expression of acetyl-coA carboxylase in n-buOH producer DC7

To further improve butanol production in strain DC7, we hypothesize that acetyl-coA might be accumulated. Therefore, we introduce one more copy of acetyl-coA carboxylase or improve acetoacetyl-coA synthase (nphT7) in DC7. Malonyl-coA is an important metabolite, which is a precursor for the fatty acid biosynthesis. Native acetyl-coA carboxylase is composed of several different subunits and how to properly express such enzyme complex remains uncertain. Therefore, we choose to introduce a single unit ACCase from *Yarrowia lypolitica* and *Saccharomyces cerevisiae* in DC7. Moreover, acetoacetyl-coA synthase uses acetyl-coA as substrate and catalyze the condensation reaction of acetyl-coA and malonyl-coA to form acetoacetyl-coA. We predict that improved expression of *nphT7* might contribute to higher butanol production, and therefore we replace the original RBS in front of nphT7 with two different synthetic RBS (Fig. 2.6) and Table 2.3.

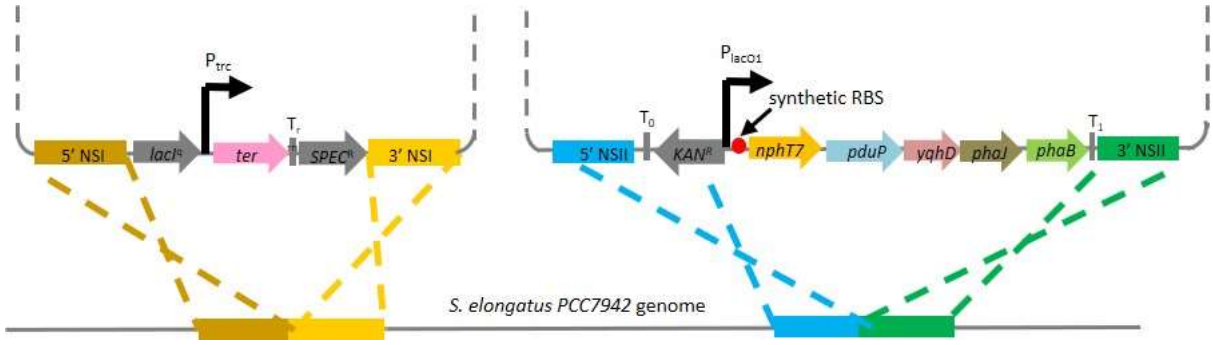

Fig. 2.6 Heterologous butanol pathway in DC7. Red dot indicates the RBS in front of *nphT7.*

Table2.3 List of RBS sequence in front of *nphT*

origin	RBS sequence
original RBS for nphT7 in DC7	AAAGAGGAGAAAGGTACC
synthetic RBS-1	CTAGACACTAGCACAGAGAAGGGGGATAAATA
synthetic RBS-2	AGGATCCTTATCCTAAAAAAACGAAGGAGGTAAAT

Our results indicated that overexpression of ACCase from *Yarrowia lypolitica* increased butanol production from 304mg/L in DC7 to 328mg/L in 8 days (Fig. 2.7), about 8% improvement. Overexpression of ACCase from *Saccharomyces cerevisiae* barely shows improvement compared to stain DC7. Furthermore, *nphT7* with synthetic RBS cannot improve butanol production even though enzyme assay indicates that synthetic RBS-1 improves nphT7 activity by 10% (Fig. 2.8).

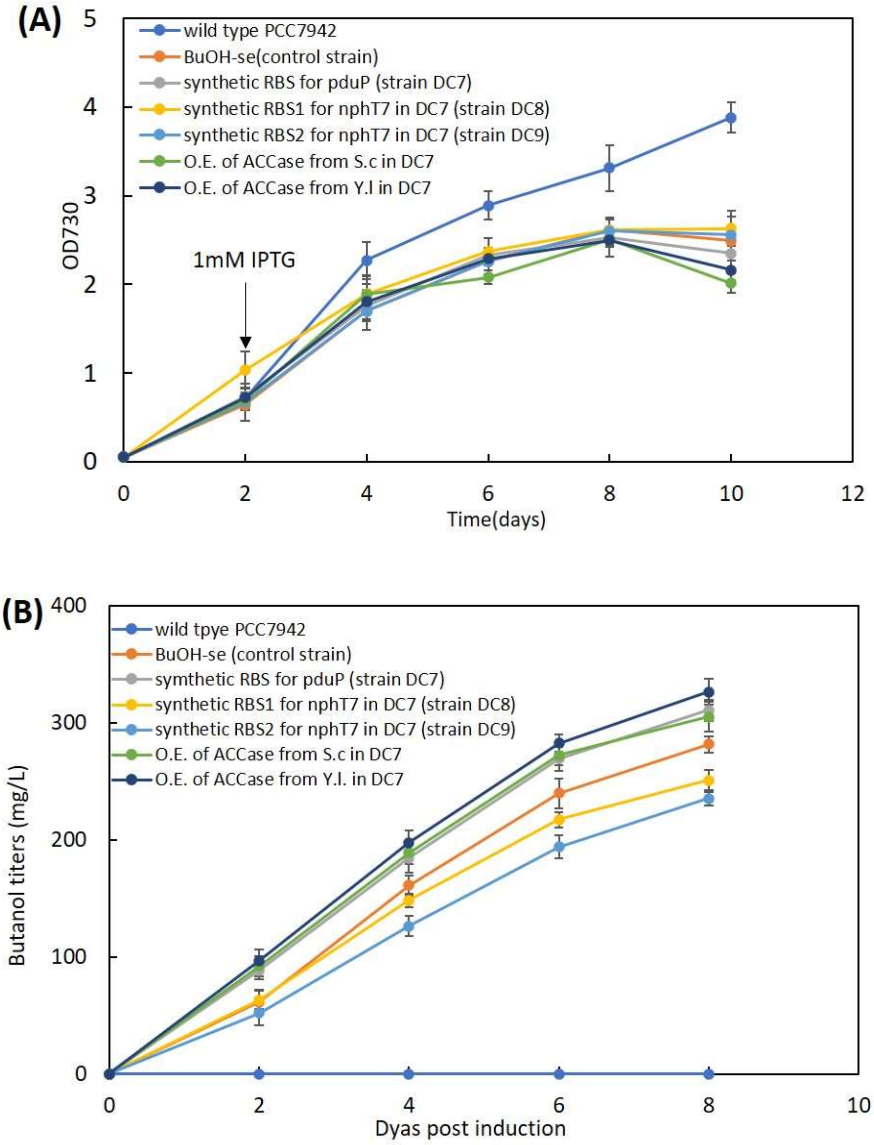

Fig. 2.7 (A) growth curve (B) butanol production with different RBS in front of nphT7 and additional single unit ACCase in DC7

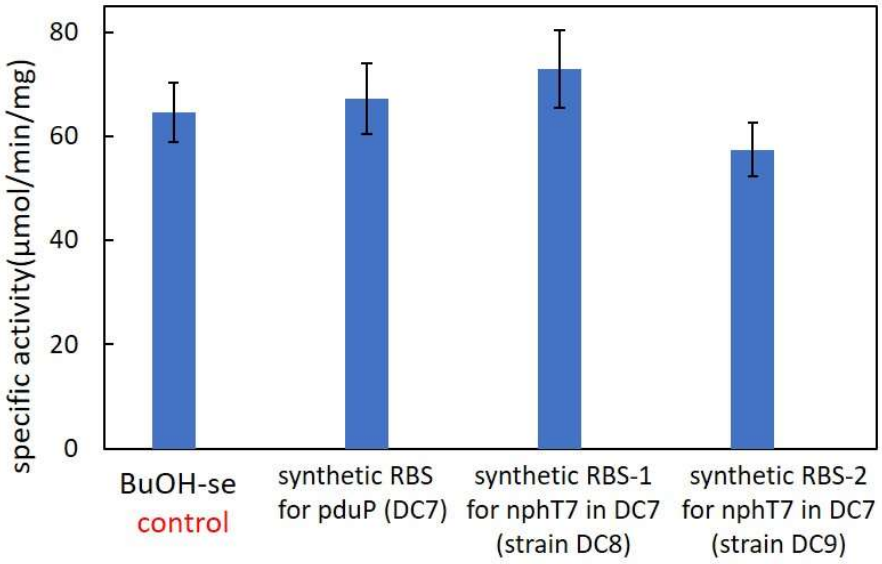

Fig.2.8 *nphT7* activity in crude extracts from strains BUOHSE, DC7 and DC8 and DC9.

To validate the expression of eukaryotic ACCase from *Yarrowia lypolitica* in PCC7942, rt-PCR was conducted. Our results indicated that PCC7942 can express eukaryotic ACCase (Fig. 2.9).

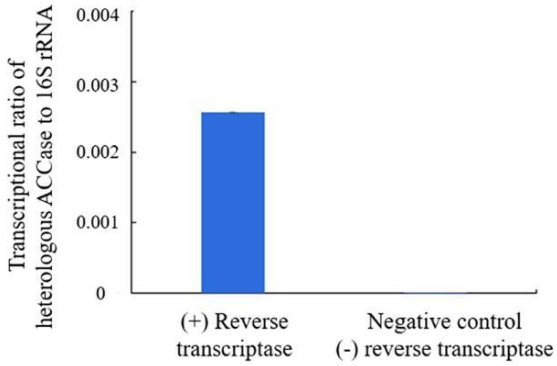

Fig. 2.9 rt-PCR results for heterologous ACCase expression in PCC7942

2.5 Pathway optimization for enhancement of n-buOH production

Our work demonstrated the importance of RBS on tuning protein expression. The combined efforts including enhancement of *pduP* expression and overexpression of ACCase successfully

increased butanol production from 285mg/L to 355mg/L in 10 days, which is about 24% improvement as shown in Fig. 2.10. These results were published in 2018 [8].

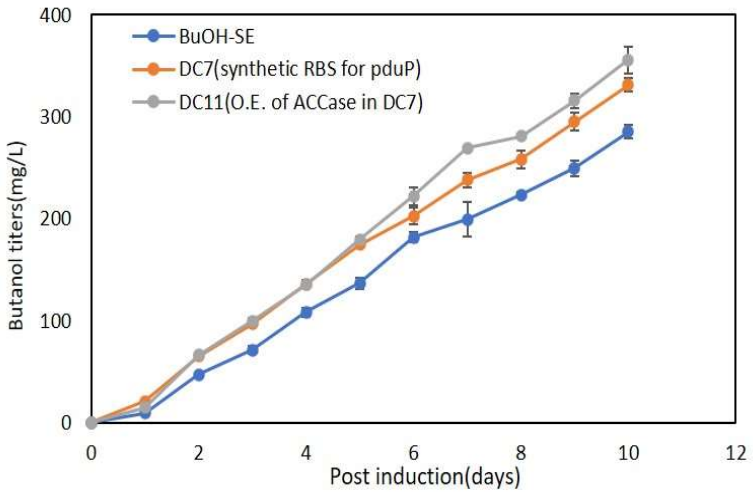

Fig. 2.10 butanol production in strain BUOHSE, DC7 and DC11 under continuous light condition(50μE/m^2s)

2.6 Reference

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3. Role of Cyanobacterial Xpk in Energy Regulation and Glucose Secretion under Dark Anaerobic and osmotic stress conditions

3.1 Introduction

Cyanobacteria have been proposed as a platform to convert atmospheric CO_2 into useful chemical products through the combined action of primary metabolic pathways such as the CBB cycle, OPP pathway, EMP pathway, and TCA cycle. Xpk is linked to all these pathways (Fig. 3.1), but its physiological role is still uncertain, except that it participates in *Synechocystis sp.* PCC6803 acetate production in dark heterotrophic conditions in the presence of glucose or xylose [1]. Xpk is a thiamine pyrophosphate (TPP) dependent enzyme that cleaves 5-carbon sugar phosphate xylulose 5-phosphate (Xu5P) or 6-carbon sugar phosphate fructose 6-phosphate (F6P) to acetyl-phosphate (AcP) and glyceraldehyde 3-phosphate (G3P) or erythrose 4-phosphate (E4P), respectively. AcP can be converted to acetate by acetate kinase (*ack*). Indeed, Xpk in some cyanobacteria is located adjacent to *ack* gene on the genome [2]. Xpk is also a key enzyme in the synthetic Non-Oxidative Glycolysis (NOG) cycle [3] that conserves carbon when converting 5 or 6 carbon sugars to acetyl-CoA. Cyanobacteria contain all the enzymes necessary (Table 3.1) for NOG cycle (Fig. 3.2) and bypassing pdhc (Fig. 3.3) for acetyl-coA production. However, little is known regarding to the physiological role of Xpk, despite its widespread presence and linkage to multiple pathways.

Xpk is one of the reactions responsible for the carbon-carbon bond cleavage in the CBB pathway besides fructose 1,6-bisphosphate aldose (FBP aldolase), transaldolase (Tal) and transketolase (TKT). Both FBP aldolase and TKT are essential in light condition for growth and responsible for the regeneration of 5-carbon sugar phosphate ribulose-5-phosphate, a precursor for the RubisCO. Tal is not essential for CO_2 fixation but a dusk-peak gene, suggesting its potential role in dark condition despite of little studies about this enzyme in cyanobacteria. FBP aldolase and

Tal cleaves C3-C4 bond in FBP and F6P respectively, which is an aldol cleavage. On the other hand, both TKT and XPK cleaves C2-C3 bond, which is a ketol cleavage. Both TKT and XPK require TPP as cofactor. The reaction mechanism is proposed by Tittmann [4] and shows in Fig. 3.4.

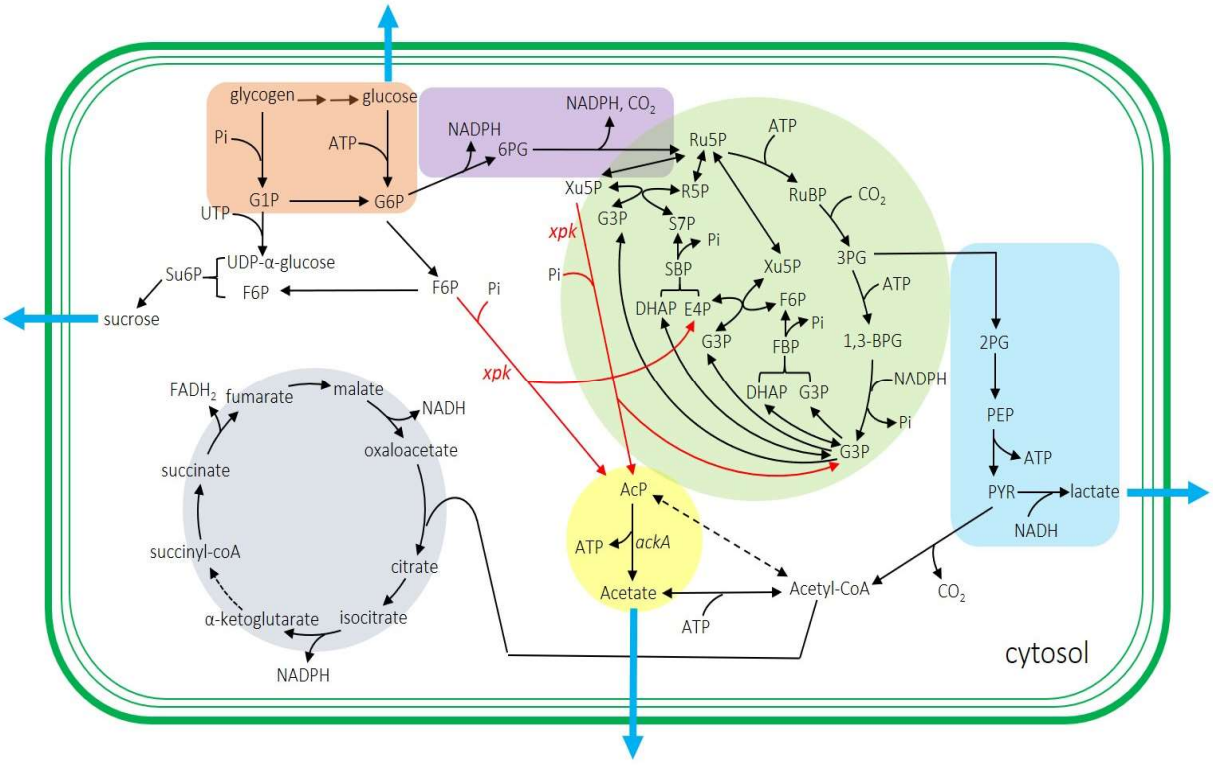
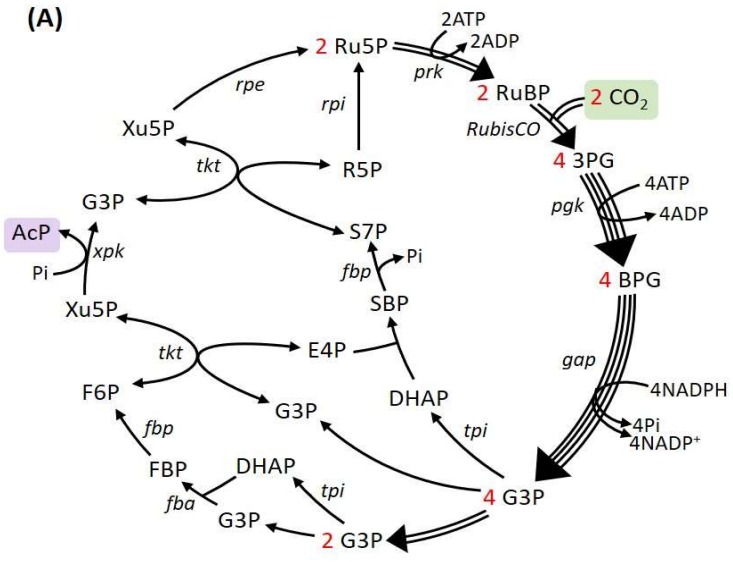


Fig.3.1 Proposed physiological role of phosphoketolase(*xpk*) in the cyanobacterium S. *elongatus* PCC7942. *ackA*, acetate kinase; G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; 6PG, 6-phosphogluconate; KDPG; 2-dehydro-3-deoxy-phosphogluconate; Ru5P, ribulose-5-phosphate; RuBP, ribulose 1,5-bisphosphate; Xu5P, xylulose-5-phosphate; R5P,ribose-5-phosphate; E4P, erythrose-4-phosphate; G3P, glyceraldehyde-3-phosphate; S7P, sedoheptulose-7-phosphate; SBP, sedoheptulose 1-7-bisphosphate; FBP. fructose 1-6-bisphosphate; 3PG, 3-phosphoglycerate; 1,3-BPG, 1,3-bisphophoglycerate; DHAP, dihydroxyacetone phosphate; 2PG, 2-phophoglycerate; PEP, phosphoenolpyruvate; PYR, pyruvate; AcP, acetylphosphate; Su6P.sucrose-6-phosphate; ATP, adenosine triphosphate; UTP, uridine triphosphate; NADPH, nicotinamide adenine dinucleotide phosphate; $FADH_2$, flavin adenine dinucleotide; Pi, inorganic phosphate.

Table 3.1 Essential genes in cyanobacteria for acetyl-coA production via NOG cycle

Synechococcus elongatus PCC 7942		*Synechocystis sp.* PCC 6803		*Anabaena variabilis* ATCC 29413	
native enzyme in PCC 7942	gene	native enzyme in PCC 6803	gene	native enzyme in ATCC 29413	gene
triosephosphate isomerase	*tpiA*	triosephosphate isomerase	*tpiA*	triosephosphate isomerase	tpiA
fructose-bisphosphate aldolase	*cbbA*	fructose-bisphosphate aldolase	*cbbA*	fructose-bisphosphate aldolase	Ava_1590
fructose-1,6-bisphosphatase	*fbp*	fructose-1,6-bisphosphatase	*fbp*	fructose-1,6-bisphosphatase	Ava_1680
Fructose-6-phosphate phosphoketolase (annotated, but not confirmed)	*xfp*	xylulose-5-phosphate phosphoketolase (annotated but not confirmed)	*Slr0453* *Sll0529*	xylulose-5-phosphate phosphoketolase (annotated but not confirmed)	Ava_0496 Ava_4264
transaldolase	*tal*	transaldolase	*talB*	transaldolase	Ava_0493
transketolase	*tktA*	transketolase	*tktA*	transketolase	Ava_1491
ribose 5-phosphate isomerase	*rpiA*	ribose 5-phosphate isomerase A	*rpiA*	ribose 5-phosphate isomerase A	Ava_4491
ribulose-phosphate 3-epimerase	*cbbE*	ribulose-phosphate 3-epimerase	*cfxE*	ribulose-phosphate 3-epimerase	Ava_4675



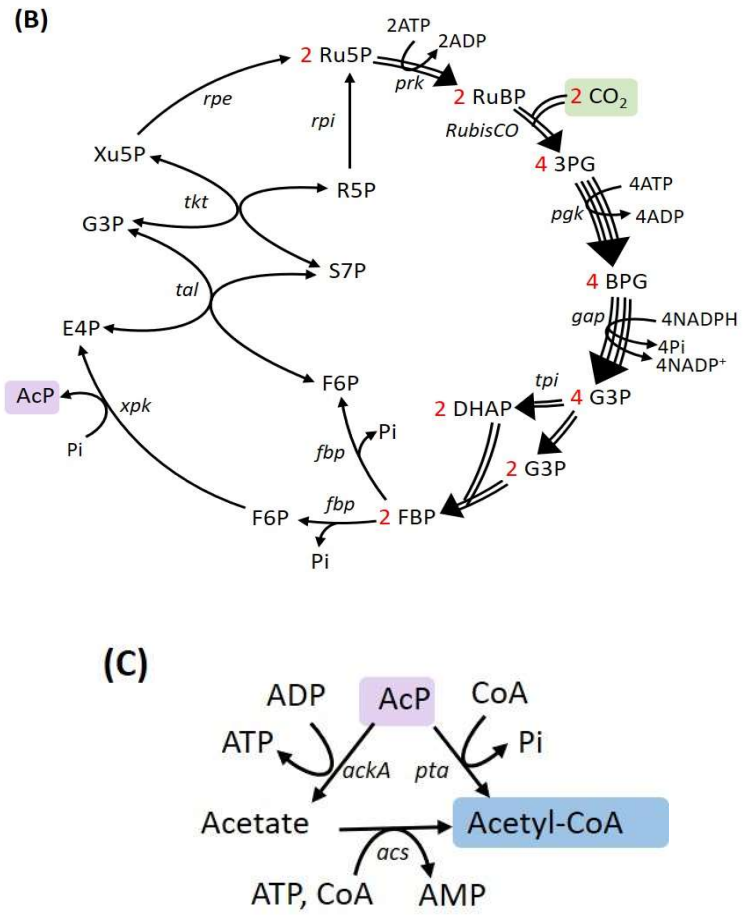


Fig. 3.2 CBB pathway coupled with phosphoketolase(*xpk*) for the production of acetyl-CoA from CO_2. The stoichiometric number is highlighted in red and the number of arrows represents the turnover in the cycle. The CO_2 uptake is highlighted in green and acetylphosphate(AcP) as intermediate product is highlighted in purple. (A)Xu5P as substrate (B)F6P as substrate (C)conversion of AcP to acetyl-CoA

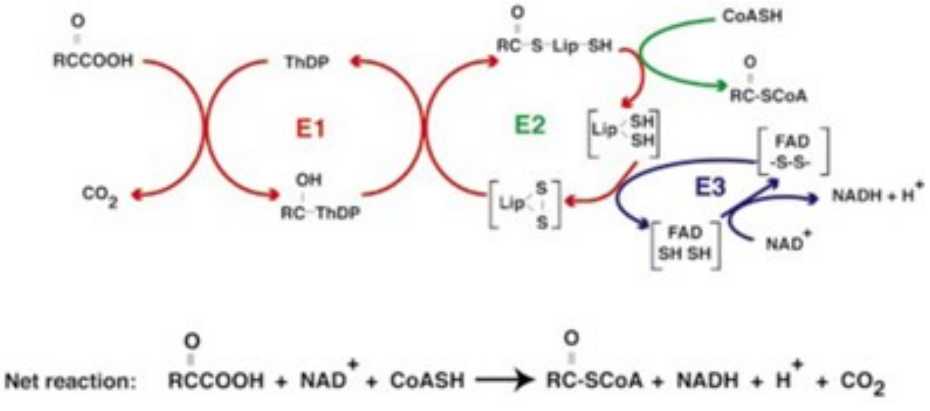


Fig. 3.3 Diagram of reaction mechanism of pdhc [5]

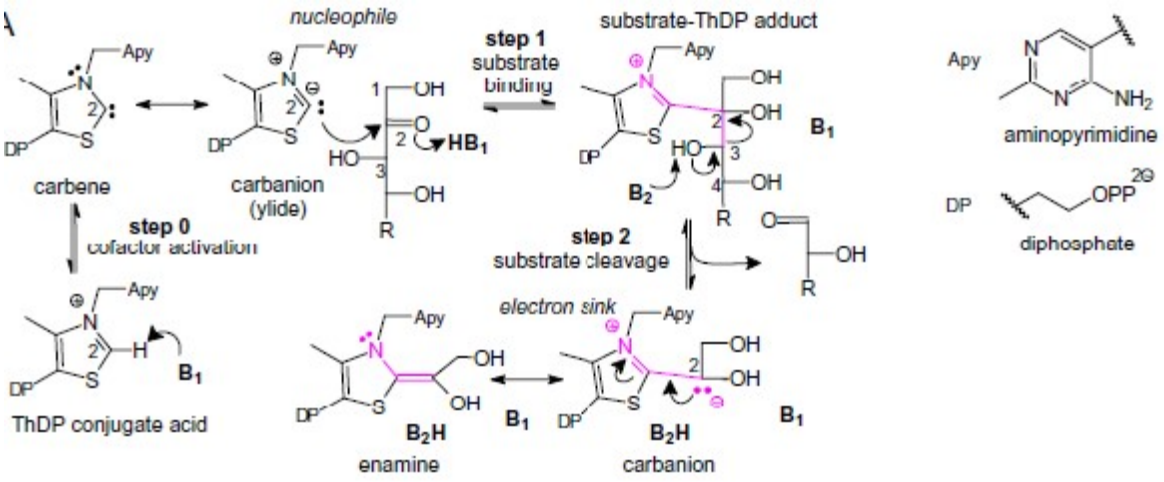


Fig. 3.4 Proposed mechanism of substrate binding and keto-cleavage of TPP dependent enzyme by Tittmann[4]

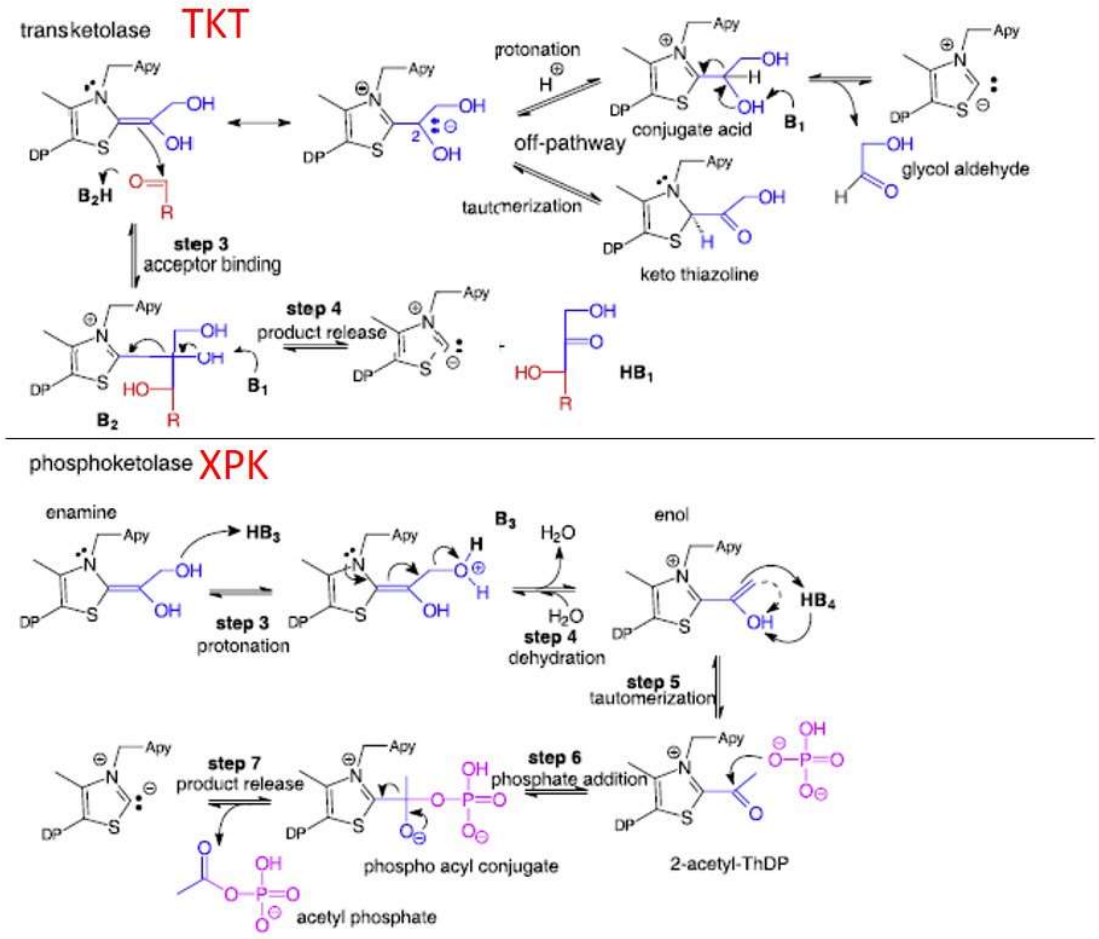


Fig.3.4(continued) reaction mechanism after step 2. TKT (top panel) and XPK (bottom panel)[4]

Pyruvate dehydrogenase complex (Pdhc), one of the most complicated enzymes, is considered the essential step to convert pyruvate to acetyl-CoA in most microorganisms. It is an enzyme complex responsible for three biochemical reactions: decarboxylation of pyruvate, CoA acetylation and regeneration of oxidized lipoyl domain. The reaction mechanism is shown in Fig. 3.3[5]. In cyanobacteria, this enzyme is composed by three different components, E1, E2 and E3 respectively. Each component is comprised of different subunits and monomers. For example, E1 component is by twelve copies of hybrid two different subunits, pdhA and pdhB, reported as $[(pdhA)(pdhB)]_{12}$. E2 component is by twenty-four copies of monomer pdhC, reported as $[(pdhC)_{24}]$. E3 component is by two copies of monomer pdhD, reported as $[(pdhD)_2]$. The active function of Pdhc is the combination of these three components as $[(pdhA)(pdhB)]_{12}[(pdhC)_{24}][(pdhD)_2]$. The complicated structure of Pdhc renders the engineering difficulty for acetyl-CoA enhancement.

In this study, we first cloned, expressed, and purified several putative sequences annotated as *xpk* from different cyanobacteria and characterized their biochemical properties. We found that cyanobacterial Xpk's are commonly inhibited by ATP, and diazotrophic cyanobacterial Xpk's are further activated by ADP. We then proposed and explored its physiological roles in *S. elongatus* PCC7942 (thereafter PCC7942). Our results suggest that Xpk serves as an energy regulator under dark and anaerobic conditions. Deletion of *xpk* reduced viability and caused the cells to secrete glucose as part of incomplete glycogen degradation.

3.2 Methods and material

3.2.1 Cultivation condition

All PCC7942 strains in this study were grown on modified BG11 medium as described in previous paper. Solid plates contained 1.5%(w/v) Bacto agar (BD bioscience) in modified BG11 medium. In the case of xpk knockout mutant, 5mg/ml gentamicin was used in the plates. For cultures grown on ambient air under day-night cycle condition, cells were inoculated from a preculture, which was grown in the same condition, with an initial OD_{730}0.01 in 50ml BG11 with 20mM HEPES in 250ml Erlenmeyer baffled flasks. Light intensity was 50μE/m^2s. An automatic control day(12hr) and night(12hr) cycles were programmed in an incubator shaker (innova 42, New Brunswick) at 30°C and 130rpm. Cell growth was measured at OD_{730}(DU800, Beckman Coulter). Gentamicin was not used under ambient air and day and night cycle conditions for Δ*xpk*.

Cultivation condition for Cyanothece ATCC51142 under ambient air and day night cycle conditions was the same as PCC7942 except that the medium for Cyanothece ATCC51142 was ASP2 without nitrate.

For cultures used in dark and anaerobic survival test, cells were inoculated from a preculture, which was grown in continuous light conditions (50μE/m^2s) in BG11 with 50mM $NaHCO_3$ for 2 days, with an initial OD_{730}0.01in 50ml BG11 in 250ml screw-capped flasks with 50mM $NaHCO_3$ in a light box(50μE/m^2s) for 10 days. Taking out 5ml from the original cultures and feeding 5ml of fresh BG11 containing 500mM $NaHCO_3$ were employed every two day until day 10. 5mg/ml of gentamicin was used in the Δxpk precultures. To conduct dark and anaerobic survival test, both W.T. and Δxpk were harvested by centrifugation at 3500rpm for 10mins at 25°C and then washing out residual bicarbonate by BG11 without $NaHCO_3$. The cell pellets

were resuspended and adjusted to OD20 in 1 ml BG11 with 20mM HEPES in a 10ml blood collection tube (BD vacutainer). 50mM of different stressors such as $NaHCO_3$, $KHCO_3$ and NaCl were added respectively. Dark conditions were made by wrapping each tube with aluminum foil. Anaerobic conditions were obtained by purging the headspace in the tubes with pure nitrogen gas in an anaerobic chamber (Coy lab). Each purging cycle started from vacuuming the headspace and then re-filling with pure nitrogen gas. Total of eight cycles were used. Condition with 10% CO_2, 5% H_2 and 85% N_2 in the headspace was obtained by replacing pure N_2 at last cycle. The flowchart for culture preparation was in the supplementary information.

3.2.2 Cloning, purification and XPK enzyme assay

Putative xpk sequence information was obtained from Biocyc and KEGG. Plasmid backbones and xpk PCR products were cloned by KOD Xtreme Hot Start DNA Polymerase, following the manufacturing's protocol. The fragments of purified xpk products and purified backbones were assembled by Gibson Assembly method. The E. coli strain BL21(DE3) was transformed by the assembly products and selected on the LB plates with 50mg/ml spectinomycin. Few single colonies were picked up and inoculated for overnight cultures. Plasmid purification was conducted by Plasmid Miniprep Kit (Zymo Research) and sequencing was conducted by Genewiz. A list of plasmids and strains was in table 3.2 and 3.3.

Table3.2 plasmids used in this study

Plasmid	Genotypes	Reference
pCDFDuet	$Spec^R$; CDF ori; P_{T7}:: MCS	Novagen
pDC91	Gen^R; colE1 ori; recombination fragment of xfp	This work
pDC158	$Spec^R$; NSI recombination site; P_{trc}:: xfp_*S. elongatus PCC7942*	This work
pDC238	$Spec^R$; CDF ori; P_{T7}:: xfp_*S. elongatus PCC7942(his tagged)*	This work
pDC268	$Spec^R$; CDF ori; P_{T7}:: cce_5158_*Cyanothece sp. ATCC 51142(his tagged)*	This work
pDC269	$Spec^R$; CDF ori; P_{T7}:: Oscil6304_5661_O. acuminate PCC6304*(his tagged)*	This work
pDC270	$Spec^R$; CDF ori; P_{T7}:: RTCIAT899_PC08935_R. tropici CIAT899*(his tagged)*	This work
pDC271	$Spec^R$; CDF ori; P_{T7}:: slr0453_*Synechocystis sp. PCC 6803(his tagged)*	This work
pDC272	$Spec^R$; CDF ori; P_{T7}:: BAD_0687_B.adolescentis ATCC 15703*(his tagged)*	This work
pDC273	$Spec^R$; CDF ori; P_{T7}:: Ava_0496_A. variabilis ATCC29413*(his tagged)*	This work
pDC274	$Spec^R$; CDF ori; P_{T7}:: Ava_4264_A. variabilis ATCC29413*(his tagged)*	This work
pDC279	$Spec^R$; CDF ori; P_{T7}:: xpkA_L. plantarum WCFS1 *(his tagged)*	This work
pDC305	$Spec^R$; CDF ori; P_{T7}:: Cyagr_2442_Cyanobium gracile PCC6307 *(his tagged)*	This work
pFD1000	$Spec^R$; CDF ori; P_{T7}:: cce_2225_*Cyanothece sp. ATCC 51142(his tagged)*	This work
pFD1001	$Spec^R$; CDF ori; P_{T7}:: cce_3607_*Cyanothece sp. ATCC 51142(his tagged)*	This work
pFD1002	$Spec^R$; CDF ori; P_{T7}:: sll0529_*Synechocystis sp. PCC 6803(his tagged)*	This work
pPL150	Amp^R; ColE1 ori; P_{LlacO1}:: CA_C1343_ C. acetobutylicum ATCC 824(his tagged)	This work

Table3.3 strain list

Strain	Relevant genotypes	Plasmid used for gene insertion	Reference
PCC7942	Wild-type *S. elongatus* PCC7942		Lab collection
ATCC51142	Wild-type Cyanothece sp ATCC51142		ATCC
DC18	P_{trc}::xfp, $spec^R$ inserted into NSI site	pDC158	This work
DC19	ΔXpk	pDC91	This work

Protein purification was performed by His-Spin Protein miniprep purification kit (Zymo Research), following the manufacturing's protocol. In general, single colonies with the correct sequences for xpk were inoculated for overnight cultures in LB with 50mg/ml spectinomycin. The overnight culture(200µl) was inoculated in 200ml LB media at 30°C. The 0.1mM IPTG was used when OD_{600} reached 0.6 and then incubated overnight at 30°C for protein expression. The cultures were harvested by centrifugation at 4300rpm at 4°C for 20mins and cell pellets were resuspended in 2ml Zymo his-binding buffers. The suspended cultures were homogenized by sonicator. After purification, Bradford assay was used to determine protein concentration.

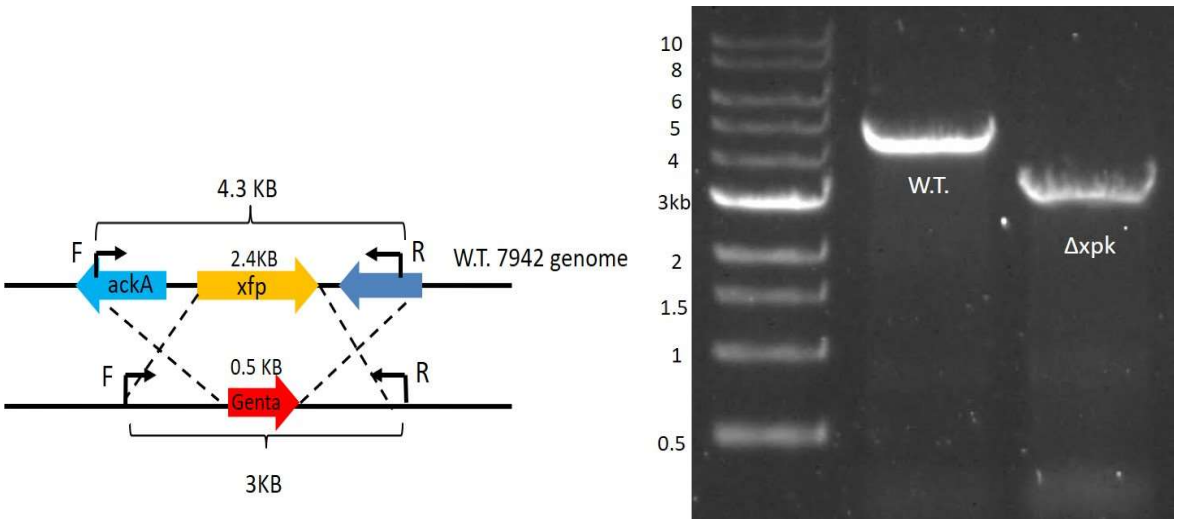
Xpk activity was measured by hydroxamate method. It was a colorimetric assay based on the reaction of AcP and hydroxyamine. The resulting hydroxamate then reacted with $FeCl_3$ to form

ferric hydroxamate complex, which showed brownish yellow color and can be detected at 505nm. In brief, a reaction mixture was 250µl including 1mM TPP, 5mM magnesium chloride in 50mM MES buffer (pH6). Different concentrations of Pi, F6P, R5P and effectors such as ATP, ADP, AMP were used. Xu5P activity was measured by converting R5P into Xu5P with excessive amounts of rpi and rpe in the reaction mixture. The reaction was incubated at 40°C and started upon adding purified Xpk. Each time point was recorded by taking out 40µl of reaction mixtures, which was quenched by mixing with 60µl of 2M hydroxyamine at 40°C for 10mins on a 96-well plate shaker and then following by adding 40µl of 15% trichloroacetic acid, 40µl of 25% HCl and 40µl of 3% $FeCl_3$ in 0.1M HCl at room temperature. The final mixture was measured at 505nm.

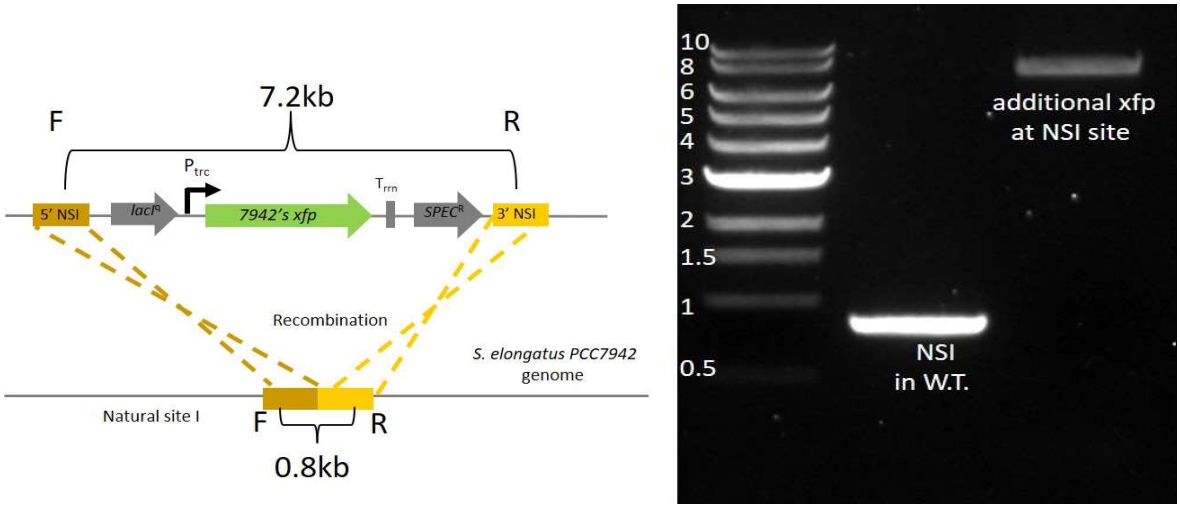
3.2.3 Strain construction and transformation

For constructing Δxpk strain, native xpk in PCC7942 were knocked out by replacing with a selection marker, gentamicin. Homologous flanking sequences of 1000bps on both sides were cloned from the genomes. For constructing xpk O.E. strain, additional copy of native xpk was introduced at neutral site I (NSI) on the PCC7942 genome. W.T. PCC7942 was transformed by incubating cells with plasmids overnight in the dark. The cells were spread on BG11 plates supplemented with 5µg/ml gentamicin for the knockout or 20µg/ml spectinomycin for the O.E. strain. The resulting single colonies were streaked on BG11 plates. Segregation is checked by colony PCR.

Colony PCR: Δ*xpk* in PCC7942



Colony PCR: additional xpk at NSI site in PCC7942



3.2.4 RNA extraction and RT-PCR

Samples were taken after 7 days of day(12hr) and night(12hr) cycles. Each sample was 800µl. RNA extraction was conducted by RiboPure RNA extraction kit (Invitrogen), following the

manufacturing's protocol. The isolated RNA products were further treated by DNase I (Invitrogen) at 37°C for 60mins. The reagents for reverse transcriptase PCR was iTaq Universal SYBR Green One Step kit (Bio Rad). NCBI primer-blast-tool was used to generate specific primers for the Xpk and 16s RNA in PCC7942 and *Cyanothece* ATCC51142 respectively. Primer sequence information is in the following table 3.4. RT-PCR was conducted by the following conditions: 50°C for 10 min, 95°C for 1 min, 40 cycles of 95°C for 10s and 60°C for 30s. The melting curve was obtained from 65°C to 95°C by the increase of 0.5°C. The cycle threshold (Ct) value was acquired and analyzed by the CFX Manager software (BIO-RAD).

Table 3.4 Primer list for rt-PCR

Target gene	Primer sequence (5'->3')	Reference
Xpk in 7942	F: ACAATCACAACAACTTGCAC	This work
	R: TCGTGGATTTGATCCTTGAG	This work
16s RNA in 7942	F: GGAATCGCTAGTAATCGCA	(21)
	R: GCTACCTTGTTACGACTTCA	(21)
Xpk(cce_5184) in 51142	F: ATCTGCAAAAATGAAGTGCC	This work
	R: CAGTTGACATCAGGAGGAAA	This work
Xpk(cce_3607) in 51142	F: GAACCAGATGAACCTGATGT	This work
	R: GTATCAAAATCCCGATCCGA	This work
16s RNA in 51142	F: TGAAGGAGGAATCGCTAGTA	This work
	R: GCTACCTTGTTACGACTTCA	This work

3.2.5 Spot assay

In dim light room (<0μE/m^2s), samples were taken from cultures under dark and anaerobic conditions from day zero, day one and then every other day until day7. As cells were sedimented on the bottom, we gently shake the cultures to make it homogenous before taking out 100μl each time by needle-syringe. Serial dilutions were prepared from 10^0, 10^{-1}, 10^{-2}. 10^{-3}, 10^{-4} to10^{-5}. Each spot is 2μl and dilution solution is the same as their original conditions. Spotted plates were incubated for five days under light condition (50μE/m^2s) and then recorded by photos.

3.2.6 Metabolite extraction

Samples were taken on day 10 in continuous light condition from 50ml cultures. Each sample contained cell numbers equal to OD10. In dim light room (<0μE/m^2s), samples were taken from parallel cultures under dark and anaerobic conditions. After sampling, cells were loaded on the membrane (EMD Millipore), which was connected to a vacuum system. The reactions were quenched in a pre-cooled well(-20°C) with 400μl extraction solution (methanol:acetonitrile:H_2O,40:40:20 by volume)(21) for 10mins on ice. Additional 100μl of extraction solution was used as the 2nd extraction to collect the residual metabolites in the well. Clear supernatants were obtained by centrifugation at 3500rpm at 4°C for 10mins. Samples were analyzed at UCLA Metabolomics Center.

3.2.7 HPLC measurement

An Agilent HPLC system with UV-detector and refractive index (RI) detector was performed to measure acetate, lactate and sugar content in the samples. The column was Aminex HPX-87H (Bio Rad). The mobile phase was 30mM H_2SO_4 and the flow rate was 0.4ml/min.

3.2.8 Glycogen measurement

Glycogen was measured by using glycogen colorimetric assay kit (BioVision). Samples were taken in dim light room. Each sample contained cell numbers equal to OD 1. After centrifugation at 13000rpm at 4°C for 10mins, cells pellets were resuspended in autoclaved water. The suspension was homogenized by bead beater for 10 mins. The homogenates were boiled in a boiling water bath for 5mins to inactive enzyme activity. The boiled samples were put on ice and centrifuged at 13000rpm for 5mins at 4°C to remove the insoluble parts. The clear supernatants were used to measure glycogen content, following the manufacturing's protocol.

3.2.9 13C bicarbonate measurement

200µl of samples were taken from O.E. Xpk cultures after IPTG induction day 1 and day 2 in light conditions. The samples were centrifuged at 4300rpm for 10 mins at room temperature. The clear supernatants were diluted 5 folds by autoclaved H_2O. The diluted samples were mixed with 5% deuterium oxide (v/v). The final samples were analyzed by NMR600 at UCLA-DOE facility center.

3.3 Results and discussion

3.3.1 Characterization of putative XPK from different bacteria

According to phylogenetic analysis (5), bacterial Xpk can be divided into three groups: Group 1 contains Xpk from fungi and bacteria such as bifidobacteria, cyanobacteria, rhizobia and lactobacillus; Group 2 contains Xpk from proteobacteria; Group 3 contains Xpk from other cyanobacteria. Although cyanobacteria are known to have more than one copy of putative *xpk* sequences in the genomes, little information was available to support their Xpk activity. Therefore, we cloned the putative *xpk* genes based on sequence information from BIOCYC and KEGG and purified the gene products for enzyme assay. Our results indicated that PCC7942 *xpk* annotated as SYNPCC7942_2080 has both Xu5P and F6P activity. The activity was 4-fold higher for Xu5P than for F6P under unsaturated conditions (Fig. 3.5ABCD), while 1.5-fold higher under saturation conditions. The pseudo-Km for Xu5P was 7.68mM, which we use excess amounts of ribose-5-phosphate isomerase (rpi) and ribulose-phosphate epimerase (rpe) to convert R5P into Xu5P. The Km for F6P was 17.7mM. Interestingly, we found that PCC7942 Xpk was strongly inhibited by ATP (Fig. 3.5ABCD). The activity dropped to 20% by 1mM ATP compared to no effector condition.

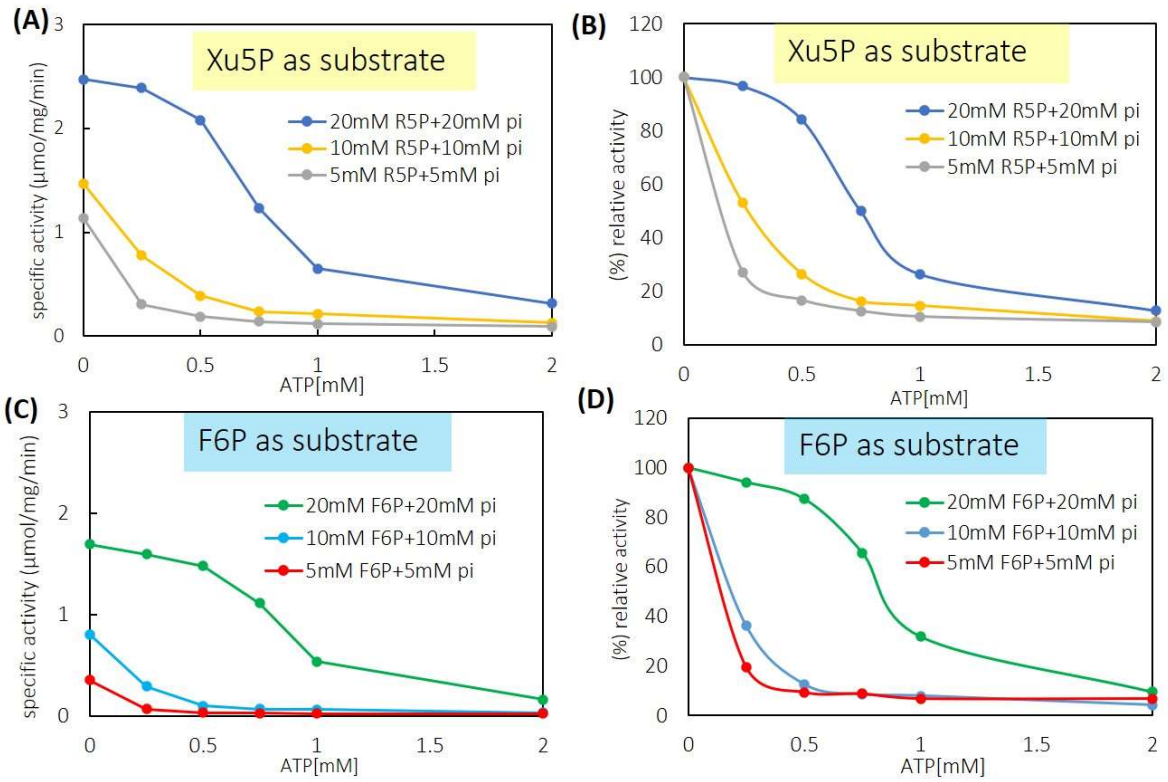


Fig.3.5 Enzyme assay by purified phosphoketolase from S. *elongatus* PCC7942. (A) specific activity using Xu5P as the substrate by using ribose-5-phosphate isomerase and ribulouse-5-phosphate epimerase to convert R5P into Xu5P at different ATP concentrations. (B) relative activity re-plotted from (A). (C) specific activity using F6P as the substrate. (D) relative activity re-plotted from (C).

Among all the cyanobacterial Xpk we examined, Group 1 Xpk's all had Xu5P activity while Group 3 had no Xu5P or F6P activity. ATP inhibition property is conserved among all cyanobacteria tested including *Synechocystis sp.* PCC6803, *Cyanobium gracile* PCC6307, *Cyanothece* ATCC51142, *Oscillatoria acuminata* PCC 6304, *Anabaena variabilis* ATCC29413 and *Nostoc sp.* PCC7120 (Fig. 3.6AB). Furthermore, we found that ADP is a strong activator for Xpk from nitrogen-fixing cyanobacteria *Cyanothece* ATCC51142, suggesting that Xpk played a more important role in energy regulation in nitrogen fixation. The non-heterocyst-forming cyanobacteria showed more pronounced ADP activation than the heterocyst-forming organisms (Fig. 3.6B). AMP did not show any significant effect except for the Xpk from *Nostoc sp.*

PCC7120, which exhibited AMP inhibition by 25%. On the other hand, non-cyanobacteria Xpk including *bifidobacterim*, *clostridium* and *lactobacillus* were insensitive to ATP, ADP or AMP except for the Xpk from *rhizobia*, which was also inhibited by ATP, suggesting its enhanced role in energy regulation in nitrogen fixation (Fig. 3.6C).

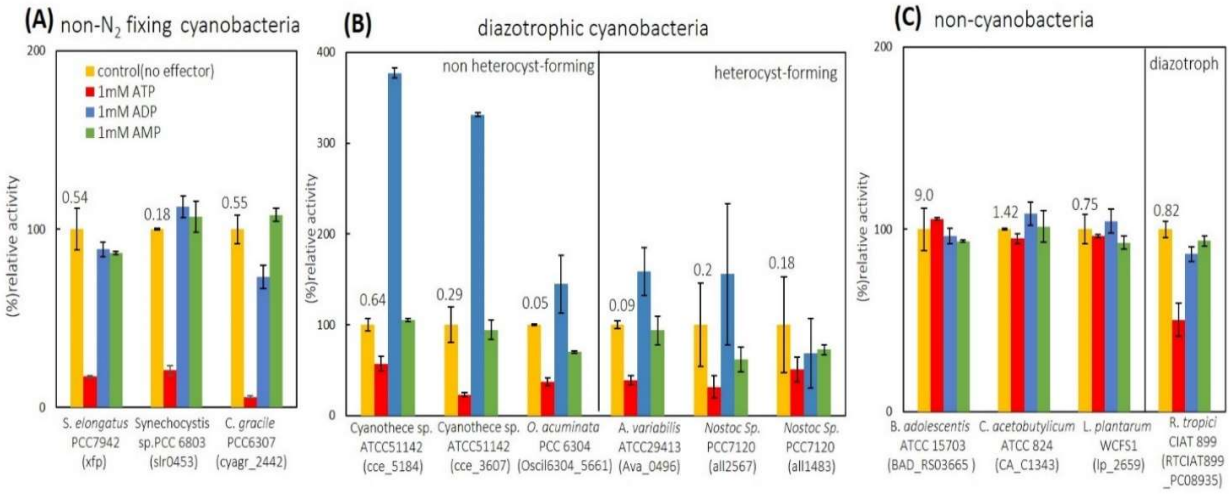


Fig.3.6 Surveying the properties of xpk from various bacteria. Relative activity of 1mM ATP, 1mM ADP and 1mM AMP to control (no effector). The number above each control indicates specific activity (μmol/mg/min). (A) non-N_2 fixing cyanobacteria (B) N_2-fixing cyanobacteria (C) other bacteria

3.3.2 XPK are dusked peak genes in PCC7942 and *Cyanothece* ATCC51142

Cyanobacteria are the only prokaryotes known to possess a robust circadian clock [6]. It is a self-bolstering oscillation, which enables them to adapt specific physiological functions to the correct time. For example, to accommodate two incompatible processes: carbon fixation and nitrogen fixation, in the unicellular diazotrophic *Cyanothece* ATCC51142, transcriptions of RubisCO and nitrogenase are temporally separated [7]. RubisCO reaches peak at the beginning of the day, while oxygen-sensitive nitrogenase reaches peak at the beginning of the night. The timing when transcription reaches peaks shows the preparedness in anticipation of the coming events. Our results indicated that *xpk* in PCC7942 and *Cyanothece* ATCC51142 were dusk-peaked genes in

day-night (12hr/12hr) cycle grown cultures (Fig. 3.7BCD), suggesting their potential roles in dark conditions. Knocking out *xpk* in PCC7942 did not affect growth under this condition (Fig. 3.7A) and all other conditions tested. Acetate production was less than 0.2mM in dark cycle in both W.T. strain and *Δxpk* strain.

One explanation is that ATP might be still high enough to inhibit Xpk in dark cycle due to respiratory activity. Unfortunately, we are unable to knock out Xpk in *Cyanothece* ATCC51142 due to lack of reported approaches available for transformation (8).

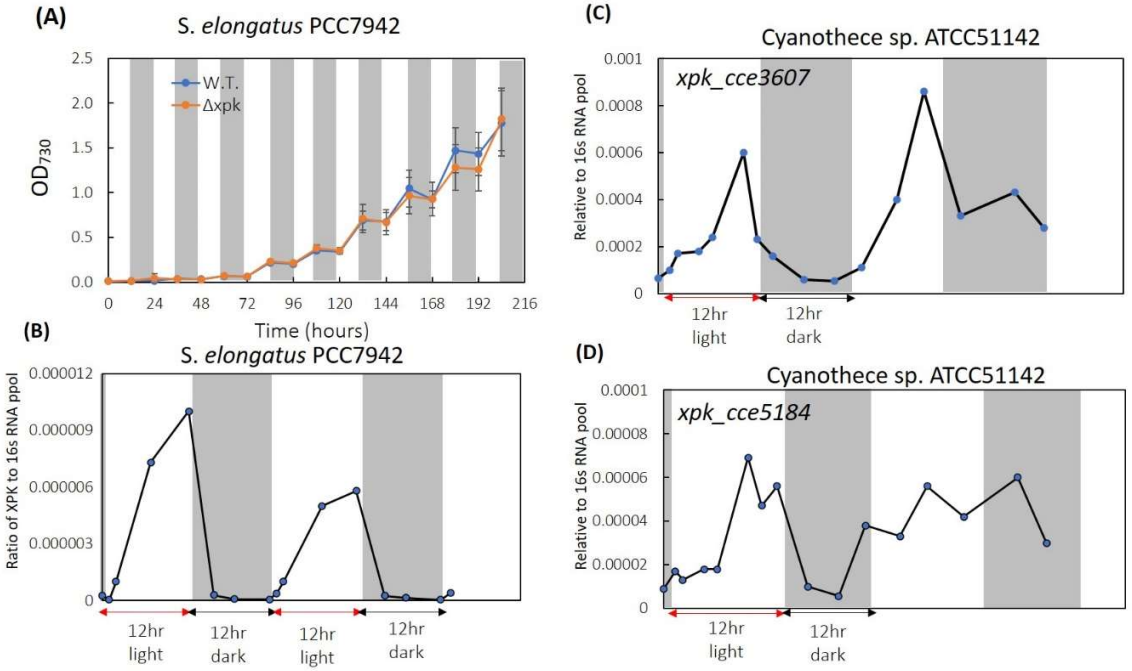


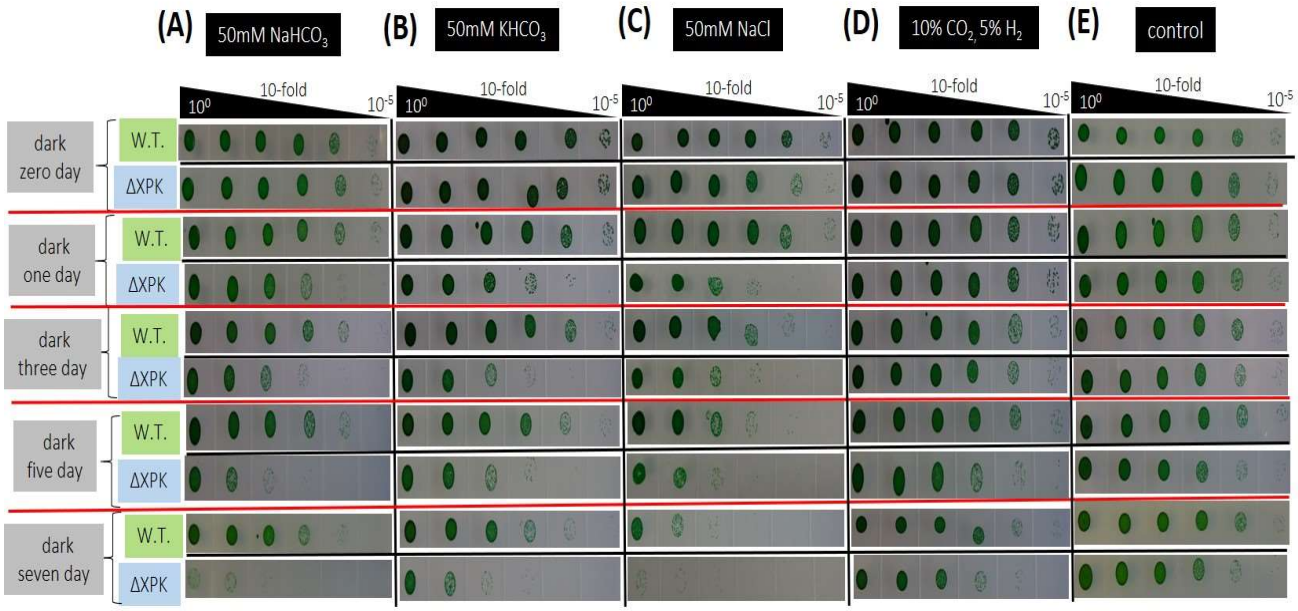
Fig.3.7 (A)Growth curves of W.T. and Δxpk mutant under ambient CO_2 condition with day and night cycle(12hr/12hr). Transcriptional kinetics of xpk in PCC7942 under ambient air and Cyanothece 51142 under ambient air and N_2 fixing condition in LD (12hr/12hr) conditions. Dark period is in grey. (B)xpk in PCC7942 (C) *xpk_cce3607* (D) *xpk_cce5184.*

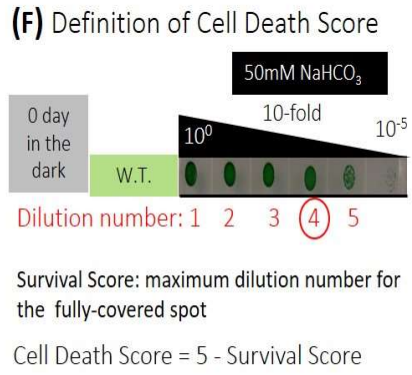
3.3.3 XPK is important in response to osmotic shock in high OD cultures under dark anaerobic conditions

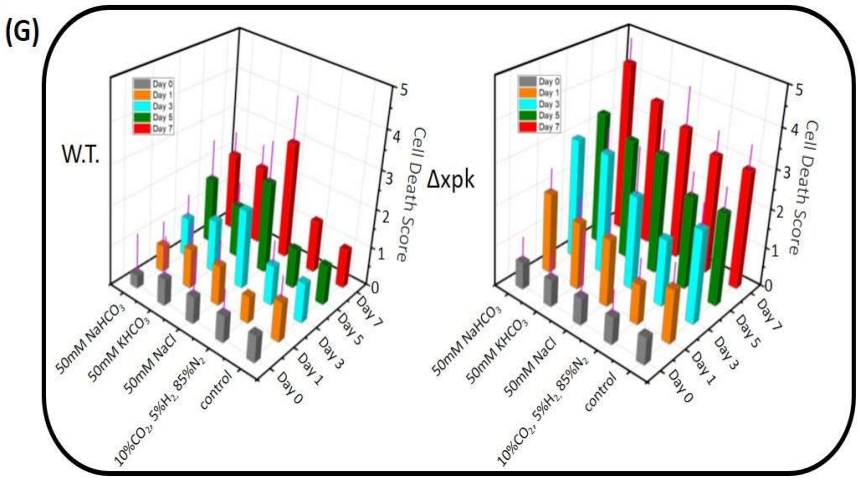
The ATP inhibition and dusk-peaked expression suggested that Xpk is important under low ATP and dark conditions. In dark condition, PCC7942 uses glycogen as an energy and carbon source to produce ATP through respiration [9]. Without oxygen, acetate production becomes the major pathway for ATP production. We therefore hypothesize that Xpk is important in dark, anaerobic survival and that Xpk is responsible for ATP production via the glycogen-acetate pathway under this condition. Therefore, we compared W.T. and *Δxpk* cell survival and acetate production under dark, anaerobic and high cell density conditions. The high cell density conditions will drain local oxygen and help achieving oxygen starvation. Additionally, acetate production in high cell density conditions will be more pronounced and can be used as a readout of Xpk activity. To generate more stress to the cells, we added 50 mM of NaCl, $NaHCO_3$, or $KHCO_3$. Additionally, 10%CO_2, 5%H_2 and 85%N_2 in headspace were used as one of the conditions to compare with the control with headspace filled by 100% nitrogen without additional salts.

Our results indicated that the viability of *Δxpk* decreased greatly with osmotic effectors such as 50mM $NaHCO_3$, or $KHCO_3$, or NaCl after incubation in continuous dark condition (Fig. 3.8ABC). In contrast, significant difference was not observed under no salt conditions (Fig.3.8DE). Summary of viability was shown in Fig.3.8G. Interestingly, glucose secretion (Fig.3.8H) was observed in the *Δxpk* strain as a result of incomplete glycogen degradation (Fig.3.9). Acetate was not detectable in *Δxpk* strain (Fig. 3.9I). Metabolomics analysis (Fig.3.10) showed that ATP was barely detectable in the *Δxpk* strain and sugar phosphates such as R5P, H6P, S7P accumulated inside the cells. 13C $NaHCO_3$ consumption was measured as the formation of 13C 3PG(m+1) over (m+0) ratio. Our results indicated that W.T. incorporates more 13C $NaHCO_3$ than the *Δxpk* strain (Fig. 3.11), possibly due to the higher ATP pool able to produce more substrate

RuBP for RubisCO. The metabolite pools such as 3PG, PEP and acetyl-CoA were higher in W.T. than *Δxpk* strain except for pyruvate.







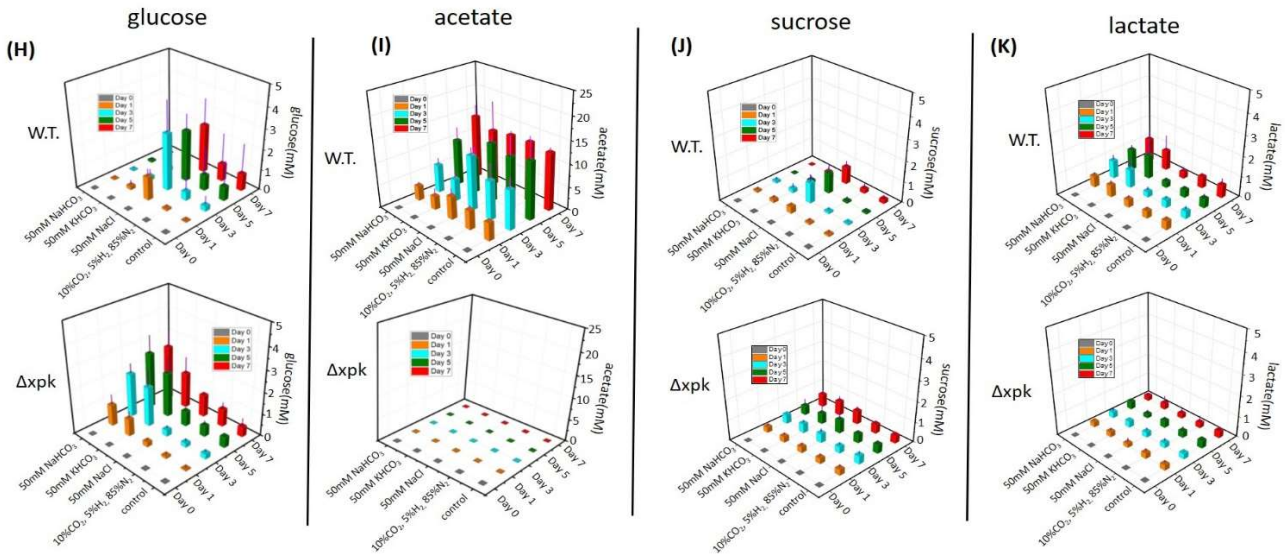


Fig.3.8 Characterization of W.T. and Δxpk mutant with different stressors under dark and anaerobic condition and summary of productions. All conditions contain 20mM HEPES in BG11 to maintain pH. Except for the condition with 10%CO_2, 5%H_2 and 85%N_2 in the headspace, all the others are purged by 100% N_2. Control is without additional salts in BG11. Spot assays on solid BG11 agar plates (A) 50mM $NaHCO_3$ (B)50mM $KHCO_3$ (C)50mM NaCl (D)10%CO_2, 5%H_2, 85%N_2 (E)control (F) definition of cell death score (G) summary of viability by cell death score. Production in the supernatants (H) glucose (I) acetate (J) sucrose(K) lactate

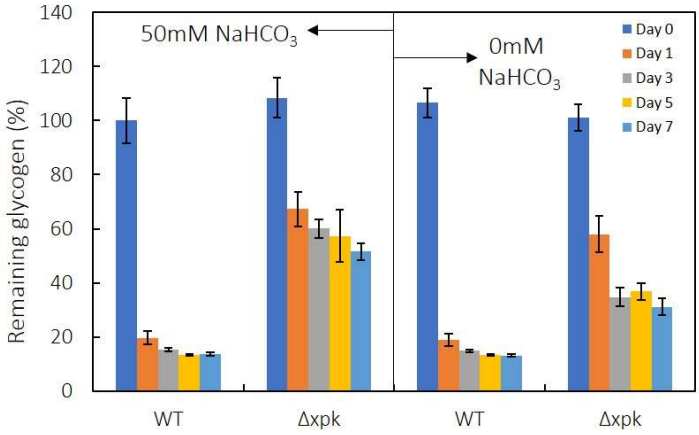


Fig.3.9 Remaining glycogen (%) in W.T. and Δxpk mutant respectively under dark and anaerobic condition.

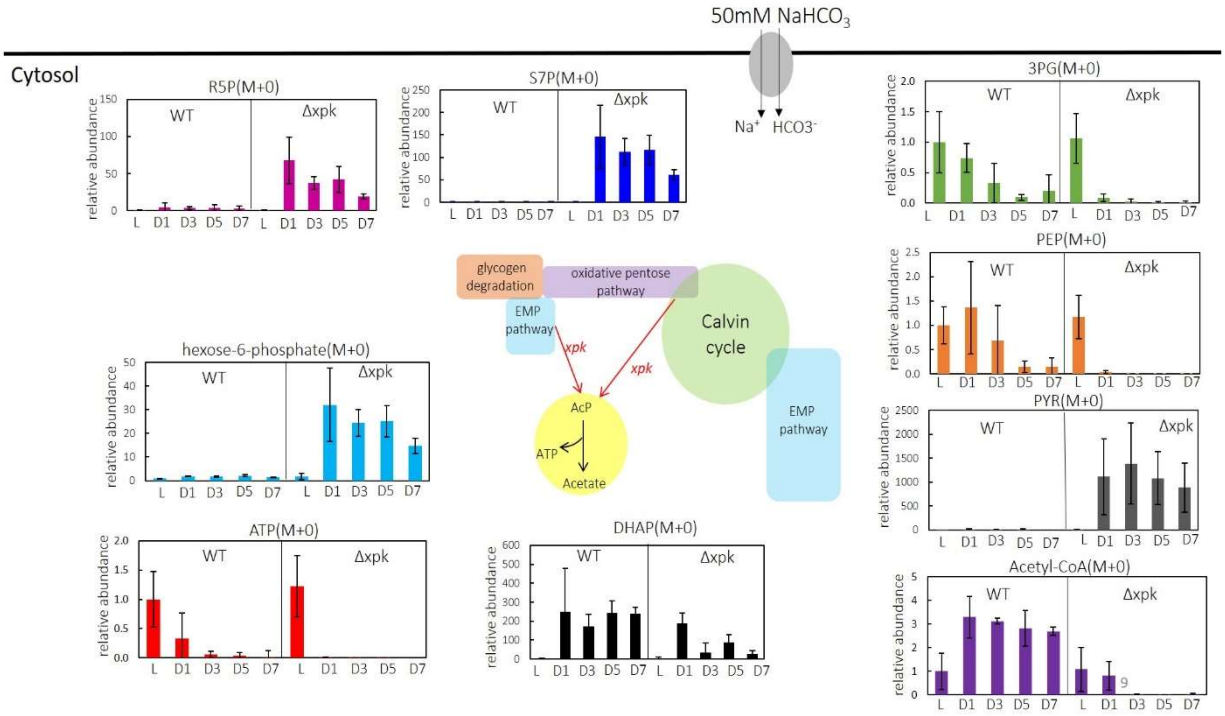


Fig.3.10 Relative abundance of metabolites in W.T. and Δxpk in light conditions and dark and anaerobic conditions. All the pool sizes are relative to W.T. in light conditions. L is light condition. D1 is day 1 in dark condition in high OD cultures under anaerobic conditions etc.

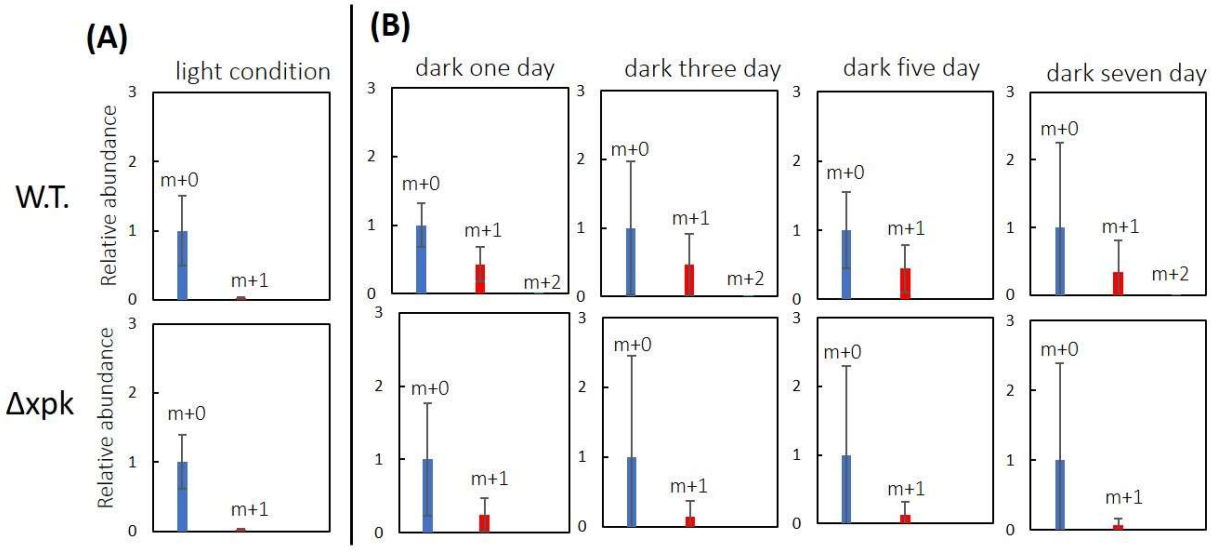


Fig. 3.11(A) natural abundance of 3PG ratio(m+1/m+0) in light condition in W.T. and Δxpk (B) 13C tracer from $NaHCO_3$ to 3PG via RubisCO using W.T. and Δxpk respectively under dark and anaerobic conditions.

Furthermore, the viability of W.T. also greatly decreased with 50mM NaCl and significant glucose secretion was observed (Fig. 3.8CH). NaCl is a well-known osmotic effector to fresh-water cyanobacteria. When treating PCC7942 with high concentrations of NaCl in light condition, growth rate decreased [10] while synthesizing non-ionic osmolytes such as sucrose from photosynthesis. Sucrose acted as an osmoprotectant against the outside osmotic shock [11]. In high OD cultures under dark and anaerobic condition, the sucrose production (Fig. 3.8J) as protection mechanism is also triggered by NaCl. The carbon source for sucrose biosynthesis came from glycogen degradation. Part of sucrose synthesis required energy (UTP) to produce UDP-α-glucose from G1P. The other part competed with Xpk for substrate F6P. However, such protection mechanism became detrimental under this condition because glycogen was not only used for energy production but also involved in sucrose synthesis. After constant dark condition, the energy required for cell maintenance was probably depleted and resulted in decreased viability. Interestingly, in most of the conditions we tested, glucose was secreted more than sucrose, particularly in the *Δxpk* strain (Fig. 4H). Secretion of glucose presumably was a result of ATP depletion, which prevented the formation of G6P from glucose to enter the glycogen-acetate pathway. This effect may be explored for glucose production from CO_2 by cyanobacteria.

3.3.4 Overexpression (O.E.) of native Xpk in PCC7942

To investigate the effect of Xpk activity to growth under continuous light condition, we introduced one more copy of native Xpk in the PCC7942 chromosome. The additional copy was integrated at neutral site I (NSI) and controlled by an IPTG inducible promoter. Our results indicate that overexpression of Xpk inhibits growth and reduces bicarbonate incorporation (Fig.3.12), suggesting that overexpressed Xpk can be used as a brake to stop carbon fixation by competing with RubisCO for the substrate precursor Xu5P. This result was consistent with our previous

report obtained by overexpression of bifidobacterial Xpk (12). Moreover, using Ensemble Modeling for Robustness Analysis (EMRA) (13), we found that the overexpression of Xpk forms a kinetic trap in the CBB cycle, and drains the substrates from a metabolic branching point within the cyclic pathway. The perturbation by overexpressed Xpk caused instability in the CBB cycle. This result was consistent with our previous prediction using EMRA.

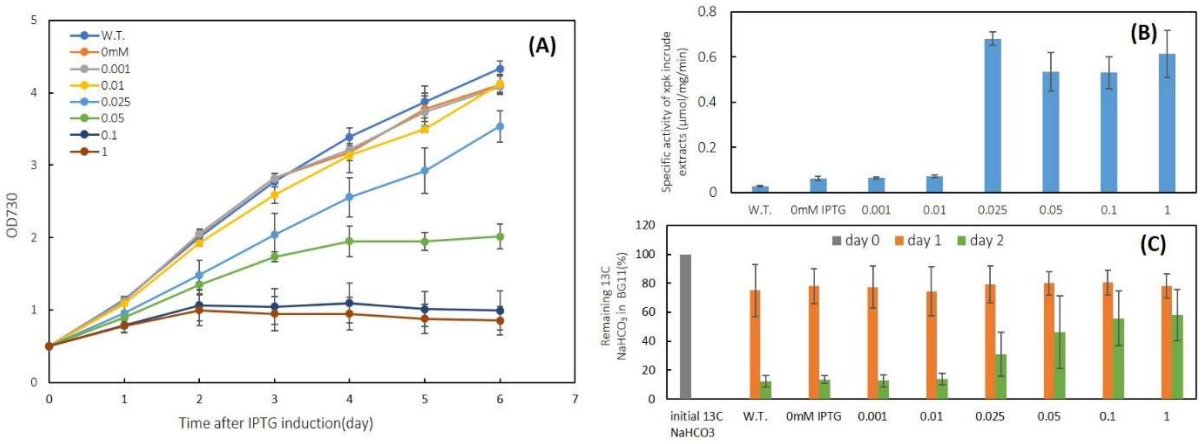


Fig.3.12 Overexpression of native phosphoketolase(*xpk*) in the Cyanobacterium S. *elongatus* PCC7942 in light condition(50μE/m^2s). (A) growth curves of wild type and engineered S. *elongatus* PCC7942 expressing native *xpk* upon different concentrations of IPTG induction at the beginning (B) specific activity of *xpk* in crude extracts in wild type and engineered S. *elongatus* PCC7942 expressing native *xpk* by different concentrations of IPTG induction. *Xpk* activities are measured day 6 after IPTG induction (C) remaining 13C bicarbonate in BG11 in the first two days after IPTG induction. Initial 13C $NaHCO_3$ concentration is 50mM.

3.3.5 Discussion

Xpk is a widespread enzyme found in many types of microbes (14,15,16). It is best characterized in gastrointestinal *Bifidobacteria* as the key enzyme in the bifid shunt (17). It is also a key enzyme in the synthetic NOG, which conserves carbon when converting various sugar to C2 intermediates, bypassing pyruvate dehydrogenase complex or pyruvate-formate lyase. Interestingly, cyanobacteria have all the enzymes required for NOG, and Xpk was suspected to function in this "natural" NOG to conserve carbon during photosynthesis. However, we found that overexpression of Xpk stops photosynthesis (12) by draining the CBB pathway intermediate,

Xu5P. In addition, its expression level is relatively low and peaked at dusk, unlikely to function in photosynthetic conditions. Although a recent report using isotopic nonstationary 13C metabolic flux analysis in the fast-growing strain *Synechococcus elongatus* UTEX2973 predicts that this strain entirely depended on the Xpk pathway instead of pyruvate dehydrogenase complex (PDHc) for acetyl-CoA biosynthesis under autotrophic condition (18), further investigations such as gene knockout might be needed to support the prediction.

Interestingly, we found that cyanobacterial Xpk's are all inhibited by ATP, in contrast to non-cyanobacterial Xpk. Furthermore, Xpk's in nitrogen-fixing cyanobacteria are further activated by ADP. These properties strongly suggest that cyanobacterial Xpk's serves as an energy regulator. Interestingly, the ADP activation effect is more pronounced in non-heterocyst forming cyanobacteria, such as *Cyanothece*, than hetercyste-forming cyanobacteria, such as *Nostoc sp.* These results are consistent with the fact that non-heterocyst forming diazotrophic cyanobacteria require more stringent energy regulation.

We then search for conditions where Xpk's physiological role can be observed. Marine cyanobacteria often encounter dynamic light conditions in natural environment. One type of changes follows the rise and fall of sunlight as a 12hr light and 12hr dark pattern. The other type of light shortage occurs by shading of objects in water. In dark conditions, cyanobacteria use stored glycogen as the energy source and produces ATP through respiration. In deep sea or other dark anaerobic limiting conditions, both ATP-producing photosynthesis and respiration are not possible. Cyanobacteria then use Xpk in the glycogen-acetate pathway to produce ATP. Indeed, the *⊿xpk* strain of PCC7942 did not produce acetate and showed a decreased survival rate under dark anaerobic conditions. Therefore, we conclude that under dark and anaerobic conditions, Xpk is the major ATP production pathway in PCC7942 and serves as an important irreversible step to

pull out sugar phosphates from the CBB cycle. Xpk in the diazotrophic *Cyanothece* ATCC51142 might play an enhanced role in energy regulation in nitrogen fixation because nitrogenase is an oxygen-sensitive enzyme and nitrogen fixation usually occurs in dark and low O_2 conditions where it allows nitrogenase to be active. The condition is similar to our findings in PCC7942.

Glycogen is one of the major carbon sinks where cyanobacteria deposit the fixed carbon in light conditions. When cyanobacteria encounter darkness or stressed conditions such as nitrogen starvation, they start to use the storage glycogen and produce energy for survival. Knocking out one of the glycogen synthesis genes, glucose-1-phosphate adenylyltransferase *(glgC*), impaired growth in PCC7942 in light condition and the growth defect can be rescued by introduction of isobutanol pathway as the alternative carbon sink (19). Moreover, knocking out glycogen phosphorylation (*glgP2*) in *Synechocystis sp.* PCC6803 impaired the ability of chlorotic cells to exit dormancy (20). Our results show that Xpk is an important pathway in the glycogen degradation and knocking out *xpk* in PCC7942 results in incomplete glycogen degradation under dark anaerobic condition.

Compared to plants, cyanobacteria have a shorter growth cycle and are more resistant to harsh conditions. Glucose is the simplest monosaccharide and one of the most important feedstocks. It is usually produced from degradation of starch or glucosides following by enzymatic degradation or acid conversion or both. Our work can skip such processes and directly harvest them under dark and anaerobic condition, which is relatively straightforward to scale-up.

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3.5 Supplementary information

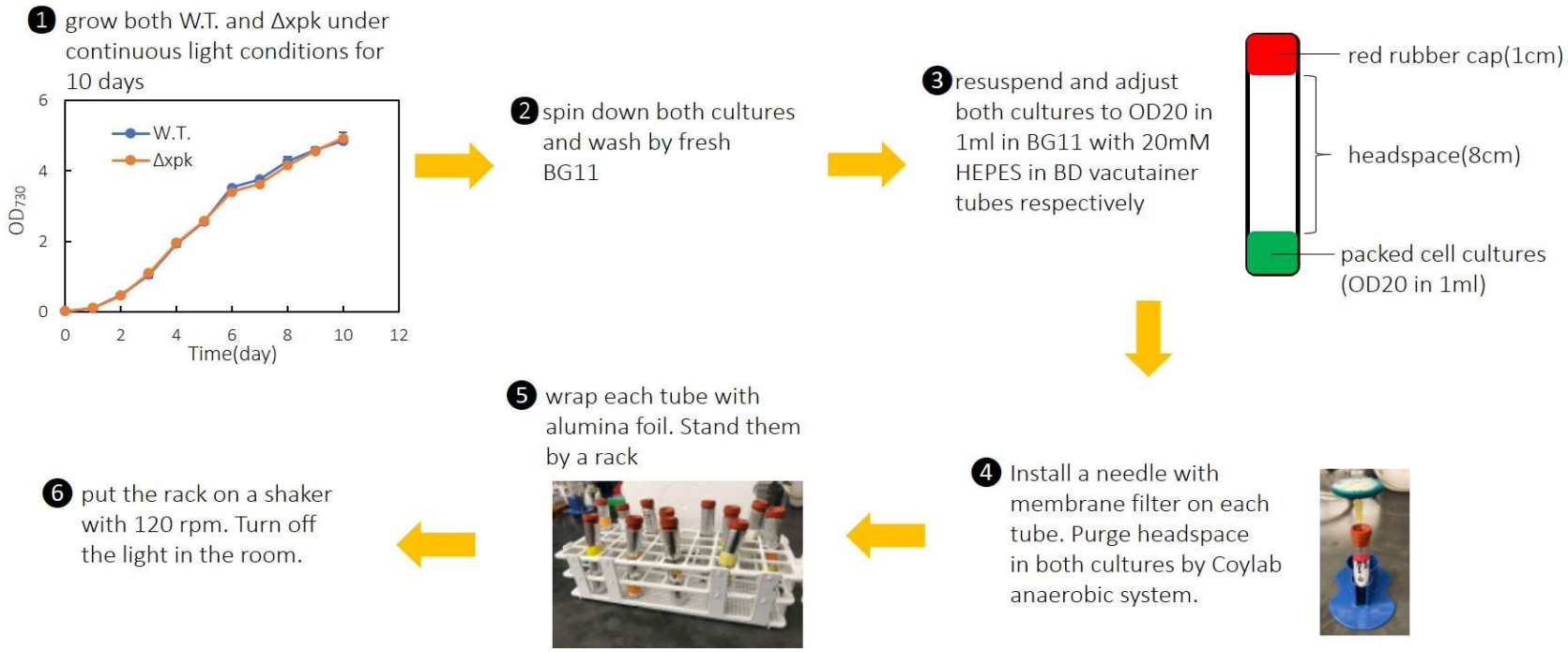

Fig. Preparation procedure for cultures under dark and anaerobic condition

4. Future work

Unfortunately, due to covid-19 and the closure of Liao lab at UCLA, I cannot make very much progress on increasing glucose secretion under dark and anaerobic condition. Here are some potential directions that might lead to higher glucose secretions in PCC7942 (Fig. 4.1). Hopefully, the results will benefit our society.

1. Knocking out glycogen consumption competing pathway: *glgP*

2. Knocking our glucose kinase: *glk*

3. Overexpression of glycogen debranching enzyme and/or 4-alpha-glucanotransferase: *glgX* and *malQ*

4. Overexpression of glucose-1-phosphate adenylyltransferase to increase glycogen pool in light condition: *glgC*

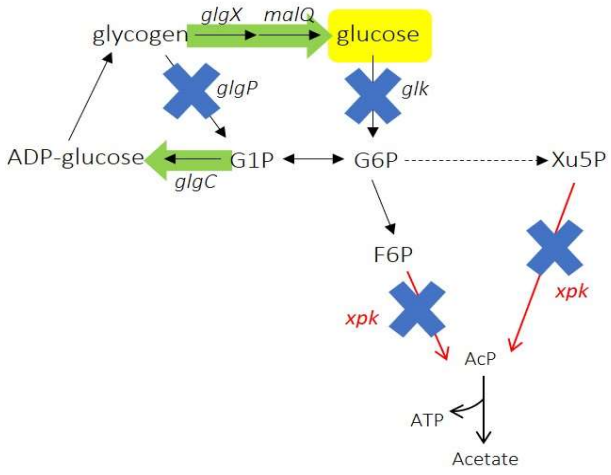

Fig. 4.1 Proposed strategy for improved glucose secretion under dark and anaerobic condition