EFFECT OF TRANSHYDROGENASE AND PHOSPHOGLUCOSE ISOMERASE KNOCKOUTS AND CITRATE SYNTHASE VARIANTS ON GROWTH AND PRODUCT FORMATION IN *ESCHERICHIA COLI*

by

HAYDEN SCOTT LIPPELMAN

(Under the Direction of Mark Andrew Eiteman)

ABSTRACT

NADPH and NADH serve as electron carriers for various reactions in organisms. Transhydrogenases provide cells to interchange these electron carriers. *Escherichia coli* has two transhydrogenases, PntAB and SthA. In this study, *pgi*, coding for phosphoglucose isomerase, and *sthA*, coding for soluble transhydrogenase, were deleted in *Escherichia coli* to direct more carbon into the pentose phosphate pathway and increase NADPH production. These strains were examined for their growth on glucose, fructose or sucrose. A 3-hydroxybutyrate (3HB) pathway was introduced into *Escherichia coli*, and experiments were completed to determine whether *pgi* or *sthA* deletions affected 3HB formation. In addition, F383M and M327S substitutions in citrate synthase, coded by the *gltA* gene, were evaluated for their effect on 3HB formation. The Δ*sthA* Δ*pgi* strain grown on glucose generated 3HB at a yield of 0.057±0.001 g/g, greater than the wild-type strain. The F383M variants were generally able to produce more 3HB than M372S variants.

INDEX WORDS: *Escherichia coli*, deletions, variants, acetyl-CoA, NADPH, SthA, GltA, PGI, Glucose, Fructose, Sucrose, 3-hydroxybutyrate, Acetate, Batch process

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INTRODUCTION AND LITERATURE REVIEW

Organisms rely on cofactors such as ATP, CoASH, NADH, and NADPH to metabolize carbon sources. In heterotrophs, carbon sources provide energy needed for anabolic reactions which synthesize the biomolecules needed for cellular processes (Shimizu, 2013). Cofactors facilitate biochemical reactions by serving as intermediaries to capture energy or electrons. NAD(H) and NADP(H) are key electron carriers in microbes such as *Escherichia coli*, and they capture electrons which are generated during the oxidation of carbon sources such as glucose and glycerol, and these electrons (as the reduced forms NADH and NADPH) are available to other cellular processes. Although equivalent in terms of the currency of redox potential, NADPH is primarily found in anabolic reactions whereas NADH is associated with catabolic reactions and energy metabolism. Central metabolism comprises the pathways through which most of the carbon flows, and in *E. coli* during growth on glucose and other common saccharides, central metabolism comprises three pathways: the Embden-Meyerhof-Parnas (EMP) pathway or glycolysis, the tricarboxylic acid (TCA) cycle, and the pentose phosphate (PP) pathway. These pathways produce energy, cofactors, and precursor molecules leading to biochemicals needed to form cells (e.g., amino acids, fatty acids). The fluxes in these pathways are distributed to match the cell demands for energy, NADH/NADPH and precursor molecules. The growth rate of *E. coli* depends on the carbon source being used such as glucose, fructose, or sucrose.

Using glucose as the carbon source under aerobic conditions, *E. coli* directs about 70% of glucose towards glycolysis through the phosphotransferase system (PTS), phosphoglucose isomerase (PGI), and phosphofructokinase (PFKA), while 30% of the glucose goes to the PP

pathway (Haverkorn van Rijsewijk et al., 2011). *E. coli* and many other organisms use phosphoglucose isomerase in glycolysis to isomerize glucose-6-phosphate (glucose-6P) into fructose-6P (Canonaco et al., 2001). In either the EMP or the PP pathways, the carbon is channeled into pyruvate and acetyl-CoA which then enters the TCA cycle. During growth on glucose at a rate of 0.2 h^{-1} approximately 41% of the NADH is generated by glyceraldehyde-3P dehydrogenase, 28% by the conversion of pyruvate to acetyl-CoA and 31% within the TCA cycle (Zhao et al., 2004). NADPH is primarily generated by isocitrate dehydrogenase (64%) and the PP pathway (36%) (Zhao et al., 2004). In the PP pathway the *zwf* gene encodes glucose-6-phosphate dehydrogenase while the *gnd* gene encodes 6-phosphogluconate dehydrogenase that both generate NADPH (Rowley et al., 1991). The process of aerobic glucose metabolism forms acidic by-products, such as acetate. Acetate limits cell growth of *E. coli* in batch and fed-batch experiments (Luli and Strohl, 1990). One way to diminish levels of acetate in *E. coli* is by using a different carbon source, such as fructose (Aristidou et al., 1999).

E. coli utilizes alternative enzymes for the first steps of glycolysis when using fructose as a carbon source. Figure 1 shows fructose can be phosphorylated into fructose-6P by fructokinase (*mak*) or into fructose-1P by a fructose-specific PTS, (EIIBC components coded by *fruAB* gene). Fructose-1P is then converted to fructose-6P by 1-phosphofructokinase (*fruK*) (Fraenkel, 1968). *E. coli* thus uses glycolysis in the absence of glucose when fructose is the carbon source. Although much less research has focused on fructose metabolism in *E. coli* for the generation of biochemical products, fructose has been used as an alternative carbon source in place of glucose to diminish levels of acetate during aerobic batch growth, and fructose grown cultures had a higher cell density than glucose grown cultures (Aristidou et al., 1999).

Sucrose is another carbon source that *E. coli* can utilize for central metabolism. This disaccharide is abundant in sugarcane. Sugar beets are also an abundant source for sucrose, and 20% of the sugar beet weight is comprised of sucrose (Gruska et al., 2022). Only a few *E. coli* strains can metabolize sucrose, such as *E. coli* W and EC3132 (Mohamed et al., 2019). *E. coli* W is able to utilize sucrose because it expresses the *csc* regulon. CscA is sucrose hydrolase/invertase that converts sucrose into glucose and fructose. Because CscA is present both intracellularly and extracellularly (Erian et al., 2018), fructose and glucose can accumulate in the medium during growth on sucrose (Moxley and Eiteman, 2023), which each must subsequently enter the cell. CscB is a permease that transports sucrose inside the cell, and CscK is a fructokinase to generate fructose-6P (Sabri et al., 2013). Figure 1 shows that sucrose provides necessary components for glycolysis. In contrast to glucose metabolism, the metabolism of fructose bypasses PGI (Fig. 1) while sucrose metabolism essentially commences with an equimolar mixture of glucose and fructose.

E. coli and other bacteria express transhydrogenases to interconvert NAD(H) and NADP(H). These enzymes balance the consumption and production of NADPH and NADH and respond to changing cellular demands (Sauer et al., 2004). *E. coli* has two transhydrogenases to regulate this balance: PntAB, a membrane-bound, proton-translocating transhydrogenase encoded by *pntAB*, and SthA (or UdhA), a soluble transhydrogenase encoded by *sthA* (*udhA*) (Boonstra et al., 1999). SthA is a cytosolic, energy-independent transhydrogenase (Haverkorn van Rijsewijk et al., 2016). In *E. coli*, PntAB primarily catalyzes the reaction:

$$NADP + NADH \rightarrow NADPH + NAD$$

while SthA primarily catalyzes the reaction:

$$NADPH + NAD \rightarrow NADP + NADH$$

The NADPH/NADP and NADH/NAD balances are influenced by the cellular requirement for redox balance based on the carbon/energy source and the culture growth rate (Haverkorn van Rijsewijk et al., 2016). The transhydrogenases appear to play a greater role during maximum exponential growth of *E. coli* (0.67 h^{-1}) approximately 42% of the NADPH is generated by PntAB, while 37% is generated by the PP pathway and 21% is generated by isocitrate dehydrogenase in the TCA cycle (Sauer et al., 2004).

The membrane bound transhydrogenase PntAB provides *E. coli* a source of NADPH. The physiological role of this protein is to provide anabolic reductions by reducing NADP to NADPH with NADH (Fuhrer and Sauer, 2009). The growth rates of a wild-type, *pntAB*:Tn5 (deleted by an insertion mutation of a transposon with kanamycin resistance), Δ*zwf*, and Δ*zwf* Δ*pntAB*::Tn5 were compared under aerobic conditions with glucose as the sole carbon source: the wild-type strain had a growth rate of 0.68 h^{-1}, *pntAB*:Tn5 had an essentially identical growth rate of 0.65 h^{-1}, Δ*zwf* with 0.80 h^{-1} and Δ*zwf* Δ*pntAB*::Tn5 with 0.39 h^{-1} (Hanson and Rose, 1980). Zwf and PntAB both catalyze reactions which generate NADPH, and the deletion of both genes, but not either one alone, significantly affects the cell's ability to generate NADPH. The PntAB counterpart, SthA, converts NADPH/NAD into NADP/NADH, thereby allowing the cell to maintain the redox balance. An *E. coli* Δ*sthA* strain grown under aerobic conditions on glucose as the sole carbon source showed 50% of the NADPH-dependent transhydrogenase activity compared to the wild-type (Sauer et al., 2004). Deleting SthA increases the NADPH pool, while a deletion in phosphoglucose isomerase has been shown to increase NADPH.

A deletion in the *pgi* gene coding phosphoglucose isomerase has several effects on the physiology of *E. coli*. During growth of a Δ*pgi* strain on glucose, glucose-6P is directed into the PP pathway, resulting in the formation of more NADPH than is necessary for the cell (Canonaco

et al., 2001). As a consequence, a Δ*pgi* strain showed an increased activity of SthA compared to the wild-type strain (Canonaco et al., 2001), an observation attributed to a cellular response when excess NADPH is generated. SthA responds to and limits NADPH accumulation, and thus its presence could undermine approaches to elevate NADPH availability as a means to synthesize NADPH-dependent products. That is, any attempt to increase the cellular availability of NADPH could lead to increased expression of *sthA*, which would offset the attempted improvement in the NADPH pool sought. In contrast, the use of fructose or sucrose in a Δ*pgi* strain should show minimal growth defects because fructose is able to bypass phosphoglucose isomerase (Fig. 1).

While *pgi* is crucial for glycolysis when using glucose, another crucial enzyme for cellular metabolism is citrate synthase. *E. coli* and other bacteria express citrate synthase to convert acetyl-CoA and oxaloacetate into citrate as an entry into the TCA cycle (Fig. 1). Type II citrate synthase found in Gram-negative bacteria occurs as a hexamer and is allosterically inhibited by NADH (Duckworth et al., 2013). A *gltA* deletion facilitates increased formation of products derived from acetyl-CoA (Wu et al., 2019). However, a deletion of *gltA* prevents the growth of *E. coli* on glucose as the sole carbon source, and therefore a Δ*gltA* strain requires supplementation of glutamate or another biochemical entry into the TCA cycle to grow on glucose (Parimi et al., 2017).

Given the growth defects caused by a *gltA* deletion, another approach to modulating the flux of acetyl-CoA into the TCA cycle is by reducing the activity of *gltA*. One approach is by controlling the expression of *gltA* by modifying the promoter region (Park et al., 1994). Another approach is to alter the activity of the expressed citrate synthase directly by making targeted amino acid substitutions in the enzyme (Wu et al., 2020). Substitutions that alter the active site by affecting acetyl-CoA binding, such as F383M, have shown significant changes in metabolism

(Pereira et al., 1994). In a Δ*poxB* strain, the F383M showed a 28% decrease in growth rate but a 3.6-fold increase in acetate yield (Tovilla-Coutiño et al., 2020). Another study in a strain with multiple deletions, showed the F383M variant led to a 25% decrease in growth rate but a 15% increase in citramalate yield in shake flasks with 5 g/L glucose (Wu et al., 2020). The F383M variant increased the intracellular concentration of acetyl-CoA compared to the wildtype. Other citrate synthase substitutions have less impact on metabolism. For example, the M372S variant had no significant impact on the growth and an 80% increase in acetate yield (Tovilla-Coutiño et al., 2020).

Many introduced and synthetic pathways to products such as chiral alcohols, polymers, and antibiotics, require NADH or NADPH (Jan et al., 2013). An example of a biochemical requiring NADPH during its synthesis is (R) 3-hydroxy-butyrate (3HB). *E. coli* does not have native pathway to produce 3HB (Chanprateep, 2010). The IPTG-inducible plasmid (pnK5) expressing *phaA* and *phaB* from *Cupriavidus necator* has been used previously for butyrate formation (Kataoka et al., 2017). Considering the conversion of (R)-3-hydroxybutyryl-CoA to 3HB by a native *E. coli*, the stoichiometric reaction from acetyl-CoA is:

$$2\text{Acetyl-CoA} + \text{NADPH} \rightarrow\rightarrow\rightarrow 3\text{HB} + 2\ \text{CoA} + \text{NADP}$$

The reaction equation suggests that 3HB formation would be improved by increasing the availability of acetyl-CoA and/or NADPH. A deletion of the *pgi* when grown on glucose, would direct carbon utilization through the PP pathway to overproduce NADPH (Kabira and Shimizu, 2003). Knocking out *pgi* and *sthA* offers a means to force cells growing on glucose to generate more NADPH. Previous research shows that *sthA* is upregulated in *pgi* knockouts (Canonaco et al., 2001). Thus, knocking out *sthA* in a *pgi* knockout could limit the cells ability to restore NADPH concentration. One hypothesis of this work is that *E. coli* having knockouts in the *pgi*

and *sthA* genes would show elevated formation from glucose of 3HB, a product requiring NADPH in its synthesis.

The formation of 3HB also requires acetyl-CoA, which is the limiting reagent (Fig. 1). As noted previously, although a *gltA* knockout has increased the formation of acetyl-CoA derived products, this knockout prevents growth using glucose as the sole carbon source unless supplemented with glutamate (Parimi et al., 2017). Instead of deleting *gltA*, modulating *gltA* activity should improve 3HB formation. The introduction of a synthetic pathway which relies on acetyl-CoA as a precursor might relieve excess NADPH and acetyl-CoA caused by deletions or modifications of *pgi*, *sthA*, and *gltA*. Different carbon sources such as glucose, fructose, and sucrose can be studied. A *pgi* knockout would have limited effect on fructose because metabolism bypasses *pgi* (Fig. 1). In the case of sucrose, a portion of the molecule (glucose) would be directed to the PP pathway but a portion (fructose) would not (Fig. 1).

The goal of this study is to examine *E. coli* W, a strain that has native pathways to utilize sucrose. Comparisons are made between the wild-type strain W, W Δ*sthA*, W Δ*pgi* and W Δ*sthA* Δ*pgi*. In addition to wild type GltA, I also examine two GltA variants, GltA[F383M] and GltA[M372S]. As measured by impact on growth rate and acetate yield, GltA[F383M] is a relatively severe substitution mutation, while GltA[M372S] is a less severe substitution (Tovilla-Coutiño et al., 2020). The first goal is to compare the strains for growth on glucose, fructose, or sucrose as the sole carbon source. Strains with a *pgi* deletion are expected to show severe growth defects on glucose, but minimal growth defects on fructose or sucrose. Also, increasing citrate synthase mutation severity is expected to reduce growth rate on any carbon source.

The second goal is to use these strains to generate the NADPH-dependent product 3HB. Increasing mutation severity (wild-type GltA versus GltA[M372S] versus GltA[F383M]) is

expected to increase the yield of 3-HB. Furthermore, Δ*sthA*, Δ*pgi*, and Δ*sthA* Δ*pgi* strains will attain greater 3HB yields as a result of greater NADPH availability, but will show lower productivity (rate of 3HB generation) as a result of the mutations. The wild-type strain is expected to show no difference in 3HB yield when grown on glucose, fructose, or sucrose. Finally, fructose or sucrose as carbon sources compared to glucose will lead to greater rate of 3HB generation in Δ*pgi* strains.

MATERIALS AND METHODS

Strains

E. coli W strains were used in this study (Table 1). Gene knockouts were constructed with the λ-red recombinase system using the pKD46 plasmid to remove the target gene and integrate a Kan^R cassette into the genome (Datsenko and Wanner, 2000). The Kan^R cassette was removed by using pCP20, a flippase (FLP) recombinase plasmid with ampicillin resistance (Cherepanov and Wackernagel, 1995). Colony PCR was used to verify the integration and removal of Kan^R cassette and confirm gene deletions. The plasmid pNK5, expressing the *phaA* and *phaB* genes from *Cupriavidus necator*, was transformed into strains (Kataoka et al., 2017).

Media

Lysogeny Broth (LB) was used for routine maintenance of cells. Experiments using the strains without 3HB production comprised the defined medium shown in Table 2. Experiments involving 3HB production used the same medium supplemented with 2 g/L casamino acids. Media were supplemented with 150 mg/L ampicillin (Amp) and/or 40 mg/L kanamycin (Kan) as appropriate for maintaining plasmids and/or strains with antibiotic resistances.

Genetic Modifications

The Kan^R locus from pKD4 was amplified by using primers with homology to the gene to be knocked out and extracted to electroporate into electrocompetent cells (Datsenko and Wanner, 2000). *E. coli* W was grown at 37°C at 250 rpm for 24 h in 3 mL LB to an optical density (OD) of 4.5-5.0. Electrocompetent cells were grown in 5 mL LB in test tubes at 37°C to an OD of 0.35-0.50. Cultures were then centrifuged (2°C at 3000 × g) and washed with 10%

glycerol. Electrocompetent cells were electroporated with pKD46 and recovered in a 1 mL LB test tube for 2-3 h at 30°C before being spread plated and grown for 24 h at 30°C. Once colonies formed, they were patched and grown for 24 h at 30°C to verify ampicillin resistance.

A culture of a W/pKD46 colony was grown in 3 mL LB with ampicillin at a final concentration of 0.15 mg/mL for 24 h at 30°C to an OD of 4.0-5.0. Electrocompetent cells were grown to an OD of 0.35-0.40, and then induced with 0.01 M L-arabinose and incubated for 15 min before centrifugation and washing. The electrocompetent cells were then electroporated with the extracted PCR product of the Kan^R locus from pKD4 and recovered at the same conditions. The recovery culture was spread plated onto LB-Kan plates and grown for 24 h at 30°C to verify presence of successful integration of the Kan^R locus. Cells were patched onto LB, LB-Amp, and LB-Kan plates to verify phenotypic presence of the Kan^R locus. PCR was performed to verify the knockout using primers external to the desired gene knockout. Once confirmed, a colony was cured of the pKD46 plasmid by incubating at 42°C for 24 h and spread onto an LB-Kan plate. This plate was incubated at 42°C for 24 h and colonies patched onto LB, LB-Amp, and LB-Kan plates to verify phenotypic the Kan^R knock out and removal of pKD46. A colony was then used to inoculate an overnight medium of LB for 24 h at 37°C to extract the gDNA. The Kan^R locus from the gDNA was amplified and extracted to be used to construct knockouts. The process to obtain pKD46, remove the plasmid, and integrate the Kan^R locus from the gDNA was performed using the same method when making the deletion in W. After confirming the gene knockout replaced with Kan^R, a colony was grown overnight in 3 mL LB with kanamycin, at a final concentration of 0.04 mg/mL, for 24 h at 37°C to an OD of 4.0-5.0. Electrocompetent cells were made as described above except kanamycin at a final concentration of 0.07 mg/mL was added to the medium. These cells were electroporated with pCP20 and recovered at 30°C. Cultures were

spread onto LB ampicillin plates and grown for 24 h at 30°C. After colonies formed, colonies were patched onto LB, LB-Kan, and LB-Amp plates and grown for 24 h at 30°C to confirm growth. Cells from a single colony were grown in 3 mL LB for 24 h at 42°C to cure pCP20 and remove the Kan cassette. The culture was then spread onto LB plates and grown for 24 hours at 42°C. Cells were patched onto LB, LB-Kan, and LB-Amp plates and grown for 24 h at 30°C to confirm removal of Kan cassette and pCP20. PCR was used to confirm the deletion of the gene. To generate *gltA* variants, site-directed mutagenesis and a NEBuilder® assembly was performed on plasmids with a Kan cassette and a *gltA* gene. Variant plasmids (pkS1-*gltA*[F383M-Kan], pkS1-*gltA*[M372S-Kan]) were sequenced to confirm variants. A linear DNA fragment was generated that had the *gltA* gene with the Kan cassette and 50 bp of homology upstream and down stream of the *gltA* gene. The F383M and M372S variants were generated in a similar process in constructing knockout strains using pKD46 and pCP20. Once a suspected *gltA* variant-FRT was shown by PCR, colonies strains were sequenced from ACGT, Inc. for confirmation of the desired *gltA* variant.

Growth Rate Experiments

A single colony was transferred to 5 mL LB in a test tube and was grown overnight at 37°C and 250 rpm. When the OD reached 3.0-5.0, 1 mL was transferred to a 250 mL baffled shake flask containing 50 mL defined medium grown at 37°C and 250 rpm. When the OD reached 2.0-3.0, 1 mL was inoculated into triplicate 250 mL baffled shake flasks containing 50 mL defined medium. Samples were periodically withdrawn for measurement of OD to calculate growth rate.

Batch Process Experiments

A single colony was transferred to 3 mL LB in a test tube and was grown at 37°C and 300 rpm for 5 h. This culture was transferred to an initial OD of 0.05 in a test tube with 3 mL defined medium 37°C and 300 rpm for 5 h. This culture was inoculated into triplicate 250 mL baffled shake flasks containing 20 mL defined medium to an initial an OD of 0.05. When the OD reached 0.5-0.8, 20 μM IPTG was added for induction. This procedure was adapted from Moxley 2022. Samples were withdrawn for OD measurement and analysis of soluble analytes. High performance liquid chromatography (HPLC) using 4 mN H_2SO_4 at 60°C and 0.6 mL/min with a Coregel 64-H ion-exclusion column (Transgenomic Ltd., Glasgow, United Kingdom), and refractive index detector was used to quantify glucose, fructose, 3HB, and organic acids (Eiteman and Chastain, 1997). The quantification of sucrose required 5.5 mN sulfuric acid and a flowrate of 0.4 mL/min at 30°C.

Batch Bioreactor Experiments

A single colony was transferred to a 5 mL LB test tube and grown overnight at 37°C at 250 rpm to an OD of 3.0-5.0. This culture was transferred to an initial OD of 0.01 into a 250 mL baffled shake flask with 50 mL defined medium with the selected carbon source. This culture was grown in the same conditions to an OD of 1.5-3.0, and then transferred to a 2 L bioreactor containing 1.2 L of defined medium. When the OD reached 0.7-0.9, 20 μM IPTG was added for induction. The bioreactor (1 liter, Bioflow 2000) operated at 30°C and at an agitation of 400 rpm. Air supplemented with oxygen was introduced at an air flowrate of 1.2 L/min to maintain dissolved oxygen (DO) above 40%. The pH was maintained at 7.0 using 30% KOH or HCl. Antifoam 204 was added to the bioreactor to control foaming. Samples were taken periodically to measure OD and pH and for HPLC analysis.

RESULTS

Growth Rate Experiments

E. coli W uses different routes to metabolize glucose, fructose and sucrose. The metabolism of glucose via glycolysis includes the enzyme phosphoglucose isomerase (coded by *pgi* gene). Thus, a *pgi* knockout would severely curtail the maximum specific growth compared to a wild-type strain. However, because fructose or sucrose (through fructose) synthesizes fructose-1,6P_2 via 1-phosphofructokinase, these substrates bypass phosphoglucose isomerase (Fig. 1). Also, the soluble transhydrogenase (*sthA*) acts as a pressure relief valve to regulate the intracellular supply of NADPH. A strain lacking this transhydrogenase is anticipated to have less flexibility in controlling NADPH levels. Because a Δ*pgi* strain would generate significantly more NADPH via the PP pathway than a strain expressing phosphoglucose isomerase, a Δ*pgi* Δ*sthA* strain would be expected not only generate more NADPH, but would also be particularly constrained by having limited ability to oxidize excess NADPH.

Therefore, the growth of W, W Δ*sthA*, W Δ*pgi* and W Δ*pgi* Δ*sthA* was examined in defined medium containing one of these substrates at 8 g/L. Wild-type W attained a growth rate of 0.901±0.007 h^{-1} on glucose while W Δ*sthA* showed a growth rate of 0.917±0.015 h^{-1}, not significantly different than the wild-type strain ($p>0.05$, Fig. 2). However, W Δ*pgi* attained a growth rate of 0.104±0.008 h^{-1} while W Δ*sthA* Δ*pgi* had a growth rate of 0.067±0.005 h^{-1}. The presence of a *pgi* deletion severely limited the growth of *E. coli* W during growth on glucose, as expected.

In contrast, the growth rate of each of the four strains on fructose as the sole carbon source was between 0.750-0.875 h^{-1} (Fig. 2). That is, a deletion in the *pgi* gene led to no significant difference in growth rate on fructose (wild-type growth rate 0.756 ±0.026 h^{-1} compared to a growth rate of 0.765 ±0.019 h^{-1} for the Δ*pgi* strain). Surprisingly, an *sthA* knockout *increased* the growth rate slightly but significantly ($p<0.05$) for both the wild-type strain (i.e., W Δ*sthA* compared to W) and the Δ*pgi* strain (i.e., W Δ*sthA* Δ*pgi* compared to W Δ*pgi*). Similarly, growth on sucrose as the sole carbon source resulted in a narrow range of growth rates 0.49-0.67 h^{-1} (Fig. 2). Furthermore, like the observations during growth on fructose, an *sthA* knockout surprisingly increased the growth rate on sucrose for both the wild-type strain (i.e., W Δ*sthA* compared to W) and the Δ*pgi* strain.

3HB Production with Different Carbon Sources

The deletion of *pgi* directs flow of carbon to the PP pathway which would tend to generate NADPH, and a deletion of the *sthA* gene would eliminate one route for NADPH oxidation. Therefore, we anticipated that the production of a NADPH-dependent product, such as 3HB, would correlate with anticipated NADPH availability. When grown on glucose, the wild-type strain produced 3HB at a yield of 0.035±0.002 g/g, W Δ*sthA* at a yield of 0.036±0.000 g/g, W Δ*pgi* at 0.046±0.003 g/g, and W Δ*sthA* Δ*pgi* at 0.057±0.001 g/g (Fig. 3). The W Δ*sthA* Δ*pgi* strain had the most 3HB produced on glucose. When grown on fructose, the W Δ*sthA* Δ*pgi* strain had the most 3HB produced compared to the other strains at 0.052±0.007 g/g (Fig. 3). When grown on sucrose, W Δ*sthA* Δ*pgi* had the most 3HB produced compared to the other strains at 0.040±0.003 g/g (Fig. 3).

3HB Yield with gltA Variants on Sucrose

Sucrose is converted into both glucose and fructose. Δ*pgi* and Δ*sthA* Δ*pgi* knockouts were able to have higher growth rates when compared to growing on glucose and fructose (Fig. 3). Therefore, sucrose was chosen to observe 3HB formation. In addition, *gltA* variants F383M and M372S were chosen to be used in this study. Both variants are different in how citrate synthase is affected in making 3HB. Fig. 4 shows that W strains with no *sthA* or *pgi* deletions had the F383M variant producing the greatest amount of 3HB produced at 0.065±0.003 compared to the wild-type and M372S variant. Wild-type had the lowest amount of 3HB at 0.007±0.002 g/g (Fig. 4). When variants were introduced into *sthA* knockout strains, F383M had the greatest amount of 3HB at 0.028±0.006 g/g. When variants were introduced into *pgi* knockout strains, F383M had the greatest amount of 3HB at 0.067±0.006 g/g. When variants were introduced into *sthA pgi* knockouts, F383M had the greatest amount of 3HB at 0.061±0.006 g/g. Surprisingly, the F383M with the *pgi* knockout produced the greatest amount of 3HB compared to the other F383M strains. F383M strains produced more 3HB than the wild-type GltA or the M372S strains (Fig. 4). Acetate yield was also measured in this study. The W strain with a wild-type GltA had an acetate yield of 0.008±0.003 g/g, and the Δ*sthA* strain had an acetate yield of 0.011±0.007 g/g. The Δ*pgi* and Δ*sthA* Δ*pgi* strains produced no acetate. The W M372S GltA variant strain had a yield 0.009±0.012 g/g of acetate, and the Δ*sthA* strain with the M372S GltA had a yield 0.008±0.006 g/g of acetate. The Δ*pgi* and Δ*sthA* Δ*pgi* strains had an acetate yield of 0.005±0.008 g/g and 0.000±0.000 g/g, respectively. For strains with the F383M GltA variant, all strains did not produce acetate.

3HB Production with F383M Variant on Glucose

After observing the effect of the citrate synthase variants, F383M was chosen over M372S due to higher yields of 3HB. The F383M variant was then grown on glucose to determine the 3HB yield since strains generally had higher yields of 3HB on glucose than on fructose and sucrose (Fig. 3). W with the F383M substitution generated significantly more 3HB (0.052±0.003 g/g) than the wild-type (0.035±0.002 g/g) (Fig. 5). The F383M substitution also significantly increased the 3HB yield in W Δ*sthA* and W Δ*pgi* compared to the wild-type GltA by 20-35%. In contrast, W Δ*sthA* Δ*pgi* with the F383M GltA substitution generated less 3HB than the same strain with the wild-type GltA. Acetate yield was recorded for this experiment. For strains with a wild-type GltA, the W strain had an acetate yield of 0.033±0.001 g/g, Δ*sthA* with 0.018±0.001 g/g, Δ*pgi* with 0.000±0.000 g/g, and Δ*sthA* Δ*pgi* with 0.003±0.003 g/g. For strains with a F383M GltA variant, the W strain had an acetate yield of 0.151±0.009g/g, Δ*sthA* with 0.188±0.004 g/g, Δ*pgi* with 0.210±0.003 g/g, and Δ*sthA* Δ*pgi* with 0.247±0.004 g/g. In all cases, the Δ*pgi* strains with the F383M substitution accumulated more acetate than other strains.

Batch Bioreactor Experiment on Glucose

The W Δ*sthA* Δ*pgi* was chosen for batch fermentation experiment since both deletions combined showed increased yield in 3HB compared to other strains. Glucose was chosen as the carbon source since higher yields of 3HB were being produced from this strain (Fig. 3,5). The strain consumed 30 g/L glucose at 85.5 h (Fig. 6). After glucose was depleted, the concentration of 3HB was only 0.4 g/L (Fig. 6), corresponding to a yield of about 0.013 g/g, much lower than the yield observed with the same strain under shake flask conditions (Fig. 5).

DISCUSSION

In this study, knockouts of *sthA* and *pgi* with citrate synthase (GltA) variants were studied to examine their effect of growth and product formation in *E. coli*. 3HB was selected as an example product because its formation requires both NADPH and acetyl-CoA. When grown on glucose, *E. coli* W Δ*sthA* Δ*pgi* and W Δ*pgi* had the lowest growth rates (Fig. 2). This result was expected since a *pgi* knockout would force metabolism to redirect carbon to PP pathway, and NADPH would accumulate in the process (Canonaco et al., 2001). In contrast, the Δ*pgi* knockout had no effect on the growth rate on fructose or sucrose, again as expected because both fructose and sucrose can essentially bypass phosphoglucose isomerase (Fig. 1, 2). A previous study reported no effect on the growth rate on fructose of an *E. coli* W Δ*pgi* strain, but the growth rate on glucose was affected for the strain (Ahn et al., 2011). Other work has not studied the effect of growth rates for Δ*pgi* strains on sucrose. The hypothesis stating that the Δ*sthA* Δ*pgi* strain would have severe growth defects on glucose but not fructose or sucrose was supported. For future work, an experiment generating a NADPH-dependent product using Δ*pgi* or Δ*sthA* Δ*pgi* strains on fructose or sucrose could be conducted in a two-phase batch fermentation process. The first phase would have the strains grow on fructose or sucrose for fast growth. Once the fructose or sucrose is depleted, glucose would be added to direct the carbon toward the product of interest.

Glucose, fructose, and sucrose were selected due to their different metabolisms in *E. coli* (Fig. 1). 3HB formation was observed to see which carbon source would produce the most 3HB. Strains growing on glucose produced the most 3HB. For example, the wild-type strain growing

on glucose generated more 3HB than strains growing on fructose or sucrose ($p<0.05$). Similar work has been studied with *Corynebacterium glutamicum*. A *C. glutamicum* strain growing on fructose had a growth rate of 0.48 h^{-1}, and 0.59 h^{-1} on glucose (Domniguez et al., 1998). Similar growth rate behaviors were observed in this study (Fig. 2). In *C. glutamicum*, the reduced growth rate on sucrose was due to overflow metabolites such as dihydroxyacetone and lactate and increased levels of CO_2 compared to growth on glucose (Domniguez et al., 1998). The glucose flux used glycolysis and the PP pathway to generate anabolic cofactors (Domniguez et al., 1998). Fructose metabolism does not use the PP pathway, so flux was directed to glycolysis (Domniguez et al., 1998). Less NADPH would be present for strains using fructose. Increased NADH/NAD was observed, and these cofactors in excess can inhibit glyceraldehyde-3-phosphate dehydrogenase and pyruvate dehydrogenase (Domniguez et al., 1998). The repression of key enzymes in glycolysis for *C. glutamicum* could prevent the growth and reduce central metabolites, such as acetyl-CoA. *E. coli* W strains could be affected by similar repression. Lactate and dihydroxyacetone were not measured in this studied, but future work could investigate if these products were formed on fructose. Limited acetyl-CoA availability was not from acetate accumulation because all strains produced less than 0.015 g/g (data not shown) of acetate. Lactate is synthesized from lactate dehydrogenase, *ldhA*, and converts pyruvate into lactate (Bunch et al., 1997). The low levels of 3HB on fructose growth than glucose growth could be from limited acetyl-CoA availability. Future work could make deletions in *ldhA* to mitigate unnecessary side products from being formed. The low 3HB yield on sucrose was most likely due to a reduced flux in the PP pathway and increased flux in the TCA cycle (Arifin et al., 2014). The reduced flux in the PP pathway decreased the amount of NADPH present, and the increased flux in the TCA cycle decreasing the amount of acetyl-CoA available. The

combination of both fluxes reduced the amount of reagents needed to generate 3HB. Additionally, acetate formation on sucrose was below 0.0012 g/g, so the low 3HB yields was not from acetate formation. Metabolic flux analysis of strains growing on glucose, fructose, and sucrose with the 3HB synthesis pathway expressed should be studied for future work. Other carbon sources should be tested, such as xylose, to increase the 3HB yield. A part of lignocellulose biomass contains xylose, which *E. coli* can utilize (Nduko et al., 2013). A study making poly(lactate-co-3-hydroxybutyrate) used *E. coli* strains growing on xylose to produce this product (Nduko et al., 2013). Additionally, the hypothesis stating that the wildtype strain would have no difference in 3HB yield and the Δ*sthA* Δ*pgi* strain would have higher 3HB yields on fructose or sucrose was not supported.

sthA and *pgi* knockouts were examined to increase NADPH availability for 3HB formation. The Δ*sthA* Δ*pgi* strain made the most 3HB compared to all the strains (Fig. 3). Growth on glucose or sucrose was not significantly different ($p>0.05$). However, growth on fructose and glucose produced more 3HB than growth on sucrose ($p<0.05$). The low yield from sucrose could be due to reduced flux of the PP pathway and increased flux of the TCA cycle (Arifin et al., 2014). NADPH availability increased with the *sthA* and *pgi* knockouts, but acetyl-CoA availability was not affected. However, growth on all three carbon sources had acetate levels less than 0.005 g/g. The increased flux of the TCA cycle could be affecting 3HB formation. 3HB generation was performed aerobically in this study, but 3HB formation in anaerobic conditions was not studied. One study used an *E. coli* BW25113 strain to produce 3HB in anaerobic and aerobic conditions on glucose (Liu et al., 2007). The 3HB yield in anaerobic conditions was 0.47 g 3HB/g glucose, and aerobic conditions was 0.32 g 3HB/g glucose (Liu et al., 2007). Future work could grow these strains anaerobically to observe 3HB formation. The

repressed TCA cycle could increase the acetyl-CoA availability in the cell. Additionally, the hypothesis that Δ*sthA* Δ*pgi* fructose or sucrose will show a higher 3HB yield than on glucose was not supported.

To further increase 3HB yield, *gltA* variants F383M and M372, severe and non-severe, were constructed. On sucrose, GltA[F383M] strains were able to produce more 3HB than GltA[M372S] strains. These findings were similar to one study that had a wild-type GltA strain with an acetate yield of 0.05 g/g, a GltA[M372S] strain with 0.09 g/g, and a GltA[F383M] strain with 0.19 g/g (Tovilla-Coutiño et al., 2020). These findings suggest that GltA[F383M] strain had more acetyl-CoA available than the other strains from the high acetate yield. The GltA[F383M] strains in this study had greater 3HB yields due to the reduced citrate synthase activity resulting in more acetyl-CoA availability. On glucose, Δ*pgi* Δ*gltA*::*gltA*[F383M] Δ*sthA* strain had a low yield of 3HB compared to the same strain growing on sucrose. The difference in yields was due to acetate levels. High levels of acetate were formed for the GltA[F383M] strains compared to the wild-type GltA strains when growing on glucose. *E. coli* growing on sucrose has been reported to have little acetate formation (Arifin et al., 2014). Acetyl-CoA was used to generate both acetate and 3HB, and the Δ*pgi* Δ*gltA*::*gltA*[F383M] Δ*sthA* strain made more acetate than 3HB. Competition exists for acetyl-CoA between biomass formation and 3HB production (Li et al., 2007). When grown in aerobic conditions on glucose, *E. coli* has an phosphotransacetylase and acetate kinase (*pta-ackA*) that converts acetyl-CoA into acetate, and a pyruvate oxidase (*poxB*) that converts pyruvate into acetate (De Mey et al., 2007). While *phaAB* utilizes acetyl-CoA, *pta-ackA* can use acetyl-CoA to form acetate. These enzymes could be limiting the 3HB production of the Δ*pgi* Δ*gltA*::*gltA*[F383M] Δ*sthA* strain. Knockouts in *pta-ackA* and *poxB* could be made in the Δ*pgi* Δ*gltA*::*gltA*[F383M] Δ*sthA* strain for more availability of acetyl-CoA in the

3HB synthesis pathway. Other work could limit the number of citrate synthase available in the cell, so *gltA* variants do not have to be constructed. Additionally, the hypothesis that the GltA[F383M] strains will attain greater 3HB yields than wild-type GltA strains was supported for strains growing on sucrose. However, this hypothesis was not supported for the Δ*pgi* Δ*gltA*::*gltA*[F383M] Δ*sthA* strain growing on glucose due to acetate formation.

In conclusion, knockouts in *sthA*, *pgi*, and reducing *gltA*, specifically F383M, resulted in slow growth on glucose, but minor growth defects were shown for strains growing on sucrose and fructose. In addition, 3HB levels increased when both *sthA* and *pgi* were knocked out on the three carbon sources. However, the Δ*pgi* Δ*gltA*::*gltA*[F383M] Δ*sthA* strain had a lower 3HB yield than the Δ*pgi* Δ*gltA*::*gltA*[F383M] strain on sucrose and glucose. With these genes modified, NADPH-dependent products can be increased. For future directions, the W Δ*sthA* Δ*pgi* strain will be studied on sucrose for batch fermentation runs for faster growth. In addition, the Δ*pgi* Δ*gltA*::*gltA*[F383M] Δ*sthA* strain will be studied in batch fermentation runs with glucose and sucrose as sole carbon sources where pH can be maintained. The *gltA* variant experiments in shake flasks were not pH controlled, and this setting would allow for more 3HB to accumulate.

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Table 1. Strains used in this study. The locus of each deleted gene is replaced with an FRT after the removal of the Kanamycin cassette.

Strain	Relevant Characteristics
ATCC9637	*Escherichia coli* W
MEC1450	W Δ*sthA*::Kan
MEC1451	W Δ*sthA*
MEC1433	W Δ*pntAB*::Kan
MEC1434	W Δ*pntAB*
MEC1459	W Δ*pntAB* Δ*sthA*::Kan
MEC1460	W Δ*pntAB* Δ*sthA*
MEC1503	W Δ*gltA*::*gltA*[F383M]
MEC1524	W Δ*pgi*::Kan
MEC1525	W Δ*pgi*
MEC1526	W Δ*sthA* Δ*pgi*::Kan
MEC1541	W Δ*sthA* Δ*pgi*
MEC1586	W Δ*sthA* Δ*gltA*::*gltA*[F383M]-Kan
MEC1587	W Δ*sthA* Δ*gltA*::*gltA*[F383M]
MEC1588	W Δ*pgi* Δ*gltA*::*gltA*[F383M]-Kan
MEC1589	W Δ*pgi* Δ*gltA*::*gltA*[F383M]
MEC1592	W Δ*pgi* Δ*gltA*::*gltA*[M372S]-Kan
MEC1593	W Δ*pgi* Δ*gltA*::*gltA*[M372S]
MEC1594	W Δ*sthA* Δ*pgi* Δ*gltA*::*gltA*[M372S]-Kan
MEC1595	W Δ*sthA* Δ*pgi* Δ*gltA*::*gltA*[M372S]
MEC1596	W Δ*sthA* Δ*gltA*::*gltA*[M372S]-Kan
MEC1597	W Δ*sthA* Δ*gltA*::*gltA*[M372S]
MEC1634	W Δ*gltA*::*gltA*[M372S]-Kan
MEC1635	W Δ*gltA*::*gltA*[M372S]
MEC1636	W Δ*pgi* Δ*gltA*::*gltA*[F383M] Δ*sthA*::Kan

Table 2. Defined medium used for shake flask experiments. Components were sterilized in compatible mixtures by autoclave or sterile filter as appropriate.

Composition of Defined Medium	
Component	**Concentration**
Glucose, fructose or sucrose	8 g/L
NH_4Cl	8 g/L
KH_2PO_4	1.2 g/L
$K_2HPO_4 \cdot 3H_2O$	1 g/L
K_2SO_4	2 g/L
$Na_2(EDTA) \cdot 2H_2O$	20 mg/L
$MgSO_4 \cdot 7H_2O$	0.6 g/L
$ZnSO_4 \cdot 7H_2O$	0.25 mg/L
$CuCl_2 \cdot 2H_2O$	0.125 mg/L
$MnSO_4 \cdot H_2O$	1.25 mg/L
$CoCl_2 \cdot 6H_2O$	0.875 mg/L
H_3BO_3	0.06 mg/L
$Na_2MoO_4 \cdot 2H_2O$	0.25 mg/L
$FeSO_4 \cdot 7H_2O$	5.5 mg/L
Citric acid	50 mg/L
Thiamine·HCl	20 mg/L
MOPS	31.35 g/L

Table 3. Defined medium used for batch fermentation experiments. Components were sterilized in compatible mixtures by autoclave or sterile filter as appropriate.

Composition of Defined Medium	
Component	**Concentration**
Glucose, fructose or sucrose	30 g/L
Casamino acid	2 g/L
NH_4Cl	8 g/L
KH_2PO_4	1.2 g/L
$K_2HPO_4 \cdot 3H_2O$	1 g/L
K_2SO_4	2 g/L
$Na_2(EDTA) \cdot 2H_2O$	20 mg/L
$MgSO_4 \cdot 7H_2O$	0.6 g/L
$ZnSO_4 \cdot 7H_2O$	0.25 mg/L
$CuCl_2 \cdot 2H_2O$	0.125 mg/L
$MnSO_4 \cdot H_2O$	1.25 mg/L
$CoCl_2 \cdot 6H_2O$	0.875 mg/L
H_3BO_3	0.06 mg/L
$Na_2MoO_4 \cdot 2H_2O$	0.25 mg/L
$FeSO_4 \cdot 7H_2O$	5.5 mg/L
Citric acid	50 mg/L
Thiamine·HCl	20 mg/L
MOPS	5.23 g/L

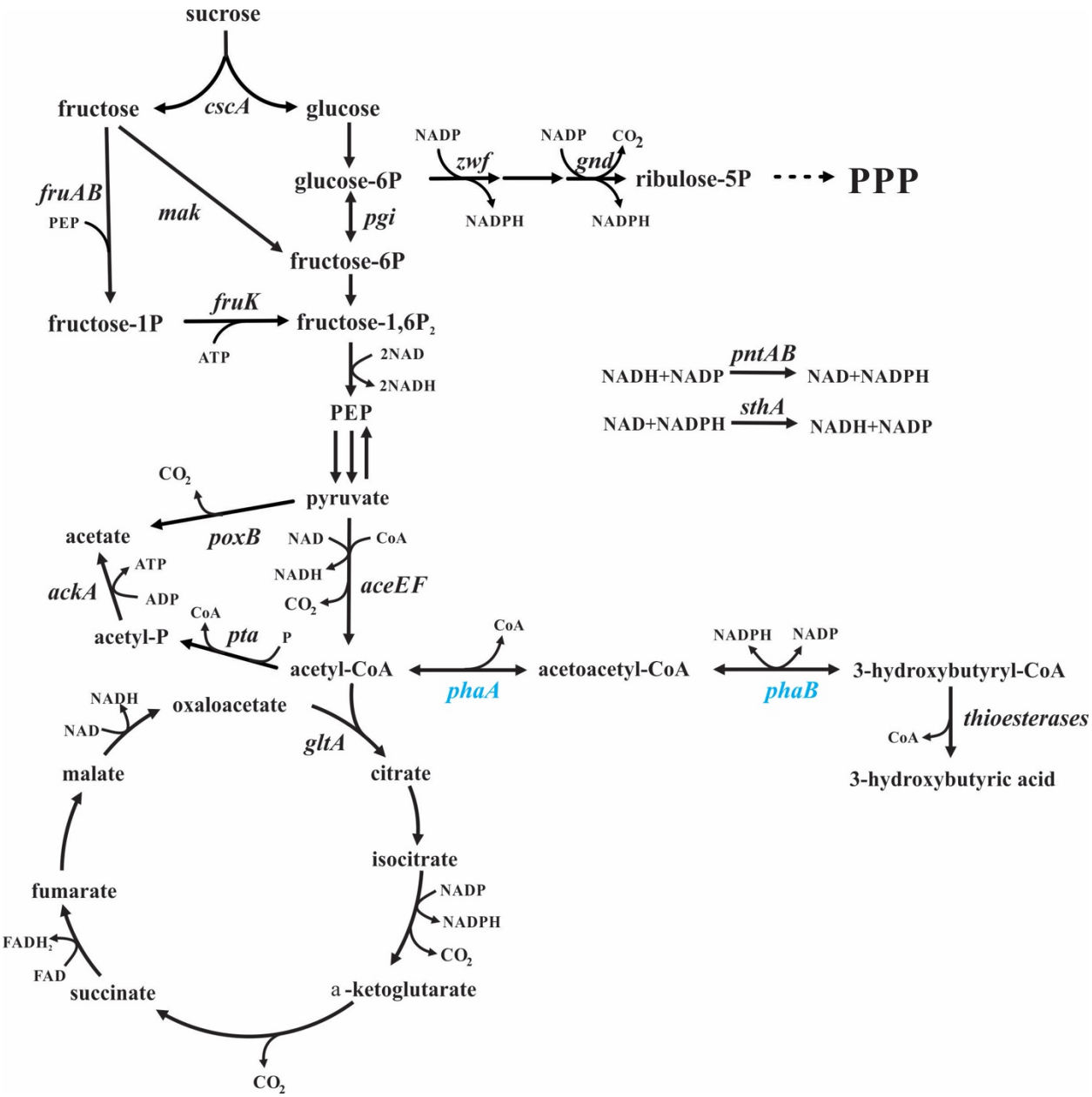

Figure 1. Metabolism of glucose, fructose, and sucrose in *E. coli* with 3HB production. The pathway to generate 3HB is expressed on the pNK5 (Kataoka et al., 2017) plasmid. Genes: *cscA* (invertase), *pgi* (phosphoglucose isomerase), *zwf* (glucose-6-phosphate dehydrogenase), *gnd* (6-phosphogluconate dehydrogenase), *fruAB* (fructose PTS permease), *mak* (fructokinase), *fruK* (fructose-1-phosphate kinase), *pntAB* (proton-translocating nucleotide transhydrogenase), *sthA* (soluble transhydrogenase), *aceEF* (pyruvate dehydrogenase [E1], dihydrolipoamide acetyltransferase [E2]), *poxB* (pyruvate dehydrogenase), *pta* (phosphate acetyltransferase), *ackA* (acetate kinase), *gltA* (citrate synthase), *phaA* (acetyl-CoA acetyltransferase), *phaB* (acetoacetyl-CoA reductase). Abbreviations: PEP (phosphoenolpyruvate), PPP (pentose phosphate pathway).

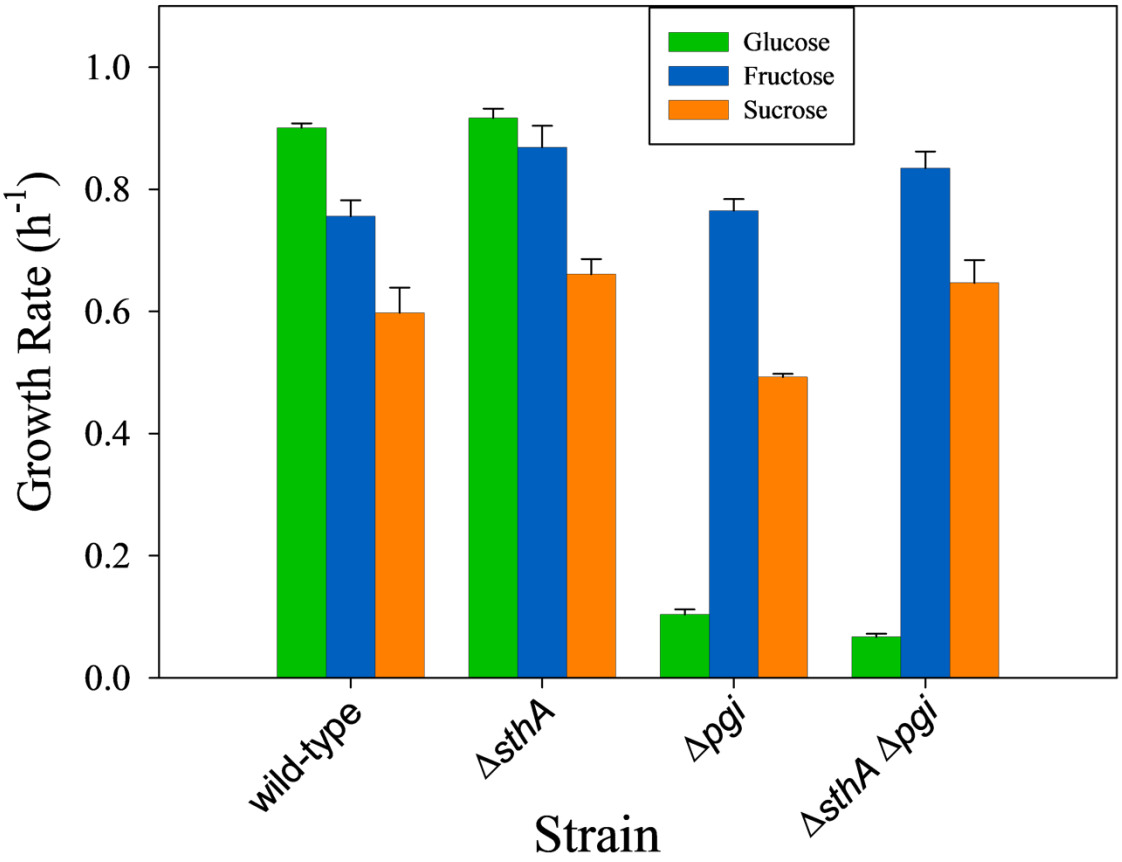

Figure 2. Growth rates of strains on different carbon sources. Data represent averages of triplicate shake flask experiments.

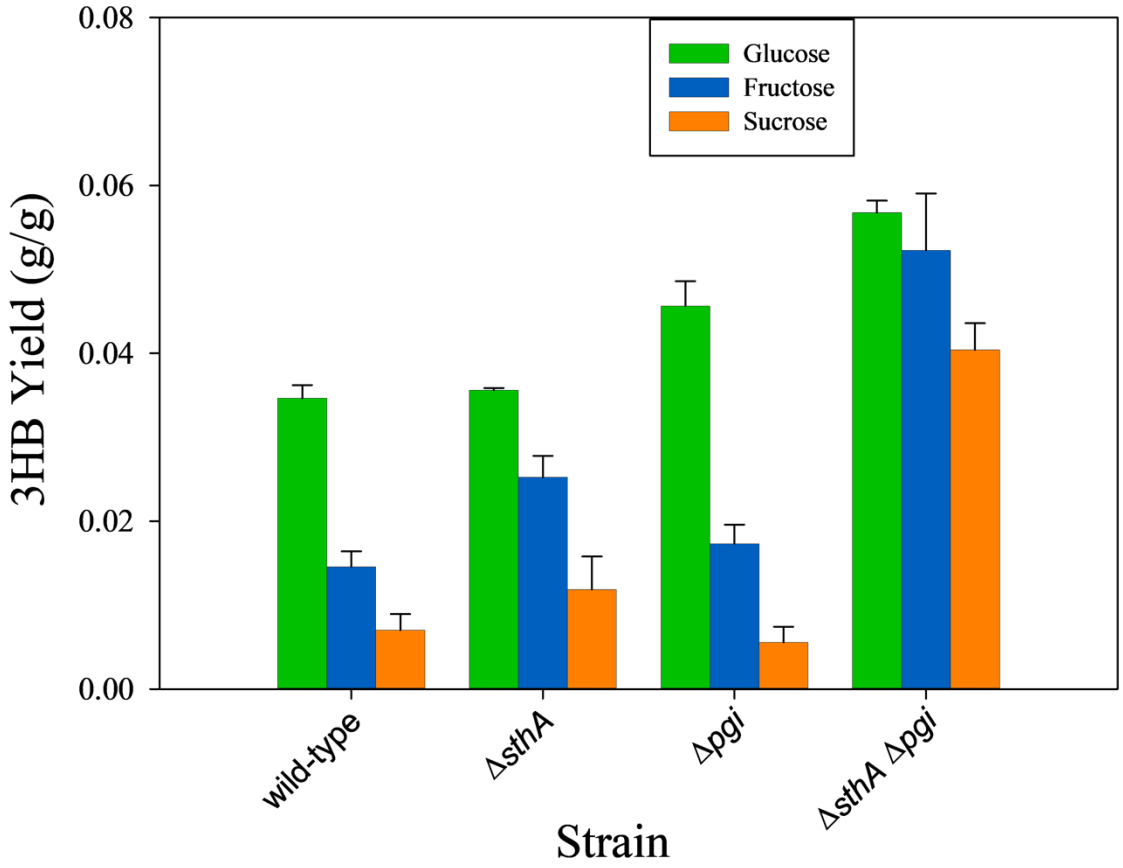

Figure 3. 3HB yield on different carbon sources with different strains. Data represent averages of triplicate shake flask experiments.

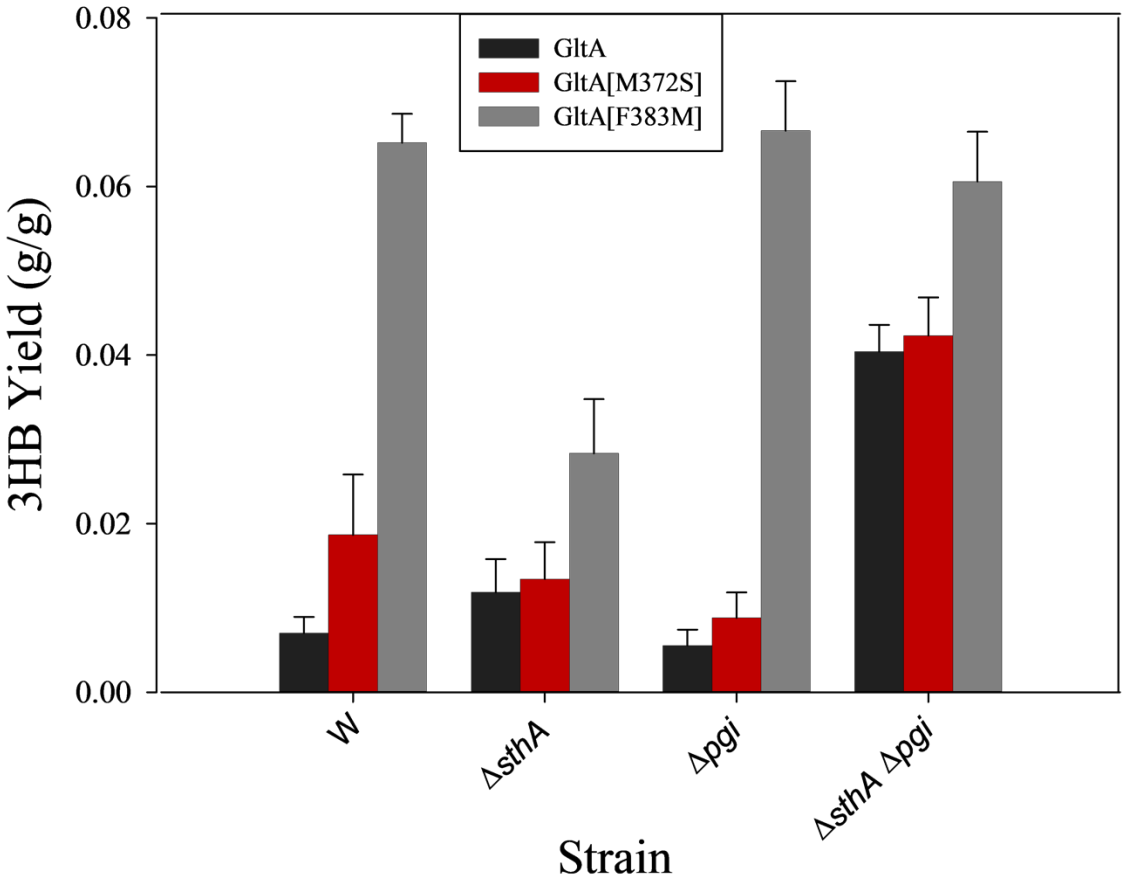

Figure 4. 3HB yield on sucrose with GltA, GltA[M372S], and GltA[F383M] strains. Data represent averages of triplicate shake flask experiments.

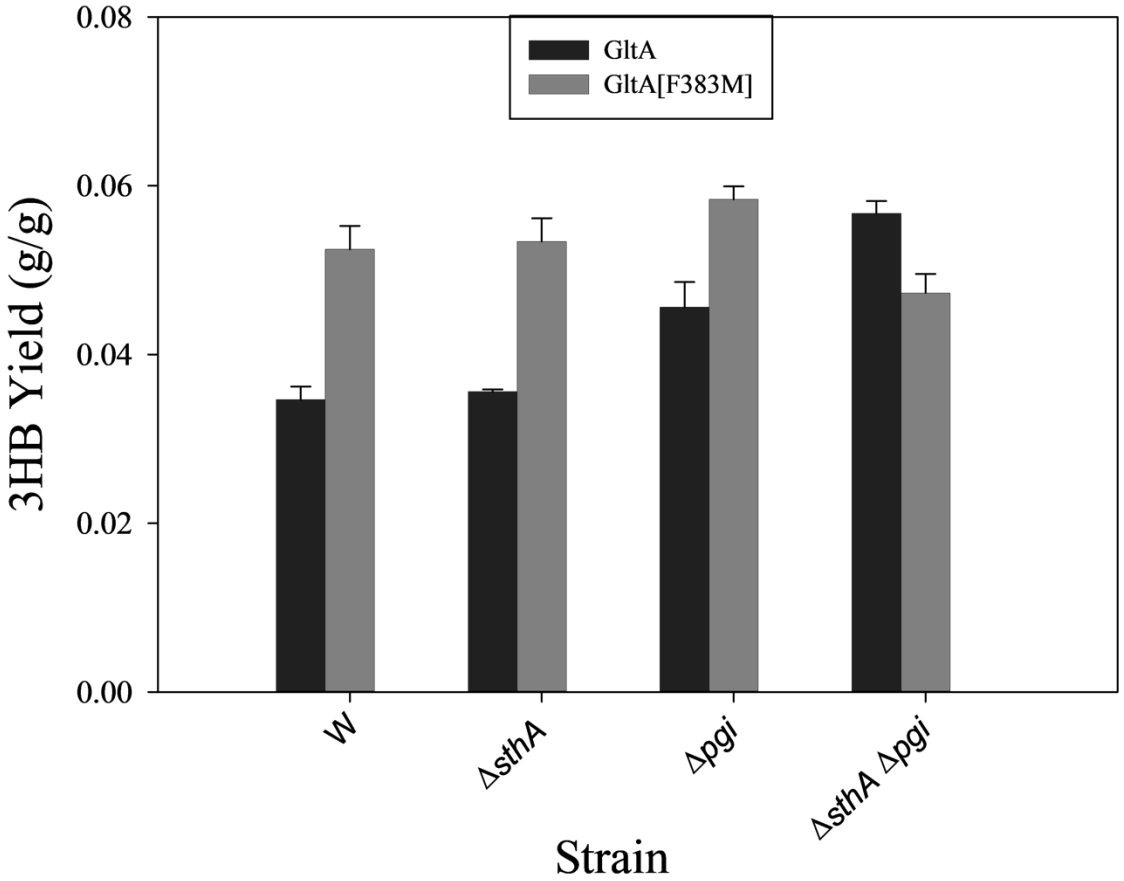

Figure 5. 3HB production on glucose for GltA and GltA[F383M] strains. Data represent averages of triplicate shake flask experiments.

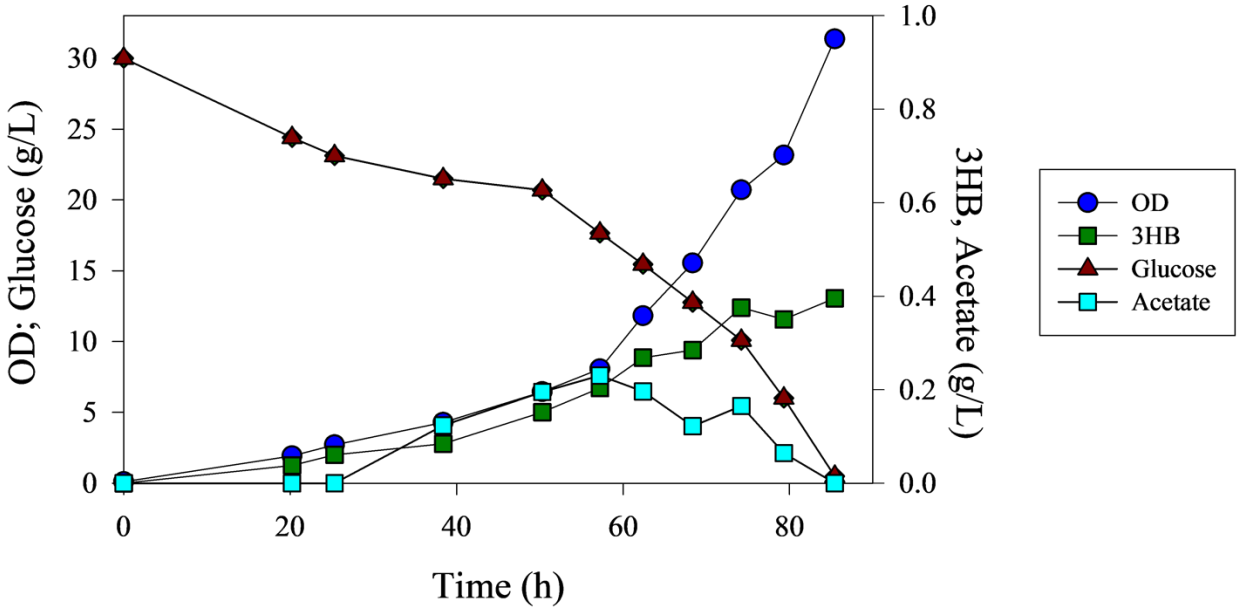

Figure 6. Batch fermentation run of W Δ*sthA* Δ*pgi*. 30 g/L glucose and 2 g/L casamino acid was supplemented into the medium.