1 **Functional proteomics reveals that Slr0237 is a SigE-**

2 **regulated glycogen debranching enzyme pivotal for**

3 **glycogen breakdown**

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7 Running title:

8 GlgX1 is necessary for glycogen breakdown

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ABBREVIATIONS:

Synechocystis: *Synechocystis* sp. PCC 6803

WT: Wild type

F6P: Fructose-6-phosphate

FBP: Fructose-1,6-bisphosphate

GAP: glyceraldehyde-3-phosphate

1,3-BPG: 1,3-Bisphosphoglycerate

PCR: Polymerase chain reaction

RT-PCR: Reverse transcription-polymerase chain reaction

OD: Optical density;

OPP: Oxidative pentose phosphorylation

ORF: Open reading frame

DEPs: differentially expressed proteins

Chl: Chlorophyll

MS: Mass spectrometry

TMT: Tandem mass tag

RP-HPLC: Reversed phase-high performance liquid chromatography

Significance Statement:

The SigE factor was important role in regulating central carbon metabolism in cyanobacteria. However, the biological processes regulated by SigE remain poorly understood. Proteomics studies showed that SigE depletion induces differential protein expression for sugar catabolic pathways including glycolysis, oxidative pentose phosphate (OPP) pathway, and glycogen catabolism. Two glycogen debranching enzyme homologues Slr1857 and Slr0237 were found differentially expressed in ΔsigE. Biochemistry study indicates that Slr0237 plays the major role as the glycogen debranching enzyme in Synechocystis. Our study offers new clues for discovering the regulatory mechanism of sugar metabolism in cyanobactiera.

Abstracts:

The group 2 σ factor for RNA polymerase SigE plays important role in regulating central carbon metabolism in cyanobacteria. However, the regulation of SigE for these pathways at a proteome level remains unknown. Using a *sigE*-deficient strain (ΔsigE) of *Synechocystis* sp. PCC 6803 and quantitative proteomics, we found that SigE depletion induces differential protein expression for sugar catabolic pathways including glycolysis, oxidative pentose phosphate (OPP) pathway, and glycogen catabolism. Two glycogen debranching enzyme homologues Slr1857 and Slr0237 are found differentially expressed in ΔsigE. Glycogen determination indicated that Δslr0237 accumulated glycogen under photomixotrophic conditions but was unable to utilize these reserves in the dark, whereas Δslr1857 accumulates and utilize glycogen in a similar way as the WT strain does in the same conditions. These results suggest that Slr0237 plays the major role as the glycogen debranching enzyme in *Synechocystis*. To our knowledge, this is the first study to report the functional difference of two glycogen debranching enzyme in *Synechocystis* and the research highlights the intricate regulation of glycogen breakdown.

1 | INTRODUCTION

Cyanobacteria are a group of Gram-negative bacteria that can perform photosynthesis similar to the higher plants. They can use sunlight to fix carbon dioxide in the air through the Calvin cycle and provide energy and carbon sources for cellular metabolism (De Marais, 2000; Kasting & Siefert, 2002). Among the photosynthesis organisms, the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) is one of the important model stains that has been widely used for studying photosynthesis and carbon metabolism because it has clear genomic information and is amenable to genetic manipulation (Gao et al., 2014; Kaneko et al., 1996; Vermaas, 1996). *Synechocystis* can grow autotrophically under light conditions and sustain life by breaking down stored carbohydrates such as glycogen in the dark. The glucose-tolerant strain of *Synechocystis* can even utilize exogenous organic carbon to maintain cell growth (Fang et al., 2016; Williams, 1988).

Carbon metabolism is central to cyanobacteria in cooperation with energy and reducing equivalent producing photosynthesis while providing carbon skeleton for synthesis of the other metabolites. In dark, the accumulated carbon reserves such as glycogen can be degraded to supply energy, reducing power, and precursors for biosynthesis of macromolecules necessary for the survival of cyanobacterial cells. Thus, the carbon metabolism needs to be precisely regulated, in both time and space. So far, the expression of genes involved in carbon metabolism was reported to be regulated by multiple proteins such as histidine kinase Hik31 and its plasmid paralog Slr6041 (Kahlon et al., 2006; Nagarajan et al., 2014), the response regulator Rre37 (Tabei et al., 2007), and the Group 2 σ Factor SigE (Osanai et al., 2005). Of these SigE-regulated genes are mainly involved in sugar catabolism as revealed by microarray-based transcriptomics and a targeted proteomics study (Azuma et al., 2011; Osanai et al., 2005). Sugar catabolism in *Synechocystis* cells is roughly divided into glycogen catabolism, Embden-Meyerhof-Parnas pathway (Glycolysis), oxidized pentose phosphate (OPP) pathway, and the tricarboxylic acid (TCA)

108 cycle. A number of the metabolic reactions in these pathways might require the

109 catalysis from enzymes encoded by multiple genes. Some of these reactions

110 are bidirectional while the others are unidirectional. For the bidirectional

111 reactions usually one direction is involved in carbon anabolism and the other in

112 carbon catabolism. Usually multiple isoenzymes are involved in such a

113 bidirectional reaction, with each predominately catalyzing the reaction towards

114 a specific direction. For example, the conversion of glyceraldehyde-3-

115 phosphate (GAP) to 1,3-Bisphosphoglycerate (1,3-BPG), a reaction integral to

116 glycolysis, was reported to be catalyzed by glyceraldehyde 3-phosphate

117 dehydrogenase 1 (GAP1), whereas the conversion of 1,3-BPG to GAP, a

118 reaction involved in gluconeogenesis, was reported to be catalyzed by GAP2

119 (Koksharova et al., 1998). For such biodirectional reactions the enzyme

120 catalyzing the catabolic reactions is more likely regulated by SigE. For the

121 unidirectional multiple isoenzyme-involved reactions, it is conceivable to

122 presume that the one regulated by SigE could play the major role in catalyzing

123 the reaction. Two steps in glycogen breakdown are such unidirectional reaction,

124 one is catalyzed by glycogen phosphorylase (GlgP) and the other is catalyzed

125 by glycogen debranching enzyme (GlgX). Both GlgP and GlgX have two

126 isoenzymes. The difference of functional regulation and specificity of the two

127 isoemzymes of GlgP have been characterized more or less (Fu & Xu, 2006),

128 and SigE is not involved in the regulation of either one. For GlgX, the functions

129 and specificities of the two isoenzyme Slr0237 (GlgX1) and Slr1857 (GlgX2)

130 have not been characterized so far. However, GlgX1 is reported to be regulated

131 by SigE based on the transcriptomics study (Osanai et al., 2005). A reasonable

132 postulation based on this information is that GlgX1 but not GlgX2 is the major

133 glycogen debrancing enzyme responsible for glycogen breakdown in

134 *Synechocystis*.

135   Herein, we first generated a *sigE* knockout mutant strain of *Synechocystis*

136 and then quantitatively profiled its proteome in an attempt to systematically

137 identify SigE-regulated proteins. Although SigE-regulated genes have been

identified in large scale by microarray-based transcriptomics studies using both *sigE* knockout and overexpressing strains of *Synechocystis*, and by targeted proteomics for a set of 112 proteins (Tokumaru et al., 2018). These studies are not sufficient to comprehensively catalog the SigE-regulated proteins. For the microarray studies, the microarray library only contains 3264 ORFs and 408 ORFs harbored by seven endogenous plasmids were not included. The proteins encoded by the plasmid-borne ORFs may be functionally important, particularly in regulation of carbon metabolism, as demonstrated by Slr6041, the paralog of the histidine kinase Hik31 (Nagarajan et al., 2014). Besides, the mRNA level may not be well correlated with protein level as previously reported (Griffin et al., 2002). After confirming that GlgX1 but not GlgX2 is regulated by SigE through the quantitative proteomics, we sought to investigate the distinct role of the two GlgXs, and demonstrated that GlgX1 is the major glycogen debranching enzyme for its breakdown.

2 | MATERIALS AND METHODS

2.1 | Cell Culture

The WT or mutant strains of *Synechocystis* were cultured in liquid BG-11 medium in moderate light (50 µmol m^{-2} s^{-1} photons) in a shaker. The cells were collected by centrifugation (4,000 g for 10 min) for biochemical and proteomic analyses at the exponential phase ($OD_{730 nm}$ = 1.0). The harvested cells were stored at -80°C until use.

2.2 | Glycogen Content Determination

Glycogen was determined as described (Xu et al., 2013). *Synechocystis* cells were harvested, resuspended in 400 µl 30% (w/v) KOH, and incubated at 95°C for 2 h. Glycogen was then precipitated with 75% (v/v) ice-cold ethanol, pelleted via centrifugation at 10,000 g for 10 min, sequentially washed with 70% and 98% (v/v) ethanol, and finally dried at 60°C for 10 min. The isolated glycogen was resuspended in 100 mM sodium acetate (pH 4.5) and enzymatically hydrolyzed to glucose with 2 mg/ml amyloglucosidase (Sigma-Aldrich) at 60°C for 2 h. The

168 glycogen content was determined with a glucose assay kit (Sigma-Aldrich)

169 according to the manufacturer's instructions.

170 **2.3 | Protein Preparation**

171 Cell pellets were lysed in a buffer containing 0.4 M sucrose, 50 mM 3-(N-

172 morpholino) propanesulfonic acid, pH 7.0, 10 mM NaCl, 5 mM EDTA, and 0.5

173 mM phenylmethanesulfonyl fluoride (PMSF, Sigma-Aldrich) with a bead beater.

174 The cell lysates were centrifuged at 4°C to remove insoluble debris (5000 × g,

175 30 min). Total proteins were precipitated with ice-cold 10% trichloroacetic acid

176 in acetone at -20°C, and then washed with acetone. The proteins were then

177 dried with a vacuum and resolubilized with 4% SDS in 0.1 M Tris-HCl, pH 7.6.

178 The protein concentrations were determined using a BCA protein assay kit

179 (Thermo Scientific, Rockford, IL).

180 **2.4 | Protein Digestion and TMT Labeling**

181 Proteins were digested using the filter-aided sample preparation (FASP)

182 method according to a previously described method with slight

183 modifications(Ge et al., 2017; Ge et al., 2018; Wiśniewski et al., 2009). Briefly,

184 the lysates (100 μg protein for each sample) were reduced with 10 mM DTT at

185 37°C for 1 h and alkylated with 55 mM iodoacetamide (IAA, Sigma-Aldrich,

186 Saint Louis, MO) at room temperature for 1 h in the dark. The alkylated lysates

187 were transferred into the Microcon YM-30 centrifugal filter units (EMD Millipore

188 Corporation, Billerica, MA), where the denaturing buffer was replaced by the

189 0.1 M triethylammonium bicarbonate (TEAB, Sigma-Aldrich, Saint Louis, MO),

190 and then digested with sequencing grade trypsin (Promega, Madison, WI) at

191 37°C overnight. The resulting tryptic peptides were collected and labeled with

192 6-plex TMT reagents (Thermo Scientific, Rockford, IL) by incubating peptides

193 with ethanol-dissolved TMT reagents for 2 h at room temperature in dark. The

194 labeling reaction was inactivated by addition of 5% hydroxylamine, and the

195 labeled samples were mixed together with equal ratios before fractionated with

196 reversed phase (RP)-high performance liquid chromatography (HPLC).

197 **2.5 | RP-HPLC**

198 Offline basic RP-HPLC was performed using a Waters e2695 separations

199 HPLC system coupled with a phenomenex gemini-NX 5u C18 column (250 x

200 3.0 mm, 110 Å) (Torrance, CA). The sample was separated with a 97 min basic

201 RP-LC gradient as previously described (Udeshi et al., 2013). A flow rate of 0.4

202 mL/min was used for the entire LC separation. The separated samples were

203 collected into 15 fractions, and completely dried with a SpeedVac concentrator

204 (Thermo Scientific, Rockford, IL) and stored at -20°C for further analysis.

205 **2.6 | Mass Spectrometry Analysis**

206 For MS analysis, the peptides were resuspended in 0.1% formic acid (FA) and

207 analyzed by a LTQ Orbitrap Elite mass spectrometer (Thermo Scientific,

208 Rockford, IL) coupled online to an Easy-nLC 1000 in the data-dependent mode.

209 Briefly, 2 μL of peptide sample (1 μg/μL) was injected into a 15-cm length, 75-

210 μm inner diameter capillary analytic column packed with C18 particles of 5-μm

211 diameter (SunChrom, Friedrichsdorf, Germany). The mobile phases for the LC

212 include buffer A (2% acetonitrile, 0.1% FA) and buffer B (98% acetonitrile, 0.1%

213 FA). The peptides were separated using a 90-min non-linear gradient consisting

214 of 3%-8% B for 10 min, 8%-20% B for 60 min, 20%-30% B for 8 min, 30%-100%

215 B for 2 min, and 100% B for 10 min at a flow rate of 300 nl/min. The source

216 voltage and current were set at 2.5 KV and 100 μA, respectively. All MS

217 measurements were performed in the positive ion mode and acquired across

218 the mass range of 300-1800 m/z. The fifteen most abundant precursor ions

219 from each MS scan were isolated and fragmented by high-energy collisional

220 dissociation (HCD) for MS/MS analysis.

221 **2.7 | Database Search**

222 The raw MS files were searched against the *Synechocystis* proteome sequence

223 database using the software MaxQuant (Cox & Mann, 2008). The database

224 containing 3,672 entries was downloaded from the CyanoBase

225 (ftp://ftp.kazusa.or.jp/pub/ CyanoBase/ *Synechocystis*, released on 5/11/2009).

226 The type of search was set to report ion MS2 and the 6-plex TMT was chosen

227 for isobaric labels, the minimum reporter parent ion interference (PIF) was set

to 0.75. Trypsin was chosen as the protease for protein digestion, and the maximum of 2 was set as the allowable miscleavages. N-terminal acetylation and methionine oxidation were included as the variable modifications. Cysteine carbamidomethylation was chosen as the fixed modification. The mass tolerances were set to 4.5 ppm for the main search and 20 ppm for precursor and fragment ions. The minimum score for unmodified peptides and modified peptides were set to 15 and 40, respectively. Other parameters were set up using the default values. The false discovery rate (FDR) was set to 0.01 for both peptide and protein identifications.

2.8 | Bioinformatics and Statistics

Bioinformatic and statistical analyses were mainly performed using the software Perseus (version 1.6.2.3) (Cox & Mann, 2012) . Student's *t*-test was used to determine the significance of differential expression of proteins between the WT and ΔsigE, and Fisher's-exact test was used for the functional enrichment analysis. A *p*-value<0.05 was used as the cut-off for all statistical analyses.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD038472 (Perez-Riverol et al., 2022).

3 | RESULTS

3.1 | Disruption of *sigE* in *Synechocystis*

Using an insertional mutation approach we generated the *sigE*-deletion mutant (Figure 1A, Left panel) (Gao et al., 2014). An about 2.0-kb spectinomycin resistance gene cassette was inserted into the *sigE* by homologous recombination and the complete segregation of the mutant was confirmed by PCR. Using the same pair of primers for *sigE* ORF fragment amplification which covers the insertion site of the resistance gene cassette, a 1.0-kb and a 3.0-kb PCR products were amplified from the WT and the mutant, respectively, indicating the complete segregation of *sigE* in the mutant (Figure 1A, Right panel). To investigate whether the insertional mutation of *sigE* affects the

258 transcription of its up-or down-stream flanking genes, RT-PCR was used to

259 confirm the transcriptional levels of the ORFs *sll1687*, *sll1688*, *sll1691*, *sll1692*,

260 and *sll1819* which are closely located up- or down-stream of *sigE* (Figure 1B,

261 Left panel). The RT-PCR result (Figure 1B, Right panel) demonstrates that the

262 transcriptional levels of the neighboring ORFs were not affected by the

263 disruption of *sigE*.

264 **3.2 | Depletion of SigE Leads to Impaired Heterotrophic Growth and Dark**

265 **Survival of *Synechocystis***

266 To investigate the effect of *sigE* knockout on the growth of *Synechocystis*, the

267 growth rates of the WT and the mutant were measured in photoautotrophic and

268 mixotrophic conditions under medium light intensity (50 μmol m^{-2} s^{-1} photons)

269 or in heterotrophic condition in the dark (Figures 2A-B). The results show that

270 there is almost no difference in growth rate between the WT and the ΔsigE

271 cultured in light conditions, except that the mutant strain grew slightly slower

272 than the WT under the photoautotrophic condition (Figure 2A, Left panel).

273 Photographs of the cell cultures at the indicated time points after inoculation

274 show that there is no significant color difference between the two strains under

275 light conditions, indicating that the chlorophyll content was not affected by *sigE*

276 mutation (Figure 2A, Right panel). However, the mutant can hardly grow in dark

277 in the presence of 5 mM glucose, a typical heterotrophic growth condition for

278 *Synechocystis* (Figure 2B). To investigate whether the inhibited growth of the

279 mutant in the heterotrophic condition is due to cell death or repressed

280 proliferation of live cells, the viability of the mutant and the WT in the

281 heterotrophic condition was assayed. The cells were photoautotrophically pre-

282 cultured to the exponential phase (OD730 = ~1.0), and then incubated in dark

283 up to 96h in the presence or absence of 5mM glucose. The dark-incubated cells

284 were sampled every 24h and spotted onto the solid BG-11 plates, which were

285 subsequently incubated in light for up to 96h and photographed every 24h. The

286 result shows that after 24h-incubation in dark in the presence of glucose, the

287 viability of the mutant was significantly impaired compared with that of the WT

as indicated by the inhibited growth of the cells (Figure 2C). After 72h dark incubation, no growth of the mutant was observed indicating all mutants were dead. Instead, the WT cells grew almost equally well compared with those incubated in dark for shorter duration. For the cells dark-incubated without glucose, only minor difference in growth was observed for the WT and the mutant even after 96h dark incubation. Together, these results suggest that viability of the mutant is impaired in the heterotrophic condition, namely the combination of darkness and presence of glucose, but not in dark along, and the latter is worthy of highlighting because the dark viability assay of cyanobacteria has been performed by different reports using or not using glucose (Özkul & Karakaya, 2015), and the consequence is different.

3.3 | Quantitative Identification of Differentially Expressed Proteins in ΔsigE

To decipher the proteomic basis of the observed ΔsigE phenotype, a systematic quantitative comparison of the proteomes between the WT and ΔsigE was performed using a 6-plex TMT labeling-based approach (Figure 3). In total, 2,193 proteins or protein groups were identified, covering about 60% of the *Synechocystis* proteome (Supplemental Table S1). Grouping the identified proteins according to the functional categories annotated by CyanoBase reveals that identification coverages of all functional categories are higher than 50% except for the unknown and other categories (Figure 4A). High coverage identification ensures comprehensive characterization of subsequent quantitative proteomics and bioinformatics analysis.

Among all identified proteins 1,925 proteins contain TMT reporter ion intensities (Supplemental Table S2), which were used for further quantification. Student's t-test with a threshold $p < 0.05$ was used to filter for 424 proteins quantified with high confidence (Figure 4B), which is indicated by the hierarchy clustering analysis that correctly clustered all three biological replicates of the WT and the mutant. A fold change of 1.4 was used to further filter for the differentially expressed proteins (DEPs) between the two strains, which

318   resulted in 42 upregulated and 95 downregulated proteins in the mutant
319   (Supplemental table S2). Comparisons of the current proteomics data with the
320   transcriptomics data of a previously reported sigE-knockout strain and sigE-
321   overexpressing strain reveal that the differential gene expression patterns at
322   protein and mRNA levels are overall positively correlated between the two sigE-
323   knockout strains (Figure 4C), and is negatively correlated between ΔsigE and
324   the sigE-overexpressing strain (Figure 4D). Nevertheless, a limited number of
325   outliers were also observed such as BioF, Hik37, Amt2, KaiA and FbaA (Figures
326   4C-D), and the inconsistency of differential expression between a mRNA and
327   its protein counterpart is also a well-documented observation (Griffin et al.,
328   2002). In addition, the expression of a number of genes located on the
329   endogenous plasmids of *Synechocystis* was significantly down-regulated in
330   ΔsigE, which was not reported in the transcriptomics data because they were
331   not included in the microarray library (Figures 4C-D; Figure 5).

332   **3.4 | Depletion of SigE Significantly Induces Differential Expression of**
333   **Proteins Involved in Carbon Metabolism**

334   Functional enrichment assay was performed for proteins upregulated or
335   downregulated in ΔsigE using Fisher's exact test. No function was significantly
336   enriched among the upregulated proteins. Manual inspection reveals that some
337   proteins associated with stress responses for *Synechocystis*, such as GgsP,
338   GlpD, sll1483, and sll1863 are upregulated in ΔsigE (Figure 5). Up-regulation
339   of these proteins could implicate SigE in the regulating the acclimation to abiotic
340   stress (Hagemann, 2011), particularly salt and Osmotic stress.

341   Among the downregulated proteins carbon metabolism network and
342   unknown proteins are significantly enriched functional categories (Figure 5;
343   Supplemental Figure 2). In addition, proteins encoded by the genes on the
344   endogenous plasmid pSYSA such as Sll7062, Sll7063, Sll7085, Sll7087, and
345   Sll7089 are also enriched (Figure 5; Supplemental Figure 2). Downregulation
346   of proteins involved in carbon metabolism is consistent with previous
347   observations, whereby depletion of SigE resulted in inhibition of glycolysis, OPP

348 and Glycogen metabolism pathways (Miyuki et al., 2011; Osanai et al., 2005;

349 Tokumaru et al., 2018). Mapping the DEPs to the carbon metabolism network

350 reveals that proteins involved in carbon catabolism but not anabolism were

351 downregulated (Figure 6). This mainly includes Zwf, Gnd, and Tal in the OPP

352 pathway, Gap1, PfkA1, Pyk1, ppsA, and GlgX in the glycolysis and glycogen

353 metabolism pathway (Figures 5-6). Downregulation of these key enzymes in

354 mutant cells indicates that SigE positively regulates sugar metabolism in

355 *Synechocystis*. Among the carbon metabolic pathways a few reactions are

356 bidirectional with one direction for carbon catabolism and the other for carbon

357 anabolism, and each direction is catalyzed by a distinct enzyme. These include

358 the reactions converting Fructose-6-phosphate (F6P) to Fructose-1,6-

359 bisphosphate (FBP) and GAP to 1,3-BPG. Interestingly, the enzymes catalyzing

360 the catabolic direction are downregulated in ΔsigE, underscoring the

361 importance and specificity of SigE in regulating carbon catabolism, and it can

362 be postulated, based on this observation, that a downregulated enzyme

363 involved in carbon metabolism is more likely catalyzing a catabolic reaction.

364 **3.5 | Glycogen Debranching Enzyme Slr0237 (GlgX1) Plays the Major Role**

365 **in Glycogen Breakdown in *Synechocystis***

366 Glycogen debranching is necessary for its breakdown, in *Synechocystis*

367 *slr0237* (*glgX1*) and *slr1857* (*glgX2*) encode putative isoamylases that

368 debranch glycogen. Previous studies have shown that two transcriptional

369 regulators, SigE and Rre37, regulate the transcriptional expression of *glgX1*

370 and *glgX2*, respectively (Azuma et al., 2011; Osanai et al., 2005). Regulation

371 by different transcription factors indicates that these two GlgXs may play

372 different roles in glycogen metabolism. Down-regulation of GlgX1 in ΔsigE,

373 based on the above assumption, indicates that GlgX1 may play the major role

374 for glycogen debranching and breakdown. To test this hypothesis and to

375 investigate the specific function of the two GlgXs, we generated the knockout

376 mutants for each of the two coding genes (ΔglgX1 and ΔglgX2) in

377 *Synechocystis* by using the insertional mutation method. The completely

segregated mutants were confirmed by PCR (Supplemental Figure 1). Growth experiments were performed to determine the effect of the *glgX1* or *glgX2* mutation on cell growth. For photoautotrophic growth, both mutants grew slightly slower than the WT, while the photomixotrophic growth of the two mutants is almost identical with that of the WT (Figures 7A-B, Left panel). The whole cell absorption spectra of the photoautotrophically-growing cells were also measured. Compared with the WT, the spectra of the two glgX mutants showed slightly reduced absorption at ~435 nm, ~485 nm, and ~625 nm, indicating slightly lower abundancy of Chl a, carotenoids and phycobilin content, respectively (Supplemental Figure 3). Together, these results suggest that deletion of glgX (*slr0237* or *slr1857*) only slightly repressed growth and pigment synthesis of *Synechocystis* under the photoautotrophic condition.

In dark, cyanobacteria mainly catabolize glycogen to provide energy, reducing equivalent, and biosynthetic precursors. To investigate the specific functions of the two GlgXs in glycogen breakdown, we compared the glycogen breakdown rates of the mutants with that of the WT. The cells were precultured to the exponential phase (OD_{730} =~1) in the light to allow glycogen accumulation, and then transferred to darkness and incubated for 7 days to boost glycogen breakdown. Seven-day dark-incubation did not cause an apparent change of the cells as indicated by the photographed cultures (Figure 7C). Glycogen contents were measured for cells immediately before and after the dark-incubation. For the cells measured before dark-incubation, there is no significant deference of glycogen contents among the three strains (Figure 7D). For dark-incubated cells, the glycogen contents of WT and ΔglgX2 were both dramatically reduced, though the amount in ΔglgX2 is higher than that in the WT. In contrast, the glycogen content of ΔglgX1 remained almost constant after dark-incubation, indicating glycogen in the mutant was barely catabolized (Figure 7D). Together, these results suggest that GlgX1 but not GlgX2 is mainly responsible for glycogen debranching, which is pivotal for glycogen breakdown.

4 | DISCUSSION

In the present study, we showed that SigE plays an important role in sugar catabolism and is critical for the dark survival of *Synechocystis* in the presence of glucose. Using a SigE-deficient mutant and quantitative proteomics, we showed that SigE depletion significantly reduced protein expression for sugar catabolic pathways including glycolysis, OPP pathway, and glycogen catabolism.

Two glycogen debranching isoenzymes GlgX1 and GlgX2 were found differentially expressed in ΔsigE. Glycogen content determination indicates that ΔglgX1 accumulated glycogen in light but was unable to utilize these reserves in dark. However, ΔglgX2 and WT cells can accumulate and break down glycogen under the same condition. These results indicate that GlgX1 plays the major role as the glycogen debranching enzyme in *Synechocystis*.

In *Synechocystis* glycogen is broken down mainly through glycogen phosphorylase (encoded by *glgP*) and glycogen debranching enzyme. *Synechocystis* possesses two *glgP* genes (sll1356 and slr1367) and two *glgX* genes (slr0237 and slr1857) in its genome (Kaneko et al., 1996). Though the specific functions of the two GlgPs have been elucidated (Fu & Xu, 2006), the functional distinction between the two GlgXs is unknown. In this study, we demonstrated that GlgX1 is the major debranching enzyme in glycogen breakdown. The fact that glycogen can be catabolized in ΔglgX2 at a comparable rate with that in the WT suggests that GlgX2 play no or a minor role in glycogen breakdown in *Synechocystis*. Studies in *Escherichia coli* showed that disruption of the *glgX* gene changes the structure patterns of the glycogen produced in cells (Dauvillee et al., 2005). In cyanobacteria, it has also been shown that heterologous expression of *Synechocystis* gene *slr0237* (*glgX1*) or *slr1857* (*glgX2*) in a *glgX* mutant of *Synechococcus elongatus* PCC 7942 (*S. elongatus PCC 7942*) could recover the abnormal glycogen structure of mutant cells (Suzuki et al., 2007). When the *glgX1* or *glgX2* gene from *Synechocystis*

437   was introduced to the *glgX* mutant of *S. elongatus* PCC 7942, the level of short

438   branches in the glycogen molecules was significantly reduced in the

439   transformants as compared to the parental mutant. These results indicate that

440   the GlgXs are not only involved in glycogen breakdown but also important for

441   glycogen synthesis by shaping correct glycogen structure.

442   It was also noted that the mutation of *glgX1* and *glgX2* did not cause the

443   over-accumulation of glycogen in *Synechocystis* cells (Figure 7D), the

444   observation was similar to what had been reported for *E. coli* (Dauvillee et al.,

445   2005), *S. elongatus* PCC 7942 (Suzuki et al., 2007) and *Arabidopsis* (Delatte

446   et al., 2006; Wattebled et al., 2005). Although the mutation of *glgX1* and *glgX2*

447   did not cause critical effects on the cellular viability or the growth rate (Figures

448   7A-B), defects in these genes may be disadvantageous in a competitive or

449   stress environment.

450   In *Synechocystis*, both *glgX* genes are significantly induced by nitrogen

451   depletion (Krasikov et al., 2012; Osanai et al., 2006). GlgX1 were decreased by

452   SigE disruption (Osanai et al., 2005) and increased by SigE overexpression

453   (Takashi et al., 2011), whereas GlgX2 were decreased by Rre37 depletion

454   (Azuma et al., 2011). These results indicate that glycogen metabolism could be

455   regulated by distinct mechanisms, particularly under various environmental

456   conditions. In this context, existence of two GlgXs may allow for more flexible

457   regulation of glycogen debranching to better adapt to environmental changes.

458

459   **5 | CONCLUSION**

460   In summary, we found that SigE depletion induces differential protein

461   expression for sugar catabolic pathways especially in glycolysis, OPP pathway,

462   and glycogen catabolism. Two glycogen debranching enzyme homologues

463   Slr1857 and Slr0237 were found differentially expressed in ΔsigE. Glycogen

464   determination indicated that Slr0237 plays the major role as the glycogen

465   debranching enzyme in *Synechocystis*. Our study reports the functional

466   difference of two glycogen debranching enzyme in *Synechocystis* and

467 highlights the intricate regulation of glycogen breakdown in Cyanobacteria.

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CONFLICTS OF INTEREST:

The authors declare no conflict of interest.

475

DATA AVAILABILITY STATEMENT:

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium and can be downloaded at: http://proteomecentral.proteomexchange.org/cgi/GetDataset, with the dataset identifier PXD038472

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602

603

604   **Figure 1 |** Generation of *sigE* knockout mutant in *Synechocystis*.

605   **(A)** The diagram depicts the plasmid construct for disrupting the ORF of *sigE*

606   on the genome of the *Synechocystis*.

607   **(B)** The same pair of primers was used to amplify DNA fragments from the WT

608   and the mutant to confirm the complete segregation of the mutant.

609   **(C)** The diagram shows the genomic locus of *sigE* and its neighboring ORFs.

610   **(D)** Transcription of *sigE* and its neighboring ORFs in WT and ΔsigE was

611   detected via RT-PCR. The gene *rnpB* was used as the internal loading control.

612

613   **Figure 2 |** Disruption of *sigE* affects the heterotrophic growth of *Synechocystis*.

614   **(A-B)** Growth curves of the WT and the ΔsigE were measured in the presence

615   (+G) or absence of 5 mM glucose under 50 μ mol m^{-2}s^{-1} light intensity **(A)** or in

616   dark **(B)** (Left panels). The cell cultures were also photographed at the indicated

617   time points during the growth experiment (Right panels).

618   **(C)** Exponentially-growing WT and ΔsigE cultured in BG-11 with or without 5μ

619   m glucose were transferred to darkness, and then sampled (10 μl each) at the

620   indicated time points during dark-incubation and spotted on solid BG-11 plates.

621   The plates were incubated in continuous light and photographed every 24 hours

622   after inoculation.

623

624   **FIGURE 3 |** Schematic representation of the workflow for the quantitative

625   proteomic analysis of the ΔsigE.

626   Total proteins were extracted from the three biological replicates of the WT and

627   the ΔsigE and digested with trypsin. The tryptic peptides were labeled with 6-

628   plex TMT reagents in the order as indicated. The labeled peptides were mixed

629   together with an equal molar ratio, and separated into 15 fractions with RP-

630   HPLC. The peptides in each fraction were quantitatively analyzed by LC-

631   MS/MS using a LTQ-Orbitrap-Elite mass spectrometer.

632

633 **FIGURE 4 |** Quantitative proteomic analysis of the ΔsigE.

634 **(A)** the bar graph shows the coverage of protein identification for all functional

635 categories annotated by CyanoBase.

636 **(B)** Z-scored TMT reporter intensities of 424 proteins after Student's *t*-test

637 (*p*<0.05) were used for hierarchy clustering analysis to evaluate the

638 reproducibility of the quantitative analysis. The extents of differential protein

639 expression are color-coded and displayed on the scale bars.

640 **(C-D)** Volcano plots shows the comparison of the results in the current

641 proteomic study and previous transcriptomic studies for *sigE* knockout **(C)** and

642 *sigE* overexpression **(D)** stains. The filled cycles indicate proteins with

643 significant changes (fold change ≥1.40 or ≤0.71, *p*<0.05) in expression in the

644 current proteomic study. The red and green spots represent genes that were

645 upregulated or downregulated in the indicated transcriptomic studies.

646

647 **FIGURE 5 |** DEPs in major functional categories.

648 Volcano plots shows the differentially expressed proteins in ΔsigE. Proteins

649 involved in carbon metabolism (purple), stress response (red), and encoded by

650 endogenous plasmids (blue) are highlighted.

651

652 **FIGURE 6 |** Mapping of the DEPs in the ΔsigE to a protein network for carbon

653 metabolism.

654 The DEPs are indicated by red or green color, which indicate upregulation and

655 downregulation in the ΔsigE, respectively.

656

657 **FIGURE 7 |** Phenotypic analysis of Δslr0237 and Δslr1857.

658 **(A-B)** Growth curves of the WT and the mutant strains Δslr0237 **(A)** and

659 Δslr1857 **(B)** cultured with or without 5 mM glucose under medium light intensity

660 (Left panels). The cultures were also photographed at the indicated time points

661 (Right panels).

662 **(C-D)** The indicate WT and mutant strains were pre-cultured in light to OD$_{730}$

663   ~1.0, and then transferred to darkness and dark-incubated up to 168h, the

664   cultures were photographed at the indicated time points **(C)**. Bar graph shows

665   the glycogen contents of the WT and the mutants before and after dark-

666   incubation **(D)**.

667

668   **Supplemental Figure 1 |** PCR confirmation of the complete segregation of the

669   Δslr0237 and Δslr1857.

670   The same pair of primers were used to amplify DNA fragments from the WT

671   and the mutants genomic DNA to confirm the complete segregation of the

672   mutant.

673

674   **Supplemental Figure 2 |** Functions enriched in the downregulated proteins in

675   ΔsigE.

676   The gene ontology terms, KEGG pathways, functional categories annotated by

677   the CyanoBase and carbon metabolism network in Figure 6 were included for

678   the analysis. FDR<0.02 and the enrichment factor ≥1.5 were used as the cutoffs

679   to include all enriched categories.

680

681   **Supplemental Figure 3 |** Whole cell absorbance spectra of Δslr0237, Δslr1857

682   and the WT strains.

683   Cells were sampled during the exponential growth phase (OD_{730} = ~0.8). The

684   absorption spectra was scanned between 380 and 800 nm. The spectra were

685   normalized to the OD_{730}.

686

Figure 1

A

DNA fragment of sll1689 (sigE)

Bam HI   Bam HI

Spec^r (2.0kb)

WT   ΔsigE

– 3.0 kb

– 1.0 kb

B

sll1687   sll1689 (sigE)   sll1692

sll1688   sll1691   sll1819

WT   ΔsigE

sll1687
sll1688
sll1691
sll1692
sll1819
rpnB
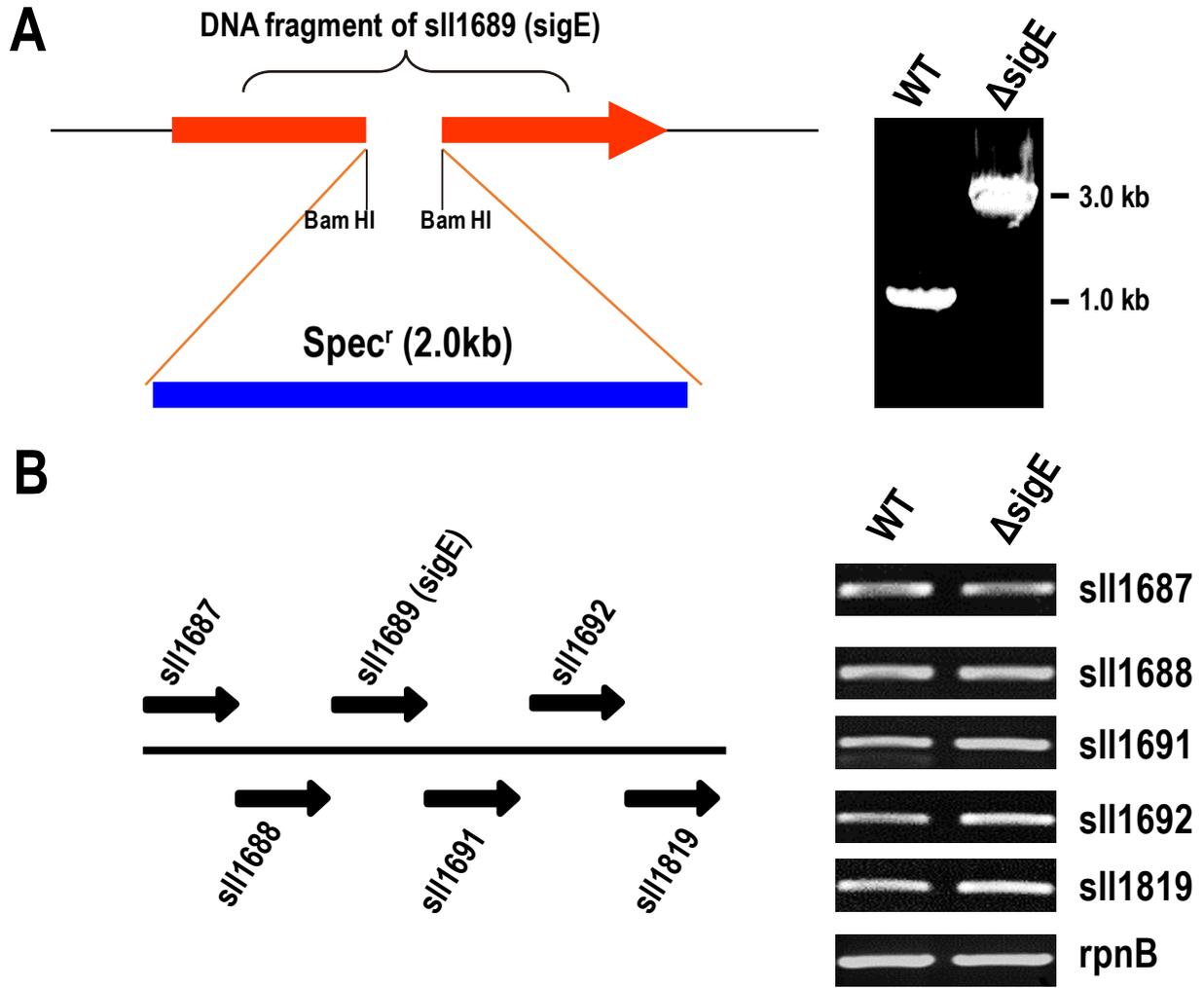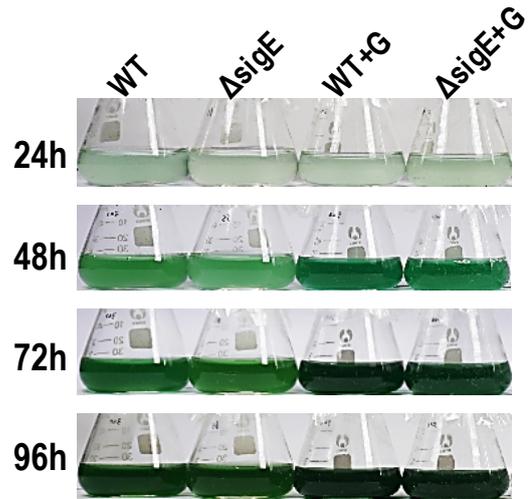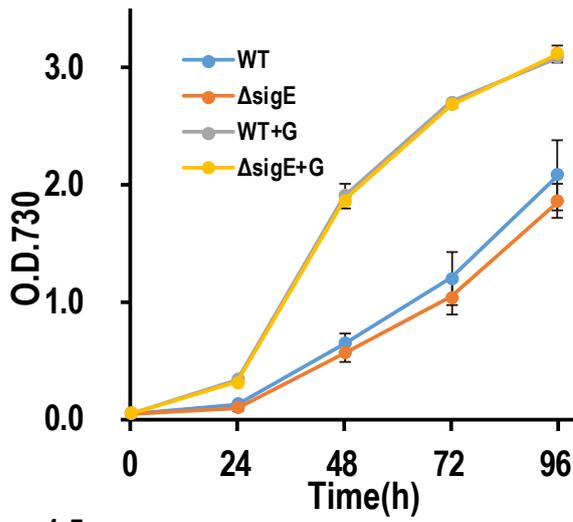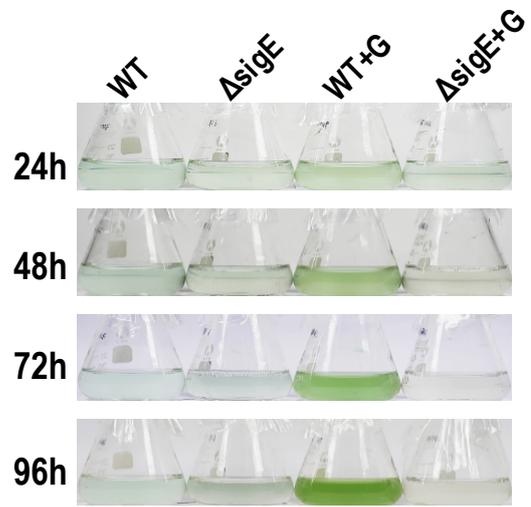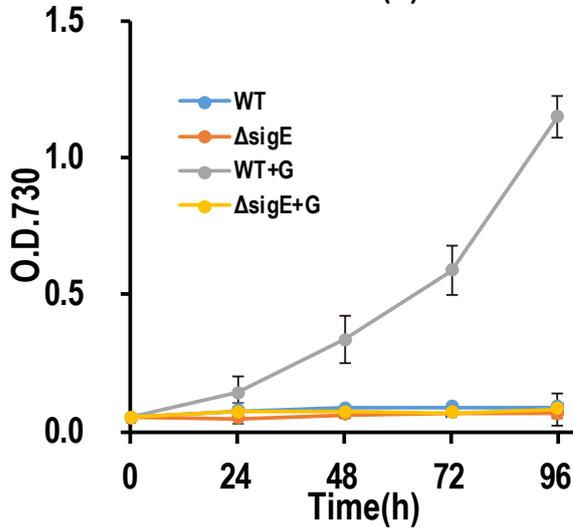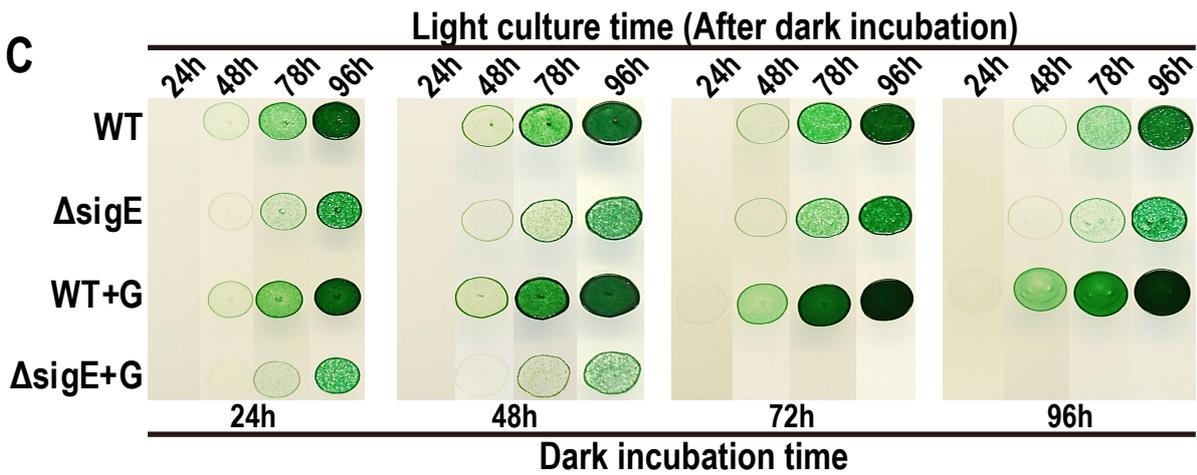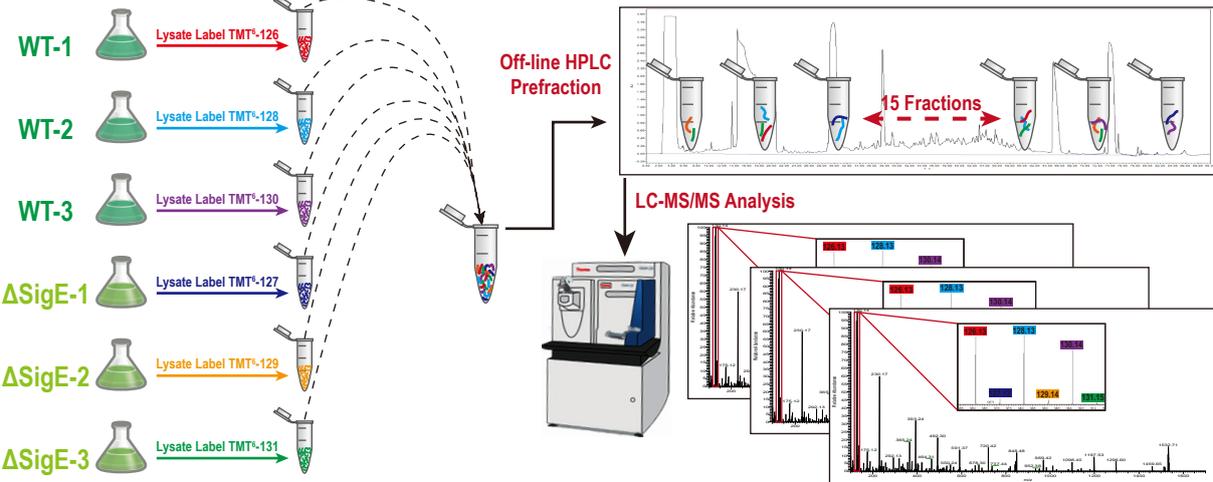
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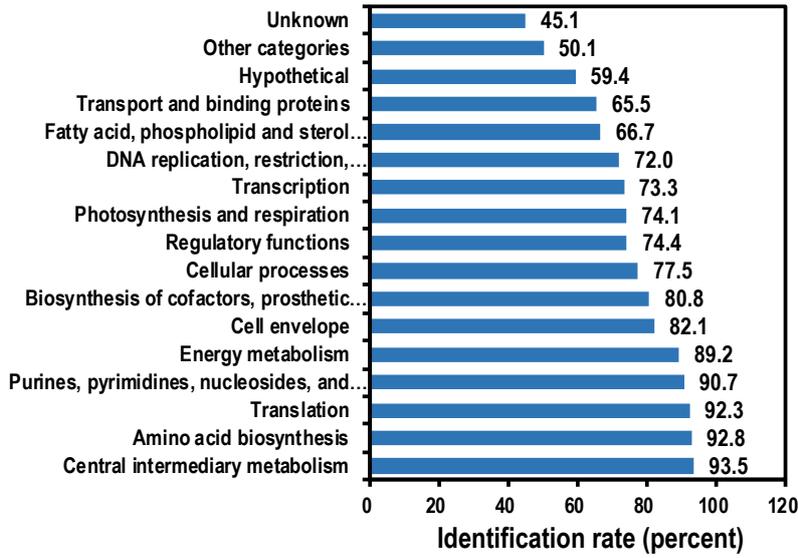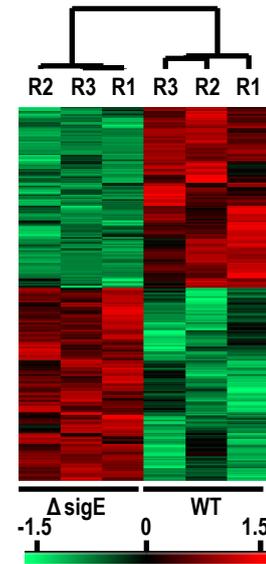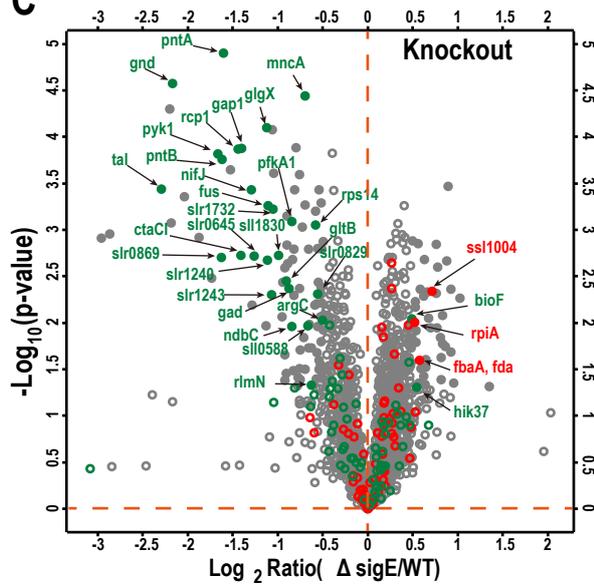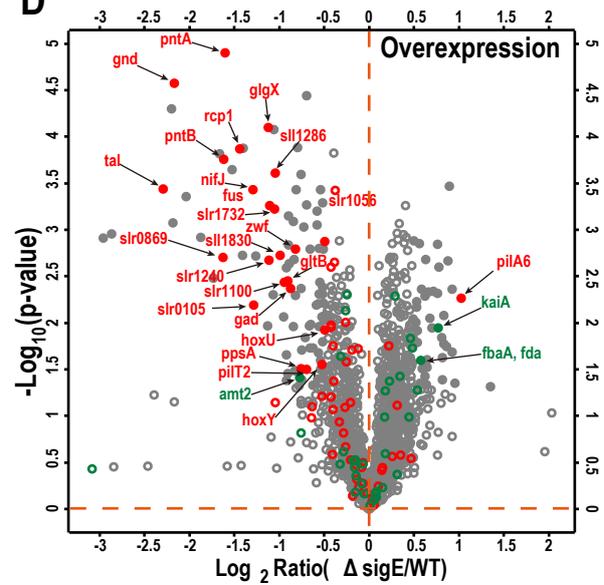
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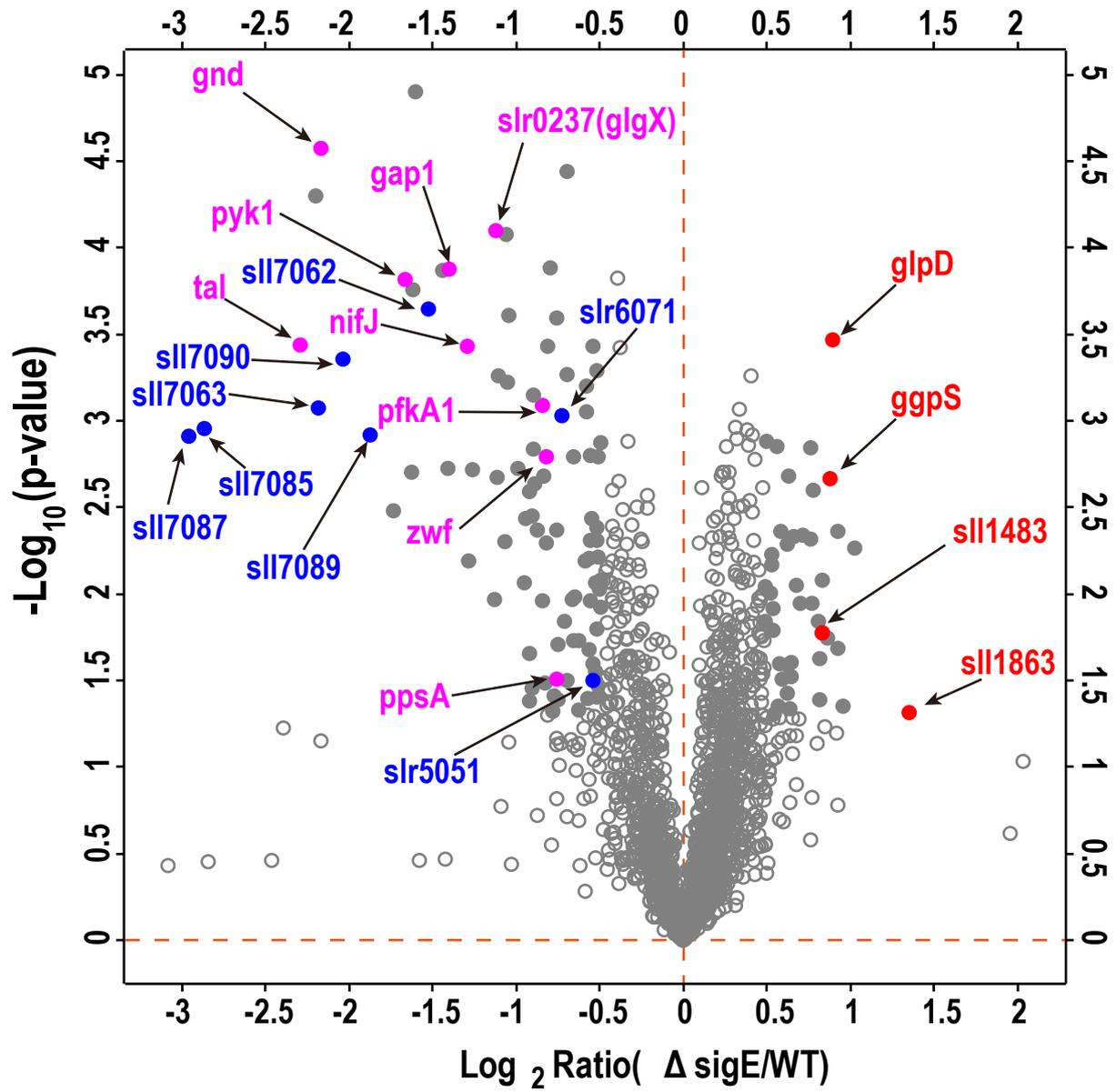
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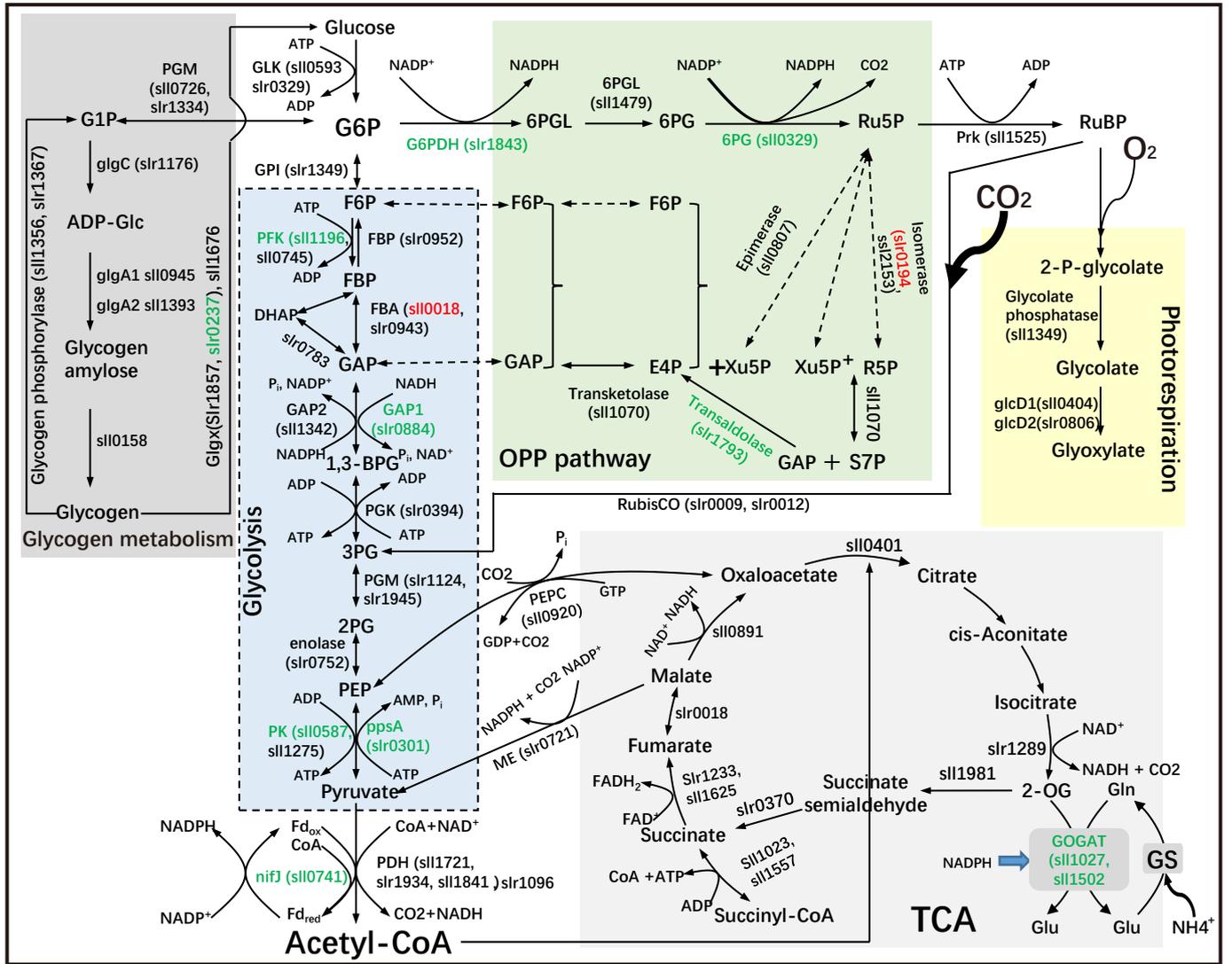
Figure 5

Figure 6

Figure 7
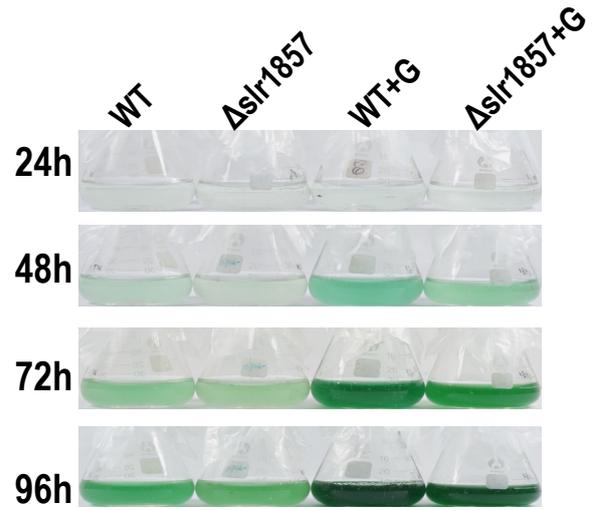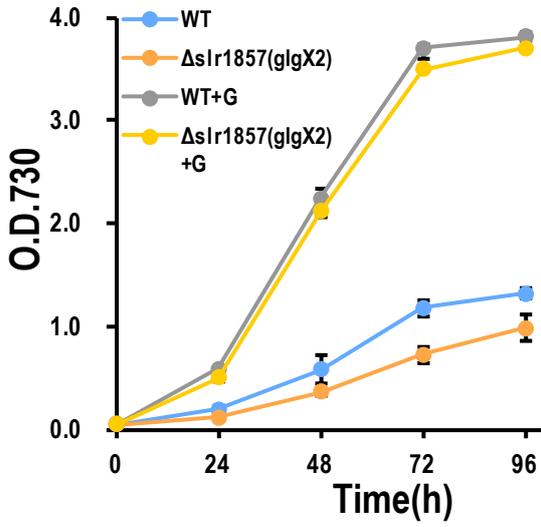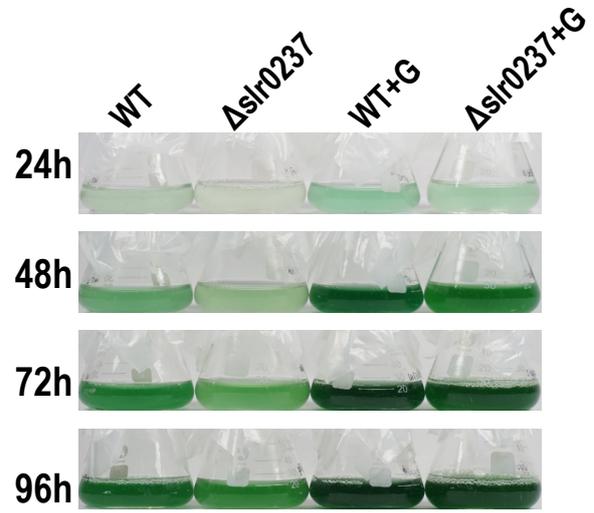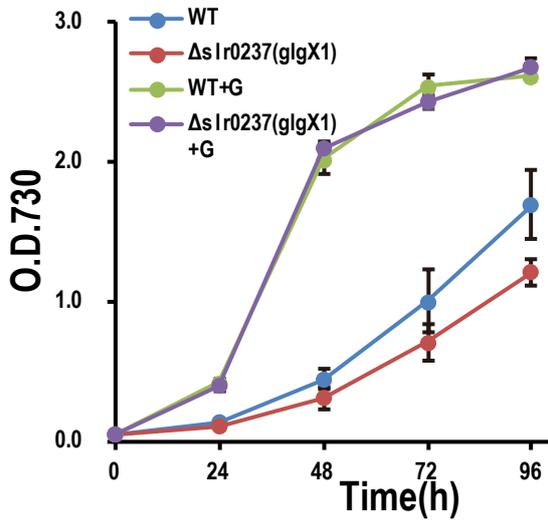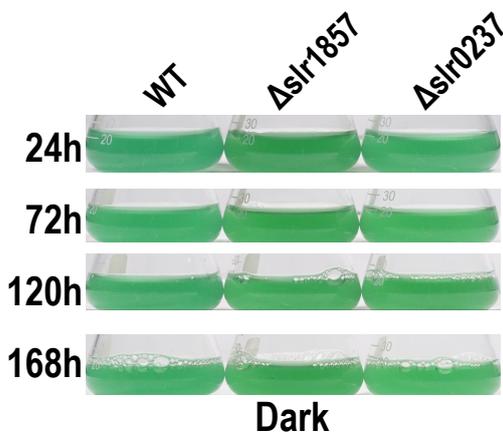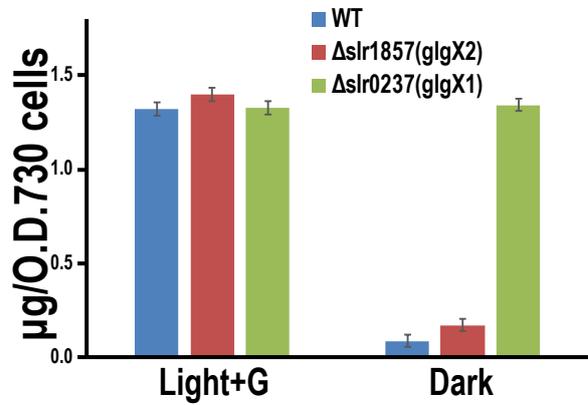
Supplemental Figure 1

A

B

Supplemental Figure 2

Horizontal bar chart with categories "Carbon network", "Unknown", and "pSYSA" on the y-axis, and x-axis labeled $-Log_{10}$(p-value) ranging from 3 to 3.6 with tick marks at 3, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6.

Supplemental Figure 3