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A third glucose uptake bypass in *Corynebacterium glutamicum* ATCC 31833

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Abstract

In *Corynebacterium glutamicum*, the phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS) has long been the only known glucose uptake system, but we recently found suppressor mutants emerging from a PTS-negative strain of *C. glutamicum* ATCC 31833 on glucose agar plates, and identified two alternative potential glucose uptake systems, the *myo*-inositol transporters encoded by *iolT1* and *iolT2*. The expression of either gene renders the PTS-negative strain WTΔptsH capable of growing on glucose. In the present study, we found a suppressor strain that still grew on glucose even after the *iolT1* and *iolT2* genes were both disrupted under the PTS-negative background. Whole-genome sequencing of the suppressor strain SPH1 identified a G-to-T exchange at 134 bp upstream of the *bglF* gene encoding an EII component of the ß-glucoside-PTS which is found in limited wild-type strains of *C. glutamicum*. Introduction of the mutation into strain WTΔptsH allowed the PTS-negative strain to grow on glucose. Reverse transcription-quantitative PCR analysis revealed that the mutation upregulates the *bglF* gene by approximately 11-fold. Overexpression of *bglF* under the *gapA* promoter in strain WTΔptsH rendered the strain capable of growing on glucose, and deletion of *bglF* in strain SPH1 abolished the growth again, proving that *bglF* is responsible for glucose uptake in the suppressor strain. Simultaneous disruption of three glucokinase genes, *glk* (Cgl2185, NCgl2105), *ppgK* (Cgl1910, NCgl1835), and Cgl2647 (NCgl2558), in strain SPH1 resulted in no growth on glucose. Plasmid-mediated expression of any of the three genes in the triple-knockout mutant restored the growth on glucose. These results indicate that *C. glutamicum* ATCC 31833 has an additional non-PTS glucose uptake route consisting of the *bglF*-specified EII permease and native glucokinases.

Key words: *Corynebacterium glutamicum*, phosphotransferase system, glucose uptake bypass, ß-glucoside-PTS, *bglF*

Introduction

Corynebacterium glutamicum is an industrially important microorganism that is widely used for the production of various amino acids (Kinoshita and Nakayama 1978; Eggeling and Bott 2005; Ikeda and Takeno 2013). This microorganism uses the phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS) to take up and phosphorylate glucose, as well as fructose and sucrose (Mori and Shiio 1987; Parche et al. 2001). The representative *C. glutamicum* wild-type strain ATCC 13032 has four sets of PTSs, one each that is specific for glucose, fructose, sucrose, and an unknown substrate (Parche et al. 2001; Moon et al. 2007). The glucose-PTS consists of the glucose-specific EIIABC component (EII^{Glc}) encoded by *ptsG* (Cgl1360, NCgl1305) and the two general components EI and HPr encoded by *ptsI* (Cgl1933, NCgl1858) and *ptsH* (Cgl1937, NCgl1862), respectively. Disruption of any of the genes in strain ATCC 13032 results in a phenotype exhibiting little growth on glucose (Parche et al. 2001). Exceptionally, another wild-type strain *C. glutamicum* R can consume glucose not only via the glucose-PTS but also via the β-glucoside-PTS because *C. glutamicum* R, unlike *C. glutamicum* ATCC 13032, has two sets of β-glucoside-PTSs that are redundantly responsible for the uptake of the β-glucosides, such as salicin and arbutin, and also glucose (Kotrba et al. 2003; Tanaka et al. 2009, 2011).

Although glucose uptake and subsequent phosphorylation in wild-type *C.*

glutamicum depends on the PTS, glucokinase activity catalyzing ATP-dependent phosphorylation of glucose into glucose-6-phosphate has been reported for this organism. The enzyme GLK, which is encoded by *glk* (Cgl2185, NCgl2105), has been shown to be involved in the metabolism of the disaccharide maltose, which is hydrolyzed to free glucose inside the cell (Park et al. 2000). An additional glucokinase encoded by *ppgK* (Cgl1910, NCgl1835) has been reported (Lindner et al. 2010). The enzyme PPGK has been shown to strongly prefer polyphosphate over ATP as a phosphoryl donor and is therefore assumed to be especially important under growth conditions depending on high intracellular polyphosphate concentrations. Recently, we identified a third enzyme that can be involved in the phosphorylation of intracellular glucose (Ikeda 2012). The enzyme CGL2647, which is encoded by the Cgl2647 (NCgl2558) gene, shows approximately 29% and 25% sequence identity with GLK and PPGK, respectively, while GLK and PPGK share 28% identity with each other.

Our laboratories aim at a better understanding of the entire cellular system of *C. glutamicum* and also at the discovery of new methods allowing us to fully draw out the potential of this microorganism as a workhorse for the production of useful compounds from renewable feedstocks (Ikeda et al. 2013a; Takeno et al. 2010, 2013). Recently, we happened to find that colonies emerged on glucose agar plates from WTΔptsH, a *ptsH*-disrupted strain of the *C. glutamicum* wild-type strain WT-96 (ATCC 31833), at a frequency of 10^{-5} (Ikeda et al. 2011). Since the suppressor strain SPH2 (Fig. 1), unlike the wild-type strain, exhibited a phenotype of resistance to 2-deoxyglucose which is known to be a toxic substrate for the glucose-PTS of this organism (Mori and Shiio 1987), we predicted that the suppressor strain would utilize glucose via a different system involving a particular permease and the native glucokinases. Our analysis following these observations

eventually led to the finding that two *myo*-inositol transporters encoded by *iolT1* (Cgl0181, NCgl0178) and *iolT2* (Cgl3058, NCgl2953) mediate glucose uptake in this organism and that glucose phosphorylation depends on glucokinase activity (Ikeda 2012; Ikeda et al. 2011). We also showed that inactivation of *iolR*, a putative transcriptional regulator gene, enables strain WTΔptsH to grow on glucose through the derepression of *iolT1*, as was the case with the suppressor strain SPH2. Therefore, strain SPH2 loses the ability to grow on glucose when *iolT1* is disrupted (Fig. 1). Based on these results, we established a strategy for engineering *C. glutamicum* to express the *iolT1*-specified glucose uptake bypass instead of the original PTS, and demonstrated the usefulness of this strategy using lysine fermentation as a model (Ikeda 2012; Ikeda et al. 2011). In keeping with our results, a German group has independently identified two *myo*-inositol transporters as alternatives to the PTS in this organism and shown the advantage of expressing the non-PTS route(s) in lysine production (Lindner et al. 2011a, b).

During the course of our studies, we found another suppressor strain that still grew on glucose even after the *iolT1* and *iolT2* genes were both disrupted under a PTS-negative background. The suppressor strain SPH1 (Fig. 1), like strain SPH2, was identified from among the colonies that emerged from strain WTΔptsH on glucose agar plates; it exhibited a phenotype of resistance to 2-deoxyglucose. This finding led us to predict that a different glucose uptake bypass was operating in strain SPH1. Here, we describe the identification of a third bypass route which is expressed in strain SPH1, as well as the suppressor mutation which renders a PTS-negative strain capable of growing on glucose. Finally, an overall picture of potential glucose uptake bypasses in *C. glutamicum* is summarized.

Materials and methods

Bacterial strains

The wild-type strain of *C. glutamicum* used in this study is strain WT-96, a single-colony derivative of *C. glutamicum* ATCC 31833. Strains WTΔptsH, WTΔptsI, and WTΔptsHI are WT-96-derived PTS-negative mutants which have deletions in *ptsH*, *ptsI*, and both of them, respectively. Strain SPH2 is one of the suppressor mutants that arose spontaneously from the PTS-negative strain WTΔptsH on an MM agar plate with glucose (Ikeda et al. 2011). This strain carries a suppressor mutation leading to inactivation of *iolR*, a putative transcriptional regulator gene. Strain SPH1 used in this study is another suppressor mutant that arose from the PTS-negative strain. This suppressor strain, unlike strain SPH2, was able to grow on glucose even after the *iolT1* and *iolT2* genes were both deleted. Strain SPHΔggg is a SPH1-derived mutant which has triple deletions of the three glucokinase genes *glk*, *ppgK*, and Cgl2647. *Escherichia coli* DH5α was used as a host for DNA manipulation.

Plasmids

Plasmid pCS299P (Mitsuhashi et al. 2004), a *C. glutamicum-E. coli* shuttle vector, was used to construct a genomic library and also to clone the PCR products. Plasmid pESB30 (Mitsuhashi et al. 2004), which is nonreplicative in *C. glutamicum*, is a vector for gene replacement in *C. glutamicum*. Plasmids pCbglF, pCglk, pCppgK, and pC2647 were used to constitutively express the *bglF*, *glk* (Cgl2185, NCgl2105), *ppgK* (Cgl1910, NCgl1835),

and Cgl2647 (NCgl2558) genes, respectively, under the promoter of the endogenous *gapA* gene. For the construction of pCbglF, the coding region of *bglF* was PCR amplified using primers bglFgapFusF and bglFdown100RBglII with WT-96 genomic DNA as a template. On the other hand, the genomic region from -1 to -558 bp upstream of the *gapA* gene, which comprises its promoter, was amplified using primers PgapAKpBgF and bglFgapFusR. These two fragments were fused by PCR with the primers PgapAKpBgF and bglFdown100RBglII. The resulting 2.6-kb fragment was digested with *Bgl*II and then ligated to *Bam*HI-digested pCS299P to yield pCbglF. Similarly, for the construction of pCglk, the coding region of *glk* was PCR amplified using primers Cgl2185gapFusF and Cgl2185down200RBglII with WT-96 genomic DNA as a template. The genomic region upstream of the *gapA* gene was amplified using primers PgapAKpBgF and Cgl2185gapFusR. These two fragments were fused by PCR with the primers PgapAKpBgF and Cgl2185down200RBglII. The resulting 1.8-kb fragment was digested with *Bgl*II and then ligated to *Bam*HI-digested pCS299P to yield pCglk. For the construction of pCppgK, the coding region of *ppgK* was PCR amplified using primers Cgl1910gapFusF and Cgl1910down500RKpnI with WT-96 genomic DNA as a template. The genomic region upstream of the *gapA* gene was amplified using primers PgapAKpBgF and Cgl1910gapFusR. These two fragments were fused by PCR with the primers PgapAKpBgF and Cgl1910down500RKpnI. The resulting 1.8-kb fragment was digested with *Kpn*I and then ligated to *Kpn*I-digested pCS299P to yield pCppgK. For the construction of pC2647, the coding region of Cgl2647 was PCR amplified using primers Cgl2647gapFusF and Cgl2647down200RBglII with WT-96 genomic DNA as a template. The genomic region upstream of the *gapA* gene was amplified using primers PgapAKpBgF and Cgl2647gapFusR. These two fragments were fused by PCR with the primers PgapAKpBgF

and Cgl2647down200RBglII. The resulting 1.7-kb fragment was digested with *Bgl*II and then ligated to *Bam*HI-digested pCS299P to yield pC2647. The sequences of the primers used in this study are listed in Table S1 in the supplemental material. Primers used for the construction of plasmids pCBglF, pCBglF134^{up} (see below), and pCbglFd (see below), and for the qPCR analysis of the *bglF* gene expression, were designed on the basis of the genomic sequence of *C. glutamicum* WT-96. The other primers were designed on the basis of the genomic sequence of *C. glutamicum* ATCC 13032 (BA000036), which is publicly available at http://www.genome.jp/kegg/genes.html (Ikeda and Nakagawa 2003).

Media and culture conditions

Complete medium BY and minimal medium MM were used for growth of *C. glutamicum* strains (Takeno et al. 2007). MM medium contained 1% glucose as the sole carbon source. When required, kanamycin was added at a final concentration of 20 mg per liter. For growth test in liquid culture, 0.05 mL of the seed culture grown aerobically at 30ºC in BY medium for 12 h was inoculated into 5 mL of medium in a L-type test tube and cultivated at 30ºC using a Monod shaker at 40 strokes/min.

Recombinant DNA techniques

Standard protocols (Sambrook and Russell 2001) were used for the construction, purification, and analysis of plasmid DNA and for the transformation of *E. coli*. Extraction of *C. glutamicum* chromosomal DNA and transformation of *C. glutamicum* by electroporation were carried out as described previously (Takeno et al. 2007). PCR was

192 performed using a DNA thermal cycler (GeneAmp PCR System 9700, Applied Biosystems,
193 Carlsbad, CA) using Phusion High-Fidelity DNA Polymerase (New England BioLabs,
194 Ipswich, MA).
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196 Identification of the suppressor mutation in strain SPH1
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198 The suppressor mutation in strain SPH1 was identified via a comparative genome
199 analysis using the strain WT-96 genome as a reference. Whole-genome sequencing of
200 strains SPH1 and WT-96 was performed by Takara Bio Inc (Shiga, Japan) using an
201 Illumina Genome Analyzer IIx (Illumina, San Diego, CA).
202
203 Strain construction
204
205 For the generation of a strain carrying the specific mutation at 134 bp upstream of the *bglF*
206 gene of strain WTΔptsH, plasmid pCbglF134^{up} was constructed as follows. A region
207 containing the mutation, in addition to the intact *bglF* gene, was PCR amplified using
208 primers bglFup800FBglII and bglFdown100RBglII with strain SPH1 genomic DNA as a
209 template. After verification by DNA sequencing, the 2.8-kb PCR product was digested with
210 *Bgl*II and then ligated to pESB30 to yield pCbglF134^{up}. This plasmid was used to replace
211 the wild-type sequence with the mutated sequence as described previously (Ohnishi et al.
212 2002). The presence of the mutation was confirmed by allele-specific PCR and DNA
213 sequencing. The nucleotide sequence of the 2.8-kb DNA fragment from *C. glutamicum*
214 ATCC 31833 was deposited in DDBJ under accession number LC009376.
215 For the chromosomal deletion of *bglF*, *ptsI* (Cgl1933, NCgl1858), *glk*, *ppgK*,

and Cgl2647, plasmids pCbglFd, pCptsId, pCglkd, pCppgKd, and pC2647d which contained internally deleted copies of the corresponding genes were used to replace the wild-type chromosomal genes with the deleted genes. For the construction of plasmid pCbglFd, the 5'-regions of the *bglF* gene were amplified by PCR using primers bglFup800FBglII and bglFdelFusR with SPH1 genomic DNA as a template. Similarly, the 3'-region of the gene was amplified using primers bglFdelFusF and bglFdown700RBglII. Fusion PCR was then performed using the purified 5'- and 3'-region fragments as templates and the primers bglFup800FBglII and bglFdown700RBglII. The resulting 1.5-kb fused fragment was digested with *Bgl*II and then ligated to *Bam*HI-digested pESB30 to yield pCbglFd. Likewise, for the construction of plasmid pCptsId, the 5'- and 3'-regions of the *ptsI* gene were amplified using two pairs of primers with WT-96 genomic DNA: the pair comprising ptsIup800F and ptsIFusR and the pair comprising ptsIFusF and ptsIdown800RBamHI, respectively. Two resulting fragments were fused by PCR, digested with *Bam*HI and then ligated to *Bam*HI-digested pESB30 to yield pCptsId. Similarly, for the construction of plasmid pCglkd, the 5'- and 3'-regions of the *glk* gene were amplified using two pairs of primers with WT-96 genomic DNA: the pair comprising Cgl2185up800FBglII and Cgl2185delFusR and the pair comprising Cgl2185delFusF and Cgl2185down800RBglII, respectively. Two fragments were fused by PCR, digested with *Bgl*II and then ligated to pESB30 to yield pCglkd. Plasmid pCppgKd was constructed as follows. The 5'- and 3'-regions of the *ppgK* gene were amplified using two pairs of primers with WT-96 genomic DNA: the pair comprising Cgl1910up800FBamHI and Cgl1910delFusR and the pair comprising Cgl1910delFusF and Cgl1910down900RBamHI, respectively. Two resulting fragments were fused by PCR, digested with *Bam*HI and then ligated into

240 *Bam*HI-digested pESB30 to yield pCppgKd. For the construction of plasmid pC2647d,
241 the 5'- and 3'-regions of the Cgl2647 gene were amplified using two pairs of primers
242 with WT-96 genomic DNA: the pair comprising Cgl2647up600FBglII and
243 Cgl2647delFusR and the pair comprising Cgl2647delFusF and Cgl2647down800RBglII,
244 respectively. Two resulting fragments were fused by PCR, digested with *Bgl*II and then
245 ligated to *Bam*HI-digested pESB30 to yield pC2647d. Defined chromosomal deletion of
246 the individual gene was accomplished using each plasmid via two recombination events
247 as described previously (Ohnishi et al. 2002).
248
249 RNA extraction, cDNA synthesis, and qPCR
250
251 Extraction of total RNAs from *C. glutamicum* strains and subsequent purification were
252 performed as described previously (Hayashi et al. 2002). Synthesis of cDNA was
253 performed with 300 ng of RNA as described by Kind et al. (2011). Quantitative PCR
254 (qPCR) analysis was performed by the method described by Katayama et al. (2013). The
255 gene expression levels were standardized to the constitutive level of 16S rRNA expression
256 and calculated according to the comparative cycle threshold method (Schmittgen and Livak
257 2008).
258
259 Analysis
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261 Cell growth was monitored by measuring the optical density of the culture broth at 660 nm
262 (OD_{660}) with a Miniphoto 518R spectrophotometer (Taitec, Saitama, Japan).
263

Results

Identification of the suppressor mutation in strain SPH1

Strain SPH1 is one of the suppressor mutants that arose spontaneously from the PTS-negative strain WTΔptsH on an MM agar plate with glucose (Fig. 1). Since this strain still grew on glucose even after the *iolT1* and *iolT2* genes were both disrupted, we assumed that a third glucose uptake bypass was operating in the strain. To identify the bypass route, we first attempted transcriptome analysis using DNA microarrays because the relevant gene(s) was thought to be upregulated in the suppressor strain SPH1. When total RNAs from mid-exponential phase cultures on glucose were used to study differential transcription profiles between strain SPH1 and its parental wild-type WT-96, a single-colony derivative of *C. glutamicum* ATCC 31833, we failed to find any candidate genes that were upregulated in strain SPH1 compared to the wild-type levels (data not shown). As it turned out afterwards, the failure was not due to our hypothesis itself but rather, to the design of our microarrays (see the Discussion section). We then conducted whole-genome sequencing of strains SPH1 and WT-96 to identify the suppressor mutation carried by strain SPH1. By comparing both genome sequences, we found a specific mutation (designated mutation *bglF134*^{up}), a G-to-T exchange at 134 bp upstream of the *bglF* gene encoding an EII component of the ß-glucoside-PTS (Fig. 2). Genome sequencing also revealed that the *bglF* gene was located in the cluster *bglF-bglA-bglG* which has previously been identified as the ß-glucoside utilization system in another wild-type *C. glutamicum* R (Kotrba et al. 2003; Tanaka et al. 2009, 2011). Interestingly, a search for *C. glutamicum* genome databases revealed that the *bglF-bglA-bglG* DNA region

288 including the upstream mutation site was missing from the corresponding position in the
289 genome of *C. glutamicum* R and ATCC 13032 (Fig. 2). Introduction of the *bglF134*^{up}
290 mutation into strain WTΔptsH actually allowed the PTS-negative strain to grow on
291 glucose: the growth profile in MM medium with glucose as the sole carbon source was
292 almost the same as that of strain SPH1 (Fig. 3). These results indicate that the suppressor
293 mutation in strain SPH1 is *bglF134*^{up}.
294
295 Effects of the *bglF134*^{up} mutation on the transcript level of *bglF* expression
296
297 Since the *bglF134*^{up} mutation is located just near the ribonucleic antiterminator (RAT)
298 sequence to which the *bglG*-encoded antiterminator protein binds in response to the
299 presence of ß-glucoside (Kotrba et al. 2003; Tanaka et al. 2009) (Fig. 2), the mutation was
300 considered to affect the transcript level of the downstream *bglF* gene. Accordingly, we
301 used reverse transcription (RT)-qPCR to investigate the transcript levels of *bglF* in the
302 suppressor strain SPH1 and strain WTΔptsH carrying the *bglF134*^{up} mutation, using the
303 wild-type strain WT-96 as a control. As shown in Fig. 4, the two strains that carried the
304 *bglF134*^{up} mutation exhibited approximately 11-fold increased transcript levels of *bglF*
305 compared to the wild-type. Considering that the mutation generates a mismatch in the
306 base-paired stem, it is likely that the mutation would interfere with the formation of the
307 terminator structure and allow RNA polymerase to transcribe the downstream *bgl* gene
308 cluster, thereby leading to overexpression of the *bglF* gene.
309
310 Effects of *bglF* modifications on growth
311

312 To substantiate the glucose-uptake capability of the *bglF* gene product, we examined the
313 effect of its expression on growth on glucose in strain WTΔptsH. For this purpose, the
314 coding region of *bglF* was cloned on a multi-copy vector so as to be constitutively
315 expressed under the promoter of the endogenous *gapA* gene encoding glyceraldehyde
316 3-phosphate dehydrogenase. We then introduced the resulting plasmid pCbglF into strain
317 WTΔptsH. As shown in Fig. 5a, the plasmid could confer the ability to grow in MM
318 medium with glucose as the sole carbon source on the PTS-negative strain, proving that the
319 *bglF* gene product plays a role in glucose uptake. When a *ptsI*-disrupted strain, WTΔptsI,
320 and a double-disrupted strain of both *ptsH* and *ptsI*, WTΔptsHI, were used as PTS-negative
321 hosts instead of strain WTΔptsH, similar recoveries of growth on glucose were observed
322 (Fig. 5bc). On the other hand, in-frame deletion of the *bglF* inner sequence in strain SPH1
323 thoroughly abolished growth on glucose (Fig. 5a), which reinforced our conclusion.
324
325 Involvement of glucokinase in growth of strain SPH1
326
327 Considering the results mentioned above, strain SPH1 was assumed to take up glucose by
328 the *bglF*-specified permease and then phosphorylate by any of the three known native
329 glucokinases, namely, GLK, PPGK, and CGL2647, encoded by *glk*, *ppgK*, and Cgl2647,
330 respectively. If so, simultaneous defects of all three glucokinases must result in defective
331 growth on glucose. To verify this, we disrupted all three genes through in-frame deletion of
332 the corresponding inner sequences in strain SPH1. The resulting triple-knockout mutant,
333 designated SPHΔggg, actually showed no growth on glucose (Fig. 6). Plasmid-mediated
334 expression of any of the three genes under the *gapA* promoter in strain SPHΔggg restored
335 the growth of the mutant on glucose (Fig. 6), proving that strain SPH1 undoubtedly

depends on glucokinase(s) for its growth on glucose (Fig. 7).

Discussion

In our previous report (Ikeda et al. 2011), we showed that the *myo*-inositol transporters IolT1 and IolT2 function as alternatives to the glucose-PTS in *C. glutamicum*. In this study, we identified a third glucose uptake bypass as the *bglF*-specified EII component of the ß-glucoside-PTS in *C. glutamicum* ATCC 31833. The *bglF* gene product EII^{Bgl} is known to be a member of the PtsG family of EII proteins (Schnetz et al. 1990), and it shows significant sequence identity (29.9%) with PtsG, a glucose-specific EII^{Glc} component, while it shares only 17.1% and 15.2% with IolT1 and IolT2, respectively. The presence of a ß-glucoside-PTS in *C. glutamicum* was first described in another wild-type *C. glutamicum* R strain (Kotrba et al. 2003), and the PTS is known to mediate the uptake of ß-glucosides such as salicin and arbutin. Actually, the wild-type strain WT-96 used in this study showed growth in MM medium with the ß-glucoside arbutin as the sole carbon source, whereas disruption of *bglF* or *ptsH* in the wild-type strain thoroughly abolished growth on arbutin (data not shown), confirming that *bglF* functions as the ß-glucoside-PTS, as it does in *C. glutamicum* R. It is already known (Kotrba et al. 2003; Tanaka et al. 2009) that the ß-glucoside utilization system consisting of the *bglF-bglA-bglG* gene set is missing from the representative wild-type strain *C. glutamicum* ATCC 13032. This finding is worth noting because the ATCC 13032 genomic information has generally been used to design *C. glutamicum* DNA microarrays. So did our microarrays. This seems to be a reason why our first attempt to identify the bypass gene using the microarrays resulted in failure.

The ß-glucoside-PTS is known to mediate the uptake not only of the ß-glucosides

but also of glucose in *E. coli, Bacillus subtilis*, and *C. glutamicum* (Schnetz et al. 1990; Schilling et al. 2006; Tanaka et al. 2011). Nevertheless, our study has shown that the EII component alone can mediate glucose uptake enough to allow normal cell growth on glucose. This conclusion is based on our observations that plasmid-mediated expression of the *bglF* gene in the PTS-negative strains with deletions of *ptsH*, *ptsI*, and both allowed any of the mutants to grow in MM medium with glucose as the sole carbon source (Fig.5). These results indicate that the *bglF*-specified EII component of the ß-glucoside-PTS plays a role in glucose uptake independently from the other two general components EI and HPr, encoded by *ptsI* and *ptsH*, respectively. This finding is noteworthy because carbohydrate transport by the bacterial PTS occurs by tightly coupling carbohydrate translocation and phosphorylation, and it is believed that EII alone is unable to catalyze carbohydrate transport in the absence of either or both of the two phosphoryl-transferring components EI and HPr (Postma and Stock 1980). To the best of our knowledge, there seems no previous report demonstrating that a wild-type EII component alone functions as a sugar permease at a level allowing normal cell growth. If there is any, the transport potency would be insufficient for cell growth, as in the case of, e.g., the *treB*-specified EII component of the trehalose-PTS in an *E. coli* W strain (Steen et al. 2014).

Although bacterial EII^{Glc} components require the general PTS components EI and HPr to catalyze glucose transport, some specific mutations that lead to the uncoupling of glucose transport from phosphorylation have been isolated in the EII^{Glc} of enteric bacteria (Postma 1981; Ruijter et al. 1992). Contrary to the wild-type protein, the uncoupled EII^{Glc} derivatives are able to transport glucose in the absence of EI and HPr. In these cases, it has been suggested that the mutant EII^{Glc} allows translocation of glucose across the membrane via a mechanism of facilitated diffusion (Ruijter et al. 1990, 1992). Although the

mechanism of glucose uptake in our newly discovered glucose uptake bypass remains to be clarified, it seems possible that the wild-type EII^{Bgl} of *C. glutamicum* functions via a similar mechanism of facilitated diffusion even under a PTS-negative background. This implies that there would be no energetic demands for glucose transport via the *C. glutamicum* EII^{Bgl} protein alone, as has been suggested for glucose transport via the uncoupled EII^{Glc} derivatives of enteric bacteria (Ruijter et al. 1991). In relation to this, a specific mutation that leads to the uncoupling of glucose transport from phosphorylation has been reported for *E. coli* EII^{Bgl} (Schnetz et al. 1990).

The third glucose uptake bypass identified in this study, like the previously-identified bypasses IolT1 and IolT2, is a non-PTS route in which growth on glucose depends on endogenous glucokinase(s) (Fig. 7). This was genetically proved in this study, but is also supported by our previous observation that strain SPH1 shows clear resistance to 2-deoxyglucose, a glucose analog which is known to be a toxic substrate for the glucose-PTS of *C. glutamicum* (Mori and Shiio 1987). The same phenotype has been observed in the other bypass strain SPH2, but not in the wild-type strain expressing the native PTS (Ikeda et al. 2011). These results indicate that strain SPH1, like strain SPH2, takes up and phosphorylates glucose via a 2-deoxyglucose-resistant non-PTS system, undoubtedly involving the endogenous permease and glucokinase(s). Although the PTS uses phosphoenolpyruvate (PEP) to phosphorylate glucose, the non-PTS routes do not; they therefore have the practical advantage of increased availability of PEP for other biosynthetic reactions, e.g., for amino acid biosynthesis, as has been demonstrated in aromatic production by *E. coli* (Flores et al. 1996). Although data are not shown, we have confirmed that significant yield improvement can be obtained when a defined lysine-producing strain of *C. glutamicum* expresses the non-PTS route(s) instead of the

original PTS (Ikeda 2012; Ikeda et al. 2011). This positive effect has also been explained by increased availability of PEP, which contributes to increased supply of carbon from central metabolism into the lysine-biosynthetic pathway through the anaplerotic reaction involving PEP carboxylase. Based on these original results, we have applied for an international patent, and currently hold a patented technology in the U.S. (Ikeda et al. 2013b). Considering the known characteristics of the *C. glutamicum* PTS, e.g., its relative sensitivity to increased osmolality (Gourdon et al. 2003), the potential practical advantage of the non-PTS routes seems to go beyond simply saving PEP for other biosynthetic reactions. Switching the glucose transport system from the original PTS to a non-PTS route(s), or co-expressing the PTS and the non-PTS route(s), could impact the industrial fermentation processes that use *C. glutamicum*. The discovery of the third non-PTS route for glucose uptake in this study will surely expand the options for the development of more efficient production strains in this industrially important microorganism.

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540 antiterminator proteins is a determinant for the difference in glucose repression of
541 two β-glucoside phosphotransferase system gene clusters in *Corynebacterium*
542 *glutamicum* R. J Bacteriol 193:349-357
543

544 **Figure legends**

545

546 **Fig. 1** Phylogeny of the mutants derived from the *C. glutamicum* wild-type strain
547 WT-96, a single-colony derivative of *C. glutamicum* ATCC 31833. Strains SPH1 and

548 SPH2 (Ikeda et al. 2011) are independently isolated from WTΔptsH, a *ptsH*-disrupted
549 strain, as suppressor mutants that grew on glucose agar plates spontaneously. The
550 typical phenotypes of each strain are indicated in parentheses. Glc^+ and Glc^- indicate
551 growth and no growth, respectively, in MM medium containing 1% glucose as the sole
552 carbon source. $2DG^s$ and $2DG^r$ indicate sensitivity and resistance to 0.1%
553 2-deoxyglucose, respectively, in MM medium containing 1% ribose or acetate as the
554 sole carbon source.
555
556 **Fig. 2** The *bglF134*^{up} mutation identified in the suppressor strain SPH1. The *bglF134*^{up}
557 mutation, a G-to-T exchange at 134 bp upstream of the *bglF* gene, is located just near
558 the putative ribonucleic antiterminator (RAT) sequence to which the *bglG*-encoded
559 antiterminator protein binds in response to the presence of ß-glucoside (Kotrba et al.
560 2003; Tanaka et al. 2009). The putative RAT sequence and transcriptional terminator
561 sequence are indicated by the gray line and stem-loop, respectively. The -10 and -35
562 regions of the potential promoter of *bglF* are boxed. The corresponding genomic
563 regions in *C. glutamicum* ATCC 13032 and *C. glutamicum* R are also shown. The
564 former strain ATCC 13032 lacks the ß-glucoside utilization system consisting of the
565 *bglF-bglA-bglG* gene cluster while the latter strain R has two functional sets of the gene
566 cluster at different positions in its genome (Tanaka et al. 2009).
567
568 **Fig. 3** Growth of suppressor strain SPH1 (*solid triangles*) and strain WTΔptsH carrying
569 the *bglF134*^{up} mutation (*open triangles*) in MM medium containing 1% glucose as the
570 sole carbon source. For comparison, the profiles of wild-type strain WT-96 (*solid*
571 *circles*), PTS-negative strain WTΔptsH (*solid squares*), and the other suppressor strain

SPH2 (*solid diamonds*) are shown as controls. The values are means of replicated cultures, which showed differences of <5% from each other. OD_{660}, optical density at 660 nm.

Fig. 4 Relative mRNA levels of the *bglF* gene in wild-type strain WT-96, suppressor strain SPH1, and strain WTΔptsH carrying the *bglF134*^{up} mutation. Total RNAs were prepared from cells grown to the early exponential phase (OD_{660} = approximately 2.5) in MM medium. Aliquots of RNAs were reverse-transcribed, and subjected to quantitative PCR. The relative mRNA levels were standardized to the constitutive expression level of 16S rRNA. The transcript level in wild-type WT-96 was set to 1.0. Data represent mean values from three independent cultures, and the standard deviation from the mean is indicated as error bars.

Fig. 5 Effect of *bglF* expression on growth of different PTS-negative strains. **a** Strain WTΔptsH carrying vector pCS299P (*solid squares*) and plasmid pCbglF (*open circles*), as well as strain SPH1 (*solid triangles*) and its *bglF*-deleted mutant (*open triangles*), were cultivated in MM medium containing 1% glucose as the sole carbon source. **b** Strain WTΔptsI carrying vector pCS299P (*solid squares*) and plasmid pCbglF (*open circles*) were cultivated in the same conditions. **c** Strain WTΔptsHI carrying vector pCS299P (*solid squares*) and plasmid pCbglF (*open circles*) were cultivated in the same conditions. The values are means of replicated cultures, which showed differences of <5% from each other.

Fig. 6 Growth of strain SPHΔggg carrying vector pCS299P (*solid squares*), plasmid pCglk (*open squares*), pCppgK (*open triangles*), and pC2647 (*open circles*) in MM medium

containing 1% glucose as the sole carbon source. Strain SPHΔggg is a SPH1-derived mutant with triple deletions of the three glucokinase genes *glk*, *ppgK*, and Cgl2647. For comparison, the profile of strain SPH1 carrying vector pCS299P (*solid triangles*) is shown as a control. The values are means of replicated cultures, which showed differences of <5% from each other.

Fig. 7 The glucose-PTS and potential glucose uptake bypasses in *C. glutamicum* ATCC 31833. As exemplified by strain SPH2, inactivation of the putative transcriptional regulator IolR under a PTS-negative background leads to a strain expressing the *iolT1*-specified glucose uptake bypass (IolT1) instead of the native PTS (Ikeda et al. 2011). On the other hand, introduction of the *bglF134*^{up} mutation into a PTS-negative background results in a strain expressing the *bglF*-specified glucose uptake bypass (EII^{Bgl}) instead of the native PTS, as exemplified by strain SPH1. In these PTS-independent glucose uptake bypasses, the ATP- or polyphosphate (PolyPn)-dependent phosphorylation of intracellular glucose depends on any of the three known native glucokinases GLK, PPGK, and CGL2647, encoded by *glk*, *ppgK*, and Cgl2647, respectively.

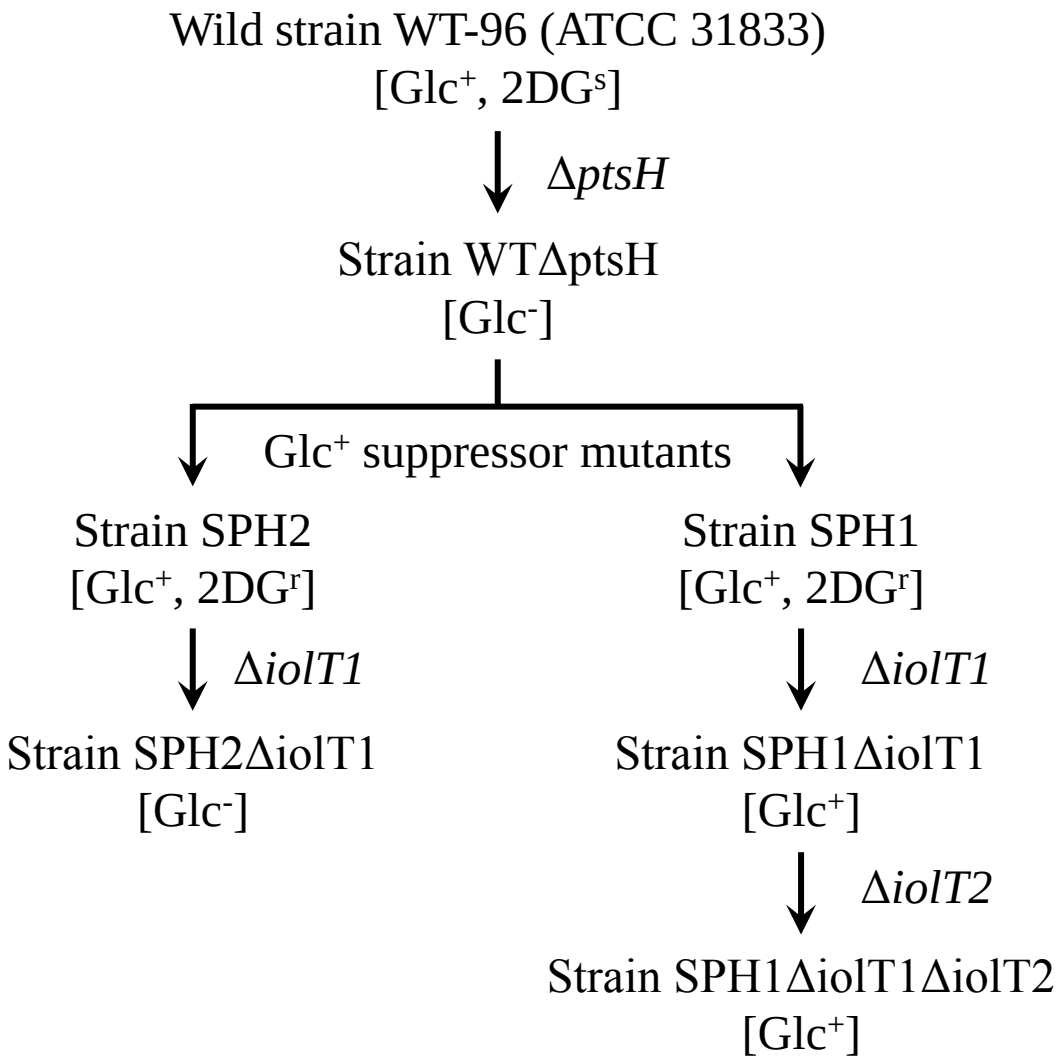


Fig. 1 Ikeda et al.

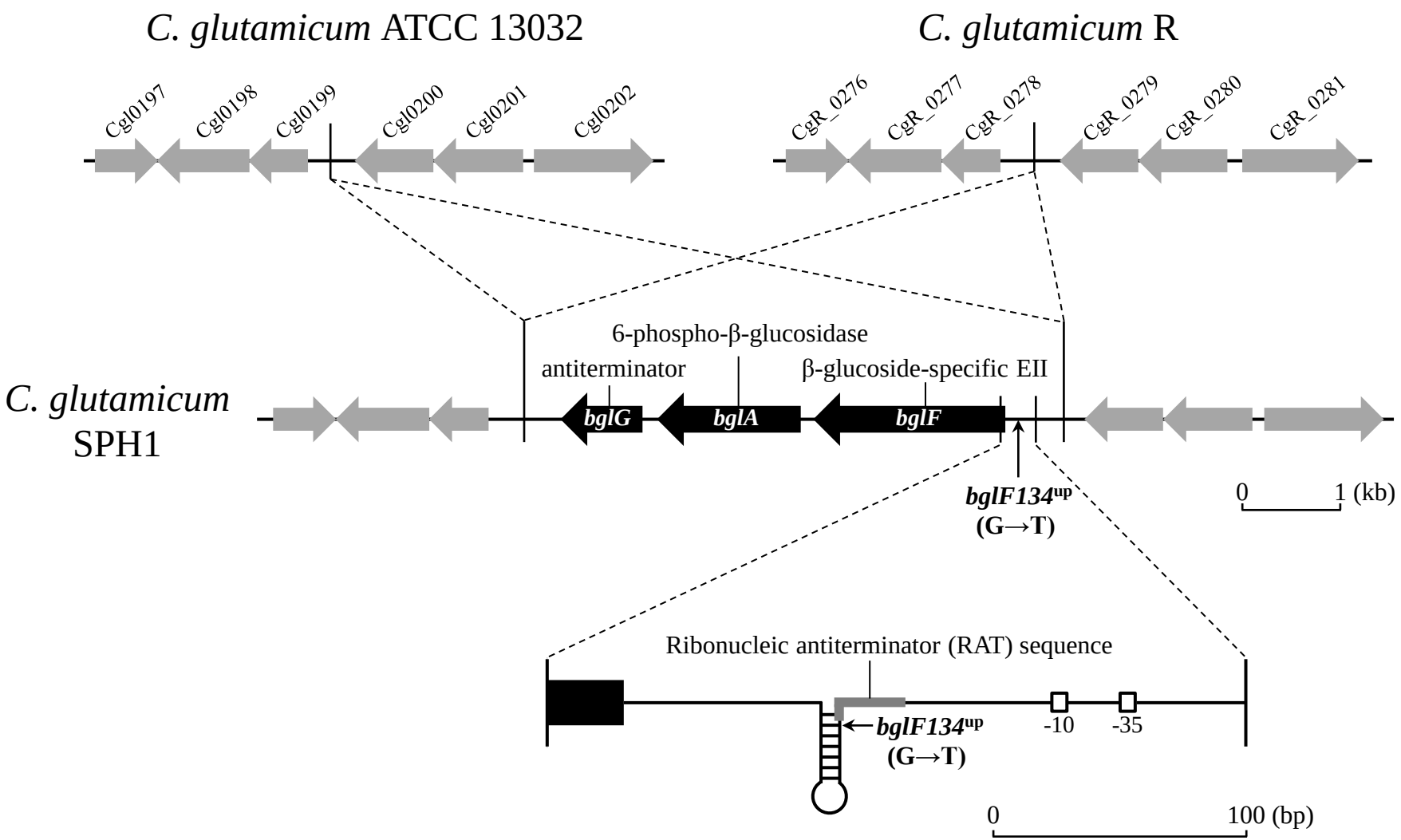
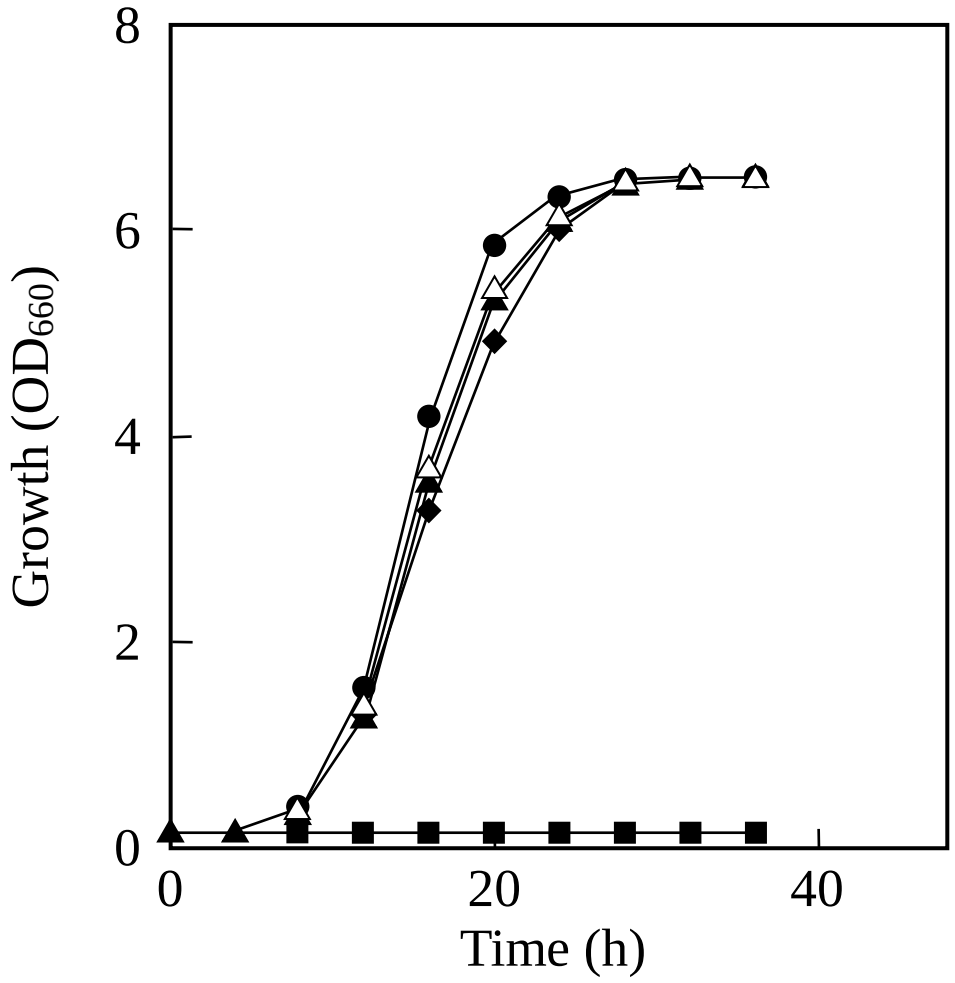


Fig. 2 Ikeda et al.



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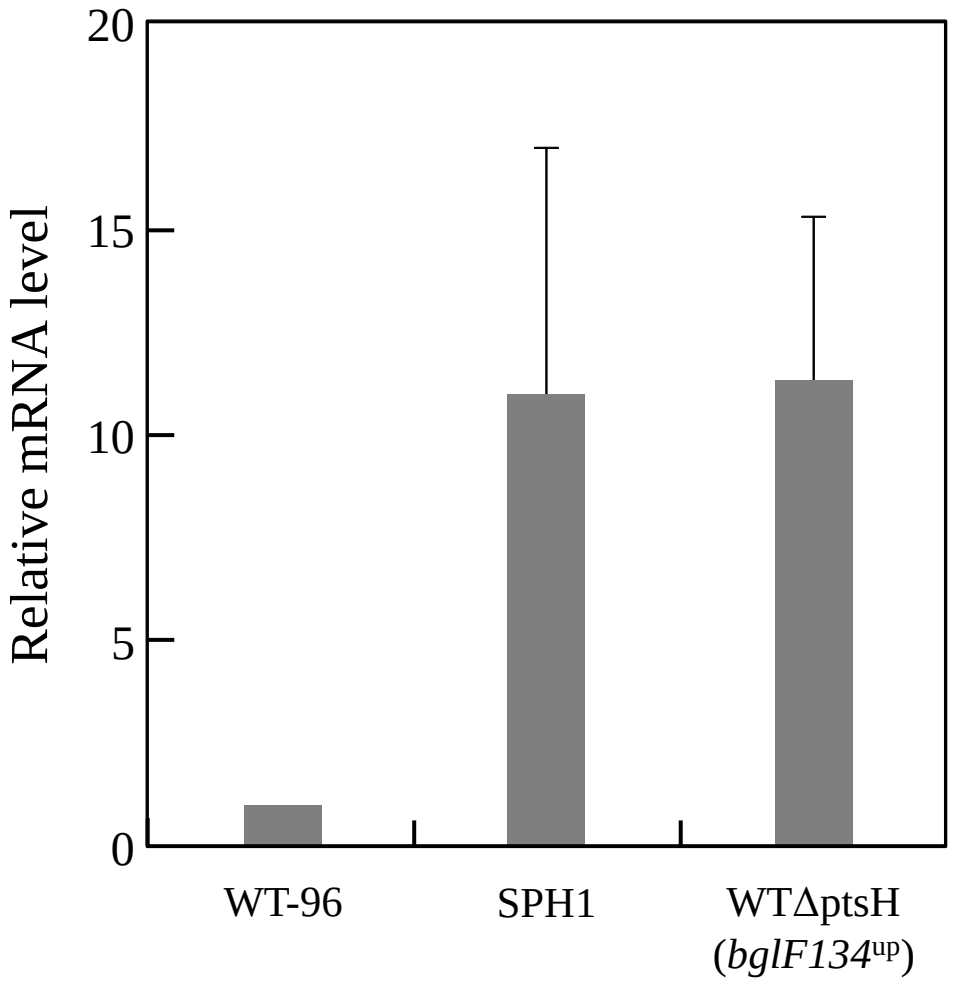

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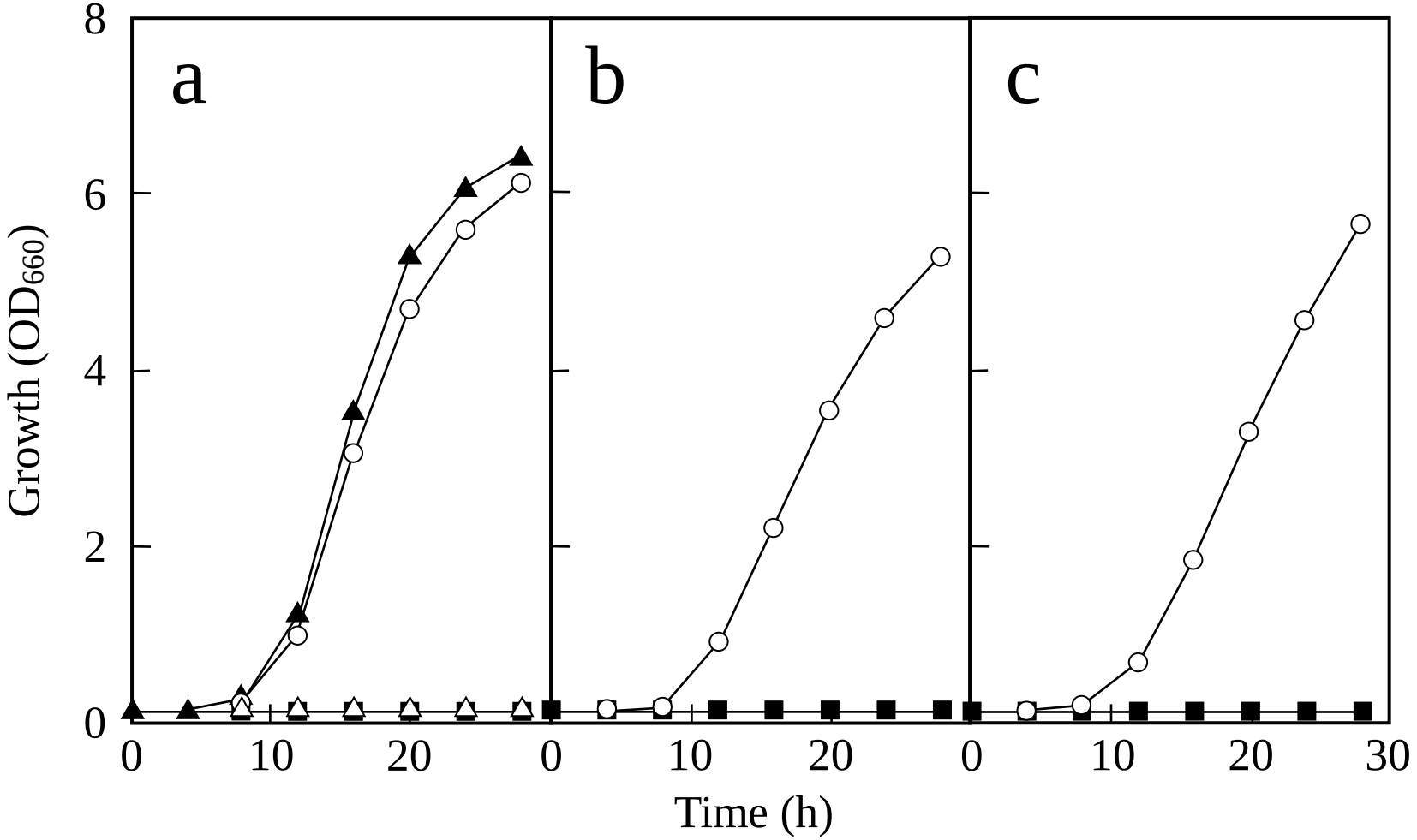

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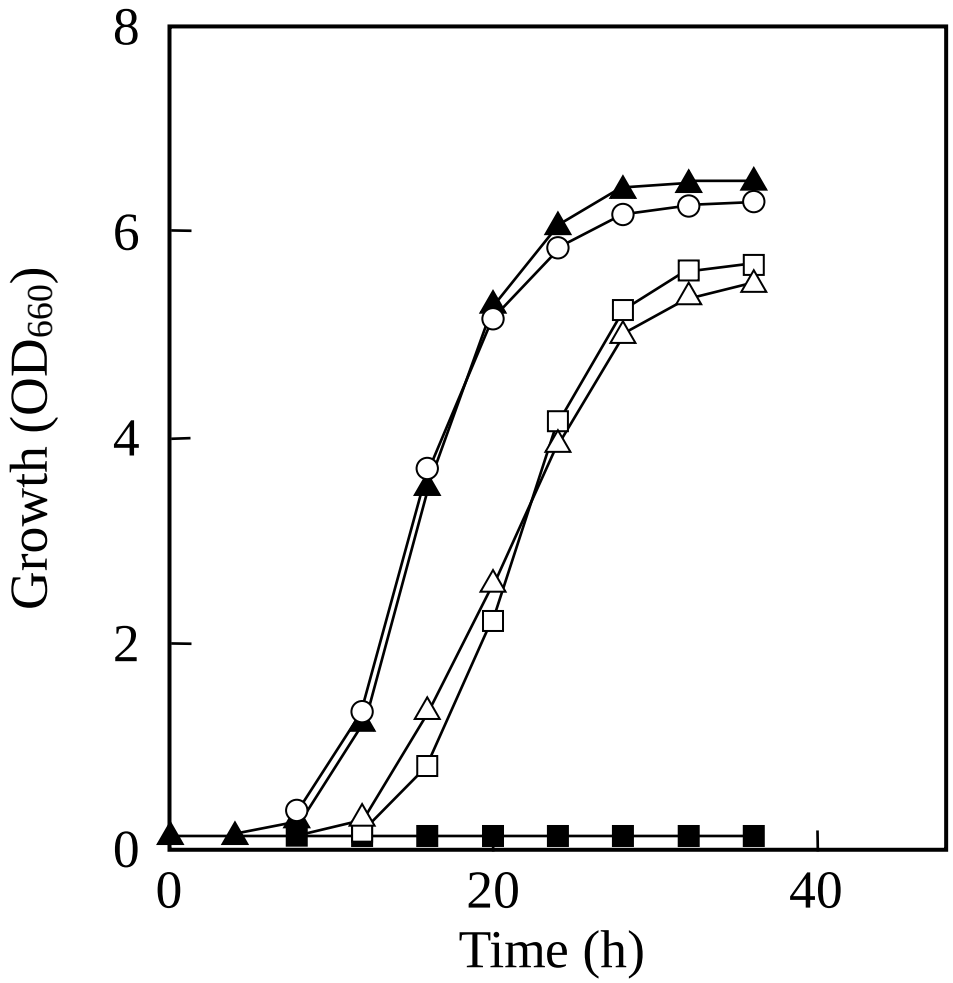

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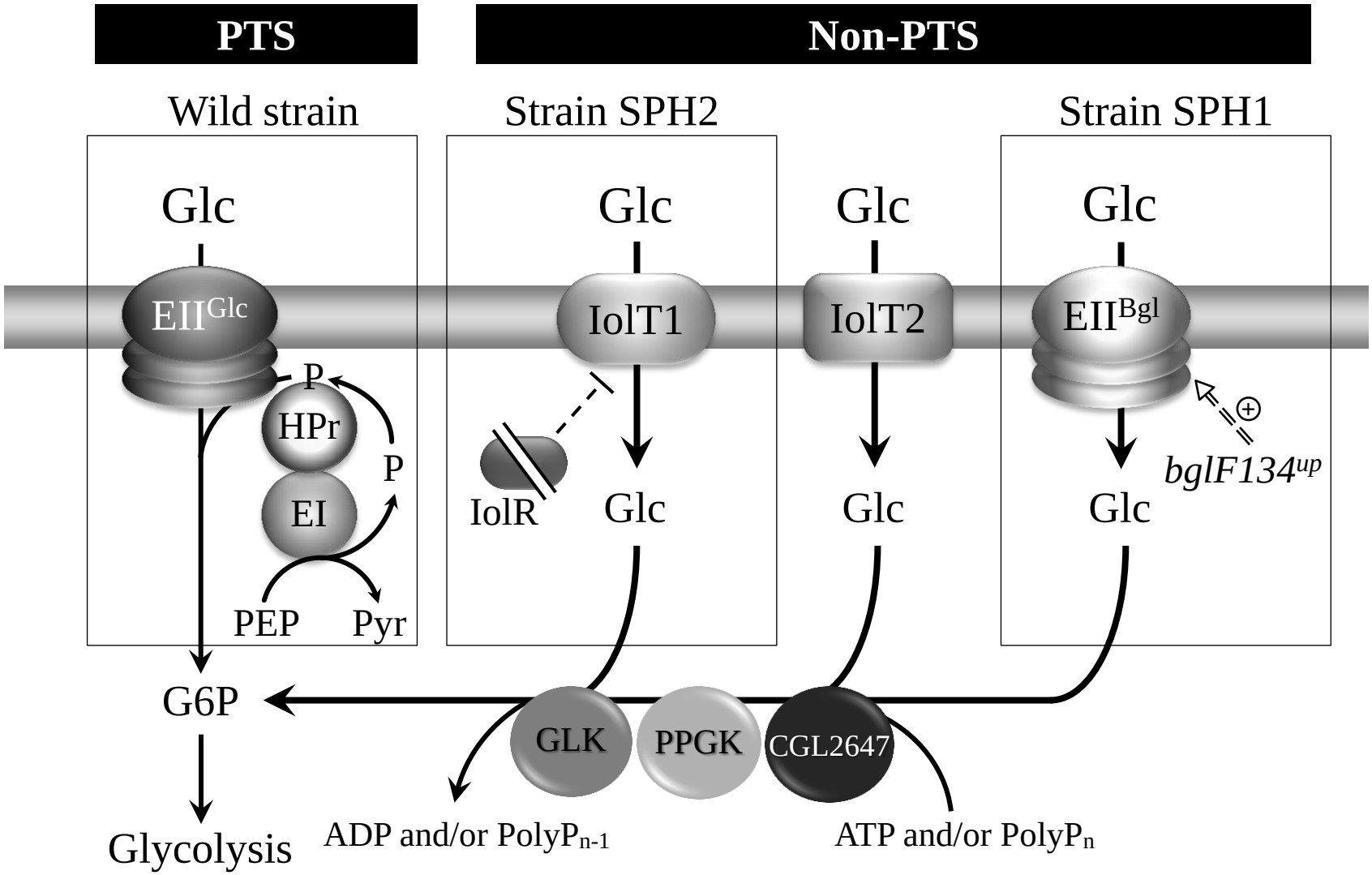

Fig. 7 Ikeda et al.