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Sugar transport systems in *Corynebacterium glutamicum*: features and applications to strain development

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23 **Abstracts**

24 *Corynebacterium glutamicum* uses the phosphoenolpyruvate (PEP)-dependent sugar
25 phosphotransferase system (PTS) to take up and phosphorylate glucose, fructose, and sucrose,
26 the major sugars from the agricultural crops that are used as the primary feedstocks for
27 industrial amino acid fermentation. This means that worldwide amino acid production using
28 this organism has depended exclusively on the PTS. Recently, a better understanding not only
29 of PTS-mediated sugar uptake but also of global regulation associated with the PTS has
30 permitted the correction of certain negative aspects of this sugar transport system for amino
31 acid production. In addition, the recent identification of different glucose uptake systems in
32 this organism has led to a strategy for the generation of *C. glutamicum* strains that express
33 non-PTS routes instead of the original PTS. The potential practical advantages of the
34 development of such strains are discussed.

35

36 **Keywords**

37 Sugar transport systems, PTS, amino acid production, Strain improvement, *Corynebacterium*
38 *glutamicum*

39

40 **Introduction**

41

42 During the half century following its discovery (Udaka 2008), the L-glutamate-producing
43 microorganism *Corynebacterium glutamicum* has played a leading role in the amino acid
44 fermentation industry. Due to its importance as an amino acid producer and a potential
45 producer of other commodity chemicals and heterologous proteins, *C. glutamicum* is also one
46 of the best-investigated microbes, as evidenced by the extensive body of relevant literature

47 and patents (Kinoshita and Nakayama 1978; Ikeda 2003; Hermann 2003; Eggeling and Bott
48 2005; Wendisch 2007; Burkovski 2008). During the last decade, genomic and other "omics"
49 data have accumulated for *C. glutamicum*, revealing new regulatory networks and functions
50 that had not previously been identified in this organism, and thereby providing a global
51 understanding of this organism and profoundly affecting strain development methods
52 (Wittmann and Heinzle 2002; Ikeda and Nakagawa 2003; Kalinowski et al. 2003; Strelkov et
53 al. 2004; Yukawa et al. 2007). Currently, metabolic engineering strategies in *C. glutamicum*
54 have been expanding from the core biosynthetic pathways to include central metabolism,
55 cofactor-regeneration systems, product transport systems, energy metabolism, global
56 regulation, and stress responses; through these methods, strain improvement is bound to
57 optimize entire cellular systems within the organism (Ikeda and Takeno 2013). Yet in spite of
58 numerous basic studies on them, the sugar transport systems of *C. glutamicum*, including the
59 phosphoenolpyruvate (PEP)-dependent sugar phosphotransferase system (PTS), have not yet
60 been thoroughly engineered for efficient amino acid production. Only recently have
61 PTS-mediated sugar transport and related global regulation gained importance as the targets of
62 strain improvement.

63 The feedstocks used for industrial fermentation by *C. glutamicum* are sugars from
64 agricultural crops, such as cane molasses, beet molasses, and starch hydrolysates from corn and
65 cassava. Because these sugars include glucose, fructose, and sucrose, the PTS by which this
66 bacterium takes up and phosphorylates each of these sugars is of primary importance. Starting
67 with the first report on the presence of the PTS in *C. glutamicum* by Mori and Shiio (1987), this
68 sugar transport system has been intensively studied and reviewed (Parche et al. 2001; Yokota and
69 Lindley 2005; Moon et al. 2007; Arndt and Eikmanns 2008; Blombach and Seibold 2010).

70 This mini-review first describes the essence of the sugar transport systems in *C.*

71 *glutamicum*, focusing on the PTS and global regulation associated with the PTS. Applications
72 of such basic information to strain improvement are then summarized, including strategies to
73 improve the negative aspects of the PTS for efficient amino acid production. Furthermore, the
74 potential practical advantages of switching from the original PTS to the recently identified
75 non-PTS glucose transport systems are discussed. Approaches to broadening the spectrum of
76 assimilable carbon sources of this organism from an environmental point of view have been
77 omitted because discussion of this issue can be found in other publications (Wendisch et al.
78 2006; Blomback and Seibold 2010; Jojima et al. 2010).
79

80 **Essence of sugar transport systems in *C. glutamicum***

81

82 The representative *C. glutamicum* strain ATCC 13032 has been reported to have four sets of
83 PTSs, one each that is specific for glucose, fructose, sucrose, and an unknown substrate (Parche
84 et al. 2001; Moon et al. 2007). The glucose-PTS consists of the glucose-specific EIIABC
85 component (EII^{Glc}) encoded by *ptsG* (Cgl1360, NCgl1305) and the two general components EI
86 and HPr encoded by *ptsI* (Cgl1933, NCgl1858) and *ptsH* (Cgl1937, NCgl1862), respectively.
87 The fructose-PTS consists of the fructose-specific EIIABC component (EII^{Fru}) encoded by *ptsF*
88 (Cgl1936, NCgl1861), as well as the two general components, while the sucrose-PTS consists of
89 the sucrose-specific EIIABC component (EII^{Suc}) encoded by *ptsS* (Cgl2642, NCgl2553). A fourth
90 PTS belongs to the L-ascorbate-type family and its EII permease consists of EIIAB and EIIC
91 components encoded by the *Escherichia coli sgaBA*-like Cgl3037 gene (NCgl2934) and the *E.*
92 *coli sgaT*-like Cgl3036 gene (NCgl2933), respectively (Hvorup et al. 2003). The *E. coli sgaTBA*
93 gene products are involved in L-ascorbate uptake and phosphorylation (Hvorup et al. 2003), but
94 the function of their homologues in *C. glutamicum* remains to be elucidated. Another wild-type

95 strain *C. glutamicum* R, unlike *C. glutamicum* ATCC 13032, has two sets of β-glucoside-PTSs
96 that are redundantly responsible for the uptake of the β-glucosides, such as salicin and arbutin,
97 and also glucose (Kotrba et al. 2003; Tanaka et al. 2009, 2011). The two β-glucoside-specific
98 EIIABC components are encoded by *bglF* (cgR_2729) and *bglF2* (cgR_2610). The PTS genes
99 and their genomic organization in *C. glutamicum* have been summarized in reviews (Moon et al.
100 2007; Teramoto et al. 2010).
101 In wild-type *C. glutamicum*, glucose uptake depends on the glucose-PTS (Parche et al.
102 2001; Yokota and Lindley 2005). However, due to residual growth on glucose and the residual
103 glucose-transporting activity of PTS-negative mutants, and due to a discrepancy between
104 measured levels of PTS activity and the observed specific glucose consumption rates during a
105 glucose-limited chemostat culture, glucose uptake by diffusion or minor contributions of
106 unidentified permease(s) to glucose uptake has been postulated (Cocaign-Bousquet et al.
107 1996; Parche et al. 2001; Gourdon et al. 2003). This secondary glucose uptake was estimated
108 to represent up to 15% of the total glucose transport in *C. glutamicum* ATCC 17965 at high
109 growth rates (Cocaign-Bousquet et al. 1996) and up to 25% in the case of the
110 glutamate-producing strain *C. glutamicum* 2262 under non-stress conditions (Gourdon et al.
111 2003). Recently, our laboratory and a German group independently showed that two
112 *myo*-inositol transporters encoded by *iolT1* (Cgl0181, NCgl0178) and *iolT2* (Cgl3058,
113 NCgl2953) mediated glucose uptake in *C. glutamicum* (Ikeda et al. 2010, 2011; Lindner et al.
114 2011ab), indicating that these transporters could be candidates for the secondary glucose
115 permeases. Exceptionally, the *C. glutamicum* R strain can consume glucose not only via the
116 glucose-PTS but also via the β-glucoside-PTS because *C. glutamicum* R has two sets of genes
117 for the β-glucoside-PTS on its genome as described previously (Tanaka et al. 2011).
118 Accordingly, the *ptsG*-deficient strain still consumed glucose well, though additional defects

119 in both *bglF* and *bglF2* resulted in an almost complete loss of glucose consumption ability
120 (Tanaka et al. 2011).

121 As for PTS-independent glucose uptake, glucokinase activity catalyzing ATP-dependent
122 phosphorylation of glucose into glucose-6-phosphate was long since reported for this organism
123 (Mori and Shiio 1987). The enzyme GLK, which is encoded by *glk* (Cgl2185, NCgl2105), has
124 been shown to be involved in the metabolism of the disaccharide maltose, which is hydrolyzed to
125 free glucose inside the cell (Park et al. 2000). Recently, an additional glucokinase encoded by
126 *ppgK* (Cgl1910, NCgl1835) has been identified (Lindner et al. 2010). The enzyme PPGK was
127 shown to highly prefer polyphosphate over ATP as a phosphoryl donor, and thus is assumed to
128 be important especially under growth conditions depending on high intracellular polyphosphate
129 concentrations. It was also shown that the polyphosphate-dependent PPGK played a role in
130 maltose catabolism, as with the ATP-dependent GLK. Very recently, our laboratory has
131 identified a third enzyme, described below, that takes part in glucose phosphorylation in *C.*
132 *glutamicum*.

133 Fructose uptake by wild-type *C. glutamicum* depends mainly on the fructose-PTS, but
134 the glucose-PTS plays an assisting role. Therefore, a *ptsF* mutant showed weak growth on
135 fructose, while an additional defect in *ptsG* resulted in no growth (Moon et al. 2005).
136 According to Kiefer et al. (2004), 92.3% of fructose was taken up by the *ptsF*-specified EII^{Fru},
137 forming fructose-1-phosphate to be channeled into glycolysis at the level of
138 fructose-1,6-bisphosphate, whereas only 7.7% of fructose was taken up by the *ptsG*-specified
139 EII^{Glc}, resulting in fructose-6-phosphate. Consequently, fructose enters different points of
140 glycolysis via two PTS enzyme IIs, and these different fructose entry points influence the flux
141 through the pentose phosphate pathway, as described below. Fructose can also be taken up by
142 the two *myo*-inositol transporters encoded by *iolT1* and *iolT2* described above (Baumchen et

143 al. 2009), but unlike glucose, intracellular fructose cannot be further metabolized due to a lack
144 of fructokinase activity in *C. glutamicum* (Dominguez and Lindley 1996).
145 Sucrose uptake in wild-type *C. glutamicum* depends exclusively on the sucrose-PTS
146 (Moon et al. 2005). Intracellular sucrose-6-phosphate is then hydrolyzed to glucose-6-phosphate
147 and fructose by sucrose-6-phosphate hydrolase encoded by *scrB* (Cgl2643, NCgl2554)
148 (Dominguez and Lindley 1996; Engels et al. 2008). Free fructose generated inside the cell is
149 exported outside by an unidentified carrier and re-imported by the fructose-PTS to generate
150 fructose-1-phosphate (Dominguez and Lindley 1996).
151 In addition to the typical carbon sources glucose, fructose, and sucrose, *C. glutamicum*
152 efficiently uses gluconate and ribose as sole carbon sources, but their uptake is mediated by
153 non-PTS systems. Gluconate is taken up by the specific gluconate permease GntP encoded by
154 *gntP* (Cgl2908, NCgl2808), then phosphorylated by the gluconate kinase GntK encoded by *gntK*
155 (Cgl2485, NCgl2399) to 6-phosphogluconate, an intermediate of the pentose phosphate pathway.
156 A defect in either *gntP* or *gntK* abolished growth on gluconate (Letek et al. 2006). Ribose, on the
157 other hand, is incorporated into cells by the specific ATP-binding cassette transporter RbsACBD
158 encoded by *rbsACBD* (Cgl1252-1255, NCgl1204-1207), then phosphorylated by the ribokinases
159 RbsK1 and RbsK2, encoded by *rbsK1* (Cgl1366, NCgl1311) and *rbsK2* (Cgl2325, NCgl2243),
160 respectively, to ribose-5-phospate which is channeled through the pentose phosphate pathway
161 (Nentwich et al. 2009). Double knockouts of *rbsK1* and *rbsK2* resulted in defective growth on
162 ribose (Brinkrolf et al. 2008).
163 An overall picture of the typical carbohydrate transport systems in *C. glutamicum* ATCC
164 13032 is depicted in Fig. 1. It should be noted that maltose transport has been omitted: although
165 *C. glutamicum* efficiently uses the disaccharide maltose, a product of starch degradation by
166 amylase (Seibold et al. 2009) considered to be taken up via a non-PTS system (Moon et al. 2005),

167 the processes of its uptake and metabolism remain to be elucidated.

168

169 **Essence of regulatory systems involved in sugar transport in *C. glutamicum***

170

171 In contrast to many other bacterial species, *C. glutamicum* simultaneously metabolizes glucose
172 with other carbon sources such as sucrose, fructose, ribose, pyruvate, and acetate, and exhibits
173 monophasic growth (Cocaign et al. 1993; Dominguez et al. 1997; Wendish et al. 2000; Arndt and
174 Eikmanns 2008). When grown on glucose, fructose, or a mixture of these, *C. glutamicum* can
175 maintain an approximately constant level of total PTS activity in response to the PTS-mediated
176 sugars available (Dominguez et al. 1997). In *C. glutamicum* grown on a mixture of glucose and
177 acetate, the glucose uptake rate is reduced by about half compared to that in *C. glutamicum*
178 grown on only glucose, indicating that the two different carbon sources are coordinately
179 incorporated into cells and metabolized (Wendisch et al. 2000). Similarly, *C. glutamicum*
180 consumes glucose at a reduced rate when gluconate is mixed into the glucose medium (Frunzke
181 et al. 2008). Such coordinated utilization of different carbon sources depends heavily on several
182 recently identified transcriptional regulators, such as SugR, FruR, GntR1, and GntR2 (Arndt and
183 Eikmanns 2008; Teramoto et al. 2010, 2011). Carbohydrate-dependent regulation mediated by
184 these regulators is depicted in Fig. 1.

185 The DeoR-type transcriptional regulator SugR, encoded by *sugR* (Cgl1931, NCgl1856),
186 represses expression of the PTS genes during growth on gluconeogenic carbon sources such as
187 acetate and pyruvate; during growth on sugars, however, when high levels of the intracellular
188 PTS-sugar metabolites glucose-6-phosphate, fructose-1-phosphate, fructose-6-phospate, and
189 fructose-1,6-bisphosphate are being generated, the SugR-mediated repression is relieved due to
190 the inhibition of SugR activity by those metabolites, allowing cells to adapt sugar uptake to the

191 carbon source available (Gaigalat et al. 2007; Engels and Wendisch 2007; Tanaka et al. 2008b).
192 Overexpression of the *sugR* gene during growth on glucose caused a reduced expression of *ptsG*,
193 resulting in decreased glucose utilization, while a *sugR*-deficient mutant showed several-fold
194 higher expression of *ptsG* on gluconeogenic carbon sources (Engels and Wendisch 2007).
195 Another DeoR-type transcriptional regulator is FruR, encoded by *fruR* (Cgl1934,
196 NCgl1859), which represses expression of the PTS genes *ptsI*, *ptsH*, and *ptsF*. Since *fruR*
197 expression itself is negatively controlled by SugR, FruR can modulate the SugR-mediated
198 repression of the PTS genes. A *fruR*-deficient mutant showed higher expression levels of *ptsI*,
199 *ptsH*, and *ptsF* during growth on fructose, indicating that FruR attenuates the induction of these
200 *pts* genes by fructose (Tanaka et al. 2008a).
201 The GntR-type regulators GntR1 and GntR2, encoded by *gntR1* (Cgl2527, NCgl2440)
202 and *gntR2* (Cgl1718, NCgl1650), respectively, are involved in the coordinated regulation of
203 gluconate catabolism and PTS-sugar uptake (Frunzke et al. 2008). In the absence of gluconate,
204 both regulators act as transcriptional activators of *ptsG* and *ptsS* and, at the same time, as
205 repressors of *gntP* and *gntK*, two genes involved in gluconate utilization. During growth in the
206 presence of gluconate, GntR1- and GntR2-mediated regulation is relieved due to inhibition of the
207 regulator activity by gluconate and glucono-δ-lactone, allowing cells to coordinately utilize
208 different carbon sources. Double knockout of the two regulator genes enhances expression of the
209 genes involved in gluconate utilization. In contrast, expression of *ptsG* and *ptsS* is
210 down-regulated in the mutant, resulting in glucose uptake and growth rates that are both 60%
211 lower than those in wild-type *C. glutamicum* during growth on glucose (Frunzke et al. 2008).
212 In addition to the carbohydrate-dependent regulation of the PTS via transcriptional
213 regulators, the PTS itself may play a regulatory role in response to carbohydrate availability. The
214 first discovery of PTS-associated catabolite repression in *C. glutamicum* was that of the

215 repression of glutamate uptake by the PTS sugars glucose and fructose (Krämer et al. 1990;
216 Krämer and Lambert 1990; Kronemeyer et al. 1995; Parche et al. 2001). For this reason, this
217 organism exhibits diauxic growth when grown on a mixture of glucose and glutamate; this
218 pattern results from the sequential utilization of the two carbon sources (Krämer et al. 1990). The
219 gluconate-catabolic genes *gntP* and *gntK* are also subject to catabolite repression by the PTS
220 sugars glucose, fructose, and sucrose (Letek et al. 2006), although *C. glutamicum* can utilize
221 gluconate and glucose simultaneously.

222 The molecular mechanism of carbon catabolite repression in this organism remains to be
223 fully elucidated. Also direct involvement of the PTS itself in the catabolite repression is not
224 verified experimentally. However, the presence of one putative *cya* gene (Cgl0311, NCgl0306)
225 encoding adenylate cyclase, which catalyzes the synthesis of cyclic AMP (cAMP), and three
226 putative *crp* genes (Cgl0291, NCgl0286; Cgl1174, NCgl1127; Cgl2970, NCgl2868) encoding
227 cAMP receptor protein (CRP) in the *C. glutamicum* genome has suggested that a cAMP-CRP
228 system similar to that seen in *E. coli* may be involved in this organism (Kim et al. 2004; Brune et
229 al. 2005). A *cya*-deficient mutant showed decreased levels of intracellular cAMP, resulting in
230 altered growth patterns on various carbon sources, as typified by a pattern of defective growth on
231 acetate (Cha et al. 2010). At the same time, the *cya* mutant exhibited derepression of *aceA* and
232 *aceB*, which encode the glyoxylate bypass enzymes isocitrate lyase and malate synthase,
233 respectively (Cha et al. 2010). Similar derepression of *aceA* and *aceB* was observed regardless of
234 the carbon sources when *glxR* (Cgl0291, NCgl0286), one of the three *crp* genes, was disrupted
235 instead of *cya* (Park et al. 2010). In this *glxR* mutant, derepression of the *gluA* gene of the
236 glutamate uptake systems on glucose medium was also observed. These results suggest that
237 cAMP-CRP plays a role as a global regulator controlling both carbon catabolite repression and
238 acetate metabolism in *C. glutamicum*. Yet the cAMP-CRP regulatory system in *C. glutamicum*

239 seems to have a different mode of action from that in *E. coli*, because the response of adenylate
240 cyclase activity to PTS-sugar availability is opposite to that in *E. coli* (Dumay et al. 1995). In fact,
241 contrary to the case for *E. coli*, levels of cAMP are higher in *C. glutamicum* cells grown on
242 glucose than in those grown on acetate (Kim et al. 2004). Catabolite repression of the *C.*
243 *glutamicum gnt* genes described above has been demonstrated to occur through the binding of the
244 *glxR* gene product GlxR to the *gnt* promoters in the presence of cAMP (Letek et al. 2006). This
245 indicates that the *C. glutamicum* GlxR protein acts as a transcriptional repressor in the presence
246 of cAMP while the *E. coli* cAMP-CRP acts as a transcriptional activator. Recently, a
247 genome-wide study of *C. glutamicum* has identified 209 regions as *in vivo* GlxR-binding sites
248 (Toyoda et al. 2011). *In vitro* binding assays and promoter-reporter assays have demonstrated
249 that GlxR acts as both a transcriptional activator and a repressor, suggesting a more complicated
250 mode of action of the regulator protein. At present, limited information is available concerning
251 global catabolite repression involving the PTS in *C. glutamicum*. Accordingly, further studies are
252 required to illuminate the whole picture of PTS-dependent catabolite repression in this organism.
253
254 **Approaches to modifying PTS-dependent sugar transport and phosphorylation**
255
256 Engineering to alter the entry point of fructose into glycolysis
257
258 In *C. glutamicum*, the problem of using fructose as a carbon source for amino acid production
259 could be that less of the reducing power NADPH is available for fructose than for glucose.
260 This is because fructose is mainly taken up by the fructose-PTS to form fructose-1-phosphate,
261 which then channels into glycolysis at the level of fructose-1,6-bisphosphate, which requires
262 gluconeogenetic fructose-1,6-bisphosphatase activity in order to direct its carbon into the

263 pentose phosphate pathway. This problem also has some relevance for sucrose, because the
264 fructose unit of sucrose follows the same metabolic fate as free fructose (Fig. 2). To solve this
265 problem, direct phosphorylation of intracellular fructose produced by sucrose hydrolysis has
266 been attempted through the heterologous expression of the fructokinase gene from
267 *Clostridium acetobutylicum* (Moon et al. 2005). This enzyme can phosphorylate intracellular
268 fructose into fructose-6-phosphate rather than fructose-1-phosphate. Therefore, this form of
269 engineering is expected to shift the entry point of fructose from fructose-1,6-bisphosphate to
270 its upstream fructose-6-phosphate and thus increase the flux through the pentose phosphate
271 pathway (Fig. 2). Another potential strategy could be engineering to increase the ratio of
272 fructose taken up by the glucose-PTS to that taken up by the fructose-PTS; this would be
273 effective because the glucose-PTS plays an assisting role in fructose uptake and
274 fructose-6-phosphate formation (Kiefer et al. 2004).
275
276 Release from the SugR-mediated repression of the PTS genes
277
278 Amino acid production from sugar is sometimes conducted in the presence of gluconeogenic
279 carbon sources such as acetate. As described above, however, when *C. glutamicum* is
280 cultivated on a mixture of glucose plus acetate, the glucose uptake rate is reduced by about
281 half compared to when it is cultivated on only glucose, probably due to acetate-mediated
282 repression of the PTS genes by the global regulator SugR (Fig. 3). Such is the case in valine
283 production by a pyruvate dehydrogenase-deficient *C. glutamicum* strain which requires acetate
284 for its growth (Blombach et al. 2009). This valine producer exhibits reduced glucose
285 metabolism and a concomitant nonproduction phenotype in the growth phase where acetate
286 coexists with glucose. This drawback has been overcome by inactivating SugR (Fig. 3),

287 allowing valine production in the growth phase (Blombach et al. 2009). This result is in
288 agreement with the previous findings that, in the absence of SugR, the proportion of glucose
289 and acetate co-utilization was shifted towards glucose, while the overexpression of SugR
290 shifted the proportion towards acetate (Engels and Wendisch 2007).

291 The replacement of acetate by ethanol without the inactivation of SugR also results in
292 efficient valine production in the growth phase (Blombach et al. 2009; Fig. 3). Ethanol is
293 oxidized via acetaldehyde to acetate, and then metabolized to acetyl-CoA in *C. glutamicum*
294 (Arndt et al. 2008). Unlike the case of glucose and acetate co-utilization, however, *C.*
295 *glutamicum* on a glucose and ethanol mixture has been shown to exhibit a sequential
296 utilization of glucose before ethanol, possibly due to the carbon catabolite repression (Arndt et
297 al. 2008). This glucose-dependent regulation of ethanol catabolism is likely to allow cells to
298 circumvent the SugR-mediated regulation of the PTS genes.
299
300 Use of maltose to increase glucose utilization
301
302 When *C. glutamicum* is cultivated on a mixture of glucose plus maltose, both sugars are
303 consumed in parallel. Such co-utilization of substrates is a well-known phenomenon in *C.*
304 *glutamicum* as mentioned above, but a unique point in this case is that the presence of maltose
305 increases the glucose consumption rate; this is reflected by the concurrent increase in the
306 growth rate (Krause et al. 2010). In this respect, *ptsG* transcription in cells grown on a mixture
307 of glucose plus maltose has been shown to be about twice as high as that observed in cells
308 grown on only glucose, and this effect was SugR-independent (Engels and Wendisch 2007).
309 This stimulative effect of maltose on glucose utilization via raising the *ptsG* transcription was
310 also observed when acetate coexisted with glucose. The findings were applied to improve

311 valine production by a pyruvate dehydrogenase-deficient *C. glutamicum* strain (Krause et al.
312 2010; Fig. 3). This strain had the drawback of producing little valine during the growth phase
313 when acetate coexisted with glucose, because only minor amounts of glucose were
314 metabolized in this phase. When this strain was cultured in glucose plus acetate medium
315 supplemented with an optimized concentration of maltose, however, glucose utilization was
316 improved during growth, resulting in a significant increase in overall valine productivity.
317 Krause et al. (2010) have suggested that starch hydrolysates, a mixture of dextrin, maltose,
318 and glucose, could be used instead of the more expensive pure maltose to trigger the maltose
319 effect in large-scale industrial amino acid production with *C. glutamicum*.
320
321 **Approaches to activating a PTS-independent glucose uptake route**
322
323 Identification of different glucose uptake systems that function as alternatives to the PTS
324
325 In industrial amino acid fermentation using *C. glutamicum*, various sugars such as cane
326 molasses, beet molasses, and starch hydrolysates are used as the feedstocks, but among these,
327 starch hydrolysates (mainly glucose) have been used preferentially because they have fewer
328 impurities and less variation among lots compared with molasses. Because this organism uses
329 the PTS to take up and phosphorylate glucose, worldwide amino acid production from glucose
330 by this organism is assumed to depend exclusively on the PTS. Recently, however, our
331 laboratory identified potential glucose uptake systems that function as alternatives to the PTS
332 (Ikeda et al. 2010, 2011). The motivation behind the study came from the observation that
333 colonies emerged from a PTS-negative mutant of *C. glutamicum* on glucose agar plates. More
334 specifically, a suppressor mutant that grew on glucose but not on fructose and sucrose was

335 spontaneously isolated from a *ptsH*-disrupted strain of wild-type *C. glutamicum* ATCC 31833.
336 The suppressor strain, unlike the wild-type strain, exhibited a phenotype of resistance to
337 2-deoxyglucose, which is known to be a toxic substrate for the glucose-PTS system of this
338 organism (Mori and Shiio 1987). Homology search against *C. glutamicum* whole genome
339 sequence using the *E. coli galP* gene encoding galactose permease, which can transport
340 glucose, led to the identification of two candidate genes, *iolT1* and *iolT2*, both of which have
341 been reported as *myo*-inositol transporters (Krings et al. 2006). Overexpression of *iolT1* alone
342 and that of *iolT2* alone under the *gapA* promoter in the *ptsH*-disrupted strain rendered the
343 strain capable of growing on glucose, proving that each transporter plays a role in glucose
344 uptake. Disruption of *iolT1* in the suppressor strain abolished growth on glucose whereas
345 disruption of *iolT2* did not, revealing that *iolT1* is responsible for glucose uptake in the
346 suppressor strain. Sequence analysis of the *iol* gene cluster and its surrounding region
347 identified a single-base deletion (320delA) in the putative transcriptional regulator gene *iolR*
348 (Cgl0157, NCgl0154) of the suppressor strain (Fig. 4). Introduction of the frameshift mutation
349 or in-frame deletion of the *iolR* inner sequence allowed the *ptsH*-disrupted strain to grow on
350 glucose, but further deletion of *iolT1* abolished growth again, indicating that the inactivation
351 of *iolR* enables a PTS-negative strain to grow on glucose through the *iolT1*-specified glucose
352 uptake system. In the *ptsH*-disrupted strain carrying the *iolR* mutation, the expression of the
353 *iol* gene cluster including *iolT1*, but not *iolT2*, was indeed derepressed by around 100-fold
354 compared to the wild-type levels (Fig. 4).

355 During the course of this study, our laboratory also found that, in addition to the two
356 known glucokinases GLK and PPGK, a third enzyme was involved in the phosphorylation of
357 intracellular glucose in the suppressor strain (data not shown). The newly identified kinase is
358 encoded by the uncharacterized Cgl2647 (NCgl2558) gene. The gene product shows

359 approximately 29% and 25% sequence identity with GLK and PPGK, respectively, while
360 GLK and PPGK share 28% identity with each other. Double knockouts of *glk* and *ppgK* in the
361 suppressor strain still grew well on glucose, whereas an additional defect in the Cgl2647 gene
362 resulted in no growth on glucose. Plasmid-mediated overexpression of any of the three genes
363 under the *gapA* promoter in the triple-knockout mutant (Δ*glk*, Δ*ppgK*, ΔCgl2647) restored the
364 growth of the mutant on glucose, proving that each gene product plays a role in glucose
365 phosphorylation in *C. glutamicum*. The glucose uptake and phosphorylation system of the
366 suppressor strain is depicted in Fig. 5.
367
368 Impact of the *iolT1*-specified non-PTS route on lysine production
369
370 The results mentioned above have established a strategy for engineering *C. glutamicum* to
371 express the *iolT1*-specified glucose uptake bypass instead of the original PTS. This is
372 comprised of just two steps: (1) disruption of *ptsH* and (2) introduction of the frameshift
373 mutation (320delA) or deletion of *iolR*. When this strategy was applied to the defined lysine
374 producer *C. glutamicum* AHP-3, which carries three specific mutations (*hom59*, *lysC311*, and
375 *pyc458*) on its genome (Ohnishi et al. 2002), the engineered non-PTS strain
376 AHPΔptsH320delA displayed approximately 20% increased lysine production from glucose
377 (Fig. 6). As has been demonstrated in aromatic production by *E. coli* (Flores et al. 1996),
378 replacement of the PTS by a non-PTS system can be a general strategy for increasing the
379 availability of PEP. This effect is likely to contribute to a better-balanced supply of carbon
380 from the central metabolism into the lysine-biosynthetic pathway through the two anaplerotic
381 reactions involving pyruvate carboxylase and PEP carboxylase. In keeping with our results, a
382 German group has shown that plasmid-mediated overexpression of *iolT1* or *iolT2* with *ppgK*

383 in a *ptsH*-disrupted lysine producer of *C. glutamicum* led to 10 to 20% higher lysine yield than
384 the parental PTS strain (Lindner et al. 2011a). This non-PTS lysine producer accumulated
385 smaller amounts of by-products such as lactate and alanine, both of which derive from
386 pyruvate, indicating a decreased ratio of pyruvate to PEP in the cells. Based on these findings,
387 applications of this engineering strategy to the production not only of lysine but also of other
388 amino acids, diamines, and diacids have been proposed (Lindner et al. 2011b).
389

390 **Conclusions and perspectives**

391

392 In this mini-review, the sugar transport systems in *C. glutamicum*, especially PTS-mediated
393 sugar transport and its related global regulation, have been described and briefly summarized
394 in Fig. 1. Applications of such basic information to strain improvement have subsequently
395 been illustrated with relevant examples. Despite the importance of the PTS in metabolite
396 production from sugar, this review has emphasized approaches to generating *C. glutamicum*
397 strains that express PTS-independent glucose uptake routes instead of the original PTS,
398 because the discovery of endogenous non-PTS routes in this organism is expected to introduce
399 new options for the development of more efficient production strains. Considering the known
400 characteristics of the *C. glutamicum* PTS system, *e.g.*, its relative sensitivity to increased
401 osmolality (Gourdon et al. 2003), the potential practical advantage of a non-PTS route for
402 glucose uptake seems not to be limited to saving PEP for other biosynthetic reactions; on the
403 contrary, other advantages can be reasonably expected. For example, we have obtained
404 original preliminary data indicating that the engineered non-PTS strain of *C. glutamicum*
405 showed a somewhat higher tolerance to some environmental stresses such as elevated
406 temperatures and high concentrations of glucose than the wild-type strain in minimal medium

407 containing glucose as the sole carbon source (data not shown). This means that glucose uptake
408 via the PTS is likely to be the step that limits the normal glucose metabolism under such
409 stressful conditions. Based on these findings and implications, switching the glucose transport
410 system from the PTS to the non-PTS route, or possibly co-expressing the PTS and the
411 non-PTS route, could offer an advantage in terms of cell robustness in large-scale industrial
412 fermentation processes using *C. glutamicum.*
413
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419

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624

625 **Fig. 1** Typical carbohydrate transport systems and regulation in *C. glutamicum* ATCC 13032.
626 Transcriptional regulators, SugR, FruR, and GntR1/R2, are all inside a cell although they are
627 depicted outside a cell due to space limitation. *The blue and red dotted lines* indicate positive (+)
628 or negative (-) regulation, respectively. The percentages *92.3%* and *7.7%* indicate the proportions
629 of fructose taken up by the *ptsF*-specific EII^{Fru} and the *ptsG*-specific EII^{Glc}, respectively (Kiefer
630 et al. 2004). It remains unclear whether the positive effect of gluconeogenic carbons on SugR is
631 direct or not. The excretion process of fructose also remains to be elucidated. The involvement of
632 the PTS itself in the catabolite repression through cAMP-GlxR is not verified experimentally.
633 *Glc* glucose, *Fru* fructose, *Suc* sucrose, *Gnt* Gluconate, *Rib* ribose, *G6P* glucose-6-phosphate,
634 *F1P* fructose-1-phosphate, *F6P* fructose-6-phosphate, *FBP* fructose-1,6-bisphosphate, *S6P*
635 sucrose-6-phosphate, *6PGnt* 6-phosphogluconate, *R5P* ribose-5-phosphate, *PEP*
636 phosphoenolpyruvate, *Pyr* pyruvate, *Ace* acetate
637
638 **Fig. 2** Engineering of the entry point of fructose into glycolysis to increase the flux through the
639 pentose phosphate pathway. *Fructokinase* from *Clostridium acetobutylicum* can phosphorylate
640 fructose into fructose-6-phosphate, instead of fructose-1-phosphate. Thus, the heterologous
641 expression of the gene in *C. glutamicum* has allowed direct phosphorylation of intracellular
642 fructose produced by sucrose hydrolysis into fructose-6-phosphate, which is expected to shift the
643 entry point of fructose from fructose-1,6-bisphosphate to its upstream fructose-6-phosphate and
644 increase the flux through the pentose phosphate pathway (Moon et al. 2005). Abbreviations are
645 as described in the legend to Fig. 1
646
647 **Fig. 3** Strategies to increase the glucose uptake rate in a pyruvate dehydrogenase-deficient
648 valine-producing *C. glutamicum* strain. Glucose uptake rate is negatively affected by acetate

649 through SugR-mediated repression of the PTS genes although it remains unclear whether the
650 positive effect of acetate on SugR is direct or not. The reduced glucose utilization under the
651 coexistence of acetate, which was the drawback of the valine producer, has been overcome either
652 by the inactivation of SugR or by the replacement of acetate by ethanol (Blombach et al. 2009).
653 Supplementation with maltose can also be a solution to the drawback due to the stimulative
654 effect of maltose on glucose utilization (Krause et al. 2010). The uptake system of maltose and
655 its metabolism remain to be elucidated. *Mal* maltose, *Val* valine, *AceCoA* acetyl-CoA, *ADH*
656 alcohol dehydrogenase, *ALDH* acetaldehyde dehydrogenase. Other abbreviations and *the dotted*
657 *lines* are as described in the legend to Fig. 1
658
659 **Fig. 4** Ratio of mRNA levels of the *iol* genes in the *ptsH*-disrupted strain carrying the frameshift
660 mutation (320delA) in *iolR* (*black arrow*) to those in the wild type. Total RNAs from
661 mid-exponential phase cultures on glucose were used to study differential transcription profiles.
662 Transcriptome analysis and the relevant experimental approaches were performed as described
663 previously (Ikeda et al. 2009). *Grey arrows* the genes that are assumed to be relevant to
664 *myo*-inositol biosynthesis, *White arrows* the genes for hypothetical proteins or genes that are
665 assumed to be irrelevant to *myo*-inositol biosynthesis
666
667 **Fig. 5** The *iolT1*-specified glucose uptake route in the *ptsH*-disrupted strain carrying the *iolR*
668 mutation. Inactivation of *iolR* under a PTS-negative background leads to a strain expressing the
669 *iolT1*-specified glucose uptake bypass instead of the native PTS. *Glk* ATP-dependent
670 glucokinase encoded by Cgl2185, *PPGK* polyphosphate (*PolyPn*)-dependent glucokinase
671 encoded by Cgl1910, *CGL2647* the Cgl2647gene product that takes part in glucose
672 phosphorylation in *C. glutamicum*. Other abbreviations and *the dotted line* are as described in the

673 legend to Fig. 1

674

675 **Fig. 6** Comparison of the defined lysine producer AHP-3 (*PTS strain*) and the newly developed

676 strain AHPΔptsH320delA (*Non-PTS strain*) in terms of ability to produce lysine from glucose.

677 Fermentation was carried out at 30ºC in LFG1 medium containing 5% glucose (*w/v*) using

678 500-ml Sakaguchi flasks as described previously (Ikeda et al. 2011)

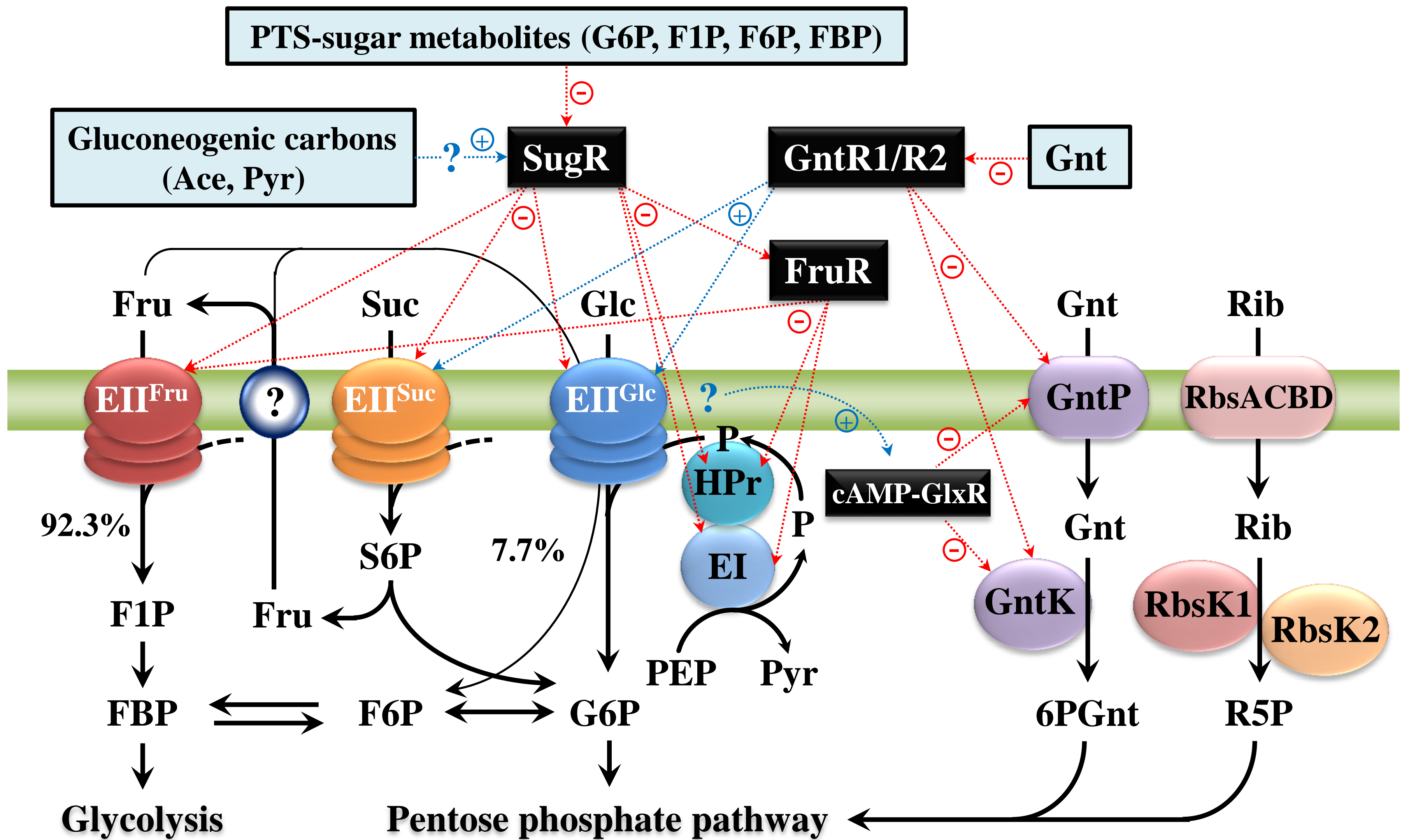

Fig. 1 Ikeda

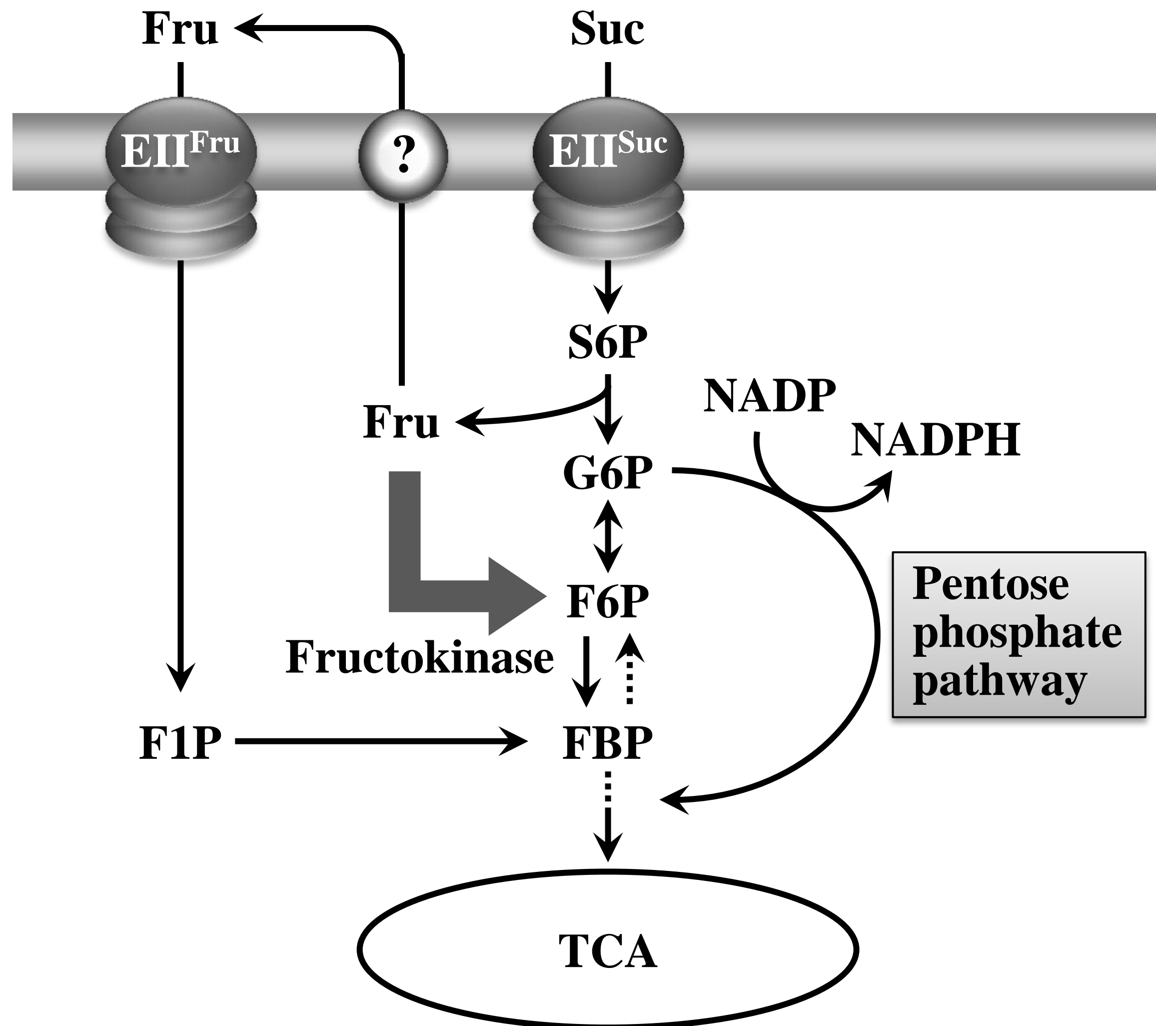

Fig. 2 Ikeda

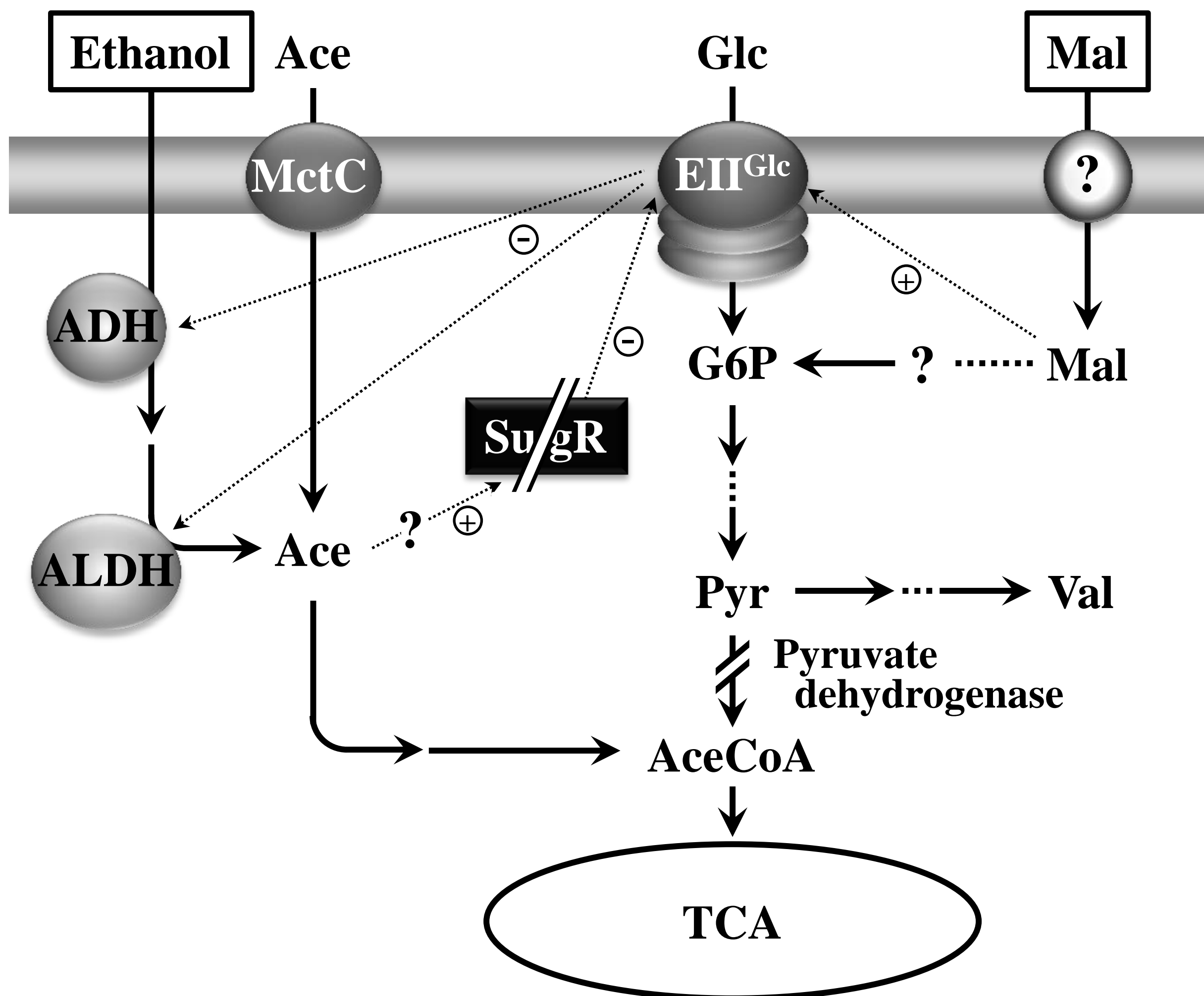

Fig. 3 Ikeda

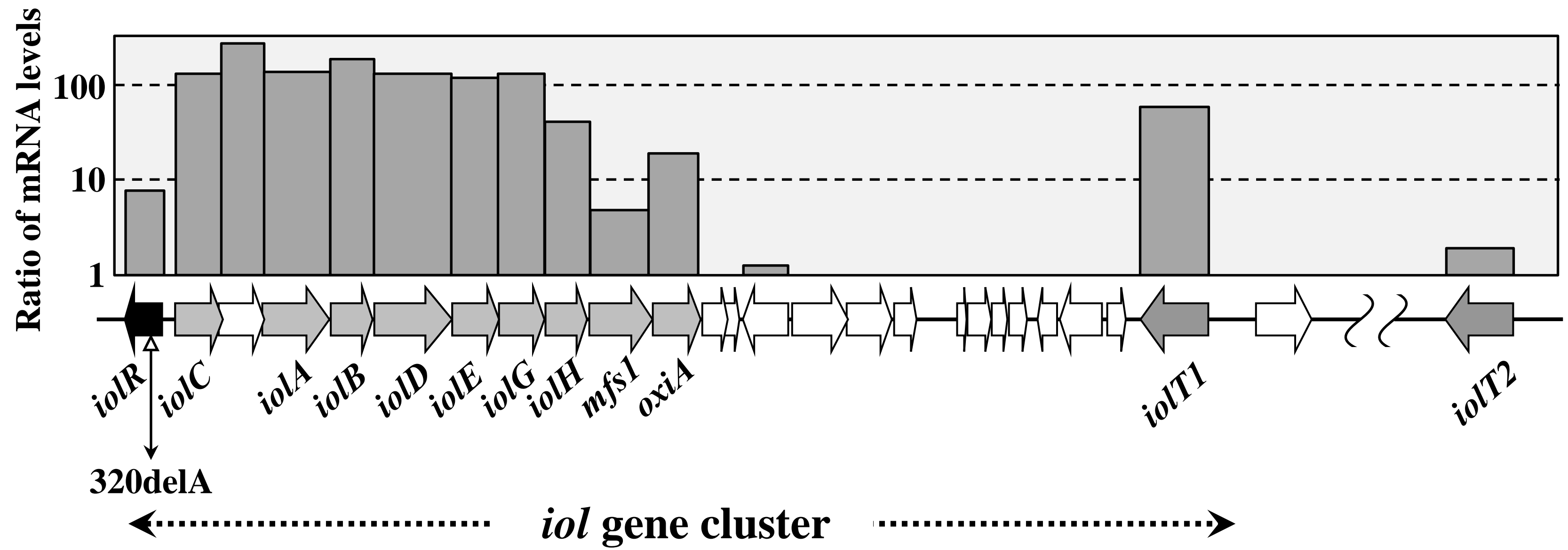

Fig. 4 Ikeda

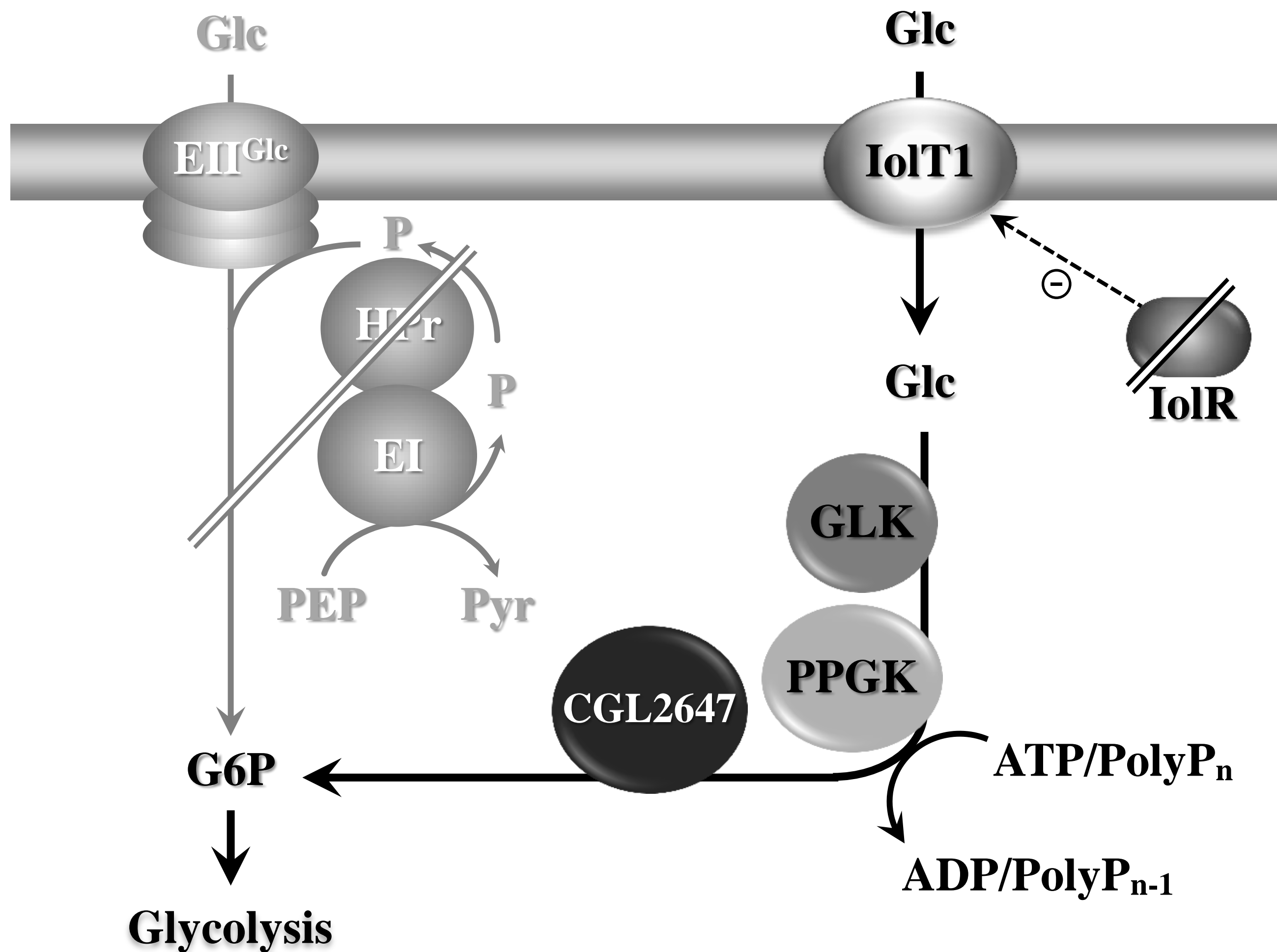

Fig. 5 Ikeda

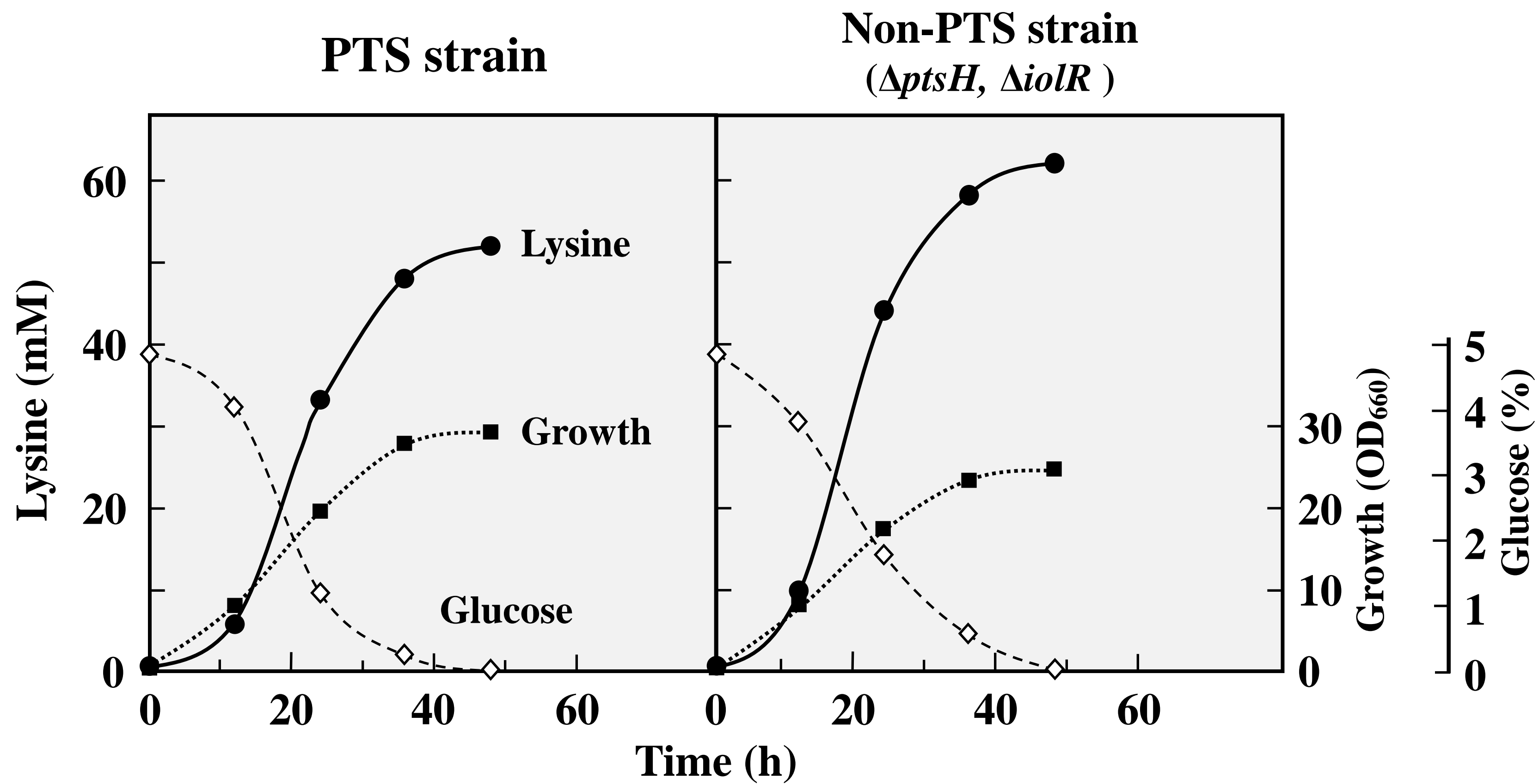

Fig. 6 Ikeda