Identification of Key Regulators in Glycogen Utilization in E. coli Based on the Simulations from a Hybrid Functional Petri Net Model

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Abstract. Glycogen and glucose are two sugar sources available during the lag phase of *E. coli*, but the mechanism that regulates their utilization is still unclear. Attempting to unveil the relationship between glucose and glycogen, we propose an integrated hybrid functional Petri net (HFPN) model including glycolysis, PTS, glycogen metabolic pathway, and their internal regulatory systems. By comparing known biological results to this model, basic regulatory mechanism for utilizing glucose and glycogen were identified as a feedback circuit in which HPr and $EIIA^{Glc}$ play key roles. Based on this regulatory HFPN model, we discuss the process of glycogen utilization in *E. coli* in the context of a systematic understanding of carbohydrate metabolism.

Keywords: metabolic pathway, glycogen, hybrid functional Petri net, PTS, HPr and $EIIA^{Glc}$ proteins, gene regulation

1 Introduction

The carbohydrate pathway occupies a central position in a cell's metabolism. In our previous paper [1], we proved that glycogen plays an important role in the lag phase of *E. coli*. But how the cell regulates the utilization of these carbon sources, intracellular glycogen and extracellular glucose, was yet to be clarified. In a cell, glycogen works as a sugar store or a sugar supply depending on different nutrition conditions, under the regulation of enzymes expressed by *glg* gene clusters (*glgBXCAP*) [2]. Uptake of extracellular glucose is conducted via the phosphotransferase system (PTS) in *E. coli*, whose enzymes are expressed from two operons, *ptsHIcrr* and *ptsG* [3]. Although several shared regulators of PTS and glycogen metabolism, such as ppGpp, Cra, CsrA and cAMP/CRP, have

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been studied [2, 4–10], a basic regulation system for the utilization of glucose and glycogen has not been studied yet.

Computer modeling is a general and effective method for the integration of biological systems. The purpose of this paper is to construct an integrated model for the systematic understanding of the carbohydrate pathway system of *E. coli*. In this work we first transposed into the hybrid functional Petri net (HFPN) [11] two published models controlling different aspects of the central carbohydrate pathway: glycolysis and pentose phosphate (PP) pathway [12], and PTS [13]. These models have then been assembled together with a newly developed general mass action model of the glycogen metabolic pathway into a single comprehensive HFPN model.

By applying metabolic regulatory mechanisms in our combined HFPN model, a basic control system regulating the utilization glucose and glycogen was identified, in which HPr::GlgP complex [14–16], EIIA^{Glc}&cAMP system [8, 17], EI dimerization [18, 19], FDP&Cra mutual feedback [6], HPr subcellular location [2, 16, 20] etc. are working as regulators. In this paper, with the support of simulation results from the HFPN model, we clarify functions of HPr and EIIA^{Glc} as key regulators of glucose and glycogen utilization.

2 Molecular mechanism for regulating glucose and glycogen utilization

2.1 Regulators

Fig. 1 shows possible regulators that control glucose and glycogen utilization, in which these components are classified into five levels, labeled 0-4, according to regulation "source" and "target". These regulators constitute a circuit that gives a whole view of the regulation of glucose and glycogen utilization as shown in Fig. 2 .

Level-1 P2P (Regulation from protein to protein). HPr regulates consumption of both glucose and glycogen by its phosphorylation state and concentration [8]. HPr is a member enzyme of PTS, which is involved in glucose uptake in *E. coli*. (From here on, PHPr denotes the phosphorylated form of HPr, HPr denotes the unphosphorylated form, and (P)HPr denotes either phosphorylated or unphosphorylated Hpr). The phosphorylation state of the (P)HPr::GlgP complex controls glycogen decomposition. The glycogen decomposing activity of HPr::GlgP is about five times higher than that of PHPr::GlgP [14]. In PTS, HPr transfers phosphate groups from (P)EI to (P)EIIA^{Glc}. Thus (P)HPr regulates the speed of carbohydrate decompositions from both glycogen and glucose.

Level-2 F2P (Regulation from molecule flux speed to protein). EI dimerization is thought to be the limiting step in the process of PTS transfer phosphate from PEP to G6P via PTS, which is regulated by PEP [19]. Whether EI acts as a dimer or a monomer to transfer phosphate from PEP to HPr is still under discussion [19, 21, 22]. Different phosphorylation states of HPr result from the balance of phosphate influx into PTS from PEP and outflux to G6P, in which phosphate influx is under the regulation of EI dimerization.

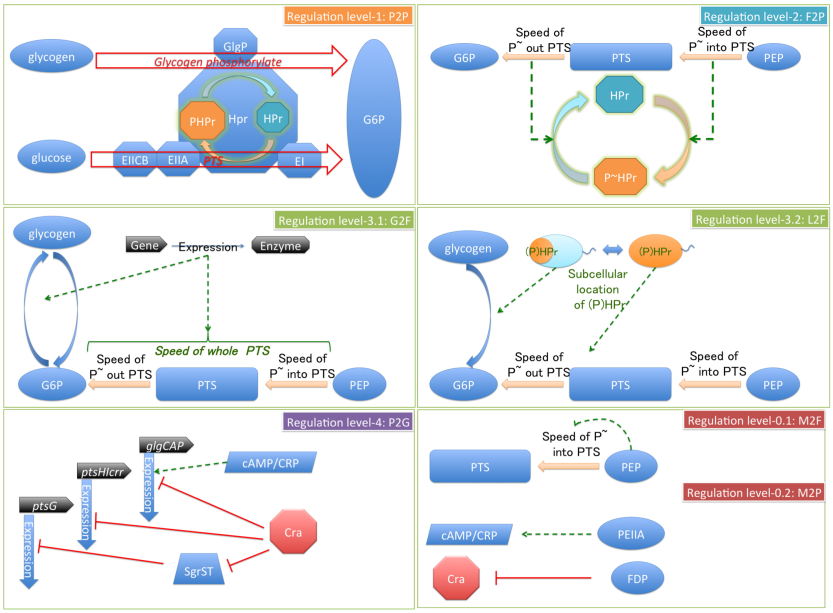

Fig. 1. Regulation mechanisms controlling glucose and glycogen utilization. **Level-1**: HPr controls glucose phosphorylation (as a PTS enzyme) and glycogen decomposition (in complex with GlgP). **Level-2**: Phosphorylation of HPr is controlled by the balance between phosphate groups influx into and outflux from the PTS. **Level-3.1**: Gene expression regulates PTS the speed of transportation of phosphate and the process of glycogen metabolism. **Level-3.2**: In HPr function, either for PTS or for glycogen degradation, depends on its subcellular location. **Level-4**: PTS and *glg* genes expression are controlled by cAMP/CRP and Cra. **Level-0.1**: PEP concentration controls the amount of phosphate entering the PTS. **Level-0.2**: Cra expression is controlled by FDP amount, and cAMP/CRP is enhanced by PEIIA^{Glc}.

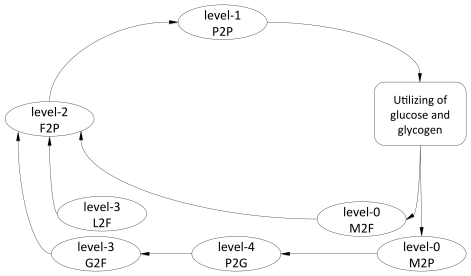

Fig. 2. Regulatory circuit system for glucose and glycogen utilization. **M2F** is a regulation from metabolite to molecule flux speed; **M2P** is a regulation from metabolite to protein; **P2G** is regulation from protein to gene expression; **G2F** is a regulation from gene expression to molecule flux speed; **L2F** is a regulation from molecule subcellular location to molecule flux speed; **F2P** is a regulation from molecule flux speed to protein; **P2P** is a regulation from protein to protein.

Level-3.1 G2F (Regulation from gene expression level to protein). PTS enzymes for glucose uptake in *E. coli* include EI, HPr, EIIA^{Glc} and EIICB^{Glc}, the former three enzymes are expressed from *ptsHIcrr* gene cluster and EIICB^{Glc} is from *ptsG*. After an exponential increasing, when an enzyme concentration increases above a certain threshold, its catalyzed reaction speed will remain in a high level [23]. Here we assumed that, when PTS enzymes are expressed above a certain threshold, the whole PTS reaction speed would be extremely accelerated.

Level-3.2 L2F (Regulation from molecule subcellular location to molecule flux speed): When (P)HPr is located at the cell's poles, it mainly functions for glycogen phosphorylation. And when (P)HPr is scattered in cytosol, it serves for the function of PTS, which is responsible for glucose uptake. The deduction of subcellular location of (P)HPr controlling system will be explained in **Subsection 2.2**.

Level-4 P2G (Regulation from protein to gene expression). Cra is a global regulator of the genes for carbon metabolism in *E. coli* [6], which directly regulates *glgC* and *glgA* and *ptsHIcrr* operon, or indirectly influences *ptsG* transcription via SgrST pathway [6, 24]. The function of upregulation of *glgC* and *glgA* by cAMP/CRP complex is confirmed by experiments of [17]. Comprehensively say, when Cra levels decreases, it releases the inhibition of *glgC* and *glgA*; as a consequence cAMP/CRP activates extremely strong expression of *glgC* and *glgA*.

Level-0.1 M2F (Regulation from metabolite to molecule flux speed). High enough PEP levels activate the phosphate influx into PTS by stimulating EI dimerization [18, 19]. This reaction EI+EI⇒EIEI has been thought to be the limiting speed of PTS.

Level-0.2 M2P (Regulation from metabolite to protein): When fructose-1,6-bisphosphate (FDP) reaches a high level, Cra expression is repressed, which releases its inhibition of *glgC* and *glgA* [6]. High concentration PEIIA^{Glc} leads to the accumulation of cAMP [8].

2.2 HPr role in glycogenolysis or PTS depends on its subcellular localization

Lopian et al. (2010) described the spatial and temporal organization of PTS enzymes in *E. coli*, especially HPr and EI [20]. According to their study, HPr and EI mainly stay in the poles of a cell independently, and if HPr is released to the cytosol, it should be phosphorylated by PEI in the presence of glucose. Genobase also shows a photo of HPr scattering in the cytosol [25].

In the glycogen metabolism, interestingly, glycogenesis enzymes (GlgC, GlgA) and glycogen granules locate at the poles, while GlgP is scattered in the cytosol [2]. GlgP is considered always bound in a complex with HPr, since the concentration of HPr is much higher than that of GlgP in *E. coli* [14, 15].

Based on these studies, we hypothesize that HPr controls the priority in glucose and glycogen utilization in *E. coli*: (1) If there is no glucose, HPr cannot get phosphate from EI, keeping its location at the poles. Hence, this pole-located

HPr mainly serves for glycogen decomposition, whose speed is regulated by phosphorylation state of (P)HPr:GlgP as described in **Subsection 2.1**. (2) If there is a little glucose supply, at the very beginning of lag phase, glucose uptake takes place at poles areas for a very short time until all the phosphates are removed from these PTS enzymes including HPr (See ***Early lag phase (1)*** in **Subsection 4.1**). Note that the pole-located HPr also has the ability of exchanging phosphate with other PTS enzymes. (3) If glucose is abundant, HPr gets phosphate from PEI, causing its release to the cytosol. Cytosol-scattered HPr works as a PTS protein, but not for glycogenolysis, hence, transporting phosphate from EI to $EIIA^{Glc}$.

3 Construction of a dynamic simulation model of central metabolic pathway with HFPN

Central metabolic pathway in *E. coli* is constituted by the glycolysis, the PP pathway, and the tricarboxylic acid cycle (TCA cycle). Most glycolysis models are based on ordinary differential equation (ODE) [12, 26, 27]. Chassagnole et al. (2002) constructed an integrated ODE model of glycolysis and PP pathways [12], which is often used as a base model in many studies [26–29]. By assembling TCA cycle with the model of [12], Kadir et al. (2010) set up an ODE model together with six pieces of logical controlling rules [27], and Usuda et al. (2010) included gene regulation in [26]. Kinetic parameters of these ODE model has been stored in many databases, such as BRENDA [30], SABIO-RK [31], and BioModels [32], and a number of works focused on parameter optimization [33, 34]. PTS are usually represented by one or a few equations in these ODE models. Rohwer at el (2000) set a mass balance theory model of PTS, by using experimentally tested mass action constant for each elementary biochemical reaction within PTS enzymes [13], and some studies are based on it [9, 35].

The simulation of our HFPN models are conducted on Cell Illustrator 4.0 [36]. Before realizing a whole model, we have first set up two independent HFPN models based on these published, ODE models of glycolysis and PP pathway [12, 32] and mass balance theory models of PTS [13, 35]. Subsequently, these two HFPN models are combined into one.

This HFPN model was further extended by incorporating glycogen metabolism pathway and basic regulatory mechanisms, and finally we got an extended HFPN model of carbohydrate metabolism, as shown in Fig. 3. We employed general mass action method to construct this integrated HFPN model, in which mass action constants were manually fitted so as to meet biological data of glycogen and other metabolites concentrations from our former study [1]. From this URL[0], a complete HFPN model, lists of places, transitions, and arcs can be obtained.

[0] `http://ds0n.cc.yamaguchi-u.ac.jp/~mzemi/etchp/ecoli_doc/MatsunoLab.htm`

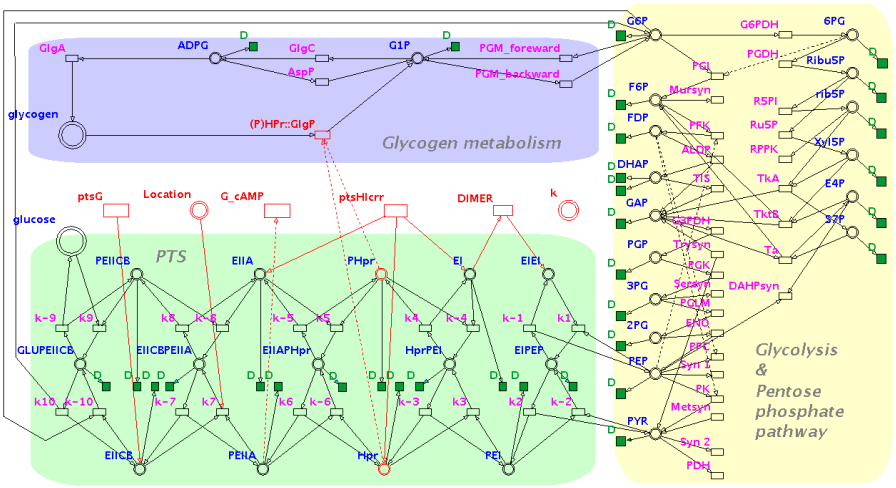

Fig. 3. HFPN model of extent center metabolism pathway in *E. coli*. This model includes 3 major parts: glycolysis and pentose phosphate pathway part is in **bright yellow area**, PTS part is in **bright green area** and glycogen metabolism pathway part is **bright blue area**. **Green filed transitions** are degradation or dilution processes of their connected metabolites or enzymes. **Black border components** are of the 3 major parts. **Red border components** are of regulatory mechanisms, in which transition *ptsG* and *ptsHIcrr* represent PTS genes expression process; **G_cAMP** is the process of PEIIA^{Glc} activating cAMP production; **Dimer** is the process of EI dimerization; ***k*** is the parameter controlling the whole PTS reaction speed; **Location** is of the molecular subcellular localization regulation mechanism; **(P)HPr::GlgP** represent the process of the binding of (P)HPr::GlgP catalyzing glycogen decomposition. This is the snapshot of main part of our HFPN model, other components can be found in Suppl. documents in the URL[0]

The integrated HFPN model produced the correct behavior of metabolite concentrations of G6P, PEP, FDP etc. in a batch culture as well as the concentrations of glycogen and extracellular glucose in Fig. 4, which can be confirmed by comparing with their experimental data in Supplementary data of [1]. Further, PTS enzymes level are also illustrated in this figure, and the noisy behaviors in EIEI and PEI come from the rapid alternation of phosphate between these molecules. Although more evaluations are required, we can say that our simulation results express similar behavior to the experimental data.

4 Confirmation of the role of HPr and EIIA^{Glc} as key regulators by simulation

4.1 Biological analyses based on the simulation results

With running simulations on the constructed HFPN model, we are able to systematically understand the process of carbohydrate metabolism in a batch cul-

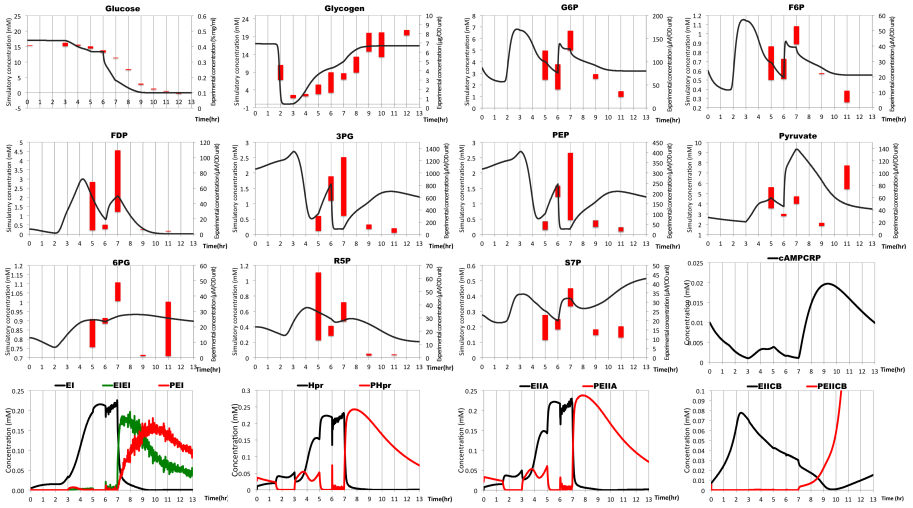

Fig. 4. Calculation results of HFPN model of extended central metabolism pathway in *E. coli*. Solid-curve is simulation result of this work. **Red-bar** denotes experimental data of our previous work [1].

ture in *E. coli* along its lifetime, which consists of 5 phases, early lag phase, late lag phase, early log phase, late log phase, and stationary phase. Simulated concentrations of glucose, glycogen, FDP, HPr ($EIIA^{Glc}$), PHPr ($PEIIA^{Glc}$), and cAMP are shown in the left panel of Fig. 5.

Early lag phase (1). At the beginning of this phase, *E. coli* begins its growth just after being put into a fresh medium. At this point, (P)HPr is mainly present at the poles and causes a little glucose uptake locally. Glycogen is not utilized well in this phase, because it is surrounded by PHPr. Indeed the higher affinity of PHPr than HPr isolates GlgP from glycogen, resulting in a very slow speed decomposition rate of glycogen.

Early lag phase (2). Although this phase begins with PHPr, this protein slowly loses its phosphate. Because glycolytic pathway is not working in this phase, so phosphate cannot be provided through PTS. As HPr dephosphorylation completes, glycogen catalysis by HPr::GlgP begins, and *E. coli* uses glycogen as its main carbon source. Along with the quick consumption of glycogen, HPr is moved to the cytosol by the function of PEI [20]. Meanwhile, glycogen supplied phosphate flows into the central metabolic pathway, causing PEP accumulation. Distribution of (P)HPr in the cytosol will be finished at almost the same time.

Late lag phase. This is a period of slow glucose uptake, which is caused by a relevant lower level of PEP, due to a low speed EI dimerization [18]. This means that metabolites produced from glycogen support the transportation of phosphate for glucose uptake. During this period, (P)HPr has been distributed in the cytosol, whose major role is to work for PTS not for glycogen, and this

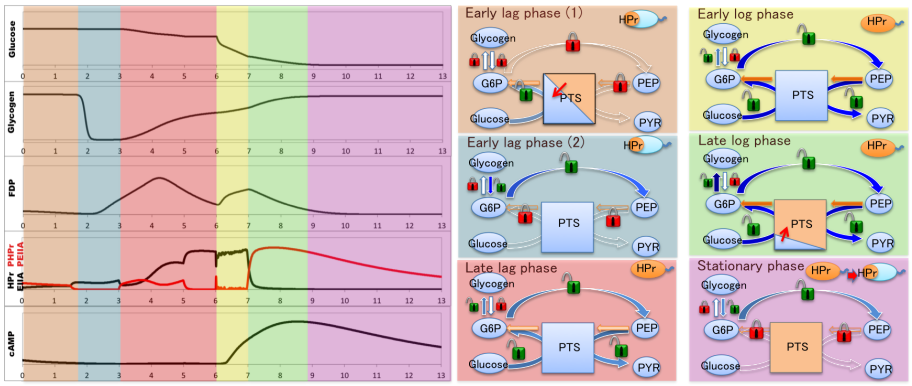

Fig. 5. Systematic understanding of the phases of extended central metabolism in an *E. coli* along its whole lifetime based on this works HFPN model. In the figure, **left panel** illustrates experiment and simulation behaviors of some major metabolites and enzymes, **right panel** shows sugar and phosphate flux processes of *E. coli* utilizing glucose and glycogen, and same colored area of left panel and right panel are of the same phase. In right panel: **Blue colored PTS** represents unphosphorylated PTS, **orange colored PTS** is phosphorylated PTS. **Blue filled arrows** indicate carbon flux routes, in which deeper blue color represents more flowing amount; **orange color filled arrows** indicate phosphate flux routes, in which deeper orange color represents more flowing amount. **Closed red locks** means inactivated pathway; **open green locks** means activated pathway. **The whole orange colored *E. coli* marked with "HPr"** indicates HPr is scattered in cytosol; **only head area orange colored *E. coli* marked with "HPr"** indicates HPr is at poles.

also causes the start of glycogen accumulation. Meanwhile in this phase more PTS enzymes are expressed, preparing for the impending log phase.

Early log phase. Uptake of glucose is very fast in this phase due to the highly expressed PTS proteins and the active transportation of phosphate by these PTS proteins. Glucose is the main sugar source in this phase.

Late log phase. In this phase, under the combined regulation of PEIIA^{Glc} (via cAMP/CRP), and FDP (via Cra), *glgC* and *glgA* are expressed at extremely high levels [2, 6, 8], causing efficient glycogen accumulation. Due to the lower speed of phosphate output from the PTS comparing with its input speed from PEP, high level of PHPr are working for glucose uptake. (P)HPr is mainly expressed in the cytosol, so it can hardly contribute to glycogen decomposition.

Stationary phase. When cells come to a stationary phase, glycogen is in its slow speed catalyzing state. Since (P)HPr is maintained in phosphorylated state, it concentrates towards the poles, where glycogen is located. In the post stationary phase, there is no glucose supplied outside, glycogen is used as a carbon source for cells to survive. Glycogen low speed catalyzation is regulated by surrounding PHPr in poles. Next, if the *E. coli* is put into another culture, a new lag phase begins.

Table 1. Multi-valued formulation of the regulation in utilizing glycogen and glucose in *E. coli*. α represents α (*glgC* & *glgA* activation), β represents β (*glgC* & *glgA* activation), γ represents γ (glycogen composition). The meaning of multi-values are 0 (no/off), 1 (on/slow) , and 2 (fast).

α *glgC* & *glgA* activation	β *glgC* & *glgA* activation	γ glycogen composition
0	0	0
1	0	1
0	1	1
1	1	2

4.2 Logical expressions of regulator states throughout the phases

Multi-valued logic rule. *glgC* and *glgA* are the genes that forms an operon with *glgP* [2, 17, 37]. According to experimental result, *glgC* and *glgA* are regulated by cAMP [2, 17] and FDP [6], respectively. Hence we can consider that the transcription of this two genes is regulated by the combination of FDP amount and cAMP level, which are distinguished α (*glgC* & *glgA* activation) and β (*glgC* & *glgA* activation), respectively. Actually, from the biological literature [2, 6, 17], it is known that the composition speed of glycogen varies depending on the expression pattern of α and β. If either α or β is expressed, glycogen is composed in slow speed, but if both α and β are expressed, glycogen is composed in high speed. This function can be expressed by multi-valued logic as presented in Table 1.

Phase transitions based on the regulatory factors. According to aforementioned analysis, the importance of HPr and EIIA^{Glc} on glycogen regulation is pointed out from a biological point of view. To make it more precise, we will express this regulatory system from an engineering point of view, presenting logical representation of this system as shown in Table 2. Glycogen process is controlled by the regulators FDP, EIIA^{Glc}, and HPr in the left column of this table. Among them, FDP and EIIA^{Glc} are involved in glycogen synthesis, and HPr in its decomposition. In the following, we will show, phase by phase, how composition and decomposition take place on the controls with these regulators in this table.

Early lag phase. Because of "very slow" uptake speed of glucose, FDP amount is in "low" level, resulting in "off" expression of *glgC* & *glgA* genes (α). EIIA^{Glc} and HPr display the same behavior, changing these phosphorylation states, "yes $\rightarrow$ no". In addition, *glgC* & *glgA* activation (β) is influenced by this state transition as "on $\rightarrow$ off" in Table 2. Glycogen composition, however, is not influenced by these regulations, because the uptake speed of glucose is too slow to produce glycogen. On the other hand, glycogen decomposition takes place in this phase, with changing its speed "slow $\rightarrow$ fast" according to the phosphorylation

Table 2. Behaviors of key regulators (HPr and EIIA^{Glc}) adjusting glucose and glycogen utilization in an *E. coli*. In this table, the five proliferation phases (e.g. Late lag phase) are corresponding with their processes of experiment and simulation data in Fig. 5 .

Regulator		Lag phase		Log phase		Stationary phase
		Early	Late	Early	Late	
speed of glucose uptake		very slow	slow	very fast	fast	no
FDP amount		low	high	low→high	high→low	no
α(*glgC*&*glgA* activation)		off	on	off→on	on→off	off
EIIA^{Glc} phosphorylation		yes→no	no	no	yes	yes
(regulated cAMP level)		high→low	low	low	high	high
β(*glgC*&*glgA* activation)		on→off	off	off	on	on
glycogen	**γ(composition)**	no	slow	slow	fast→slow	no
	(decomposition)	slow→fast	no	no	no	slow
HPr	**(phosphorylation)**	yes→no	no	no	yes	yes
	(localization)	pole	cytosol	cytosol	cytosol	pole

state transition of HPr "yes → no". Hence, glycogen is the major sugar source in this phase.

Late lag phase. Since *E. coli* have not consumed much energy yet in this phase, FDP accumulates in "high" levels despite the "slow" glucose uptake speed. Hence *glgC* & *glgA* (α) is "on". On the contrary, *glgC* & *glgA* (β) is "off", resulted from "no" phosphorylation state of EIIA^{Glc} via "low" cAMP level. According to the rule (if α=1 and β=0 then γ=1) in Table 1, glycogen is composed (γ) in "slow" speed. On the other hand, glycogen decomposition does not take place in this phase, because HPr is not located at the poles but distributed in the cytosol, which does not satisfy the requirement for glycogen decomposition.

Early log phase. Due to "very fast" speed of glucose uptake, FDP is accumulated in *E. coli*, despite its high metabolic activity, changing its amount as "low → high". Accordingly, the state of *glgC* & *glgA* (α) activation is changed as "off → on". In this stage, HPr is not phosphorylated, then the expression of *glgC* & *glgA*(β) is "off"; consequently the composition speed of glycogen (γ) is "slow", though it temporally drops to "no" level. On the other hand, "no" decomposition of glucose takes place in this phase from the same reason as late lag phase above.

Late lag phase. Because much glucose was consumed in the previous phase, its uptake speed is going to be slow down. Accordingly, for the phosphate flow in PTS, the input speed of phosphate from PEP becomes faster than the output speed to G6P, causing EIIA^{Glc} phosphorylation "yes" and cAMP level "high". As a result, *glgC* & *glgA* activation (β) turns "on". In addition, because, in the early half of this phase, FDP is in a high level, *glgC* & *glgA* activation (α) is also turned "on". Hence, both α and β regulations are working. In this case, according to Table 1, glycogen composition (γ) should be marked at "fast"

speed. Accompanying with decreasing glucose amount, FDP concentration drops later in this phase, that is "high → low", resuling in *glgC* & *glgA* activation (α) as "on → off". As a result, in the later part of this phase, the speed of glycogen composition (γ) changes as "fast → slow". On the other hand, in this phase, HPr is still in cytosol working for PTS, not for glycogenolysis. In all, since "fast" composition and "no" decomposition are conducted, glycogen accumulates quickly in this period.

Stationary phase. In this period, because extracellular glucose has been totally consumed off, the speed of glycogen is marked as "no" despite the "on" state of *glgC* & *glgA* activation (β). Hence there is "no" glycogen composition (γ). Because of the inactive PTS and the high amount glycogen, (P)HPr is concentrated at the "poles", decomposing glycogen (γ) in a "slow speed" for long survival of cells.

5 Conclusion

Some works focus on modeling glycolysis, pentose phosphate pathway, TCA cycle etc. [12, 26, 27], and some focus on the calculation of PTS performance with a protein mass balance theory method [13, 35]. And also some of them set up ODE models by combining PTS into their glycolysis pathways [26, 27]. But none of them take the glycogen metabolic pathway into account. In this work we firstly integrated general mass action based glycogen metabolism model and mass balance theory based PTS model into a computational model with HFPN.

By applying this model, basic regulators for *E. coli* to utilize extracellular glucose and intracellular glycogen were identified. That is, (P)HPr not only works as a member of PTS enzymes but also functions to realize different catalyzing speeds of glycogen by its phosphorylation state combined with GlgP. Actually, phosphorylation state of (P)HPr is controlled by the phosphate flux speed influx and outflux of PTS, and this flux speed is controlled by gene expression, subcellular localization, and metabolite concentration (glucose, PEP, FDP). HPr and EIIA^{Glc} are considered to be key roles among these regulators during the utilization of glycogen and glucose by *E. coli*.

Based on the model with regulatory systems in this work, we provided a systematic view of glucose and glycogen utilization by *E. coli*. This confirms our previous conclusion that glycogen plays an important role as a primary carbon source in lag phase [1].

In our model, the behavior after log phase does not correspond well to experimental data. The reasons might be inconsistencies in the referenced ODE and PTS that were modeled so as to function in a short time course (50 s) or steady stat context, and the difficulty in controlling its flux speed dynamically in an hour time scale. One of our future tasks is to address this limitation.

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