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Metabolic regulation of *Escherichia coli* cultivated under anaerobic and aerobic conditions in response to the specific pathway gene knockouts

Yu Matsuoka[1], Kazuyuki Shimizu[1,2*]

[1]Department of Bioscience and Bioinformatics, Kyushu Institute of Technology, Fukuoka, Japan
[2]Institute of Advanced Bioscience, Keio University, Tokyo, Japan
Email: *shimi@bio.kyutech.ac.jp

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ABSTRACT

Effect of the specific gene knockout on the main metabolism in *Escherichia coli* was reviewed, and the regulation mechanisms were clarified based on different levels of information such as gene expressions, enzyme activities, intracellular metabolite concentrations, and metabolic fluxes together with fermentation data. The effects of the knockout of such genes as *pflA*, *pta*, *ppc*, *pykF*, *adhE*, and *ldhA* on the metabolic changes were analyzed for the case under anaerobic condition. The effects of the knockout of such genes as *pgi*, *zwf*, *gnd*, *ppc pck*, *pyk*, and *lpdA* on the metabolic changes were also analyzed for the case under aerobic condition. The metabolic regulation analysis was made focusing on the roles of transcription factors.

Keywords: Metabolic Regulation; Single-Gene Knockout Mutant; *Escherichia coli*

1. INTRODUCTION

Escherichia coli is a Gram-negative, facultative anaerobic and non-sporulating bacterium. The *E. coli* cells are typically rod-shaped with length of about 1 - 2 μm having a cell volume of 0.6 - 0.7 μm^3. This organism has been identified by Theodor Escherich in 1885, and is now classified as part of *Enterobacteriaceae* family of gamma-proteobacteria [1]. This bacterium grows optimally at 37˚C and can survive even at higher temperature up to about 50˚C. *E. coli* is commonly found in the intestine of warm-blooded organisms. Although most *E. coli* strains are harmless to host organisms, and those are part of normal flora of the gut, and can benefit the host organisms by providing some vitamins, and by preventing the establishment of pathogenic bacteria within the intestine, some strains such as O157:H7 cause serious poisoning to human beings [2,3]. *Escherichia coli* is now the most popular bacterial model organism due to its well-known characteristics and easy for gene manipulation, and the research on such organism is useful for medical, pharmaceutical, and fermentation applications.

The most typical wild type strain *E. coli* K-12 has about 4605 open reading frame (ORF) in a 4.6 Mbp long [1]. The genes are located in 3386 transcription units, where it is operative unit for gene expression based on promoter and terminator regions, which correspond to the start and terminus of transcription, respectively. Of the total genes, about 300 are considered to encode transcription factors (TFs) and 7 for σ factors [4,5]. The detailed information is updated as documented in RegulonDB [6] and EcoCyc [7]. The hierarchical structure of such TFs and the regulated genes have been identified to some extent [8], and some of the metabolic regulation mechanism has been investigated [9,10].

As mentioned above, *E. coli* has been often utilized to produce useful metabolites by metabolic engineering. However, the success of pathway engineering is limited, where the expected result is rarely obtained for strain improvement. The main reason is due to limited knowledge on the metabolic changes caused by gene level and enzyme level regulation. Here, an attempt is made to clarify the regulation mechanisms for the several specific gene knockout mutants under both anaerobic and aerobic conditions based on different levels of information focusing on the roles of transcription factors.

2. REGULATION OF THE METABOLISM BETWEEN ANAEROBIC AND AEROBIC CONDITIONS

Global regulators such as Fnr and ArcAB are mainly responsible for the regulation of the metabolism under oxygen limited condition, where Fnr regulates the expressions of metabolic pathway genes under anaerobic

*Corresponding author.

condition [11], while ArcAB regulates under both anaerobic and microaerobic conditions [12,13]. It has been shown that ArcA/B system exerts more significant effect on the cell metabolism under microaerobic condition than under aerobic or anaerobic condition. The effect of ArcAB system on the flux distribution at pyruvate node has been investigated based on the extracellular metabolite concentrations [14-16]. It was shown that lactate can be overproduced by *arcA*/*fnr* double mutant [14] in a similar way as *pfl* gene knockout [17,18].

Reoxidation of the reducing equivalents such as NADH generated by the oxidation of the energy source occurs in the respiratory chain under aerobic or microaerobioc condition. In *E. coli*, NADH is oxidized in the respiratory chain via a coupled NADH dehydrogenage NDH-1 encoded by *nuo* or an uncoupled dehydrogenase NDH-2 encoded by *ndh*, and the electron flows into quinone and quinol pool. Quinol is then oxidized by either the cytochrome bo or the cytochrome bd terminal oxidase complex, which in turn passes the electrons to oxygen with concomitant production of water. The *cyoABCDE* operon is represses by both ArcA and Fnr, while *cydAB* operon is activated by ArcA and repressed by Fnr [19].

The reducing equivalents such as NADH are reoxidized in the respiratory chain, where oxygen, nitrate, fumarate, and dimethyl sulfoxide etc. are the electron acceptors. This process is coupled to the formation of a proton mortive force (PMF), which is utilized for ATP generation from ADP and P_i. In the absence of oxygen, or other electron accepters, ATP is generated via substrate level phosphorylation through the process of degradation of carbon source in the metabolic pathways. The expressions of *pfl* genesis activated by ArcA and Fnr, and it becomes higher at lower oxygen concentrations, whereas *aceE*,*F* which encode α and β subunits of PDHc is repressed by ArcA under oxygen limited condition. At the branch point of AcCoA, the product of both Pfl and PDHc reactions, is converted to either acetate and ethanol or subsequently undergo further oxidation in the TCA cycle.

The Arc (anoxic respiration control) system, composed of ArcA, the cytosolic response regulator, and ArcB, the membrane bound sensor kinase, regulates the TCA cycle genes depending on the oxygen level or redox state. Upon stimulation by the redox state, ArcB undergoes autophosphorylation, and the phosphoryl group is transferred to ArcA. The phosphorylated ArcA binds to the promoter regions of the TCA cycle and other genes. It has been reported that ArcA, when phosphorylated, represses the expressions of the genes involved in the TCA cycle and the glyoxylate shunt genes such as *glt*A, *acn*AB, *icd*A, *suc*ABCD, *sdh*CDAB, *fum*A, *mdh*, and *aceA*,*B* (**Appendix A**). Moreover, the genes which encode the primary dehydrogenases such as *glpD*, *lctPRD*, *aceE*,*F* and *lpdA* are also repressed by ArcA (**Appendix A**).

Alexeeva *et al*. [13] investigated the effects of different oxygen supply rates on the catabolism in *arc*A mutant. A strong link is demonstrated between redox ratio ($NADH/NAD^+$) and acetate overflow in *E. coli* [20]. It was shown that the commencement of acetate overflow occurred above the critical $NADH/NAD^+$ ratio of 0.06 [20]. Moreover, acetate overflow is delayed by the expression of heterologous NADH oxidase (NOX), an enzyme that serves to reduce the $NADH/NAD^+$ ratio [20]. The redox state has been reported to trigger the Arc regulon [21,22].

Since phosphorylated ArcA represses TCA cycle genes, the *arcA* gene deletion activates the TCA cycle, resulting in the reduction in the acetate formation [20]. The NADH oxidation by the expression of NOX in the *arc*A gene knockout mutant further reduced the acetate formation, resulting in the increased recombinant protein production [20]. Since TCA cycle is the main source of energy generation and provides important precursors for amino acids such as glutamate, lysine etc., it is of practical interest to enhance the TCA cycle activity. As stated above, the *arc*A/B genes knockout in *E. coli* transcriptionally activates the TCA cycle and overproduces NADH, which may in turn repress the TCA cycle by its allosteric regulation. Moreover, it has been reported that ArcAB does not control the TCA cycle under aerobic condition due to the fact that oxidized quinone electron carriers inhibit autophosphorylation of ArcB, and it cannot transphosphorylate ArcA [21]. As expected from the above mentioned regulation, the TCA cycle is activated by *arcA/B* gene knockout, which then causes higher NADH/NAD ratio, which in turn represses TCA cycle activity [23]. Vemuri *et al*. [24,25] considered to express heterologous *nox* gene to oxidize NADH, and in turn activate TCA cycle, while nicotinic acid and Na nitrate may also activate TCA cycle [26].

Since the TCA cycle is the source of energy generation and provide some of the precursors for the cell synthesis, the activation of the TCA cycle may lead to the improvement of ATP production for the cell growth and/or the TCA cycle-related metabolite productions in practice. Moreover, the activation of TCA cycle reduces the acetate production rate, which is the common obstacle for the metabolite production using *E. coli*. However, the activation of the TCA cycle causes the decrease in the cell yield due to higher production of CO_2 in the TCA cycle.

Respiration is a fundamental cellular process utilizing different terminal electron acceptors such as oxygen and nitrate etc. The ability to sense these electron acceptors is a key for the cells to survive. *Escherichia coli* is a me-

tabolically versatile chemoheterotroph grown on a variety of substrate under various oxygen concentrations with fumarate or nitrate, replacing oxygen as terminal electron acceptor under anaerobic condition [27]. Many bacteria utilize oxygen as the terminal electron acceptor, but they can switch to other acceptors such as nitrate under oxygen limitation. In *E. coli*, this switch from aerobic to anaerobic respiration is controlled by Fnr (fumarate and nitrate reduction). Under oxygen limitation, Fnr binds a $[4Fe\text{-}4S]^{2+}$ cluster, and becomes a transcriptionally active dimeric form.

The metabolic regulation is made by the binding of dimeric Fnr to the promoter regions of the relevant genes with affinities depending on the redox state [28]. The ability of Fnr to bind DNA is regulated by the change in equilibrium between monomeric apo Fnr (inactive) and dimeric Fnr (active) *in vivo*. The active form of Fnr binds to DNA to regulate the corresponding genes under anaerobic condition. Molecular oxygen can oxidize the ion-sulfur cluster of the corresponding region, resulting in monomerization of the protein and subsequent loss of its ability to bind DNA [29].

Since Fnr is known to activate *frd* and *pfl* genes, the *fnr* mutant produced less succinate and formate [30]. Although *arcA* is known to be activated by Fnr, the regulation mechanism is somewhat complicated. Namely, *cyo* and *cyd* genes are repressed by Fnr, while *cyo* is repressed and *cyd* is activated by ArcA. The *fnr* mutant shows decreased gene expression of *arcA*, and increased gene expressions of both *cyoA* and *cydB*. This implies that the activated cytochrome oxydase increases quinone pool, which inhibits ArcB phophorylation, and in turn decreases phosphorylation of ArcA, where *arcA* gene expression also decreases due to *fnr* gene knockout.

Under anaerobic condition, TCA cycle is the branched pathway patterns, while *arcA* mutant or nitrate was used as electron accepter, TCA cycle functions as the case under aerobic conditions [31].

3. EFFECT OF A SPECIFIC GENE KNOCKOUT ON THE METABOLISM UNDER ANAEROBIC CONDITION

Here, we consider the metabolic regulation of *E. coli* cultivated under anaerobic condition in response to the specific pathway gene knockout on the metabolic fluxes as well as other levels of information (**Figure 1**). Let us first consider the fermentation characteristics and some enzyme activities for *pflA*, *pta*, *ppc*, *adhE*, and *pykF* mutants, as compared to the wild type BW25113 [18]. It was shown that the *pflA* mutant gives much higher activities of GAPDH and LDH, which imply the coupling between the NADH production and consumption between the two corresponding reactions. Similarly to the *pflA* mutant, the simultaneous up-regulations of GAPDH and LDH are also observed in the *pta* mutant. Interestingly, Pfl activity is repressed in this mutant, which implies a common regulatory mechanism for these two strains. The significant difference between *pta* and *pflA* mutants is the specific activity of Ppc. In the *pta* mutant, this enzyme activity changed little compared to the parent strain, but thismutant produced more succinate than the *pflA* mutant, despite lower Ppc activity. This implies that the intracellular pool size of PEP plays another important role in the metabolic regulation, where PEP concentration is higher for *ppc* mutant, while it is low for *pflA* mutant [18].

The enzyme activities of Ack and ADH, both of which are the AcCoA assimilation pathway enzymes, increased in the *ppc* mutant. However, the enzyme activity of Pfl, which supplies AcCoA for these two reactions, is much lower than that of the parent strain, and less acetate and ethanol are formed in the *ppc* mutant than those of the parent strain. Although the activity of LDH is lower, lactate production is higher in the *ppc* mutant compared to that of the parent strain. The activity of Pyk in the *ppc* mutant is about one-quarter of that in the parent strain. Pyk activity in the *adhE* mutant is also down-regulated. The activities of LDH and Pfl are both down-regulated in the *adhE* mutant, which is consistent with relatively lower lactate and formate yield in thisstrain. The *pykF* mutant shows higher Ppc activity, while the activities of LDH and ADH are both down-regulated as compared to the parent strain [18].

The pool sizes of G6P, FDP, and PYR increase, while PEP concentration is lower in the *pflA* mutant than the wild type strain. The low PEP concentration causes less succinate production, even with high Ppc activity, where this may be caused by the allosteric activation by the increased FDP. The increased FDP concentration also allosterically activated Pyk. The metabolite concentrations in the *pta* mutant are similar to those in the *pflA* mutant, except for significantly accumulated PEP. In the case of *pykF* mutant, all intracellular metabolites in the glycolysis are significantly accumulated, as compared to the parent strain and other mutants. Note that the value of ATP/AXP (ATP + ADP + AMP) is less in the mutants as compared to the parent strain [18].

Among the different levels of information, the metabolic flux distribution is the most important, where it is located on top of the hierarchy of informations [32]. Metabolic flux is defined as the intracellular reaction rate per cell (weight) and per time ($mmol \cdot gDCW^{-1} \cdot h^{-1}$). The information on the metabolic flux distribution is particularly useful in metabolic engineering. Although one of the catabolic pathways is blocked by the specific gene knockout, the higher glucose uptake rate, followed by higher glycolytic flux, may be seen in such strains as *pflA*, *pta*, and *pykF* mutants, as compared to the parent

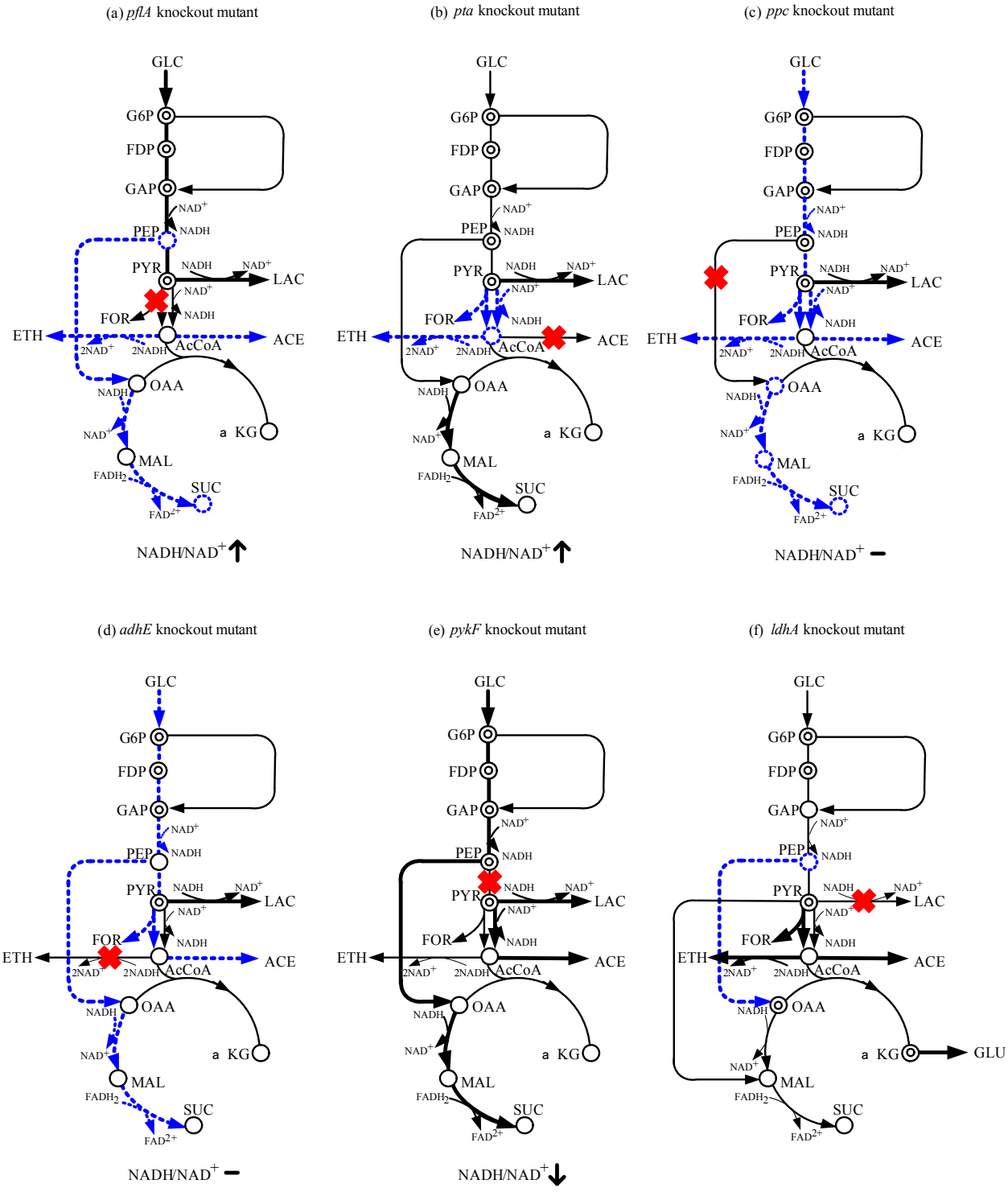

Figure 1. Schematic illustration of the specific pathway gene knockout on the metabolism under anaerobic condition: (a) *pflA*; (b) *pta*; (c) *ppc*; (d) *adhE*; (e) *pykF*; and (f) *ldhA* knockout mutants.

strain [18]. The flux through GAPDH is shown to be regulated by the intracellular NADH/NAD^+ ratio [33,34]. It has been shown that NADH competitively combines with GAPDH and inhibits the reaction through this enzyme. The GAPDH activities in the *pflA*, *pta*, and *pykF* mutants were all higher than that of the parent strain. The intracellular NADH/NAD^+ ratio was significantly lower in the *pykF* mutant, and therefore, the higher glycolysis flux is not restricted by the competitive inhibition of NADH on the reaction through relatively high levels of

GAPDH. The high glycolytic flux of *pflA* and *pta* mutants results in a high intracellular NADH/NAD^+ ratio, and subsequently lactate is produced to regenerate NAD^+ so that the glycolysis continues to work [18].

The lactate producing flux in the *ppc* mutant is lower than those of the *pflA* and *pta* mutants. However, as compared to the parent strain, the lactate production rate in the *ppc* mutant is significantly higher. The glycolytic flux in the *ppc* mutant is about 17% of that in the wild type strain. The flux through Ppc in the *pykF* mutant was higher than that of the parent strain. From enzyme activity data, Ppc activity in the *pykF* mutant is up-regulated as compared to the parent strain. The reason for the high Ppc flux may be due to high PEP concentration and synergistic activation by FDP [35,36]. Since the product of the reaction through Ppc is OAA, which is the precursor for biosynthesis, the *ppc* gene knockout causes low biomass synthesis flux [18].

Pyruvate is competed by the reactions through Pfl and LDH under anaerobic condition. In wild-type *E. coli*, the LDH reaction is not as competitive as the reaction through Pfl, and therefore, acetate and formate are the main metabolites instead of lactate. The knockout of the *pflA* gene blocks the pyruvate assimilation through the Pta-Ack and ADH pathways, which are commonly used for ATP production and NADH re-oxidation, respectively, in the wild-type strain. Since the glycolytic flux is promoted by anaerobiosis with a higher ATP requirement, lactate is produced to satisfy both the stoichiometric and intracellular redox balances [17].

Note the higher acetate production in the *pykF* mutant as compared to the parent strain. The reaction from PEP to OAA through Ppc is the main pathway to replenish OAA in *E. coli* under oxygen-limited conditions. Succinate is derived from OAA, and two equivalents of NADH are required for 1 mole of succinate production. In PTS (phosphotransferase system), PEP is used to transport phosphate for glucose utilization. The reaction through Pyk also uses PEP to produce PYR and ATP. When the *pykF* mutant was cultivated under anaerobic condition, the specific glucose uptake rate increased as compared to the parent strain. Flux through glycolysis is reported to be controlled by the ATP requirement in *E. coli*. By optimizing additional ATP hydrolysis, the glycolytic flux increases significantly [37].

Consider next the effect of the *ldhA* gene knockout on the metabolism of *E. coli* under anaerobic conditions. It was shown that that the extracellular formate, acetate, ethanol, pyruvate, and glutamate production rates increased in the *ldhA* mutant as compared to the parent strain [38]. Intracellular metabolite concentrations of G6P, F6P, 6PG, FBP, PYR, OAA, and α-KG are increased in the *ldhA* mutant, as compared to those of the parent strain. The enzyme activities of Pfk, GAPDH, Pgk, Pyk, and PDHc were up-regulated significantly in the *ldhA* mutant as compared to the parent strain. Citrate synthase (CS) was up-regulated in the *ldhA* mutant compared to the parent strain. This up-regulation caused an increase in the carbon flux from citrate to glutamate via α-KG, as is evidenced by the up-regulation of ICDH, and high concentration of intracellular α-KG in response to *ldhA* gene deletion [38]. The anaplerotic enzyme Ppc is down-regulated in the *ldhA* mutant compared to the parent strain. This is mainly due to the shortage of PEP in the *ldhA* mutant, as evidenced by the measurement of intracellular PEP concentration [38]. The reason for the decrease in PEP concentration is mainly due to the significant up-regulation of Pyk and PDH activities that channel more carbon flux toward the formate, acetate, and ethanol pathways, from the PYR and AcCoA pool, as well as increased NAD^+-Mez (Sfc) activity. The activity of $NADP^+$-specific malic enzyme (Mez) remains unchanged, whereas the NAD^+-specific malic enzyme (Sfc) is up-regulated significantly in the *ldhA* mutant compared to that in the parent strain, implying that the inactivation of the *ldhA* gene leads to the production of malate from pyruvate.

Deletion of the LDH pathway blocks carbon flow from pyruvate and results in a higher pyruvate excretion rate. Metabolic flux analysis shows that most of the carbon flux in *ldhA* mutant *E. coli* is forced through formate, acetate, and ethanol production pathways, resulting in a concomitant increase in these fluxes. When PEP is converted to MAL via OAA by Ppc and MDH, the free energy is wasted. In contrast, one ATP is produced when PEP is directly converted to pyruvate by Pyk. Therefore, a large fraction of PEP is channeled through the Pyk pathway and this might be why the *ldhA* mutant induces the Sfc pathway to supply the C4 intermediates in the TCA cycle for conserving the free energy of PEP, as evidenced by the significant up-regulation of Sfc.

The product of the global regulatory gene *cra* (*fruR*) is known to control the transcriptional expressions of numerous genes concerned with carbon and energy metabolism. The genes *ptsHI*, *pfkA*, *adhE*, and *pykF* are regulated negatively, whereas the genes *ppsA*, *pckA*, *icdA*, and *cydAB* are regulated positively by Cra (**Appendix A**). Gene expression analysis shows a slight up-regulations of *ptsH*, *pfkAB*, *pykF*, and *adhE* genes in the *ldhA* mutant, compared to those in the parent strain. The reason is due to the higher concentration of FDP in the *ldhA* mutant, since the effect of the Cra protein on the transcription is counteracted by a high concentration of FDP (**Appendix A**). The deletion of the *ldhA* gene may result in a reducing power imbalance in *E. coli*. However, the overall NADH balance calculated, based on the metabolic flux distribution, indicates that NADH production and consumption are almost the same in both the *ldhA* mutant

and the parent strain. The reason is mainly due to the increased carbon flux through the ethanol pathway in the *ldhA* mutant from pyruvate node via AcCoA.

Based on the study of gene expressions, enzyme activities, and intracellular metabolite concentrations, together with metabolic flux analysis, the overall regulation mechanisms for *E. coli* under anaerobic conditions in response to the specific gene deletion are shown in **Figure 1**.

4. EFFECT OF DIFFERENT CARBON SOURCES ON THE METABOLISM OF *PFL* MUTANT

Consider next the effects of other carbon sources such as gluconate, pyruvate, fructose, and glycerol on the metabolism of *pfl* mutant under anaerobic conditions, where *pflA,B* mutants are useful for the lactate production in practice [17]. A large amount of acetate is produced together with lactate, when gluconate or pyruvate is used as a carbon source. In particular, acetate is the major product when pyruvate is used as a carbon source. The fermentation patterns, when using fructose or glycerol as a carbon source, are similar to those when using glucose. However, fructose and glycerol uptake rates are significantly lower than glucose uptake rates. Compared to the case of using glucose as a carbon source, Ack activity is significantly higher for the case of using gluconate or pyruvate as a carbon source, while this enzyme activity is reduced when using glycerol or fructose as a carbon source. Ppc activity is the highest when using glucose as a carbon source, but it is significantly lower when using fructose or glycerol as a carbon source. It was shown that NADH/NAD^+ratio was highest when using glycerol as a carbon source, and this ratio was relatively low when using gluconate or pyruvate as a carbon source [17]. In *E. coli*, the Pta-Ack pathway is related to the AcCoA pool. Since AcCoA formation through Pfl is deficient in the two *pfl* mutants, Pta-Ack reactions may occur in the direction of forming AcCoA using acetate and ATP. The significant induction of Ack in the *pfl*A mutant may be related to the activation of PoxB, which produces acetate, and the cell utilizes the Ack-Pta and ACS pathway to supplement the AcCoA pool.

The cell will produce less NADH when using gluconate or pyruvate as a carbon source. This is confirmed by the lower NADH/NAD^+ ratio when using gluconate or pyruvate as a carbon source than when using glucose. The activity of GAPDH is significantly higher when using gluconate as a carbon source, which indicates the partial release of NADH inhibition on this enzyme. Correspondingly, the lactate yields, when using gluconate or pyruvate, are lower. The results using gluconate or pyruvate as a carbon source may also be distinguished from those using other carbon sources, because of the significantly lower ATP/AMP ratio in the *pfl*A mutant, which is significantly less than that when using glucose as a carbon source. One reason may be due to the difference in the metabolic networks; when using gluconate as a carbon source, some of the carbon atoms will go directly to PYR through the Entner-Doudoroff pathway, which reduces the flux through glycolysis where ATP is generated.

5. CATABOLITE REGULATION OF WILD TYPE *E.COLI* UNDER AEROBIC CONDITION

Let us consider how the specific growth rate (dilution rate in the chemostat culture) affects the global regulators and metabolic pathway genes of wild type *E.coli* (BW25113) [39]. It was shown that the specific acetate production rate, and the specific CO_2 evolution rate (CER) increase as the dilution rate was increased [39]. **Figure 2** shows the effect of the dilution rate (the specific growth rate) on gene transcript levels, where it indicates that in accordance with the increased specific glucose consumption rate, the transcript levels of *ptsG*, *ptsH*, and *pfkA* are increased as the dilution rate increased, where *cra* transcript level decreased and *crp* as well as *mlc* decreased accordingly. The transcript levels of *zwf*, *gnd*, *edd*, and *eda* increased as the dilution rate increased. The transcript level of *ppc* increased while *pckA* decreased as the dilution rate was increased. Moreover, the transcript levels of *fadR* and *iclR* increased, and *aceA* and *aceB* decreased as the dilution rate increased [39].

The central players in carbon catabolite regulation in *E. coli* are the transcriptional activator Crp (cyclic AMP (cAMP) receptor protein; also called as catabolite gene-activator protein (CAP)), the signal metabolite cAMP, adenylate cyclase (Cya), and the PTSs, where these systems are involved in both transport and phosphorylation of carbohydrates. The PTS in *E. coli* consists of two common cytoplasmic proteins, EI (enzyme I) encoded by *ptsI* and HPr (histidine-phosphorylatable protein) encoded by *ptsH*, as well as carbohydrate-specific EII (enzyme II) complexes. The glucose-specific PTS in *E. coli* consists of the cytoplasmic protein $EIIA^{Glc}$ encoded by *crr* and the membrane-bound protein $EIICB^{Glc}$ encoded by *ptsG*, which transport and concomitantly phosphorylate glucose as explained before. The phosphoryl groups are transferred from PEP via successive phosphorelay reactions in turn by EI, HPr, $EIIA^{Glc}$ and $EIICB^{Glc}$ to glucose. The cAMP-Crp complex and the repressor Mlc are involved in the regulation of *ptsG* gene and *pts* operon expressions. It has been demonstrated that unphosphorylated $EIICB^{Glc}$ can relieve the expression of *ptsG*

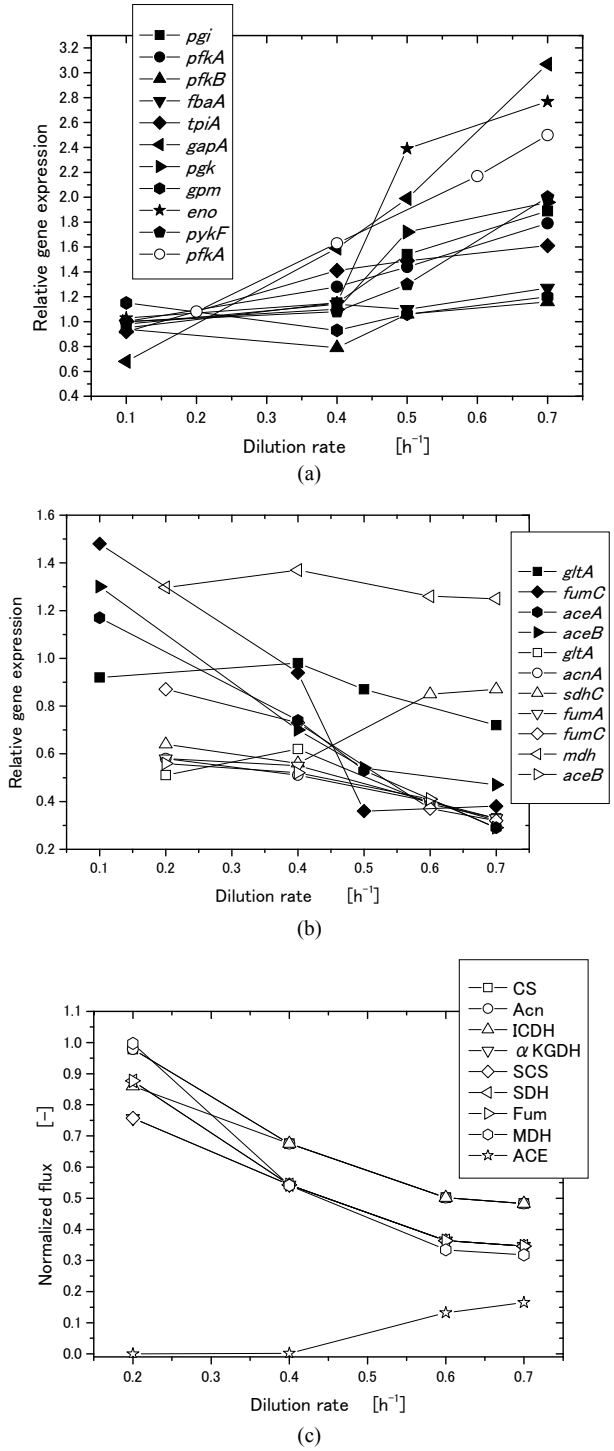

Figure 2. Effect of the growth rate (dilution rate) on the glycolysis and TCA cycle gene expressions and fluxes: (a) glycolysis genes; (b) TCA cycle genes; (c) TCA cycle fluxes and acetate formation rate.

gene expression by sequestering Mlc from its binding sites through a direct protein-protein interaction in response to glucose concentration. In contrast to Mlc, where it represses the expressions of *ptsG*, *ptsHI* and *crr* (**Appendix A**), cAMP-Crp complex activates *ptsG* gene expression (**Appendix A**).

Since intracellular cAMP levels are low during growth on glucose, these two antagonistic regulatory mechanisms guarantee a precise adjustments of *ptsG* expression levels under various conditions [40]. It should be noted that unphosphorylated $EIIA^{Glc}$ inhibits the uptake of other non-PTS carbohydrates by the so-called inducer exclusion [41], while phosphorylated $EIIA^{Glc}$ ($EIIA^{Glc}$-P) activates adenylate cyclase (Cya), which generates cAMP from ATP and leads to an increase in the intracellular cAMP level [42]. Note that if the concentration ratio between PEP and PYR(PEP/PYR) is high, $EIIA^{Glc}$ is predominantly phosphorylated,whereas if this ratio islow, then $EIIA^{Glc}$ is predominantly dephosphorylated [43,44]. $EIIA^{Glc}$ is preferentially dephosphorylated when *E. coli* cells grow rapidly with glucose as a carbon source [43,44]. Note also that cAMP levels are low during growth with non-PTS carbohydrates, where PEP/PYR ratio is the key factor that controls phosphorylation of $EIIA^{Glc}$, which explains dephosphorylation of $EIIA^{Glc}$, resulting in low cAMP pool [43,44].

In addition to cAMP-Crp, which acts depending on the level of glucose concentration, the catabolite repressor/activator protein (Cra) originally characterized as the fructose repressor (FruR) plays an important role in the control of carbon flow in *E. coli* [45-47]. The carbon uptake and glycolysis genes such as *ptsHI*, *pfkA*, *pykF*, *zwf* and *edd-eda* are reported to be repressed, while *ppsA*, *fbp*, *pckA*, *icdA*, *aceA* and *aceB* are activated by Cra (**Appendix A**). The gluconeogenic pathway is deactivated by the knockout of *cra* gene, and the carbon flow toward catabolism and the glucose consumption rate are expected to increase, since glycolysis pathway genes such as *ptsHI*, *pfkA* and *pykF* are activated by the *cra* gene knockout. It has been shown that *cra* gene knockout enables the increase of the glucose consumption rate and thus improve the rate of metabolite production under certain culture conditions [48]. However, the regulation mechanism is complex and it must be careful since *icdA*, *aceA*, *B*, and *cydB* genes are repressed, while *zwf* and *edd* gene expressions are activated, and thus ED pathway is activated by *cra* gene knockout [48].

6. EFFECT OF THE SPECIFIC PATHWAY GENE KNOCKOUT ON THE METABOLISM UNDER AEROBIC CONDITION

6.1. Effect of *pgi* Gene Knockout on the Metabolism

If *pgi* gene was knocked out, the primary route for glucose catabolism is the pentose phosphate (PP) pathway together with Entner Doudoroff (ED) pathway [49-52].

The NADPH is over-produced, which inhibits the activity of G6PDH, and thus the glucose consumption rate becomes significantly low in this mutant [52]. This in turn causes glycolysis fluxes as well as intermediate concentrations of the glycolysis to be decreased [52]. The decreases in FDP and PEP concentrations cause Ppc activity to become lower by allosteric regulation, which causes OAA concentrations to be significantly decreased, which further activated glyoxylate pathway [51-53]. The acetate formation becomes significantly low due to the activation of glyoxylate pathway [52]. The activation of glyoxylate pathway reduces NADPH production at ICDH, where some of the NADPH overproduced may be converted to NADH by transhydrogenase such as Udh [51].

Referring to **Figure 3**, note that the decreased glucose consumption rate may cause cAMP-Crp to be increased, which in turn activates TCA cycle genes (**Appendix A**). Moreover, the decrease in FDP concentration may cause Pyk and Ppc activities to be decreased by allosteric regulation, and the decreased FDP concentration activates Cra, which in turn represses glycolysis gene expressions, while activates gluconeogenetic pathway gene expressions as well as *icdA* and *aceA/B* genes. Those caused the acetate production to be decreased.

6.2. Effect of *zwf* and *gnd* Genes Knockout on the Metabolism

In the case of *zwf* gene knockout, the cell growth phenotype is little affected, where the non-oxidative PP pathway flux is reversed [51,54]. This mutant shows significant overflow metabolism, and some of the NADPH, which cannot be produced at the oxidative PP pathway,

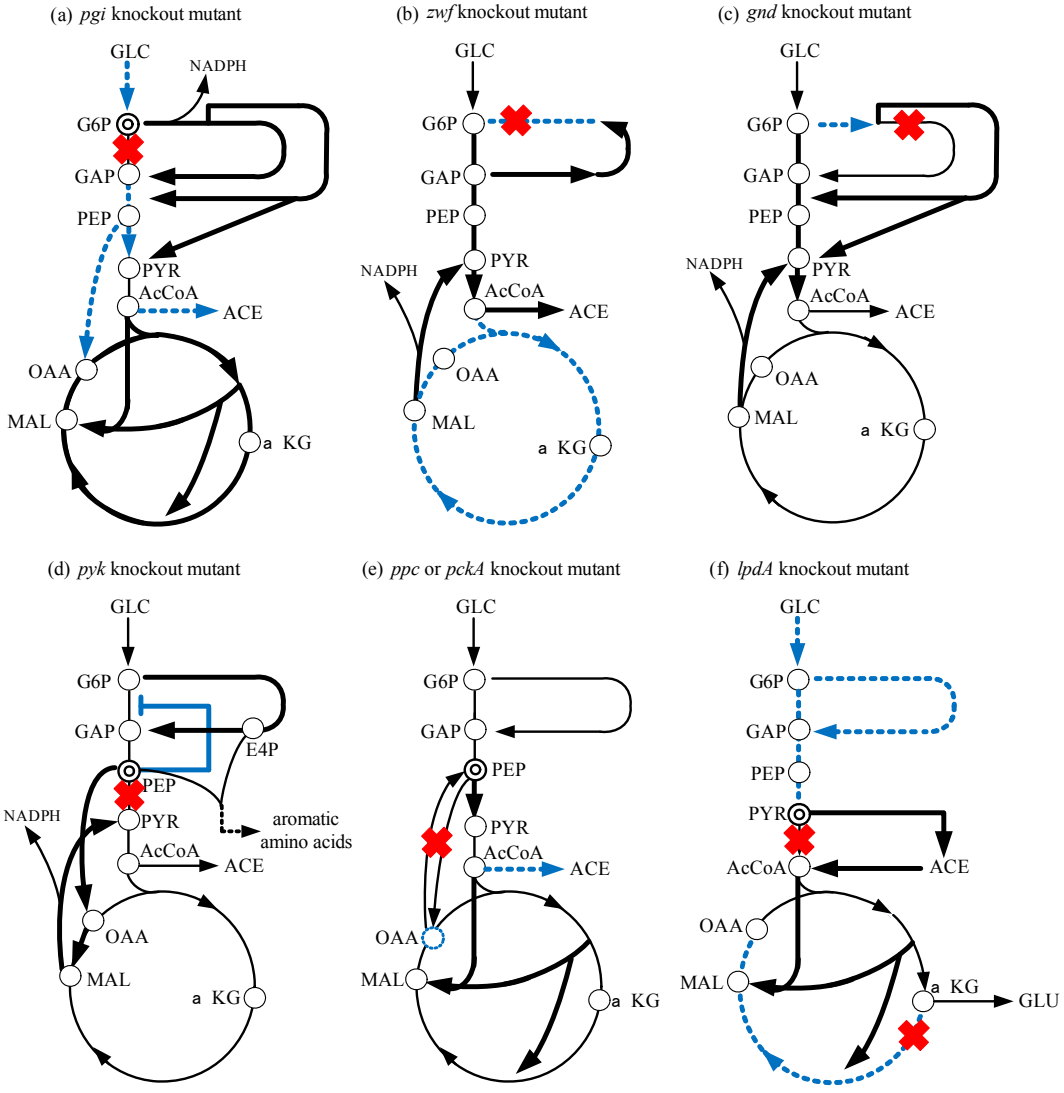

Figure 3. Schematic illustration of the specific pathway gene knockout on the metabolism under aerobic condition: (a) *pgi*; (b) *zwf*; (c) *gnd*; (d) *pyk*; (e) *ppc* or *pckA*; (f) *lpdA* knockout mutants.

is backed up by activating Mez (**Figure 3**), and by the transhydrogenase Pnt from NADH [51]. Note that *zwf* gene knockout causes the increase in glycolysis flux, and thus EIIA-P decreases and in turn cAMP-Crp decreases. This causes TCA cycle to be repressed. Moreover, as glycolysis flux increases, FDP concentration also increases, and in turn represses Cra activity, and thus TCA cycle is repressed by the down regulation of *icdA* and *aceA/B* genes. This is the reason why more acetate overflow metabolism was observed as compared to wild type strain [51,54].

The effect of *gnd* gene knockout on the metabolism is different from *zwf* gene knockout mutation [55]. The *gnd* gene knockout activates the ED pathway and causes a decrease in the flux through G6PDH, which reduces NADPH production throughthe oxidative PP pathway. This decreased NADPH production is backedup by activating Mez using MAL together with transhydrogenase Pnt. The conversion of carbon skeletons from the TCA cycle through Mez enables the cells to respond to TCA carbon depletion by regulating the carbon flux through Ppc. This can be shown in the positive correlation between the Mez and Ppc pathways.

Growth characteristics indicate that neither *zwf* nor *gnd* is essential. However, further inspection of the metabolic parameters indicates a unique alteration in the utilization of the metabolic pathways for optimal growth of the mutants under different culture conditions. The carbon can be fed into the ED pathway through two routes in *E. coli* grown on glucose. It is clear that the ED pathway, which is not possessed by *S. cerevisiae* serves as the route to relieve the toxic level of 6PG in the *E. coli* mutant grown on glucose. Because of the activation of this potential bypass reaction, the flux via G6PDH is reduced but not blocked. The activity of the oxidative PP pathway is therefore partly maintained. ICDH is important in producing NADPH [56]. Although the shortage of NADPH due to disruption of the oxidative PP pathway can be partially compensated for by an increased flux through ICDH, it appears that the flux via ICDH alone cannot enable the apparent shortage of NADPH to be met under certain circumstances. For this, malate is deviated out of the TCA cycle through Mez to function as the route in which an adequate supply of NADPH is generated to meet the biosynthesis requirements. The drain of carbon skeletons from the TCA cycle through Mez enables the cells to replenish the OAA pool by regulating the carbon flux through Ppc. To increase the synthesis of OAA from PEP, Ppc is therefore up-regulated in accordance with the activity of the Mez pathway. In *zwf* and *gnd* mutants, all directions of the fluxes through on oxidative PP pathways are reversed, indicating that the mutant tries to compensate for the lack of E4P and R5P through the glycolytic metabolites GAP and F6P. In thisway, the non-oxidative branch can function as an important metabolic route without the participation of the oxidative PP pathway.

6.3. Effect of *ppc* and *pck* Gene Knockout on the Metabolism

The decrease in OAA concentrations due to the *pckA* gene knockout causes the phosphorylation/inactivation of ICDH, which results in an increase in isocitrate concentration, and the flux through Icl also significantly increases [53]. Moreover, the *pckA* gene knockout causes accumulation of PEP, which in turn inhibits Pfk activity, and thus reduces the glucose consumption rate. This mutant produces less acetate and CO_2, resulting in higher cell yield with less growth rate than the wild type [53]. It is shown that *in vivo* regulation of the Pck flux occurs mainly by modulation of enzyme activity and by the changes in PEP and OAA concentrations rather than by the ATP/ADP ratio under aerobic condition. This indicates that the reaction catalyzed by Pck can respond flexibly to the availability of PEP and OAA, which may form the metabolic cycle at low glucose concentrations [50]. PEP is an important intermediate in the metabolism, since it alone directly regulates the phosphotransferase (PTS) system but also affects Pfk and Pyk activities. Since PEP can be formed gluconeogenetically through Pck from the TCA cycle, Pck serves to maintain the relative balance between OAA and PEP pools and drain off excess carbon of the TCA cycle to supply PEP for cellular requirements [53].

In relation to the *pckA* gene knockout, consider the effect of the *ppc* gene knockout on the metabolism. Among the central metabolic pathway enzymes of *E. coli*, Ppc plays an anaplerotic role in replenishing OAA consumed in biosynthetic reactions and keeping the TCA cycle intermediates from starvation. Farmer and Liao [57] overexpressed Ppc and/or induced a glyoxylate shunt by the *fadR* gene knockout [58], to elevate TCA cycle and glyoxylate pathway activities for the reduction of acetate excretion under aerobic conditions, since acetate excretion is a major obstacle in recombinant protein production. The specific cell growth rate and specific glucose consumption rate of the *ppc* mutant are lower than those of the wild type [59]. The CO_2 evolution rate (CER) is also reduced in the *ppc* mutant. Note that little acetate is excreted in the *ppc* mutant during cultivation as compared to the wild-type *E. coli*. As a consequence, an improvement of biomass yield on glucose can be observed in the *ppc* mutant. The activity of Pck, which catalyzes the reverse reaction to Ppc, is considerably lower in the *ppc* mutant. The activities of glycolytic enzymes, such as Pgi, Pfk, Fba, and GAPDH and the pentose phosphate (PP) pathway enzymes, such as G6PDH, 6PGDH, and

Tal, are all significantly decreased in the *ppc* mutant [59]. These data correspond to the slower growth and lower glucose utilization rates of the *ppc* mutant.

The up-regulation of Pyk is expected to channel more carbon flux from PEP to pyruvate due to the blockage of Ppc in the *ppc* mutant. Accordingly CS, the first enzyme of the TCA cycle, is up-regulated in the *ppc* mutant [59]. This up-regulation is expected to increase the carbon flux from pyruvate into the TCA cycle for the replenishment of OAA in response to Ppc deficiency. The activities of Acn and MDH, but not ICDH, in the TCA cycle, increase in a co-ordinated manner. Note that Icl, encoded by *aceA* involved in the glyoxylate shunt, is significantly induced in the *ppc* mutant [59]. This regulation pattern clearly demonstrates that the *ppc* mutant utilizes an alternative anaplerotic pathway, the glyoxylate shunt, to replenish OAA in response to the blockage through Ppc. It was shown that the glycolytic intermediates, such as G6P, F6P, F1,6BP (FDP), and PEP, and the PP pathway intermediate, such as 6PG, accumulate in the *ppc* mutant. In contrast, the intracellular concentration of AcCoA decreases in the *ppc* mutant, implying higher activity of the TCA cycle (or glyoxylate pathway) relative to glycolysis.

PEP accumulates in the *ppc* mutant and allosterically inhibits some of the glycolytic enzymes, such as Pgi and Pfk [60]. Inhibition of these enzymes leads to higher intracellular concentrations of their intermediates, such as F6P and F1,6BP (FDP), which in turn affect some other enzymes. For instance, G6PDH is allosterically inhibited by FDP and PRPP and induced by glucose, while 6PGDH is inhibited by FDP, PRPP, GAP, Ru5P, E4P, and NADPH, and induced by gluconate [61]. Apparently, the higher concentrations of intracellular F1,6BP (FDP) in the *ppc* mutant partially cause down-regulation of both enzymes. Although Cra activity may be repressed by the increased FDP concentration, and *icdA* and *aceA* gene expressions may be repressed, PEP/PYR ratio decreased, and the phosphorylated EIIA increased, and in turn cAMP-Crp increased, which may have caused TCA cycle to be activated. The higher flux through the TCA cycle produces more NADPH in the *ppc* mutant, which may also be considered the reason for the down-regulation of 6PGDH. The transcript of the glucose transport gene, *ptsG*, is also associated with accumulation of the glycolytic intermediates, such as G6P and F6P, which degrade the mRNA of *ptsG* by activating the RNaseP enzyme [62]. Both down-regulation of the glycolytic and PP pathway enzymes results in slower growth rates and lower glucose uptake rates in the *ppc* mutant. The remarkably reduced Pck activity in the *ppc* mutant may be caused by the higher intracellular concentration of PEP, since Pck is allosterically inhibited by nucleotides ATP and PEP [63]. Note that the activation of the glyoxylate shunt contributes to the reduction in CO_2 production in the *ppc* mutant.

6.4. Effect of *pyk* Gene Knockout on the Metabolism

Let us consider how the cell regulates upon *pyk* gene knockout [52,64,65]. The flux through the anaplerotic reaction catalyzed by Ppc in the *pykF* mutant is high in the *pykF* mutant as compared to the wild-type strain. It should be noted that the flux through Pck is also high. It can also be seen that the flux through Mez is high for the *pykF* mutant. Moreover, the glycolytic flux from G6P to F6P is low for the *pykF* mutant as compared to the wild-type strain, while the flux through the oxidative PP pathway is higher for the *pyk* mutant as compared to the wild type strain.

In short, the blockage of PEP to the PYR pathway activitivates both Ppc and Mez to supply PYR by forming an alternative pathway, and increases the PEP pool in the mutant, which might inhibit Pfk enzyme activity in the glycolytic pathway. The accumulation of PEP and the activation of the PP pathway (or increased E4P concentration) may activate the aromatic amino acids synthetic pathways [66].

6.5. Effect of *lpdA* Gene Knockout on the Metabolism

The lipoamide dehydrogenase (LPD) encoded by the *lpdA* gene is a component of PDHc, KGDH, and glicine cleavage multi-enzyme (GCV) system. This *lpdA* gene knockout produces more pyruvate and L-glutamate under aerobic conditions than the wild-type strain [67]. It is shown that AcCoA is considered to be formed by the combined rerouting pathways through PoxB, Acs, Ack, and Pta in the *lpdA* mutant. Metabolic flux analysis of the *lpdA* gene knockout mutant indicates that the ED pathway and glyoxylate pathway are activated, while the glycolysis and the oxidative PP pathway, as well as the TCA cycle, are down-regulated [67]. If only KGDH was blocked by the *sucA* gene knockout, metabolism is a little different from the *lpdA* gene knockout mutant [68]. The regulation of the anaplerotic pathway, such as the glyoxylate shunt, plays a role in these mutants. It was shown that the cell growth of the *lpdA* mutant is slow compared to its parent strain, and that a fair amount of pyruvate is produced during exponential growth phase of *lpdA* mutant. The accumulated pyruvate begins to decrease rapidly after glucose exhaustion. Then the cell starts to grow again using pyruvate as another carbon source, and produces acetate, which is subsequently utilized after the pyruvate is consumed. Although cell growth is depressed in the mutant, the final cell concentrations were higher than that in the parent strain.

The activity of the oxidative PP pathway enzyme, such as G6PDH, is up-regulated in the mutant and also the activity of the ED pathway enzymes and the glyoxylate shunt enzyme. It was shown that intracellular PYR concentration was higher in the *lpdA* mutant as compared to that in the parent strain. In addition, accumulation of L-Glu was higher than in the parent strain. It was shown by the continuous culture that the biomass yield of the *lpdA* mutant is higher than that of the parent strain, while glucose uptake rates are lower for the mutant and the specific CO_2 evolution rate is much reduced in the mutant.

To maintain growth, the cell can generate AcCoA from PYR using another less efficient route by first converting PYR to acetate using PoxB, and then convert acetate to AcCoA via Acs and Ack-Pta. The activation of PoxB can be detected in the PDHc-deficient mutant by *in vitro* PoxB enzyme assay [69]. It has been shown that *poxB* gene transcription depends on the *rpoS* gene product, σ^{38} (**Appendix A**). It is assumed that the PYR assimilation in the *lpdA* mutant might be done via PoxB and Ppc. It has been reported that the double mutants lacking the PDHc and Pps, or the PDHc and Ppc, could not metabolize PYR accumulated during growth on glucose [70]. Thus, the direct utilization of PYR is made by the combining reactions catalyzed by PoxB and Pps-Ppc. The inactivation of KGDH in the *lpdA* mutant causes the TCA cycle to be interrupted, which may activate the glyoxylate shunt to supply the precursor for cell growth. The low concentration of AcCoA causes down-regulation of CS in the TCA cycle. The synthesis of Glu needs NADPH, which may be supplied by the overproduction of NADPH in the PP pathway due to the upregulated activity of the oxidative PP pathway enzymes.

7. CONCLUSION

In the present article, an attempt was made to clarify the metabolic regulation mechanisms in response to the specific pathway gene knockout. It is shown to be quite important to focus on the roles of transcription factors, which gives insight into the essential metabolic regulation.

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APPENDIX A

Regulation of global regulators on the metabolic pathway gene.

Global regulator	Metabolic pathway gene
Cra	+:*aceBAK*, *cydB*, *fbp*, *icdA*, *pckA*, *pgk*, *ppsA* −:*acnB*, *adhE*, *eda*, *edd*, *pfkA*, *pykF*, *zwf*
Crp	+:*acnAB*, *aceEF*, *focA*, *fumA*, *gltA*, *malT*, *manXYZ*, *mdh*, *mlc*, *pckA*, *pdhR*, *pflB*, *pgk*, *ptsG*, *sdhCDAB*, *sucABCD*, *ugpABCEQ* −:*cyaA*, *lpdA*, *rpoS*
ArcA/B	+:*cydAB*, *focA*, *pflB* −:*aceBAK*, *aceEF*, *acnAB*, *cyoABCDE*, *fumAC*, *glpD*, *gltA*, *icdA*, *lctPRD*, *lpdA*, *mdh*, *nuoABCDEFGHIJKLMN*, *pdhR*, *sdhCDAB*, *sodA*, *sucABCD*
IclR	−:*aceBAK*, *acs*
FadR	+:*iclR*
Mlc	−:*crr*, *manXYZ*, *malT*, *ptsG*, *ptsHI*
PdhR	−:*aceEF*, *lpdA*
Fnr	+:*acs*, *focA*, *frdABCD*, *pflB*, *yfiD* −:*acnA*, *cyoABCDE*, *cydAB*, *fumA*, *fnr*, *icdA*, *ndh*, *nuoA-N*, *sdhCDAB*, *sucABCD*
RpoS	+:*acnA*, *acs*, *adhE*, *fumC*, *gadAB*, *talA*, *tktB*, *poxB*, *asmC* −:*ompF*

