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Metabolic Regulation of *Escherichia coli* in Response to Culture Environment such as Phosphate Concentration and Others

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Dedication

................To my beloved two sweet little angels Shafqat and Muntaha

ABSTRACT

It is quite important to understand the effect of culture environment on the cell metabolism, where carbon, nitrogen, phosphate etc. as well as oxygen level, pH, temperature etc. may be considered as important environmental factors. In the present study, the effects of phosphate (P)-limitation as well as acidic condition and nitrogen limitation on the metabolism in *Escherichia coli* were investigated. Moreover, the effects of several genes knockout on the metabolism were also investigated.

In the present study, the effect of phosphate (P) concentration on the metabolism in *Escherichia coli* was first investigated in terms of fermentation characteristics and gene transcript levels for the aerobic continuous culture at the dilution rate of 0.2 h^{-1}. The result indicates that the specific glucose consumption rate and the specific acetate production rate significantly increased, while the cell concentration decreased at low P concentration (10% of the M9 medium). The increase in the specific glucose uptake rate may be due to ATP demand caused by limited ATP production under P-limitation. The lower cell concentration was also caused by less ATP production. The less ATP production by H^+-ATPase may have caused less cytochrome reaction affecting in quinone pool, and caused up-regulation of ArcA/B, which repressed TCA cycle genes and caused more acetate production. In the case of *phoB* mutant (and also *phoR* mutant), the fermentation characteristics were less affected by P-limitation as compared to the wild type where the PhoB regulated genes were down-regulated, while *phoR* and *phoU* changed little. The *phoR* gene knockout caused *phoB* gene to be down-regulated as well as PhoB regulated genes, while *phoU* and *phoM* changed little. The effect of pH together with lower P concentration on the metabolic regulation was also investigated. In accordance with up-regulation of *arcA* gene expression, the expressions of the TCA cycle genes such as *sdhC* and *mdh* were down-regulated at acidic condition. The gene expression of *rpoS* was up-regulated, and the expression of *gadA* was up-regulated at pH 6.0. In accordance with this, PhoB regulated genes were up-regulated in the wild type under P-rich and P-limited conditions at pH 6.0 as compared to those at pH 7.0. Moreover, the effect of nitrogen limitation on the metabolic regulation was investigated, where the result indicates that *phoB* gene was up-regulated, and PhoB regulated genes were also up-regulated under N-limitation, as well as nitrogen-regulated genes.

The effect of *fnr* gene knockout on the metabolism of *Escherchia coli* also investigated under microaerobic condition based on gene expressions, enzyme activities, and intracellular metabolic fluxes. Under micro-aerobic condition, the flux through Pfl and Frd were reduced for the mutant, which are due to *fnr* gene knockout. The decreased flux through Pfl may have caused accumulation of PYR, which increased the flux through LDH. The *fnr* gene knockout caused *arcA* to be down-regulated, and thus the TCA cycle was activated, and *cyoA* and *cydB* genes were up-regulated. The down-regulation of *arcA* caused *lpdA* to be up-regulated where the flux through PDHc increased. The *fnr* gene knockout indirectly affected *cra* gene transcript level to

be decreased, which in turn caused the glycolysis genes to be up-regulated, which corresponded to the increase in the specific glucose consumption rate. The *fnr* gene knockout also caused *crp* transcript level to be increased, where there might be some relationship between the two from the gene structural similarity point of view.

The effect of pH on the fermentation characteristics was investigated by the batch and continuous culture of *Escherichia coli* at both aerobic and anaerobic conditions. It was found that more acetate was formed with lower biomass yield and less specific glucose consumption rate at lower pH as compared to the case at pH 7.0. The gene expressions indicate that the down-regulation of the glucose uptake rate corresponds to the down-regulation of *ptsG* gene expression caused by the up-regulation of *mlc* gene which is under positive control of Crp. In accordance with up-regulations of *arcA* gene expression at acidic condition, the expressions of the TCA cycle-related genes such as *icdA* and *gltA*, and the respiratory chain gene *cyoA* were down-regulated, whereas *cydB* gene expression was up-regulated. The decreased activity of TCA cycle caused more acetate formation at lower pH. Under micro-aerobic condition, the fermentation changed in such a way that formate and lactate were more produced at lower pH due to up-regulations of *pflA*, *yfiD* and *ldhA* genes, whereas ethanol was less produced due to down-regulation of *adhE* gene at lower pH as compared to the neutral pH. It was also found that *pflA* gene knockout mutant produced higher amount of lactate at lower pH compared to the case at pH 7.0 under micro-aerobic condition. The effects of both pH and temperature were also investigated. The overall metabolic regulation mechanisms under both pH down-shift and temperature up-shift were clarified in view of gene expressions.

Based on these results, it was implied that the effects of culture conditions are interrelated, and it is strongly desirable to uncover the basic principal which govern the cell metabolism. Moreover, the present result can be extended to other organisms than *E.coli*.

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Chapter 1 General Background

1.1 Introduction

Escherichia coli is an excellent free-living, single-celled, model organism for metabolic and biological studies as DNA replication and repair, DNA transcription, metabolic pathways, adaptive stress responses, signal transduction, and genetic regulation, since it is one of the best-characterised prokaryotes, served as a biological replica for cellular processes. Since the entire genome of *E. coli* has been sequenced (Blattner et al., 1997), several kinds of analyses have been applied in order to reveal metabolic regulation. Metabolic engineering can be defined as the directed improvement of product formation or cellular properties through modification of the specific biochemical reaction(s) or the production of new one(s) with the use of recombinant DNA technology. In many of these analyses, the aim has been to determine how the different genes interact with each other, enabling the cells to consume nutrients, grow, divide, regulate, and respond to different stimuli. The different approaches that have been used for these purposes can be classified as transcriptome, proteome, metabolome analyses and metabolic pathway analysis, depending on the type of compounds measured. So, it is quite important to understand how the culture environment affects cell metabolism. With those backgrounds in mind, the aim of present thesis was to reveal the metabolic regulation of *Escherichia coli* by using some mutants as well as by changing the culture condition.

1.2 Signal Transduction and Two-Component Regulatory Systems

Signal transduction systems transmit environmental signals to the cell. In prokaryotes, most of the signal transduction systems are two-component regulatory systems (Madigan and Martinko, 2006), by which prokaryotes regulate cellular metabolism in response to environmental fluctuations. This two-component regulatory system made up of

two different proteins such as

a) Sensor kinase: (cytoplasmic membrane) detects environmental signal and autophosphorylates

b) Response regulator: (cytoplasm) DNA-binding protein that regulates transcription (absent in bacteria that live as parasites of higher organisms)

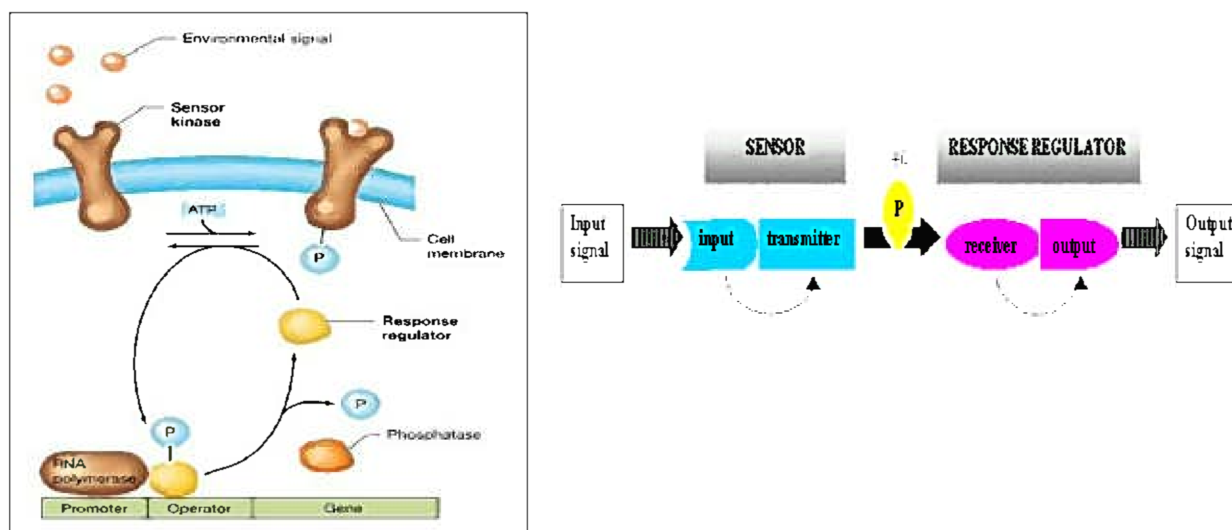

Fig. 1.1 Signal transduction and two-component regulatory system in *E.coli*

Table 1.1 Examples of two-component regulatory systems that regulate transcription in *Escherichia coli* (Madigan and Martinko, 2006).

System	Environmental signal	Sensor kinase	Response regulator	Activity of response regulator[a]
Arc system	Oxygen	ArcB	ArcA	Repressor/Activator
Nitrate and nitrite (anaerobic regulation) (*Nar*)	Nitrate and nitrite	NarX and NarQ	NarL NarP	Activator/Repressor
Nitrogen utilization (*Ntr*)	NH_4^+ (Shortage of organic nitrogen)	NR_{II} the product of *glnL*	NR_I, the product of *glnG*	Activates RNA polymerase at promotores requiring σ^{54}
Pho regulon	Inorganic phosphate	PhoR	PhoB	Activator
Porin regulation	Osmotic pressure	EnvZ	OmpR	Activator/Repressor

[a] Note that several of the response regulator proteins act as both activators and repressors depending on the genes being regulated. Although ArcA can function as either an activator or a repressor, it functions as a repressor on most operons that it regulates.

1.3 Role of Culture Condition on Metabolic Regulation

It is important to understand the cellular responses emanating from the environmental perturbations to redesign the networks for practical applications as well as for theoretical studies (Fuhrer and Sauer, 2009; Yuan et al., 2009; Hua et al., 2004). Microorganisms such as *Escherichia coli* live in environments which are subject to rapid changes in the availability of phosphate, carbon (C) and nitrogen (N) sources (Magasanik, 2000; Zimmer et al., 2000). The phosphate metabolism, carbon metabolism, nitrogen assimilation, and energy generation are integrated to maintain the cellular integrity. The limitation of such nutrients stimulates hunger state responses in bacteria, which turns the emphasis on scavenging substrates and induction for stimulating nutrient acquisition (Peterson et al., 2005; Ferenci, 2001; Harder and Dijkhuizen, 1983). Microbes adapt to the low nutrient conditions by maintaining high metabolic fluxes that may reduce the energetic efficiency of overall metabolism (Molenaar et al., 2009; De Mattos and Neijssel, 1997).

In the case of phosphate regulation, the phosphorus compounds serve as major building blocks of many biomolecules, and have important roles in signal transduction. The phosphate is involved in many biochemical reactions by the transfer of phosphoryl groups. All living cells sophisticatedly regulate the phosphate uptake, and survive even under phosphate-limiting condition, and thus phosphate metabolism is closely related to the diverse metabolism including energy and central carbon metabolism. In particular, phosphorylation may play important roles in the metabolic regulation at acidic condition and nitrogen limiting condition, which typically appears at the late growth phase in the batch culture. Moreover, phosphate starvation is a relatively inexpensive means of gene induction in practice, and the *phoA* promoter has been used for overexpression of heterologous genes. A better understanding of phosphate regulation would allow for optimization of such processes.

In the case of N-assimilation, energy independent glutamate dehydrogenase (GDH) pathway is used when sufficient amount of nitrogen is present, while energy dependent

glutamine synthetase-glutamate synthase (GS-GOGAT) pathway is used under N-limitation (Yuan et al., 2006; 2009; Reitzer, 2003; Kumar and Shimizu, 2011). The ATP required for the nitrogen assimilation using GS/GOGAT cycle under N-limiting condition accounts for 15% of the total requirement in *E.coli*. The bulk of energy is generated by the respiratory chain in *E. coli* under aerobic condition, and its efficiency depends upon the cumulative activity of various elements (Pramanik and Keasling, 1997; Neijssel et al., 1996; Varma et al., 1993). The aerobic respiratory chain consists of multiple elements such as NADH dehydrogenases, catalyzing the generation of proton motive force during NADH oxidation, and quinine pool containing terminal oxidases, transferring electrons to oxygen (Kumar and Shimizu, 2011). It is also crucial for the maintenance of redox balance (Bekker et al., 2009; Vemuri et al., 2006).

Oxygen is another major factor to affect the metabolic activities. A variety of pathways for carbon and electron flow in *E.coli* are differentially expressed depending on whether molecular oxygen is present in the cell environment. Different sets of enzymes are induced or repressed by two major oxygen-responsive regulatory proteins: Fnr and ArcA. Thirty one transcriptional units (including over 70 genes) have been recognized as members of the Fnr modulon. All of the genes in the Fnr modulon encode proteins that are involved in cellular adaptations to growth in an anoxic environment (Lynch and lin, 1996). Fnr functions as a transcriptional activator of *frdABCD*, *dmsABC*, and *narGHJI* expression under anerobic conditions (Gunsalus, 1992).

The tricarboxylic acid (TCA) cycle of *E.coli* is highly operative only in aerobically grown cells, with the key regulatory control responsible for determining the levels of TCA cycle enzymes exerted at the transcriptional level. The principal elements of this regulation are encoded by the ArcB-ArcA two-component signal transduction system (Lynch and Lin, 1996). The system comprises ArcB as the membrane bound sensor kinase and ArcA as the cognate response regulator. Under anaerobic or microaerobic conditions, ArcB undergoes

autophosphorylation and then catalyzes the trans-phosphorylation of ArcA. Under aerobic conditions, oxidized forms of quinine electron carriers in the membrane inhibit the autophosphorylation of ArcB and therefore its mediation of the Arc metabolic response (Geogellis et al., 2001). About 30 operons are known to be controlled by phosphorylated ArcA (ArcA-P), most of which are involved in respiratory metabolism (Liu and Wulf, 2004).

The role of pH in gene expression in *E.coli* and related enteric bacteria has been studied extensively, but it has been studied largely under aerobic conditions. Relatively few studies have addressed the relationship between pH and anaerobiosis, the predominant condition of bacterial growth; the best-studied cases include anaerobic acid induction of amino acid decarboxylase. In the present study, we investigated how gene expressions pattern changes in *E.coli* at low pH under aerobic and microaerobic conditions.

1.4 Research Target

The present investigation clarified the effect of phosphate limitation, nitrogen limitation, acidic condition and anerobic condition on the metabolism in view of gene transcript levels. Moreover, the present study implies that these metabolic regulations are highly interconnected and complex (Chapter 3-Fig 3.7, Chapter 4-Fig 4.4, and Chapter 5-Fig 5.5). These phenomena occur at the late growth phase in the batch culture. The present result is useful for the analysis of the metabolism changes during late growth phase and/or stationary phase.

1.5 Thesis outline

In the present work, some single gene knockout *E.coli* strains were used for the investigation of the overall metabolic regulation mechanisms. The metabolic regulations corresponding to environmental and genetic perturbations were studied by measuring gene expression analysis, enzyme activities and fermentation data. The detailed works are

presented in the following chapters:

Chapter 3: Metabolic regulation of *Escherichia coli* and its *phoB* and *phoR* genes knockout mutants under phosphate and nitrogen limitations as well as at acidic condition was studied in this chapter. By measuring gene expression and fermentation data analysis under different phosphate, pH and nitrogen concentration for different strains (*E.coli* wild type and its *phoB*, *phoR* mutant), the metabolic effects of different nutrient-limitation conditions on the metabolism were studied.

Chapter 4: This chapter studied on metabolic regulation of *fnr* gene knockout *Escherichia coli* under oxygen limitation. The regulation mechanism for these metabolic changes was studied based on enzyme activities, metabolic flux distributions, fermentation data and gene expression analysis on wild type *E.coli* and its *fnr* mutant.

Chapter 5: The effect of pH on the metabolic regulation of *Escherichia coli* was studied based on fermentation data and gene expression analysis under different pH condition. The metabolic regulation mechanism was studied on *E.coli* wild type and its *phoB, fnr, pflA* mutant under acidic and neutral condition.

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Chapter 2 Materials and Methods

2.1 Bacterial strains used and culture conditions

The strains used in the present study were *Escherichia coli* BW25113 (*lacI^q* $rrnB_{T14}$ $\Delta lacZ_{WJ16}$ *hsdR514* $\Delta araBAD_{AH33}$ $\Delta rhaBAD_{LD78}$), its *phoB* gene knockout mutant (JW0389), *phoR* mutant (JW0390), *fnr* mutant (JW1328), *pflA* mutant (JW0885). The mutants were constructed by one-step inactivation of chromosomal *phoB*, *phoR*, *fnr*, *pflA* genes, respectively (Baba et al. 2006). Batch and continuous cultivations were carried out in a 2- L fermentor (M-100, Tokyo, Rikakiki Co., Tokyo, Japan), where the temperature was maintained at 37°C or more at 42°C. The pH of the broth was maintained either at 7.0 ± 0.1 or less at 4.0 ± 0.1 with a pH controller by automatic addition of 2.0 M HCl or 2.0 M NaOH. The aerobic condition was ascertained by controlling the stirring speed at 350 rpm with the constant air flow rate of 1 L min^{-1}, which has been shown to be 30-40% of air saturation. The CO_2 and O_2 concentrations were monitored using an off-gas analyzer (BMJ-02 PI, ABLE Co., Japan). The inoculum was prepared by transferring cells from a glycerol stock (0.1 ml) to 50 ml L-shaped test tube containing 10 ml of LB medium. The culture was incubated overnight, and 1 ml of culture broth was then transferred to a 500 ml T- shaped flask containing 100 ml LB medium. The M9 minimal medium was used where it contained 10 g of glucose per liter, 48 mM Na_2HPO_4, 22 mM KH_2PO_4, 10 mM NaCl, and 30 mM $(NH_4)_2SO_4$. The following components were filter sterilized and then added (per liter of final medium): 1 ml of 1 M $MgSO_4$, 1 ml of 0.1 mM $CaCl_2$, 1 ml of 1 mg of vitamin B1 per liter, and 10 ml of trace element solution containing (per liter) 0.55 g of $CaCl_2.2H_2O$, 1.67 g of $FeCl_3$, 0.10 g of $MnCl_2.4H_2O$, 0.17 g of $ZnCl_2$, 0.043 g of $CuCl_2.2H_2O$, 0.06 g of $CoCl_2.6H_2O$ and 0.06 g $Na_2MoO_4.2H_2O$. Continuous cultivations were performed at the dilution rate of 0.2 h^{-1}, where the feed glucose concentration was 10 g/L, and several different phosphate concentrations were considered: P-rich or 100% P concentration (2.99 g/L of KH_2PO_4 and 6.81 g/L of

Na_2HPO_4) and P-limitation or 10% of P concentration (0.229 g/L of KH_2PO_4 and 0.681 g/L of Na_2HPO_4). Several other P concentrations in-between were also investigated. For the nitrogen limited condition, 6.0 mM of $(NH_4)_2SO_4$ was used instead of 30.0 mM. The continuous (chemostate) culture was controlled by adjusting the rotation speed of input and output pumps of the fermentor, where the rotation speed of output pump was adjusted to keep the broth volume constant, while the rotation speed of input pump was adjusted to set the dilution rate. In the present investigation, the dilution rate was set at 0.2 h^{-1}, where glucose limitation was not observed at such dilution rate. If the dilution rate was decreased less than about 0.1 h^{-1}, the glucose concentration becomes undetectable level, and both glucose and phosphate limitation may occur, and the changes of the transcript levels may be direct or indirect. To avoid such situation, we set the dilution rate to be at 0.2 h^{-1}. The microaerobic cultivation was initiated by aerobic cultivation for 2 h followed by the microaerobic condition without supplying air and slowing down the agitation speed to around 100 rpm, so that the cultivation was nearly anaerobic.The triplicate samples were taken after 4-5 residence times where the steady state was ascertained.

2.2 Measurements of biomass and extracellular metabolite concentrations

Cell concentration was measured by the optical density (OD) of the culture broth at 600 nm wave length with a spectrophotometer (Ubet-30, Jasco Co., Tokyo, Japan), and then converted to dry cell weight (DCW) per liter based on the relationship between OD_{600nm} and DCW previously obtained (Peng and Shimizu, 2006). Glucose concentration was measured using enzymatic kit (Wako Co., Osaka, Japan). Acetate, formate, lactate, succinate, and ethanol concentrations were also measured using enzymatic kits (Boehringer Co., Mannheim, Germany). Triplicate measurements were made for each sample to compute the standard deviation.

2.3 RNA preparation, design of PCR primers

Total RNA was isolated from *E. coli* cells by Qiagen RNeasy Mini Kit (QIAGEN K.K., Japan) according to the manufacturer's recommendation. The quantity and purity of the RNA were determined by the optical density measurements at 260 and 280 nm and by 1% formaldehyde agarose gel electrophoresis. Criteria for the design of the gene-specific primer pairs were followed according to Sambrook and Russel (2001). The primers used in this study were described elsewhere (Kabir and Shimizu, 2003; Kumar and Shimizu, 2010), except those as given in Table 2.1 and Table 2.2. The primers used in this study were synthesized at Hokkaido System Science Co. (Sapporo, Hokkaido Japan). In all cases, the primer-supplied company confirmed the absolute specificity of the primers.

2.4 cDNA synthesis and PCR amplification

RT-PCR reactions were carried out in a TaKaRa PCR Thermal Cycler (TaKaRa TP240, Japan) using Qiagen One Step RT-PCR Kit (QIAGEN K.K., Japan). The reaction mixture was incubated for 30 min at 50°C for reverse transcription (cDNA synthesis) followed by 15 min incubation at 95°C for initial PCR activation. Then the process was subjected to 30 cycles of amplification which consisted of a denaturing step (94°C for 1 min), annealing step (approximately 5°C below melting temperature, of primers for 1 min), and an extension step (72°C for 1 min), and finally the reaction mixture of 25 μl was subjected for 10 min at 72°C for final extension. To check for nucleic acid contamination, one negative control was run in every round of RT-PCR. This control lacks the template RNA in order to detect possible contamination of the reaction components. 5 μl of amplified products were run on 1.8 % agarose gel. Gels were stained with 1 mg ml^{-1} of ethidium bromide, photographed using a Digital Image Stocker (DS-30, FAS III, Toyobo, Osaka, Japan) under UV light and analyzed using Gel-Pro Analyzer 3.1 (Toyobo, Osaka, Japan) software. In order to determine the optimal amount of input RNA, the two-fold diluted template RNA was

amplified in RT-PCR assay under identical reaction condition to construct a standard curve for each gene product. When the optimal amount of input RNA was determined for each gene product, RT-PCR was carried out under identical reaction condition to detect differential transcript levels of genes. The gene *dnaA*, which encodes *E. coli* DNA polymerase and is not subjected to variable expression, i.e. abundant expression at relatively constant rate in most cells, was used as an internal control in the RT-PCR determinations. The gene expressions are given as relative values to that of *dnaA*. The selection of genes was made based on global regulator-metabolic pathway gene relationships (Appendix A)

To calculate the standard deviation, RT-PCR was independently performed three times under identical reaction condition. To ensure that the observed expression changes were statistically significant, the Student's t-test was applied.

Table 2.1: A List of additional primers

phoB:	Left primer TTAAACCACGTCTGGGGAAC Right primer TAAAAGCGGGTTGAAAAACG
phoA:	Left primer ACGAAAAAGATCACCCAACG Right primer GATCCTTTTCCGCCTTTTTC
phoE:	Left primer TGGGGCCTATACCAACTCAG Right primer GCCAGTTATTGGCGTCATTT
phoH:	Left primer AAGGAAGCCAACCCTCTGAT Right primer GGCCATACCAATGGCTTCTA
phnC:	Left primer CGTACTGGAGAACGTGCTGA Right primer GTTCATCGGCCAGAATCACT
pstS:	Left primer AGCTACCTGGCGAAAGTGAA Right primer GGTGTACGCCAGGTTGTTCT
ugpB:	Left primer CAAAGCAGCGTATGACCTGA Right primer GGTGTCTTCTTACCGGTCCA
phoM (*creC*):	Left primer CTGGATAACGCCATCGATTT Right primer ATTTGCACGAGGCAAAGAGT
phoR:	Left primer TGGAATTTATTGCGCCTTTC Right primer ACCAGAAAATACCGCCCTCT
phoU:	Left primer CGTCAACATGATGGAAGTGG Right primer CCAGCGACTCCAGACTTACC
asr:	Left primer CGCTGCTATGGGTCTGTCTT Right primer TTTTGTTCAGGGGCTTTCTG

Table 2.2: A List of additional primers (cont.)

mlc:	left primer AGCAGACCAACGCGGGCGCG right primer GACTATACGCAGGAAGGGCC
gadA:	left primer CGGATAAACCAAACCTGGTG right primer GAATTTATCCAGCGCATCGT
yfiD:	left primer AACTCTTTCTGGCTGCTGG 3 right primer GATGGTCAGCTGCGGATAT
ompR:	left primer GGTGAAGATGGCTTGTCGAT right primer AGCAATTACCGCCTCTTCCT
envZ:	left primer ATATGGCGGCTGGTGTTAAG right primer TAAGATCCGCCATTTCCATC
ompC:	left primer CTACATGCGTCTTGGCTTCA right primer CGACCGTAGTCGAAAGAACC
ompF:	left primer CAATGGCGACATGACCTATG right primer TATTTAAGACCCGCGAATGC
gadX:	left primer ATTGCGCGAAGAAGAGACAT right primer ATACTCTGTGGGCGTCATCC
gadC:	left primer CTAAAGTGGGCACCCTGGTA right primer TCGCAATAGACAAACCACCA

Chapter 3 Metabolic regulation of *Escherichia coli* and its *phoB* and *phoR* genes knockout mutants under phosphate and nitrogen limitations as well as at acidic condition

3.1 Introduction

The phosphorus compounds serve as major building blocks of many biomolecules, and have important roles in signal transduction (Wanner, 1996). The phosphate is contained in lipids, nucleic acids, proteins, and sugars, and is involved in many biochemical reactions by the transfer of phosphoryl groups (Lamarche et al., 2008). Moreover, phosphate metabolism is closely related to the diverse metabolisms such as energy and central carbon metabolisms (Ishige et al., 2003). All living cells sophisticatedly regulate the phosphate uptake, and survive even under phosphate-limiting condition (Baek and Lee, 2006; Wendisch, 2006). *Escherichia coli* contains about 15 mg of phosphate (P) per g (dry cell weight) (Damoglou and Dawes, 1968). Depending on the concentration of environmental phosphate, *E. coli* controls phosphate metabolism through Pho regulon, which forms a global regulatory circuit involved in a bacterial phosphate management (Wanner, 1993, 1996). The PhoR-PhoB two-component system plays an important role in detecting and responding to the changes of the environmental phosphate concentration (Stock et al., 1989; Parkinson, 1993; Baek and Lee, 2007). It has been known that PhoR is an inner-membrane histidine kinase sensor protein that appears to respond to variations in periplasmic orthophosphate (P_i) concentration through interaction with a phosphate transport system, and that PhoB is a response regulator that acts as a DNA-binding protein to activate or inhibit specific gene transcription (Wanner, 1996; Smith and Payne, 1992; Harris et al., 2001; Blanco et al., 2002). The activation signal, a phosphate concentration below 4 μM, is transmitted by a phospho-relay from PhoR to PhoB. Phospho-PhoB in turn controls Pho regulon gene expressions. PhoB is phosphorylated by PhoR under phosphate starvation or by PhoM (or CreC) in the absence of functional PhoR (Torriani and Ludke, 1985; Makino et al., 1985; 1988; 1989; Shinagawa et al., 1987; Wanner,

1987; Amemura et al., 1990).

The *E. coli* Pho regulon includes 31 (or more) genes arranged in eight separate operons such as *eda*, *phnCDEFGHIJKLMNOP*, *phoA*, *phoBR*, *phoE*, *phoH*, *psiE*, *pstSCAB-phoU*, and *ugpBAECQ* (Hsieh and Wanner, 2010). When P_i is in excess, PhoR, Pst, and PhoU together turn off the Pho regulon, presumably by dephosphorylating PhoB. In addition, two P_i-independent controls that may be form of cross regulation turn on the Pho regulon in the absence of PhoR. The sensor CreC, formerly called PhoM, phosphorylates PhoB in response to some (unknown) catabolite, while acetyl phosphate may directly phosphorylate PhoB (Wanner, 1993). When P_i is in excess, P_i is taken up by the low affinity P_i transporter, Pit. Four proteins such as PstS, PstC, PstA and PstB form an ABC transporter important for the high-affinity capture of periplasmic inorganic phosphate (P_i) and its low-velocity transport into the cytosol (Van Dien and Keasling, 1998). These proteins are encoded together with PhoU as the *pstSCAB–phoU* operon. PstS is a periplasmic protein that binds P_i with high affinity. PstC and PstA are innermembrane channel proteins for P_i entry, while PstB is an ATP-dependent permease that provides the energy necessary for P_i transport from periplasm to cytosol. When phosphate is in excess, the Pst system forms a repression complex with PhoR, and prevents activation of PhoB. PhoU and PstB are also required for dephosphorylation of phospho-PhoB under P-rich condition (Wanner, 1997). Indeed, PhoU is essential for the repression of the Pho regulon under high phosphate condition (Wanner, 1996). It may be considered that PhoU acts by binding to PhoR, PhoB or PhoR/PhoB complex to promote dephosphorylation of phosphorylated PhoB or by inhibiting formation of the PhoR–PhoB complex (Oganesyan et al., 2005).

It has been shown that *phoB* mutant does not synthesize alkaline phosphatase (*phoA* gene product) (Nesmeianova et al., 1975; Pratt and Torriani, 1977; Zuckier et al., 1980; Guan et al., 1983; Yamada et al., 1989; Kimura et al., 1989) and phosphate binding protein (*pstS* gene product) (Pratt and Torriani, 1977; Yamada et al., 1989; Kimura et al., 1989). It was

observed that *phoU* expression changed depending on phosphate concentration of the *phoB* mutant (Nakata et al., 1984). Since the *phoA* gene mutation leads to the decreased content of membrane proteins or completely lacks them, mutations in the *phoB* gene result in the loss of alkaline phosphate and two membrane proteins (Tsfasman and Nesmeianova, 1981). Nesmeianova et al. (1975) found that *phoB* mutation leads to loss of polyphosphate kinase activity which catalyzes the synthesis of polyP in *E.coli*. Ault-Riche´et al. (1998) also found that the strains with deletion of *phoB* failed to accumulate polyP in response to osmotic stress or nitrogen limitation. Mutations in the *phoB* gene had no effect on *pepN* (Gharbi et al., 1985) and *lky* (*tolB*) expressions (Lazzaroni and Portailer, 1985).

The expressions of the genes under the control of the PhoR-PhoB two-component system were found to be affected by the duration of P-limitation in response to phosphate starvation in *E. coli*. This means that the roles of the PhoR-PhoB two-component regulatory system seem to be more complex (Baek and Lee, 2007). Although molecular level regulation by PhoR-PhoB under P-limitation has been investigated as stated above, little has been investigated about the effect of P- limitation on the overall metabolism and fermentation characteristics of *E.coli* so far. In the present study, therefore, we investigated the effect of phosphate limitation on the cell metabolism in *E.coli* in view of fermentation characteristics and gene transcript levels, since it is quite important for the development of microbial cell factories to understand the fermentation mechanism at the late growth phase in the batch culture, where nutrient starvation occurs. Moreover, the effect of *phoB* gene (and also *phoR* gene) knockout on the metabolism was also investigated under both P-rich and P-limited conditions to clarify the role of phosphate regulation. Since it has been implied that phosphate regulation is interconnected with acid tolerance and nitrogen regulation, we also investigated the effect of pH downshift and nitrogen limitation together with P-limitation on the metabolic regulation in *E.coli*, where those phenomena also occur at the late growth phase of the batch culture. Since phosphate starvation is a relatively inexpensive means of gene

induction in practice, the *phoA* promoter has been used for overexpression of heterologous genes (Shin and Seo, 1990). A better understanding of the Pho regulon would allow for optimization of such processes (Van Dien and Keasling, 1998).

3.2 Results

3.2.1 Effect of phosphate limitation on the metabolism

In order to make clear the effect of phosphate limitation on the metabolism, aerobic continuous cultivation was conducted at the dilution rate of 0.2 h^{-1} under different P concentrations. Fig. 3.1a shows the effect of P concentration on the fermentation characteristics of the wild type strain, where it indicates that the fermentation characteristics significantly changed when feed P concentration became low around 10% of the M9 medium. In particular, the specific glucose consumption rate and the specific acetate production rate became significantly higher, while cell concentration became significantly lower under such P-limiting condition. Table 3.1 also shows the detailed values.

Figure 3.2 shows the effect of P concentration on the transcript levels, where Fig. 3.2b indicates that *phoB* transcript level increased as P concentration decreases, and *phoB* regulated genes such as *phoA*, *phoE*, *phoH*, *phnC*, *pstS*, and *ugpB* were all increased in a similar fashion, and *eda* transcript level also changed in a similar fashion (Fig. 3.2c). Note that *phoU* and *phoM* changed in a similar fashion as *phoR*, and also that the transcript level of *rpoD*, which encodes the RNA polymerase holoenzyme containing σ^{70}, increased in a similar fashion as PhoB regulatory genes (Makino et al., 1993). Figure 3.2a also indicates that the transcript level of *arcA* increased as P concentration decreases, and those of *sdhC* and *mdh* genes decreased (Fig. 3.2c and Appendix A). Figure 3.2 also shows that *cra* transcript level decreased, and thus the transcript levels of *ptsH* and *pykF* increased (Appendix A). Those are consistent with the increased specific glucose consumption rate (Table 3.1 and Figure 3.1a). The decrease in *cra* transcript level may be due to higher glucose concentration. The

transcript level of *fnr* is somewhat different from *arcA* but that of *yfiD* changed in a similar fashion as *fnr* (Appendix A). Moreover, Fig. 3.2a also indicates that *soxR/S* transcript levels increased as P concentration decreases, and accordingly the transcript levels of *rpoD*, *zwf* and *sodA* changed in a similar fashion (Appendix A). The respiratory chain genes such as *atpA*, *ndh*, and *nuoA* also changed in a similar fashion, implying that the respiration is activated under P-limitation. Figure 3.2d shows that *rpoN* which encode σ^{54} increases as P concentration decreased.

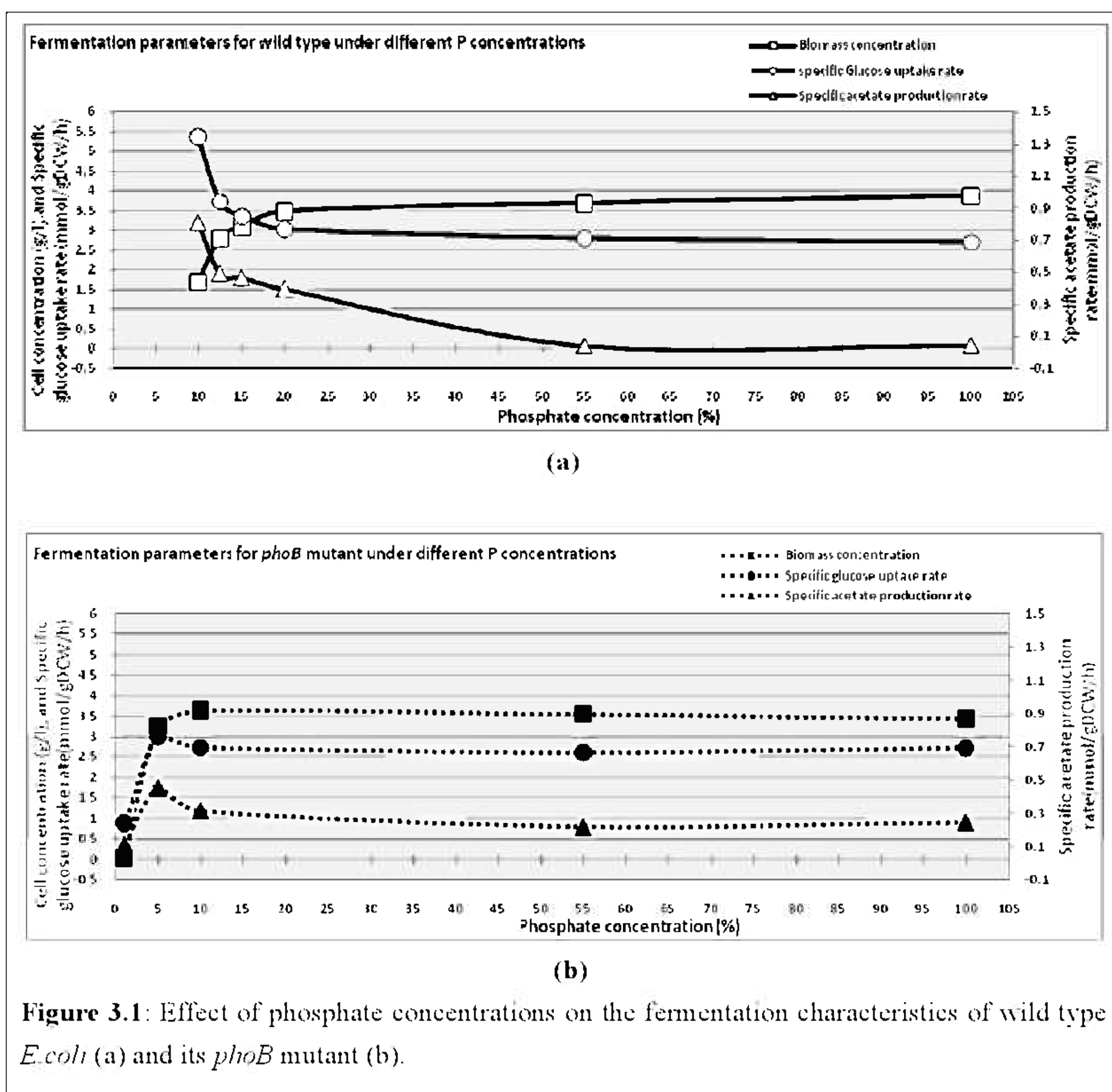

Figure 3.1: Effect of phosphate concentrations on the fermentation characteristics of wild type *E.coli* (a) and its *phoB* mutant (b).

Table 3.1: Fermentation characteristics of the wild type *E. coli* and its *phoB* and *phoR* mutants in the aerobic chemostat culture under different phosphate concentrations at the dilution rate of 0.2 h^{-1} at pH 7.0.

Fermentation parameters		P-rich (100%) condition	P-lower (55%) condition	P-limited (20%) condition	P-limited (15%) condition	P-limited (12.5%) condition	P-limited (10%) condition	P-limited (5%) condition	P-limited (1%) condition
Biomass concentration (g/l)	Wild	3.86 ± 0.03	3.68 ± 0.05	3.47 ± 0.05	3.08 ± 0.02	2.78 ± 0.03	1.69 ± 0.03	–	–
	Δ*phoB*	3.44 ± 0.04	3.560 ± 0.011	–	–	–	3.64 ± 0.01	3.24 ± 0.02	0.050 ± 0.001
	Δ*phoR*	–	–	–	–	–	3.710 ± 0.112	–	–
Glucose concentration (g/l)	Wild	0.660 ± 0.004	0.760 ± 0.004	0.557 ± 0.001	0.700 ± 0.003	0.700 ± 0.003	1.85 ± 0.01	–	–
	Δ*phoB*	1.59 ± 0.29	1.66 ± 0.23	–	–	–	1.050 ± 0.001	1.330 ± 0.001	9.960 ± 0.001
	Δ*phoR*	–	–	–	–	–	0.910 ± 0.004	–	–
Acetate concentration (g/l)	Wild	0.046 ± 0.002	0.042 ± 0.001	0.410 ± 0.001	0.43 ± 0.07	0.41 ± 0.03	0.41 ± 0.02	–	–
	Δ*phoB*	0.255 ± 0.130	0.233 ± 0.030	–	–	–	0.346 ± 0.010	0.440 ± 0.010	0.002 ± 0.002
	Δ*phoR*	–	–	–	–	–	0.0035 ± 0.0010	–	–
Specific glucose uptake rate (mmol/gDCW/h)	Wild	2.69 ± 0.05	2.79 ± 0.02	3.024 ± 0.002	3.350 ± 0.001	3.717 ± 0.002	5.36 ± 0.01	–	–
	Δ*phoB*	2.72 ± 0.09	2.60 ± 0.07	–	–	–	2.730 ± 0.003	2.970 ± 0.001	0.890 ± 0.001
	Δ*phoR*	–	–	–	–	–	2.720 ± 0.004	–	–
Specific acetate production rate (mmol/gDCW/h)	Wild	0.040 ± 0.002	0.0380 ± 0.0001	0.394 ± 0.010	0.465 ± 0.003	0.491 ± 0.030	0.81 ± 0.02	–	–
	Δ*phoB*	0.247 ± 0.080	0.218 ± 0.033	–	–	–	0.317 ± 0.001	0.452 ± 0.010	0.133 ± 0.002
	Δ*phoR*	–	–	–	–	–	0.0031 ± 0.0010	–	–

Note: " – " indicates that no data was collected for this condition. The standard deviation was obtained by triplicate measurements.

Table 3.1 also shows the effect of *phoB* gene knockout on the fermentation characteristics under both P-rich and lower P conditions, where it indicates that the glucose concentration increased and cell concentration decreased for the *phoB* mutant as compared to

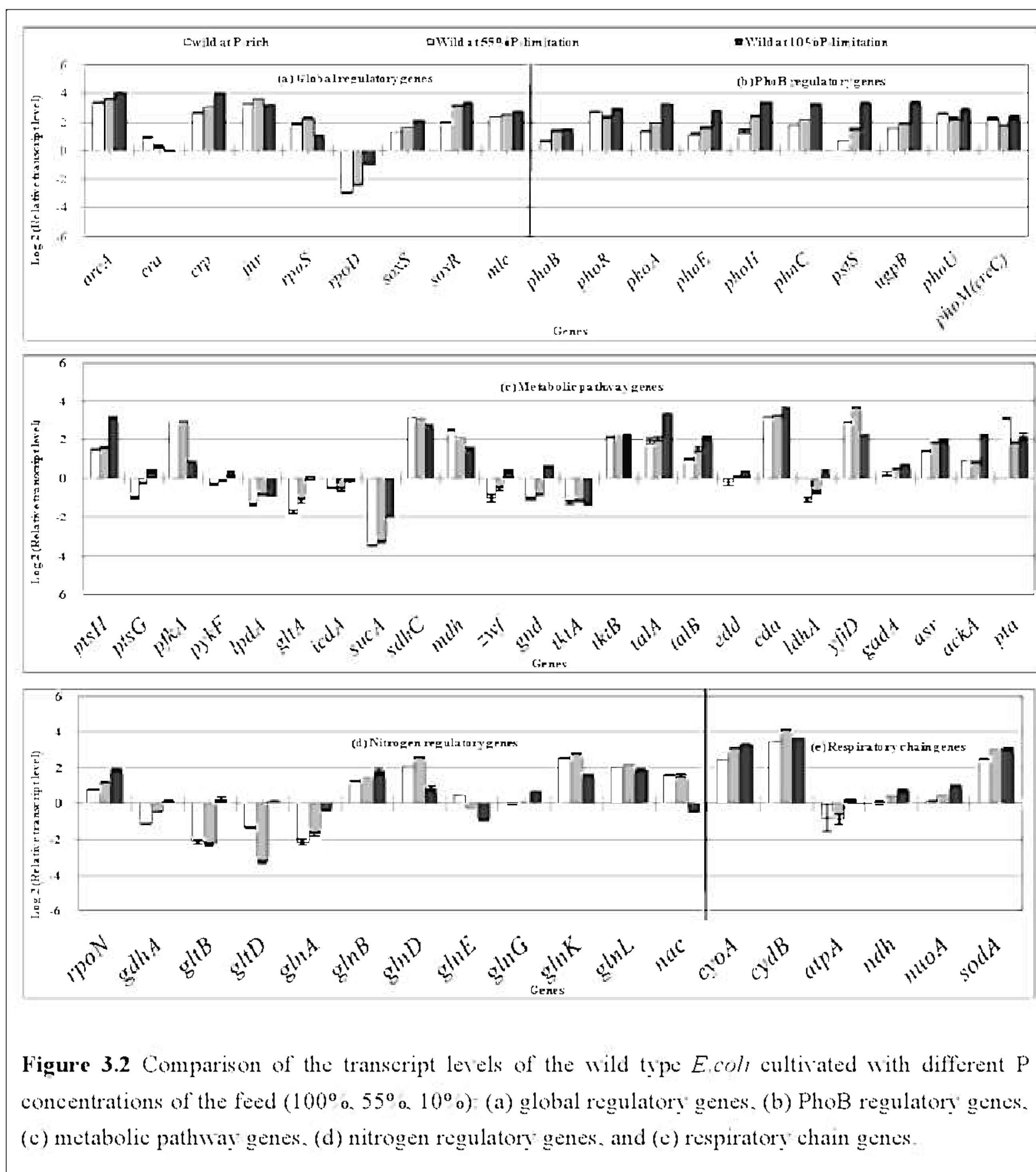

Figure 3.2 Comparison of the transcript levels of the wild type *E.coli* cultivated with different P concentrations of the feed (100%, 55%, 10%): (a) global regulatory genes, (b) PhoB regulatory genes, (c) metabolic pathway genes, (d) nitrogen regulatory genes, and (e) respiratory chain genes.

the wild type, and that the specific acetate production rate was higher at P-rich condition and 55% of P concentration for the *phoB* mutant as compared to the wild type. It is surprising that the fermentation characteristics were less affected even under P-limitation (10% and 5%) for the *phoB* mutant, whereas the wild type shows significant changes at 10% of P concentration. In the case of *phoB* mutant, cell could survive even at 1% of P concentration (Table 3.1 and Fig. 3.1b).

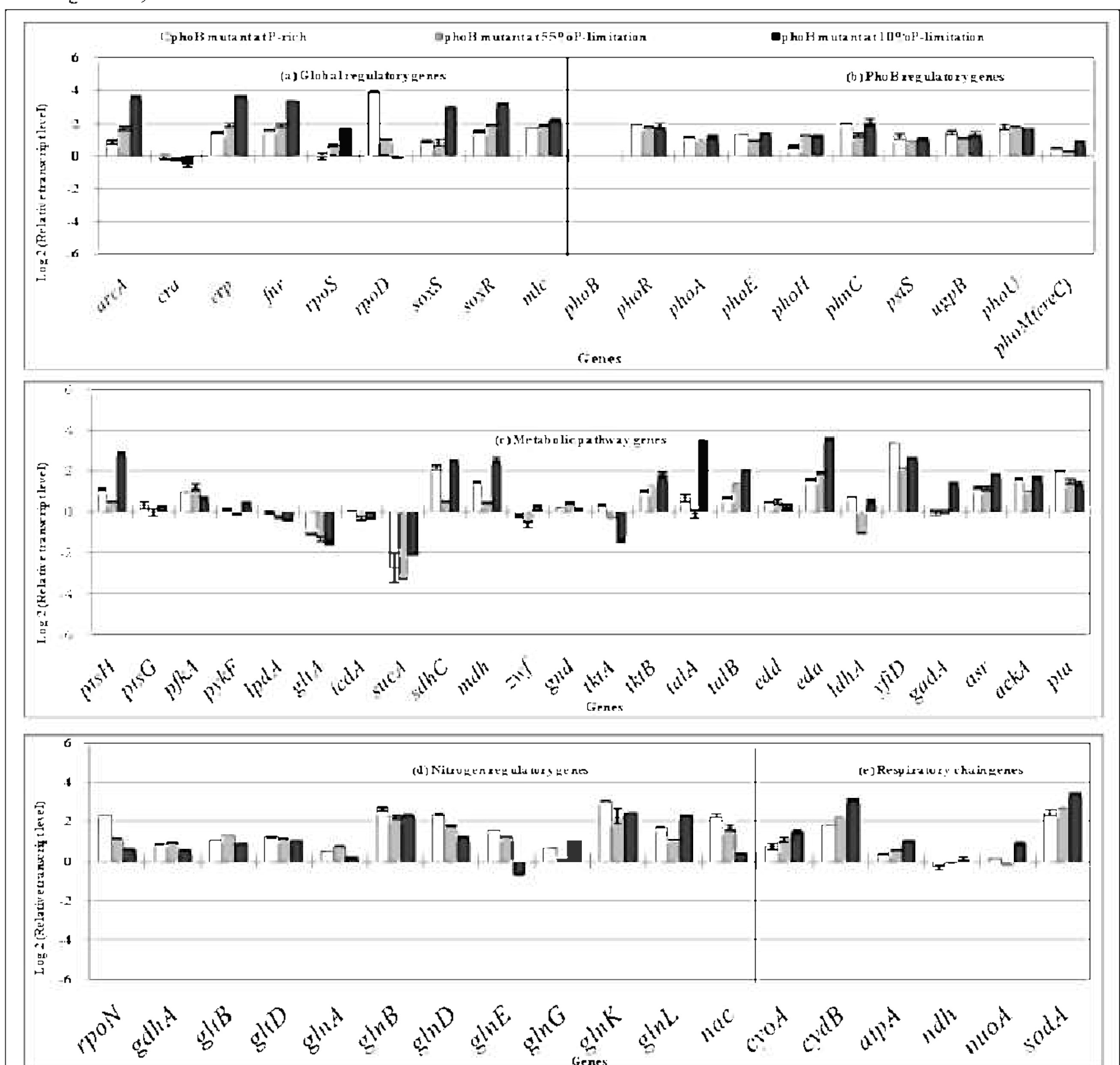

Figure 3.3 Comparison of the transcript levels of the *phoB* mutant *E.coli* cultivated with different P concentrations of the feed (100%, 55%, 10%): (a) global regulatory genes, (b) PhoB regulatory genes, (c) metabolic pathway genes, (d) nitrogen regulatory, and (e) respiratory chain genes.

Figure 3.3 indicates that the transcript levels of PhoB regulated genes such as *phoA*, *phoE*, *phoH*, *pstS*, *ugpB* and *phoM* were down-regulated, whereas *phoR* and *phoU* changed little, as compared to those of wild type. In a similar fashion as the wild type, the transcript level of *arcA* increased while *cra* decreased as P concentration decreased for the *phoB* mutant, which implies that those phenomena are *phoB* independent. The transcript levels of *soxR* and *rpoS* increased and *sodA* as well as respiratory chain genes such as *cyoA*, *ndh* and *nuoA* increased in a similar fashion as P concentration decreased for the *phoB* mutant, which implies that the activation of the respiratory chain is *phoB*-independent, but P-concentration dependent.

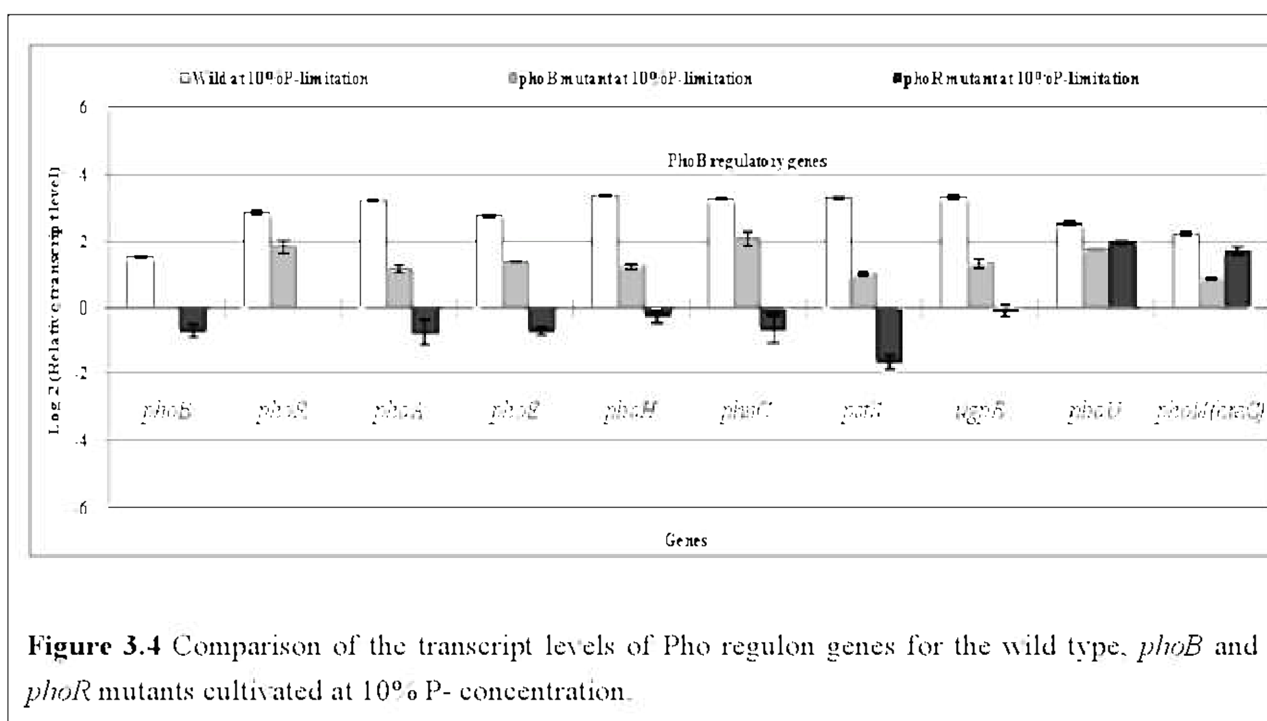

Figure 3.4 Comparison of the transcript levels of Pho regulon genes for the wild type, *phoB* and *phoR* mutants cultivated at 10% P- concentration.

In order to confirm the result for *phoB* mutant, the effect of *phoR* gene knockout on fermentation characteristics and some selected gene transcript levels were also investigated for the case of 10 % of P concentration as given in Table 3.1 and Fig. 3.4. Table 3.1 indicates that the cell concentration and the glucose concentration for *phoR* mutant are similar to those of *phoB* mutant, whereas acetate concentration became quite low at P-limiting condition for the *phoR* mutant. Figure 3.4 indicates that the *phoB* regulated genes such as *phoA*, *phoE*,

phoH, *phnC*, *pstS*, *ugpB* were more down-regulated for the *phoR* mutant as compared to *phoB* mutant, whereas *phoU* and *phoM* (*creC*) were less affected by *phoR* gene knockout.

3.2.2 Effect of culture pH and phosphate limitation on the metabolism

Table 3.2 shows the effect of pH and phosphate limitation on the fermentation characteristics in the continuous culture of *E.coli* at the dilution rate of 0.2 h^{-1}, where it indicates that more acetate was formed with higher glucose uptake rate, while the cell concentration became lower at pH 6.0 as compared to the case of pH 7.0. Note that the fermentation characteristics were different even between 100% and 55% of phosphate concentration under lower pH value. Figure 3.5 shows the effect of culture pH on the transcript levels, where it indicates that *arcA* gene was up-regulated ($P < 0.01$), and the TCA cycle genes such as *sdhC* and *mdh* were down-regulated accordingly ($P < 0.01$ and $P < 0.01$, respectively). Note that *icdA* gene was up-regulated ($P < 0.01$), which coincided with the up-regulation of *cra* gene ($P < 0.01$) (Appendix A). Figure 3.5 also shows that the transcript level of *rpoS* was up-regulated, and the expression of *gadA* (glutamate decarboxylase) gene was up-regulated at pH 6.0 (Cheville et al., 1996). The *yfiD* gene, which encodes acid-inducible protein (Wyborn et al., 2002), was also up-regulated at lower pH. Figure 3.5 also shows that *phoB* gene was up-regulated and the PhoB regulated genes such as *phoA*, *phoE*, *phoH*, *phnC*, *pstS*, and *ugpB* as well as *phoR*, *phoU*, *phoM* and *eda* were up-regulated ($P < 0.01$ for all genes). This means that acid stress and phosphate regulation are directly or indirectly interconnected (Baek and Lee, 2006).

Figure 3.5 also shows the effects of lower pH and lower P-concentration on the transcript levels (3rd bars), where *phoB* gene was further up-regulated, and the Pho regulon genes such as *phoR*, *phoH*, *phnC*, *pstS*, *ugpB*, and *phoU* were all further up-regulated at lower P concentration at pH 6.0. The *rpoS* gene further increased ($P < 0.01$), and *gadA* gene was also further up-regulated ($P < 0.01$). Figure 3.5 shows that *arcA* transcript level tended to be

up-regulated though not significant, and this may have caused down-regulations of *sdhC* and *mdh* ($P < 0.01$ and $P < 0.01$, respectively) under P-limitation as compared to P-rich condition at acidic condition. Table 3.2 shows the effects of lowering pH and P concentration on the fermentation characteristics of *phoB* gene knockout mutant as well, where it indicates that the

Table 3.2: Fermentation characteristics of the wild type *E. coli* and its *phoB* mutant in the aerobic chemostat culture under two phosphate concentrations (100% and 55%) and two pH values (7.0 and 6.0) at the dilution rate of 0.2 h^{-1}.

Fermentation parameters		P-rich condition (100%)		Lower P concentration (55%)
		pH 7.0	pH 6.0	pH 6.0
Biomass concentration (g/l)	Wild	**3.86 ± 0.03**	**3.680 ± 0.002**	**1.960 ± 0.001**
	Δ*phoB*	**3.44 ± 0.04**	_	**3.040 ± 0.001**
Glucose concentration (g/l)	Wild	**0.660 ± 0.004**	**1.053 ± 0.010**	**0.677 ± 0 .050**
	Δ*phoB*	**1.59 ± 0.29**	_	**1.145 ± 0.020**
Acetate concentration (g/l)	Wild	**0.046 ± 0.002**	**0.468 ± 0.003**	**0.519 ± 0.001**
	Δ*phoB*	**0.255 ± 0.130**	_	**0.510 ± 0.007**
Specific glucose uptake rate (mmol/gDCW/h)	Wild	**2.69 ± 0.05**	**2.700 ± 0.001**	**5.285 ± 0.031**
	Δ*phoB*	**2.72 ± 0.09**	_	**3.24 ± 0.01**
Specific acetate production rate (mmol/gDCW/h)	Wild	**0.040 ± 0.002**	**0.424 ± 0.003**	**0.882 ± 0.001**
	Δ*phoB*	**0.247 ± 0.080**	_	**0.559 ± 0.007**

Note: " – " indicates that no data was collected for this condition. The standard deviation was obtained by triplicate measurements.

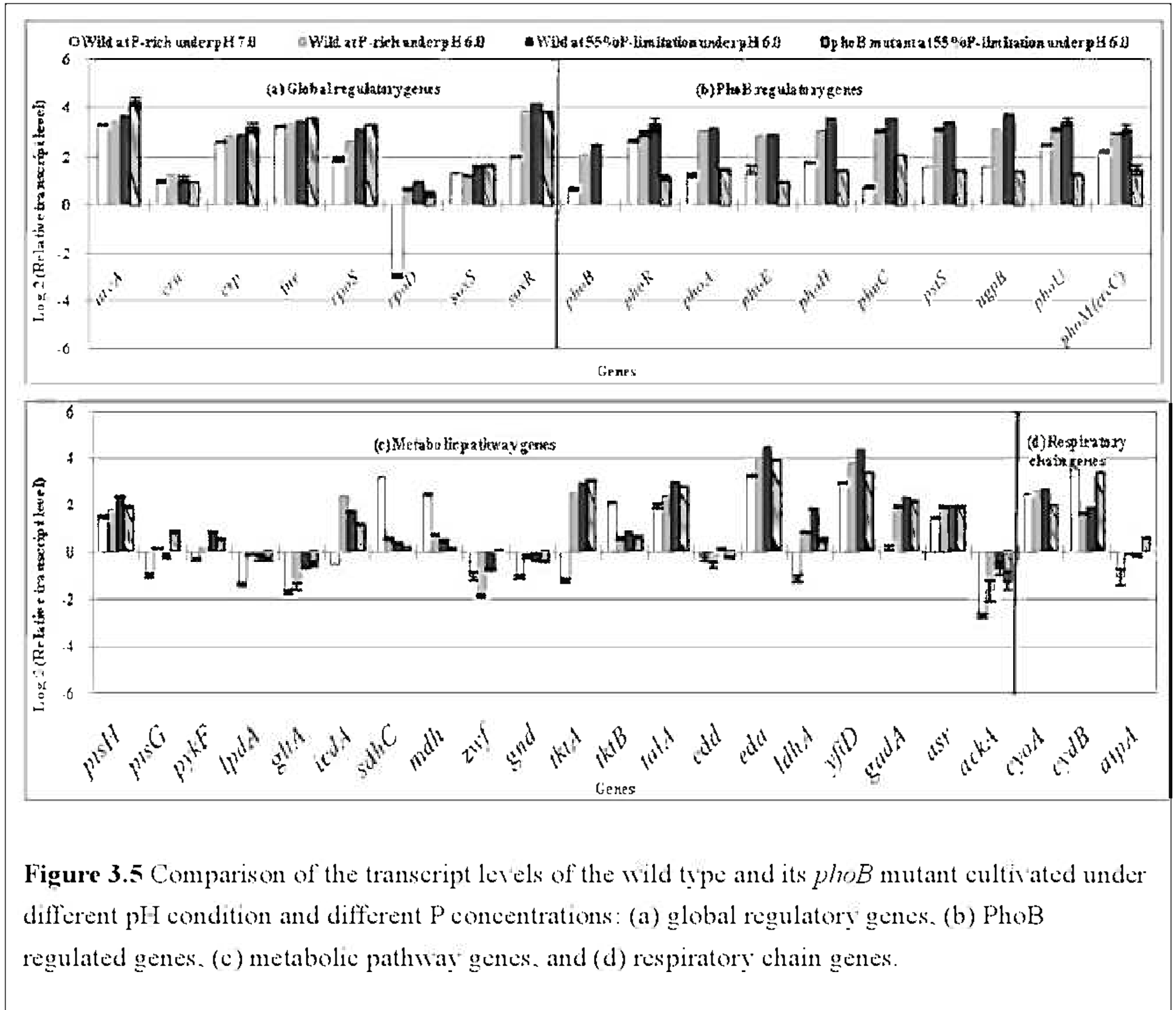

Figure 3.5 Comparison of the transcript levels of the wild type and its *phoB* mutant cultivated under different pH condition and different P concentrations: (a) global regulatory genes, (b) PhoB regulated genes, (c) metabolic pathway genes, and (d) respiratory chain genes.

glucose concentration increased for the *phoB* mutant as compared to the wild type under both pH 7.0 and 6.0. Figure 3.5 shows the comparison of the transcript levels between the wild type (3rd bars) and its *phoB* mutant (4th bars) at pH 6.0, where it indicates that the *phoB* regulated genes such as *phoA*, *phoE*, *phoH*, *phnC*, *pstS*, *ugpB* ($P < 0.01$ for all genes) as well as *phoR*, *phoU*, *phoM* and *eda* were all significantly down-regulated ($P < 0.01$ for all genes) for the *phoB* mutant. The transcript levels of TCA cycle genes such as *sdhC* and *mdh* were down-regulated for the *phoB* mutant as compared to the wild type.

3.2.3 Effect of nitrogen limitation

Table 3.3 shows the effect of nitrogen (N) limitation and lower P concentration on the fermentation characteristics, where the specific glucose consumption rate and the specific acetate production rate increased, while cell concentration decreased for the case of N-limitation as compared to N-rich condition. Those changes were further enhanced at lower P concentration. Figure 3.6 shows the effect of N-limitation on the transcript levels of several genes, where it indicates that *rpoN* transcript level increased and *glnA, L, G, gltB, glnD, glnK* and *nac* genes were up-regulated ($P < 0.01$ for all genes), while *glnE* gene was down-regulated ($P < 0.01$). Figure 3.6 also shows that the transcript level of *phoB* gene was up-regulated and PhoB regulated genes such as *phoA, phoE, phoH, phnC, pstS* were increased ($P < 0.01$ for all genes) as well as *phoR, phoU,* and *phoM* ($P < 0.01$ for all genes) under N-limitation as compared to N-rich condition. The TCA cycle genes such as *sdhC* and *mdh* decreased ($P < 0.01$ for both genes), which may have caused TCA cycle to be repressed, which corresponds to the increase in the specific acetate production rate. The *ptsG, ptsH* and *pfkA* gene expressions increased ($P < 0.01$ for all genes), which corresponds to the increase in the specific glucose consumption rate. The transcript levels of *fnr* and *yfiD* were up-regulated ($P < 0.01$for both genes) in a similar fashion. Moreover *soxR* increased and the respiratory chain genes such as *cyoA, cydB, ndh* and *nuoA* as well as *sodA* were all up-regulated under N-limitation ($P < 0.01$ for all genes).

Table 3.3 also shows the effect of lower P concentration on the fermentation characteristics of *phoB* mutant under N-limitation, where it indicates that the cell concentration decreased, while acetate and glucose concentrations increased under N-limitation as compared to N-rich condition for the *phoB* mutant. Figure 3.6 indicates (by comparison of the 2nd and 3rd bars) that phoB regulated genes such as *phoA, phoE, phnC, pstS, phoR, phoU,* ($P < 0.01$ for all genes) as well as *phoH* and *phoM* were down-regulated ($P < 0.05$ for the two genes) as compared to the wild type. Although *rpoN* transcript level changed

little, such genes as *glnD, glnG, glnL* were up-regulated ($P < 0.01$ for all genes), whereas *gltD* ($P < 0.5$), and *glnA, glnE, glnK, nac* genes were down-regulated ($P < 0.01$ for all genes).

Table 3.3: Fermentation characteristics of the wild type *E. coli* and its *phoB* mutant in the aerobic chemostat culture under different nitrogen and phosphate concentrations at the dilution rate of 0.2 h^{-1} at pH 7.0.

Fermentation parameters		N and P-rich condition (100%)	N–limited (20%) and P-rich condition	N–limited (20%) and lower P concentration (55%)
Biomass concentration (g/l)	Wild	**3.86 ± 0.03**	**1.753 ± 0.005**	**1.68 ± 0.01**
	Δ*phoB*	**3.44 ± 0.04**	**1.703 ± 0.005**	**1.59 ± 0.01**
Glucose concentration (g/l)	Wild	**0.660 ± 0.004**	**5.39 ± 0.01**	**4.10 ± 0.01**
	Δ*phoB*	**1.59 ± 0.29**	**2.890 ± 0.006**	**4.81 ± 0.01**
Acetate concentration (g/l)	Wild	**0.046 ± 0.002**	**0.486 ± 0.002**	**0.502 ± 0.004**
	Δ*phoB*	**0.255 ± 0.130**	**0.475 ± 0.004**	**0.495 ± 0.010**
Specific glucose uptake rate (mmol/gDCW/h)	Wild	**2.69 ± 0.05**	**2.92 ± 0.01**	**3.90 ± 0.01**
	Δ*phoB*	**2.72 ± 0.09**	**4.64 ± 0.01**	**3.63 ± 0.02**
Specific acetate production rate (mmol/gDCW/h)	Wild	**0.040 ± 0.002**	**0.923 ± 0.010**	**0.995 ± 0.010**
	Δ*phoB*	**0.247 ± 0.080**	**0.928 ± 0.010**	**1.037 ± 0.020**

Note: The standard deviation was obtained by triplicate measurements.

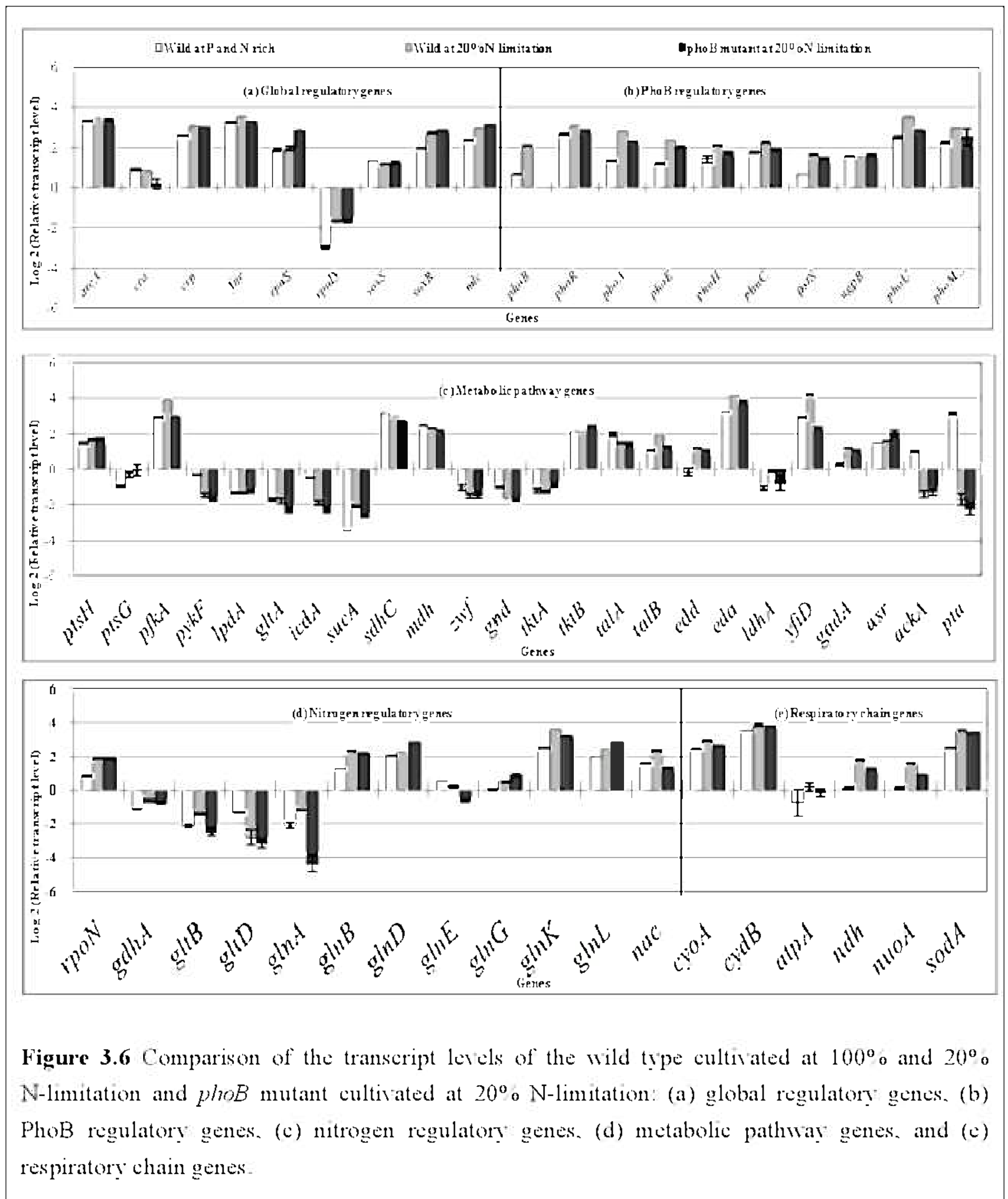

Figure 3.6 Comparison of the transcript levels of the wild type cultivated at 100% and 20% N-limitation and *phoB* mutant cultivated at 20% N-limitation: (a) global regulatory genes, (b) PhoB regulatory genes, (c) nitrogen regulatory genes, (d) metabolic pathway genes, and (e) respiratory chain genes.

3.3 Discussion

It was shown that the glycolysis was activated under phosphate limiting condition by the data of the specific growth rate (Table 3.1) and by the corresponding gene transcript levels (Fig. 3.2). This may be due to ATP demand caused by the decrease in ATP formation with limited amount of available phosphate as implied by Kobeman et al. (2002) who investigated the effect of [ATP] / [ADP] ratio on the glycolytic flux. The lower cell concentration under P-limitation may also be due to lower ATP formation as we investigated previously on the relationship between the cell growth rate and the specific ATP production rate (Toya et al., 2010; Abdul Kadir et al., 2010).

Moreover, phosphate limitation causes less ATP production by H^+-ATPase, which causes less quinol oxidation by Cyo, which in turn affected quinone pool size, and thus activate ArcA/B, which represses the TCA cycle genes, and in turn produced more acetate. The similar situation has also been seen in *cyoA* and *cydB* genes knockout mutants in our previous investigation (Kumar and Shimizu, 2011).

Figure 3.2b indicates that *phoB* gene transcript level increased as P concentration decreases in the wild type, and Fig. 3.2a indicates that *rpoD* also increased as P concentration decreases. The *phoA*, *phoE*, *phoH*, *phnC*, *pstS*, and *ugpB* were all increased in a similar fashion as that of *rpoD* as mentioned in the result section. Figure 3.2a indicates that the expression pattern of *rpoS* is somewhat different. When cells enter into P_i-starvation phase in the batch culture, the Pho regulon is activated, and σ^S starts to accumulate in the cytosol (Wanner, 1996; Gentry et al., 1993; Ruiz and Silhavy, 2003). The promoters of the Pho genes are recognized by σ^D - associated RNA polymerase. A mutation in *rpoS*, significantly increases the level of AP (Alkaline phosphatase) activity, and the overexpression of σ^S inhibits it (Taschner et al., 2004). It has been reported that in *rpoS* mutant, the expression of AP was considerably higher than in wild-type strain, implying that σ^S is involved in the regulation of AP. Other Pho genes such as *phoE* and *ugpB* are likewise affected by σ^S. The

rpoS may inhibit the transcriptions of *phoA*, *phoB*, *phoE*, and *ugpB*, but not that of *pstS* (Taschner et al., 2004). Figure 3.2ab indicates that Pho genes are highly expressed as compared to low *rpoS* transcript level in the case of P-limitation. In contrast, *pst* may be transcribed by both σ^S and σ^D. The Pho regulon is thus evolved to maintain a trade-off between cell nutrition and cell survival during P_i-starvation (Taschner et al., 2004). The previous reports suggest that the Pho regulon and the stress response are interrelated (Ruiz and Silhavy, 2003; Taschner et al., 2004; 2006; Spira et al., 1995; 1999; Schurdell et al., 2007).

E.coli cells have been demonstrated to exhibit acid resistance by such genes as *gadAB* which encode glutamate decarboxylase and *gadC* which encodes glutamate: γ-amino butyric acid (GABA) antiporter. Glutamate decarboxylase production has been shown to increase in response to acid, osmotic and stationary phase signals (Castanie-Cornet et al., 1999; De Biase et al., 1999). In the typical batch culture, organic acids are most accumulated at the late growth phase or the stationary phase. It was shown that *gadA* was PhoB-dependently up-regulated in the present study (Fig. 3.5), and this indicates that this gene is indirectly regulated by PhoB. Note that Fig. 3.5 indicates that *gadA* gene expression decreased for the *phoB* mutant under acidic condition (4th bar), while *rpoS* increased. This suggests that phosphate starvation and acid stress responses may be interconnected (Back and Lee, 2006).

Figure 3.5 also indicates that *yfiD* transcript level increased at acidic condition. It has been shown that the expression of *yfiD* gene is induced at acidic condition, and this reduces the accumulation of acidic metabolite and products (Wyborn et al., 2002). The anaerobic transcription factor Fnr (Fumarate and nitrate reduction regulator) has been shown to be the major regulator of *yfiD* expression, and ArcA was shown to enhance anaerobic *yfiD* expression (Wyborn et al., 2002). Figure 3.5 indicates that *yfiD* transcript level changed in a similar fashion as *rpoD* rather than *arcA* and *fnr*. It has been known that the transcriptional regulator Fnr of *E.coli* functions as an O_2 sensor, and the protein is in the active form and

predominately exists as a homo-dimer with one [4Fe-4S] cluster per monomer under anoxic conditions. In the presence of oxygen, [4Fe-4S] FNR is converted to [2Fe-2S] FNR cluster and finally to apoFnr, which is no longer active in gene regulation (Unden et al., 2002; Reinhart et al., 2008). Nevertheless, *fnr* gene transcript level changed, which indicates that Fnr does not play its conventional role, and may have some role under aerobiosis, but it is not clear at this stage. Figure 3.5 also indicates that *soxR* increased at acidic condition. The acidic condition may affect membrane properties such as lipid content, thus effectively changing the proton permeability. The increased expression of *soxR* regulates the removal of damaging oxidizing agents (Warnecke and Gill, 2005).

As expected, the acid inducible *asr* gene transcript level increased at pH 6.0 as compared to the case at pH 7.0 as shown in Fig. 3.5c. The *asr* gene has been reported to be under the transcriptional control of the PhoR/PhoB two component system in *E. coli* (Suziedeliene et al., 1999). Figure 3.2c indicates that *asr* gene transcript level increased as P concentration decreases in accordance with the change in *phoB* transcript level. Asr is thought to play a role similar to that of the *E. coli* periplasmic protein HdeA, which serves as a proton sink or a chaperone for protecting periplasmic proteins from the deleterious effects at lower pH (Gajiwala and Burley, 2000). As another example, the PhoR/PhoB system has been suggested to sense external acidity and regulate the transcription of genes that are important for acid shock resistance (Suziedeliene et al., 1999; Tucker et al., 2002; Šeputienė et al., 2003; 2004; 2006).

The presence of glucose or mutations in *cya* or cAMP receptor protein (*crp*) gene leads to induction of *phoA* gene in *phoR* mutant. This induction requires the sensor PhoM (CreC) and the regulator PhoB (Wanner et al., 1988). However, PhoM (CreC) may not detect glucose per se, where it may detect an intermediate in the central metabolism. Therefore, *cya* or *crp* mutation may indirectly affect PhoM (CreC) - dependent control. In addition to P_i control, two P_i-independent controls may lead to activation of PhoB. These two may be

connected to control pathways in carbon and energy metabolisms, in which intracellular P_i is incorporated into ATP. One P_i independent control is the regulation by the synthesis of AcP, where P_i is incorporated into ATP at Ack (acetate kinase) pathway. AcP may act indirectly on PhoB.

In *E.coli*, assimilation of N-source such as NH_4^+ using α-KG results in the synthesis of glutamate and glutamine. Glutamine synthetase (GS encoded by *glnA*) catalyzes the only pathway for glutamine biosynthesis. Glutamate can be synthesized by two pathways through combined actions of GS and glutamate synthase (GOGAT encoded by *gltBD*) forming GS/GOGAT cycle, or by glutamate dehydrogenase (GDH encoded by *gdhA*). Under N-limitation, ammonium enters into the cell via AmtB and is converted to Gln by GS, and UTase (encoded by *glnE*) uridylylates both GlnK (encoded by *glnK*) and GlnB (encoded by *glnB*) (Ninfa et al., 2000). Figure 3.6d (1st and 2nd bars) indicates that *rpoN* increased under N-limitation, and *glnALG*, *glnB*, *glnK* as well as *nac* genes increased as stated above. On the other hand, under N-rich condition, UTase deurydylylates GlnK and GlnB. GlnK complexes with AmtB, thereby inhibiting the transporter via AmtB, where GlnB interacts with NtrB (encoded by *glnL*) and activates its phosphatase activity leading to dephosphorylation of NtrC (encoded by *glnG*), and NtrC- dependent gene expression ceases (Ninfa et al., 2000), thus the nitrogen regulation is affected by the phosphorylation caused by the available P source.

Figure 3.6d (2nd and 3rd bars) indicates that *glnB* and *glnK* transcript levels decreased, and *glnA* transcript level became lower under P-limitation as compared to P-rich condition under N-limitation. In the case under N-limitation, C/N ratio increases where α-KG is withdrawn via GDH, which affects the TCA cycle flux. A decreased flow through the TCA cycle would be expected to cause an increase in AcCoA pool and caused more acetate overflow.

Although little research has been done, it is quite important in practice to analyze the metabolism at the late growth phase and the stationary phase in the batch culture, where the

medium is nutrient poor indicating carbon, phosphorous, and nitrogen limitations, as well as lower pH. When a particular nutrient becomes limiting, the first response is scavenging. These scavenging regulons include cAMP-Crp which allows for the use of alternative carbon sources such as acetate, and the two-component regulatory systems PhoR/PhoB and NtrB/NtrC, which control scavenging for phosphorus and nitrogen, respectively. Both Crp and Ntr systems survey nutrient status through intracellular metabolites, where Crp recognizes cAMP, while NtrC responds to glutamine. The Pho system, on the other hand, monitors inorganic phosphate levels via the activity of the Pst transport system (Wanner, 1996). The sigma factor responsible for the general stress resistance is RpoS (σ^{38}) upon starvation. Note that the housekeeping sigma factor RpoD (σ^{70}) is homologous to RpoS. Carbon starvation is one of the strongest inducers of RpoS, where regulation of RpoS occurs at the level of proteolysis by C1pXP. This regulation is made by *sprE*, which encodes a response regulator SprE (also called RssB) (Pratt and Silhavy, 1996). RpoS plays also an important role under phosphate limiting condition. However, its regulation mechanism is different. Note that while carbon starvation completely shuts down the central metabolism, it continues upon phosphate starvation (Ballesteros et al., 2001). In contrast to carbon and nitrogen starvation, the PhoR/PhoB two component system, either directly or indirectly regulates the translation of *rpoS* mRNA (Ruiz and Silhavy, 2003). Since PhoB is a transcriptional regulator, its effects on *rpoS* translation may be indirect, where small noncoding RNAs (sRNAs) are important regulators of translation of mRNA. The sRNAs require the RNA chaperone Hfq for the formation of the RNA-RNA duplex, and there are several RNAs known to affect *rpoS* translation (Peterson et al., 2006). Namely, impeding phosphorous starvation is sensed as diminished activity of the Pst transporter, which causes autophosphorylation of PhoR, which then phosphorylate PhoB. The phosphorylated PhoB directly or indirectly activates transcription of an sRNA that stimulates translation of *rpoS* mRNA, thus elevating levels of RpoS (Peterson et al., 2006). Note that the regulation may be

more complicated, since Fig. 3.3a indicates that *rpoS* level increased as P concentration decreases even for the *phoB* mutant as also noted by Peterson et al. (2006).

Upon nitrogen starvation, ppGpp levels were known to increase, and there might be some correlations between levels of ppGpp and RpoS levels. RpoS is not stabilized upon nitrogen starvation like it is upon carbon starvation or phosphate starvation, and thus the regulation mechanism may be different, suggesting an increase in the activity of RpoS (Peterson et al., 2006). Similar proteins are induced following starvation for carbon, phosphorous and nitrogen (Groat et al., 1986; Matin, 1991), where RpoS-dependent genes are induced upon starvation. Although the activity of RpoS seems to be critical for nitrogen starvation, there are many players that affect the competition between RpoS and RpoD, including Rsd, 6S RNA, and ppGpp (Peterson et al., 2006). The role that the NtrB/C nitrogen scavenging system plays in regulating RpoS is unclear.

The overall regulation mechanism may be illustrated schematically as Fig. 3.7.

Similar mechanism might exist in *phoB* mutant *E.coli*, and further investigation is needed to clarify this.

Finally, it seems to be surprising that *phoB* (and also *phoR*) mutant could survive even under strict P limiting condition as compared to wild type as shown in Table 3.1 and Fig. 3.1. Figure 3.3 indicates that Pho regulon genes were insensitive to P concentration as expected, whereas global regulatory genes (Fig. 3.3a), metabolic pathway genes (Fig. 3.3c), and respiratory chain genes (Fig. 3.3e) changed significantly. It has recently been reported that *phoB* mutant was more sensitive to hydrogen peroxide, but that *phoB* mutant was more resistant to high osmolarity and acid conditions compared to the wild type of *Vibrio cholerae* (Sultan et al., 2010).

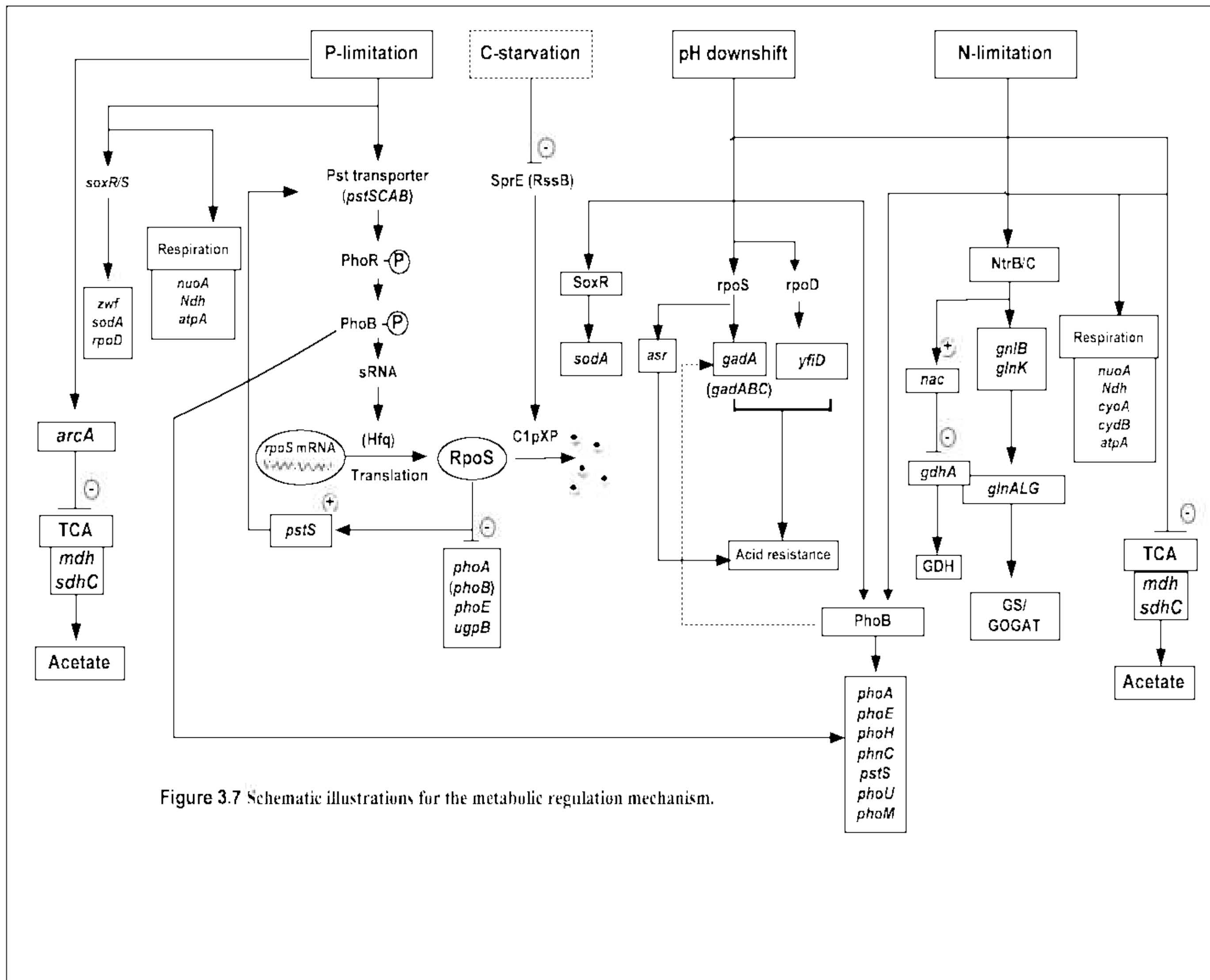

Figure 3.7 Schematic illustrations for the metabolic regulation mechanism.

3.4 Summary

The present investigation clarified the effect of phosphate limitation, nitrogen limitation, and acidic condition on the metabolism in view of gene transcript levels. Moreover, the present study implies that the metabolic regulations under phosphate limitation, nitrogen limitation and acidic condition are interconnected. These phenomena occur at the late growth phase in the batch culture. The present result is useful for the analysis of the metabolism changes during late growth phase and/or stationary phase.

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Chapter 4 Metabolic regulation of an *fnr* gene knockout *Escherichia coli* under oxygen limitation

4.1 Introduction

It is important to understand the cellular metabolism in response to environmental perturbations to redesign the metabolic networks for practical applications. Microorganisms such as *Escherichia coli* adapt to the change in culture environments such as carbon sources, nitrogen sources, oxygen availability etc. by regulating metabolic pathway genes through global regulators and signal transduction. In our laboratory previously, metabolic regulations of *E.coli* and its single-gene knockout mutants such as *gdhA*, *glnL*, *gltB*, and *gltD* mutants were investigated at various C/N ratios under aerobic condition (Kumar and Shimizu, 2010). From this study, it was found that transcript levels of terminal oxidases encoded by such genes as *cyoA* and *cydB*, and the global regulatory genes such as *fnr* and *fur* were up-regulated in particular at higher C/N ratios (Kumar and Shimizu, 2010). Then the effects of *cyoA* and *cydB* gene knockouts as well as *fnr* and *fur* genes knockout on the metabolism under aerobic condition were also investigated (Kumar and Shimizu, 2011). Given that Fnr plays important role under anerobic condition, herein, we further investigated the metabolic regulation of the *fnr* mutant under oxygen limiting condition.

Escherichia coli possesses sensing/regulation systems for the rapid response to the availability of oxygen, redox state as represented by $NADH/NAD^+$ ratio, and the presence of other electron acceptors. Those regulation systems channel electrons from donor to terminal acceptors. The pyridine nucleotides such as NADH and NAD^+ function as the important redox carriers involved in the metabolism. These coenzymes not only serve as electron acceptors in the breakdown of substrates but also provide the reducing power for the redox reactions in the anaerobic and aerobic respirations. A balance for oxidation and reduction of these nucleotides is regulated for catabolism and anabolism, since the turnover of the

nucleotides is very high compared to their concentrations (De Graef et al., 1999). Under anaerobic condition, the fermentation results in the reoxidation of NADH and the formation of reduced compounds occure, whereas NADH oxidation is coupled to the respiration by electron transfer under aerobic or nitrate respiration. In *E.coli*, the genes which code for enzymes specific to respiration and fermentation are mainly under control of three global regulators, where those exert their effects depending on the redox state of the cell. One of those is Fnr (fumarate, nitrate reduction), which is involved in the regulation of gene expressions for fermentation-related enzymes, while the others are the two-component regulatory systems such as Nar (nitrate reduction) and Arc (anoxic respiration control).

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 (Green and Guest, 1993). A model was proposed where 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-sulfer cluster of the corresponding region, resulting in monomerization of the protein and subsequent loss of its ability to bind DNA (Kiley and Reznikoff, 1991). Nar plays a role when nitrate is present, and belongs to the two component redox regulation systems, where it comprises a memebrane sensor (NarX) that may act as a kinase causing phosphorylation of the regulator (NarL) under certain condition. The Nar system activates such genes as nitrate reduction encoding nitrate and nitrite reductases, and represses such genes as fumarate reductase genes. The mechanism of Arc system involves a transphosphorylation from the sensor ArcB to the regulator ArcA. It has been reported that there exists an O_2 sensing mechanism (Georgellis et al., 2001), and that lactate and NADH stimulate the activation (phosphorylation) of Arc (Iuchi, 1993; Iuchi et al., 1994). ArcA protein is one of the important candidates for controlling the TCA cycle genes under micro-aerobic condition (Alexeeva et al., 2000; 2003; Lynch and Lin, 1996; Park et al.,

1994; Park and Gunsalus, 1995). The respiratory chain in *E.coli* can function with either of the two different memebrane-bound NADH dehydrogenase I (*nuo* operon) and NADH dehydrogenase II (*ndh*), where those generate quinones, which serve as important electron carriers for respiratory chain, where cytocrome bo_3 (*cyoABCD*) and cytochrome bd (*cydAB*) utilize them for proton motive force. Ubiquinone inhibits the kinase activity of ArcB.

Pyruvate is the terminal product of glycolysis, and a key intermediate in the catabolism, and its subsequent conversion by either pyruvate dehydrogenase complex (PDHc) and pyruvate formate lyase (Pfl) can be considered as a major switch point between oxidative routes such as TCA cycle and subsequent respiration, and the fermentative routes such as mixed acid fermentation. In *E.coli*, PDHc is regulated by the Arc system, while Pfl is regulated by Fnr and Arc systems. It has been shown that PDHc activity is not dependent on the presence of oxygen per se but rather on the external redox condition, and that E3 (Dihydrolipoamide dehydrogenase) subunit of PDHc is less affected, whereas E1 and E2 subunits are under control of Arc system (De Graef et al., 1999). If neither oxygen nor any of the alternative anaerobic electron acceptors are present, the reducing equivalent such as NADH generated during conversion of glucose to pyruvate cannot be reoxidized by electron transport reactions, and must be reoxidized through fermentative pathways. The ethanol, lactate and succinate must be formed along with the excretion of the other oxidation products that include acetate, formate, hydrogen and CO_2 (Bock and Sawers, 1996; Gunsalus and Park, 1992).

As stated above, Fnr and ArcA/B play important roles in regulating the metabolism under anaerobic and micro-aerobic conditions. The detailed regulation mechanism is, however, more complicated, since other global regulators such as Cra, Crp, RpoS, etc. may play roles. We have previously investigated on the effect of *fnr* gene knockout on the metabolism under aerobic condition to discover the metabolic regulations under nitrogen limitations (Kumar and Shimizu, 2010). Since Fnr plays essential role under anaerobic

condition, we further investigated the effect of *fnr* gene knockout on the metabolism in the present research.

4.2 Results and Discussion

Table 4.1 shows the comparison of fermentation parameters for the continuous culture at the dilution rate of 0.1 h^{-1} between wild type *Escherichia coli* BW25113 ($lacI^q$ $rrnB_{T14}$ $\Delta lacZ_{WJ16}$ *hsdR514* $\Delta araBAD_{AH33}$ $\Delta rhaBAD_{LD78}$) and its *fnr* mutant (JW1328) under micro-

Table 4.1 Comparison of the fermentation parameters for *E.coli* BW25113 and its *fnr* mutant under the microaerobic continuous cultivation at the dilution rate of 0.1 h^{-1}.

Strains	Cell yield (gDCW/g)	Specific rates (mmol/gDCW/h)				
		Glucose	Acetate	Formate	Ethanol	Lactate
BW25113	0.094±0.002	5.93±0.01	1.076±0.050	0.661±0.075	0.481±0.003	0.116±0.050
fnr mutant	0.088 ± 0.001	6.280±0.001	1.055±0.002	0.620±0.080	0.461±0.100	0.593±0.010
Strains	**Biomass concentration (g/l)**	**Concentration (g/l)**				
		Glucose	Acetate	Formate	Ethanol	Lactate
BW25113	0.726 ± 0.015	2.254±0.010	0.469±0.020	0.221±0.060	0.161±0.001	0.076±0.030
fnr mutant	0.739 ± 0.013	1.640±0.001	0.468±0.001	0.211±0.030	0.157±0.040	0.395±0.005

Note: The standard deviation was obtained by triplicate measurements.

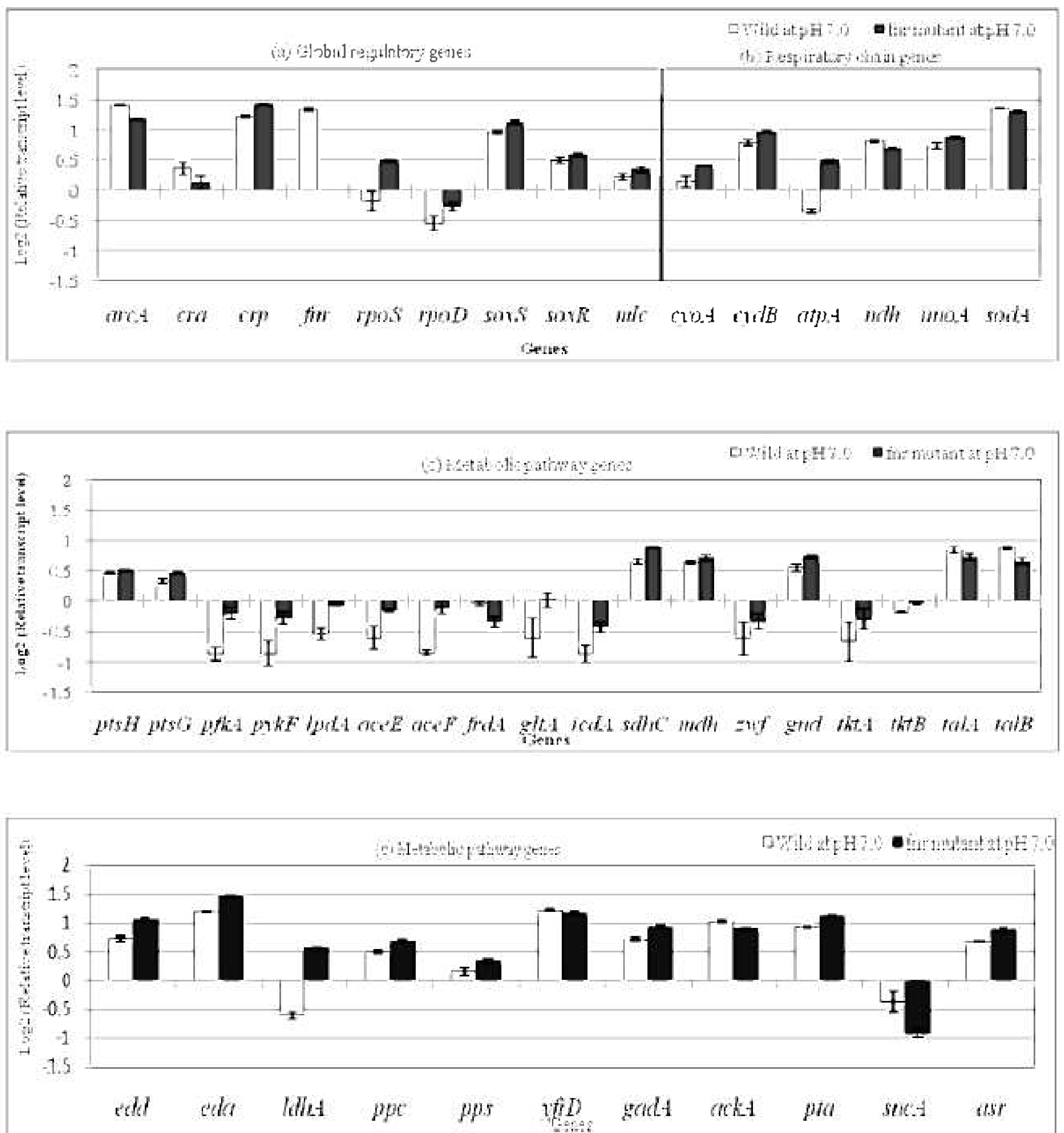

Figure 4.1 Comparison of the transcript levels between wild type and *fnr* mutant under micro-aerobic continuous culture condition: (a) global regulatory genes, (b) respiratory chain genes, and (c) metabolic pathway genes.

-aerobic condition, where it is essentially anaerobic condition without air supply at 100 rpm. The pH was controlled at 7.0 ± 0.1. Table 4.1 indicates that specific glucose consumption rate was increased, the specific lactate production rate was increased, and the specific production rates of formate, ethanol and acetate tended to decrease but not so significant for the mutant as compared to wild type. Figure 4.1 shows the gene transcript levels for the continuous culture, where it indicates that the *arcA* transcript level decreased ($p < 0.01$), and TCA cycle genes such as *gltA*, *icdA*, *sdhC*, and *mdh* were increased ($p < 0.05$, $p < 0.05$, $p < 0.01$, $p < 0.1$) for *fnr* mutant as compared to wild type. This effect is compounded by the fact that *arcA* transcription is directly activated by Fnr (Compan and Touati, 1994), which in turn is essential for oxiadation of quinol (Constantinidou et al., 2006).

It should be noted that *cyoA* and *cydB* were both increased ($p < 0.05$, $p < 0.01$), where *cyoA* is known to repressed by both ArcA and Fnr, while *cydB* is activated by ArcA but repressed by Fnr. The increased activities of cytochromes may have enhanced the oxidation of quinol to quinon, which inhibits the phophorylation of ArcB, and in turn decreased the phosphorylation of ArcA. The *lpdA* as well as *aceE, F* gene transcript levels significantly increased ($p < 0.01$, $p < 0.05$, $p < 0.01$) for the *fnr* mutant as compared to wild type, where it may be due to decreased activity of *arcA*.

Figure 4.1 also indicates that *ldhA* gene transcript level significantly increased for the *fnr* mutant as compared to wild type. The *cra* transcript level decreased ($p < 0.1$), and *ptsH*, *ptsG*, *pfkA*, *pykF*, *edd* and *eda* as well as *ppc* were increased for the *fnr* mutant as compared to wild type ($p < 0.05$, $p < 0.01$, $p < 0.01$, $p < 0.01$, $p < 0.01$, $p < 0.01$, $p < 0.01$). The *crp* transcript level increased ($p < 0.01$), which may be due to lower glucose concentration (Table 4.1). Moreover, there might be some relationship between Fnr and Crp. The sequence of the *fnr* gene revealed that it encodes a protein which shows significant homology to Cap/Crp (for catabolic activator protein). However, a number of significant differences between the two proteins have been investigated. Fnr is a monomeric protein, and it does not have the

conserved group of surface residues that interact with cyclic AMP. It contains an oxygen labile iron-sulfur center as a sensor element for anaerobiosis (Lynch and Lin, 1996; Salmon et al., 2003; Williams et al., 1991; Ziegelhoffer and Kiley, 1995). Several studies have been conducted on the structure and gene sequence for Fnr and Crp proteins. From those studies, it was found that both Fnr and Crp protein possess almost similar structure and gene sequence. The genes that are controlled by these two global regulators have similar binding sites (Kiley and Reznikoff, 1991; Lynch and Lin, 1996; Williams et al., 1991; Bell et al., 1989; Li Bo et al., 1998; Unden et al., 2002). Even if some mutation changed the structure of proteins, the mutation in Fnr protein could convert to Crp protein, and similarly Crp protein could convert to Fnr protein (Sipro et al., 1990). It may be also considered that both Crp and Fnr protein can form heterodimer, which might not allow both of them to function properly (Williams et al., 1991; Ziegelhoffer and Kiley, 1995; Bell et al., 1989). Then the absence of Fnr protein or gene allows Crp protein to bind more effectively to the target gene sequence.

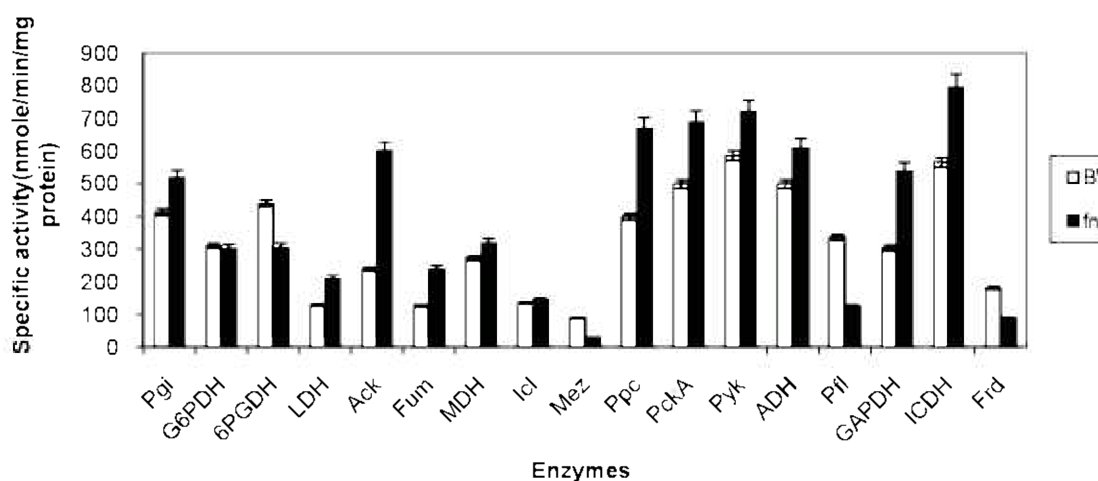

Figure 4.2 Comparison of enzymes activities during micro-aerobic batch culture between (□) *E.coli* BW25113, (■) *E.coli fnr* mutant. Glyceraldehyde 3 phosphate dehydrogenase (GAPDH), Pyruvate-formate lyase (Pfl), Isocitrate dehydrogenase (ICDH), Formate reductase (Frd) and rest of other abbreviations are similar to Figure 4.3.

Although continuous culture is suitable for comparison of gene expressions between the wild type and its mutant, batch fermentation is better for the analysis of a distribution of the metabolites formed from the practical application point of view. Batch fermentation results indicate that the production rate of extra-cellular metabolites such as acetate, formate, and ethanol were reduced, while the production rate of lactate was increased for *fnr* mutant as compared to the wild type. The enzyme activities were also measured for both strains under micro-aerobic condition as shown in Fig. 4.2. Most of the glycolytic enzymes such as Pgi, GADPH and Pyk showed higher activities in the *fnr* mutant as compared to its parent strain. The increased activities of GAPDH and ICDH in the mutant are consistent with the results of other researchers (Park et al., 1994; Park and Gunsalus, 1995; Chao et al., 1997). Since Fnr is known to act as an activator of the *pfl* gene, significant reduction of Pfl activity was observed for the *fnr* mutant. The reduction of Pfl activity caused increased activity of the other fermentative pathway enzymes such as LDH in the mutant as compared to the wild type. Other fermentative enzyme such as Frd that produces succinate from fumarate under anaerobic condition was found to be reduced in the mutant, which is consistence with lower succinate production rate in the mutant (data not shown). The flux distributions through the central metabolic pathway of the parent strain and the *fnr* mutant were then estimated based on mass balances. The analysis was performed based on the measurement of the specific rates and the pseudo-steady-state assumption for intracellular metabolites. A total number of measured flux is 7, and it is higher than the degrees of freedom for the corresponding metabolic network. As a result, the system is an over determined system. The best estimates for all of the measured and estimated fluxes were then calculated (Tsai and Lee, 1988), where the comparison of metabolic fluxes between wild type and *fnr* mutant is shown in Fig. 4.3, where it indicates the lower flux through Pfl, and the increased flux through LDH. The increase in the flux through Ack and decrease in the flux through Frd for the mutant are

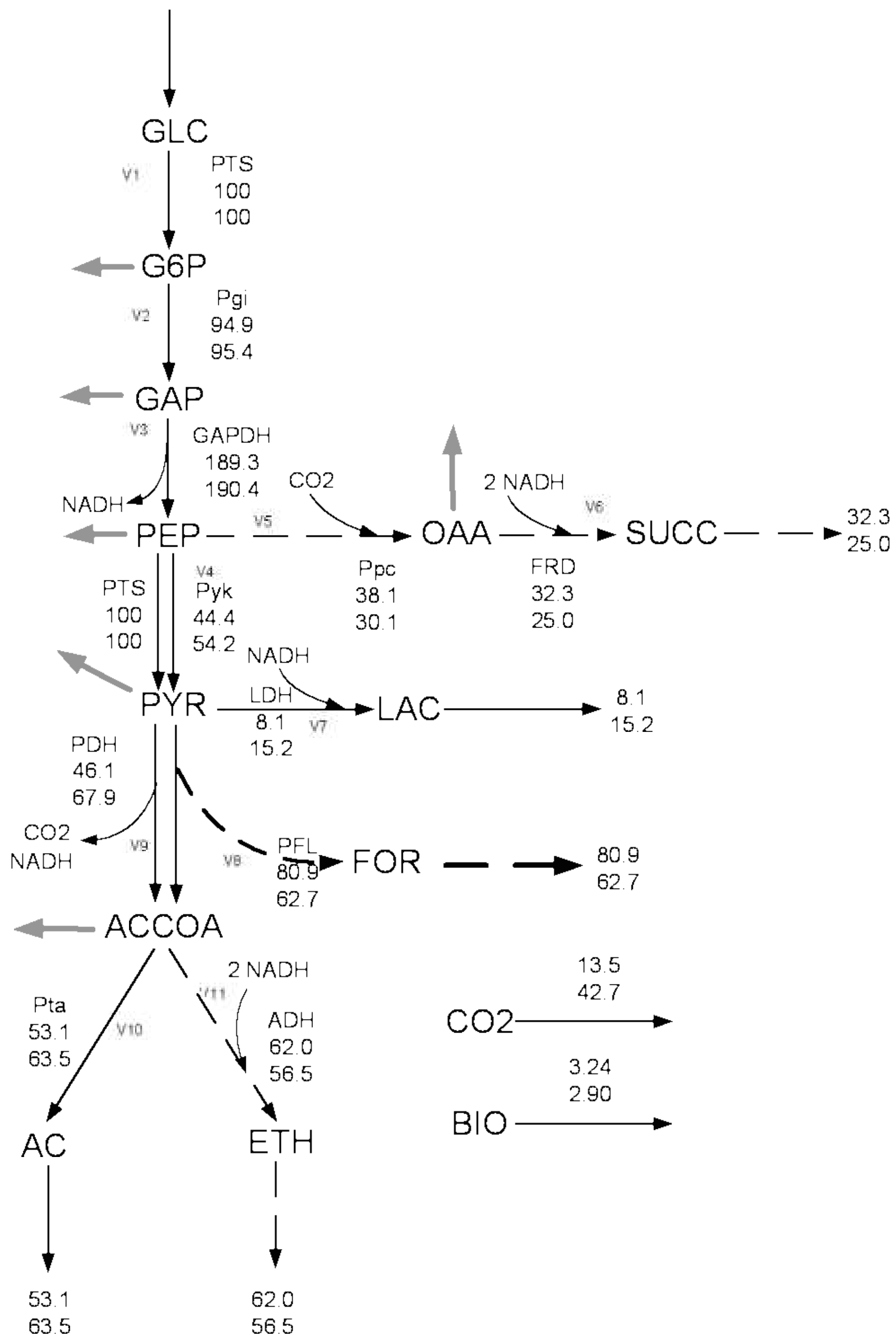

Figure 4.3 Metabolic flux distributions of wild type (upper values) and *fnr* mutant (lower values) under micro-aerobic condition. The dotted lines indicate the reduced flux. The grey arrows indicate biomass synthesis.

consistent with enzyme activities, Acetyl CoA has two alternative fates. The energy in the thioester bond can be conserved in the form of ATP by the action of Pta-Ack pathway, but its formation does not result in the consumption of any reducing equivalents. Alternatively, the energy can be sacrificed by reducing AcCoA to ethanol through two dehydrogenation reactions catalyzed by ADH. The increased activity of LDH in the mutant caused the reduce flux through ADH pathway.

In summary, the overall regulation mechanism for *fnr* mutant may be expressed as Fig. 4.4. Since Fnr is known to activate *frd* and *pfl* genes, the *fnr* mutant produced less succinate and formate as expected. Although *arcA* is known to be activated by Fnr, the regulation mechanism is somewhat complicated as shown in Fig 4. Namely, *cyo* and *cyd* genes are repressed by Fnr, while *cyo* is repressed and *cyd* is activated by ArcA. The present result indicates that 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 increased quinine pool, which inhibited ArcB phophorylation, and in turn decreased phosphorylation of ArcA, where *arcA* gene expression also decreased due to *fnr* gene knockout. The down-regulation of *arcA* caused up-regulations of TCA cycle genes as well as *lpdA* and *aceE, F* which code for PDH. Although indirect effect, *fnr* mutant caused less growth rate, which caused less biomass concentration, which in turn caused more glucose concentration to be increased. This may have caused *cra* gene to be down-regulated and thus activated glycolysis genes, and eventually caused up-regulation of the specific glucose uptake rate. The increase of glucose concentration may cause decrease of cAMP and cAMP-Crp complex for catabolite regulation, but *crp* gene expression was up-regulated. This might have been caused by *fnr* gene knockout, but it is not clear at this stage. The increased lactate formation may be due to higher NADH/NAD$^+$ ratio caused by reduced Frd activity, and higher pyruvate concentration caused by the down-regulation of Pfl and increased flux of Pyk,

although PDH activity may be increased.

In conclusion, the present research result clarified the metabolic changes caused by *fnr* gene knockout based on different levels of information, and this may contribute the efficient metabolic engineering and process optimization for culture condition.

Figure 4.4 Overall regulation mechanisms for *fnr* gene knockout under micro-aerobic condition. Flat-headed (⊣) arrows indicate negative regulation; a pointed arrowhead (→) indicates positive regulation. Dotted lines indicate that the proposed regulation may be direct or indirect.

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Chapter 5 Effect of pH on the metabolic regulation of *Escherichia coli*

5.1 Introduction

Escherichia coli is a facultative anaerobe that uses transcription factors to control gene expressions in response to the change in the growth environment such as carbon and nitrogen sources, pH, temperature, oxygen level etc. Among them, the effect of pH on the metabolism is of practical interest, since the culture pH is often not controlled in industry. Moreover, the change in the culture pH is of practical importance for the variety of applications such as heterologous protein production, simultaneous saccharification and fermentation (SSF) etc, where culture pH has to be lower in SSF due to enzymatic hydrolysis of cellulose in the ferementor (Olofsson et al., 2008). In the present research, we investigated the effect of pH on the metabolism of *Escherichia coli* under aerobic and anaerobic conditions.

The effect of pH on protein expressions has been investigated for *E. coli* (Blankenhorn et al., 1999). Bacteria have a number of strategies for surviving at low pH. Growth in a moderately acidic environment triggers the synthesis of proteins that protect the cell from more extreme acidic conditions. Acid resistance, acid tolerance, and acid habituation are used to describe survival at low-pH condition (Bearson et al., 1998). Three proteins associated with glutamate-dependent acid resistence have been identified in the past, where they are glutamate decarboxylase encoded by *gadA* and *gadB* and a putative glutamate:γ- amino butyric acid(GABA) anti porter encoded by *gadC* (Foster, 1995). It has been shown that *gadA* and *gadB* genes increased in response to the stationary phase at low pH in the batch culture (Castanie-Cornet and Foster, 2001).

Although several studies have been reported as stated above, little attention has been focused on the effect of pH on the metabolism. This is important from the practical

application point of view. In the present study, therefore, we investigated how the metabolism changes in *E. coli* at low pH in view of fermentation data and gene expressions under aerobic and microaerobic conditions.

The sequences of the primers used in the present study are given elsewhere (Kabir and Shimizu, 2003) except theose mentioned in Table 2.2 (Chapter 2: Materials and Methods section).

5.2 Results

5.2.1 Effect of culture pH on the metabolism

The aerobic continuous cultivation was conducted at the dilution rate of 0.2 h^{-1}, where Table 5.1 and Fig. 5.1 shows the effect of culture pH on the fermentation characteristics. Table 5.1 indicates that acetate was more formed ($p < 0.05$), the cell yield was lower ($p < 0.05$), and the specific glucose consumption rate was lower ($p < 0.1$) at pH 5.5 as compared to the case at pH 7.0.

In order to make clear the metabolism of *E. coli* under acidic condition, gene expressions were measured by RT-PCR, where Fig. 5.2 compares the gene expressions at two different pH values. Figure 5.2 indicates that the transcript level of *rpoS* was up-regulated ($p < 0.10$), and the expressions of *gadA* (glutamate decarboxylate gene), and *acs* were up-regulated ($p < 0.05$ and $p < 0.05$, respectively), where these genes are known to be under control of RpoS (Cheville et al., 1996). Figure 5.2 also shows the up-regulation of *arcA* gene expression ($p < 0.1$), where *arcA* gene product functions as a repressor of such genes as involved in the TCA cycle under microaerobic condition (Appendix A). Namely, in accordance with up-regulation of *arcA*, some of the TCA cycle genes such as *icdA* ($p < 0.1$) and *gltA* were down-regulated ($p < 0.05$). Figure 5.2 also indicates that the expressions of the respiratory chain gene such as *cydB* was up-regulated ($p < 0.1$), whereas *cyoA* was down

Table 5.1 Fermentation characteristics of the wild type *E. coli* and its *phoB* and *fnr* mutants in the aerobic chemostat culture under different phosphate concentrations and different pH conditions at the dilution rate of 0.2 h^{-1}.

Fermentation parameters		P-rich (100%) condition					P-limited (10%) condition	
Culture pH		7.0	6.0	5.0	4.5	4.0	7.0	5.0
Biomass concentration (g/l)	Wild	3.86 ± 0.03	3.680 ± 0.002	2.44 ± 0.03	1.204 ± 0.06	0.0475 ± 0.0160	1.69 ± 0.03	1.46 ± 0.011
	Δ*phoB*	3.44 ± 0.04	3.36 ± 0.11	2.05 ± 0.06	–	–	3.64 ± 0.01	1.53 ± 0.04
	Δ*fnr*	3.88 ± 0.03	–	2.69 ± 0.03	0.415 ± 0.01	–	–	–
Glucose concentration (g/l)	Wild	0.660 ± 0.004	1.053 ± 0.010	0.964 ± 0.08	1.59 ± 0.01	2.13 ± 0.02	1.85 ± 0.01	3.59 ± 0.02
	Δ*phoB*	1.59 ± 0.29	0.917 ± 0.324	1.07 ± 0.24	–	–	1.050 ± 0.001	4.24 ± 0.01
	Δ*fnr*	1.00 ±0.001	–	0.868±0.080	1.88 ± 0.001	–	–	–
Acetate concentration (g/l)	Wild	0.046 ± 0.002	0.468 ± 0.003	0.483 ± 0.200	0.252±0.010	0.024±0.01	0.41 ± 0.02	0.434 ± 0.001
	Δ*phoB*	0.255 ± 0.130	0.003 ± 0.02	0.497 ± 0.03	–	–	0.346 ± 0.010	0.417 ± 0.001
	Δ*fnr*	0.0003±0.10	–	0.525±0.23	0.267±0.024	–	–	–
Specific glucose uptake rate (mmol/gDCW/h)	Wild	2.69 ± 0.05	2.700 ± 0.001	4.11 ± 0.03	7.76 ± 0.01	184.1 ± 0.02	5.36 ± 0.01	4.88 ± 0.02
	Δ*phoB*	2.72 ± 0.09	3.004 ± 0.11	4.84 ± 0.13	–	–	2.730 ± 0.003	4.18 ± 0.01
	Δ*fnr*	2.58	–	3.77±0.03	21.74±0.003	–	–	–
Specific acetate production rate (mmol/gDCW/h)	Wild	0.040 ± 0.002	0.424 ± 0.003	0.659 ± 0.2	0.697±0.028	1.60±0.70	0.81 ± 0.02	0.95 ± 0.001
	Δ*phoB*	0.247 ± 0.080	0.003 ± 0.020	0.807 ± 0.04	–	–	0.317 ± 0.001	0.91 ± 0.001
	Δ*fnr*	0.00026 ± 0.080	–	0.650±0.300	2.143±0.190	–	–	–

Note: " – " indicates that no data was collected for this condition. The standard deviation was obtained by triplicate measurements.

regulated ($p < 0.1$) (Appendix A). The *fnr* gene expression was also up-regulated as well as *arcA*, which caused the down regulation of *lpdA* and *aceE* gene expressions. Figure 5.2 also indicates that the transcript level of *crp* gene, which codes for cAMP receptor protein Crp,

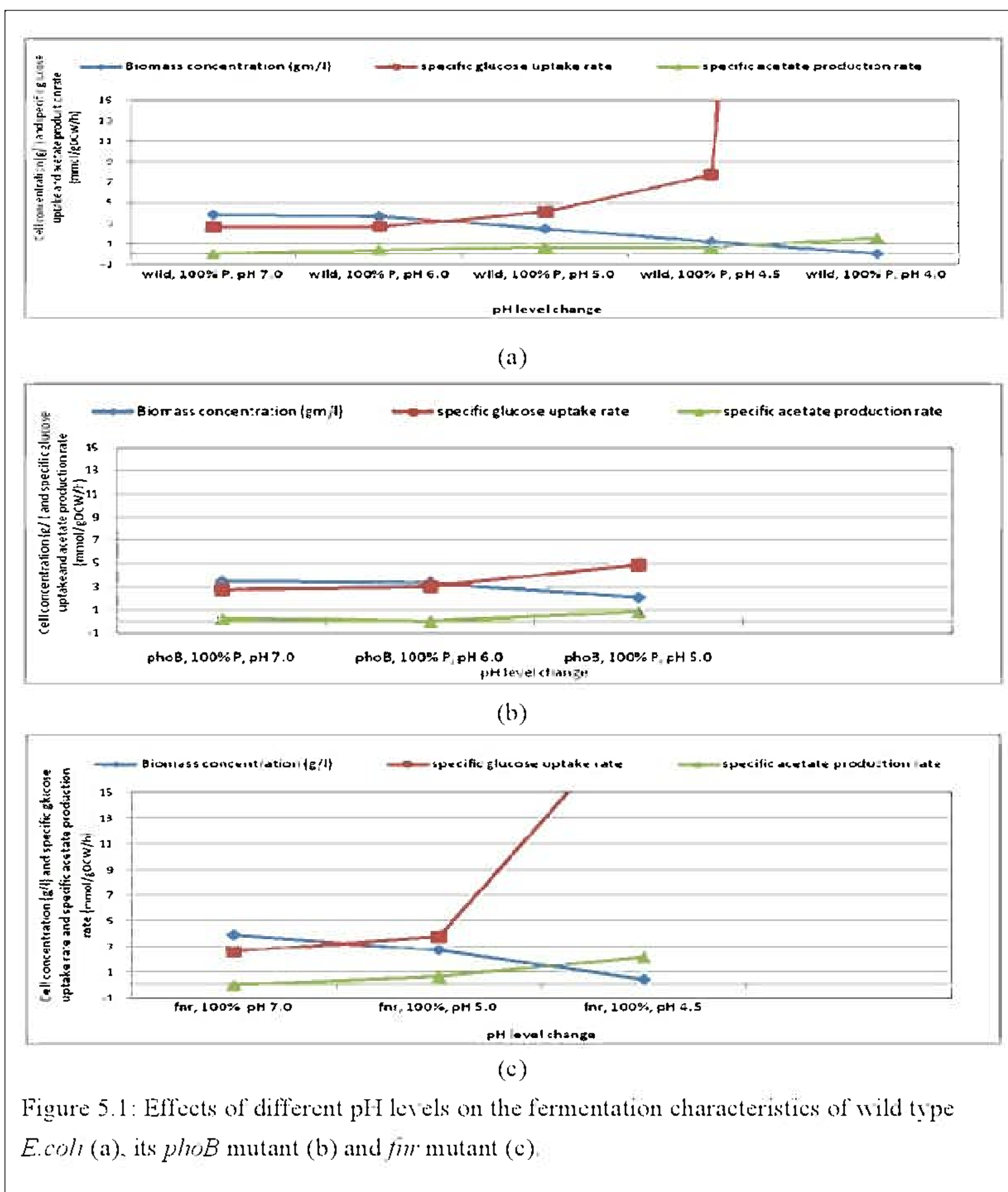

Figure 5.1: Effects of different pH levels on the fermentation characteristics of wild type *E.coli* (a), its *phoB* mutant (b) and *fnr* mutant (c).

Table 5.2 Comparison of the fermentation parameters at different culture pH in the aerobic condition.

Fermentation parameters	Culture pH		% of change
	7.0	**5.5**	
Specific glucose uptake rate (mmol/gDCW/h)	2.46 ± 0.03	2.08 ± 0.02	- 15.44
Specific acetate production rate (mmol/gDCW/h)	0.185 ± 0 .02	0.302 ± 0.04	+ 67.77
Specific CER (mmol/gDCW/h)	5.83 ± 0.04	5.12 ± 0.04	- 12.17
Specific OUR (mmol/gDCW/h)	5.74 ± 0 .05	5.06 ± 0.05	- 16.21
Biomass yield (gDCW/g substrate)	0.421 ± 0 .01	0.165 ± 0 .02	- 61.92

Note: The standard deviation was obtained by triplicate measurements.

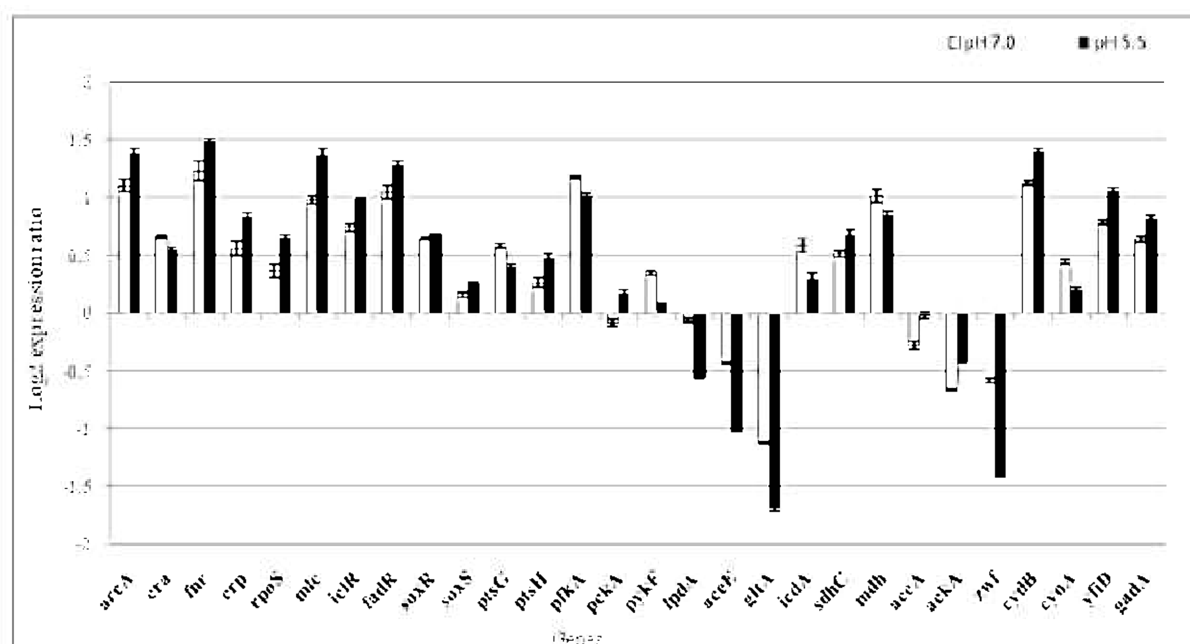

Fig 5.2 The effect of pH on gene expressions of the wild type *E. coli* BW25113 under aerobic condition

was up-regulated ($p < 0.1$), and the expression of *sdhC*, which is known to be under control of Crp (Appendix A), was also up-regulated ($p < 0.1$) (Hoffmann et al., 2002). Moreover, *mlc* gene expression was higher ($p < 0.05$), and *ptsG* gene expression was lower ($p < 0.05$), where it has been reported that *ptsG* is repressed by Mlc (Kimata et al., 1998) (Appendix A). Figure 5.2 also shows that *cra* (catabolite repressor activator) gene expression was up-regulated ($p < 0.1$), where *cra* gene product regulates the carbon flow in such a way that gluconeogenesis is activated, whereas glycolysis is repressed (Appendix A). The down-regulations of *pfkA*, *pykA* and *zwf* gene expressions were partly due to up-regulation of *cra*. The gene expressions of *fadR* and *iclR* were also higher ($p < 0.05$, $p < 0.1$), where FadR activates *iclR*, and IclR is known to repress *aceBAK*.

Table 5.3 Comparison of the fermentation parameters at different culture pH in the microaerobic condition.

Fermentation parameters	Culture pH		% of change
	7.0	**6.0**	
Specific glucose uptake rate (mmol/gDCW/h)	5.19 ± 0.03	4.25 ± 0.02	-18.11
Specific acetate production rate (mmol/gDCW/h)	0.264 ± 0 .02	0.381 ± 0.03	+44.23
Specific lactate production rate (mmol/gDCW/h)	2.23± 0 .02	3.111± 0 .02	+39.50
Specific formate production rate (mmol/gDCW/h)	1.132± 0 .02	1.27± 0 .03	+12.38
Specific ethanol production rate (mmol/gDCW/h)	0.546± 0 .04	0.501± 0 .03	-8.24
Biomass yield (gDCW/g substrate)	0.502 ± 0 .02	0.201 ± 0 .03	-59.96

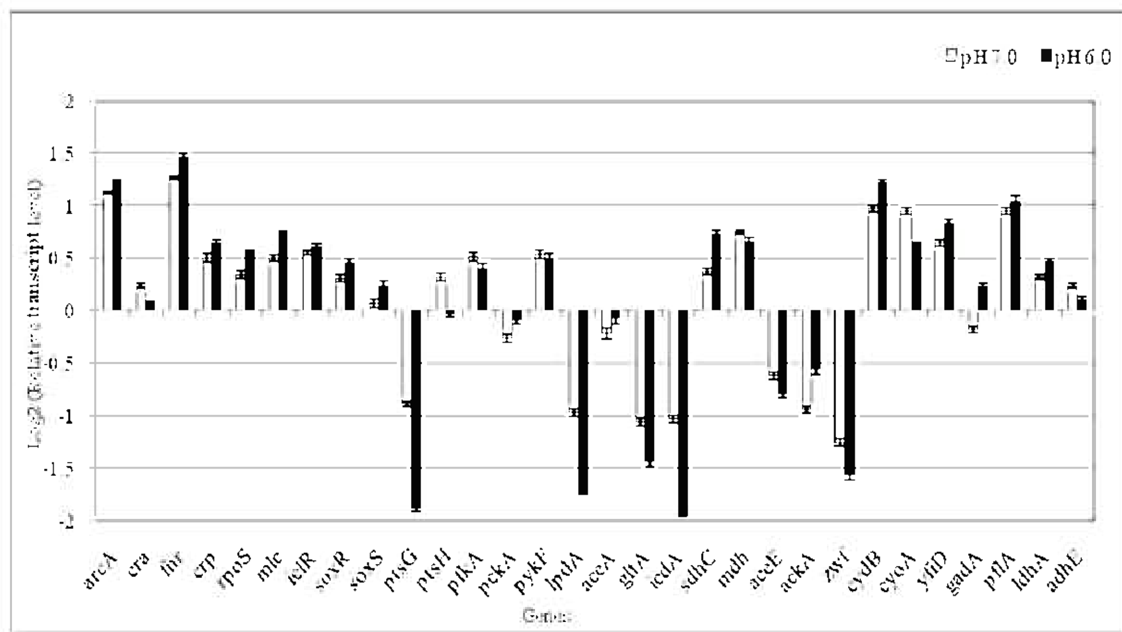

Fig 5.3 The effect of pH change on gene expressions of the wild type *E. coli* BW25113 cultivated under microaerobic condition

Table 5.3 shows the comparison of the fermentation data at different pH values under microaerobic condition, wehere it indicates that acetate, formate, and lactate were more produced, whereas ethanol was less produced at pH 6.0 as compared to the case at pH 7.0. Figure 5.3 compares the gene expressions at two different pH values, where the gene expression patterns were almost the similar as those shown in Fig.5.2, where it indicates that *gadA* gene expression increased, and *fnr* gene expression increased at pH 5.5 as compared to the case at pH 7.0 ($p<0.1$, $p< 0.05$, respectively). The *arcA* gene expression increased ($p< 0.1$) and the expressions of *icdA*, *aceE* and *mdh* genes decreased ($p< 0.05$, $p< 0.1$, $p< 0.1$ respectively). The *crp* gene expression increased ($p<0.5$), and *mlc* gene expression increased ($p< 0.05$) while the expressions of such genes as *ptsG*, *ptsH*, *lpdA* decreased ($p< 0.05$, $p<0.1$, and $p< 0.1$, respectively). In the microaerobic condition, additional changes were observed. Namely, *yfiD* and *pflA* gene expressions increased ($p< 0.05$, $p< 0.05$ respectively) where these are involved in formate formation. The *ldhA* gene

expression increased whereas *adhE* gene expression decreased at pH 6.0 as compared to those at pH 7.0 ($p < 0.05$ and $p < 0.1$ respectively).

5.2.2 Effects of both pH and temperature on the metabolism

Table 5.4 shows the effects of pH and temperature on the fermentation characteristics, where the specific glucose consumption rate decreased, and the specific acetate production rate increased. The specific CO_2 production rate decreased and the cell yield significantly decreased at pH 6.0 and 42° C as compared to those at pH 7.0 and 37°C. Since the cell growth was significantly depressed at pH 5.5 and 42°C, the culture pH was set at 6.0 at 42°C. The gene expressions were compared as shown in Fig.5.4, where it indicates that *rpoH* gene expression increased, and the heat shock genes such as *dnaK*, *groL*, *groS* and *ibpB* were all up-regulated ($p < 0.05$, $p < 0.05$, $p < 0.05$ and $p < 0.05$ respectively) at pH 6.0 and 42°C as compared to those at pH 7.0 and 37°C. The *arcA* gene expressions as well as *fnr* gene expression increased ($p < 0.5$ and $p < 0.1$) , and *icdA* and *gltA* expressions decreased ($p < 0.05$ and $p < 0.1$), and *cydB* gene expression increased ($p < 0.5$) at pH 6.0 and 42°C as compared to those at pH 7.0 and 37°C. The *crp* gene expression increased ($p < 0.05$), and *mlc* gene expression increased ($p < 0.05$), and *ptsG* gene expression decreased ($p < 0.05$), and *lpdA* and *sdhC* gene expressions increased ($p < 0.1$ and $p < 0.05$). The *pykF*, and *zwf* gene expressions were down-regulated ($p < 0.05$, and $p < 0.1$ respectively) at pH 6.0 and 42°C as compared to those at pH 7.0 and 37°C.

Table 5.4 Comparison of the fermentation parameters at pH 7 and 37° and pH 6.0 and 42°C for the aerobic condition.

Fermentation parameters	Culture pH & Temperature		% of change
	7.0 & 37°	**6.0 & 42°**	
Specific glucose uptake rate (mmol/gDCW/h)	2.46 ± 0.02	2.28 ± 0.02	- 7.31
Specific acetate production rate (mmol/gDCW/h)	0.186 ± 0 .02	0.307 ± 0.03	+ 65.05
Specific CER (mmol/gDCW/h)	5.83 ± 0.04	5.02± 0.04	- 13.89
Specific OUR (mmol/gDCW/h)	5.72 ± 0 .03	5.18 ± 0.04	- 9.44
Biomass yield (gDCW/g substrate)	0.425 ± 0 .01	0.165 ± 0 .02	- 61.17

Note. The standard deviation was obtained by triplicate measurements.

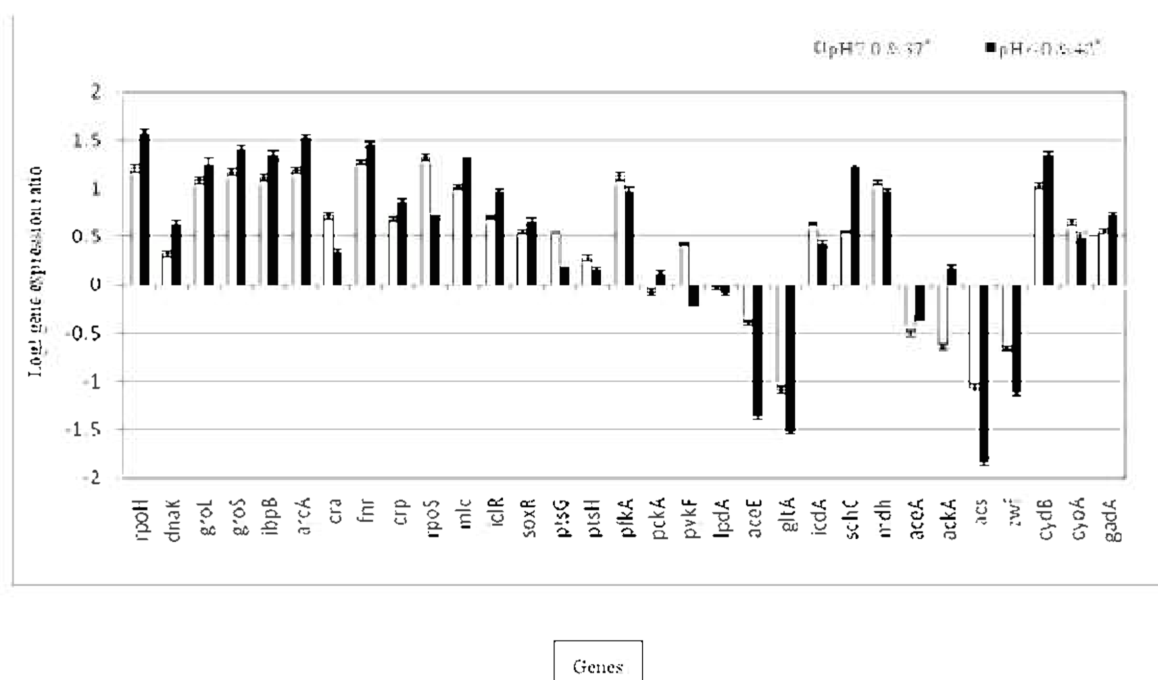

Fig 5.4 The effect of both pH and temperature change on gene expressions of the wild type *E. coli* BW25113 cultivated under aerobic condition

5.2.3 Effects of pH on the metabolism of *pflA* mutant

We have previously shown that *pflA/B* mutants overproduced lactate (Zhu and Shimizu, 2004). Here we also investigated the effect of lower pH on the fermentation characteristics of *pflA* mutant *E.coli*. Table 5.5 shows the effect of pH on the fermentation characteristics of *pflA* mutant in the continuous culture at the dilution rate of 0.2 h^{-1}, where the specific glucose consumption rate increased, and the specific lactate production rate increased whereas ethanol production rate decreased. This indicates that it is not necessary to control pH for lactate production by *pflA* mutant *E. coli*.

Table 5.5 Comparison of the fermentation parameters at different culture pH in *pflA* mutant under the microaerobic condition.

Fermentation parameters	Culture pH		% of change
	7.0	**6.0**	
Specific glucose uptake rate (mmol/gDCW/h)	5.56 ± 0.03	5.87 ± 0.02	+ 5.57
Specific acetate production rate (mmol/gDCW/h)	2.44 ± 0 .02	2.04 ± 0.03	- 16.39
Specific lactate production rate (mmol/gDCW/h)	8.77 ± 0 .02	10.92 ± 0 .02	+ 24.51
Specific ethanol production rate (mmol/gDCW/h)	2.56 ± 0 .02	2.36 ± 0 .03	-7.81
Biomass yield (gDCW/g substrate)	0.702 ± 0 .02	0.571 ± 0 .03	-18.66

Note. The standard deviation was obtained by triplicate measurements.

5.3 Discussion

Acid resistance is considered to be an important virulence factor of pathogenic *E. coli* strains such as O157:H7 (Castanie-Cornet et al., 1999). It is well known that Gad is needed for survival under low pH (Hersh, 1996; Castanie-Cornet et al., 1999). Cells possess specific defense mechanisms against acid environments in which Gad system has been studied for its major role in the detoxification of acid-induced stress in *E. coli*. Figures 5.2 and 5.3 indicate that *gadA* gene expression increased at lower pH as compared to the case at pH 7.0. GadA and GadB are known to be induced at lower pH as compared to the case at pH 7 (Castanie-Cornet et al., 1999). Under acid stress, the product GABA is exported by GadC (Castanie-Cornet and Foster, 2001). Anaerobiosis amplifies the acidic induction of amino acid decarboxylases (Meng and Bennett, 1992; Slonczewski and Foster, 1996). It has been noted that the acid-induced expression of amino acid decarboxylases is enhanced under anaerobic condition (Meng and Bennett, 1992; Neely et al, 1994). The *gad* system (GadABC) neutralizes acidity and enhances survival in extreme acid; its induction during anaerobic growth may help protect alkaline-grown cells from the acidification resulting from anaerobic fermentation. Fig. 5.2 shows that *gadA* is also up-regulated even at aerobic condition.

It has been shown that Fnr is the major activator of *yfiD* expression, but that the extent of Fnr-mediated activation can be modulated by the indirect oxygen sensor ArcA. Moreover, in accord with the proteomic analysis, the expression of *yfiD::lac* reporter fusion and the intracellular content of YfiD were found to be high during the growth at low pH for both aerobic and anaerobic cultures (Neil et al, 2002). Figures 5.2 and 5.3 show higher expression of *yfiD* at both aerobic and microaerobic conditions. Expression of *yfiD* is activated by Fnr acting at -40.5, a conventional activation position, but it is down-regulated by a second Fnr dimer at -93.5, which leads to maximal *yfiD* expression during microaerobic growth (Green

and Baldwin, 1997). It has been shown that YfiD, a homologue of pyruvate formate lyase, was induced to high levels at pH 4.4 and induced two-fold more by propionate at pH 6 (Blankenhorn et al., 1999). Both of these conditions cause internal acidification. At neutral or alkaline pH, YfiD was virtually absent and YfiD is, therefore, a strong candidate for response to internal acidification.

It has been reported that the expression of cytochrome *o* is repressed by acid stress (Cotter et al., 1990). Figures 5.2 - 5.4 also indicates the down-regulation of *cyo* gene expression, which may be also partly due to up-regulation of *arcA* gene expression (Appendix A). Therefore, there is a complex relationship between *yfiD*, *cyoA*, pH and oxygen level (Blankenhorn et al., 1999).

The up-regulation of *sdhC* is also partly due to up-regulation of *crp* gene (Appendix A). The *ptsG* gene expression was down-regulated at lower pH as compared to the case at neutral pH (Figs. 5.2 - 5.4), which may be due to up-regulation of *mlc* gene expression. This phenomenon was also observed at temperature up-shift (Hasan and K. Shimizu, 2008).

Figures 5.2 and 5.3 show that *aceA* is up-regulated under acidic condition as compared to the case at pH 7.0. This may be repressed by the up-regulation of *arcA* gene expression. One of the reason why *aceA* increased may be due to the fact that isocitrate lyase was induced and showed substantially greater induction in acid or in base condition than at pH 7 (Blankenhorn et al., 1999).

Figures 5.2 and 5.3 also indicate the up- regulation of *rpoS* at lower pH. At low pH, acetate is driven the cell by the pH gradient. Acetate and other acidic fermentation products induce stationary-phase stress proteins (Arnold and Kaspar, 1995, Kirkpatrick et al., 2001) as well as the auto inducer synthesis protein LuxS (Kirkpatrick et al., 2001, Schauder and Bassler, 2001). The growth-phase dependent sigma factor RpoS (Lange and Hengge-Aronis,

1991) regulates several components of resistance to both acid and base (Hersh et al., 1996; Lazar et al., 1998; Small et al., 1994; Waterman and Small, 2003). The *E.coli* cells initially produce fermentation products such as acetate and formate under microaerobic condition, which can reenter the cell and reach deleterious concentrations at extremely low pH (Russell and Diez-Gonzalez, 1998; Kihara and Macnab, 1981). Under acidic condition, *lpdA* was repressed, whereas *pflA* as well as *ackA* genes were activated which caused more formate and acetate production. Note that *ldhA* is induced by acid in order to produce more lactate instead of acetate (Bunch et al., 1997) (Table 5.5).

Figure 5.5 shows the overall regulation mechanism we found in the present study. Note that some are the direct effect of pH down-shift, whereas some are indirect due to the change in culture environment such as dissolved oxygen for *arcA* gene expression etc. It should be noted that Table 5.5 indicates the promising feature that lactate production can be enhanced at lower pH. This suggests that the fermentation may be started at pH 7.0 under aerobic condition to enhance the cell growth followed by decreasing pH (just without pH control) under anaerobic condition for lactate production by *pflA/B* mutants.

5.4 Conclusion

E. coli is capable of sensing difference in external pH and reacting to these changes. As shown in Fig. 5.5, the present research result clarified the mechanism of metabolic changes upon pH down shift based on gene expressions of global regulators and the metabolic pathway genes. In particular, the pH down-shift caused the up-regulation of *rpoS* gene, which in turn caused the up-regulation of *gadA* gene expression, and finally protect from intracellular acidification. It was also found that the pH down-shift also caused the up-regulation of *fnr* gene expression, and activated *yfiD* and *pflA* gene expression which caused higher formate production. Moreover, it was shown that the pH down-shift caused

other TCA cycle genes to be repressed due to up-regulation of *arcA* gene indirectly caused by lower dissolved oxygen concentration and caused higher acetate production. This information is useful for the variety of applications such as temperature-induced heterologous protein productions, SSF etc. In particular, it is suggested to use low pH and high temperature in SSF for higher lactate production as *ldhA* gene is induced upon pH down-shift.

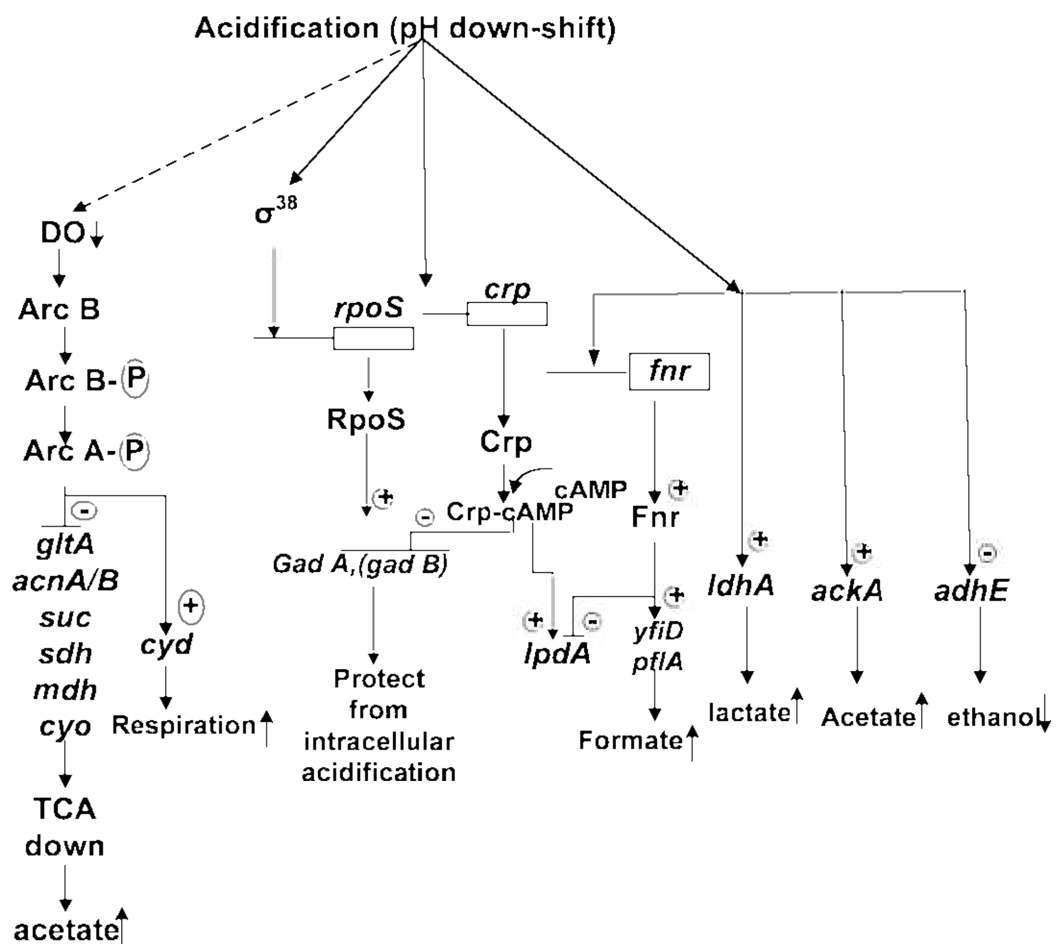

Fig 5.5 Overall metabolic regulation of *E. coli* during acidification.

5.5 References

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Appendix A: Global Regulators and their Regulated Genes

ArcA/B:

+ : *pfl, cydAB*

− : *aceBAK, aceEF, acnA, fumAC, gltA, icdA, lpdA, mdh, ptsG, sdhCDAB, cyo*

pdhR:

+ : *aceEF, ndh, yfiD*

− : *cyoABCD*

Fur:

+ : *hmp*

− : *cyoABCD, ompF*

Cra:

+ : *aceA, acnA, fbp, icdA, pckA, ppsA, cydB*

− : *acnB, eda, edd, eno, gapA, pfkA, ptsHI, pykF*

Crp/Cya:

+ : *mlc, aceEF, acnAB, crr, fumA, gltA, mdh, pckA, ptsG, ptsHI, sdhABCD, sucABCD, tpiA, ompF, rpoS*

− : *lpdA, aceBAK, acnA, cyoA, gdhA, glnAL, gltA, mdh, sdhCDAB, sodA, sucABCD, ugpA,*

Fnr:

+ : *frd, pfl, ackA, ndh, nuoA, pstSCAB-phoU, yfiD*

− : *acnA, fumAC, icdA, lpdA, ptsG, sdhCDAB, talA, cyoABCD, cydAB*

PhoB:

+ : *phoBR, phoA-psiF, asr, pstSCAB-phoU*

− : *phoH, phnCHN, ugpA, argP*

RpoS:

+ : *gadA, gadB, osmB, sodC, talA, tktB, acs, poxB, acnA, fumC*

SoxR/S:

+ : *sodA, zwf, rpoD, rpoS, fumC, tolC, micF, marA*

− : *rob*

Mlc

+ : *ldhA*

− : *crr, ptsG, ptsH1, manXYZ, malT*

GadE

+ : *gadE, gadXW*

− : *cyoABCDE, gltB*

Appendix B: Table of each Gene and its Associated Function

(a) Global regulatory genes

Gene names	Description (function as encoded protein)
arcA	Anoxic redox control
cra	Catabolite repressor activator
crp	cAMP receptor protein
fnr	Fumarate and nitrate reductase
rpoS	RNA polymerase sigma factor
rpoD	RNA polymerase, sigma 70 subunit
soxS	Dual transcriptional activator
soxR	Superoxide Response protein
mlc	Making large colonies protein
fadR	Fatty acid degradation regulator
iclR	Isocitrate lyase repressor
aceA	Isocitrate lyase
acs	AcCoA synthetase
acnB	Aconitase B
fumA	Fumerase

(b) PhoB regulatory genes

Gene names	Description (function as encoded protein)
phoB	Dual transcription regulator
phoR	Sensor kinase of the PhoRB two component signal transduction pathway
phoA	Alkaline phosphatase precursor

phoE	Outer-membrane pore protein
phoH	PhoH protein (phosphate starvation inducible protein PsiH)
phnC	ATP binding component of the alkylphosphonate ABC transporter
pstS	Subunit of phosphate ABC transporter
ugpB	Subunit of glycerol-3-P ABC transporter
phoU	Phosphate transport system regulatory protein
phoM(creC)	Sensor histidine kinase of the CreCB two-component signal transduction system

(c) Metabolic pathway genes

Gene names	Description (function as encoded protein)
ptsH	Phosphohistidinoprotein-hexose phosphotransferase
ptsG	Glucose phosphotransferase enzyme IIBC[Glc]
pfkA	6-phosphofructokinase
pykF	Pyruvate kinase
lpdA	Lipoamide dehydrogenase
gltA	Citrate synthase
icdA	Isocitrate dehydrogenase
sucA	α –ketoglutarate dehydrogenase
sdhC	Succinate dehydrogenase
mdh	Malate dehydrogenase
zwf	Glucose 6-phosphate-1-dehydrogenase
gnd	6-phosphogluconate dehydrogenase
tktA	Transketolase I
tktB	Transketolase II

talA	Transaldolase A
talB	Transaldolase B
edd	6-phosphogluconate dehydratase
eda	Entner-Doudoroff aldolase
ldhA	D-lactate dehydrogenase
yfiD	Stress-induced alternate pyruvate formate-lyase subunit
asr	Acid shock RNA
gadA	Glutamate decarboxylase A
ackA	Acetate kinase
pta	Phosphate acetyltransferase

(d) Nitrogen regulatory genes

Gene names	Description (function as encoded protein)
rpoN	RNA polymerase, sigma 54 (sigma N) factor
gdhA	Glutamate dehydrogenase
gltB	Glutamate synthase, large subunit
gltD	Glutamate synthase, small subunit
glnA	Glutamine synthetase
glnB	Protein PII-control the level and activity of glutamine synthetase
glnD	Uridylyltransferase / uridylyl-removing enzyme
glnE	Glutamine synthetase adenylyltransferase [multifunctional]
glnG	NtrC transcriptional dual regulator
glnK	Nitrogen regulatory protein
glnL	NtrB sensory histidine kinase
nac	Nac DNA-binding transcriptional dual regulator

(e) Respiratory chain genes

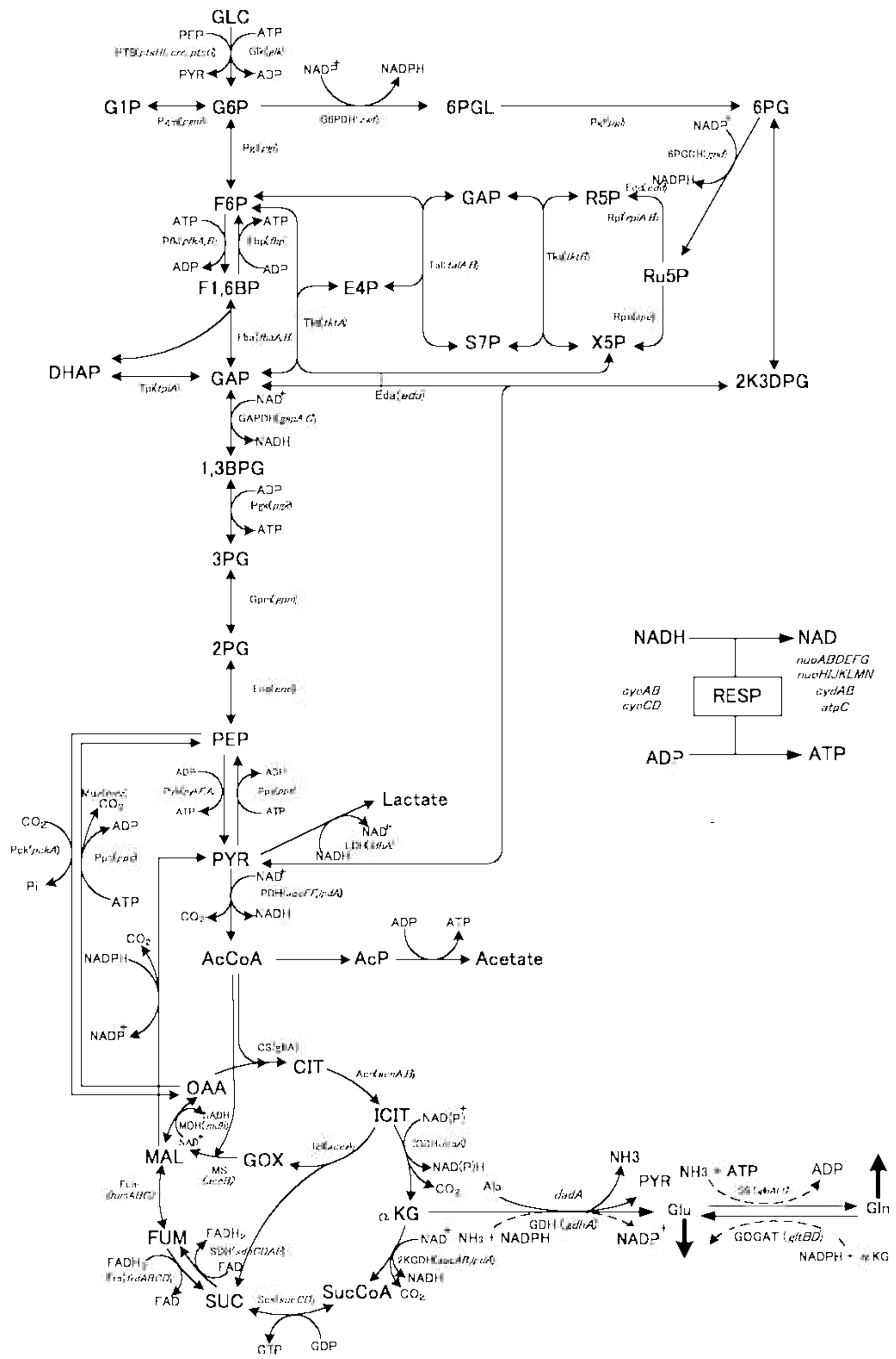
Gene names	Description (function as encoded protein)
cyoA	Cytochrome *bo* terminal oxidase subunit II
cydB	Cytochrome *bd*-I terminal oxidase subunit II
atpA	ATP synthase α- subunit
ndh	Ubiquinone oxidoreductase II
nuoA	NADH: ubiquinone oxidoreductase
sodA	Superoxide dismutase (Mn)

(f) Fermentative pathway genes

Gene names	Description (function as encoded protein)
adhE	Alchol dehydrogenase
ldhA	D-lactate dehydrogenase
pflA	Pyruvate formate lyase
poxB	Pyruvate oxidase

(g) Heat shock and related genes

Gene names	Description (function as encoded protein)
dnaK	Hsp70 molecular chaperone
groL	Chaperonin Cpn60
groS	Chaperonin Cpn10
htpG	Heat shock chaperone



Appendix C: Central metabolic pathways of *E.coli*

Appendix D: List of Abbreviations

α-KG:	2-Ketoglutarate;
AcCoA:	Acetyl-Coenzyme A;
Ack:	Acetate kinase;
AcP:	Acetyl Phosphate;
ADH:	Alcohol dehydrogenase;
AmtB:	Ammonium transport protein;
AP:	Alkaline phosphatase;
ATP:	Adenosine Tri-Phosphate;
cAMP:	Cyclic adenosine monophosphate;
cAMP-Crp:	cAMP receptor protein;
DCW:	Dry Cell Weight;
[4Fe-4S]:	In a number of iron-sulfur proteins;
Frd:	Fumarate7uh reductase;
GABA:	γ-Aminobutyric acid;
GAPDH:	D-glyceraldehyde-3-phosphate dehydrogenase;
GDH:	Glutamate Dehydrogenase;
Gln:	Glutamine;
GOGAT:	Glutamate Syntheses;
GS:	Glutamine Synthetase;
HdeA:	Periplasmic protein;
ICDH:	Icocitrate dehydrogenase;
LDH:	Lactate dehydrogenase;
N:	Nitrogen;
OAA:	Oxaloacetic Acid;
P:	Phosphate;
PDHc:	pyruvate dehydrogenase complex;
PEP:	Phospho-enol-pyruvate;
ppGpp:	Guanosine tetraphosphate;
Pta-Ack:	Phosphotransacetylase-Acetate kinase A;
Pfl:	Pyruvate-formate lyase;
PYR:	Pyruvate;
TCA:	Tri-carboxylic acid;
PTS:	Phosphotransferase System;
UTase:	Uridylyltransferase,

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PUBLICATIONS

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