3

Metabolic regulation by global regulators in response to culture environment

Abstract: Basic metabolic regulation mechanisms are explained in terms of catabolite regulation, nitrogen regulation, and phosphate regulation, as well as the effects of acidic pH, heat shock, and nutrient starvation on metabolic regulations. Attention focuses on the effects of global regulators (transcription factors with sigma factors), such as cAMP-Crp, Cra, Mlc, RpoN, ArcA/B, Fnr, SoxR/S, PhoR/B, RpoH, and RpoS on metabolism. The effects of knockout of such genes as cra, crp, mlc, arcA/B, phoR/B, soxR/S, and rpoS, on metabolic regulation are also explained.

Key words: catabolite regulation; nitrogen regulation; phosphate regulation; acidic pH; heat shock; stress oxygen; nutrient starvation.

3.1 Introduction

It is important to clarify the metabolic regulation mechanism and to understand cellular metabolism in response to changes in culture environment. Microorganisms adapt to the changes in the culture, such as carbon sources, nitrogen sources, oxygen availability, by regulating metabolic pathway genes through global regulators and signal transduction. The central metabolic pathways of a cell are controlled by a number of global regulators or transcription factors, depending on culture conditions (Figure 3.1). Biological systems are known to be robust and adaptable to the culture environment. It has become apparent that such robustness is inherent in biochemical and genetic networks. Several

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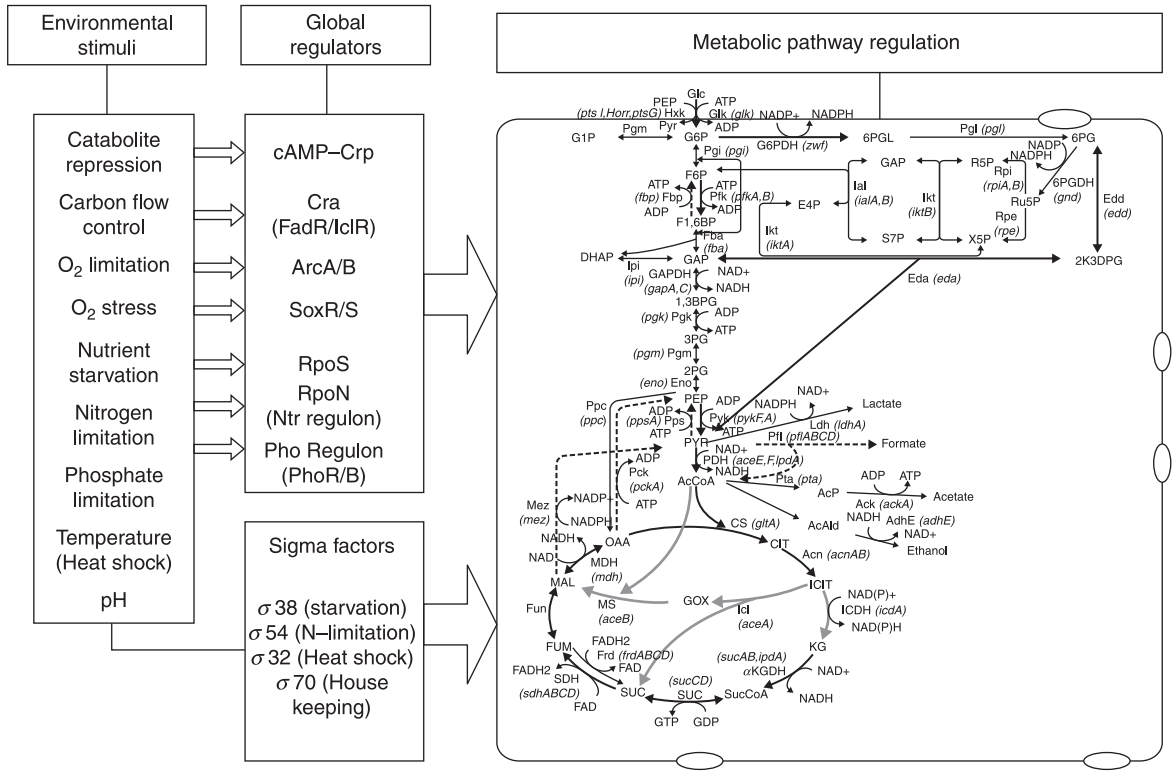

Figure 3.1 Overall metabolic regulation scheme

genes that are necessary to respond to various environmental or nutritional changes require specific recognition by RNA polymerase associated with alternative sigma factors. Here, we consider how the culture environment affects global regulators, and how the metabolic pathway genes are regulated by the corresponding global regulators. The effect of the knockout of the global regulatory gene on metabolism is also considered, so as to understand the roles of global regulators.

3.2 Carbon catabolite regulation

3.2.1 Transport of substrate molecules

The first step in the metabolism of carbohydrates is the transport of these molecules into the cell. In bacteria, various carbohydrates can be taken up by several mechanisms (Gunnewijk et al., 2001). Primary transport of sugars is driven by ATP, while secondary transport is driven by the electrochemical gradients of the translocated molecules across the membrane (Poolman and Konings, 1993), where the secondary transport systems contain the symporters which co-transport two or more molecules, uniporters that transport single molecules, and antiporters that counter-transport two or more molecules. Sugar symporters usually couple the uphill movement of the sugar to the downhill movement of the proton (or sodium ion). Namely, the electrochemical proton (or sodium ion) gradient drives the accumulation of glucose (Gunnewijk et al., 2001). Sugar uptake by group translocation is unique for bacteria and is involved in the phosphotransferase system (PTS), as will be explained in detail in this chapter.

Gram-negative bacteria such as *Escherichia coli* have two concentric membranes surrounding the cytoplasm, and the space between these two membranes is called the periplasm (Figure 3.2). The outer membrane and cytoplasmic membrane constitute a hydrophobic barrier against polar compounds. The outer membrane contains channel proteins, where the specific molecules can only move across these channels. In the outer membrane of *E. coli*, 10^8 channels are formed by the porin proteins (Nikaido and Nake, 1979). The OmpC and OmpF are the most abundant porins present under typical growth conditions, representing up to 2% of the total cellular protein (Nikaido, 1996). Their relative abundance changes, depending on such factors as osmolarity, temperature, and growth phase (Lugtenberg et al., 1976; Hall and Silhavy, 1981; Pratt and Silhavy, 1996). These porins allow glucose to enter the periplasm when

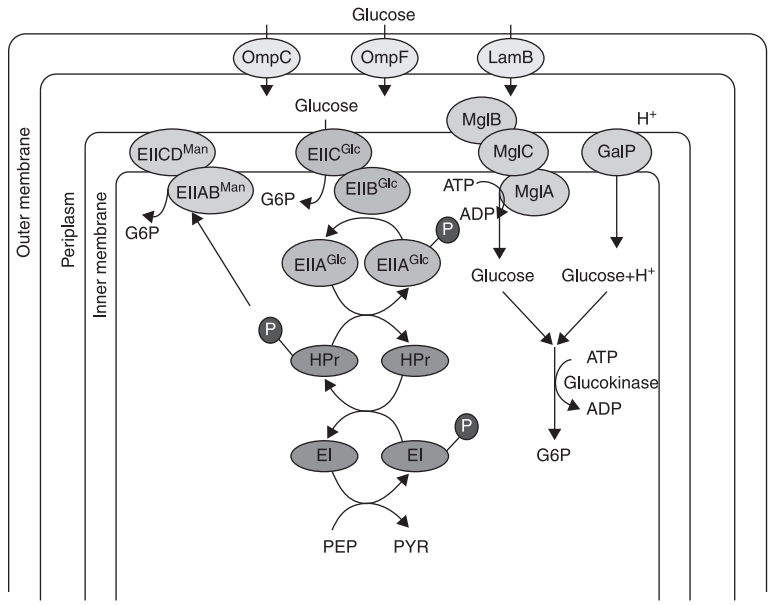

Figure 3.2 Outer and inner membrane and periplasm

glucose is present at higher concentrations than about 0.2 mM (Nikaido and Vaara, 1985; Death et al., 1993). It has been shown that the diffusion rate for glucose is found to be about 2-fold higher through OmpF than through OmpC (Nikaido and Rosenberg, 1983). Under glucose limitation, the outer membrane glycoporin LamB is induced (Death et al., 1993), where this protein permeates several carbohydrates such as maltose, maltodextrins, and glucose (von Meyenburg and Nikaido, 1977). It has also been reported that about 70% of the total glucose import capacity of the cell is contributed by LamB (Deathe et al., 1993). Glucose transport by diffusion through porins of the outer membrane is a passive process (Gosset, 2005).

Once glucose is transported into the periplasm, it can be internalized into the cytoplasm by the PTS. It may be that the glucose concentration in the periplesm is low due to active transport systems in the cytoplasmic membrane (Gosset, 2005). Once inside the periplasm, glucose can be transported into cytosol by PTS, while PTS is widespread in bacteria and absent in Archaea and eukaryotic organisms (Postma et al., 1996; Sair, 2000). PTS is composed of the soluble and non sugar-specific components Enzyme I (EI) encoded by *ptsI* and the phosphohistidine carrier protein (HPr) encoded by *ptsH*, where these transfer phosphoryl group from PEP

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to the sugar-specific enzyme IIA and IIB. Another component of PTS, enzyme IIC (in some cases also IID) is an integral membrane protein permease that recognizes and transports the sugar molecules, where these are phosphorylated by EIIB. Twenty-one different enzyme II complexes in *E. coli* have been reported, which are involved in the transport of about 20 different carbohydrates (Tchieu et al., 2001). In *E. coli*, EII^{Glc} and EII^{Man} are involved in the transport of glucose. EII^{Glc} is composed of soluble $EIIA^{Glc}$ encoded by *crr*, and the integral membrane permease $EIICB^{Glc}$ is encoded by *ptsG*. The EII^{Man} complex is composed of the $EIIAB^{Man}$ homodimer enzyme and the integral membrane permease $EIICD^{Man}$ (Figure 3.2), where these proteins are encoded in the *manXYZ* operon (Gosset, 2005). In addition to mannose, these proteins can also transport glucose, fructose, N-acetylglucosamines, and glucosamine with similar efficiency (Curtis and Epstein, 1975). In a wild-type strain growing with glucose as a carbon source, *ptsG* is induced, while the *manXYZ* operon is repressed. In the *ptsG* mutant, the glucose can be transported by the EII^{Man} complex, and the cell can grow with less growth rate than the wild type (Chou et al., 1994). When the extracellular glucose concentration is less than 1 μM, this can also be utilized, even at higher glucose concentrations of more than 2 g/L for *pts* mutants (Flores et al., 2005). The induction of these genes is caused by the intracellular galactose that functions as an auto-inducer of the system (Death and Ferenci, 1994). One of the genes induced under glucose limitation is *galP*, which codes for the low affinity galactose: H^+ symporter GalP (Figure 3.2).

The genes in the *mglABC* operon encode an ATP-binding protein, a galactose/glucose periplasmic binding protein, and an integral membrane transporter protein, respectively, forming an Mgl system for galactose/glucose (methyl galactoside) import (Gosset, 2005). This high affinity importer belongs to the ATP-binding cassette (ABC) superfamily of the primary active class of transporters (Gosset, 2005). When the extracellular glucose concentration is very low, the Mgl system, together with LamB, attains high-affinity glucose transport (Gosset, 2005). The glucose molecule transported either by GalP or Mgl systems must be phosphorylated by Glk encoded by *glk* from ATP to become G6P (Figure 3.2) (Lunin et al., 2004).

Note that PTS seems to be efficient as it consumes 1 mole of PEP for each internalized and phosphorylated glucose, where 1 mole of PEP is equivalent to 1 mole of ATP, since the conversion of PEP to PYR by Pyk would yield 1 mole of ATP by substrate-level phosphorylation. The high affinity Mgl-glucokinase system is energetically the most expensive, as it consumes 2 moles of ATP per glucose. The GalP-glucokinase system

requires 1 mol of H^+ that is internalized into the cytoplasm and 1 mol of ATP (Figure 3.2).

3.2.2 Carbon catabolite regulation

Among culture environments, carbon sources are by far the most important for the cell, from the point of view of energy generation and biosynthesis. Most living organisms, including bacteria, can use various compounds as carbon sources, where these can be either co-metabolized or selectively used with preference for the specific carbon sources selected from among those available. One typical example of selective carbon-source usage is the diauxie phenomenon observed in *E. coli*, when a mixture of glucose and lactose is used as a carbon source and this phenomenon was first observed by Monod (1942). Subsequent investigations on this phenomenon have revealed that selective-carbon source utilization is common and that glucose is the preferred carbon source in many organisms. Moreover, the presence of glucose often prevents the use of other carbon sources. This preference of glucose over other carbon sources has been named glucose repression, or more generally carbon catabolite repression (CCR) (Magasanik, 1961). CCR is observed in most heterotrophic bacteria, which include facultatively autotrophic bacteria that repress the genes for CO_2 fixation in the presence of an organic carbon source (Bowien and Kusian, 2002). Some pathogenic bacteria, such as *Chlamydia trachomatis* and *Mycoplasma pneumonia*, seem to lack CCR, where these are adapted to nutrient-rich host environments (Nicholson et al., 2004; Halbedel et al., 2007). Another phenomenon can be seen in *Corynebacterium glutamicum*, where co-assimilation of glucose and other carbon sources is made, but it is highly regulated (Wendisch et al., 2000; Frunzke et al., 2008). For some bacteria, such as *Streptococcus thermophilus*, *Bifidobacterium longum*, and *Pseudomonas aeruginosa*, glucose is not a primary carbon source, and the genes for glucose utilization are repressed when preferred carbon sources are available; this phenomenon is known as reverse CCR (Collier et al., 1996; Oarche et al., 2006). CCR is one of the most important regulatory phenomena in many bacteria (Moreno et al., 2001; Blencke et al., 2003; Liu et al., 2005). CCR is important for the cells to compete with other organisms in nature, where it is crucial to select a preferred carbon source in order to improve the growth rate, which then results in higher survival rates than other organisms. Moreover, CCR has a crucial role in the expression of virulence genes, which often enable the

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organism to access new sources of nutrients. The ability to select the appropriate carbon source that allows fastest growth may be the driving force for the evolution of CCR (Gorke and Stulke, 2008).

3.2.3 Catabolite control of sugar transporters

The *E. coli lac* operon is only expressed if allolactose (a lactose isomer formed by *β*-galactosidase) binds and inactivates the *lac* repressor. Lactose cannot be transported into the cell in the presence of glucose, because the lactose permease, LacY, is inactive in the presence of glucose (Winkler and Wilson, 1967). As shown in Figure 3.3, phosphorylated E II A^{Glc} is dominant when glucose is absent, and does not interact with LacY, whereas unphosphorylated $EIIA^{Glc}$ can bind and inactivates LacY when glucose is present (Nelson et al., 1983; Hogema et al., 1999). Note that this only occurs if lactose is present (Smirnova et al., 2007). The same mechanism may be seen in the transport of other secondary carbon sources, such as maltose, melibiose, raffinose, and galactose (Misko et al., 1987; Titgemeyer et al., 1994).

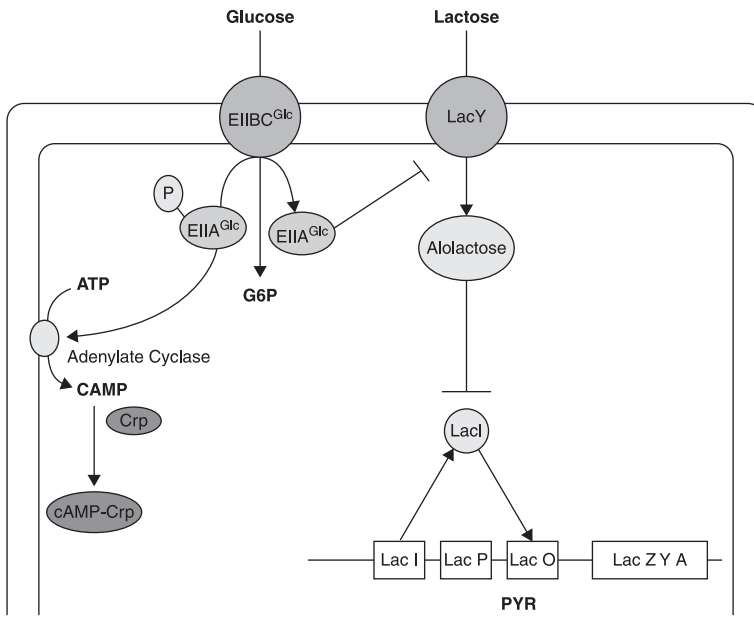

Figure 3.3 Inducer exclusion and the activation of adenylate cyclase in the glucose-lactose system

Inducer exclusion has also been reported for Gram-positive bacteria, and HPr is the major player in these organisms. In *Lactobacillus brevis*, HPr(Ser-P) is formed when glucose is present, and binds and inactivates permease (Djordjevic et al., 2001). By contrast, the lactose permease of *S. thermophilus* is controlled by HPr (His-P)-dependent phosphorylation. In the absence of glucose, HPr (His-P) can phosphorylate a PTS-like domain, thereby activating the permease for lactose transport (Poolman et al., 1995). When glucose is present, HPr becomes phosphorylated on Ser46 and can no longer activate the lactose permease (Gunnewijk and Poolman, 2000).

3.2.4 CCR in *E. coli*

The central players in carbon catabolite regulation in *E. coli* are the transcriptional activator Crp (cyclic AMP (cAMP) receptor protein, also called catabolite gene-activator protein (CAP)), the signal metabolite cAMP, adenylate cyclase (Cya), the phosphoenol pyruvate (PEP), and carbohydrate phosphotransferase systems (PTSs), where these systems are involved in both transport and phosphorylation of carbohydrates. The PTS in *E. coli* consists of two common cytoplasmic proteins, EI (enzyme I) encoded by *ptsI* and HPr (histidine-phosphorylatable protein) encoded by *ptsH*, as well as carbohydrate-specific EII (enzyme II) complexes (Figure 3.2). The glucose-specific PTS in *E. coli* consists of the cytoplasmic protein $EIIA^{Glc}$ encoded by *crr* and the membrane-bound protein $EIICB^{Glc}$ encoded by *ptsG*, which transport and concomitantly phosphorylate glucose. The phosphoryl groups are transferred from PEP via successive phosphorelay reactions in turn by EI, HPr, $EIIA^{Glc}$, and $EIICB^{Glc}$ to glucose. The cAMP-Crp complex and the repressor Mlc are involved in the regulation of the *ptsG* gene and the *pts* operon expressions. It has been demonstrated that unphosphorylated $EIICB^{Glc}$ can relieve the expression of *ptsG* gene expression by sequestering Mlc from its binding sites through a direct protein–protein interaction in response to glucose concentration. In contrast to Mlc, where it represses the expressions of *ptsG*, *ptsHI*, and *crr* (Plumbridge, 1998a,b), the cAMP-Crp complex activates *ptsG* gene expression (De Reuse and Danchin, 1988) (Figure 3.4). Since intracellular cAMP levels are low during growth on glucose, these two antagonistic regulatory mechanisms guarantee precise adjustments of *ptsG* expression levels under various conditions (Bettenbrock et al., 2006) (Figure 3.4). It should be noted that unphosphorylated $EIIA^{Glc}$ inhibits the uptake of other non-PTS carbohydrates by the so-called inducer exclusion (de Boris,

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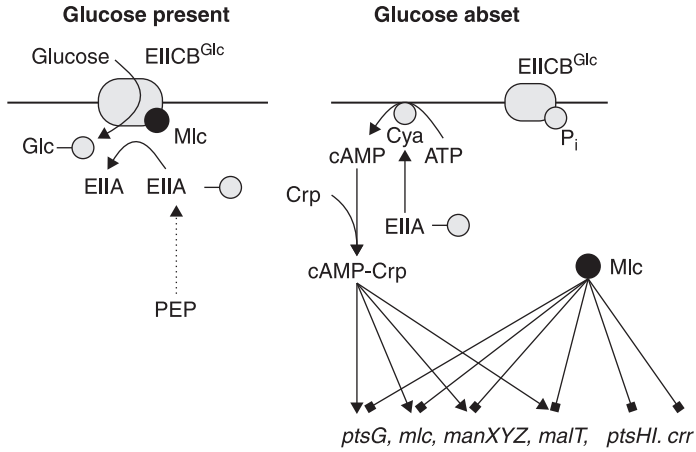

Figure 3.4 The multiple regulations by Mlc and cAMP-Crp

2008), while phosphorylated $EIIA^{Glc}$ ($EIIA^{Glc}$-P) activates adenylate cyclase (Cya), which generates cAMP from ATP and leads to an increase in the intracellular cAMP level (Park et al., 2006) (Figure 3.4). In the absence of glucose, Mlc binds to the upstream of the *ptsG* gene and prevents its transcription. If glucose is present in the medium, the amount of unphosphorylated $EIICB^{Glc}$ increases due to the phosphate transfer to glucose. In this situation, Mlc binds to $EIICB^{Glc}$, and thus it does not bind to the operator of *pts* genes (Lee et al., 2000; Tanaka et al., 2000; Bettenbrock et al., 2006). Note that if the concentration ratio between PEP and PYR (PEP/PYR) is high, $EIIA^{Glc}$ is predominantly phosphorylated, whereas if this ratio is low, then $EIIA^{Glc}$ is predominantly dephosphorylated (Hogema et al., 1998; Bettenbrock et al., 2007). $EIIA^{Glc}$ is preferentially dephosphorylated when *E. coli* cells grow rapidly with glucose as a carbon source (Hogema et al., 1998; Bettenbrock et al., 2007). Note also that cAMP levels are low during growth with non-PTS carbohydrates such as lactose, where the PEP/PYR ratio is the key factor that controls phosphorylation of $EIIA^{Glc}$, which explains dephosphorylation of $EIIA^{Glc}$, resulting in a low cAMP pool (Hogema et al., 1998; Bettenbrock et al., 2007). As stated above, inducer exclusion is the dominant factor for the glucose-lactose diauxie (Inada et al., 1996a,b; Hogema et al., 1999). The role of cAMP-Crp is then to express the *lac* operon, which is involved in CCR by activating the expression of *ptsG* and EIICB domain of the glucose-specific PTS, and therefore transport of glucose (Kimata et al., 1997).

3.2.5 Carbon flow control in E. coli

In addition to cAMP-Crp, which is dependent on the level of glucose concentration, the catabolite repressor/activator protein (Cra), originally characterized as the fructose repressor (FruR), plays an important role in the control of carbon flow in *E. coli* (Saier et al., 1997; Saier and Ramseier, 1996; Moat et al., 2002). The carbon uptake and glycolysis genes, such as *ptsHI*, *pfkA*, *pykF*, *zwf*, and *edd–eda*, are reported to be repressed, while *ppsA*, *fbp*, *pckA*, *icd*, *aceA*, and *aceB* are activated by the Cra protein (Seier and Ramseir, 1996; Moat et al., 2002) (Appendix A). It has been shown that genes, such as *pfkA*, *pyk*F, and *edd–eda*, have Cra binding sites that overlap or follow the RNAP-binding site (Chin et al., 1989; Ramseier et al., 1995; 1996; Lee et al., 2003). It is shown that a mutant defective in the *cra* gene is unable to grow on gluconeogenic substrates such as pyruvate, acetate, and lactate (Saier et al., 1997). This appears to be due to a deficiency in the gluconeogenic enzymes, such as Pps, Pck, some TCA cycle enzymes, the two glyoxylate-shunt enzymes, and certain electron transport carriers (Saier et al., 1997). Molecular level research on *cra* gene expression has been done by several researchers using *lacZ*-transcriptional fusion (Cortay et al., 1994; Ryu et al., 1995; Ramseier et al., 1996; Mikulskis et al., 1997; Prost et al., 1999). The gluconeogenic pathway is deactivated by the knockout of the *cra* gene, and the carbon flow toward catabolism and the glucose consumption rate are expected to increase, since glycolysis pathway genes such as *ptsHI*, *pfkA*, and *pykF* are activated by the *cra* gene knockout. It has been shown that multiple genes knockout, when knocked out together with *cra* gene, and can increase the consumption rate of the substrate and thus improve the rate of metabolite production under certain culture conditions (Sarkar and Shimizu, 2008). However, the regulation mechanism is complex since *icdA*, *aceA*, *B*, and *cydB* genes are repressed, while *zwf* and *edd* gene expressions are activated and thus the ED pathway is activated by the *cra* knockout gene (Sarkar and Shimizu, 2008). The details of such complex regulation networks have not yet been fully investigated for the *cra* mutant (Saier et al., 1997). Phue et al. (2005) also studied the role of the *cra* gene in relation to high density cell cultures of *E. coli B* and *E. coli K*.

3.2.6 Fermentation characteristics and gene expressions of cra gene knockout mutant

Figure 3.5 shows the aerobic batch cultivation result for the wild type and its *cra* gene knockout mutant, where acetate tends to be less consumed

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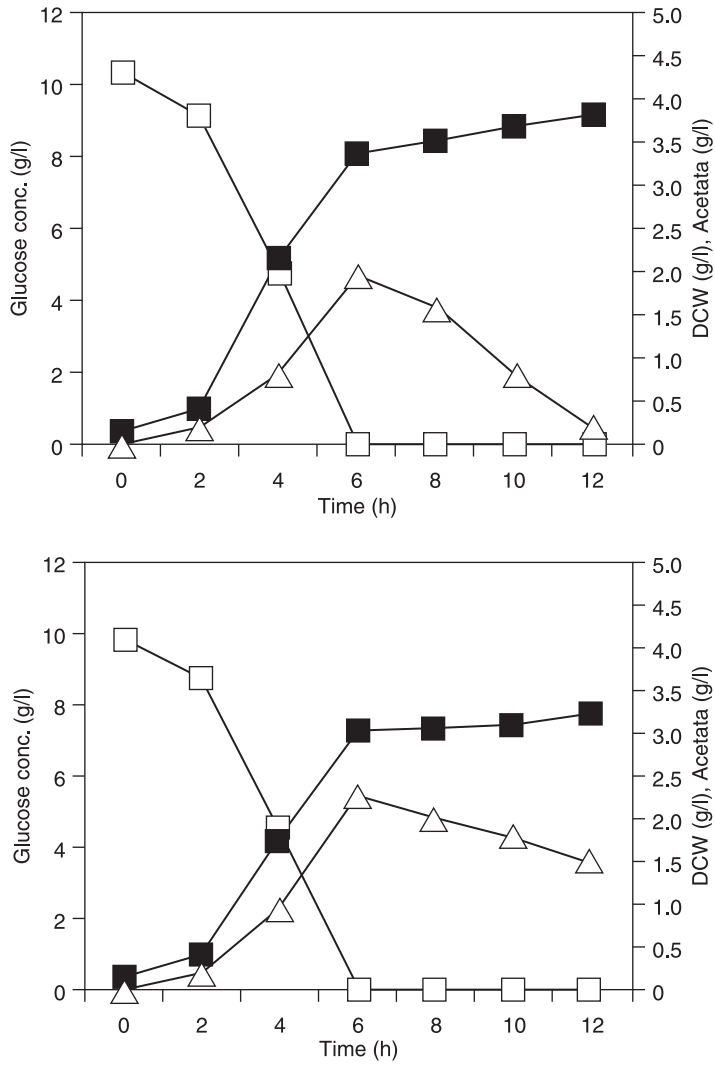

Figure 3.5 **Batch cultivation of (a) *E. coli* BW25113 and (b) its *cra* mutant: □, glucose concentration; ■, biomass concentration; Δ acetate concentration**

at the late growth phase in the case of the *cra* mutant when compared to the parent strain (Sarkar and Shimizu, 2008; Sarkar et al., 2008).

Table 3.1 shows the fermentation characteristics of *E. coli* wild type (BW25113) and its *cra* mutant cultivated in continuous cultures at a dilution rate of 0.2 h^{-1}. The results show the increases in the specific

Table 3.1 Growth parameters for *E. coli* BW25113 and its *cra* mutant cultivated at the dilution rate of 0.2 h^{-1} where feed glucose concentration was 4 g/l

Growth parameters	BW25113	*cra* mutant
Biomass yield (gg-1)	0.44 ± 0.01	0.30 ± 0.02
Glucose uptake rate (mmole.g^{-1}.h^{-1})	2.54 ± 0.11	3.61 ± 0.03
Acetate production rate (mmole.g^{-1}.h^{-1})	0.02 ± 0.01	0.84 ± 0.02
O_2 uptake rate (mmole.g^{-1}.h^{-1})	10.24 ± 0.52	8.88 ± 0.63
CO_2 evolution rate (mmole.g^{-1}.h^{-1})	8.51 ± 0.35	8.57 ± 0.8

glucose uptake rate and the acetate production rate for the mutant, when compared to the parent strain. Table 3.1 also shows that the cell yield for the mutant is reduced, when compared to the parent strain.

Table 3.2a shows that the expression of the glycolytic pathway gene *pykF* is up-regulated, and those of the gluconeogenic pathway genes, such as *ppsA* and *fbp*, are down-regulated as expected. The gene expression related to the TCA cycle, such as *acnA*, is down-regulated, and the glyoxylate pathway related genes, such as *aceA* and *aceK* were down-regulated in the mutant as expected. The pentose phosphate (PP) pathway related genes such as *zwf* are up-regulated in the mutant. The *adhE* gene is also up-regulated in the mutant, when compared to the parent strain, consistent with the results of Mikulskis et al. (1997). The respiratory pathway related genes, such as *cydA* and *cydB* genes, are down-regulated, which is known to be positively controlled by Cra (Ramseier et al., 1996). The *fru* operon of enteric bacteria is known to be regulated at the transcriptional level by Cra. In the absence of the *cra* gene, the fructose operon genes, such as *fruK* and *fruB*, are up-regulated in the mutant. Other carbohydrate related genes, such as the fucose operon genes, are also up-regulated in the *cra* mutant. Table 3.2b shows that some of the transporter genes, such as *ptsH*, *mglC*, and *xylE*, are up-regulated in the mutant. These are involved in the transport of glucose, galactose, and xylose. Other transporters for the amino acids, such as *gltL* and *proV*, which encode glutamate/aspartate and glycine betaine/l-proline transport proteins, are also up-regulated in the *cra* mutant. Table 3.2c shows that the expression level of the *fabA* gene, which encodes the enzyme that catalyzes the key reaction from unsaturated to saturated fatty acid, is up-regulated in the mutant. The *fabH* (β-ketoacyl-ACP synthase I) involved in the initial step of fatty acid elongation is also up-regulated.

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Table 3.2 Gene expressions of *cra* mutant as compared with the wild type strain

(a) Carbon and energy related genes

Gene	Log_2 (cra/parent)	Description
aceA	–1.43	Isocitrate lyase (EC 4.1.3.1)
aceK	–0.30	Isocitrate dehydrogenase kinase/phosphatase (EC 2.7.1.116) (EC 3.1.3.-).
adhE	1.39	Alcohol_dehydrogenase_(EC__1.1.1.1)
cydB	–1.09	Cytochrome_d_ubiquinol_oxidase_subunit_II_ (EC__1.10.3.-)
cydA	–1.12	Cytochrome_d_ubiquinol_oxidase_subunit_I_ (EC__1.10.3.-)
fbp	–0.81	Fructose-1,6-bisphosphatase (EC 3.1.3.11)
fruB	1.29	PTS_system,_fructose-specific_IIA/FPR_ component_(EIIA-Fru)
fruK	1.42	1-phosphofructokinase_(EC__2.7.1.56)_ (fructose_1-phosphate_kinase)
fucA	1.14	L-fuculose_phosphate_aldolase_(EC_4.1.2.17)
fuci	1.03	Fucose_Isomerase_FucI_(EC__5.-.-.-)
fucK	1.56	L-fuculokinase (EC 2.7.1.51) (L-fuculose kinase).
fucO	1.48	Lactaldehyde_reductase_(EC_1.1.1.77)
fucP	1.12	Fucose_permease
fucU	0.93	Fucose_operon_FucU_protein
gcl	–1.06	Glyoxylate_carboligase_(EC_4.1.1.47)_(tartronate-semialdehyde_synthase)
gltA	–0.36	Citrate_synthase_(EC__4.1.3.7)
gpsA	–0.58	L-glycerol_3-phosphate_dehydrogenase
mdh	–0.39	Malate_dehydrogenase_(EC__1.1.1.37)
ppsA	–0.84	Phosphoenolpyruvate synthase (EC 2.7.9.2)
pykF	1.43	Pyruvate_kinase_(EC__2.7.1.40)
tktA	1.52	Transketolase_1_(EC__2.2.1.1)_(tk_1)
xylA	1.6	Xylose_isomerase_(EC__5.3.1.5)_(version_1)
zwf	1.76	Glucose-6-phosphate_1-dehydrogenase_ (EC_1.1.1.49)

(*continued*)

Table 3.2 Gene expressions of *cra* mutant as compared with the wild type strain (*continued*)

(b) Metabolic transport related genes

Gene	Log_2 (cra/parent)	Description
gltL	1.47	Glutamate/aspartate_transport_atp-binding_protein_gltL
malK	1.46	Maltose/maltodextrin_transport_ATP-binding_protein_MalK
manX	1.65	Phosphotransferase_system_enzyme_II_(EC__2.7.1.69),_mannose-specific,_factor_III
mglC	1.34	Galactoside_transport_system_permease_protein
proV	1.41	Glycine_betaine/l-proline_transport_ATP-binding_protein_ProV
ptsH	1.57	PTS_system, Phosphocarrier protein HPr (Histidine-containing protein).
xylE	1.75	D-xylose-proton symporter (D-xylose transporter).

(c) Fatty acids, purine, and pyrimidine metabolism related genes

Gene	Log_2 (cra/parent)	Description
fabA	2.00	3-hydroxydecanoyl-[acyl-carrier-protein]_dehydratase_(EC__4.2.1.60)
fabB	1.02	3-oxoacyl-[acyl-carrier-protein]_synthase_I_(EC__2.3.1.41)
fabH	1.32	3-oxoacyl-[acyl-carrier-protein]_synthase_(EC__2.3.1.41)_III.
kdsB	–1.00	3-deoxy-manno-octulosonate_cytidylyltransferase_(EC__2.7.7.38)
nth	–2.06	Endonuclease III (EC 4.2.99.18) (DNA-(apurinic or apyrimidinic site) lyase).
purE	0.82	Phosphoribosylaminoimidazole carboxylase catalytic subunit (EC 4.1.1.21) (AIR carboxylase) (AIRC).
purF	0.85	Amidophosphoribosyltransferase_(EC__2.4.2.14)

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(d) Amino acid metabolism genes

Gene	Log_2 (cra/parent)	Description
aroC	–0.76	Chorismate_synthase_(EC__4.6.1.4).
aroG	–0.78	Phospho-2-dehydro-3-deoxyheptonate aldolase, Phe-sensitive (EC 4.1.2.15) (Phospho-2-keto-3-deoxyheptonate aldolase)
artI	–1.12	Arginine-binding periplasmic protein 1 precursor.
aroL	–0.74	Shikimate_kinase_(EC__2.7.1.71)_II
aroP	–3.06	Aromatic amino acid transport protein aroP (General aromatic amino acid permease).
cysK	–0.78	Cysteine_synthase_A_(EC__4.2.99.8)
hisI	–1.09	Histidine biosynthesis bifunctional protein hisIE [Includes: Phosphoribosyl-AMP cyclohydrolase (EC 3.5.4.19) (PRA-CH); Phosphoribosyl-ATP pyrophosphatase (EC 3.6.1.31) (PRA-PH)].
hisC	–0.92	Histidinol-phosphate_aminotransferase
ilvG	–0.78	Acetolactate_synthase_(EC__4.1.3.18)_II_large_chain
mhpE	–1.06	4-hydroxy-2-oxovalerate_aldolase_(EC_4.1.3.-)
pepD	–2.06	X-his_dipeptidase_(EC__3.4.13.3)_precursor
torA	–1.00	Trimethylamine-n oxide_reductase_precursor_ (EC__1.6.6.9)
trpE	1.59	Anthranilate_synthase_component_I_(EC__4.1.3.27)
trpC	0.96	Tryptophan biosynthesis protein trpCF [Includes: Indole-3-glycerol phosphate synthase (EC 4.1.1.48) (IGPS); N-(5′-phospho- ribosyl)anthranilate isomerase (EC 5.3.1.24) (PRAI)].
ybaS	–1.32	Probable glutaminase ybaS (EC 3.5.1.2).

(e) Global and metabolic regulatory genes

Gene	Log_2 (cra/parent)	Description
icIR	1.96	Acetate operon repressor.(Repressor_protein_IclR)
fis	1.52	DNA-binding protein fis (Factor-for-inversion stimulation protein) (HIN recombinational enhancer binding protein).
fucR	–0.47	L-fucose operon activator.
lysR	1.51	Transcriptional activator protein lysR.
purR	–0.45	Purine nucleotide synthesis repressor.
rpoS	1.62	RNA polymerase sigma-stationary_phase

Table 3.2d shows that most of the genes involved in amino acids metabolism are down-regulated, where most of these genes are related to the non-aromatic amino acids biosynthetic pathway. Although some aromatic amino acids synthetic genes, such as *trpE*, and *trpC*, are up-regulated in the mutant, the *aroL*, *aroG*, and *aroC* genes are down-regulated in the mutant. The histidine biosynthesis related genes, such as *hisI* and *hisC*, are also down-regulated in the mutant. Table 3.2e shows that the *lysR* gene, which encodes the LysR protein that participates in the control of several genes involved in lysine biosynthesis, are up-regulated. The repressor *purR* of the *pur* operon involved in the purine biosynthesis is down-regulated. This is consistent with the up-regulation of purine biosynthesis gene. The *iclR* gene is up-regulated, which is in accordance with the down-regulation of the glyoxylate pathway genes in the mutant. The stress related regulatory genes such as *rpoS* are up-regulated in the mutant. The *fis* gene, which serves as an early signal of nutritional up-shift, is also up-regulated in the mutant as compared to its parent strain. Some of the global regulatory genes, such as *arcA* and *fnr*, do not change in their expression patterns in the mutant when compared to the parent strain.

Some of the enzyme activities are also given in Figure 3.6, where EMP pathway enzymes such as Pfk and Pyk are up-regulated, as well as G6PDH and ED pathway enzyme activities. However, the activity of the gluconeogenic enzymes such as Pck and the activities of ICDH and Icl are down-regulated, and the Ack activity is up-regulated.

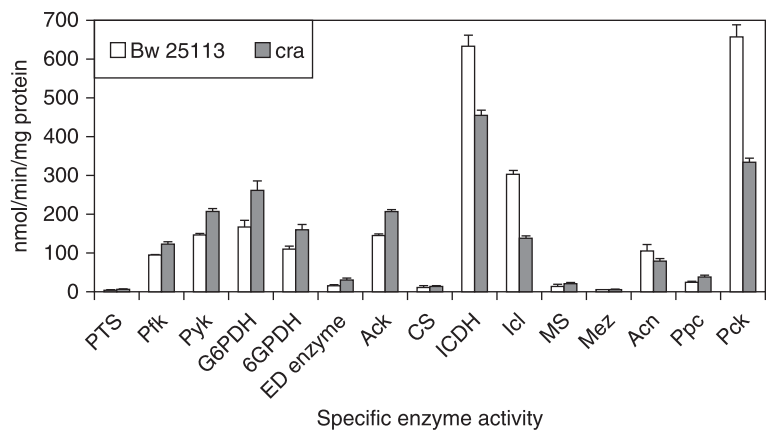

Figure 3.6 **Comparison of enzyme activities of the *cra* mutant as compared to the wild type (BW25113)**

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3.2.7 Effect of glucose concentration on gene expressions in E. coli

Let us consider how culture conditions, such as glucose concentrations, affect global regulators and metabolic pathway genes of wild-type *E. coli* (BW25113) (Yao et al., 2011). Table 3.3 shows the fermentation characteristics of the wild-type *E. coli* for the continuous culture at different dilution rates, where it indicates that the specific glucose uptake rate, acetate production rate, and the specific CO_2 evolution rate (CER) increase as the dilution rate increases. Figure 3.7 shows the effect of the dilution rate (the specific growth rate) on gene transcript levels, where it indicates that in accordance with the increased specific glucose consumption rate, the transcript levels of *ptsG*, *ptsH*, and *pfkA* increase as the dilution rate increases, where the *cra* transcript level decreases and *crp* as well as *mlc* decreases accordingly (Appendix A). The decrease in *crp* is also coincident with a decrease in *cyaA*, which encodes Cya. The transcript levels of *zwf*, *gnd*, *edd*, and *eda* increase as the dilution rate increases in accordance with the decrease in *cra*. The transcript level of *ppc* increases while *pckA* decreases as the dilution rate increases. Moreover, the transcript levels of *fadR* and *iclR* increases, and *aceA* and *aceB* decrease as the dilution rate increases. In accordance with the increase in the specific acetate production rate, the transcript levels of *pta* and *ackA* increase. The TCA cycle genes, such as *gltA*, *acnA*, *fumA*, and *fumC* decrease, while *acnB*, *icdA*, and *lpdA* increase as the dilution rate increases. In accordance with the increase in *soxR/S* transcript levels, *zwf* and *sodA* increase (except the case of dilution rate at 0.7 h^{-1}). In accordance with the decrease in the transcript level of *arcA*, the transcript level of *cyoA* increases, while *cydB* decreases as the dilution rate increases, where the latter phenomenon also coincides with the decrease in *cra* transcript levels (Appendix A). Further observations indicate that in accordance with the decrease in *rpoS* transcript level, *tktB*, *acnA*, and *fumC* decrease as the dilution rate increases.

The effect of the dilution rate (the specific growth rate) on gene transcript levels and fermentation characteristics may be explained as follows: As the dilution rate increases, the glucose concentration increases (though under detectable level in Table 3.3), which causes *cra* transcript levels to be decreased. The *crp* transcript level also decreases as the dilution rate increased, which may be due to a decrease in phosphorylated $EIIA^{Glc}$, causes by higher glucose concentration. This deactivated Cya (as implied by *cyaA* in Figure 3.7b) in turn caused a decrease in cAMP concentrations, and thus cAMP-Crp or *crp* transcript levels decreased (Figure 3.7a). It has

Table 3.3 Effects of dilution rate on fermentation characteristics of wild type *E. coli*

Dilution rate (h^{-1})	Biomass conc. (g/L)	Glucose conc. (g/L)	Specific glucose uptake rate (mmol/g/h)	Specific acetate formation rate (mmol/g/h)	Biomass yield (g/g)	Specific CER (mmol/g/h)	Carbon recovery
0.2	1.45 ± 0.06	ND*	3.07 ± 0.13	ND*	0.37 ± 0.015	9.15	94%
0.4	1.87 ± 0.09	ND*	4.75 ± 0.23	0.01	0.47 ± 0.023	11.61	98%
0.6	2.0 ± 0.09	ND*	6.67 ± 0.3	0.88 ± 0.04	0.5 ± 0.023	13.17	98%
0.7	1.93 ± 0.08	ND*	8.05 ± 0.34	1.33 ± 0.06	0.48 ± 0.02	15.83	97%

ND*: not detectable, where glucose detectable limit is 0.038 g/l, CER, the CO_2 production rate

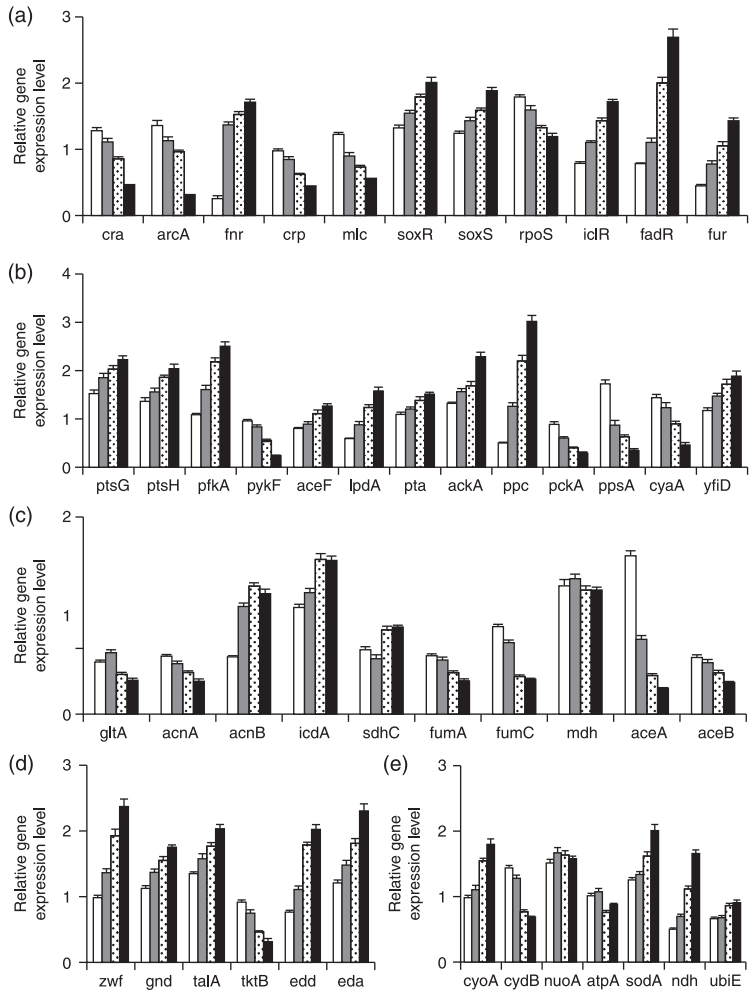

Figure 3.7 **The effect of dilution rate on the gene transcript levels. (a) Global regulator genes; (b) PTS, glycolysis, anaplerotic pathway, *cyaA* and *yfiD* genes; (c) TCA and glyoxylate pathway genes; (d) PP pathway genes; (e) Respiratory chain genes. □ : 0.2 ; ▒ : 0.4 ; ⁝ : 0.6 ; ■ : 0.7**

been reported that cAMP levels start to increase when the glucose concentration becomes less than 0.3 mM. The increases in *fadR* and *iclR* (Figure 3.7a) also coincides with the increase in the glucose concentration as the dilution rate increases. The decrease of *rpoS* (Figure 3.7a) may be also explained by less nutrient stress as the dilution rate increases.

Referring to Figure 3.7 and Appendix A, some of the metabolic pathway transcript levels may be explained with respect to the change in global regulators. The decreases of *aceA* and *aceB* transcript levels coincide with increases of those of *iclR* and *fadR* as the dilution rate increases. The decrease of the transcript levels of *aceA*, *B*, and *pckA*, *ppsA*, and *acnA* (Figure 3.7b,c) coincide with the decrease in the *cra* transcript levels (Figure 3.7a), which also causes an increase in *eda*, *edd*, *pfkA*, and *ptsH* transcript levels. The increase of *cyoA* and decrease of *cydB* coincide with the decrease in *arcA* as the dilution rate increases, where the decrease in *cydB* also coincides with the decrease in *cra*. Note that *ppc* transcript levels increase as the dilution rate increases, which may be due to increased F1,6BP (FDP), where FDP is the effector of Ppc. Although *lpdA* and *aceF* transcript levels increase as the dilution rate increases, TCA cycle gene transcript levels, such as *gltA*, *acnA*, *fumA*, and *fumC* tend to decrease, while *acnB* and *icdA* (*sdhC*) transcript levels tend to increase. The increase in *lpdA* transcript levels may be due to a decrease in *crp,* where cAMP-Crp represses such gene expression. The decrease of *acnA* and *fumC* transcript levels may be caused by the decrease in *rpoS* (Appendix A). The increase in *soxR/S* causes *zwf* and *sodA* to be increased, whereas *acnA* and *fumC* decreases. The latter may be due to a decrease in *rpoS* transcript levels (Appendix A). The increase in *yfiD* may be due to an increase in *fnr* transcript levels, though Fnr may be in an inactive form under aerobic conditions. Upon complex formation of cAMP-Crp, it activates genes encoding the glucose PTS, the TCA cycle, and gluconeogenesis (Appendix A). Although *crp* transcript levels decrease as the dilution rate increases (Figure 3.7a), as stated above, *ptsG* and *ptsH* transcript levels increases (Figure 3.7b). This may be caused by the decrease in *cra*, where *pfkA* gene expression is also increased.

3.2.8 Effect of crp gene mutation on the metabolism

Table 3.4 shows the effect of the *crp* gene knockout and *crp* enhancement (crp^+) on the fermentation characteristics at the dilution rate of 0.2 h^{-1}, where it indicates that the specific glucose uptake rate is lower and the cell concentration is higher as compared to the wild type, and the specific acetate production rate is higher for the *crp* knockout mutant (Yao et al., 2011). In the case of the crp^+ mutant, the fermentation characteristics are similar to the wild type by considering the statistical significance.

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Table 3.4 Effects of the specific gene mutation on the fermentation characteristics at the dilution rate of 0.2 h^{-1}

Strains	Biomass conc. (g/L)	Glucose conc. (g/L)	Glucose uptake rate (mmol/g/h)	Acetate formation rate (mmol/g/h)	Biomass yield (g/g)
BW25113	1.45 ± 0.06	ND*	3.07 ± 0.13	ND*	0.37 ± 0.015
Δ*crp*	1.57 ± 0.07	ND*	2.83 ± 0.13	0.35 ± 0.018	0.39 ± 0.018
crp$^+$	1.42 ± 0.07	ND*	3.12 ± 0.14	ND*	0.36 ± 0.018
Δ*pgi*	1.6 ± 0.08	ND*	2.77 ± 0.14	ND*	0.4 ± 0.021
Δ*mlc*	1.65 ± 0.08	ND*	2.69 ± 0.13	ND*	0.41 ± 0.019
Δ*mgsA*	1.55 ± 0.07	ND*	2.87 ± 0.14	0.1 ± 0.005	0.39 ± 0.018

ND*: not detectable, where glucose detectable limit was 0.038 g/l.

Figure 3.8a shows the transcript levels of the *crp* knockout mutant and the *crp*$^+$ mutant as compared to the wild type, where it indicates that *mlc* is down-regulated in the *crp* knockout mutant, and up-regulated in the *crp*$^+$ mutant. Figure 3.8b indicates that *ptsG* and *ptsH* transcript levels change in a similar fashion to *mlc* and *crp*, which corresponds to the lower glucose uptake rate for the *crp* knockout mutant and similar to the wild type for the *crp*$^+$ mutant (Table 3.4). Figure 3.8 also indicates that *acnA*, *gltA*, *fumA*, *mdh*, *pckA*, and *sdhC* all change in a similar fashion to *crp* (Appendix A). The *cydB* transcript level changes in a similar fashion to *arcA*, while *cyoA* gene changes in a reverse fashion (Appendix A). The *sodA* and *fur* changes in a similar fashion to *soxR/S*. The *tktB* and *fumC* changes in a similar fashion to *rpoS* (Appendix A). The changing patterns of *aceA*, *icdA*, and *pckA* transcript levels are similar to *cra*, while reverse patterns may be seen for *fadR* and *iclR*.

As stated above, the specific glucose consumption rate decreased in the *crp* knockout mutant, as compared to the wild type, is almost the same in the *crp*$^+$ mutant. The *ptsG* gene is activated by Crp, but also repressed by Mlc. Note that the changing pattern of *crp* transcript levels (Figure 3.8a) coincides with that of *mlc* (Figure 3.8a) as well as *cyaA*. It has been reported that there is a Crp binding region in the promoter region of the *mlc* gene.

In the case of the *crp* knockout mutant, the decrease in the glucose consumption rate may be caused by down-regulation of *ptsG* and *ptsH*, whereas *pfkA* and *pykF* are up-regulated. The former may be caused by the *crp* knockout gene, while the latter may be due to down-regulation

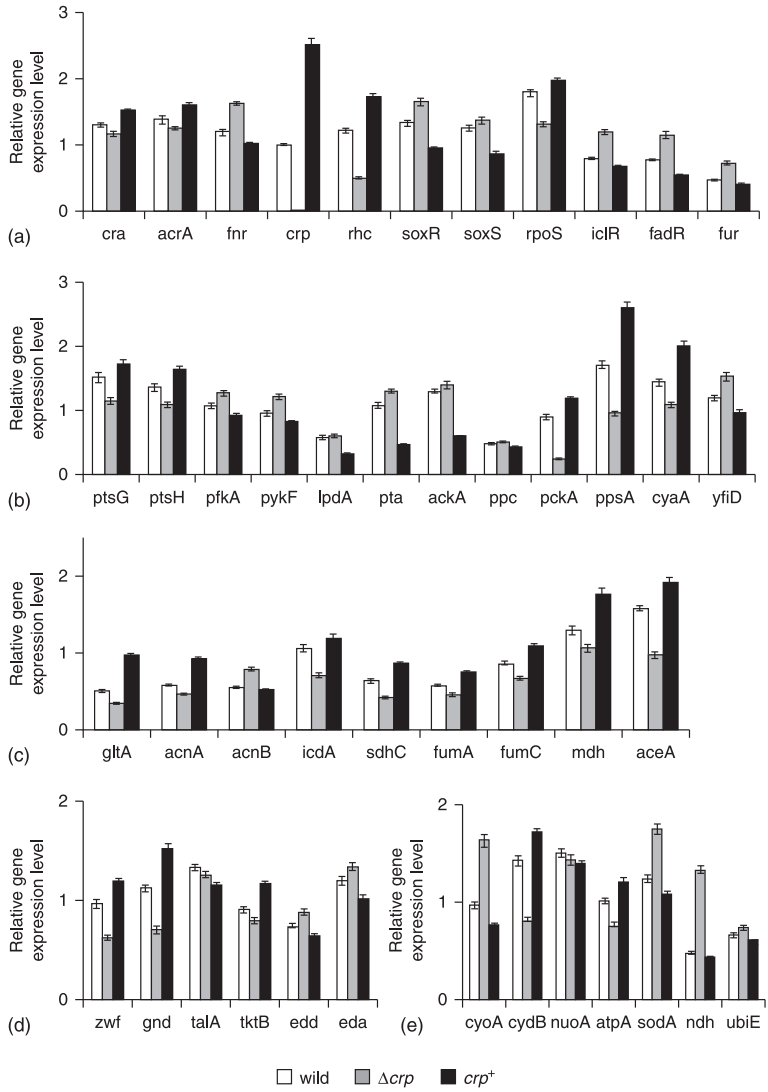

Figure 3.8 **Comparison of gene transcript levels of the wild type, *crp* knockout mutant, and *crp*$^+$ mutant. (a) Global regulator genes; (b) PTS, glycolysis, anaplerotic pathway, *cyaA* and *yfiD* genes; (c) TCA and glyoxylate pathway genes; (d) PP pathway genes; (e) Respiratory chain genes**

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of *cra* (Figure 3.8a), where *mlc* may not be dominant. The *crp* gene knockout causes the TCA cycle genes, such as *gltA*, *acnA*, *sdhC*, *fumA*, and *mdh*, to be down-regulated. Figure 3.8a shows *cra* to be down-regulated, which may be caused by a higher glucose concentration, though it is less than a detectable level. The down-regulation of *cra* also causes *icdA* gene as well as gluconegogenic pathway genes *pckA* and *ppsA* to be down-regulated (Figures 3.8a–c). The decrease in the glyoxylate pathway gene *aceA* (Figure 3.8c) may be caused by the increases of *fadR* and *iclR*, which may be due to higher glucose concentrations. The decreased activities of the TCA cycle and glyoxylate pathway may cause acetate to accumulate, where this is also reflected by the up-regulation of *pta* and *ackA* transcript levels (Figure 3.8b). The batch culture of the Δ*crp* mutant indicates that the acetate formed during cell growth phase cannot be consumed during the stationary phase. The decreased transcript levels of *acnA*, *fumC*, and *tktB* may also be due to down-regulation of *rpoS*, which may be due to less nutrient stress caused by an increase in glucose concentration. The *sodA* gene transcript levels increase (Figure 3.8e), consistent with up-regulation of *soxR/S* (Figure 3.8a), which might be due to an increase in oxygen concentration. The decrease of *arcA* (Figure 3.8a) is also reflected by this hypothesis, whereas its effect on the TCA cycle genes may be minor and not dominant.

In the case of the *crp*$^{+}$ mutant, the *ptsG* and *ptsH* transcript levels are increased, whereas *pfkA* and *pykF* are decreased. It should be noted that *crp* gene enhancement causes an increase in the *mlc* gene (Figure 3.7a), and thus Mlc may repress *ptsG* and *ptsH*. This seems to be dependent on the culture conditions. This may happen when glucose concentration is higher, as will be shown for the batch culture. Different from the case of the Δ*crp* mutant, the TCA cycle genes and glyoxyalte pathway genes, as well as gluconeogenic pathway genes, are up-regulated. The activation of the TCA cycle may cause the decrease in cell yield.

3.2.9 Glucose PTS and other PTS such as fructose PTS in E. coli

As stated above, PTS is a transport system that catalyzes the uptake of a variety of carbohydrates and their conversion into their respective phosphoesters during transport (Deutscher et al., 2006). PTS is composed of EI, HPr, and EII, where these accept a phophoryl group from a donor and transfer it to an acceptor, thus cycling between the phosphorylated and unphosphorylated states (Deutscher et al., 2006). EI and HPr are common

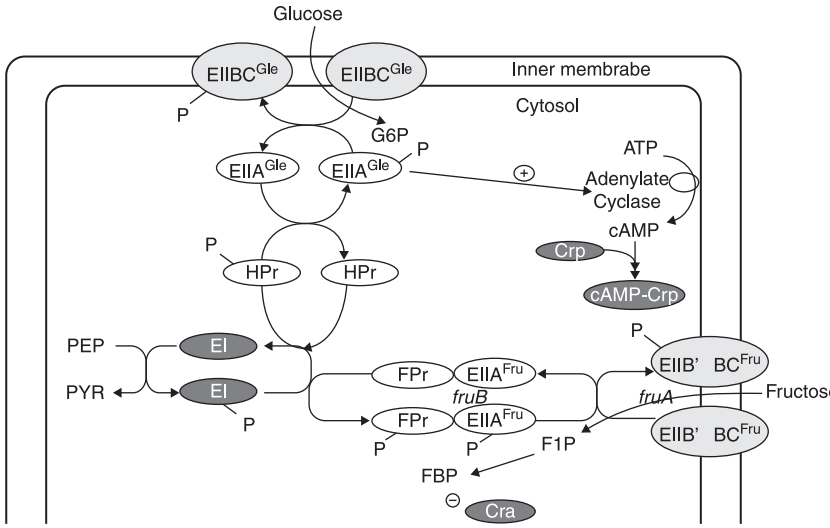

Figure 3.9 **Glucose PTS and fructose PTS**

to all PTS carbohydrates, while EII is carbohydrate specific. Thus, bacteria usually contain many different E IIs. Each E II complex consists of one or two hydrophobic integral membrane domains (C and D) and two hydrophilic domains (A and B), which together are responsible for the transport of the carbohydrate across the membrane as well as its phosphorylation. *E. coli* contains 15 different E II complexes, where the E II complexes are formed either by distinct proteins that contain EI and/or HPr domains exist (Deutscher et al., 2006). A prominent example for the latter is FPr, which consists of HPr and E IIA domains and mediates the phosphotransfer in the uptake of fructose by *E. coli*. As shown in Figure 3.9, fructose can be transferred and phosphorylated by the fructose PTS (E II BC^{Fru}) or ATP-dependent mannofruct kinase Mak (Aulkemeyer et al., 1991). The E II BC^{Fru} encoded by *fruA* phosphorylate fructose is concomitant with transport to fructose 1-phosphate, which is further converted to FDP by an ATP-dependent Pfk (Kornberg, 2001) (Chapter 1).

3.2.10 CCR in B. subtilis and other Firmicutes

The key players in CCR in *Bacillus subtilis* are the pleiotropic transcription factor CcpA (catabolite control protein A), the Hpr protein of the PTS, the bifunctional Hpr kinase/phosphorylase (HPrK), and the glycolytic intermediates such as FDP and G6P (Henkin et al., 1991; Titgemeyer and

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Hillen, 2002; Warner and Lolkema, 2003). Unlike *E. coli*, HPr phosphorylation plays an important role, where phosphorylated HPr serves as the effector for the dimeric CcpA, which controls the expressions of CCR genes (Gorke and Stulke, 2008). The phosphorylation of HPr is catalyzed by HPrK that binds ATP, and its activity is triggered by the availability of FDP as an indicator of high glycolytic activity (Galinier et al., 1998; Reizer et al., 1998; Jault et al., 2000). By contrast, phosphorylase activity prevails under nutrient limitation, and the activation is stimulated by the inorganic phosphate in the cell (Jault et al., 2000; Mijakovic et al., 2002). Under nutrient-rich conditions, HPrK acts as a kinase and phosphorylates HPr, and the cofactor for CcpA is formed. The interaction between CcpA and the phosphorylated HPr is enhanced by FDP and G6P (Seidel et al., 2005; Schumacher et al., 2007).

With the exception of the mycoplasmas, firmicutes also use HPr, HPrK, and CcpA for CCR (Titgemeyer et al., 2002). CcpA in lactic acid bacteria, such as *Lactococcus lactis*, not only represses genes of carbon metabolism but controls metabolic pathway genes such as glycolysis and lactic acid formation pathway genes (Gorke and Stulke, 2008).

3.2.11 CCR in actinobacteria

In *Streptomyces coelicolor* and related species, glucose kinase is the key player of CCR, where it is independent of the PTS (van Wezel et al., 2007). *Corynebacterium glutamicum* is important in the industrial production of amino acids, where it prefers to use multiple carbon sources simultaneously. Diauxic growth is observed when using a mixture of glutamate or ethanol and glucose, where the repressor protein RamB is activated when glucose is present, and binds to the promoter regions of the genes involved in acetate and ethanol catabolism (Gestmeir et al., 2004; Arndt and Eikmanns, 2007). The *ramB* expression is regulated by a feedback of RamB and RamA, where RamA is activated when acetate is present.

3.2.12 CCR in Pseudomonas putida

P. putida can assimilate various aromatic and aliphatic hydrocarbons, where it has been reported that the use of hydrocarbons is repressed by succinate, and this seems to be a general feature of CCR in this organism (Collier et al., 1996; Muller et al., 1996). Under CCR, the translation of

operon-specific regulators is inhibited by the binding of an RNA-binding protein Crc to mRNAs of the regulator transcript, and thus CCR seems to be governed by an RNA-binding protein at the level of post-transcriptional control rather than by a DNA-binding transcriptional regulator (Moreno et al., 2007; Moreno and Rojo, 2008).

3.2.13 The impact of CCR on bacterial virulence

CCR is crucial for the expression of virulence genes and for pathogenicity in many pathogenic bacteria. Note that the primary aim of pathogenic bacteria is to gain access to nutrients rather than to cause damage to the host, and that the expression of virulent genes is linked to the nutrient supply of the bacteria (Gorke and Stulke, 2008).

In many firmicules, the mutants devoid of the HPr kinase grow significantly slower than wild-type cells. Therefore, it is suggested that HPr kinase, which generates the cofactor for CcpA, might be a suitable drug target, where the compound that inhibits the kinase activity of HPr has been identified, and this compound inhibits the growth of *B. subtilis* but not of *E. coli*, where *E. coli* does not contain HPr kinase (Gorke and Stulke, 2008).

Crp and cAMP are essential for the expression of virulence genes in enteric bacteria, and therefore, the corresponding *crp* and *cya* mutant strains of *S. enterica* and *Y. enterocolitica* can be used as live vaccines in mice and pigs (Curtiss and Kelly, 1987; Petersen and Young, 2002; Ramströme et al., 2004).

3.3 Nitrogen regulation

Next to carbon (C) source metabolism, nitrogen (N) metabolism is also important in understanding the metabolic regulation. In *E. coli,* assimilation of an *N*-source, such as ammonia/ammonium (NH_4^+) using α-KG, results in the synthesis of glutamate and glutamine (Figure 3.10). Glutamine synthetase (GS, encoded by *glnA*) catalyzes the only pathway for glutamine biosynthesis. Glutamate can be synthesized by two pathways through the combined actions of GS and glutamate synthase (GOGAT, encoded by *gltBD*) forming GS/GOGAT cycle, or by glutamate dehydrogenase (GDH encoded by *gdhA*) (Yan, 2007). The GS/GOGAT cycle has a high affinity for NH_4^+ (K_m <0.2 mM for GS), and therefore is

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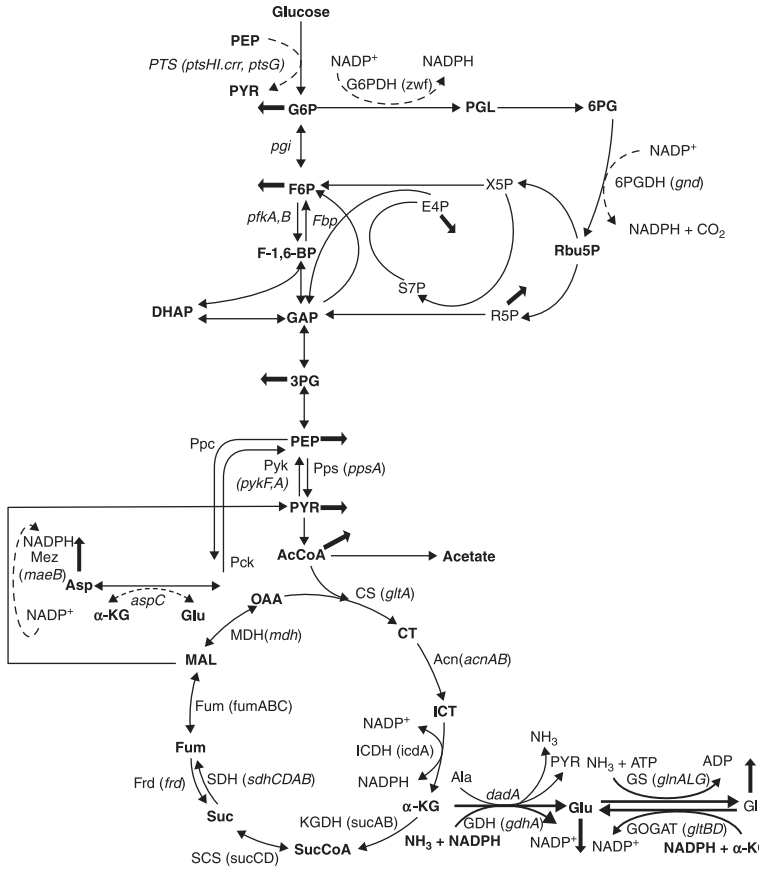

Figure 3.10 Central metabolic pathways and NH_3-assimilation pathways

dominant when nitrogen is scarce in the medium, whereas GDH has a low affinity for NH_4^+ (K_m >1 mM) and is utilized when a sufficient nitrogen source is available in the medium. When extracellular NH_4^+ concentration is low at around 5 μM or less, ammonium enters the cell via AmtB and is converted to glutamine by GS, and UTase uridylylates both GlnK and GlnB (Ninfa et al., 2000) (Figure 3.11). When the extracellular NH_4^+ concentration is more than 50 μM, the metabolic demand for the glutamine pool rises, and UTase deuridylylates GlnK and GlnB. GlnK complexes with AmtB, thereby inhibiting the transporter via AmtB, where GlnB interacts with NtrB and activates its phosphatase activity leading to dephosphorylation of NtrC, and NtrC-dependent gene expression ceases (Ninfa et al., 2000) (Figure 3.11). The nitrogen

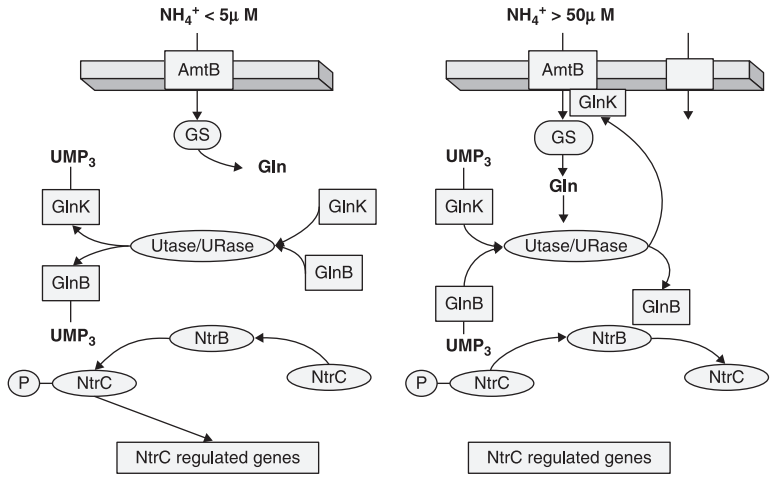

Figure 3.11 **Ammonia assimilation under different NH_4^+ concentration**

intermediates, such as glutamate and glutamine, provide nitrogen for the synthesis of all the other N-containing components. About 88% of cellular nitrogen comes from glutamate, and the rest from glutamine (Reitzer, 2003). The ATP required for the nitrogen assimilation, using the GS/GOGAT cycle under N-limiting conditions, accounts for 15% of the total requirement in *E. coli*. A significant amount of NADPH is also required for nitrogen assimilation (Reitzer, 2003; Yan, 2007). The other pathways involved in maintaining cellular nitrogen balance under specific conditions include aspartate-oxaloacetate and alanine-pyruvate shunts (Fischer and Sauer, 2003; Zhang et al., 2007).

It should be noted that carbon metabolism is not only controlled by carbon-derived signals, but also by the availability of nitrogen and other nutrients (Commichau et al., 2006). From studies on interdependence of different metabolic routes, two of the major signal transduction systems of nitrogen and carbon metabolism have been identified as P_{II}, a small nitrogen regulatory protein and PTS. Because of the important roles in the regulatory functions, P_{II} and PTS can be regarded as the central processing units of N and C metabolism, respectively. The P_{II} protein senses αKG and ATP, thus linking the state of central carbon and energy metabolism for the control of N assimilation (Commichau et al., 2006). The glucose catabolism is modulated by global regulators, such as Cra, Crp, Cya, Mlc, as stated in the previous sections, while N assimilation is regulated by the P_{II}-Ntr system together with global regulators, such as

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Crp, providing a novel regulatory network between C and N assimilation in *E. coli* (Mao et al., 2007). The effects of C and N limitations on *E. coli* metabolism have been investigated for continuous culture (Hua et al., 2003, 2004; Sauer and Eikmanns, 2005; Nanchen et al., 2008; Kumar and Shimizu, 2010).

C and N metabolisms may be linked by energy metabolism, where it has been demonstrated that the glycolytic flux in *E. coli* is controlled by the demand for ATP (Koebman et al., 2002). It has been reported that the P_{II} protein controls N assimilation by acting as a sensor of adenylate energy charge, which is the measure of energy available for metabolism. The signal transduction requires ATP binding to P_{II}, which is synergistic with the binding of αKG. Moreover, αKG serves as a cellular signal of C and N status, and strongly regulates P_{II} functions (Jiang and Ninfa, 2007). The studies on the C and N pathway interdependence have so far focused on the conversion of αKG to glutamate (Ninfa and Jiang, 2005). It is evident that the regulatory mechanism of this conversion is critical for the interdependence of C and N assimilation.

Figure 3.12 shows the effect of the C/N ratio on fermentation characteristics during aerobic continuous culture where the dilution rate was 0.2 h^{-1}, and where the C/N ratio was based on the feed content. Figure 3.12 indicates that the glucose concentration increases, whereas the cell concentration decreases as the C/N ratio increases. Figure 3.12 also shows that the glucose concentration is very low at 100% and 60% of N concentrations (C-limitation), whereas its concentration is high at 20% and 10% of N concentrations (N-limitation). It is also shown that the specific glucose consumption rate, as well as the specific acetate and CO_2 production rates, tends to increase as the C/N ratio increases.

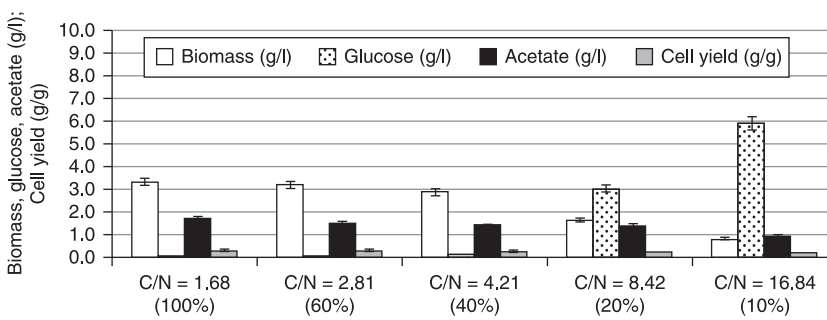

Figure 3.12 **Effect of C/N ratio on the fermentation characteristics for the continuous culture at the dilution rate of 0.2 h^{-1}**

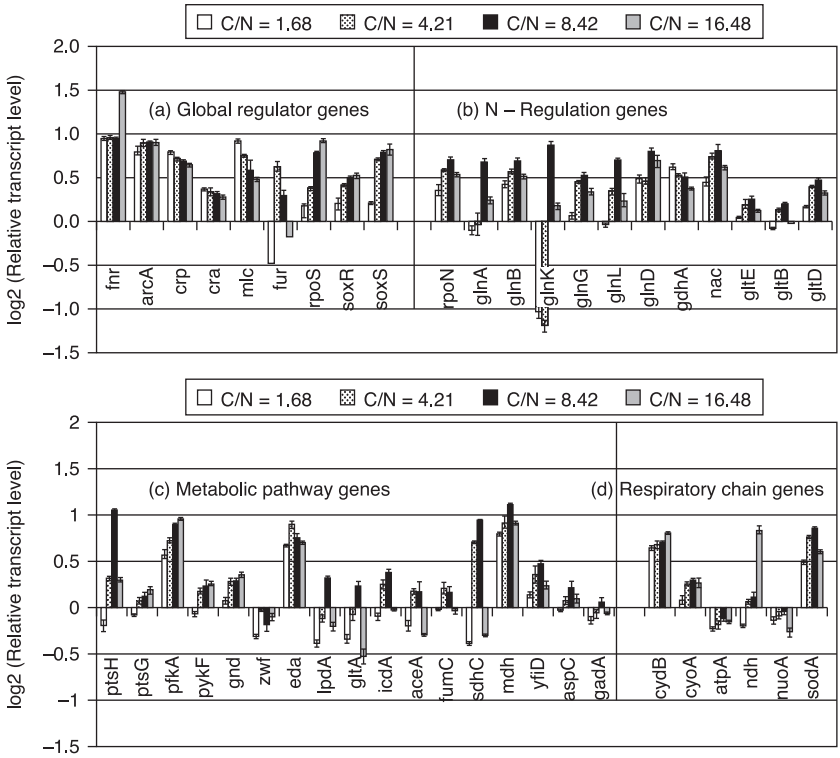

Figure 3.13 **Schematic illustration of the interaction among several metabolic regulations. Comparison of the transcriptional mRNA levels of the wild type *E.coli* genes cultivated at 100% (C/N = 1.68), 40% (C/N = 4.21), 20% (C/N = 8.42) and 10% (C/N = 1.68) N^- concentration: (a) global regulatory, (b) N^- regulatory, (c) metabolic pathway, (d) respiratory chain**

In order to interpret the fermentation characteristics, the relative mRNA levels are shown for different C/N ratios in Figure 3.13. Figure 3.13a shows that *crp* transcript levels become lower as C/N ratio increases, which corresponds to the fact that the cAMP-Crp level decreases as glucose concentration increases. In accordance with the change in *crp* transcript levels, the *mlc* level changes in a similar fashion (Gosset et al., 2004). Figure 3.13a also shows that the transcript levels of such genes as *soxR/S* and *rpoS* become higher as the C/N ratio increases, which may be

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due to oxygen stress caused by higher respiratory activity for the former (Hua et al., 2003), along with nutrient stress for the latter (Rahman et al., 2008). In relation to the up-regulation of *soxR/S*, the *sodA* transcript level increases as the C/N ratio increases, except at the highest C/N ratio (Figure 3.13d). Figure 3.13a shows a high expression of anaerobic regulator *fnr* at the highest C/N ratio, while the transcript level of *arcA* changes little (Kumar and Shimizu, 2010).

The transcript level of *rpoN*, which encodes σ^{54}, increases as the C/N ratio increases (Figure 3.13b). Figure 3.13b also shows that the expressions of *glnA*, *glnL*, *glnG*, and *gltD* genes change in a similar fashion to *rpoN*, indicating the activation of the GS-GOGAT pathway under N-limitation. The *glnB* gene, which codes for P_{II}, also changes in a similar fashion, while *glnD*, which controls uridylylation and deuridylylation, appears somewhat different but the trend seems to be similar (Figure 3.13b). The P_{II} paralogue encoding gene, *glnK*, shows high expressions at 20% and 10% of N-limitation (Figure 3.13b). The expression pattern of *nac* is similar to that of *rpoN*, whereas *gdhA* shows a reverse pattern, implying that *gdhA* is repressed by Nac (Figure 3.13b).

As the C/N ratio increases, the transcript level of the *crp* gene as well as the *mlc* gene decreases, which then causes the transcript level of *pts*G gene to be increased (Figure 3.13c). In relation to the decrease in the transcript level of *cra*, the transcript levels of such genes as *ptsH*, *pfkA*, and *pykF* increase as the C/N ratio increases (Figure 3.13c). These correspond to the increased specific glucose consumption rate as the C/N ratio increases. Moreover, respiratory chain genes, such as *cyoA*, *cydB*, and *ndh*, together with TCA cycle genes, such as *gltA*, *icdA*, *fumC*, *sdhC*, and *mdh*, show increased expressions as the C/N ratio increases (Figure 3.13d), which corresponds to the increase in the specific CO_2 production rate. Part of the reason for this may be due to the accumulation of α-KG caused by the decreased activity of GDH. Since ferric uptake regulator Fur activates some of the TCA cycle genes, such as *sdh*, *suc*, and *fum* (Zhang et al., 2005), part of the reason may be due to up-regulation of the transcript levels of the *fur* gene (Figure 3.13a).

The glucose concentration in the fermentor increases with the increase in the C/N ratio (Figure 3.12). The glucose uptake is made via PTS in *E. coli*, where phosphate of PEP is transferred by phosphorelation via enzyme I (EI) encoded by *ptsI*, histidine phosphorylatable protein HPr encoded by *ptsH*, glucose specific enzyme II, $EIIA^{Glc}$ encoded by *crr*, and membrane bound $EIICB^{Glc}$ encoded by *ptsG*, as stated in the

previous section. When glucose is present in excess, the phosphorylated $EIIA^{Glc}$ transfers phosphate to $EIICB^{Glc}$ for the glucose uptake with phosphorylation, and the unphosphorylated $EIIA^{Glc}$ is dominated in the cytosol (Deutscher et al., 2006). Since unphosphorylated $EIIA^{Glc}$ does not activate Cya, the cAMP level decreases under N-limitation together with the *crp* gene (Figure 3.13a). Since *mlc* is under control of *crp*, the transcript level of *mlc* gene also decreases (Figure 3.13a), which causes up-regulation of transcript levels of *ptsH* and *ptsG* genes (Appendix A). Moreover, an increase in the glucose concentration at a higher C/N ratio may cause down-regulation of *cra,* which causes up-regulation of the glycolysis genes, such as *ptsH*, *ptsG*, *pfkA*, and *pykF*, together with *zwf* (Appendix A).

The GDH pathway is favored when the organism is stressed for energy, because GDH does not use ATP as does the GS pathway (Helling, 1998). Figure 3.13b shows the decreased expression of *gdhA* as the C/N ratio increases. Liang and Houghton (1981) investigated the effect of NH_4Cl concentration on GDH and GS activities, and showed the up-regulations of GDH and transhydrogenase activities at lower NH_4Cl concentration.

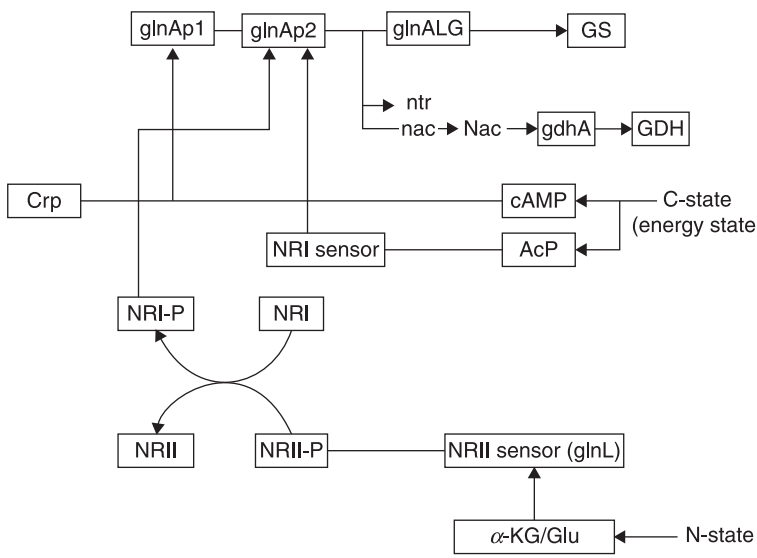

Figure 3.14 The interaction between nitrogen regulation and catabolite regulation

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The availability of nitrogen is sensed by the P_{II} protein at the level of intracellular glutamine, where glutamine is synthesized by glutamine synthetase (GS) encoded by *glnA*, and is transported mainly by GlnHPQ. The *glnHPQ* operon is under the control of tandem promoters, such as *glnHp1* and *glnHp2*, where the former is σ^{70}-dependent, and the latter is σ^{54}- and NtrC-P dependent (Willis et al., 1975; Claverie-Martin and Magasanik, 1991). It has been shown that as the major transcriptional effector of the glucose effect, Crp affects nitrogen regulation (Mao et al., 2007). Namely, *glnAp1* is activated by Crp with glutamine as the N-source (Figure 3.14). Through *glnHPQ*-dependent signaling, Crp acts to decrease the amount of the phosphorylated NtrC activator, which in turn causes a decrease in *glnAp2* expression (Mao et al., 2007). However, this regulation is more complex. It has been suggested that σ^{54}-dependent Ntr genes of *E. coli* form a gene cascade in response to N-limitation (Blauwkamp and Ninfa, 2002). The central participants of Ntr response are NR_I or NtrC and NR_{II} or NtrB, and RNA polymerase complexed to σ^{54}. NR_I is the transcriptional activator of σ^{54}-dependent promoters, while NR_{II} is a bifunctional protein that can either transfer phosphate to NR_I or control the dephosphorylation of NR-phosphate. N-limitation results in the phosphorylation of NR_I, which in turn stimulates the expression of *glnALG* operon. The expression of the *glnALG* operon is controlled by tandem promoters, such as *glnAp1* and *glnAp2*, where *glnAp1* is a σ^{70}-dependent weak promoter and its transcription can be activated by Crp and blocked by Ntr-P. However, *glnAp2* is transcribed by RNA polymerase ($E\sigma^{54}$) and is activated by Ntr-P. Therefore, *glnAp2* is responsible for activating *glnA* transcription under N-limitation (Magasanik, 1996). Figure 3.13b shows that the expressions of the *glnA*, *L,* and *G* genes change in similar fashion as *rpoN* gene expression.

It has been reported that there are no NR_I-P binding sites in the *gdhA* regulatory region (Riba et al., 1988), and it is unlikely for NR_I to directly repress the *gdhA* promoter (Camarena et al., 1998). As it has been shown that Nac is involved in the transcriptional repression of the *gdhA* gene under N-limitation (Camarena et al., 1998), Nac seems to repress *gdhA* gene (Figure 3.13). This figure shows that the transcript level of *gdhA* gene is lower, while *gltB* and *D* genes are higher under N-limitation as compared to C-limitation. NADPH is an important cofactor in GDH and (GS)-GOGAT activities and it has been reported that transhydrogenase plays some role in the regulation of these pathways (Liang and Houghton, 1981). Under N-limitation, the glutamate and

glutamine synthetic pathways are expected to be repressed due to shortage of NH_3 for these reactions, and thus NADPH is less utilized, resulting in overproduction of NADPH. Part of this may be converted to NADH by transhydrogenase, and the converted NADH together with other NADH formed may be utilized for ATP production through the respiratory chain. Overproduction of NADPH represses such pathways as G6PDH, 6PGDH, and ICDH in *E. coli*. However, *zwf* is activated in Figure 3.13c, which may be due to SoxR/S encoded by *soxR/S* caused by higher respiratory activity. The ICDH activity is reported to be insensitive to N concentration, where Figure 3.13c also shows little change in *icdA* gene expression.

E. coli possesses two closely related P_{II} paralogues, such as GlnB and GlnK, where GlnB is produced constitutively, and regulates the NtrB (NR_{II})/NtrC (NR_I) two-component system (Ninfa and Atkinson, 2000). It has been shown that the intracellular concentrations of NR_I and NR_{II} increase upon N limitation (Reitzer and Magasanik, 1985; Atkinson and Ninfa, 1993; Atkinson et al., 2002). The phosphorylated NtrC is an activator of various nitrogen-controlled genes, such as *glnA*, which codes for GS (Blauwkamp and Ninfa, 2002) and *glnK*, encoding the second P_{II} paralogues (Atkinson et al., 2002). The increased NR_I, presumably in the phosphorylated form such as NR_I-P, activates the expression of *glnK* and *nac* promoters under N-limitation (van Heeswijk et al., 1996; Pahel et al., 1982). Figure 3.13b shows that the transcript levels of *glnK* and *nac* genes increase as C/N ratio increases, while a slight decrease can be seen at the highest C/N ratio, where it has been reported that *glnK* and *nac* promoters are sharply activated when ammonia is used up (Atkinson et al., 2002).

The *gltBDF* operon has been found to have binding affinity with global regulators such as Fnr and Crp in the promoter region (Paul et al., 2007), while the transcript level of *fnr* gene is higher under N-limitation whereas *crp* gene became lower (Figure 3.13a). The up-regulation of *yfiD* (Fig. 3.13c) may be due to up-regulation of *fnr*.

The Ntr system is composed of four enzymes (Figure 3.15): a uridylytransferase/uridylyl-removing enzyme (UTase/UR) encoded by the *glnD* gene; a small trimeric protein, P_{II} encoded by *glnB*; and the two-component system composed of NtrB and NtrC. GlnD controls the activity of GS by adenylylation/deadenylylation through a bifunctional enzyme adenylyltransferase (ATase), the *glnE* gene product (Shapiro and Stadtman, 1968; Stadtman, 1990; Jaggi et al., 1997). The activity of GlnK becomes high under N-limitation (Figure 3.13b) and contributes to the regulation of NtrC-dependent genes (Maheswaran

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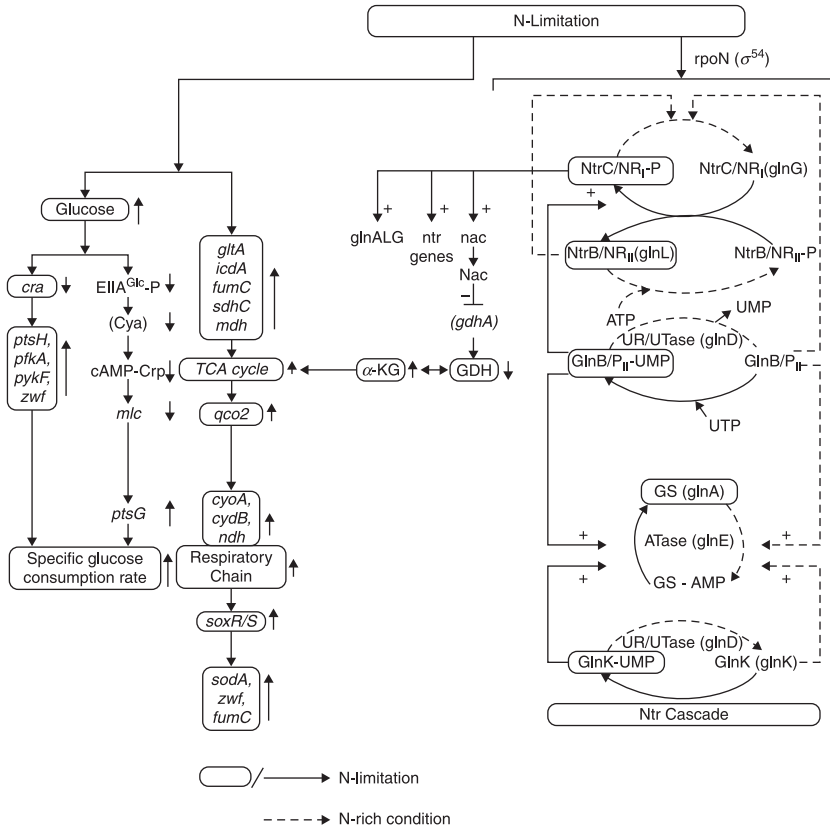

Figure 3.15 **Overall mechanism of nitrogen assimilation in *E. coli* under C-limited (N-rich) and N-limited conditions**

and Forchhammer, 2003). It has been shown that on GS adenylylation, ATase activity is regulated by UTase/UR and P_{II}, such that upon nitrogen limitation, UTase covalently modifies P_{II} by addition of a UMP group at a specific residue and the resultant uridylylated form of P_{II} promotes deadenylylation of GS by ATase (Figure 3.15). Conversely, under N-rich conditions, the uridylyl-removing activity of GlnD predominates and the deuridylylated P_{II} promotes adenylation of GS by ATase. Adenylylation by ATase is promoted by deuridylylated P_{II}, which is produced by UR action on P_{II} $(UMP)_3$ under higher N-concentration (low C/N ratio) (Figure 3.15). These indicate that UTase/UR and P_{II} acting together sense the intracellular nitrogen status (Merrick and Edwards, 1995). The P_{II} signal transduction proteins, such as GlnB and GlnK, are uridylylated/deuridylylated in response to

intracellular glutamine levels, where low intracellular glutamine level, signaling N-limitation, leads to uridylylation of GlnB (Merrick and Edwards, 1995). GlnB is shown to be allosterically regulated by α-KG, and thus GlnB may play a role in integrating signals of C/N status. The NtrB/NtrC two-component system and GlnE, which adenylylates/deadenylylates GS, are the receptors of GlnB signal transduction (Maheswaran and Forchhammer, 2003). It has been suggested that the carbon/cAMP effect is mediated through GlnB uridylylation (Maheswaran and Forchhammer, 2003).

Phosphorylated NR_I/NtrC (NR_I/NtrC-P) activates transcription from N-regulated σ^{54}-dependent promoters by binding to the enhancers (Kustu et al., 1989; Merrick and Edwards, 1995; Jiang et al., 1998; Ninfa et al., 2000). P_{II} and the related GlnK protein control the phosphorylation state of NR_{II}/NtrB by stimulating the phosphatase activity of NR_{II}. The ability of GlnK and P_{II} to regulate the activities of NR_{II} is in turn regulated by the intracellular signals of C and N availability via allosteric control (Ninfa et al., 2000).

3.4 Phosphate regulation

The phosphate (P) metabolism is also important from the energy generation and phosphorelay regulation points of view. The phosphorous 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 conditions (Baek and Lee, 2006; Wendisch, 2006). *E. 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 and Lee, 2007). It has been shown that PhoR is an inner-membrane histidine kinase sensor

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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 (Smith and Payne, 1992; Wanner, 1996; Harris et al., 2001; Blanco et al., 2002). The activation signal, a phosphate concentration below 4 μM, is transmitted by a phosphorelay 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 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 by dephosphorylating PhoB. In addition, two P_i-independent controls, which may be a 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

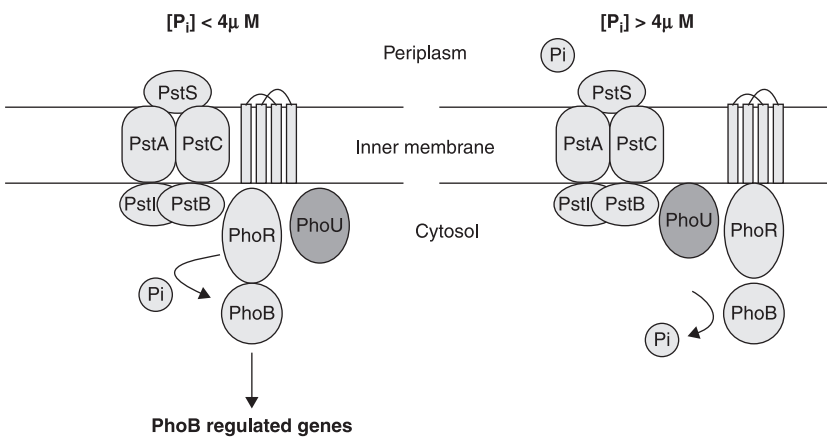

Figure 3.16 Molecular mechanism of phosphate regulation

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 inner-membrane 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 (Figure 3.16). 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 conditions (Wanner, 1997). Indeed, PhoU is essential for the repression of the Pho regulon under high phosphate conditions (Wanner, 1996). It may be considered that PhoU acts by binding to the PhoR, PhoB, or PhoR/PhoB complexes to promote dephosphorylation of phosphorylated PhoB or by inhibiting formation of the PhoR–PhoB complex (Oganesyan et al., 2005).

It has been shown that the *phoB* mutant does not synthesize alkaline phosphatase (*phoA* gene product) (Nesmeianova et al., 1975; Pratt and Torriani, 1977; Zuckier et al., 1980; Guan and Wanner, 1983; Kimura et al., 1989; Yamada et al., 1989) and phosphate binding protein (*pstS* gene product) (Pratt and Torriani, 1977; Kimura et al., 1989; Yamada et al., 1989). It is observed that *phoU* expression changes, 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-Riché et al. (1988) 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 are 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). 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).

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Table 3.5 **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%) conditions	P-limited (20%) conditions	P-limited (10%) conditions
Biomass concentration (g/l)	Wild	3.86 ± 0.03	3.47 ± 0.05	1.69 ± 0.03
	Δ*phoB*	3.44 ± 0.04	–	3.64 ± 0.01
Glucose concentration (g/l)	Wild	0.660 ± 0.004	0.557 ± 0.001	1.85 ± 0.01
	Δ*phoB*	1.59 ± 0.29	–	1.050 ± 0.001
Acetate concentration (g/l)	Wild	0.046 ± 0.002	0.410 ± 0.001	0.41 ± 0.02
	Δ*phoB*	0.255 ± 0.130	–	0.346 ± 0.010
Specific glucose uptake rate (mmol/gDCW/h)	Wild	2.69 ± 0.05	3.024 ± 0.005	5.36 ± 0.01
	Δ*phoB*	2.72 ± 0.09	–	2.730 ± 0.003
Specific acetate production rate (mmol/gDCW/h)	Wild	0.040 ± 0.002	0.394 ± 0.010	0.81 ± 0.02
	Δ*phoB*	0.247 ± 0.080	–	0.317 ± 0.001

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

A better understanding of the Pho regulon would allow for optimization of such processes (Van Dien and Keasling, 1998).

Table 3.5 shows the effect of P concentration on the fermentation characteristics of the wild-type *E. coli* BW25113, its *phoB* gene knockout mutant (JW0389), and *phoR* mutant (JW0390) in the aerobic continuous culture at a dilution rate of 0.2 h^{-1}, where it indicates that the fermentation characteristics significantly change when feed P concentration become low at around 10% of the M9 medium (Marzan and Shimizu, 2011). In particular, the specific glucose consumption rate and the specific acetate production rate become significantly higher, while cell concentration become significantly lower under such P-limiting conditions.

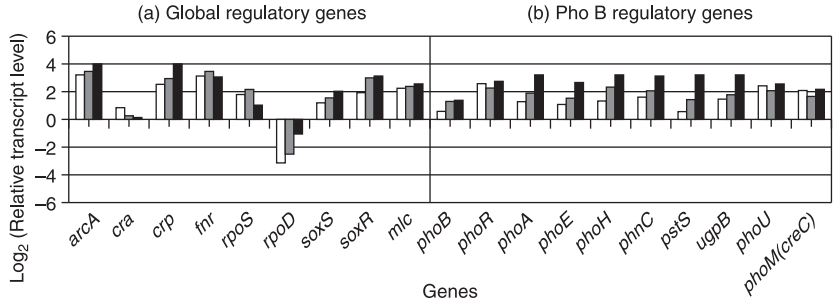

Figure 3.17 **Comparison of the transcript levels of the wild type *E. coli* cultivated with different P concentrations of the feed (100%, 55%, 10%): (a) global regulatory genes, (b) PhoB regulatory genes**

Figure 3.17 shows the effect of P concentration on the transcript levels, where Figure 3.17b indicates that *phoB* transcript level increases as P concentration decreases, and *phoB* regulated genes, such as *phoA*, *phoE*, *phoH*, *phnC*, *pstS*, and *ugpB*, are all increased in a similar fashion, (Figure 3.17b) and *eda* transcript levels also change in a similar fashion. Note that *phoU* and *phoM* change in a similar fashion to *phoR*, and also that the transcript levels of *rpoD*, which encodes the RNA polymerase holoenzyme containing σ^{70}, increases in a similar fashion to PhoB regulatory genes (Makino et al., 1993). Figure 3.17a also indicates that the transcript levels of *arcA* increase as P concentrations decrease. Figure 3.17a shows that *cra* transcript levels decrease. These are consistent with the increased specific glucose consumption rate (Table 3.4). The decrease in *cra* transcript levels may be due to higher glucose concentration. Moreover, Figure 3.17a also indicates that *soxR/S* transcript levels increase as P concentrations decrease, and accordingly the transcript levels of *rpoD*, (as well as *zwf* and *sodA*) change in a similar fashion. The respiratory chain genes, such as *atpA, ndh*, and *nuoA*, also change in a similar fashion, implying that the respiration is activated under P-limitation (Marzan and Shimizu, 2007).

Table 3.5 also shows the effect of the *phoB* gene knockout on the fermentation characteristics under both P-rich and lower P conditions, where it indicates that the glucose concentration increases and cell concentration decreases for the *phoB* mutant as compared to the wild type, and that the specific acetate production rate is higher at P-rich conditions for the *phoB* mutant as compared to the wild type.

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Figure 3.17b indicates that *phoB* gene transcript levels increase as P concentration decreases in the wild type, and Figure 3.17a indicates that *rpoD* also increases as P concentration decreases. The *phoA*, *phoE*, *phoH*, *phnC*, *pstS*, and *ugpB* are all increased in a similar fashion to that of *rpoD*, as mentioned before. Figure 3.17a indicates that the expression pattern of *rpoS* is somewhat different. When cells enter into the P_i-starvation phase in the batch culture, the Pho regulon is activated, and σ^S starts to accumulate in the cytosol (Gentry et al., 1993; Wanner, 1996; 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 the *rpoS* mutant, the expression of AP is considerably higher than that in the wild-type strain, implying that σ^S is involved in 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 3.17ab indicates that Pho genes are highly expressed as compared to low *rpoS* transcript levels 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 (Spira et al., 1995; Spira and Yagil, 1999; Ruiz and Silhavy, 2003; Taschner et al., 2004; Taschner et al., 2006; Schurdell et al., 2007).

The presence of glucose or mutations in the *cya* or cAMP receptor protein (*crp*) gene leads to induction of the *phoA* gene in the *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 metabolism, in which intracellular P_i is incorporated into ATP. One P_i independent control is regulation by synthesis of AcP, where P_i is incorporated into ATP at the Ack (acetate kinase) pathway. AcP may act indirectly on PhoB.

The overall regulation mechanism is illustrated schematically in Figure 3.18 (Marzan et al., 2011).

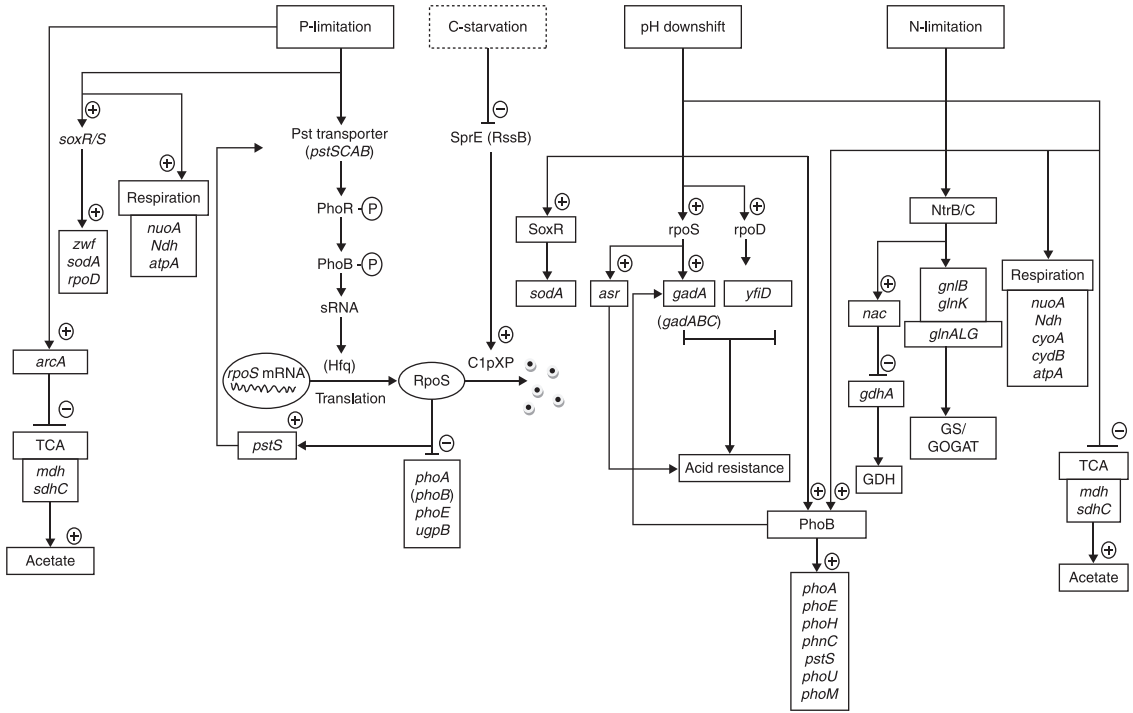

Figure 3.18 Schematic illustration of the interaction among several metabolic regulations

3.5 Oxygen level regulation

3.5.1 Effect of oxygen limitation on the metabolism

In addition to nutrient sources, oxygen levels are also important in metabolic regulation. Global regulators, such as Fnr and ArcAB, are mainly responsible for regulation to the availability of oxygen and other electron acceptors in the culture environment, where Fnr regulates the expressions of metabolic pathway genes under anaerobic conditions (Kang et al., 2005), while ArcAB regulates under both anaerobic and microaerobic conditions (Gunsalus, 1992; Alexeeva et al., 2003). Moreover, the genes that encode the primary dehydrogenases, such as *glpD*, *lctPRD*, *aceE*,*F*, and *lpdA*, are also repressed by ArcA (Iuchi et al., 1990a; Lynch and Lin, 1996b; Cunningham et al., 1998). It has been shown that the ArcA/B system exerts more significant regulation effect on the cell metabolism under microaerobic conditions than under aerobic or anaerobic conditions. The effect of the ArcAB system on the flux distribution at the pyruvate node has been investigated based on the extracellular metabolite concentrations (Shalel-Levanon et al., 2005a,b; Zhu et al., 2006). It is shown that lactate can be overproduced by the *arcA/fnr* double mutant (Zhu et al., 2006) in a similar way to the *pfl* gene knockout (Zhu and Shimizu, 2004, 2005).

Reoxidation of the reducing equivalents, such as NADH generated by the oxidation of the energy source, occurs in the respiratory chain under aerobic or microaerobic conditions. In *E. coli*, NADH is oxidized in the respiratory chain via a coupled NADH dehydrogenage NDH–1 encoded by *nuo* or an uncoupled dehydrogenase NDH–2 encoded by *ndh*, and the electron flows into quinone and the quinol pool. Quinol is then oxidized by either the cytochrome bo or the cytochrome bd terminal oxidase complex, which in turn passes the electrons to the oxygen with concomitant production of water. The two terminal oxidases differ in their affinities for oxygen as well as in their H^+/e- stoichiometries, where cytochrome bo oxidase has a low affinity for oxygen and translocates two H^+s per e^-, while cytochrome bd has a high affinity to oxygen and translocates one H^+ per e^-. The *cyoABCDE* operon is repressed by both ArcA and Fnr, while *cydAB* operon is activated by ArcA and repressed by Fnr (Alexeeva et al., 2000).

Microbial cells, such as *E. coli*, can generate energy as ATP under a wide range of redox conditions. The reducing equivalents, such as

NADH, are re-oxidized in the respiratory chain, where oxygen, nitrate, fumarate, and dimethyl sulfoxide, etc. are the electron acceptors. This process is coupled to the formation of a proton mortive force (PMF), which is utilized for ATP generation from ADP. In the absence of oxygen, or other electron accepters, ATP is generated via substrate level phosphorylation through the process of degradation of a carbon source in the metabolic pathways. Under such fermentation conditions, the cell such as *E. coli* excretes such metabolites as lactate, ethanol, succinate, formate (CO_2 and H_2 as well), as well as acetate, where the relative production rates for these metabolites are governed by the demand for redox neutrality. The succinate is formed from PEP via Ppc. Pyruvate serves as a common substrate for pyruvate formate-lyase (Pfl) and the pyruvate dehydrogenase complex (PDHc), and this branch point involves the cleavage of PYR. The expressions of *pfl* genes or the *focApfl* operon, which encode Pfl, are activated by ArcA and Fnr, and become higher at lower oxygen concentrations, whereas *aceE,F*, which encode α and β subunits of PDHc, are repressed by ArcA under oxygen limited conditions. At the branch point of AcCoA, the product of both Pfl and PDHc reactions is converted to either acetate and ethanol, or subsequently undergoes further oxidation in the TCA cycle. The interconversion of Pfl between inactive and active glycyl radical-bearing species occurs at low oxygen concentrations and is controlled by the activities of iron-sulfur protein Pfl activase and the product of the *adhE* gene Pfl deactivase (Kessler and Knappe, 1996; Sawers and Watson, 1998). The active glycyl radical form of Pfl is irreversibly destroyed by molecular oxygen and hence must be either protected from oxygen damage or converted to the inactive, oxygen-stable species during the transition between anaerobiosis and aerobiosis (Wagner et al., 2001; Alexeeva et al., 2000).

3.5.2 Regulation by ArcA/B system

The Arc system, composed of ArcA, the cytosolic response regulator, and ArcB, the membrane bound sensor kinase, regulates the TCA cycle genes, depending on the oxygen level or redox state. The ArcB protein has three cytoplasmic domains: a primary transmitter domain (H1) containing a conserved His292; a receiver domain (D1) containing a conserved Asp576; and a secondary transmitter domain (H2) containing a conserved His717 (Iuchi and Lin, 1992; Ishige et al., 1994; Tsuzuki et al., 1995; Kato et al., 1997). The primary transmitter domain of

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ArcB is autophosphorylated at His292 at the expense of ATP (Iuchi, 1993; Georgellis et al., 1997). The phosphoryl group is then sequentially transferred to Asp576 and His717 and from there to Asp54 of ArcA. However, the phosphoryl group on His292 can also be directly transferred *in vitro* to ArcA at a very low rate (Georgellis et al., 1997). The phosphoryl group from His717 can also be transferred to ArcA, but this transfer is regulated by redox conditions (Matsushika and Mizuno, 1998). Namely, upon stimulation by the redox state, ArcB undergoes autophosphorylation, and the phosphoryl group is transferred to ArcA by the His→Asp→His→Asp phosphorelay (Georgellis et al., 1999). Consequently, the phosphorylated ArcA binds to the promoter regions of the TCA cycle and other genes.

It has been reported that ArcA, when phosphorylated, represses the expressions of the genes involved in the TCA cycle and the glyoxylate shunt, such as *glt*A, *acn*AB, *icd*A, *suc*ABCD, *sdh*CDAB, *fum*A, *mdh*, and *aceA,B* (Iuchi and Lin, 1988; Park et al., 1994, 1995, 1997; Lynch and Lin, 1996a). *E. coli* possesses two terminal quinol oxidases in the respiratory chain. The genes *cyo*ABCDE, which encode cytochrome o oxidase that has a low oxygen affinity and mainly functions under aerobic conditions, are repressed by ArcA (Cotter and Gunsalus, 1992). However, the *cyd*AB genes, which encode cytochrome d oxidase that has high oxygen affinity, are activated by ArcA (Iuchi et al., 1990; Drapal and Sawers, 1995; Tseng et al., 1996).

Alexeeva et al. (2003) investigated the effects of different oxygen supply rates on the catabolism in *arc*A mutant. It is shown that the ArcAB system exerts more significant effects on the cell's catabolism under microaerobic conditions than under aerobic or anaerobic conditions. A strong link is demonstrated between redox ratio ($NADH/NAD^+$) and acetate overflow in *E. coli* (Vemuri et al., 2005). It is shown that the commencement of acetate overflow occurs above the critical $NADH/NAD^+$ ratio of 0.06 (Vemuri et al., 2005). Moreover, acetate overflow is delayed by the expression of heterologous NADH oxidase (NOX), an enzyme that serves to reduce the $NADH/NAD^+$ ratio (Vemuri et al., 2005). The redox state has been reported to trigger the Arc regulon (Georgellis et al., 2001; Malpica et al., 2004).

Since phosphorylated ArcA represses TCA cycle genes, the *arcA* gene deletion activates the TCA cycle, resulting in a reduction in the acetate formation (Vemuri et al., 2005). The NADH oxidation by the expression of NOX in the *arc*A knockout gene mutant further reduces the acetate formation, resulting in increased recombinant protein production (Vemuri et al., 2005). Since the TCA cycle is the main source of energy

generation and provides important precursors for amino acids, such as glutamate and lysine, it is of practical interest to enhance TCA cycle activity. As stated above, the *arc*A/B genes knockout in *E. coli* transcriptionally activate the TCA cycle and overproduce NADH, which may in turn repress the TCA cycle by allosteric regulation. Moreover, it has been reported that ArcB does not control the TCA cycle under aerobic conditions, due to the fact that oxidized quinone electron carriers inhibit autophosphorylation of ArcB, and it cannot transphosphorylate ArcA (Georgellis et al., 2001).

As expected from regulation, the TCA cycle is activated by the *arcA/B* gene knockout, which then causes a higher NADH/NAD ratio, which in turn represses TCA cycle activity (Nizam and Shimizu, 2009). Vemuri et al. (2006a,b) considered to express the heterologous *nox* gene to oxidize NADH, and in turn activate the TCA cycle, while nicotinic acid and Na nitrate may also activate the TCA cycle (Nizam and Shimizu, 2008).

Although the *arcB* gene knockout may be phenotypically silent under fully aerobic conditions, it may be affected under microaerobic conditions. To investigate the effect of the *arcB* gene knockout on fermentation characteristics, an aerobic batch cultivation was conducted. As shown in Table 3.6, the cell yield of *arcB* mutant is lower as compared to that of the wild type, while the specific glucose consumption rate is higher and the acetate production rate is lower for the former as compared to those of the latter strain. Figure 3.19 shows the gene expressions of the *arcB* gene knockout mutant as compared to its parent strain for the samples taken at 4 h of batch culture, where the TCA cycle genes, such as *gltA*, *icdA*, *fumA*, and *mdh*, are up-regulated compared to the parent strain (Nizam and Shimizu, 2008). The *aceA* gene of the glyoxylate pathway, and the *aceE* and *aceF* genes of PDHc (pyruvate dehydrogenase complex) pathway, are also up-regulated.

Table 3.6 Growth parameters of *E. coli* BW25113 and *arc*B mutant in aerobic batch cultures

	BW 25113	arcB mutant	arcA mutant
Cell yield (g g^{-1})	0.356 ± 0.04	0.344 ± 0.03	0.324 ± 0.04
Specific glucose consumption rate (mmol g^{-1} h^{-1})	1.45 ± 0.02	0.957 ± 0.04	1.38 ± 0.05
Specific acetate production rate (mmol g^{-1} h^{-1})	0.286 ± 0.02	0.14 ± 0.03	0.21 ± 0.03

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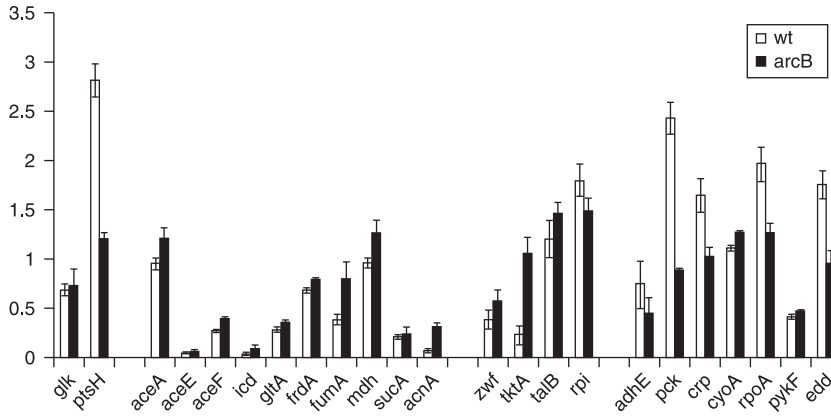

Figure 3.19 Comparison of some gene expressions for parent *E. coli* BW25113 and *arcB* mutant at 4 h of batch cultivation along with the gel picture. Open bars indicate the gene expressions in *E. coli* BW25113 and black bars indicate the gene expressions in *arcB* mutant

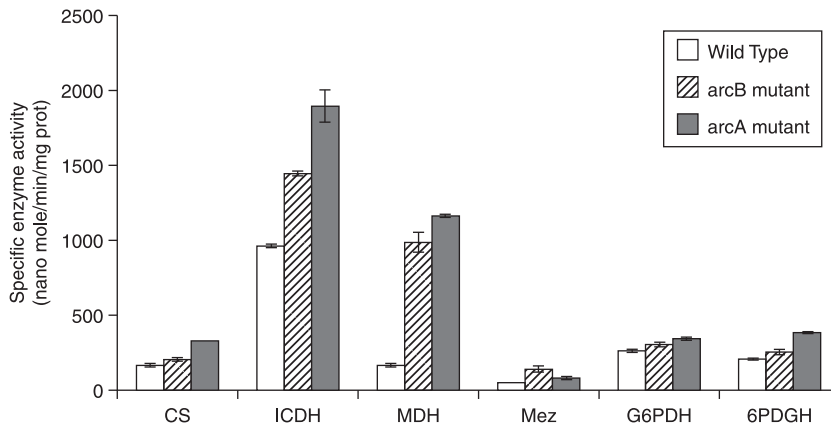

Figure 3.20 Comparison of specific enzyme activities of *E. coli* BW25113, its *arcB* and *arcA* mutant at 4 h of batch cultivation. Open bars indicate the enzyme activities in *E. coli* BW25113, hatched bars indicate *arcB* mutant and black bars indicate *arcA* enzyme activities

Under aerobic or micro-aerobic conditions, TCA cycle related genes, such as *gltA*, *fumA*, *mdh*, and *aceA*, are derepressed, resulting in activation of the TCA cycle enzymes, such as CS, ICDH, and MDH, in the *arcB* gene knockout mutant, as compared with the wild type. The *aceE* and *aceF* genes that code for PDHc, the enzyme that catabolize pyruvate dehydrogenase reactions under aerobic conditions, are up-regulated for the *arcB* mutant (Iuchi et al., 1989; Sawers and Suppmann, 1992; Suppmann and Sawers, 1994; de Graef et al., 1999; Alexeeva et al., 2000), and the increased flux of PDHc for *arcA* mutant. The *zwf* gene is known to be regulated by SoxS and MarA. SoxS can stimulate *zwf* expression for NADPH generation under oxidative stress conditions, and the induction by MarA is related to the superoxide resistance (Li and Demple, 1994; Jair et al., 1995). The reason for the up-regulation of *zwf* gene expression and the up-regulation of G6PDH and 6PGDH in the *arcB* mutant compared to the wild type, may be due to the oxidative stress caused by increased respiration, where SoxRS plays a role under oxidative stress conditions, and regulates such genes as *zwf* (Moat et al., 2002). The increased flux of the PP (Figure 3.23) pathway is consistent with the up-regulation of G6PDH and 6PDGH for the *arcA* mutant (Nizam et al., 2009).

As previously observed, *cyd* gene expression is repressed in *arcA* mutant (Tseng et al., 1996; Shalel-Levanon et al., 2005a,b), which together to TCA cycle activation causes a higher redox state when compared with the wild type. The higher redox state in turn inhibits some of the TCA cycle enzymes, such as ICDH, α-KGDH, and SDH, allosterically.

Since the TCA cycle is the source of energy generation and provides some of the precursors for cell synthesis, the activation of the TCA cycle may lead to improvement of ATP production for the cell growth and/or TCA cycle-related metabolite production in practice. It should be noted that the activation of the TCA cycle reduces the acetate production rate, which is a common obstacle for metabolite production using *E. coli*. However, it should also be noted that the activation of the TCA cycle causes a decrease in cell yield due to higher production rate of CO_2 in the TCA cycle. This may be overcome by activating the glyoxylate pathway by the *fadR* knockout gene (Peng et al., 2006). It is controversial whether cell metabolism is controlled to maximize ATP generation or cell synthesis (Schuetz et al., 2006).

3.5.3 Fnr and Nar systems

Respiration is a fundamental cellular process utilizing different terminal electron acceptors, such as oxygen and nitrate. The ability to sense these

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electron acceptors is key to the cell's survival. *E. coli* is a metabolically versatile chemoheterotroph grown on a variety of substrates, under various oxygen concentrations with fumarate or nitrate, replacing oxygen as the terminal electron acceptor under anaerobic conditions. Many bacteria utilize oxygen as the terminal electron acceptor, but can switch to other acceptors such as nitrate under oxygen limitation. In *E. coli*, this switch from aerobic to anaerobic respiration is controlled by Fnr (fumarate and nitrate reduction), where it was identified by Sipro et al. (1990). Under oxygen limitation, Fnr binds a $[4Fe–4S]^{2+}$ cluster, and becomes a transcriptionally active dimeric form.

E. coli possesses sensing/regulation systems for a rapid response to the availability of oxygen, redox state, as represented by NADH/NAD^+ ratio, and the presence of other electron acceptors. These regulation systems channel electrons from donor to terminal acceptors. The pyridine nucleotides, such as NADH and NAD^+, function as important redox carriers involved in metabolism. These cofactors not only serve as electron acceptors in the breakdown of substrates but also provide the reducing power for the redox reactions in 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 high compared to their concentrations (de Graef et al., 1999). Under anaerobic conditions, the reoxidation of NADH and the formation of reduced compounds occur, whereas NADH oxidation is coupled to respiration by electron transfer under aerobic or nitrate respiration. In *E. coli*, the genes that code for enzymes specific to respiration and fermentation are mainly under the control of three global regulators, where these exert their effects depending on the redox state of the cell. One of those is Fnr, which is involved in the regulation of gene expressions for fermentation-related enzymes, while the others are the two-component regulatory systems of Nar (nitrate reduction) and Arc (anoxic respiration control).

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). The ability of Fnr to bind DNA is regulated by a 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 conditions. Molecular oxygen can oxidize the ion-sulfur cluster of the corresponding region, resulting in monomerization of the protein and subsequent loss of its ability to bind DNA (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 membrane sensor (NarX), which may act as a kinase causing phosphorylation of the regulator (NarL) under certain conditions. The Nar system activates such genes as nitrate reduction encoding nitrate and nitrite reductases, and represses such genes as fumarate reductase.

As stated above, Fnr and ArcA/B play important roles in regulating the metabolism under anaerobic and microaerobic conditions. However, the detailed regulation mechanism is more complicated, since other global regulators, such as Cra, Crp, RpoS, may play roles.

3.5.4 Effect of fnr knockout gene on the metabolism

In order to clarify metabolic regulation by Fnr, consider the continuous culture at the dilution rate of 0.1 h^{-1} and batch cultures for the wild-type *E. coli* BW25113 and its *fnr* mutant (JW1328) under anaerobic conditions, where a M9 synthetic medium was used (Marzan et al., 2011). The fermentation result indicates that the specific glucose consumption rate is increased, the specific lactate production rate is increased, and the specific production rates of formate, ethanol, and acetate tend to decrease but not as significantly for the mutant as compared to the wild type. Figure 3.21 shows the gene transcript levels for the continuous culture, where it indicates that the *arcA* transcript level decreases, (and the TCA cycle genes, such as *gltA*, *icdA*, *sdhC*, and *mdh*, are increased) for the *fnr* mutant as compared to the wild type. This effect is compounded by the fact that *arcA* transcription

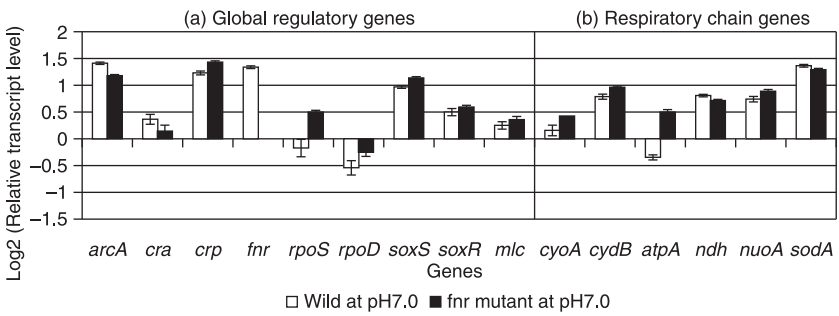

Figure 3.21 **Comparison of the transcript levels between wild type and *fnr* mutant under micro-aerobic continuous culture conditions: (a) global regulatory genes, (b) respiratory chain genes**

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is directly activated by Fnr (Compan and Touati, 1994), which in turn affects the oxidation of quinol (Constantinidou et al., 2006). The increased activities of cytochromes may enhance the oxidation of quinol to quinon, which inhibits the phophorylation of ArcB, and in turn may decrease the phosphorylation of ArcA. The *lpdA* as well as *aceE*, *F* gene transcript levels significantly increase for the *fnr* mutant as compared to the wild type, which may be due to decreased activity of *arcA*. It was also shown that the *ldhA* gene transcript levels are significantly increased for the *fnr* mutant as compared to the wild type. There might be some relationship between Fnr and Crp. Namely the sequence of the *fnr* gene reveals that it encodes a protein that 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 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 (Williams et al., 1991; Ziegelhoffer and Kiley, 1995; Lynch and Lin, 1996a,b; Salmon et al., 2003). Several studies have been conducted on the structure and gene sequence for Fnr and Crp proteins. From these studies, it is found that both Fnr and Crp proteins possess almost similar structure and gene sequences. The genes that are controlled by these two global regulators have similar binding sites (Bell et al., 1989; Kiley and Reznikoff, 1991; Williams et al., 1991; Lynch and Lin, 1996a,b; Bo et al., 1998; Unden et al., 2002). Even if some mutation changes the structure of proteins, the mutation in Fnr protein could convert to a Crp protein, and similarly Crp protein could convert to a Fnr protein (Sipro et al., 1990). It may also be considered that both Crp and Fnr proteins can form a heterodimer, which might not allow both of them to function properly

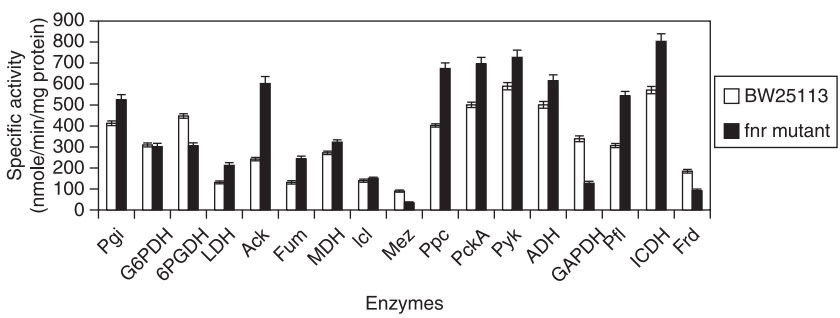

Figure 3.22 **Comparison of enzymes activities during micro-aerobic batch culture between (□) *E. coli* BW25113 and (■) *E. coli fnr* mutant**

(Bell et al., 1989; Williams et al., 1991; Ziegelhoffer and Kiley, 1995). Then the absence of an Fnr protein or gene would allow a Crp protein to bind more effectively to the target gene sequence.

Batch fermentation results indicate that the production rate of extra-cellular metabolites, such as acetate, formate, succinate, and ethanol, is reduced, while the production rate of lactate is increased for the *fnr* mutant as compared to the wild type. The enzyme activities are shown in Figure 3.22. Most of the glycolytic enzymes, such as Pgi, GADPH, and Pyk, show 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, 1995; Chao et al., 1997). Since Fnr is known to act as an activator of the *pfl* gene, significant reduction of Pfl activity is observed for the *fnr* mutant. The reduction of Pfl activity causes increased activity of the other fermentative pathway enzymes, such as LDH in the mutant as compared to the wild type. Other fermentative enzymes, such as Frd which produces succinate from fumarate under anaerobic conditions, are reduced in the mutant, which is consistent with lower succinate production rate in the mutant.

The metabolic flux distributions through the central metabolic pathway of the parent strain and its *fnr* mutant are then estimated based on mass balances. The analysis is performed based on the measurement of the specific rates and the pseudo-steady-state assumption for intracellular metabolites, as explained in Chapter 4. The total number of measured fluxes 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 are then calculated (Tsai et al., 1988), and the comparison of metabolic fluxes between the wild type and the *fnr* mutant is shown in Figure 3.23 (Marzan et al., 2011), 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 consistent with enzyme activities. AcCoA has two alternative fates. The energy in the thioester bond can be conserved in the form of ATP by the action of the 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 causes the reduce flux through the ADH pathway.

Since Fnr is known to activate the *frd* and *pfl* genes, the *fnr* mutant produces less succinate and formate. Although *arcA* is known to be activated by Fnr, the regulation mechanism is somewhat complicated. Namely, *cyo* and *cyd* genes are repressed by Fnr, while *cyo* is repressed

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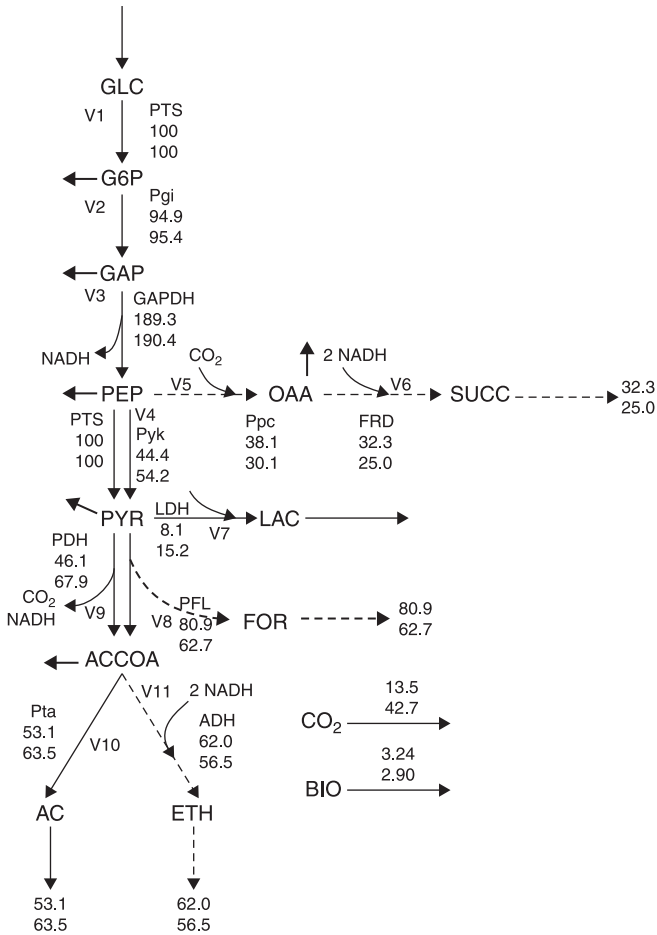

Figure 3.23 **Metabolic flux distributions of wild type (upper values) and *fnr* mutant (lower values) under micro-aerobic conditions. The dotted lines indicate the reduced flux. The arrows without destination indicate biomass synthesis**

and *cyd* is activated by ArcA. The above 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 increases the quinone pool, which inhibits ArcB phophorylation, and in turn decreases phosphorylation of ArcA, where *arcA* gene expression also decreases due to the *fnr* gene knockout. The down-regulation of *arcA* causes up-regulation of TCA cycle genes as well as *lpdA* and *aceE*, *F*, which code for PDH. Although an indirect effect,

the *fnr* mutant causes less growth rate, which causes less biomass concentration, which in turn causes glucose concentration to be increased. This may cause the *cra* gene to be down-regulated and thus activate glycolysis genes, and eventually cause up-regulation of the specific glucose uptake rate. The increased lactate formation may be due to higher NADH/NAD^+ ratios caused by the reduced Frd activity, and higher pyruvate concentrations caused by the down-regulation of Pfl and increased flux of Pyk, although PDH activity may be increased to some extent.

3.5.5 Effect of excess oxygen on the metabolism

The microbial cell responds to oxidative stress by inducing antioxidant proteins, such as superoxide dismutase and catarase (Greenberg and Demple, 1989). The well characterized pleiotropic regulators of the antioxidant responses are the OxyR and SoxR proteins (Pomposiello and Demple, 2001). The activation of both proteins results in the transcriptional enhancement of sets of genes whose products relieve the stress by eliminating oxidants and preventing or repairing oxidative damage (Pomposiello and Demple, 2001). SoxR is a member of the MerR family of metal-binding transcription factors, and exists in solution as a homodimer with each subunit containing a [2Fe–2S] cluster. These clusters are in the reduced state in inactivated SoxR, and their oxidation activates SoxR as a powerful transcription factor (Gaudu and Weiss, 1996). The active form of SoxR activates transcription of the *sox* gene. The *sox* gene product, SoxS, belongs to the AraC/XylS family of DNA-binding transcription factors (Amabile-Cuevas and Demple, 1991). SoxS regulates the expressions of more than 17 genes or operons (Liochev and Fridovich, 1992; Gruer and Guest, 1994; Fawcett and Wolf, 1995; Liochev et al., 1999; Gaudu and Weiss, 2000).

Oxygen derivatives, such as superoxide ($O_2\cdot^-$), hydrogen peroxide (H_2O_2), and the hydroxyl radical (·OH), are usually generated as toxic by-products of aerobic metabolism in a cascade of monovalent reductions from molecular oxygen. Although these are not so reactive *per se*, $O\cdot^-$ and H_2O_2 have been reported to cause severe cell damage. H_2O_2, along with Fe^{2+} via the Fenton reaction, produces ·OH, which can react with any macromolecule such as a protein, membrane constituents, and DNA (Flint et al., 1993; Liochev and Fridovich, 1994). $O_2\cdot^-$ exacerbates the Fenton reaction by increasing the intracellular pool of 'free iron', for instance by releasing iron from $O_2\cdot^-$-oxidized [4Fe–4S] clusters. $O_2\cdot^-$ may

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also react with nitric oxide (·NO), producing highly reactive peroxynitrite ($ONOO^-$), which can generate ·OH. Despite their potential toxicity, reactive oxygen species (ROS) at low concentration have been shown to be actively involved in the cell life and, therefore, should not be entirely eliminated. Potent basic defense systems maintain ROS at harmless levels, but cannot deal with sudden increases in ROS production. This creates an imbalance between production and elimination, referred to as oxidative stress.

Early studies using 2D gel electrophoresis to analyze variations in protein expressions have shown that the synthesis of more than 80 proteins are activated in response to oxidative stress (Greenberg and Demple, 1989). Some of these induced proteins are identified as possessing fundamental antioxidant functions, for example, superoxide dismutase and catalase. The search for mutants with altered antioxidant defenses has led to the isolation and characterization of pleiotropic regulators that operate as redox-regulated genetic switches (Greenberg et al., 1990; Storz et al., 1990; Amabile-Cuevas and Demple, 1991). The best characterized pleiotropic regulators of the antioxidant responses are the OxyR and SoxR proteins (Pomposiello and Demple, 2001). Both proteins have the remarkable ability of directly transducing oxidative signals to genetic regulation. Both proteins are expressed constitutively in an inactive state and are transiently activated in cells under specific types of oxidative stress. Activation of the OxyR and SoxR proteins results in the transcriptional enhancement of sets of genes (regulons), whose products relieve the stress by eliminating oxidants and preventing or repairing oxidative damage (Pomposiello and Demple, 2001).

3.5.6 Effect of soxR/S genes knockout on the metabolism

Here, the effects of *soxR* and *soxS* genes knockout on the central metabolism of *E. coli* are explained, based on fermentation characteristics and gene expressions (Kabir and Shimizu, 2006). Batch aerobic cultivations of *soxR* mutant (JW4024), *soxS* mutant (JW4023), and its parent *E. coli* BW25113 are performed, and the growth parameters are shown in Table 3.7. The specific growth rate is slightly lower for *soxS* mutant (7.9% decrease), but significantly lower for *soxR* mutant (31.6% decrease) compared to the parent strain. The glucose uptake rate is also found to be lower for both *soxR* and *soxS* mutants, as compared to the parent strain. Consequently, biomass yield is lower for mutants, as

Table 3.7 Specific rate of *soxR* and *soxS* mutants, and parent *E. coli* grown on glucose under aerobic conditions. Standard deviations are calculated from four independent measurements

Parameter	BW25113 (Parent)	JW4024 ($soxR^-$)	JW4023 ($soxS^-$)
μ [h^{-1}]	0.42 ± 0.04	0.27 ± 0.01	0.38 ± 0.03
$Y_{X/S}$ [gDCW.gGLC^{-1}]	0.37 ± 0.01	0.33 ± 0.02	0.34 ± 0.01
q_{GLC} [mM.gDCW^{-1}.h^{-1}]	6.33 ± 0.43	4.50 ± 0.71	6.16 ± 0.55
q_{ACE} [mM.gDCW^{-1}.h^{-1}]	4.16 ± 0.45	4.50 ± 0.44	8.00 ± 0.38
q_{LAC} [mM.gDCW^{-1}.h^{-1}]	0.02 ± 0.01	0.04 ± 0.01	0.09 ± 0.01
q_{CO_2} [mM.gDCW^{-1}.h^{-1}]	0.26 ± 0.03	0.17 ± 0.03	0.22 ± 0.02

μ, specific growth rate; q specific uptake/production rate; $Y_{X/S}$, biomass yield; GLC, glucose; ACE, acetate; LAC, lactate; CO_2, carbon dioxide; DCW, dry cell weight

compared to the wild type. Note that the acetate production rate is significantly increased in *soxS* mutant compared to the parent strain, whereas it is slightly increased in the *soxR* mutant.

The relative expression levels of *pgi*, *crp*, *pfkB*, *fbaA*, *pgk*, *pykA*, *pykF*, *aceE*, and *aceF* involved in the glycolysis are significantly up-regulated in both *soxR* and *soxS* mutants, compared to those in the parent strain (Figure 3.24). Relative expression level of *zwf* is significantly down-regulated in the *soxR* and *soxS* mutants, compared to the parent strain (Figure 3.24). The expression level of the *gnd* gene is significantly down-regulated in the *soxR* mutant, whereas it is slightly regulated in the *soxS* mutant strain. Fermentative genes, such as *pta* and *ackA*, are up-regulated significantly in the *soxS* mutant, whereas the *pta* gene is unchanged and the *ackA* gene is up-regulated in the *soxR* mutant, compared to those in the parent strain.

Among the TCA cycle genes, the relative expression levels of *gltA*, *acnA*, *B*, *icdA*, *sucA*, *sdhC*, and *fumA* are down-regulated, whereas the *mdh* gene is almost unchanged in both *soxR* and *soxS* mutants, as compared to those in the parent strain (Figure 3.29). The down-regulation of these genes is mainly due to the mutation in the *soxRS* system, since the *soxRS* system increases the expression of *fumC* and *acnA* (Hidalgo and Demple, 1996). The expression levels of the genes *secA* and *lepB* are significantly up-regulated, whereas the *dsbA* gene is slightly up-regulated in both the *soxR* and *soxS* mutants (Figure 3.24), indicating that these gene deletions might increase the activity of protein secretion and disulfide bond formation. The transcript levels of *rpoE, rpoH*, and *rpoS* (encoding σ^E, σ^{32}, and σ^S, respectively) are up-regulated significantly, whereas *rpoD* (encoding σ^{70}) is almost unregulated in both the *soxR* and *soxS* mutants,

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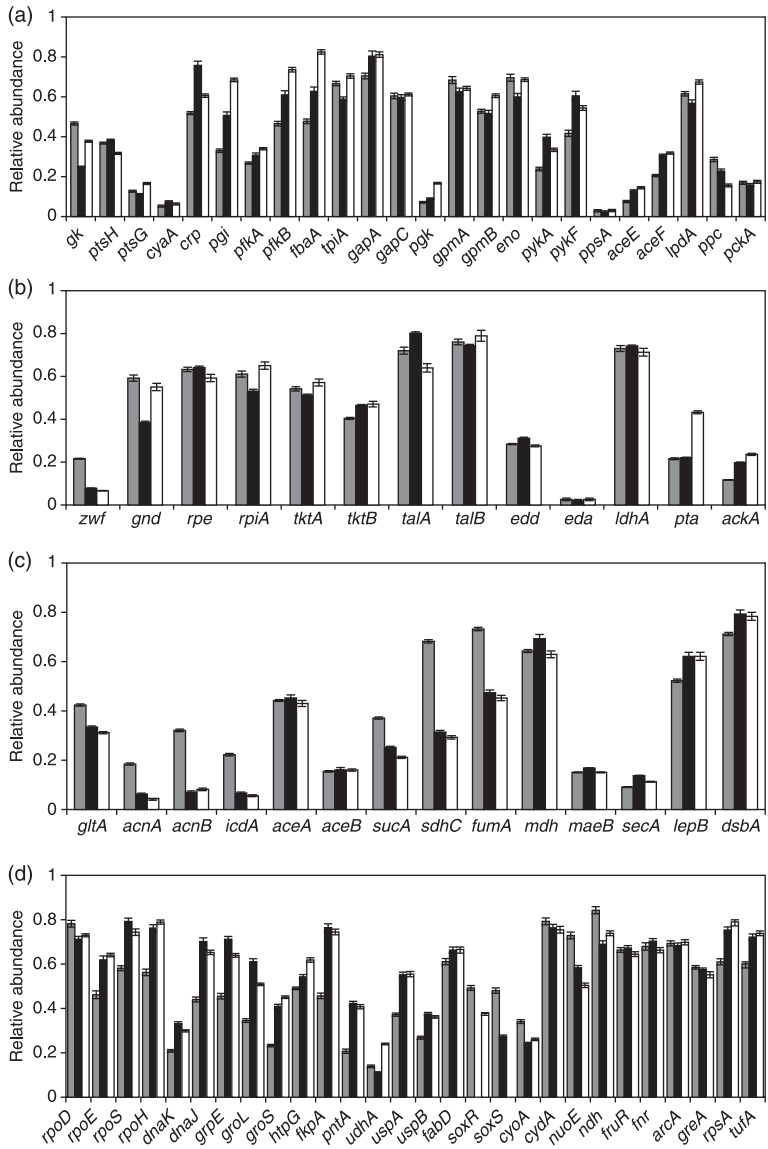

Figure 3.24 Comparison of gene expressions for parent (■), *soxR* mutant (■) and *soxS* (□) mutant *E. coli*. (a) Transport and glycolytic pathway genes; (b) PP pathway and fermentative; (c) Genes involved in TCA cycle and protein synthesis; and (d) Genes involved in sigma factor, heat shock, NADPH reoxidation, respiration, global, and other regulations

as compared to those in the parent strain (Figure 3.24). The significant up-regulation of heat shock genes, such as *dnaK*, *dnaJ*, *grpE*, *groL*, *groS*, *htpG*, and *fkpA*, in both mutants corresponds to the fact that the heat shock gene expressions are largely proportional to the amount of σ^{32}, though the level of σ^{32} activity is known to be regulated at multiple stages, including translational efficiency and protein stability (Straus et al., 1987). The expression level of the gene *pntA* is also up-regulated significantly in both mutants, and *udhA* is down-regulated in the *soxR* mutant but up-regulated in the *soxS* mutant (Figure 3.2), indicating that deletion of the *soxR* and *soxS* genes affects the transdydrogenase activity. However, expression levels of the genes involved in respiration, such as *cyoA, nuoE*, and *ndh*, are down-regulated, except for *cydA* in both the *soxR* and *soxS* mutants.

The specific activities of Pgi and Pfk are up-regulated in both mutants, as compared to the parent strain (Figure 3.25). The enzyme activities of Fba, GAPDH, Pgk, Pyk, and PDH complexes are also up-regulated significantly in the *soxS* mutant, whereas these enzymes are slightly regulated in the *soxR* mutant. The two enzymes involved in the oxidative PP pathway, G6PDH and 6PGDH, which provide NADPH for biosynthesis, are significantly affected in both the *soxR* and *soxS* mutants (Figure 3.25). The activities of G6PDH and 6PGDH decrease in both the *soxR* and *soxS* mutants, compared to the parent stain. The down-regulations of these two enzymes agree with the slower growth rates in both mutants, since these enzymes are known to be under growth rate-dependent regulation (Wolf et al., 1979).

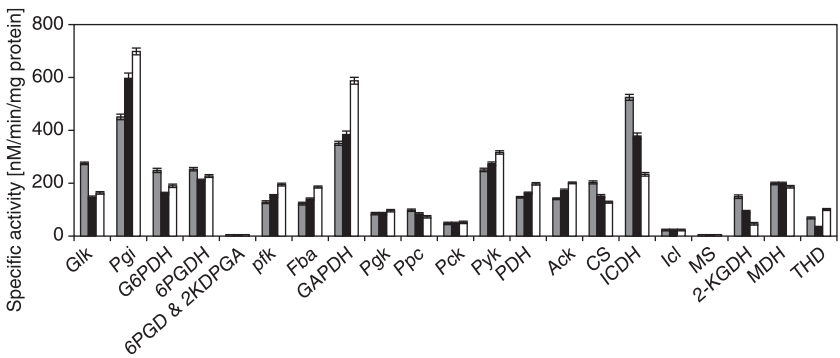

Figure 3.25 **Specific enzyme activities in cell extracts of parent (■), *soxR* mutant (■) and *soxS* (□) mutant *E. coli* grown on glucose under aerobic conditions. The mean value from four independent measurements is presented with standard deviation**

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The starting enzyme of the TCA cycle, citrate synthase (CS), which participates in the generation of cellular biosynthetic intermediates and the reduced purine nucleotides used in energy generation via an electron transport-linked phosphorylation reaction, is down-regulated in the *soxR* and *soxS* mutants, as compared to the parent strain (Figure 3.25). This down-regulation causes subsequent decreases in the other TCA cycle enzyme activities, such as ICDH and KGDH, except MDH in both mutants. As a result, a higher specific acetate secretion rate was observed in both mutants, especially in the *soxS* mutant due to the reduced TCA cycle activity. The anaplerotic enzyme Ppc is also found to be down-regulated significantly in both of the mutants compared to the parent strain (Figure 3.25). Ack activity is significantly up-regulated in both mutants compared to the parent strain, consistent with the higher acetate production rate. Moreover, the activity of soluble transhydrogenase (THD) encoded by *udhA* which catalyzes the conversion of NAD^+ and NADPH to NADH and $NADP^+$, is up-regulated in the *soxS* mutant but down-regulated in the *soxR* mutant, compared to those in the parent strain (Figure 3.25), and these results are consistent with the gene expressions (Figure. 3.24).

The activity of Pgi is up-regulated in both the *soxR* and *soxS* mutants, compared to the parent strain, and this result is consistent with the analysis of gene expression levels for *pgi* transcripts. This is mainly due to the down-regulation of the G6PDH enzyme in both of the mutants. The down-regulation of the *zwf* gene in both mutants is also due to the effects of *soxS* and *soxR* genes deletion, since *zwf* is a member of *soxRS* and multiple antibiotic resistance (*mar*) regulons. Thus, unlike *gnd, zwf* expression is transcriptionally activated by SoxS during episodes of oxidative stress (Greenberg et al., 1990; Tsaneva and Weiss, 1990).

The *pntA* (membrane bound transhydrogenase) transcripts, which are involved in NADPH generation (Hanson and Rose, 1980), are up-regulated in both the *soxR* and *soxS* mutants. This may be due to down-regulation of NADPH generating enzymes such as G6PDH and 6PGDH in the PP pathway, and ICDH in TCA cycle, since NADPH plays a significant role to reduce oxidative stress (Greenberg et al., 1990). However, *udhA* (soluble transhydrogenase) transcripts, which are involved in the reoxidation of NADPH (Canonaco et al., 2001), are found to be down-regulated in the *soxR* mutant but up-regulated in the *soxS* mutant, compared to that in the parent strain (Figure 3.25), and this result is consistent with the measurement of THD activity (Figure 3.25), indicating that the *soxR* mutant suffers from insufficient re-oxidation of reducing power. This might be one of the reasons why growth of the *soxR* mutant is severely affected.

As mentioned before, acetate production rates are increased significantly in the *soxS* mutant and slightly changed in the *soxR* mutant, as compared to the parent strain. The reason may be due to up-regulation of the glycolytic pathway (down-regulation of PP pathway) and TCA cycle. Moreover, the relative expression levels of *cyoA*, *nuoE*, and *ndh* genes are down-regulated in both mutants, indicating that the *soxR* and *soxS* genes deletion adversely affects the respiratory system and the electron transport chain.

3.6 Acid shock or the effect of pH

The acid barrier of the stomach represents a strong challenge for many pathogenic enterobacteria. Enteric bacteria that cause disease in the human intestine endure transient but extreme acid conditions in the stomach. The normal stomach presents an acid environment at around pH2, with an emptying time of about 2 h (Smith, 2003). Unlike acid sensitive *Vibrio cholerae, E. coli*, and *Shigella* have potent acid resistant systems able to withstand a low pH at around 2 for at least 2 h (Lin et al., 1995; Castanié-Cornet et al., 1999). *E. coli* possesses a level of acid resistance rivaling that of the gastric pathogen *Helicobacter Pylori* (Rektorschek et al., 2000). As such, it is important to understand cell metabolism in relation to acidic conditions, from both medical and fermentation points of view. The molecular and physiological response to acid stress has thus been the subject of intense investigation (Foster, 2004; Stincone et al., 2011).

Several acid stress response systems that can protect *E. coli* from acidic conditions have been investigated (Foster, 2004; Richard and Foster,

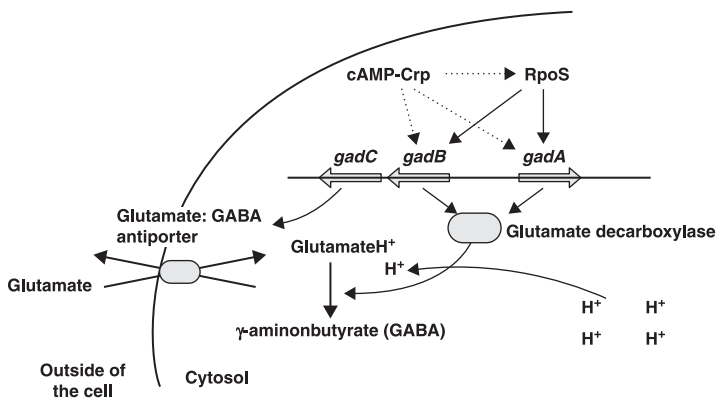

Figure 3.26 The role of glutamate decarboxylase for acid resistance

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2003, 2004; Stincone et al., 2011). Some of these depend on the available extracellular amino acids such as glutamate, arginine, and lysine, where the intracellular proton is consumed by the reductive decarboxylation of the amino acid followed by the excretion of the product, such as γ-amino butyric acid (GABA) from cytoplasm to the periplasm by a dedicated antiporter that also imports the original amino acid (Foster, 2004) (Figure 3.26). *E. coli* cells have been demonstrated to exhibit acid resistance by such genes as *gadAB*, which encode glutamate decarboxylase and *gadC*, which encode the Glutamate : GABA antiporter. Glutamate decarboxylase production has been shown to increase in response to acid, osmotic, and stationary phase signals. The *gadA* and *gadB* genes for glutamate decarboxylase isozymes form a glutamate-dependent acid response system, where the process of decarboxylation consumes an intracellular proton and helps maintain pH homeostasis. It has also been shown that there exist similar acid resistant systems using arginine instead of glutamate by arginine decarboxylase, where the antiporter is AdiC in this case (Lin et al., 1995; Castanié-Cornet et al., 1999; Gong et al., 2003; Iyer et al., 2003), and using lysine by lysine decarboxylase (Iyer et al., 2003). Note that cells grown in a media rich in amino acids such as LB are acid resistant (Foster, 2004).

In a typical batch culture, organic acids are most accumulated at the late growth phase or the stationary phase, and it has been known that GadA and GadB proteins increase in response to the stationary phase and low pH (Castanié-Cornet and Foster, 2001). The sigma factor σ^S or RpoS, which increases its amount at the late growth phase and the stationary phase, as well as Crp, are involved in acid resistance (Castanié-Cornet et al., 1999; Foster, 2004). As is implied by the involvement of Crp, the resistance system is repressed when glucose is present. Moreover, it has been shown that FoF_1 proton translocating ATPase is involved in this system (Richard and Foster, 2004). The FoF_1 ATPase is utilized as the protons in the periplasm move into the cytosol across the cell membrane, producing ATP from ADP and P_i by the negative proton motive force (PMF). Since the basic problem of acid stress is the accumulated proton in the cytosol, this proton can be pumped out through FoF_1 ATPase by hydrolyzing ATP and reversed proton movement due to positive PMF at a low pH such as pH2 or 3 (Richard and Foster, 2004). Without amino acids available in the media, this acid response system is activated by utilizing FoF_1 ATPase (Martin-Galiano et al., 2001; Richard and Foster, 2003), where the positive proton motive force (PMF) pumps extrudes protons (H^+) from the cytoplasm with consumption of ATP (Richard and Foster, 2004). Namely PMF is operated in the reverse direction, as compared to producing ATP.

Table 3.8 Regulators involved in regulating glutamate-dependent acid resistance

Protein	Descriptor	Function in acid resistance
RpoD	σ^{70}	Transcription of *gadA/BC*
RpoS	σ^{38}	Transcription of *gadX*
EvgAS	2-component signal transduction	Activates *ydeO* and *gadE* transcription
YdeO	AraC-like regulator	Activates *gadE* transcription
GadE	LuxR-related activator	Required for acid resistance, binds to Gad box, activates transcription of *gadA/BC*, autoactivates *gadE*, represses *ydeO*
GadX	AraC-like regulator	Activator of *gadE*, co-activator of *gadA/BC*, represses *gadW*
GadW	AraC-like regulator	Inhibits RpoS production, activator of *gadE*, can co-activate *gadA/BC* at pH 8
Crp	cAMP receptor protein	Inhibits RpoS production
TrmE	Era-like GTPase	Activates *gadE* mRNA production, stimulates translation of *gadA* and *gadB* mRNA
HNS	Histone-like protein	Negative regulator
TorR	Response regulator of TMAO reductase	Negative regulator of *gadE*

Table 3.8 shows 11 regulators involved in regulating glutamate-dependent acid resistance. In the typical batch culture, the *gadA/BC* loci can be induced during growth in acidic minimal media (pH 5.5) or in the stationary phase regardless of pH (Foster, 2004). However, in complex media such as LB, neither locus is induced until the culture enters into stationary phase.

The expressions of *gadA/BC* genes are under control of GadE and the response regulator RcsB (Castanié-Cornet et al., 2010), where RcsB is part of the RcsCDB phosphorelay, a signal transduction system conserved in members of the *Enterobacteriaceae*. The RcsB can also be activated independently of the phosphorelay, by binding of different co-regulators, such as RcsA (main one), RmpA, TviA, and PhoP. (Castanié-Cornet et al., 2010). As shown in Figure 3.27, EvgS is a sensor kinase and phosphorylates EvgA, where the phosphorylated EvgA activates the *gadE*

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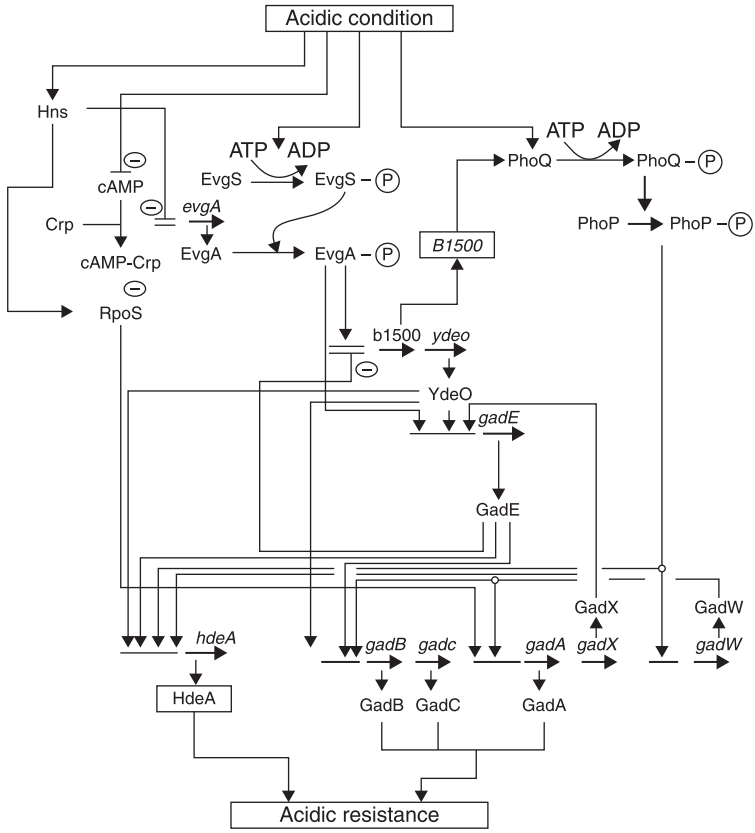

Figure 3.27 **Acid resistance mechanism under acidic conditions**

gene as well as *ydeO*, where YdeO also regulates the *gadE* gene. It has also been shown that the small membrane protein B1500 connects the signal transduction cascade between EvgS/EvgA and PhoQ/PhoP, where *b1500* is located upstream of *ydeO* and under control of EvgA (Eguchi et al., 2007) and Mg^{2+} turns off the PhoQ/PhoP system (Castelli et al., 2000). Moreover, the phosphorylated PhoP activates *gadW*, and GadW activates *hdeA* and *gadA*, where *hdeA* is under the control of GadW, PhoP-P, and GadE. EvgA regulates at least eight genes related to acid resistance, such as *ydeP*, *b1500*, *ydeO* (Matsuda and Church, 2003).

It has been shown that an acid pH lowers cAMP levels in exponentially growing cells in the minimal glucose medium. This may elevate RpoS that would drive increased expression of *gadX*. However, GadW represses RpoS synthesis under acidic conditions, and in turn GadX synthesis.

GadX, when not repressed by GadW, is acid induced due to changes in cAMP. GadW is also acid induced when it is not repressed by GadX. GadX directly binds to the *gadW* promoter region. GadX and GadW collaborate to repress *gadA* and *gadBC* expressions under alkaline conditions (Ma et al., 2003). The GadX-GadW regulon has also been investigated by DNA microarray (Tucker et al., 2003).

It has also been shown that the two-component system of EnvZ (sensor) and OmpR (regulator) regulates porin expression, where OmpR may be a key regulator for acid adaptation, and thus the *opmR* mutant is sensitive to acid exposure (Stincone et al., 2011). It has been shown that the level of OmpC increases with increased osmolarity when cells are growing in neutral or alkaline media, whereas the level of OmpF decreases at high osmolarity (Sato et al., 2000). It has also been shown that these porin proteins play important roles at acidic conditions.

The acid-inducible *asr* gene is reported to be regulated by the two-component system PhoR/B, which controls the *pho* regulon in response to phosphate starvation, and thus the PhoB-PhoR deletion mutant fails to induce *asr* gene expression (Suziedeliene et al., 1999). It has also been suggested that H^+ directly or via its acceptor might activate a sensor protein PhoR in the periplasm (Suziedeliene et al., 1999).

Under anaerobic conditions, additional genes, such as *ackA* and *lpdA*, as well as *hdeA* and *ompT*, are induced. In order to avoid deleterious concentration in the cell caused by the production by the cell at low pH, *ldhA* is induced by acid in order to produce lactate instead of the more harmful acetate plus formate (Bunch et al., 1997).

3.7 Heat shock response

Organisms respond to a sudden temperature up-shift by increasing the synthesis of a set of proteins. This phenomenon is called the heat shock response. The research on the heat shock response of a microorganism contributes to the variety of practical applications, such as temperature-induced heterologous protein production (Hockney, 1994; Hoffmann et al. 2002), and simultaneous saccharification and fermentation (SSF) (Philippidis et al., 2004). Heat shock proteins play a role in the assembly and disassembly of macromolecular complexes such as GroE (Sternberg, 1973), intracellular transport such as Hsp70 (Chirico et al., 1988), transcription such as σ^{70} (Taylor et al., 1984), proteolysis such as Lon (Goff et al., 1984), and translation such as lysil tRNA synthetase (Vanbogelen et al., 1983). The heat shock response in *E. coli* is mediated

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by $E\sigma^{32}$ (Yura et al., 1993), and it has been shown that at least 26 genes are induced by heat shock (Chuang and Blattner, 1993), where E denotes the RNA polymerase holoenzyme. Among them, *groEL*, *dnaK*, and *htpG* are the genes that code for the major chaperones, such as Hsp 60, Hsp 70, and Hsp 90. ClpP, Lon, and HtrC are involved in proteolysis. DnaK, DnaJ, GrpE, and RpoH are involved in autoregulation of the heat shock response (Tilly et al., 1983; Craig and Gross, 1991; Gamer et al., 1992; Liberek et al., 1992). It has been shown that DnaK prevents the formation of inclusion bodies, by reducing aggregation and promotion of proteolysis of misfolded proteins (Mogk et al., 2003). A bi-chaperone system, involving DnaK and ClpB, mediates the solubilization or disaggregation of proteins (Schlieker et al., 2002). GroEL operates protein transit between soluble and unsoluble protein fractions and participates positively in disaggregation and inclusion body formation. Small heat shock proteins, such as IbpA and IbpB, protect heat-denatured proteins from irreversible aggregation and have been found to be associated with inclusion bodies (Kitagawa et al., 2002; Sorensen and Mortensen, 2005).

Much has been reported on the molecular mechanisms of heat shock proteins (Richmond et al., 1999; Rosen and Ron, 2002). Hoffmann et al. (2002) investigated the metabolic adaptation of *E. coli* during temperature-induced recombinant protein production, and showed that cAMP/Crp-controlled LpdA of the pyruvate dehydrogenase complex (PDHc) and SdhA in the TCA cycle are induced four times, reaching a maximum at 1 h after temperature up-shift. It is also shown that TCA cycle enzymes, such as ICDH and MDH, are initially less produced but regain their respective preshift values about 30 min after the temperature up-shift. Gadgil et al. (2005) investigated the effect of temperature down-shift from 37°C to 33°C and 28°C on gene expressions in *E. coli*. This kind of investigation is useful in analyzing the metabolic changes and investigating the effects of gene modification for strain improvement (Kao, 1999).

Consider how gene expression pattern changes in *E. coli* for the temperature up-shift from 37°C to 42°C in relation to fermentation characteristics (Hassan and Shimizu, 2008). Table 3.9 shows the effect of culture temperature on the fermentation parameters, which indicates that acetate is more accumulated, the cell yield is lower, and the specific glucose consumption rate is lower at 42°C, as compared to the case at 37°C.

Figure 3.28 compares the gene expressions for the two different culture temperatures in continuous culture (Table 3.9), which indicates that the expression of *rpoH* is up-regulated, and the expressions of *dnaK*, *groL*, *groS*, *htpG*, and *ibpB* are up-regulated, which are known to be under the

Table 3.9 Fermentation parameters for the aerobic chemostat culture of the wild type *E. coli* BW25113 at the dilution rate of 0.2 h^{-1}

Fermentation parameters	Culture temperature		% changes
	37 °C	42 °C	
Specific glucose uptake rate (mmol/gDCW/h)	2.46 ± 0.05	2.28 ± 0.04	–7.31
Specific acetate production rate (mmol/gDCW/h)	0.18 ± 0.02	0.32 ± 0.04	+77.8
Specific CER (mmol/gDCW/h)	5.83 ± 0.05	5.87 ± 0.04	+0.69
Specific OUR (mmol/gDCW/h)	5.74 ± 0.06	5.76 ± .04	+0.35
Biomass yield (gDCW/g substrate	0.42 ± 0.01	0.34 ± 0.02	–19.0

control of the sigma factor (σ^{32}). Figure 3.28 also shows the up-regulation of *arcA* gene expression, where the *arcA* gene product functions as a repressor of such genes as are involved in the TCA cycle under microaerobic conditions. Figure 3.28 indicates that some of the TCA cycle and glyoxylate pathway genes, such as *icdA, sucA*, and *aceA*, are down-regulated. These genes are under the control of ArcA. Figure 3.28 also indicates that the expression of the respiratory chain gene, such as *cydB*, is up-regulated,

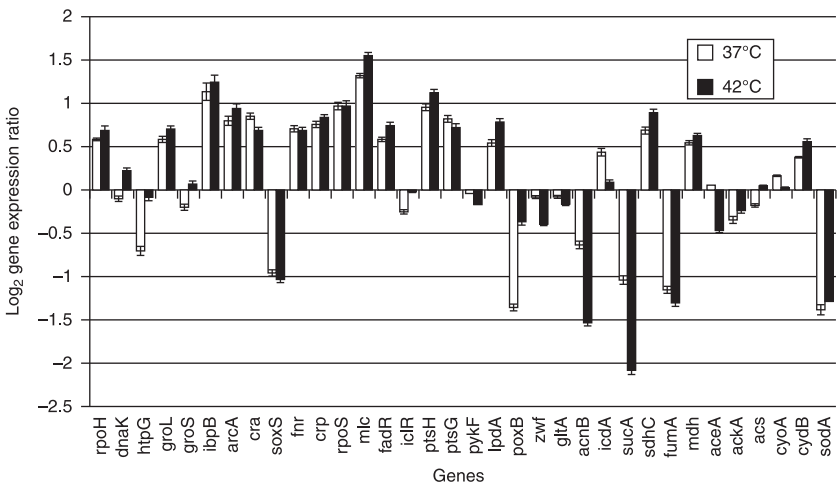

Figure 3.28 Effect of temperature up-shift on gene expressions in *E. coli* BW25113 under aerobic continuous culture at the dilution rate of 0.2 h^{-1}

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whereas *cyoA* is down-regulated. This may be due to the up-regulation of *arcA* and its gene product ArcA. Figure 3.28 also indicates that the expression of the *crp* gene, which codes for the cAMP receptor protein Crp, is up-regulated, and the expression of *lpdA*, which is under control of Crp (Appendix A), is also up-regulated (Hoffman et al., 2002). Moreover, *mlc* (making larger colonies) gene expression is higher, and the *ptsG* gene expression is lower. Figure 3.28 also shows that the *cra* gene expression is down-regulated. The gene expressions of *fadR* and *iclR* are also higher, where FadR activates *iclR*, and IclR is known to repress *aceBAK*.

To survive, cells have to control gene expressions precisely in response to changes in their growth environment. Microorganisms, such as *E. coli*, attain this primarily at the transcription level. To control the initiation of the specific transcription, the cell uses diverse mechanisms including various sigma factors. The classical heat shock regulon has been shown to be under the control of the σ^{32} transcription factor, the product of the *rpoH* gene (Gross et al., 1990). The regulation of the sigma factor (σ^{32}) is complex and depends on the feedback control loops involving the *dnaK* chaperone and temperature-induced changes in mRNA secondary structure (Yura and Nakahigashi, 1999). The relative levels of the major heat shock genes, such as *dnaK*, *groS*, *groL*, *ibpB*, *lonA*, and *htpG*, are up-regulated after the temperature up-shift. The expressions of heat shock genes, such as *dnaK*, *groL*, and *ibpB*, increase in the early induction phase (first 10–20 min) and then decline. In *E. coli*, heat shock protein synthesis rates peak at about 5~10 min after the temperature up-shift and then decline to new steady-state levels (Tilly et al., 1986). The heat shock response is made transcriptionally, where it has been known that the RNA polymerase core (E) binds to new initiation subunit σ^{32} (Skelly et al., 1987), and the resulting holoenzyme Eσ^{32} transcribes only heat shock genes (Grossman et al., 1984), which have promoter sequences that differ from those transcribed by E plus σ^{70}, the normal vegetative initiation factor (Cowing et al., 1985). The transcription factor σ^{70} is itself a heat shock protein and the increase in its concentration after heat shock may contribute to its decline in heat shock protein synthesis. Moreover, other heat shock proteins, in particular the *dnaK* gene product, contribute to the shutoff, since the mutations in their genes prolong the high level synthesis of heat shock proteins (Tilly et al., 1983). The heat shock response must be tightly regulated in order to allow rapid changes in heat shock protein synthesis rates. Although the level of mRNA transcribed from the *rpoH* gene increases after heat shock, their increase may be insufficient and too slow to be the sole explanation of the rapid effect of the heat shock. It has been shown that the concentration of

active σ^{32} limits the expression of heat shock genes, and that the stability of σ^{32} is modulated (Tilly et al., 1986).

Because of the rapid turn-over (half life <1 min), the cellular concentration of σ^{32} is very low at normal temperature and is limiting for the transcription of the heat shock gene. Upon temperature up-shift, σ^{32} becomes transiently stabilized until the beginning of the shut-off phase of the heat shock response. The heat shock response is induced as a consequence of declining σ^{32} levels and inhibition of σ^{32} activity. Stress-dependent changes in the heat shock gene are mediated by the antagonistic action of σ^{32} and negative modulators, which act upon σ^{32}. These modulators are the DnaK chaperone system, which inactivates σ^{32} by direct association and mediates its degradation by proteases (Arsene et al., 2000). Degradation of σ^{32} is mediated mainly by FtsH and ATP dependent metallo-protease within the inner membrane. The heat shock proteins increased immediately after the temperature up-shift reached a maximum 5–15 min later, and decreased to pre-shift values largely within 1 h, while the maximum induction of many heat-shock proteins, including DnaK and HtpG, was reached at least 30 min later.

The cyclic AMP (cAMP) receptor protein Crp activates transcription for more than 100 promoters. When bound to its allosteric effector cAMP, the Crp homodimer binds to the specific DNA sites near target promoters, enhancing the binding of RNA polymerase holoenzyme (RNAP) and facilitating the initiation of the transcription. The above result indicates that *crp* gene expression increases and *lpdA* gene expression follows a similar pattern after heat shock.

It is shown that *mlc* gene expression follows the same pattern as that of *rpoH* upon heat shock, which confirms that $E\sigma^{32}$ is involved in the expression of *mlc* gene. It has been shown that $E\sigma^{32}$ plays an important role in balancing the relative concentration of Mlc and EIICB in response to the availability of glucose in order to maintain inducibility of Mlc regulon at higher temperature (Shin et al., 2001). When Mlc is overproduced, it has been known to reduce acetate accumulation (Hosono et al., 1995), and to cause slow growth but give better performance for recombinant protein production (Cho et al., 2005). Mlc is a global regulator of carbohydrate metabolism, and regulates the expression of *pts* operon. It has been shown that Mlc represses *manXYZ* encoding enzyme II of the mannose PTS (Plumbridge, 1998a,b), *malT* encoding the activator of maltose operon, and *mlc* itself negatively (Kim et al., 1999). Moreover, *ptsG* encoding enzyme IICB of the glucose PTS ($EIICB^{glc}$) and the *pts* operon encoding general PTS proteins, are also known to be repressed by Mlc (Kimata et al., 1998; Tanaka et al., 1999).

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The *mlc* promoter is very weak because the nucleotide sequence of the –10 region of the promoter differs from the consensus sequence of the strong promoter of *E. coli*. In addition, Mlc expression is autoregulated by Mlc itself. Therefore, the intracellular concentration of Mlc is limited in *E. coli* (Nam et al., 2001). The *mlc* gene has been known to be transcribed by two promoters, P1 and P2, and have a binding site of its own gene product. It has been shown by *in vitro* transcription assays of *mlc* gene that the P2 promoter could be recognized by RNA polymerase containing the heat shock sigma factor σ^{32} ($E\sigma^{32}$) as well as $E\sigma^{70}$, while the P1 promoter is only recognized by $E\sigma^{70}$. The overall regulation mechanism against heat shock may be expressed as in Figure 3.29.

Let us consider the production mechanism of acetate at higher temperatures. In a typical batch cultivation, cells must switch efficiently from a rapid growth on a favored carbon source such as glucose to a much slower growth on the excreted by-products such as acetate. Acetate excretion occurs through the Pta-Ack pathway, or possibly may be by the Pox pathway. Acetate utilization occurs through AcCoA synthetase (Acs).

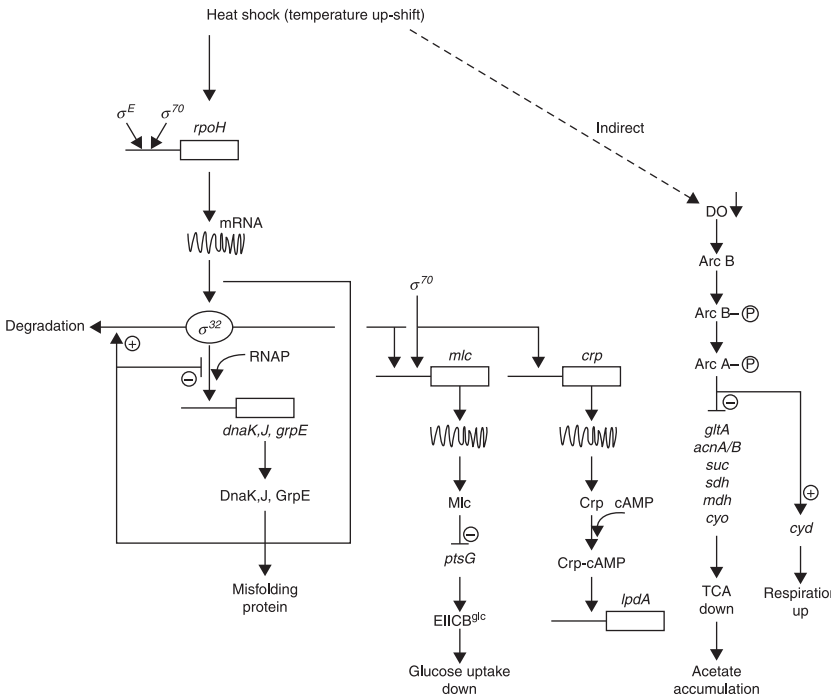

Figure 3.29 **Effect of heat shock on gene and protein expressions and the fermentation characteristics**

This high-affinity acetate-scavenging enzyme converts acetate to AcCoA, where cells introduce it into the TCA cycle to generate energy and/or the glyoxylate pathway to build cell constituents. The higher expression of *acs* accelerates acetate assimilation in the presence of acetate (Kumari et al., 2000; Lin et al., 2005), which leads to the activation of glyoxylate pathway. Transcription occurs from two σ^{70}-dependent promoters, such as the distal promoter *acs* P1 and proximal promoter *acs* P2 (Kumari et al., 2000; Browning et al., 2002). While multiple factors influence transcription, Crp appears to function directly as the critical transcription factor. Cells control this acetate switch primarily by controlling the initiation of *acs* transcription from the major promoter *acs*P2 (Kumari et al., 2000; Beatty et al., 2003). Activation of *acs* transcription depends on the cAMP-Crp. The cAMP-Crp binds two sites within the *acs* regulatory region. However, it has been shown that Fis and Ihf independently modulate Crp-dependent activation of *acs*P2 transcription (Browning et al., 2004), and the mechanism is not so simple. As such, the activation of *crp* may cause *acs* to be up-regulated. The *acs* gene is also under control of *rpoS*. It has been shown that *acs* is expressed in an *rpoS*-dependent manner during different phases of the growth (Rahman and Shimizu, 2008).

Although cellular ATP may increase for a short period after the temperature up-shift in *E. coli* (Soini et al., 2005), it eventually decreases at higher temperatures (Hoffman et al., 2002; Soini et al., 2005). It has also been reported that the specific CO_2 production rate as well as O_2 consumption rate increases upon temperature up-shift (Hoffmann and Rinas, 2001; Hoffman et al., 2002; Soini et al., 2005). As a result, cell yield decreases and cell maintenance increases (Hoffman et al., 2002; Weber et al., 2002). Although it has been reported that the TCA cycle flux increases upon temperature up-shift at the specific growth rate of 0.08 h^{-1} (Soini et al., 2005), another investigation based on ^{13}C- labeled experiment indicates that the TCA cycle flux becomes low at the dilution rates of 0.45 and 0.32 h^{-1} (Wittmann et al., 2007). It is shown that the repression of TCA cycle genes may be due to up-regulation of the *arcA* gene, while the respiratory activity becomes higher due to up-regulation of the *cyd* gene, which may be caused by up-regulation of the *arcA* gene. The *icdA* and *aceA* genes are known to be repressed by ArcA/B and activated by Cra. The up-regulation of the *arcA* gene expression and down-regulation of the *cra* gene expression both act to repress *icdA* and *aceA* genes, and thus the TCA cycle as well as the glyoxylate pathway is repressed. In accordance with this, the *fadR* and *iclR* expressions are also up-regulated, which are known to repress the *aceBAK* operon. This may cause more acetate accumulation. Moreover, the down-regulation of

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cyoA may limit respiratory activity, while it may be counteracted by the activation of *cydB* gene. It has been reported that respiration is activated during the temperature up-shift (Hoffman et al., 2002). It may be due to activation of *cydB* gene expression, since the K_m value is lower or the affinity to oxygen is higher for Cyd as compared to Cyo. The *arcA* gene shows increased expression after the temperature up-shift (especially first 30 mins) and modulates the expressions of such genes as *cydB, cyoA*, and *icdA*. The up-regulation of the *arcA* gene may not be the direct effect of heat shock but indirectly due to lower dissolved oxygen concentration caused by the lower solubility at higher temperature (Soini et al., 2005).

It has been reported that superoxide dismutase gene (*sod*) is induced in response to the oxidative stress imposed by dioxygen or by the redox active compounds such as viologens or quinones caused by the temperature up-shift (Privalle and Fridovich, 1987). It has also been reported that the exposure of a *sodA/B* null mutant *E. coli* to aerobic heat stress caused a profound loss of viability (Benov and Fridovich, 1995; Weber et al., 2002). Moreover, the *sod* gene is under the control of SoxRS, where it becomes significant under dual osmotic and heat stresses (Gunasekera et al., 2008).

3.8 Fatty acid metabolic regulation

The acetate or fatty acid metabolism is also of practical interest. Consider this by looking at the effect of the *fadR* gene knockout on the physiology of *E. coli*, where the parent and its *fadR* mutant are grown on glucose in a minimal medium under aerobic conditions (Peng and Shimizu, 2006). Batch cultivation results are shown in Table 3.10. Compared to the

Table 3.10 **Batch cultivation characteristics of the parent and the *fadR* mutant *E. coli* in glucose minimal medium under aerobic conditions**

Strain	μ_{max} (h^{-1})	q_{glc} (mmol $gDCW^{-1}$ h^{-1})	$Y_{A/G}$ (g_{ace} $ggIc^{-1}$)	$Y_{X/G}$ ($g_{biomass}$ $ggIc^{-1}$)
Wild type strain	0.52 ± 0	5.61 ± 0.07	0.33 ± 0.02	0.47 ± 0.01
fadR mutant	0.54 ± 0.01	5.89 ± 0.11	0.24 ± 0.03	0.51 ± 0.01

μ_{max}: the maximum specific growth rate; q_{glc}: the specific glucose consumption rate; $Y_{A/G}$: yield of acetate on glucose; $Y_{X/G}$: yield of biomass on glucose

parent strain, acetate production is reduced in the final concentration, and $Y_{A/G}$ is also reduced, whereas the biomass yield is enhanced in the *fadR* mutant. The specific growth rate and the specific glucose consumption rate of the *fadR* mutant are slightly higher than those of the wild-type strain during exponential growth. This cultivation phenomenon is similar to the results of Farmer and Liao (1997).

Table 3.11 shows the comparison of protein expressions measured by 2DE with MALDI-TOF MS for *fadR* mutant and the wild type, which indicates that the proteins involved in glucose transport and energy metabolism, including PtsH, PtsI, Crr, GapA, Pgk, SucA, FumA, AtpA, AtpC, AtpD, and CyoD, show increased abundance. The levels of amino acids biosynthesis proteins, such as TrpD, AsnB, AroG, and the cell process proteins, such as GreA and RplI, are also higher in the *fadR* mutant. However, the proteins involved in fatty acids biosynthesis pathways, such as AccB and FabD, are down-regulated. Apart from these proteins, the expressions of UspA, the universal stress protein, and DnaK,

Table 3.11 **Differentially expressed proteins in the *fadR* mutant *E. coli* compared to the parent strain**

Protein name	AC Swiss prot no.	Protein description	Ratio (*fadR*/ parent)
Glucose transport and energy metabolism			
PtsI	P08839	Phosphoenolpyruvate-protein phosphotransferase	1.8
PtsH	P07006	Phospho carrier protein HPR	1.5
Crr	P08837	PTS system, glucose-specific IIA component	1.4
GapA	P06977	Glyceraldehyde-3-phosphate dehydrogenase	1.5
Pgk	P11665	Phosphoglycerate kinase	1.3
SucA	P07015	2-oxoglutarate dehydrogenase e1 component	2.0
FumA	P00923	Fumerase hydratase class 1	1.5
AtpA	P00822	ATP synthase alpha chain	1.4
AtpD	P00824	ATP synthase beta chain	1.2
AtpC	P00832	ATP synthase epsilon chain	∞
CyoD	P18403	Cytochrome O ubiquinol oxidase protein cyoD	2.2

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Amino acid biosynthesis			
AsnB	P22106	Arsperagine synthetase B	1.4
TrpD	P00904	Anthranilate synthase component II	1.7
AroG	P00886	Phospho-2-dehydro-3-deoxyheptonate aldolase	1.3
Fatty acid biosynthesis			
FabD	P25715	Malonyl CoA-acyl carrier protein transacylase	0.6
AccB	P02905	Biotin carboxyl carrier protein of acetyl coenzyme A carboxylase	0.7
Cell process			
RplI	P02418	50S ribosomal protein L9	1.4
GreA	P21346	Transcription elongation factor greA	1.2
Others			
AceK	P11071	ICDH kinase/phosphotase	1.7
DnaK	P04475	DnaK protein	1.6
UspA	P28242	Universal stress protein	1.4
OppA	P23843	Periplasmic oligopeptide-binding protein	0.6
SgaH	P39304	Probable hexulose-6-phosphate synthase	0.2

the molecular chaperone responsive to heat shock, increases. Both proteins are known to protect the cell from stress conditions. The level of AceK, the bifunctional protein catalyzing phosphorylation/inactivation protein, is higher in the *fadR* mutant. Acetate induced periplasmic transporter OppA shows lower expression levels in the *fadR* mutant as compared to wild type.

Table 3.12 shows the comparison of enzyme activities, where it indicates that Icl and MS, the two enzymes of the glyoxylate shunt, are significantly induced in the *fadR* mutant. CS, Acn, Fum, and MDH were coordinately up-regulated to some extent. However, the activity of ICDH is slightly decreased. Moreover, $NADP^+$-dependent malic enzyme (Mez) is up-regulated, whereas NAD^+-dependent malic enzyme (Sfc) is down-regulated in the *fadR* mutant, as compared to the wild type. In addition, Ppc shows lower activity in the *fadR* strain, whereas Pck activity increases.

Table 3.12 **Specific enzyme activities in cell extracts of the parent and the *fadR* mutant *E. coli* at the exponential phase grown in glucose minimal medium under aerobic conditions**

Enzymes	Activities (nmol mg protein^{-1} min^{-1})		
	Parent	*fadR* mutant	Ratio (*fadR*/ parent)
Isocitrate lyase (Icl)	62.3 ± 3.1	231.9 ± 9.5	3.7
Malate synthase (MS)	8.0 ± 0.7	14.9 ± 1.8	1.9
Citrate synthase (CS)	113.0 ± 6.3	144.6 ± 9.1	1.3
Aconitase (Acn)	121.5 ± 7.5	174.7 ± 10.2	1.4
Isocitrate dehydrogenase (ICDH)	1312 ± 59	918.5 ± 50.2	0.7
Fumarase (Fum)	19.7 ± 1.1	64.6 ± 2.8	3.3
Malate dehydrogenase (MDH)	59.3 ± 3.3	75.4 ± 4.3	1.3
NADP+-specific malic enzyme (Mez)	66.3 ± 2.9	112.5 ± 3.6	1.7
NAD+-specific malic enzyme (Sfc)	68.7 ± 1.5	52.8 ± 1.7	0.8
Acetate kinase (Ack)	1075 ± 42.2	767.3 ± 33.5	0.7
Glucokinase (Glk)	123.0 ± 7.1	131.6 ± 7.5	1.1
Glucose phosphate isomerase (Pgi)	2227 ± 56.0	2338 ± 59.2	1.1
Phosphofructose kinase (Pfk)	392.4 ± 13.1	509.7 ± 13.9	1.3
Fructose bisphosphate aldolase (Fba)	857.6 ± 34.3	989.5 ± 47.0	1.2
Triose phosphate isomerase (Tpi)	2474 ± 66.4	2671 ± 59.8	1.1
Glyceraldehyde-3-P dehydrogenase (GAPDH)	136.3 ± 7.4	217.4 ± 8.5	1.6
3-Phosphoglycerate kinase (Pgk)	55.1 ± 4.7	70.6 ± 4.0	1.3
Pyruvate kinase (Pyk)	240.6 ± 13.0	223.8 ± 12.1	0.9
Phosphoenolpyruvate carboxylase (Ppc)	571.5 ± 17.7	473.1 ± 15.2	0.8
Phosphoenolpyruvate carboxykinase (Pck)	78.4 ± 4.2	120.7 ± 9.4	1.5

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Glucose-6-P dehydrogenase (G6PDH)	357.6 ± 23.2	439.1 ± 26.1	1.2
6-Phosphogluconate dehydrogenase (6PGDH)	443.2 ± 27.7	465.6 ± 34.0	1.1
Transaldolase (Tal)	218.0 ± 11.5	259.1 ± 15.3	1.2
E-D pathway (Edd and Eda)	6210 ± 110	5490 ± 109	0.9

In the fermentative pathways, Ack activity is reduced in the *fadR* mutant as compared to the parent, which is in agreement with the decrease of acetate production by the *fadR* mutant. These trends of the enzyme activities indicate that the *fadR* mutant utilizes the glyoxylate shunt for the replenishment of OAA for biosynthesis. The activation of the glyoxylate shunt by-passes the TCA cycle and thus prevents the loss of carbons as CO_2 in ICDH and KGDH catalyzed reactions, which leads to the increased utilization of AcCoA.

Table 3.13 shows the comparison of intracellular metabolite concentration for the two strains, where it indicates that PYR and AcCoA concentrations decease, whereas the concentrations of isocitrate (ICIT), αKG, MAL, OAA, and aspartate (ASP) increase in the *fadR* mutant as compared to the parent. These results are in agreement with those reported by van de Walle and Shiloach (1998), who found that the operation of the glyoxylate shunt in *E. coli* BL21 results in accumulation of TCA cycle intermediates and higher biosynthesis fluxes. Similar to PYR, PEP concentration is also reduced in the *fadR* mutant, which may be partially due to the elevated glucose uptake that needs more PEP for PTS. These variations reflect the action of the glyoxylate shunt in the *fadR* mutant. It is also observed that the intracellular concentrations of intermediates in the glycolysis and the PP pathway, such as G6P and 6PG, are reduced in the *fadR* mutant strain, implying the accelerated dissimilation of glucose. Apart from these changes, the ratio of NADPH/$NADP^+$ is lower, while that of NADH/NAD^+ is higher in the *fadR* mutant as compared to wild type.

It has been reported that FadR is a transcriptional regulator with a Helix-turn-Helix motif (van Aalten et al., 2001), regulating metabolic pathways such as the fatty acid biosynthesis and degradation, glyoxylate shunt, and possibly playing a role in the regulation of amino acid biosynthesis directly or indirectly (DiRusso et al., 1992; Gui et al., 1996; Cronan and Subrahmanyam, 1998; DiRusso and Nystrom, 1998). The results show that the *fadR* mutant reduces acetate production and

Table 3.13 **Intracellular metabolite concentrations in the parent and the *fadR* mutant *E. coli* at the exponential phase grown in glucose minimal medium under aerobic conditions**

Metabolites	Intracellular concentrations (mM)		
	Parent	fadR mutant	Ratio (fadR/parent)
AcCoA	0.25 ± 0.026	0.063 ± 0.006	0.3
ICIT	<0.03	0.062 ± 0.007	2.1
α-KG	1.85 ± 0.36	2.22 ± 0.40	1.2
MAL	0.088 ± 0.009	0.38 ± 0.03	4.3
OAA	0.061 ± 0.005	0.17 ± 0.03	2.8
ASP	2.43 ± 0.29	4.47 ± 0.38	1.8
G6P	0.24 ± 0.022	0.16 ± 0.015	0.7
PEP	0.10 ± 0.008	0.08 ± 0.009	0.8
PYR	1.58 ± 0.23	1.027 ± 0.17	0.6
6PG	0.35 ± 0.06	0.33 ± 0.05	0.9
NADH	0.13 ± 0.012	0.12 ± 0.011	0.9
NAD+	2.53 ± 0.188	1.62 ± 0.154	0.6
NADPH	0.032 ± 0.003	0.025 ± 0.003	0.8
NADP+	0.11 ± 0.006	0.10 ± 0.005	0.9

Cell volume: 2.55 μl $mg(DCW)^{-1}$

enhances biomass yield. The activities of Icl and MS, which are involved in the glyoxylate shunt, increase and AceK expression is up-regulated. The overall effect of the *fadR* mutant is illustrated in Figure 3.30 (Peng and Shimizu, 2006).

Induction of the glyoxylate shunt leads to better utilization of AcCoA by increasing the carbon flow through this anaplerotic pathway, which is inferred from the significantly reduced intracellular concentration of AcCoA. The decrease of the intracellular AcCoA pool is therefore suggested to be responsible for the reduced acetate excretion in the *fadR* mutant. In contrast, the higher level of PYR in the parent is directed to acetate due to less TCA cycle enzyme activity. For example, KGDH or accumulation of NADH restricts the carbon flow through the TCA cycle (van de Walle and Shiloach, 1998). Meanwhile, the pools of PEP and PYR are cojointly reduced in the *fadR* mutant due to the draining of carbon into the TCA cycle and the glyoxylate shunt. PEP is known to be a critical

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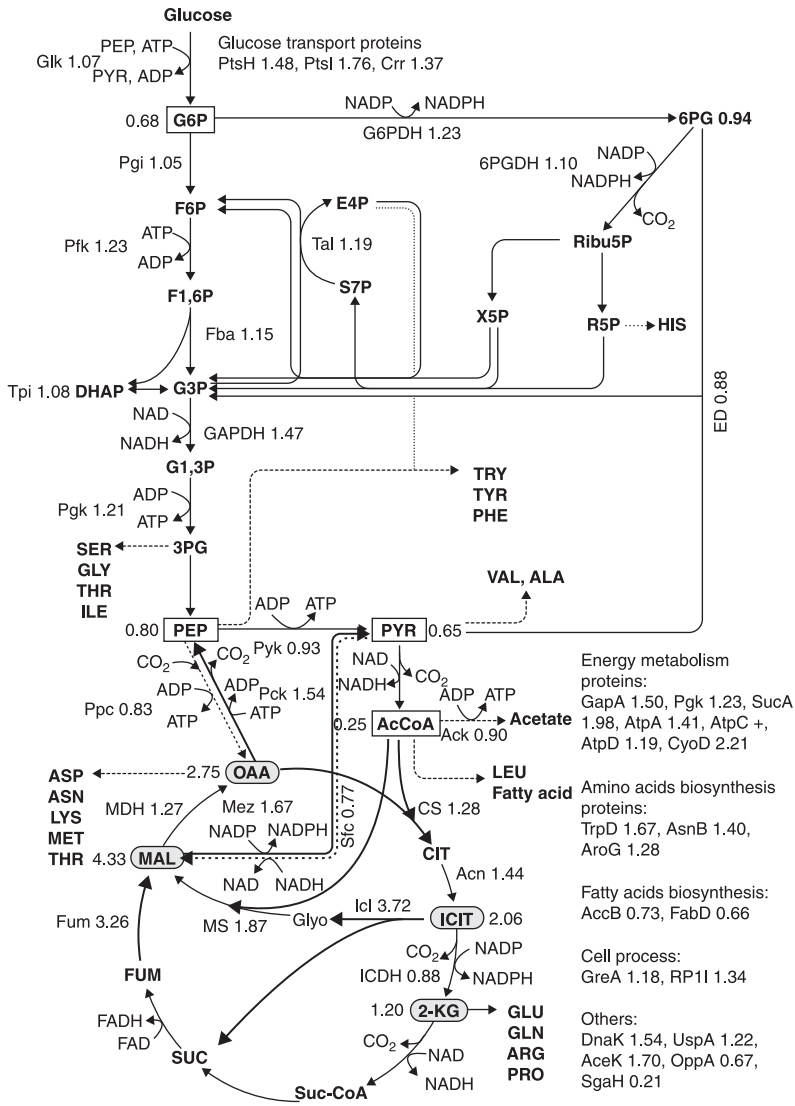

Figure 3.30 Metabolic pathways showing levels of enzymes (or proteins) and intracellular metabolite concentrations in the *fadR* mutant *E. coli* relative to those in the parent at the exponential phase grown in glucose minimal medium under aerobic conditions. The numbers beside the protein names and the metabolites represent the ratios. Oval and boxed metabolites mean increased and decreased intracellular concentrations in *fadR* mutant *E. coli*

metabolite in *E. coli*. It involves not only in the PTS as a phosphoryl donor but also in the regulation of many enzymes as an effector (Fraenkel and Nidhardt, 1999). Therefore it is considered that the up-regulation of the glycolytic enzymes, such as Pgi and Pfk in the *fadR* mutant, are associated with the release from the inhibition due to the lower PEP concentration, since PEP is an inhibitor of both enzymes. Decrease of G6P concentration is responsible for faster glucose uptake in the *fadR* mutant by up-regulation of PTS proteins, since G6P degrades the mRNA of PTS proteins by activating RNaseP enzyme (Morita et al., 2003). Other glycolytic enzymes, such as Fba, Tpi, GAPDH, and Pgk are concurrently up-regulated in the *fadR* mutant to some extent to form more PEP and PYR, which are consistent with D'Alessio and Josse's (1971) results that these enzymes are always regulated proportionally in *E. coli*.

Concomitant with the induction of the glyoxylate shunt, some of the TCA cycle enzymes, such as CS, Acn, Fum, and MDH, are coordinately up-regulated. Besides, SucA, a component of KGDH, and FumA show higher expression levels. These up-regulations are expected to fulfill the role in driving the increased carbon flux due to the action of the glyoxylate shunt. It is reported that CS and Acn, but not ICDH, are regulated in a coordinate mode, which may be due to the fact that citrate is an activator of Acn (Nakano et al., 1998). However, ICDH activity is subject to phosphorylation/inactivation control at the branch of isocitrate to force the carbon flux toward the glyoxylate shunt. The phosphorylation/inactivation of ICDH is exerted by AceK, which is induced in the *fadR* mutant. Decreased carbon flux via ICDH, therefore, restricts the production of NADPH in the TCA cycle, as shown by the NADPH concentration, which is much lower in the mutant than in the parent strain. NADPH is an important cofactor for biosynthesis and mainly formed in the TCA cycle. Up to 60% of the total NADPH is produced in the TCA cycle in the parent strain under aerobic conditions (Hua et al., 2003). To meet the need for biosynthesis, NADPH has to be generated by other NADPH producing pathways. One way is through the NADPH-dependent malic enzyme, Mez, which is up-regulated in the *fadR* mutant. The up-regulation of Mez is probably related to the reduced intracellular AcCoA concentration, as this enzyme is repressed by glucose and AcCoA (Murai et al., 1971). This up-regulation also plays a role in supplying AcCoA from MAL via PYR in the *fadR* mutant. Another route to produce NADPH is via G6PDH and 6PGDH in the PP pathway. Indeed, both enzymes are up-regulated in the *fadR* mutant to some extent. Accelerated cell growth concomitant with the reduction of acetate may cause the shortage of energy in the *fadR* mutant.

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Proteome analysis demonstrates that the protein expressions in amino acids biosynthesis, such as AsnB, TrpD and AroG, F_1F_2 proton-translocating ATPase, such as AtpA, AtpC and AtpD, and the ribosomal protein RplI, as well as the transcription elongation factor GreA, are up-regulated in the *fadR* mutant, implying the capacity of biosynthesis of the *fadR* mutant is enhanced. These genes are clustered and show growth rate dependent expression (Yoon et al., 2003). On the contrary, the levels of AccB and FabD involved in fatty acids biosynthetic pathways are negatively affected by the *fadR* mutant, which is consistent with previous studies which indicate that the *E. coli* FadR functions as a repressor of the fatty acid degradative (*fad*) pathways and can also act as an activator of unsaturated fatty acid synthesis (*fab*) (Farewell et al., 1996; Gui et al., 1996; Campbell and Cronan, 2001). In addition, DnaK, the heat shock protein and UspA, the universal stress protein, are induced in the *fadR* mutant. These proteins are known to protect cells from stressful conditions, such as heat shock, starvation, and stress stimulons, thus *fadR* mutation seems to be a stress to the *E. coli* cell. The *uspA* is a member of the *fadR* regulon, and transcription of *uspA* is depressed during exponential growth in *fadR* null mutants (Farewell et al., 1996). Previous studies reveal that the RpoS-regulated genes, periplasmic transporters for amino acids, and peptides and metabolic enzymes, are induced either by acetate or at low pH (Arnold et al., 2001; Kirkpatrick et al., 2001). Of these, it is considered that the down-regulation of OppA is related to the lesser accumulation of acetate in the *fadR* mutant compared to the parent strain.

3.9 Response to nutrient starvation

3.9.1 Metabolic regulation by RpoS

Although many industrial fermentations are conducted in the batch mode, most of the studies have focused on the cell growth phase, and little attention has been paid to the late growth and stationary phases. Since the important metabolites are produced at the early stationary or stationary phases, it is important to clarify the metabolic regulation mechanisms that occur during these phases. During batch fermentation, cultural conditions change from glucose rich to acetate rich conditions, and change further to carbon starvation conditions. The presence of several global regulators, such as RpoD, SoxRS, Cra, FadR, and IclR, have been reported to help *E. coli* to cope with different kinds of metabolic

stresses (Cortay et al., 1991; Liochev and Fridovich, 1992; Jordan et al., 1999; Tang et al., 2002; Lu et al., 2003; Varghese et al., 2003; Perrenoud and Sauer, 2005). Apart from these regulators, RpoS, the master regulator of the stationary phase or stress-induced genes in *E. coli*, regulates such genes as those for the carbohydrate PTS, *crr*, glycolytic pathway genes such as *fbaB* and *pfkB*, the acetate-forming gene *poxB*, the non-oxidative PP pathway genes such as *talA* and *tktB*, and TCAcycle genes such as *acnA* and *fum*C. In addition, some of the genes relating to the amino acid and fatty acid metabolic pathways such as a*rgH*, *aroM* and *yhgY*, and energy metabolism genes such as *narY*, *appB*, and *ldcC*, have also been identified as being regulated in an *rpoS*-dependent manner (Aronis, 1996; Wei et al., 2000; Aronis, 2002a,b; Lacquor and Landini, 2004; Vijaykumar et al., 2004, Rahman et al., 2006).

E. coli cells are exposed to different stress conditions such as oxidative stress, acid stress, or stresses from particular ion or carbon limitation at different phases of growth. Fortunately, *E. coli* cells possess several regulatory proteins, which through the regulation of a large number of genes, help the bacteria to cope with a continuously changing environment under different stress conditions, including acid stress, or the other stresses mentioned above (Moat et al., 2002). Of these stress conditions, acid stress, particularly stress from acetate, is one of the major points to be discussed, as this is the major fermentative product of glucose metabolism under aerobic conditions of growth. In addition, acetic acid has been recognized as a problem in recombinant protein production, as it easily passes through the thin lipid layer of the bacterial cell wall and causes damage to protein production (Booth, 1985; Aronis, 1996).

It has been reported that the stress regulatory protein RpoS regulates the expression of approximately 78 genes in *E. coli* during acid stress (Aronis, 1996; Weber et al., 2005). The glyoxylate pathway is regulated by the global regulators, such as Cra, IclR, and FadR (Gui et al., 1996; Perrenoud and Sauer, 2005). There could be an important relationship among the RpoS and Cra or the other regulators that protect *E. coli* cells from acid stress, etc.

In general, the bacterial culture medium is considered to be rich in carbohydrate or glucose as the sole carbon source during the exponential growth phase. As the cell such as *E. coli* utilizes the glucose, acetate is produced as the major fermentative product under aerobic conditions, and the cell exhibits a diauxic shift, which causes termination of the exponential growth phase and stimulation toward the stationary phase. Then *E. coli* utilizes acetate as a carbon source during the early stationary

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phase of growth. When acetate is used up, *E. coli* starts to utilize amino acids as carbon and nitrogen sources during the stationary phase. The complex changes occurring among the major metabolic pathway enzymes, their respective genes, and the intermediary metabolites, during a shift from carbon-rich to carbon-limited conditions, have been a major topic of interest in metabolic regulation analyses. RpoS is a well-known global regulator that regulates the expressions of many genes at the onset of the stationary phase or carbon starved conditions and other stress conditions in *E. coli* (Aronis, 2002a; Wei et al., 2000; Vijaykumar et al., 2004).

RpoS is a sigma factor of RNA polymerase. It is known that the core RNA polymerase consists of four subunits, such as two α, one β, and one β'. Part of the RNA polymerase that recognizes the promoter-binding site is generally known as sigma factor (σ). Without this sigma factor, RNA polymerase remains inactive (Maeda et al., 2000). *E. coli* possesses seven different σ factors (Maeda et al., 2000) (Figure 3.31). Depending on the environmental conditions, different sigma units bind with the RNA polymerase so that particular gene expressions are initiated. Of these seven different sigma factors, *rpo*S or σ^{38} has become an important part in bacterial metabolism, as this transcription factor has been shown to be associated with different kinds of stresses in *E. coli* (Maeda et al., 2000; Aronis, 2002).

Unlike other regulators, expressions of which are stimulated by certain effector molecules and these regulators then function by binding to the promoter sites of particular genes, *rpo*S itself is a transcription factor and regulates the expressions of genes at the transcriptional levels. However, once the transcription starts, the sigma factor dissociates from the RNA polymerase (Figure 3.32).

RpoS has been shown to stimulate the expressions of several oxidative stress response genes such as *katE*, *katG*, *sodC*, *dps*, and osmotic stress response genes such as *osmE*, *osmY*. Strains lacking a functional *rpoS* gene also failed to express the genes for acid resistance, such as *gadA* and *gadB*, near-UV resistance gene *nuv*, and acid phosphatase genes *appAR*. (Aronis, 2002). The intracellular level of RpoS itself is regulated by various mechanisms, depending on the stress type and growth conditions. For example, *rpo*S transcription is stimulated by a reduction in growth rate, whereas translation is stimulated by osmotic shock, low temperature, or pH downshifts (Aronis, 2002; Weber et al., 2005) (Figure 3.33). The third mechanism that controls the RpoS level is through proteolysis. While under normal situation, RpoS is rapidly degraded by ClpXP proteases, the proteolytic activity of this enzyme is considerably

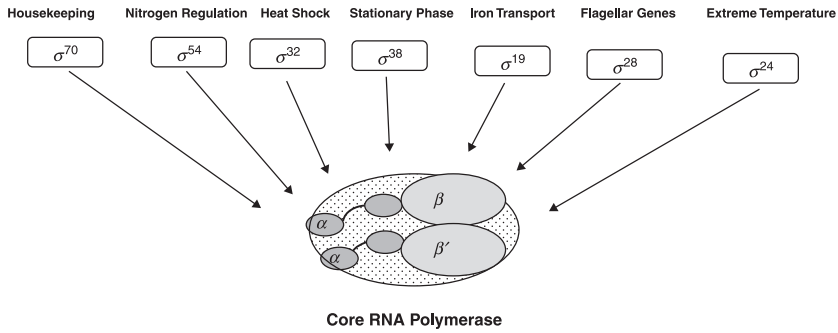
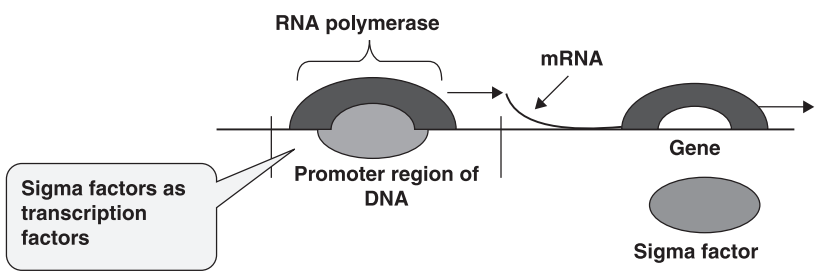


Figure 3.31 Different kinds of sigma factor in *E. coli* (Maeda et al., 2009)

reduced under stress conditions (Lowen et al., 1993; Kusano et al., 1996; Aronis, 2002a).

Although the roles of RpoS are originally described for various types of stress response, it has been demonstrated that the regulatory roles of *rpo*S are not restricted to stress response genes only. In *E. coli*, RpoS-dependent genes are found all over the chromosome, whose function ranges from DNA repair and protein synthesis to the transport, biosynthesis, and metabolism of sugars, amino acids, and fatty acids. Notably, RpoS is found to regulate the expression of DNA repair enzymes, such as the exonuclease encoded by *xth*A and the methyl transferase encoded by *ada*, the gene that determines the cell morphology such as *bol*A, the genes encoding transport and binding proteins, such as *gab*P and *ugp*EC. In addition, a considerably large number of unknown proteins are invariably affected by *rpo*S mutation (Lacour and Landini,



(Maeda et al., 2000; Stryer 1986)

Figure 3.32 Schematic diagram on the function of sigma factor as a transcription factor

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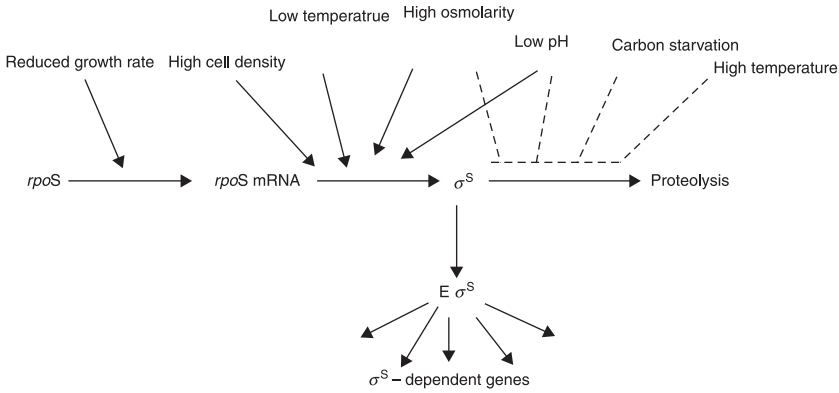

Figure 3.33 **Various levels of σ^S regulation are differentially affected by various stress conditions (Aronis, 2002a)**

2004; Weber et al., 2005). Considering the wide range of activities of RpoS, it seems obvious that RpoS could make a significant contribution in the maintenance of *E. coli* metabolic pathways at the stationary phase under carbon-starved conditions.

The complexity of the metabolic system of *E. coli* is exemplified by the fact that many metabolic pathway genes are found to be regulated by more than one global regulatory protein. For example, *icd* of the TCA cycle is regulated by RpoD and Cra, *acn*A and *fum*C of the TCA cycle are regulated by SoxRS and RpoS, and *ace*A and *ace*B of the glyoxylate pathways are regulated by Cra and IclR, etc. (Cortay et al., 1991; Liochev and Fridovich, 1992; Jordan et al., 1999; Tang et al., 2002; Lu et al., 2003; Varghese et al., 2003). Moreover, the metabolic pathway of *E. coli* consists of many genes that possess iso-genes. These iso-genes are known to encode back-up enzymes in response to certain environmental stimuli, and the expressions of these enzymes are often regulated by one or more of the global regulators. For example, the *fum*C and *acn*A genes of the TCA cycle are known to encode back-up enzymes at the stationary phase of growth and, as mentioned earlier, they are regulated by both *rpo*S and *sox*RS (Cunningham et al., 1997). It appears that the stress inducible metabolic pathway genes constitute the functional units through which different global regulators co-ordinate metabolic activities in the face of changing culture environment.

In *E. coli*, transketolase is encoded by *tktA* and *tktB* genes, and transaldolase is encoded by *talA* and *talB* genes, respectively. TktA and TalB are reported to be the major enzymes to catalyze transketolase and transaldolase reactions, and TktB and TalA are the minor enzymes

of the non-oxidative PP pathway (Zhao and Winkler, 1994; Sprenger et al., 1995). The non-oxidative PP pathway is important as E4P, the important precursor of the aromatic amino acids, and S7P, which is the cell wall components of *E. coli*, are synthesized only through the non-oxidative branch. E4P and S7P are produced through the consecutive reactions catalyzed by Tkt and Tal (Datta and Racker, 1961; Sprenger et al., 1995). While the physiological roles of the major and minor enzymes have been elucidated, the reports on the positive regulation of the minor transketolase (encoded by *tkt*B) and transaldolase (encoded by *tal*A) of the non-oxidative PP pathway by the stress regulator *rpoS*, indicate that these genes might play a significant role under carbon starved conditions (Lacour and Landini, 2004; Jung et al., 2005; Weber et al., 2005).

However, the TCA cycle genes, such as *fum*C and *acn*A, encode FumC and AcnA enzymes of the TCA cycle, respectively. While fumarase catalyzes the conversion of FUM to MAL by the hydration reaction, Acn catalyzes the conversion of CIT to cis-aconitate to ICIT through dehydration and hydration reactions (Stryer, 1988). However, both FumC and AcnA have iso-enzymes such as FumA and AcnB, which are encoded by the genes *fumA* and *acnB*, respectively. It has been reported that FumA and AcnB play major roles of fumarase and aconitase when the cell grows under optimum growth conditions. Both FumA and AcnB possess Fe-S clusters that make these enzymes vulnerable to oxidative stress, and under such conditions, FumC and AcnA act as back-up enzymes (Jordan et al., 1999; Chen et al., 2001; Tang et al., 2002; Varghese et al., 2003). It has been reported that RpoS regulates the expressions of *fum*C and *acn*A at the stationary phase of growth. It is also reported that the expressions of both *fumC* and *acnA* genes are also regulated by the oxidative stress regulators SoxRS (Park and Gunsalus, 1995a; Krapp et al., 2002).

In summary, RpoS plays an important role at the late growth phase and the stationary phase, as explained in Chapter 2 (see Section 2.3) (Rahman and Shimizu, 2008; Rahman et al., 2008).

3.9.2 Effect of rpoS gene knockout on the metabolism

Figure 3.34 shows the comparison of the batch cultivation characteristics for the wild-type (*E. coli* BW25113) and the *rpoS* mutant (JW2711) strains, where it indicates that the mutant cells enters the stationary phase

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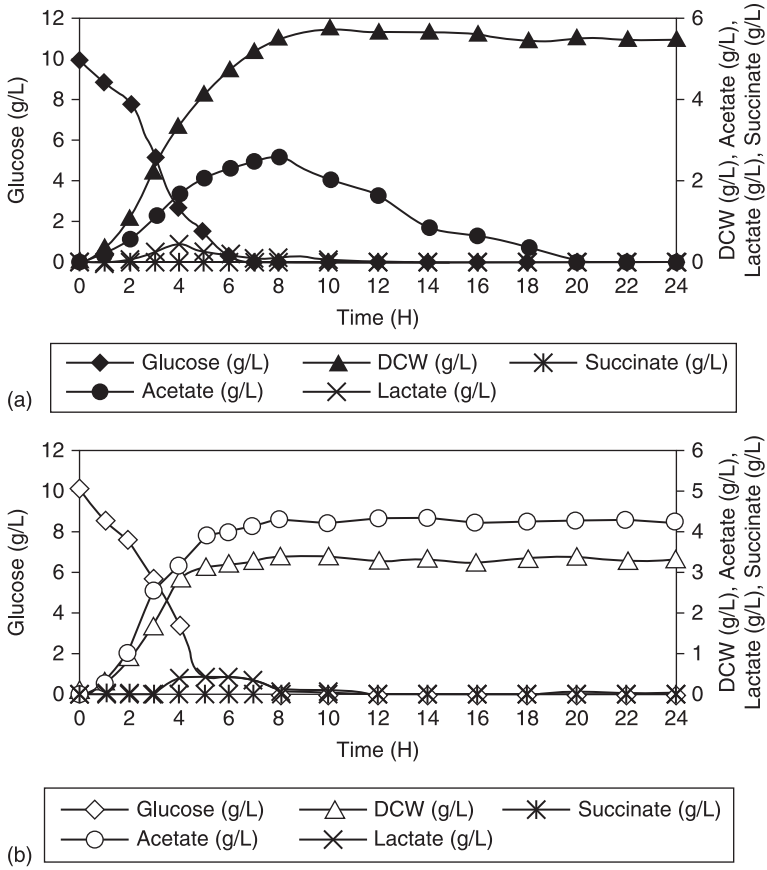

Figure 3.34 **Growth curves of: (a) *E. coli* BW25113 (parent strain); and (b) *E. coli* JWK 2711 (*rpo*S mutant). Strains were grown for 24 h in LB media containing 10g/L of glucose at 37°C, at pH 7.0 under aerobic conditions**

much earlier than the wild-type cells. Although the glucose consumption by both strains is similar, acetate accumulation/consumption patterns are significantly different. The extracellular acetate concentration at the mid-exponential phase is more than 2-fold higher in the case of the mutant compared to that of the wild type. Moreover, the acetate consumption rate is significantly lower for the mutant throughout the cultivation period. In contrast, lactic acid is found to be utilized by the mutant during the stationary phase. The growth parameters of the wild type and *rpo*S knockout mutant are given in Table 3.14. As shown in the table, biomass yield is significantly lower in the mutant, and the specific O_2 uptake rate

(OUR) and the specific CO_2 evolution rate (CER) are considerably higher for the mutant compared to the wild-type strain.

In order to determine how the mutant copes with the absence of a vital gene such as *rpo*S, it may be identified that such genes are up-regulated at the early stationary phase from the microarray data (Rahman et al., 2006). A total of 208 genes are up-regulated in the mutant, excluding the genes for hypothetical proteins. Among these genes, 25 genes (~12%) are up-regulated in both phases of growth. Microarray data reveals that, of the central metabolic pathways, significant reduction of the expression is observed for several genes during the early stationary phase, with the exception of *fum*C, which is up-regulated. Interestingly, at the exponential growth phase, a substantial up-regulation of the TCA cycle genes is observed. An exceptionally higher level of expression of certain acetate producing genes such as *cys*DEK and down-regulation of acetate utilization pathway genes such as *acs*, *aceBAK*, coincide with the accumulation of acetate throughout the fermentation period. However, the lactate permease gene is up-regulated, thereby making lactate available for use during the stationary phase. Among the genes for amino acid metabolism, the *rpo*S mutation results in significant down-regulation of certain genes involved in the metabolic pathways of valine, isoleucine, serine, asparagine, methionine, and arginine, whereas some of the genes for alanine, leucine, threonine, cysteine, lysine, histidine, and glutamate metabolism are up-regulated during early stationary phase.

A significant down-regulation is also observed for molybdenum uptake transport protein, vitamin B_{12}, and ferric ion transporter proteins, and

Table 3.14 **Growth parameters of *E. coli* BW25113 (parent strain) and *E. coli* JW 2711 (*rpo*S mutant) under aerobic growth conditions in LB media**

Growth parameters	BW25113	JW2711
Maximum specific growth rate (μ)	0.57 ± 0.05	0.57 ± 0.04
Specific glucose uptake rate (mM/g DCW/h)	5.8 ± 0.03	6.09 ± 0.05
Specific acetate Production rate (mM/g DCW/h)	20 ± 0.05	54.51 ± 0.03
Biomass yield (g DCW/g Substrate)	0.55 ± 0.05	0.34 ± 0.04
Specific CER (mM/g DCW/h)	17.56 ± 0.05	22.34 ± 0.06
Specific OUR (mM/g DCW/h)	18.11 ± 0.06	21.55 ± 0.05

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most of the glucose or carbohydrate transport proteins of the phosphotransferase (PTS) system. Expressions of several important enzymes of fatty acid biosynthesis and phospholipid biosynthesis are suppressed in the mutant, whereas some of the genes involved in fatty acid degradation are up-regulated. In contrast to this situation, the key fatty acid degradation regulator *fadR* is also up-regulated during the stationary phase. Table 3.15 shows the expression levels obtained by RT-PCR, which are consistent with the corresponding data in the microarray analysis, as shown in the parentheses.

In order to further investigate the metabolism of the *rpo*S mutant at the protein level, the specific activities of several enzymes of the central metabolic pathways are shown in Table 3.16. The enzymes catalyze three important reactions in the glycolytic pathway, such that Pgi, Pfk, and Pyk are down-regulated in the early stationary phase, whereas these activities are higher in the exponential growth phase compared to the parent strain. However, the relative activity of Ppc is higher, even during the stationary phase. The enzyme activities of two of the TCA cycle enzymes, such as ICDH and MDH of the mutant, are not significantly different from the parent strain at the early stationary phase, but these are

Table 3.15 **Growth parameters of *E. coli* BW25113 (parent strain) and *E. coli* JW2711 (*rpo*S mutant) under aerobic growth conditions in LB media**

Genes	Ratio (JW2711/BW25113)	
	Exponential phase	Early stationary phase
Glycolytic pathway		
*pts*H	1.5 ± 0.036 (1.789)	0.67 ± 0.014 (0.669)
*pts*G	1.2 ± 0.019 (1.092)	0.24 ± 0.003 (0.187)
*pts*I	1.58 ± 0.017 (1.578)	0.17 ± 0.011 (0.106)
glk	1.47 ± 0.021 (1.765)	1.9 ± 0.006 (1.956)
pgi	1.5 ± 0.025 (1.565)	0.24 ± 0.004 (0.184)
*pfk*A	2.3 ± 0.011 (2.218)	0.09 ± 0.001 (0.002)
*tpi*A	2.0 ± 0.011 (1.921)	0.4 ± 0.007 (0.237)
*pyk*A	1.2 ± 0.013 (1.210)	0.8 ± 0.017 (0.834)
*pyk*F	1.0 ± 0.005 (0.789)	0.08 ± 0.001 (0.058)
eno	1.2 ± 0.007 (1.223)	0.03 ± 0.001 (0.021)
ppc	1.2 ± 0.015 (1.197)	1.9 ± 0.042 (2.863)

(*Continued*)

Table 3.15 Growth parameters of *E. coli* BW25113 (parent strain) and *E. coli* JW2711 (*rpo*S mutant) under aerobic growth conditions in LB media (*Continued*)

Genes	Ratio (JW2711/BW25113)	
	Exponential phase	Early stationary phase
Pentose phosphate pathway		
zwf	1.2 ± 0.017 (1.197)	1.2 ± 0.011 (1.230)
gnd	1.4 ± 0.009 (1.368)	1.8 ± 0.003 (1.810)
*tal*A	0.5 ± 0.001 (0.368)	0.08 ± 0.013 (0.086)
*tal*B	2.5 ± 0.025 (1.796)	1.7 ± 0.003 (1.22)
*tkt*A	1.3 ± 0.011 (1.144)	1.4 ± 0.005 (1.366)
*tkt*B	0.3 ± 0.005 (0.315)	0.08 ± 0.001 (0.058)
Gluconeogenic pathway		
*sfc*A	1.7 ± 0.019 (1.671)	9.25 ± 0.259 (21.597)
*mae*B	1.6 ± 0.032 (1.38)	0.85 ± 0.035 (0.87)
TCA cycle and glyoxalate shunt		
*glt*A	4.5 ± 0.057 (4.842)	0.58 ± 0.054 (0.23)
*icd*A	5.5 ± 0.179 (7.144)	1.2 ± 0.007 (1.210)
mdh	4.0 ± 0.027 (4.296)	0.25 ± 0.004 (0.263)
*sdh*C	5.3 ± 0.09 (6.421)	0.2 ± 0.09 (0.210)
Glyoxylate shunt		
*ace*A	0.8 ± 0.015 (0.765)	0.3 ± 0.031 (0.368)
*ace*B	1.79 ± 0.011 (1.789)	0.09 ± 0.024 (0.034)
Global regulators		
*rpo*D	1.42 ± 0.007 (1.231)	0.09 ± 0.001 (0.002)
*sox*R	0.43 ± 0.009 (0.355)	0.32 ± 0.04 (0.280)
*sox*S	1.5 ± 0.006 (1.236)	0.24 ± 0.07(0.165)

Values in brackets represent the microarray data of the respective genes

significantly higher at the exponential growth phase. The enzyme activity of NAD^+ specific malic enzyme of the mutant is significantly higher at the early stationary phase. It can be seen that PoxB is active only in the wild-type strain during the early stationary phase of growth, while in the mutant the enzyme activity is not detected in both phases of cell growth.

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Table 3.16 Ratio of specific activities of enzymes of *E. coli* BW25113 (parent strain) and *E. coli* JW2711 (*rpoS* mutant) during exponential and early stationary phases of growth

Enzymes	Specific activity at exponential phase (μmole/min/mg of protein)		Specific activity at early stationary phase (μmole/min/mg of protein)	
	BW25113	JWK2711	BW25113	JW2711
Glycolytic pathway				
Pgi	3.52 ± 0.005	4.96 ± 0.09	2.56 ± 0.008	1.1 ± 0.005
Pfk	4.39 ± 0.003	10.91 ± 0.005	0.24 ± 0.07	0.07 ± 0.002
Ppc	0.23 ± 0.01	0.28 ± 0.04	0.11 ± 0.01	0.26 ± 0.003
Pyk	6.28 ± 0.07	7.17 ± 0.1	0.91 ± 0.013	0.38 ± 0.009
Pentose phosphate pathway				
G6PDH	0.15 ± 0.18	0.2 ± 0.1	0.12 ± 0.006	0.2 ± 0.02
PGDH	0.15 ± 0.06	0.21 ± 0.09	0.09 ± 0.001	0.18 ± 0.02
TCA cycle and glyoxylate shunt				
ICDH	3.45 ± 0.09	7.02 ± 0.04	1.77 ± 0.011	2.47 ± 0.09
MDH	0.22 ± .05	0.44 ± 0.09	1.99 ± 0.08	1.54 ± 0.002
ICl	0.048 ± 0.08	0.04 ± 0.05	0.68 ± 0.04	0.19 ± 0.03
Gluconeogenic pathway				
Fbp	0.48 ± 0.011	0.59 ± 0.09	0.63 ± 0.14	1.16 ± 0.13
PckA	0.39 ± 0.08	0.59 ± 0.1	1.46 ± 0.07	0.89 ± 0.15
Mez (NAD+ specific malic enzyme)	0.22 ± 0.13	0.36 ± 0.015	0.03 ± 0.09	0.31 ± 0.011
Malic Enzyme (NADP+ specific malic enzyme)	0.07 ± 0.07	0.10 ± 0.05	0.31 ± 0.06	0.33 ± 0.01
Fermentative pathway				
LDH	1.75 ± 0.11	2.79 ± 0.09	0.82 ± 0.09	0.69 ± 0.14
Ack	2.37 ± 0.09	3.13 ± 0.06	1.42 ± 0.07	0.71 ± 0.09
PoxB	ND	ND	0.11 ± 0.05	ND

ND: not detected

Intracellular metabolite concentrations are also important indicators of carbon flow and metabolic regulation in bacteria. The intracellular metabolite concentrations of four important intermediates of the central metabolism, such as AcCoA, OAA, PYR, and PEP, are shown in Figure 3.35, which indicates that the intracellular AcCoA concentration is very low in the mutant as compared to the wild strain during both phases of growth. The concentrations of three other metabolites in the mutant are lower than the wild-type strain in the exponential phase of growth, but these are increased at the early stationary phase.

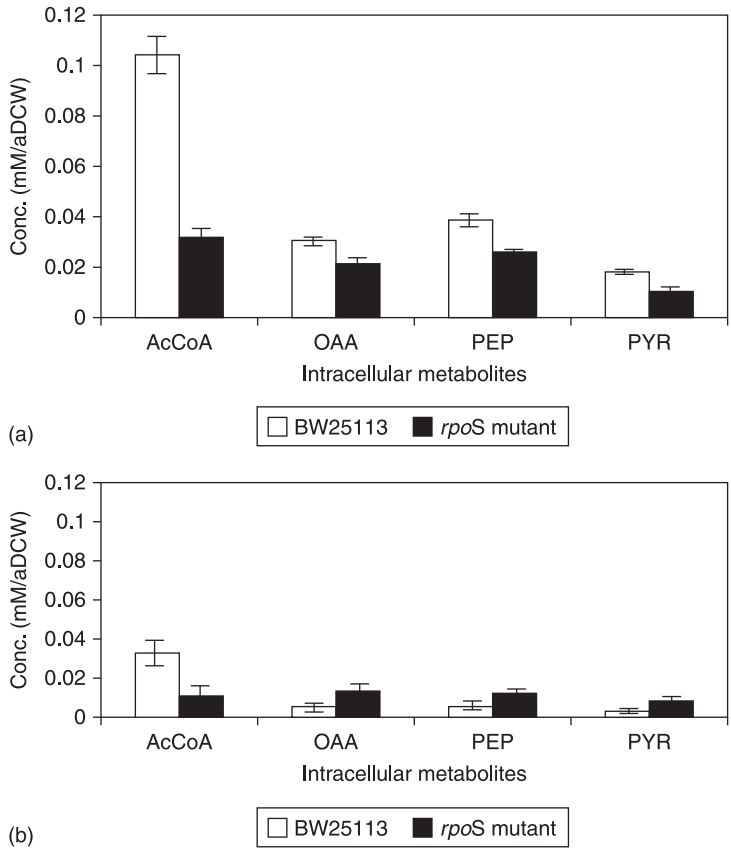

Figure 3.35 **Intracellular metabolite concentrations of *E. coli* BW25113 (parent strain) and *E. coli* JWK 2711 (*rpo*S mutant) at: (a) exponential; and (b) early stationary phases of cell growth**

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When the cell enters into the stationary phase of growth at low glucose concentrations, the *rpoS* gene is known to cause up-regulation of the expression of the carbohydrate metabolism genes of the PTS, such as *crr*, or the glycolytic enzymes encoded by *fbaB* and *pfkB* (Lacour and Landini, 2004). Complying with these observations, several genes, such as *fba*, *pgi*, *pfkB* of the carbohydrate utilization pathway, are down-regulated in the *rpoS* mutant during the early stationary phase of growth. Because of the low level of substrate in the medium at the stationary phase, reduced activity of the glycolytic pathway is expected in the parent strain, and a further decline of this activity in the mutant implies the role of *rpoS* in the regulation of this pathway. The down-regulation of several genes, such as *glgA* and *glgS* involved in glycogen synthesis, is also an indicator of the bacterial adaptive response to carbon-limited conditions in the absence of the *rpoS* background.

Among the PP pathway genes, significant down-regulation is observed for the FAD and FMN synthesizing gene, *ribA*, during the early stationary phases of growth in the mutant, where *ribA* encodes GTP cyclohydrolase II that catalyzes the first committed step in riboflavin biosynthesis from ribulose 5-P and GTP (Zubay, 1993; Eberhardt et al., 2000). Significant down-regulation is also observed for the non-oxidative PP pathway genes. For example, *tktB* and *talA* are significantly down-regulated in the mutant during both phases of growth, although previous studies report the regulation of these two genes by *rpoS* at the stationary phase of growth only (Vijaykumar et al., 2004). Down-regulation of the *tktB* and *talA* genes does not cause any profound change to other pathways that are linked to the non-oxidative PP pathway during the exponential growth phase. However, during the early stationary phase of growth, significant down-regulation is observed among the aromatic amino acid biosynthesis genes, such as *aroM*, *aroL*, and *wrbA*. Among these, the latter two genes are reported to be positively regulated by *rpoS*, whereas *aroM* is known to be co-transcribed with *aroL* (DeFeyter et al., 1986; Lacour and Landini, 2004; Vijaykumar et al., 2004). The reduced activity of the non-oxidative branch of the PP pathway also interrupts the synthesis of a number of vitamins during the early stationary phase of growth. For example, the vitamin B_6 biosynthetic enzyme, such as E4P dehydrogenase (*epd*) that catalyzes the initial step of pyridoxal phosphate synthesis from E4P, is significantly down-regulated (Zubay, 1993; Yang et al., 1998). While the non-oxidative PP pathway branch is affected by the *rpoS* knockout gene during the early stationary phase, some other complementary pathways are induced, which might supply the necessary pentoses for the cell. The expression levels of such genes as *rbsA* and

rbsC, which encode ribose transport proteins and *rbsK*, and which encode ribokinase are up-regulated. These are known to contribute to the maintenance of the cellular essential level of R5P through increased uptake of D-ribose and subsequent conversion to R5P, respectively (Iida et al., 1984; Sprenger, 1995; Moat et al., 2002). In addition to R5P, which is a precursor of histidine, the histidine biosynthetic pathway genes, such as *hisG*, *hisA*, and *hisB*, are significantly up-regulated.

Knockout of the *rpoS* gene also affects the TCA cycle significantly. The gene expression analysis, enzyme activity data, and intracellular metabolite concentration data demonstrate higher activity of the TCA cycle during the exponential growth phase. Most of the TCA cycle genes, such as *gltA*, *icdA*, *mdh*, *sdhA*, and *sdhC*, are up-regulated, and the concentrations of intracellular metabolites, such as PYR, PEP, OAA, and Ac-CoA, are lower compared to the wild-type strain, indicating rapid consumption of these metabolites by the activated TCA cycle enzymes. However, TCA cycle activity in the stationary phase is considerably lower with a higher accumulation of PYR, PEP, and OAA. The vegetative sigma factor *rpoD* (an ortholog of *rpoS* gene), which contains promoter binding consensus regions for most of the TCA cycle genes, could be involved with these characteristics of the TCA cycle (Farewell et al., 1998; Maeda et al., 2000; Aronis, 2002b).

As mentioned earlier, apart from TCA cycle activity, accumulation of acetate throughout the cultivation period is another notable feature of the *rpoS* mutant. While two genes, *ackA* and *poxB*, involved in acetate production, are down-regulated in the mutant at the early stationary phase, microarray data indicate that acetate production could be stimulated by the up-regulation of L-cysteine biosynthesis genes, such as *cysD*, *cysE*, and *cysK*, catalyzing the reactions that generate acetate as a by-product (Moat et al., 2002). Moreover, enzymes involved in acetate catabolism, such as AcCoA synthetase encoded by *acs*, the glyoxylate shunt enzymes encoded by the *aceBAK* operon, and the TCA cycle genes such as *gltA*, *mdh*, *sdhC*, are down-regulated during the stationary phase (Cronan et al., 1996; Shin et al., 1997; Wei et al., 2000). Of the acetate utilizing pathway genes, *acs* is reported to be regulated by *rpoS* (Shin et al., 1997).

Down-regulation of *acs* during the early stationary phase results in a decrease in the intracellular pool of AcCoA in the mutant compared to the wild-type strain. While the major route for AcCoA formation is less expressed, the other pathways for AcCoA formation rely on fatty acid degradation pathway (Moat et al., 2002). Several genes that participate in β-oxidation of fatty acids, particularly *fadA* and *fadB*, are significantly up-regulated, and the fatty acid biosynthesis genes, such as

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accB, *accC*, *accD*, and *fabF*, are also up-regulated in the mutant. The expression of the fatty acid degradation regulator, *fadR*, is significantly high at the early stationary phase of growth. It is known that *fadR* regulates fatty acid metabolism by binding to the DNA that contains *fadB* promoter binding sites, and in this way *fadR* controls fatty acid metabolism (Marrakchi, 2002; DiRusso, 1993). Down-regulation of *aceA* and *aceB* genes corresponds to the higher expression of *fadR*, where FadR indirectly represses the glyoxylate shunt encoded by *aceBAK* by directly regulating the activation of the glyoxylate shunt repressor, *iclR* (Maloy and Nunn., 1982; Gui et al., 1996). The higher expressions of *fadR* and *iclR* also cause acetate accumulation.

One exception among the TCA cycle genes, at the stationary phase, is the significant up-regulation of a fumarase gene, such as *fumC*, an isoenzyme of *fumA* (Chen et al., 2001). Despite the activation of *fumC*, down-regulation of *mdh* directs the carbon flux toward PYR formation with the up-regulation of the anapleoretic NAD^+ dependent enzyme encoded by *sfcA* (Phue et al., 2005). The accumulation of PYR is also due to down-regulation of the PDH enzyme complex encoded by *aceE*, *aceF*, and *lpdA* (Stephens et al., 1983). Other possible reasons for intracellular pyruvate accumulation could be due to the reduced activity of LDH and PoxB enzyme activities, as observed by enzyme activity measurement and gene expression profiles (Yang et al., 2001; Weber et al., 2005).

Again, as mentioned earlier, OAA accumulates during the early stationary phase of cell growth. Accumulation of intracellular OAA ultimately leads carbon flux toward threonine production from aspartate (Kim et al., 2004). This is indicated by more than 30-fold higher expressions of two major enzymes of threonine biosynthesis pathway encoded by *thrA* and *asd* genes. In addition, the arginine biosynthesis and transport systems are strongly affected in the mutant. Previous works show that some of the arginine transport proteins encoded by *artPM* are under positive control of *rpoS* (Lacour and Landini, 2004). It is demonstrated that the arginine transport protein *artJ*, a periplasmic protein, is significantly up-regulated during the stationary phase, but other proteins of this complex transport system, such as *artQ*, are significantly down-regulated.

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