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Doctor Thesis

Metabolic engineering approaches
for glutamate production
in *Corynebacterium glutamicum*

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Department of Biotechnology

Graduate School of Engineering

OSAKA UNIVERSITY

2010

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Metabolic engineering approaches
for glutamate production
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(代謝工学的アプローチによるコリネ型細菌の

グルタミン酸生産に関する研究)

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Chapter 1

General introduction

Metabolic engineering is generally referred to as the targeted and purposeful alteration of cellular metabolic pathways in order to achieve a desired goal. For an engineering strategy to be successful, a better understanding of the cellular state is necessary to determine the types of genetic modifications needed to achieve the desired goal. As a glutamate-producing bacterium, *Corynebacterium glutamicum* is one of the world's most important microorganisms for use in biotechnology; the discovery of *C. glutamicum* gave birth to the fermentation industry. Due to its industrial importance, with an annual amino acid production by *C. glutamicum* of more than two million tons, it has been studied extensively for improvement of glutamate production. Recently, application of molecular biology techniques has made *C. glutamicum* one of the best investigated and understood model industrial microorganisms. Recent achievements in metabolic engineering of *C. glutamicum* by modification of the cell to achieve the desired goal mark the beginning of development toward sustainable biotechnological production. Knowledge of the genomic DNA sequences of *C. glutamicum* and recently accumulated achievements have made it possible to improve glutamate production by metabolic engineering approaches, using recombinant DNA technologies. Therefore, the comprehensive analysis and integrated metabolic engineering approaches were applied in order to understand the mechanism of glutamate overproduction in *C. glutamicum*.

1.1 Metabolic engineering

Metabolic engineering is an approach to understanding and use of metabolic processes. As the name implies, metabolic engineering is generally referred to as the targeted and purposeful alteration of metabolic pathways in an organism in order to better understand and utilize cellular pathways for chemical transformation, energy transduction, and supramolecular assembly (Lessard 1996). Essentially, it is the application of engineering principles of design and analysis in order to achieve a particular desired goal. This goal may be to increase process productivity, such as that for antibodies, biosynthetic precursors, or polymers, or to extend metabolic capability by addition of extrinsic activities for production of biological and chemical materials (Yang et al. 1998).

For an engineering strategy to be successful, a better understanding of the host cell is necessary to determine the types of genetic modifications needed to achieve the final goal (Yang et al. 1998). Moreover, rational modification by metabolic engineering for improvement of cellular properties is crucial to successful processes, and is based on efficient genetic tools and detailed knowledge of metabolic pathways and their regulation (Wendisch et al. 2006). Wendisch et al. (2006) summarized recent advances in metabolic engineering of the industrial model bacteria *Escherichia coli* and *C. glutamicum*, which led to efficient recombinant biocatalysts for production of acetate, pyruvate, ethanol, D- and L-lactate, succinate, L-lysine, and L-serine. Moreover, they also suggested that new tools for genetic manipulation of *E. coli* and *C. glutamicum*, such as modulation of expression of chromosomal genes (Meynial-Salles et al. 2005) or creation of multiple chromosomal deletions (Vertès et al. 2005), will certainly improve the efficiency and fine-tuning of metabolic engineering. Furthermore, it is expected that

an increasing number of targets for strain development will be identified as the result of genome, transcriptome, proteome, metabolome, flux, and other systems biology analyses (Hermann 2004; Wendisch et al. 2006). The examples, as well as the principles and methodologies of metabolic engineering, have been extensively reviewed (Bailey 1991; Stephanopoulos and Vallino 1991; Eggeling and Sahm 1999; Stephanopoulos 1999; Sahm et al. 2000; Nielsen 2001; Shimizu 2002).

1.2   Monosodium glutamate and Glutamate

Monosodium glutamate (MSG; Figure 1.1), the major ingredient of seaweeds, is a unique flavor enhancer commonly added to food, which exhibits the special attentive taste of "*Umami*" (well-tasting) in Japanese (Shimizu and Hirasawa 2007). It was discovered by Dr. Kikunae Ikeda and isolated from hydrolyzates of "*Kombu,*" a kind of seaweed, which has been used traditionally in Japanese cuisine.
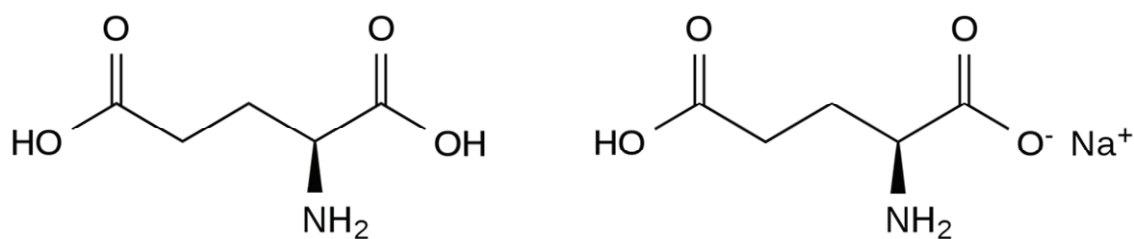
Figure 1.1   Chemical structures of glutamate (*left*) and monosodium glutamate (*right*)

Glutamate is one of the most abundant free amino acids in bacterial cytoplasm (Figure 1.1). It is synthesized from 2-oxoglutarate by a one-step reaction catalyzed by glutamate dehydrogenase (GDH). 2-Oxoglutarate is the substrate of both the 2-oxoglutarate dehydrogenase complex (ODHC) and GDH, and is located at an

important branching point of metabolism because it can either be oxidized in the tricarboxylic acid (TCA) cycle or be converted to glutamate either by GDH under abundant nitrogen conditions or by the combined reactions of glutamine synthetase and glutamate synthase under nitrogen limitation conditions.

Among amino acids produced by microbial fermentation, glutamate is one of the prominent amino acids. L-Glutamate is used mainly as not only a flavor enhancer but also as a drug and as the precursor of drugs, cosmetics, and further pharmaceutical compounds, and is in demand as a commercially useful chemical. The amount of glutamate produced worldwide by fermentation using microorganisms has increased to more than 1.5 million tons per year, with an annual growth rate of 5-7% (Leuchtenberger et al. 2005; Shimizu and Hirasawa 2007).
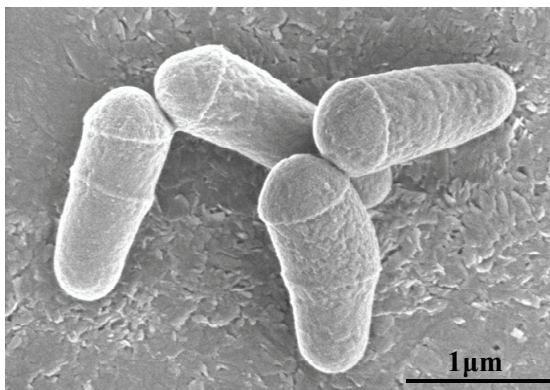
Figure 1.2   *Corynebacterium glutamicum*

1.3   *Corynebacterium glutamicum*

As shown in Figure 1.2, a coryneform bacterium, *C. glutamicum*, was originally isolated as a glutamate-producing bacterium in a screening program from nature (a soil sample contaminated with avian feces), collected at the Ueno Zoo in

Tokyo, Japan (Kinoshita et al. 1957; Udaka 1960) by Dr. Kinoshita and his colleagues in 1956. As a member of the *Actinobacteria* in the genus *Corynebacterium* (originally named *Micrococcus glutamicus*), *C. glutamicum* has been regarded as one of the most effective producers. Even though various glutamate-producing bacteria have been isolated and classified as *Arthrobacter*, *Brevibacterium*, or as members of other genera (Kinoshita 1999), a recent study has shown that most of these strains belong to the genus *Corynebacterium* (Liebl et al. 1991).

 C. glutamicum is a non-pathogenic, Gram-positive, and facultatively anaerobic bacterium with a high G+C content, inhabiting diverse ecological niches, such as soil, vegetables, sewage, etc. It has been used as one of the most biotechnologically important bacterial species for prosperous industrial fermentation of various amino acids, such as glutamate, lysine, and threonine (Nakayama et al. 1961; Shiio and Nakamori 1970) and serves as a model organism for the suborder *Corynebacterineae* within the order *Actinomycetales*. Because of the great importance of more efficient production of amino acids, *C. glutamicum* has been enthusiastically studied in the last decade by many companies and academic associations in order to cope with the fast growing market for amino acids. Genomic DNA sequences of *C. glutamicum* were determined by three independent research groups in Japan and Europe (Ikeda and Nakagawa 2003; Kalinowski et al. 2003; Yukawa et al. 2007). Moreover, several molecular biology tools for genetic manipulation of *C. glutamicum* are well established (Vertès et al. 2005).

1.4 Glutamate production by *C. glutamicum*

 Glutamate production by *C. glutamicum* is induced by depletion of biotin,

which is an essential element for growth (Shiio et al. 1962); by addition of fatty acid ester surfactants, such as Tween 40 (polyoxyethylene sorbitan monopalmitate; Takinami et al. 1965); by addition of β-lactam antibiotics, such as penicillin, which inhibits peptidoglycan biosynthesis (Nara et al. 1964; Nunheimer et al. 1970); or by addition of ethambutol, an antimycobacterial drug, which inhibits biosynthesis of mycolic acid arabinogalactan (a cell wall component of *C. glutamicum*) in Mycobacteria (Radmacher et al. 2005). Since biotin is a cofactor for acetyl-CoA carboxylase, one of the enzymes necessary for synthesis of fatty acid, it was thought that cell membrane permeability is increased when biotin was depleted in the culture medium. Similarly, addition of Tween 40, penicillin, or ethambutol affects the composition of the cell membrane and cell wall of this microorganism. During the 1960s-70s, because these triggers affect the cell surface structure of *C. glutamicum*, it was believed that glutamate overproduction was achieved by passive leakage of glutamate through the cell membrane and cell wall due to increased permeability of the cell membrane and cell wall caused by these triggering agents (Shibukawa et al. 1970; Shibukawa and Ohsawa 1966; Shiio et al. 1963).

However, in the late 1980s and 1990s, many researchers wondered about the "leak model" as an explanation for glutamate secretion in *C. glutamicum* in terms of the material balance of extracellular and intracellular glutamate. The leak model itself could not explain the high accumulation of extracellular glutamate. Consequently, it was thought that permeability of the cell membrane or cell wall should be changed. Therefore, many researchers (Hoischen and Krämer 1989; Gutmann et al. 1992) proposed the presence of a specific glutamate export system in the membrane, which differs from the earlier-mentioned leak model.

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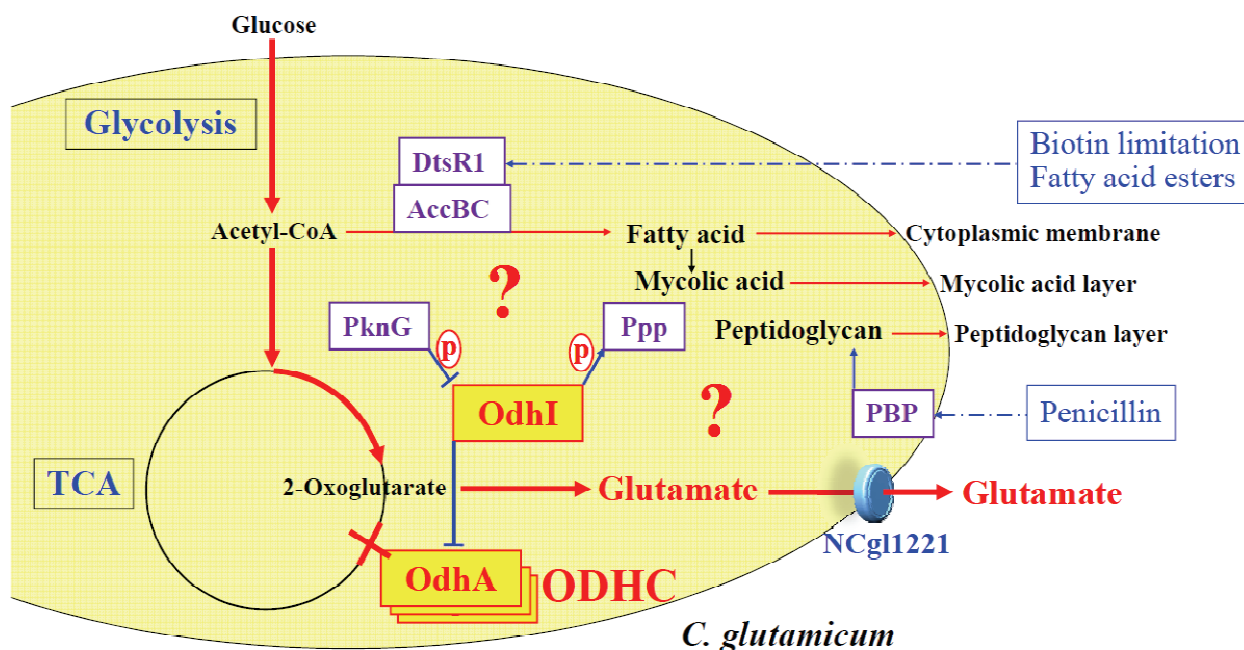
Figure 1.3   A possible molecular regulatory mechanism of glutamate overproduction in

C. glutamicum

1.5   Regulatory mechanism of glutamate overproduction by *C. glutamicum*

A current possible molecular mechanism for regulation of glutamate production in *C. glutamicum* is shown in Figure 1.3. In this Figure, the relationship among important factors, including OdhA, OdhI, DtsR1, PknG, Ppp, and NCgl1221 for glutamate overproduction in *C. glutamicum* is schematically shown. These proteins can be considered as the target of metabolic engineering for glutamate production in *C. glutamicum*.
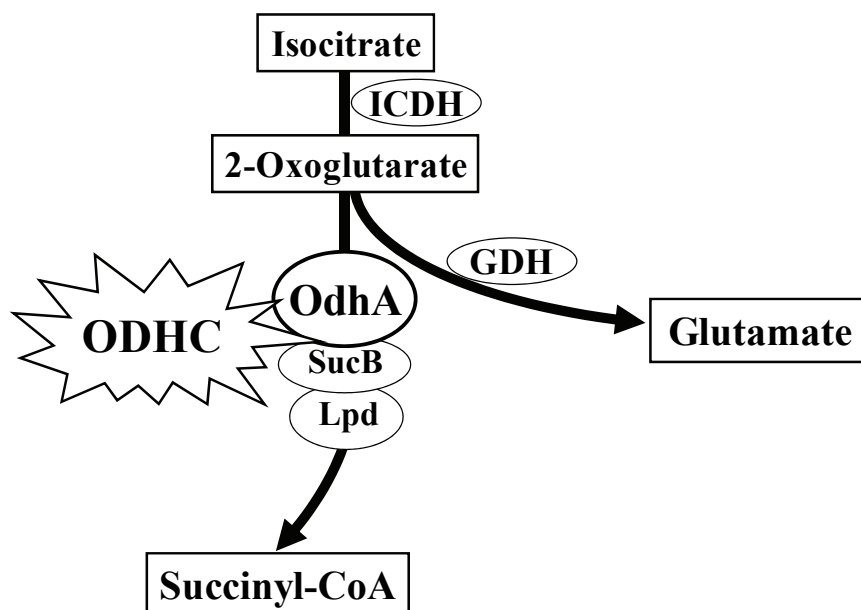
Figure 1.4   The 2-oxoglutarate branching point between the TCA cycle and the glutamate biosynthesis pathway

1.5.1   Effect of 2-oxoglutarate dehydrogenase complex activity on glutamate production

The 2-oxoglutarate dehydrogenase complex (ODHC), which is located at the branching point between the TCA cycle and the glutamate biosynthesis pathway, is thought to be one of the key enzymes in glutamate production (Figure 1.4). ODHC consists of three subunits: 2-oxoglutarate dehydrogenase (E1o; EC 1.2.4.2), coded by *odhA* (Usuda et al. 1996); dihydrolipoamide *S*-succinyltransferase (E2; EC 2.3.1.61), coded by *sucB* (found from the genome sequencing); and dihydrolipoamide dehydrogenase (E3; EC 1.8.1.4), coded by *lpd* (Schwinde et al. 2001). Shingu and Terui (1971) found that ODHC activity is reduced during glutamate overproduction, resulting from metabolic change at the branching point of 2-oxoglutarate in the TCA cycle for production of glutamate synthesis. To further understand the relationship between

changes of enzyme activities and glutamate overproduction by *C. glutamicum*, Kawahara et al. (1997) measured the enzyme activities of ODHC as well as isocitrate dehydrogenase (ICDH) and glutamate dehydrogenase (GDH) of *C. glutamicum*, both of which are also located at the 2-oxoglutarate branching point, during glutamate production conditions under biotin limitation, Tween 40 addition, and penicillin addition. Activities of ICDH and GDH did not change; however, that of ODHC significantly decreased during glutamate production under these conditions. These results indicate that glutamate overproduction by *C. glutamicum* is highly correlated with change in metabolic flows from the TCA cycle to glutamate production due to the decrease in ODHC activity and that the change in ODHC activity is one of the key factors in glutamate overproduction by *C. glutamicum*.

1.5.2   Metabolic flux distribution at the 2-oxoglutarate branching point

Metabolic flux distribution at the 2-oxoglutarate branching point was analyzed in *C. glutamicum* by Shimizu et al. (2003). It was shown that enhancement of ICDH and GDH activities by overexpression of *icd* and *gdh* genes, respectively, did not significantly affect flux distribution at the 2-oxoglutarate branching point. Even though ICDH and GDH activities were enhanced 3.0- and 3.2-fold, respectively, more than 70% carbon flux still flowed into the TCA cycle. On the other hand, when ODHC activity was decreased to about 52% after biotin limitation, marked changes in fluxes of GDH and ODHC were observed. More than 75% carbon was used for glutamate production, showing quantitatively that ODHC activity has the most significant responsibility for glutamate production (Shimizu et al. 2003; Heijnen et al. 2004).

Furthermore, to clarify the enzyme-to-substrate affinity, the Michaelis Menten

constants (K_m) of the enzymes around the 2-oxoglutarate branching point, including ICDH, GDH, and ODHC were also determined (Shirai et al. 2005), as shown in Table 1.1. The K_m value of GDH was 50- to 100-fold higher than the K_m values of ICDH and ODHC. Therefore, the affinity of 2-oxoglutarate for GDH was much lower than that for ODHC. For this reason, glutamate biosynthesis flux was not observed prior to the decrease in ODHC activity, even though the K_m value of GDH was determined to be sufficient. After the decrease in ODHC activity, the flux to glutamate biosynthesis occurred due to the increased 2-oxoglutarate pool.

Almost all 2-oxoglutarate is converted to succinyl-CoA catalyzed by ODHC without any triggers; glutamate was not overproduced in *C. glutamicum*. Although sufficient GDH activity was observed, the flux catalyzed by GDH was very small because the K_m of GDH was much higher than that of ODHC. Therefore, once ODHC activity is decreased after any triggers, 2-oxoglutarate is accumulated; glutamate is then overproduced as catalyzed by GDH (Shimizu and Hirasawa 2007).

Table 1.1 K_m values of enzymes around 2-oxoglutarate in *C. glutamicum* (Shirai et al. 2005)

Enzyme	K_m value (mM)
ICDH	0.03
ODHC	0.08
GDH	2

K_m of ICDH for isocitrate and those of ODHC and GDH for 2-oxoglutarate are shown.

1.5.3   Involvement of the OdhA protein on glutamate production

The *odhA* gene, encoding the E1o subunit (2-oxoglutarate dehydrogenase OdhA) of the ODHC in *C. glutamicum*, has been identified as a homologous counterpart of the *E. coli sucA* and *Bacillus subtilis odhA* genes (Usuda et al. 1996).

Mutant strains of *C. glutamicum*, obtained after treatment with the mutagen, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, showed lower ODHC activity (10 to 1000 times less) than that of the wild-type strain and then produced a high amount of glutamate in the presence of excess biotin (Nakazawa et al. 1994). Asakura et al. (2007) constructed *C. glutamicum* strains in which the *odhA* gene was deleted. The *odhA* knockout strain was able to produce high amounts of glutamate in the presence of excess biotin; however, the *odhA* knockout strain showed a severe growth defect. To solve the problem of the growth defect in the *odhA* knockout strain, other *odhA* mutants were constructed using *in vitro* mutagenesis (Nakamura et al. 2006). *odhA* mutants produced glutamate in the presence of excess biotin with less than half of the ODHC activity, compared to the wild-type; however, a growth defect was also observed, meaning that it was not suitable for industrial glutamate production.

1.5.4   Involvement of the DtsR1 protein on glutamate production

Kimura et al. (1996 and 1997) reported that *dtsR*1 is a multicopy suppressor gene in the *C. glutamicum* mutant with sensitivity to Tween 40, whose product shows high homology to the β subunits of some biotin-containing acyl-CoA carboxylase complexes responsible for fatty acid biosynthesis, but does not have any biotin-binding motif. DtsR1 is thought to form a complex with AccBC, the biotin-carboxyl-carrier protein/biotin carboxylase protein, which is a subunit of acyl-CoA carboxylases (Jäger

et al. 1996). The *dtsR2* (*accD2*) gene shows high sequence homology to *dtsR1* and is located downstream of *dtsR1*. Recently, further homologs of *dtsR1* and *dtsR2* (*accD2*), named *accD3* and *accD4*, were identified from the genome sequence of *C. glutamicum* (Gande et al. 2004; Portevin et al. 2005). The *dtsR2, accD3,* and *accD4* genes also encode the β subunits of acyl-CoA carboxylase, and their products form complexes with AccBC and are involved in biosynthesis of mycolic acids, which are fatty acids found in the cell wall of *C. glutamicum* and its related species, such as *Mycobacterium* and *Rhodococcus*.

Disruption of *dtsR1* resulted in fatty acid auxotrophy, such as oleic acid, oleate ester (Tween 80), or laurate ester (Tween 20), and produced high amounts of glutamate in the presence of excess biotin (Kimura et al. 1997). Glutamate overproduction by addition of Tween 40 is completely suppressed by *dtsR1* overexpression, whereas glutamate overproduction by biotin limitation and addition of penicillin is partially suppressed in *dtsR1* disruption (Kimura et al. 1999). With the reduction in ODHC activity, the expression level of the DtsR1 protein was decreased by both addition of Tween 40 and biotin limitation, but not by addition of penicillin (Kimura et al. 1999). Moreover, the link among the expression levels of *dtsR1,* levels of ODHC activity, and glutamate overproduction induced by biotin limitation and addition of Tween 40 is clearly proven.

1.5.5   Involvement of serine/threonine protein kinases and the OdhI protein

The genome sequence of *C. glutamicum* (Ikeda and Nakagawa 2003; Kalinowski et al. 2003) has revealed the presence of four genes encoding serine/threonine protein kinases (STPKs), designated PknA (cg0059), PknB (cg0057),

PknG (cg3046), and PknL (cg2388) and a single gene (*ppp*, cg0062) coding for a phospho-serine/threonine protein phosphatase. Moreover, the genomic organization of *pknA, pknB, pknG, pknL*, and *ppp* in *C. glutamicum* and the domain architecture of the corresponding proteins, PknA, PknB, PknG, PknL, and Ppp were depicted by Schultz et al. (2009). Niebisch et al. (2006) reported that the *pknG* deletion mutant had a strong defect in utilization of L-glutamine as a sole carbon and nitrogen source. Moreover, the intracellular glutamate content was twice as high in the *pknG* deletion mutant than in the wild-type, indicating that the *pknG* deletion mutant has a defect in glutamate catabolism.

Niebisch et al. (2006) identified the phosphorylation status of the novel protein OdhI, a 15-kDa protein, comprising 143 amino acid residues, of *C. glutamicum*. OdhI is composed of an N-terminal domain of 42 amino acid residues, which is followed by a forkhead-associated (FHA) domain (residues 43-143) (Barthe et al. 2009). FHA domains are known to bind to phosphothreonine epitopes of proteins (Pallen et al. 2002; Liang and Van Doren 2008). The phosphorylation status of OdhI is important for inhibition of ODHC specific activity. Unphosphorylated OdhI specifically binds to the E1o subunit, OdhA, resulting in inhibition of ODHC activity. Phosphorylation of OdhI at the 14th amino acid residue of OdhI (Thr) by PknG relieves the inhibition of ODHC activity. Identification of the OdhI protein as a target of PknG was identified by the proteome comparison between the wild-type and pknG deletion mutant, followed by *in vitro* phosphorylation studies. In addition, deletion of the *odhI* gene abolished glutamate overproduction induced by biotin limitation or by addition of Tween 40 and penicillin, suggesting that the OdhI protein might play a central role in glutamate production by *C. glutamicum* (Schultz et al. 2007). These results suggest that inhibition of ODHC

specific activity by the OdhI protein is an important factor in glutamate overproduction by *C. glutamicum*.

The OdhA protein was copurified with an unphosphorylated OdhI mutant protein expressed in the Δ*odhI* mutant; only a low amount of OdhA was copurified with the wild-type OdhI protein, which was predominantly phosphorylated (Niebisch et al. 2006), suggesting that the OdhA protein may interact with the unphosphorylated OdhI protein. Moreover, it was confirmed that the unphosphorylated OdhI protein can function as an inhibitor of ODHC by *in vitro* enzyme assay. Furthermore, the OdhA protein was also copurified with SucB (E2o subunit of ODHC), Lpd [(E3o subunit of ODHC and pyruvate dehydrogenase complex (PDHC)], and AceE (E1 subunit of PDHC), suggesting that the mixed complexes are formed by OdhA, AceE, SucB, and Lpd, having ODHC and PDHC activities (Niebisch et al. 2006). Surprisingly, OdhA or AceE, copurified with DtsR1, DtsR2, and AccBC are involved in fatty acid biosynthesis, indicating that the OdhA, unphosphorylated OdhI, and DtsR1 proteins might be included in the same protein complex. Interaction of these proteins might be involved in glutamate overproduction in *C. glutamicum*.

By the study of two-dimensional (2D) gel electrophoresis, Niebisch et al. (2006) revealed the presence of three OdhI protein spots of similar molecular mass but different isoelectric point (p*I*). The diphosphorylated OdhI form was absent in the pknG deletion mutant; however, a small fraction of OdhI still migrated as the monophosphorylated OdhI form. Recently, the purified kinase domains of PknA and PknB were shown to phosphorylate OdhI *in vitro* (Fiuza et al. 2008) and threonine at the position of the 15th residue was identified as the phosphorylation site of these two kinases (Barthe et al. 2009). Relative amounts of three OdhI spots representing

unphosphorylated, monophosphorylated, and presumably diphosphorylated forms were calculated *in vivo* by densitometric analysis using the in-frame deletion mutants Δ*pknA,* Δ*pknB,* Δ*pknL,* Δ*pknAG,* Δ*pknAL,* Δ*pknBG,* Δ*pknBL,* Δ*pknLG,* Δ*pknALG,* and Δ*pknBLG*, revealing that all four STPKs can contribute to OdhI phosphorylation, with PknG being the most important one (Schultz et al. 2009). Moreover, Schultz et al. (2007) reported that OdhI is only present in the phosphorylated status in the *ppp* deletion mutant. The phosphatase domain of the phospho-serine/threonine protein phosphatase Ppp catalyzed the dephosphorylation of OdhI *in vitro*, confirming that OdhI is a substrate of Ppp (Krawczyk et al. 2010). They revealed that the entire FHA domain of OdhI and the C-terminal dehydrogenase domain of OdhA are required for interaction using copurification and surface plasmon resonance experiments with different OdhI and OdhA length variants. The FHA domain was also sufficient for inhibition of ODHC activity; however, phosphorylated OdhI was binding-incompetent, relieving the inhibition of ODHC activity.

1.5.6   Involvement of the NCgl1221 protein in glutamate production

Recently, Nakamura et al. (2007) identified a possible L-glutamic acid exporter coded by the NCgl1221 gene in *C. glutamicum*. They revealed that glutamate production is derived from the function of the mutated NCgl1221 protein and concluded that the NCgl1221 protein is important for glutamate production in *C. glutamicum*. It was also reported that the NCgl1221 protein, a mechanosensitive channel homolog, has an N-terminal region similar to that of YggB in *E. coli*. Moreover, the NCgl1221 protein is confined to the cytoplasmic membrane and is a membrane protein with four transmembrane segments within its C-terminal region in the cytoplasm (Yao et al. 2009).

Nakamura et al. (2007) reported that disruption of the NCgl1221 gene abolished glutamate production, and that specific mutations in the NCgl1221 gene induced constitutive glutamate secretion in *C. glutamicum* without any triggers. It has been proposed that treatments that induce glutamate overproduction alter membrane tension and trigger conformational changes in the NCgl1221 protein, thereby enabling *C. glutamicum* cells to secrete glutamate into the medium.

1.6   Objective of this study

The objective of this study is to apply metabolic engineering approaches to glutamate production in *C. glutamicum* in order to understand the regulatory mechanism of glutamate overproduction in *C. glutamicum* and to improve glutamate production. Moreover, for a better understanding of the cellular condition in *C. glutamicum*, the change in cellular protein production in *C. glutamicum* was also analyzed during glutamate production. First, the effect of chloramphenicol, an inhibitor of *de novo* protein synthesis on penicillin-induced glutamate production was investigated, implying that *de novo* protein synthesis after addition of penicillin is an absolute requirement for glutamate overproduction. To identify the proteins responsible for glutamate production by *C. glutamicum*, proteome analysis was performed using two-dimensional gel electrophoresis under penicillin addition conditions. Among the 13 proteins that were upregulated after addition of penicillin, the most upregulated OdhI protein was investigated by glutamate production assay in order to determine whether or not OdhI synthesis is necessary for penicillin-induced glutamate overproduction in *C. glutamicum*. In terms of DNA techniques used in metabolic engineering, amplification of the *odhI* gene has been used for modification to improve the synthesis of the *odhI* gene or its

products with the *odhI* overexpressing strain. Moreover, based on the importance of decreased ODHC specific activity during glutamate overproduction in *C. glutamicum*, the relationship between changes in ODHC specific activity and glutamate production were focused on examination of the effect of modulation of ODHC activity on Tween 40-triggered glutamate overproduction in *C. glutamicum*. Therefore, recombinant strains of *C. glutamicum*, in which expression of *odhA* and its product can be controlled by *odhA* overexpression or *odhA* antisense RNA expression as metabolic engineering approaches for metabolic pathway control, were constructed and glutamate production was investigated to elucidate the role of the *odhA* gene and its product in glutamate overproduction by *C. glutamicum*. Furthermore, protein expression changes, such as OdhA and OdhI proteins, were also investigated during glutamate overproduction in *C. glutamicum*. Throughout this study, comprehensive analysis and integrated metabolic engineering must be applied in order to understand the cellular mechanism of glutamate overproduction by *C. glutamicum*.

1.7   Overview of the thesis

This thesis consists of five chapters.

Chapter 1 deals with the general introduction of this research. It contains the background for this research and a review of previous literature regarding the regulatory mechanism of glutamate overproduction in *C. glutamicum*.

Chapter 2 and **Chapter 3** deal with the application of metabolic engineering approaches for glutamate production in *C. glutamicum* based on an understanding of cellular conditions of regulatory mechanism during glutamate production.

Chapter 2 deals with the requirement of *de novo* synthesis of the OdhI protein in penicillin-induced glutamate overproduction by *C. glutamicum*. First, using proteomic analysis of two-dimensional gel electrophoresis under penicillin addition, protein production change was investigated in *C. glutamicum* during glutamate overproduction for identification of the *de novo* protein(s). Of more than 500 proteins detected, 13 proteins, including OdhI (an inhibitory protein for ODHC), were significantly increased upon penicillin treatment. To determine whether *de novo* OdhI synthesis is necessary for penicillin-induced glutamate overproduction in *C. glutamicum*, the *odhI*-overexpressing strain in which amplification of the *odhI* gene was used as a metabolic engineering approache was constructed and examined. Moreover, continuous glutamate overproduction was also conducted by the *odhI*-overexpressing strain without any triggers.

Chapter 3 deals with the effects of metabolic engineering by *odhA* overexpression and *odhA* antisense RNA expression during glutamate production in *C. glutamicum*. Because of the binding specificity of the OdhI protein to the E1o subunit OdhA of ODHC, resulting in inhibition of ODHC activity and the result obtained from Chapter 2, in which *de novo* synthesis of the OdhI protein is required for glutamate overproduction in *C. glutamicum*, further investigation focused on the role of the *odhA* gene and its product, which is adjacent to the OdhI protein in the regulatory mechanism in glutamate overproduction by *C. glutamicum*. Expression of the *odhA* gene and its product was controlled by metabolic engineering approaches, such as *odhA* overexpression or *odhA* antisense RNA expression in order to examine the effect of modulation of ODHC activity on Tween 40-triggered glutamate overproduction in *C. glutamicum*.

Chapter 4 deals with the expression change of OdhA and OdhI proteins during glutamate overproduction in *C. glutamicum.* Because of the importance of OdhA and OdhI proteins during glutamate overproduction in *C. glutamicum*, the correlation between protein expression changes and glutamate overproduction under various glutamate production conditions was investigated; overexpression of the *odhI* gene in both ATCC 31831 and ATCC 13032, and Tween 40-triggered glutamate production conditions.

Chapter 5 deals with the general conclusion obtained in this research.

Chapter 2

Requirement of *de novo* synthesis of the OdhI protein in penicillin-induced glutamate production by *Corynebacterium glutamicum*

2.1 Introduction

L-Glutamate is used as a flavor enhancer and is in demand as a commercially useful chemical. The amount of glutamate produced worldwide by fermentation using microorganisms has increased to more than 1.5 million tons per year (Leuchtenberger et al. 2005; Shimizu and Hirasawa 2007). *C. glutamicum* is used for the industrial production of glutamate and also used to produce other amino acids such as lysine and threonine (Nakayama et al. 1961; Sano and Shiio 1970; Shiio and Nakamori 1970).

Glutamate production by *C. glutamicum* is induced by the limitation of biotin, which is an indispensable vitamin for growth (Shiio et al. 1962), and the addition of fatty acid ester surfactants such as polyoxyethylene sorbitan monopalmitate (Tween 40) (Takinami et al. 1965), β-lactam antibiotics such as penicillin (Nara et al. 1964), and inhibitors of biosynthesis of arabinogalactan (a cell wall component of *C. glutamicum*) such as ethambutol (Radmacher et al. 2005). Metabolic change at the branch point of 2-oxoglutarate in the tricarboxylic acid cycle is important for glutamate production due to the reduced activity of the 2-oxoglutarate dehydrogenase complex (ODHC) (Kawahara et al. 1997; Shigu and Terui 1971).

Proteomics analysis of *C. glutamicum* has been performed by many researchers (Bendt et al. 2003; Hermann et al. 2001; Li et al. 2007; Schaffer et al. 2001; Schleusener et al. 2005). Niebisch et al. (2006) identified the novel protein OdhI, which regulates ODHC activity, by proteomics analysis. Unphosphorylated OdhI binds to OdhA and inhibits ODHC activity. In addition, Schultz et al. (2007) showed that deletion of the *odhI* gene abolishes glutamate overproduction induced by biotin limitation or by the addition of Tween 40 and penicillin, suggesting that the OdhI protein might play the central role in glutamate production by *C. glutamicum*.

In this chapter, for a better understanding of the cellular state, we analyzed protein production change during penicillin-induced glutamate production in *C. glutamicum*. First, we examined the effect of addition of chloramphenicol, an inhibitor of *de novo* protein synthesis, on penicillin-induced glutamate production, and showed that *de novo* protein synthesis after addition of penicillin was required for glutamate production. Accordingly, we analyzed protein production change using two-dimensional gel electrophoresis and identified proteins that were upregulated after addition of penicillin using a peptide mass fingerprinting method. Our results revealed that production of several proteins, including OdhI, is induced after addition of penicillin. Moreover, we newly found that *de novo* synthesis of the OdhI protein after penicillin addition is necessary for glutamate overproduction. Therefore, in terms of recombinant DNA techniques used in metabolic engineering, amplification of the *odhI* gene has been used for metabolic modification to improve the synthesis of the *odhI* gene.

2.2 Materials and methods

2.2.1 Bacterial strains, media, and culture conditions

C. glutamicum wild-type strain ATCC 31831 was used in this study. For recombinant DNA techniques, *Escherichia coli* JM109 [*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*)/F'(*traD36 proAB⁺ lacI�q lacZΔM15*)] was used. For the transformation of *C. glutamicum*, plasmids were isolated from the *dam dcm* mutant of *E. coli* SCS110 [*rpsL* (Str^r) *thr leu endA thi-1 lacY galK galT ara tonA tsx dam dcm supE44* Δ(*lac-proAB*)/F' (*traD36 proAB⁺ lacI�q lacZΔM15*)] (Stratagene, Cedar Creek, TX, USA) to escape the restriction system of *C. glutamicum* (Vertès et al. 1993).

For the recombinant DNA experiments, *E. coli* and *C. glutamicum* were cultured in Lennox medium consisting of 1% polypeptone, 0.5% Bacto yeast extract, 0.5% NaCl, and 0.1% glucose (pH 7.2). For the glutamate production studies, CM2B agar comprising 1% polypeptone, 1% Bacto yeast extract, 0.5% NaCl, 10 μg/l of D-biotin and 1.5% agar, pH 7.2 (Miwa et al. 1985) and synthetic medium comprising (per liter) 80 g of glucose, 30 g of $(NH_4)_2SO_4$, 3 g of Na_2HPO_4, 6 g of KH_2PO_4, 2 g of NaCl, 3.9 mg of $FeCl_3$, 0.9 mg of $ZnSO_4 \cdot 7H_2O$, 0.3 mg of $CuCl_2 \cdot 2H_2O$, 5.56 mg of $MnSO_4 \cdot 5H_2O$, 0.1 mg of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.3 mg of $Na_2B_4O_7 \cdot 10H_2O$, 0.4 g of $MgSO_4 \cdot 7H_2O$, 40 mg of $FeSO_4 \cdot 7H_2O$, 84 mg of $CaCl_2$, 500 μg of vitamin B1·HCl, 0.1 g of ethylenediaminetetraacetic acid, 20 μg of biotin, and 25 g of $CaCO_3$, pH 7.2 (Shirai et al. 2006) were used. If necessary, ampicillin (50 μg/ml for *E. coli*) or kanamycin (20 μg/ml for *E. coli* and 10 μg/ml for *C. glutamicum*) was added to the culture medium to select the antibiotic-resistant transformants.

E. coli was cultivated at 37°C and *C. glutamicum* was cultivated at 30°C or 31.5°C, depending on the experiment. Cell growth was monitored by measuring the

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optical density of the culture at 660 nm (OD_{660}) with a spectrophotometer (U-2000; Hitachi High-Technologies Co., Tokyo, Japan). Cultures were diluted with 0.2 N HCl to dissolve $CaCO_3$ prior to OD_{660} measurements.

2.2.2 Construction of the *C. glutamicum* strain overexpressing the *odhI* gene

The *odhI* gene fragment, including the possible promoter region, was amplified by polymerase chain reaction (PCR) from *C. glutamicum* ATCC 31831 genomic DNA using *Z-Taq* polymerase (Takara Bio Inc., Shiga, Japan) and the set of primers 5′-GAATTCAACCCACTTGCGGGTAGTGG-3′ and 5′-GAATTCTTAGGCATTCTATACACAAAACG-3′; the PCR fragment was cloned in a pGEM-T Easy Vector (Promega Co., Madison, WI, USA). Since the *odhI* gene from the ATCC 31831 strain could not be directly compared with that from the *C. glutamicum* ATCC 13032 strain (whose genome DNA sequence has been already determined), the sequences of five independently cloned PCR fragments were determined using the BigDye Terminator Cycle Sequencing Kit ver. 1.1 (Applied Biosystems, Foster City, CA, USA) and an ABI Prism genetic analyzer 310NT (Applied Biosystems) and compared with each other. The PCR product in the pGEM-T Easy was recloned into the *Eco*RI site of the *E. coli-C. glutamicum* high-copy-number shuttle vector pHT1 (Hirasawa et al. 2003). The resulting plasmid was introduced into *C. glutamicum* ATCC 31831.

2.2.3 Glutamate production by *C. glutamicum*

C. glutamicum cells were grown at 30°C for 24 h on a CM2B plate for seed culture preparation. For the preculture, cells were collected from the CM2B plate,

inoculated into 40 ml of synthetic medium in a Sakaguchi flask, and then incubated at 31.5°C for 17 h with reciprocal shaking at 120 strokes per minute. Then, 1 ml of the preculture was inoculated into 40 ml of the fresh synthetic medium in a Sakaguchi flask, and the culture was incubated at 31.5°C with reciprocal shaking. After cell growth reached the early exponential phase (OD_{660} = approximately 15), penicillin G was added to the culture to induce glutamate production. Cultivation was continued until glucose in the culture medium was depleted.

2.2.4   Measurement of glucose and glutamate concentrations and ODHC specific activity

Glucose and glutamate concentrations in the culture supernatant were measured by the Glucose CII-Test Wako (Wako Pure Chemicals Inc., Osaka, Japan) and an F-kit glutamate (R-Biopharm AG, Darmstadt, Germany), respectively.

ODHC specific activity of *C. glutamicum* was determined according to the method described by Kim et al. (2009) and Shiio and Ujigawa-Takeda (1980). *C. glutamicum* cells were harvested, washed twice with 0.2% KCl, and suspended in 3 ml of 0.1 M *N*-tris(hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES)·NaOH buffer (pH 7.7) containing 30% (*v/v*) glycerol. Cells were disrupted by sonication and then centrifuged to remove the cell debris. One milliliter of the supernatant, applied to the gel filtration column (PD-10 Desalting Column; GE Healthcare UK, Buckinghamshire, UK) to remove low-molecular-weight compounds, was used for the enzyme assay.

The reaction mixture was prepared as follows: 100 mM TES·NaOH (pH 7.7), 5 mM $MgCl_2$, 3 mM cysteine, 0.3 mM thiamine pyrophosphate, 0.2 mM coenzyme A, 1

mM 3-acetylpyridine adenine dinucleotide (APAD$^+$) instead of oxidized form of nicotinamide adenine dinucleotide (NAD$^+$), and 150 µl of the cell extract. After addition of 1 mM 2-oxoglutarate to the reaction mixture, the initial increase in the absorbance of APADH at 365 nm was measured at 31.5°C for 3 min with 15-s intervals. ODHC specific activity was defined as the amount of enzyme required for the conversion of an equivalent of 1 µmol of NAD$^+$ into reduced form of nicotinamide adenine dinucleotide (NADH) in 1 min. The concentration of total protein in the crude extract was determined by Bradford's method (Bradford 1976) using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

2.2.5   Two-dimensional gel electrophoresis

C. glutamicum cells harvested by centrifugation at 9,500 × *g* for 5 min at 4°C were suspended in 50 mM sodium phosphate buffer (pH 7.0) containing 12.5% (*v/v*) protease inhibitor cocktail for use with bacterial cell extracts (Sigma, Saint Louis, MO, USA) and disrupted by ultrasonic homogenizer (UH-50, SMT Co. Ltd., Tokyo, Japan) at 50 W for 15 s with 15 s intervals for six times on ice. Cell debris was removed by centrifugation at 9,500 × *g* for 10 min at 4°C, and the supernatant was used for two-dimensional gel electrophoresis.

Isoelectric focusing was performed using a Multiphor II electrophoresis unit (GE Healthcare UK) and a commercially available immobilized pH gradient (IPG) gel strip (Immobiline Drystrip, p*I* range of 4–7, 13 cm, linear gradient; GE Healthcare UK). The protein sample (30 µg) in 250 µl of rehydration buffer consisting of 6.4 M urea, 2% 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonate, 2% protease inhibitor cocktail, 2% IPG buffer (GE Healthcare UK), 0.96% destreak reagent (GE Healthcare

UK), and 0.01% bromophenol blue was loaded onto a IPG strip, which was then rehydrated for 15 h. Isoelectric focusing and two-dimensional electrophoresis and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were carried out according to the protocols reported by Hirasawa et al. (2009).

The gel was stained with SYPRO red protein gel stain (Invitrogen Co., Carlsbad, CA, USA) according to the manufacturer's protocol and scanned using a Typhoon 9210 (GE Healthcare UK) to detect the protein spots on the gel. The Image Master 2D Elite (GE Healthcare UK) was used to quantify the volume of each spot. To allow comparison of the two gel images, each spot volume on the gels was normalized to the summation of the volume of all the detected spots on the two gels.

As reported previously (Hirasawa et al. 2009), to evaluate the significant changes in protein abundance in two samples, the same samples were independently examined by two-dimensional gel electrophoresis. This analysis revealed that if the ratio of the amounts of protein obtained from one sample compared to another is more than 3 or less than 0.33, then the ratio is significant (data not shown).

2.2.6 Peptide mass fingerprinting for protein identification

Protein spots were identified by peptide mass fingerprinting analysis using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF MS) and liquid chromatography mass spectrometry (LC-MS/MS).

For the identification of proteins on the two-dimensional electrophoresis gels, the gels were stained with Coomassie brilliant blue (CBB) in CBB-staining solution (2.5 g/l of CBB R-250 in 50% methanol and 10% acetate). Each CBB-stained protein spot was excised and placed in a 1.5-ml centrifuge tube (Treff AG, Degersheim,

Switzerland). The excised spot was destained by incubation in 100 µl of destaining solution consisting of 50% methanol and 40 mM ammonium bicarbonate for 30 min with vigorous shaking and then dried at 60°C with evaporation. In-gel digestion of the protein in the excised gel and purification of the digested peptides were performed by the method reported by Hirasawa et al. (2009). Purified peptides were mixed with matrix solution (α-cyano-4-hydroxycinnamic acid-buffered 33% acetonitrile containing 0.1% trifluoroacetic acid) and spotted onto a target plate for MALDI-TOF MS. MALDI-TOF MS analysis was carried out using an Autoflex (Bruker Daltonics K. K., Bremen, Germany) in the measurement range m/z of 800–4600. Calibration was achieved using a peptide calibration standard (Bruker Daltonics). The m/z data from the detected peptides were used to search the *C. glutamicum* ATCC 13032 genome in the NCBInr database with Mascot 2.0 (Matrix Science Inc., Boston, MA, USA). The proteins identified by peptide mass fingerprinting were evaluated and compared with each molecular mass, and the p*I* was calculated from the amino acid sequence.

Peptide mass fingerprinting was also conducted by LC-MS/MS analysis. After extracting the trypsin-digested peptides from the gel as described above, the peptide solutions were purified through a membrane filter (GV4, Millipore, Billerica, MA, USA) and analyzed by LC-MS/MS. High-performance liquid chromatography (HPLC) was performed using a Pepmap C18 (3 µm, 100 Å, 150 × 0.3 mm) HPLC column (LC Packings, Amsterdam, The Netherlands). The elution was carried out with 0.1% formic acid in 95% acetonitrile (buffer A) and 0.1% formate in 5% acetonitrile (buffer B) at a flow rate of 3 µl/min. The elution conditions were as follows: A/B = 100:0 (0 min), 98:2 (5 min), 70:30 (45 min), 2:98 (50 min), 2:98 (65 min), 98:2 (67 min), and 98:2 (80 min). For MS/MS analysis, the eluted peptides were diverted to an ion trap mass

spectrometer (Esquire, Bruker Daltonics) equipped with positive electrospray mode. The measurement was performed in the mass range m/z of 50–3,000. The m/z data from the detected peptides were analyzed with Mascot 2.0 in a manner similar to that used in the MALDI-TOF MS analysis.

2.3   Results and discussion

2.3.1   Effect of chloramphenicol addition on penicillin-induced glutamate production

For an engineering strategy to be successful, a better understanding of a cellular state is necessary. To analyze whether *de novo* protein synthesis is required for glutamate production by *C. glutamicum*, we examined the effect of chloramphenicol, an inhibitor of *de novo* protein synthesis, on penicillin-induced glutamate production. *C. glutamicum* wild-type strain ATCC 31831 was cultured in synthetic medium, and 10 μM of penicillin G [100 × minimal inhibitory concentration (MIC)] and/or 77 μM of chloramphenicol (20 × MIC) were added to the culture medium after cell growth reached the early exponential phase (Figure 2.1).

Cell growth of the ATCC 31831 strain stopped or decreased after the addition of penicillin G and/or chloramphenicol (Figure 2.1A). When penicillin G alone was added, approximately 20 g/l of glutamate was produced in 48 h (Figure 2.1C). Simultaneous addition of chloramphenicol with penicillin G almost completely abolished glutamate overproduction (about 2 g/l). On the other hand, when chloramphenicol was added to the culture 2 h after the addition of penicillin G, approximately 13 g/l of glutamate production was achieved in 48 h. When chloramphenicol was added 4 h after the addition of penicillin G, approximately 20 g/l of glutamate was produced, which was comparable to that obtained upon addition of penicillin G alone. These results suggested that *de novo* protein synthesis during the first 4 h after the addition of penicillin might be required for glutamate overproduction by *C. glutamicum*.
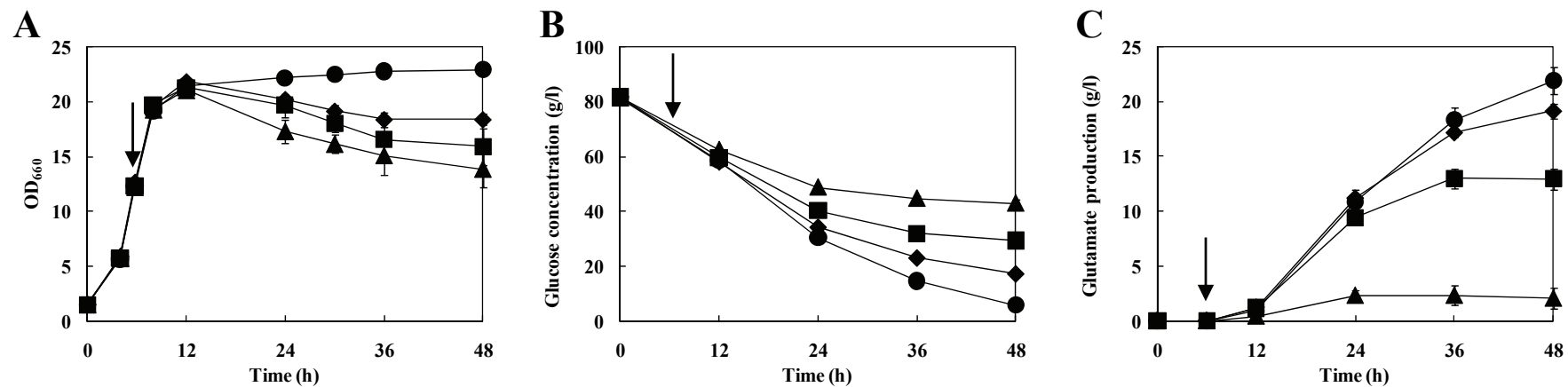
Figure 2.1   Effect of chloramphenicol addition on penicillin-induced glutamate production by *C. glutamicum*. *Arrows* indicate the additions of penicillin G or chloramphenicol, respectively. Averages with standard deviations in three independent experiments are shown. Cell growth (A), glucose consumption (B), and glutamate production (C) are shown. *Circles* Addition of 10 μM penicillin G alone; *triangles* simultaneous addition of 10 μM penicillin G and 77 μM chloramphenicol; *squares* 77 μM chloramphenicol addition at 2 h after 10 μM penicillin G addition; *diamonds* 77 μM chloramphenicol addition at 4 h after 10 μM penicillin G addition

2.3.2 Proteome analysis of *C. glutamicum* during penicillin-induced glutamate production

On the basis of the above results, we expected that the pattern of protein production for *C. glutamicum* would be changed after penicillin addition. We speculated that the proteins whose amount increased followed by penicillin addition could be responsible for glutamate production because glutamate production was abolished by the simultaneous addition of chloramphenicol with penicillin G. Thus, proteomics analysis was carried out to analyze the global protein production pattern and to identify proteins required for penicillin-induced glutamate production.

Proteins obtained from the ATCC 31831 strain at 1, 2, 3, and 4 h after the addition of 10 μM of penicillin G were separated by two-dimensional gel electrophoresis, and the protein production patterns in each sample were compared with that in the sample obtained from cells before penicillin G addition. Culture experiments were independently performed five times, and the obtained protein samples were applied to two-dimensional gel electrophoresis.

Approximately 500 proteins in *C. glutamicum* ATCC 31831 were detected by two-dimensional gel electrophoresis with SYPRO red staining (Figure 2.2). Of these ~500 detected proteins, the amounts of 13 proteins increased after the addition of penicillin G (Figure 2.2 and Table 2.1). Surprisingly, one protein (no. 572) was remarkably overproduced: a tenfold increase in its amount upon penicillin addition was observed.
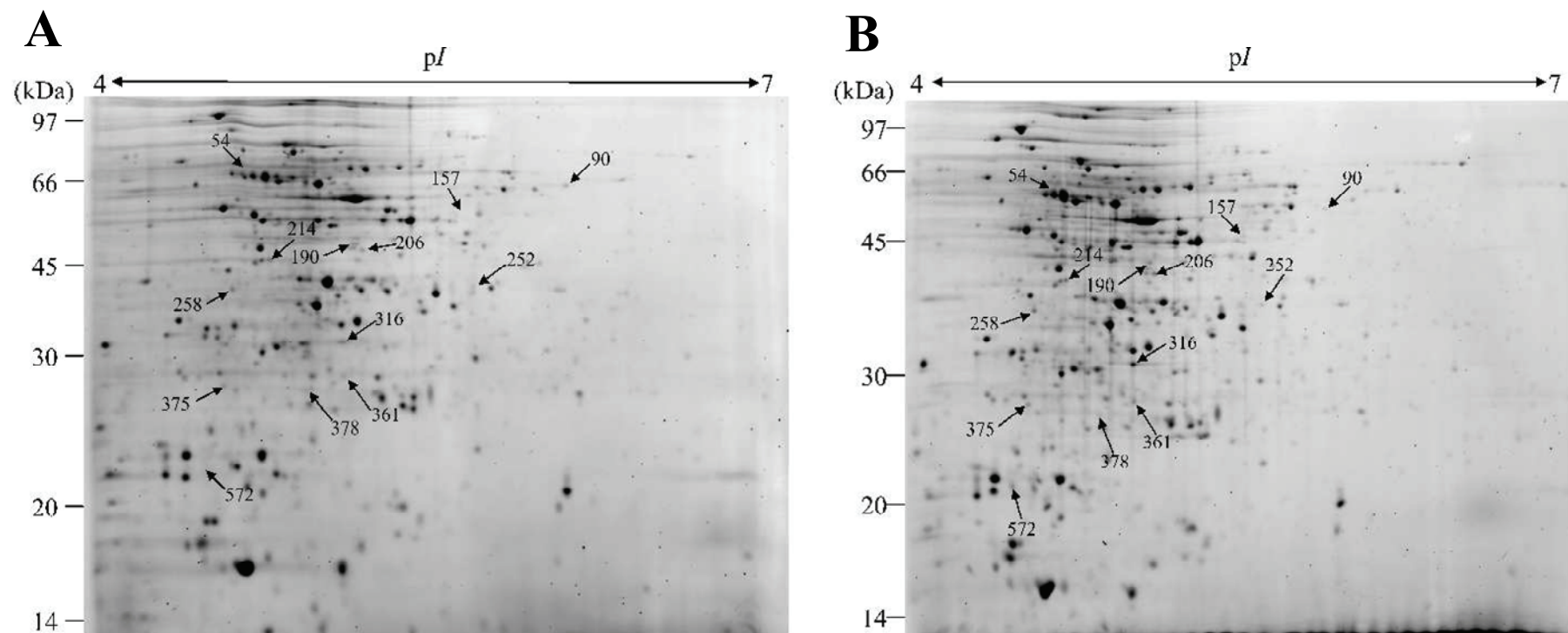
Figure 2.2 Gel image of *C. glutamicum* ATCC 31831 proteins separated by two-dimensional gel electrophoresis. The gel images of proteins obtained from *C. glutamicum* cells before (A) and 4 h after addition (B) of 10 µM penicillin G are shown. Each spot indicated by *arrows* corresponds to a protein whose amount was increased by penicillin G addition. *Numbers* represent the spot numbers shown in Table 2.1.

Table 2.1   Changes in the production of proteins significantly upregulated by penicillin G addition

Spot number	Protein name	Locus tag[a]	Gene	pI[b]	Molecular mass (kDa)[b]	Change in protein amount after penicillin addition[c]			
						1 h	2 h	3 h	4 h
54	Phosphoglucomutase	Cgl2514/cg2800	*pgm*	4.76	59.2	1.3 ± 0.8	1.9 ± 0.5	1.6 ± 0.1	3.1 ± 1.5
90	*ND*					1.1 ± 0.9	3.1 ± 4.0	2.0 ± 1.1	2.7 ± 3.6
157	Putative acetyl-CoA acetyltransferase	Cgl2392/cg2625	*pcaF*	5.20	42.7	2.7 ± 1.0	2.6 ± 2.0	4.9 ± 4.0	1.8 ± 1.3
190	Acetylornithine aminotransferase	Cgl1397/cg1583	*argD*	5.05	41.4	3.3 ± 2.5	1.3 ± 0.9	5.2 ± 2.0	2.4 ± 2.2
206	*ND*					1.7 ± 0.5	1.7 ± 1.1	3.2 ± 1.6	2.4 ± 1.9
214	Probable oxidoreductase	Cgl0936/cg1068	-	4.70	35.4	2.1 ± 1.4	2.2 ± 1.8	3.3 ± 1.5	2.1 ± 1.4
252	*ND*					1.6 ± 1.0	2.5 ± 2.3	3.1 ± 1.8	1.5 ± 1.1
258	Thioredoxin reductase	Cgl3090/cg3422	*trxB*	4.64	34.4	2.2 ± 1.5	2.7 ± 1.9	1.1 ± 0.1	3.0 ± 2.7
316	*ND*					1.3 ± 0.3	2.8 ± 2.2	4.0 ± 2.8	3.8 ± 3.8
361	UMP-kinase	Cgl2024/cg2218	*pyrH*	5.10	26.4	2.2 ± 0.7	2.0 ± 1.3	4.9 ± 3.0	1.9 ± 1.0
375	Nicotinamidase/Pyrazinamidase	Cgl2487/cg2734	*pncA*	4.60	20.3	1.6 ± 0.7	2.6 ± 1.1	3.7 ± 0.9	2.9 ± 1.5
378	Dethiobiotin synthetase protein	Cgl2605/cg2886	*bioD*	4.65	23.8	3.2 ± 3.8	2.6 ± 1.7	0.8 ± 0.1	0.7 ± 0.8
572	FHA-domain-containing protein[d]	Cgl1441/cg1630	*odhI*[d]	4.76	15.4	2.6 ± 1.7	7.9 ± 2.2	5.5 ± 2.3	12.8 ± 5.4

ND not determined by peptide mass fingerprinting

[a]Locus tag corresponds to the ORF name defined by Ikeda and Nakagawa (2003) and Kalinowski et al. (2003), respectively

[b]pI and molecular mass are theoretical values calculated from the amino acid sequence.

[c]Change in protein amount was defined as the ratio of the protein amount after penicillin addition to that before penicillin addition. Average values with standard deviation in 5 independent culture experiments are shown

[d]The FHA-domain-containing protein Cgl1441 was annotated as OdhI, an inhibitory protein for ODHC (Niebisch et al. 2006)

2.3.3 Identification of proteins whose amount was increased by penicillin addition

We hypothesized that the genome sequence of the *C. glutamicum* ATCC 31831 strain is similar to that of the ATCC 13032 strain whose genome sequence was determined. Therefore, to identify protein spots in peptide mass fingerprinting analysis, we used the protein database for the ATCC 13032 strain.
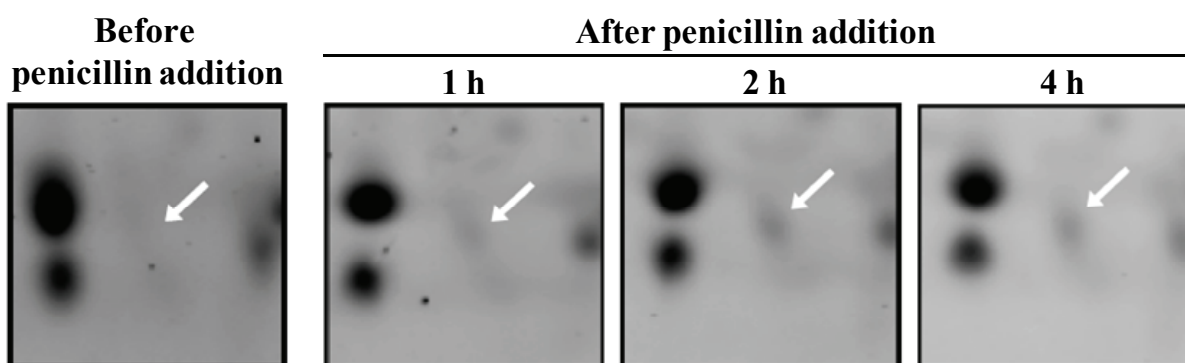
Figure 2.3 Change in the abundance of Cgl1441 (OdhI) protein after penicillin G addition in *C. glutamicum* ATCC 31831. The spots corresponding to the Cgl1441 protein on gel images obtained before and 1, 2, and 4 h after 10 μM penicillin G addition are shown. *Arrows* indicate the spots corresponding to the Cgl1441 protein.

Peptide mass fingerprinting was performed using MALDI-TOF MS and LC-MS/MS to identify the proteins whose amount was increased by penicillin addition. Nine of the 13 proteins could be identified (Table 2.1). The estimated p*I* and molecular mass of each protein on the gel matched well with those calculated from the corresponding amino acid sequence (date not shown). The functions of the identified proteins are related to energy metabolism, amino acid metabolism, and stress response. Interestingly, the protein corresponding to spot no. 572, which showed a tenfold

increase in its amount followed by penicillin addition (Table 2.1 and Figure 2.3), was identified as the Cgl1441 protein. Cgl1441 was annotated as OdhI, an inhibitory protein for ODHC (Niebisch et al. 2006).

2.3.4 Requirement of *de novo* synthesis of the OdhI protein in penicillin-induced glutamate production by *C. glutamicum*

It was revealed that OdhI protein, one of the several identified upregulated proteins, is significantly induced after penicillin addition as a 10-fold increase, compared to before penicillin addition, suggesting that *de novo* synthesis of the OdhI protein is absolutely necessary for penicillin-induced glutamate overproduction by *C. glutamicum*. Moreover, Schultz et al. (2007) reported that the deletion of the *odhI* gene abolishes penicillin-induced glutamate production. Thus, to determine whether the OdhI protein is indeed necessary for penicillin-induced glutamate production, we planned to examine glutamate production in a strain overexpressing the *odhI* gene without any triggers. Therefore, we constructed the *odhI* overexpressing stain by the amplification of *odhI* gene as metabolic modification. The overproduction of the OdhI protein in the *odhI*-overexpressing strain throughout the cultivation was confirmed by SDS-PAGE (Figure 2.4).

The pHT1-transformed ATCC 31831 strain produced a small amount of glutamate in the absence of any triggers (Figure 2.5C). On the other hand, the *odhI*-overexpressing strain, as the purposeful amplification of *odhI* gene, resulted in significantly increased glutamate production without any triggers (Figure 2.5C). It should be noted that glutamate production by the *odhI*-overexpressing strain was initiated after cell growth reached the late exponential and early stationary phase.

Moreover, the level of ODHC specific activity in the *odhI*-overexpressing strain was significantly lower than that in the pHT1-transformed ATCC 31831 strain throughout the cultivation (Figure 2.5D). These phenomena were consistent with the result of SDS-PAGE analysis for the *odhI*-overexpressing strain; i.e., the overproduction of OdhI protein in the *odhI*-overexpressing strain was shown throughout cultivation (Figure 2.4), and the significantly high amount of OdhI was detected at the stationary phase (data not shown). These results indicate that increasing the amount of the OdhI protein alone because of *odhI* gene overexpression as a metabolic engineering strategy can induce glutamate overproduction in *C. glutamicum*. Taken together with the results reported by Schultz et al. (2007), these results led us to conclude that *de novo* synthesis of the OdhI protein is necessary for penicillin-induced glutamate overproduction.
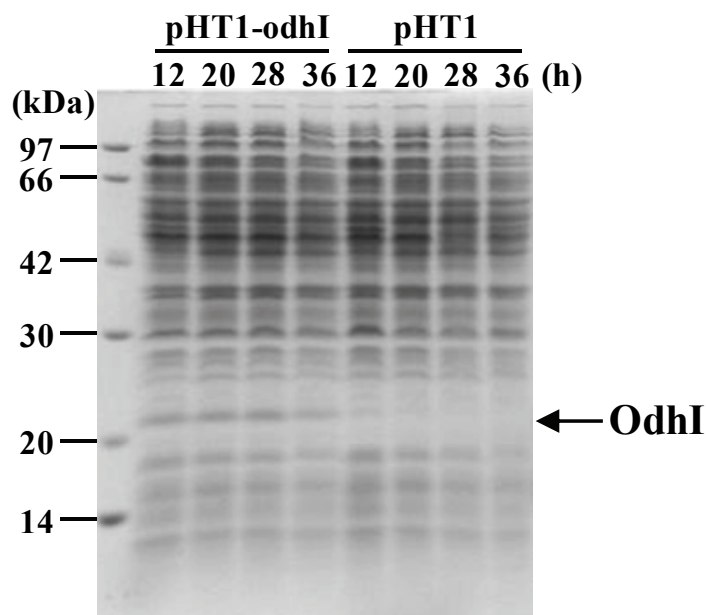
Figure 2.4   Confirmation of OdhI protein overproduction in pHT1-odhI by SDS-PAGE. The ATCC 31831/pHT1-odhI (*odhI*-overexpressing strain) and ATCC 31831/pHT1 strains were cultivated in Sakaguchi flask at 31.5ºC with shaking, and then total cellular protein (20 μg) of each cell was obtained at 12, 20, 28, 30, and 38 h.
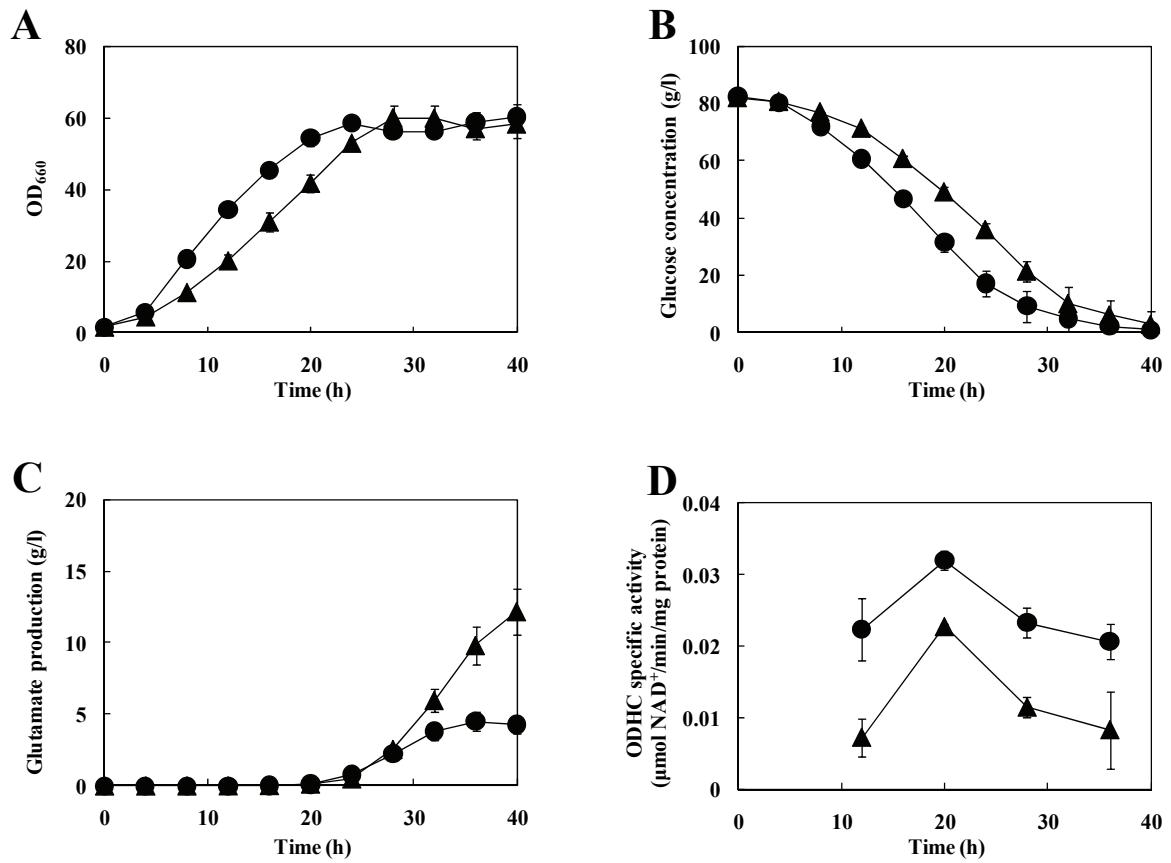
Figure 2.5 Glutamate production and change in ODHC specific activity in the *odhI*-overexpressing strain of *C. glutamicum* without any triggers. ATCC 31831/pHT1 and the *odhI*-overexpressing ATCC 31831 were, respectively, cultured, and then glutamate production was observed. Cell growth (A), glucose consumption (B), glutamate production (C), and ODHC specific activity (D) in ATCC 31831/pHT1 (*circles*) and in the *odhI*-overexpressing strain (*triangles*) are represented. Averages with standard deviation in three independent experiments are shown.

2.3.5 Continuous glutamate production in the *odhI*-overexpressing *C. glutamicum* by supplying a carbon source

Glutamate production by the *odhI*-overexpressing strain begins in the late exponential or early stationary phase, as described above, suggesting that the cellular state of this strain reaches the glutamate production phase at that phase. To investigate whether glutamate production by this *odhI*-overexpressing strain continues in the absence of any triggers after the cells reach the late exponential phase, additional glucose was supplemented to the culture medium after most of the initial glucose was depleted. As shown in Figure 2.6, glutamate production by the *odhI*-overexpressing strain was observed after glucose addition. After glucose addition, cell growth stopped, but the cells continued to consume glucose. The supplemental glucose consumption and glutamate production occurred together. These results suggest that the *odhI*-overexpressing strain can continue to produce glutamate even during the early stationary growth phase. These results also suggest that glutamate can be efficiently produced using a simple carbon source without any additional biomass formation.
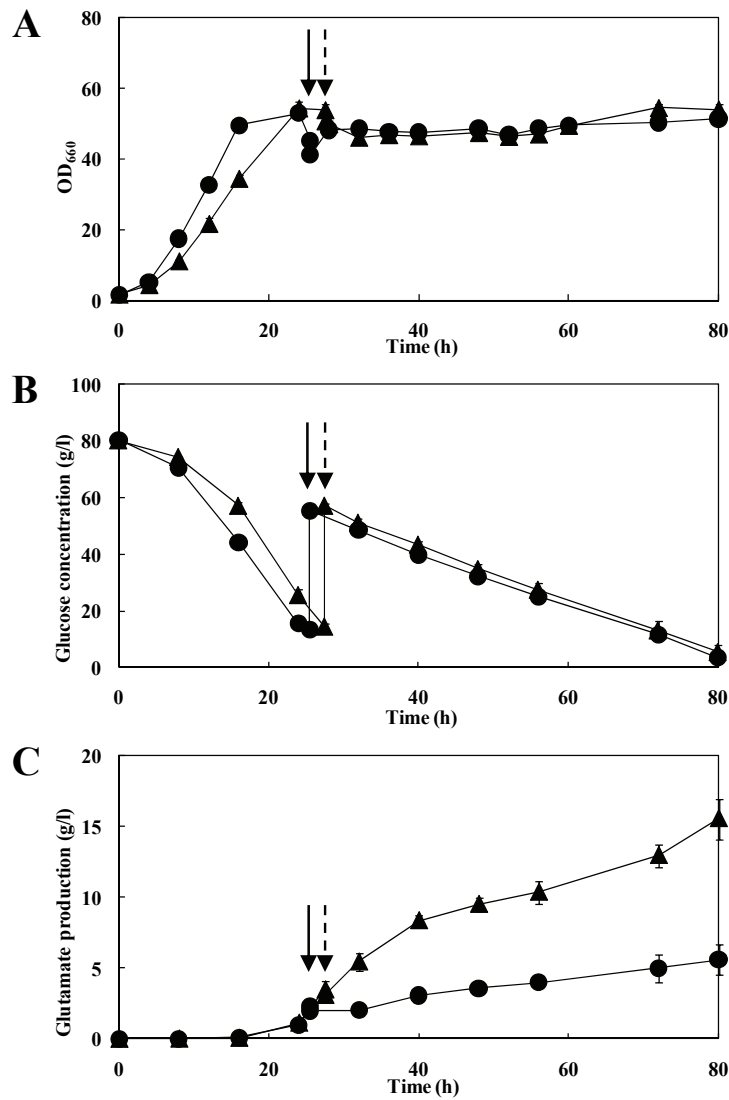
Figure 2.6 Continuous glutamate productions by the *odhI*-overexpressing strain without any triggers. The ATCC 31831/pHT1 (*circles*) and the *odhI*-overexpressing strains (*triangles*) were cultured in Sakaguchi flasks containing synthetic medium, respectively. Glucose as a carbon source was added when most of the initially added glucose was depleted and cell growth had reached the late exponential phase. Cell growth (A), glucose consumption (B), and glutamate production (C) were then measured. *Arrows with solid and dashed lines* in each graph represent glucose supplementation in ATCC 31831/pHT1 and the *odhI*-overexpressing strains, respectively. Averages with standard deviations in three independent experiments are shown.

2.4   Discussion

Many researchers have reported analyses of the glutamate production mechanism of *C. glutamicum* at the metabolic and molecular levels, as described in "Introduction" section because a better understanding of a cellular state is required for a metabolic engineering strategy to be successful. To identify the important protein(s) for glutamate production, proteome analysis of *C. glutamicum* followed by penicillin addition was performed with two-dimensional gel electrophoresis.

Firstly, to confirm whether *de novo* protein synthesis is required for penicillin-induced glutamate production, we examined the effect of the addition of chloramphenicol during penicillin-induced glutamate overproduction. The results shown in Figure 2.1 indicate that *de novo* protein synthesis within 4 h after penicillin addition is absolutely required for penicillin-induced glutamate production. Therefore, we performed proteomic analysis, considering it as an effective approach for a better understanding of a cellular state to identify proteins required for penicillin-induced glutamate production.

We could detect approximately 500 protein spots of the *C. glutamicum* ATCC 31831 strain by two-dimensional gel electrophoresis (p*I* range, 4–7) with SYPRO red staining (Figure 2.2). The comparison of the protein patterns before and after penicillin G addition revealed that, of the approximately 500 protein spots detected, the amounts of 13 proteins were significantly increased by penicillin G addition (Table 2.1). Among the increased proteins, we found that the increase in the amount of the OdhI protein directly contributed to penicillin-induced glutamate production by *C. glutamicum*. Recently, Schultz et al. (2007) reported that *odhI* deletion abolishes penicillin-induced glutamate overproduction by *C. glutamicum*. In this regard, our results indicate that *de*

novo synthesis of the OdhI protein is absolutely necessary for penicillin-induced glutamate overproduction by *C. glutamicum*. However, Schultz et al. (2007) also reported that the introduction of the plasmid carrying the *odhI* gene with its native promoter into the *odhI* deletion strain of *C. glutamicum* could not induce glutamate overproduction without any triggers, which is totally inconsistent with our results. In our case, since the *odhI* gene with its native promoter was cloned under the *lac* promoter of *E. coli* on the pHT1 shuttle vector and the *lac* promoter is constitutively active in *C. glutamicum* cells, it is thought that the expression of the *odhI* gene on pHT1 might be achieved by both the native promoter for the *odhI* and *lac* promoter. Therefore, the expression level of *odhI* from our plasmid might be higher than that from the plasmid reported by Schultz et al. (2007), and the increased expression level of *odhI* in the overexpressing strain might be similar to the expression level after penicillin addition. As a result, we successfully induced glutamate overproduction by introducing the *odhI*-carrying plasmid alone as the amplification of metabolic pathway.

In addition, as shown in Figure 2.3, the expression of the OdhI protein in the strain ATCC 31831 was very low before penicillin G addition. This phenomenon was inconsistent with the result reported by Schultz et al. (2007). They detected the OdhI protein by Western blotting, whereas we detected it by SYPRO red staining. Since the amplification magnitude of protein abundance for detection in Western blotting is generally larger than that in SYPRO red staining, very small amounts of protein(s) can be detected by Western blotting. Thus, the abundance of the OdhI protein was shown to be very low in SYPRO red staining. The reason why OdhI protein has to be expressed after penicillin G addition has been still obscure. However, the disruption of *odhI* gene does not induce glutamate overproduction after triggers as reported by Schultz et al.

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(2007) and the decrease in ODHC specific activity is an important factor for glutamate overproduction as reported by Kawahara et al. (1997). Therefore, the induction of *de novo* synthesis of OdhI protein by penicillin G addition, which causes the decrease in ODHC specific activity, is considered to be a reasonable phenomenon.

Nakamura et al. (2007) showed that the ODHC specific activity in the mutant strain of NCgl1221 gene encoding a possible glutamate exporter was half lower than that in the parent strain, indicating that both activation of NCgl1221 protein, which is probably caused by conformational change, and the decrease in ODHC specific activity occurred in *C. glutamicum* during glutamate overproduction. In addition, they also suggested that the inhibition of ODHC-catalyzed reaction by disruption of *odhA* gene, encoding one of the subunits of ODHC, does not activate NCgl1221 protein, but the activation of NCgl1221 protein decreases the ODHC specific activity in *C. glutamicum*. Since the overproduction of OdhI protein by metabolic amplification of *odhI* gene resulted in glutamate overproduction as shown in Figure 2.5, the overproduced OdhI protein might be able to activate NCgl1221 protein. The mechanism of penicillin-induced glutamate production, including NCgl1221, is speculated as follows: (1) penicillin treatment, (2) OdhI production and NCgl1221 activation, (3) decrease in ODHC specific activity, and (4) glutamate overproduction.

In order for OdhI to exhibit an inhibitory effect on ODHC activity (i.e., binding to the E1o subunit of ODHC, OdhA), the dephosphorylation of the specific amino acid residue in OdhI is necessary (Barthe et al. 2009; Niebisch et al. 2006; Schultz et al. 2007). In our experiments, phosphorylated OdhI protein could not be found on the two-dimensional gel as shown in Figure 2.2 and 2.3. Moreover, the serine/threonine protein kinase (STPK) and phosphatase for OdhI have been already identified: the

kinase is encoded by the *pknG* gene and the phosphatase is encoded by the *ppp* gene (Niebisch et al. 2006; Schultz et al. 2007). On the other hand, Fiuza et al. (2008) reported that OdhI is also phosphorylated by other STPKs such as PknA and PknB and PknL and that the phosphorylation of OdhI by PknG depends on the phosphorylation of PknG by PknA. Although the abundance of STPKs and Ppp proteins might be changed by penicillin addition, we could not detect these proteins in this study. Analysis of not only the phosphorylation status of OdhI but also the expression of the genes encoding STPKs and quantification of the amount of their products during glutamate production will be required for a better understanding of glutamate overproduction by *C. glutamicum*.

Since the *odhI*-overexpressing strain produced glutamate after cell growth reached the late exponential or early stationary phase, we examined the effect of supplemental glucose on glutamate production by the *odhI*-overexpressing strain when the initial glucose was almost consumed, without any triggers. Glucose addition as a carbon source during the late exponential phase resulted in continuous glucose consumption, although cell growth stopped. Importantly, using the *odhI*-overexpressing strain of *C. glutamicum*, continuous glutamate production without another biomass formation was successfully achieved by supplemental glucose (Figure 2.6). This result indicates that glucose added at the late exponential phase was efficiently converted to glutamate. However, glucose consumption and glutamate production rates after glucose addition were slightly lower than those before glucose addition (Figure 2.6), suggesting that high amounts of glucose might be stress for cells. Thus, the level of glutamate production in this experiment was slightly increased compared to that shown in Figure

2.5. The decrease in glutamate production rate might be overcome by continuous glucose feeding to achieve a low glucose concentration in the culture.

In conclusion, the *de novo* synthesis of the OdhI protein is absolutely required for penicillin-induced glutamate overproduction by *C. glutamicum*, and *odhI* overexpression as one of metabolic engineering methods for amplification results in continuous glutamate overproduction without any triggers. However, it is thought that other factors such as the phosphorylation status of OdhI, expression of kinases and phosphatase for OdhI, expression of other genes and/or proteins, and metabolic regulation are also involved in glutamate overproduction by *C. glutamicum*. Moreover, we could identify eight additional proteins whose amounts were increased followed by penicillin G addition (Table 2.1). The effect of artificial overexpression or deletion of these genes on glutamate production by *C. glutamicum* should be further analyzed. Furthermore, since we could not identify four proteins, further analyses are needed to assess the contribution of these unidentified proteins in glutamate production by *C. glutamicum*.

Chapter 3

Effect of *odhA* overexpression and *odhA* antisense RNA expression on Tween 40-triggered glutamate production by *Corynebacterium glutamicum*

3.1 Introduction

A coryneform bacterium, *C. glutamicum*, is a Gram-positive and facultatively anaerobic bacterium with a high G+C content. It was originally isolated as a glutamate-producing bacterium (Kinoshita et al. 1957; Udaka 1960) and has been used for the industrial fermentation of various amino acids such as lysine and threonine (Nakayama et al. 1961; Shiio and Nakamori 1970).

Among those produced by microbial fermentation, glutamate is one of the prominent amino acids. Glutamate overproduction by *C. glutamicum* is induced by the depletion of biotin, which is an essential element for growth (Shiio et al. 1962), by the addition of fatty acid ester surfactants such as Tween 40 (polyoxyethylene sorbitan monopalmitate; Takinami et al. 1965), by the addition of penicillin, which inhibits peptidoglycan biosynthesis (Nara et al. 1964; Nunheimer et al. 1970), or by the addition of ethambutol, an antimycobacterial drug which inhibits the biosynthesis of mycolic acid in Mycobacteria (Radmacher et al. 2005).

The 2-oxoglutarate dehydrogenase complex (ODHC) is a key enzyme in glutamate production, which is located at the branch point between the tricarboxylic acid (TCA) cycle and the glutamate biosynthesis pathway. ODHC consists of three subunits: 2-oxoglutarate dehydrogenase (E1o; EC 1.2.4.2), coded by *odhA* (Usuda et al.

1996); dihydrolipoamide *S*-succinyltransferase (E2; EC 2.3.1.61), coded by *sucB* (found from the genome sequencing); and dihydrolipoamide dehydrogenase (E3; EC 1.8.1.4), coded by *lpd* (Schwinde et al. 2001). Shingu and Terui (1971) and Kawahara et al. (1997) found that ODHC specific activity is dramatically reduced during glutamate overproduction. Moreover, metabolic flux analysis revealed that attenuation of ODHC activity has a great impact on glutamate production by *C. glutamicum* (Shimizu et al. 2003; Shirai et al. 2005). These results suggest that decrease in ODHC specific activity is an important factor for producing glutamate by *C. glutamicum*, leading to increase in the metabolic flux for glutamate biosynthesis**.**

The *odhA* deletion strain could produce glutamate in the presence of excess biotin; however, this strain showed a severe growth defect under the aerobic conditions, implying the necessity of the *odhA* gene in the cell growth of *C. glutamicum* (Asakura et al. 2007). To solve the problem of cell growth defect in the *odhA* deletion strain, the mutant strains carrying the missense mutation in the *odhA* gene were constructed using *in vitro* mutagenesis (Nakamura et al. 2006). Although these mutants could produce glutamate with less than half of ODHC specific activity of the wild-type strain in the presence of excess biotin, they also showed a moderate cell growth defect. Therefore, the *odhA* deletion strain and the *odhA* mutant strains might not be suitable for industrial glutamate production. As an alternative method for down-regulation of the expression of the *odhA* gene and its product, antisense RNA technology was considered as one of metabolic engineering approaches for the control of metabolic pathway in this chapter.

The antisense RNA technology is recognized as one of the effective methods for regulating gene expression at the posttranscriptional level. Almost all of the naturally occurring cases of antisense RNA have been found in bacteria (see Wagner

and Simons 1994). It forms a complementary duplex between the antisense RNA and target messenger RNA (mRNA); thus, translation from the target mRNA is blocked. This technology can be also applied to metabolic engineering to control the flow of specific metabolic pathways. Therefore, this technology shows promise for application in industrial production processes. Among several antisense RNA methods such as antisense oligonucleotides, antisense RNA expression, and small interfering RNA (Lee and Roth 2003), the antisense RNA expression system was used because of its advantages: it is possible to control antisense RNA expression in *trans* at a certain time according to the requirements**.** Moreover, down-regulation of protein expression by antisense RNA offers ease of implementation and flexibility, which are not seen in gene deletion or knockout technologies. For example, some studies have demonstrated the effectiveness of antisense RNA in the metabolic engineering of *Clostridium acetobutylicum* (Desai and Papoutsakis 1999; Tummala et al. 2003a; Tummala et al. 2003b).

In this chapter, we examined the effect of modulation of ODHC specific activity by *odhA* overexpression and *odhA* antisense RNA expression as metabolic engineering approaches for metabolic pathway control of glutamate production by *C. glutamicum*. Moreover, the relationship between changes in ODHC specific activity levels and glutamate production due to *odhA* overexpression or *odhA* antisense RNA expression was also investigated.

3.2 Materials and methods

3.2.1 Bacterial strains, plasmids, and media

All bacterial strains and plasmids used in this study are listed in Table 3.1. For the recombinant DNA techniques, *Escherichia coli* and *C. glutamicum* were cultivated in Lennox medium (1% polypeptone, 0.5% Bacto yeast extract, 0.5% NaCl, and 0.1% glucose; pH 7.2). To escape the restriction system of *C. glutamicum*, the plasmids obtained from the *dam dcm* mutant strains of *E. coli* SCS110 were used for the transformation (Vertès et al. 1993).

For glutamate production assay of *C. glutamicum*, two culture media were used. For seed preparation, a CM2B plate (1% polypeptone, 1% Bacto yeast extract, 0.5% NaCl, 10 μg/l D-biotin, and 1.5% agar; pH 7.2; Miwa et al. 1985) was used. For the preculture, the synthetic medium (Shirai et al. 2006) used was 80 g glucose, 30 g $(NH_4)_2SO_4$, 3 g Na_2HPO_4, 6 g KH_2PO_4, 2 g NaCl, 3.9 mg $FeCl_3$, 0.9 mg $ZnSO_4 \cdot 7H_2O$, 0.3 mg $CuCl_2 \cdot 2H_2O$, 5.56 mg $MnSO_4 \cdot 5H_2O$, 0.1 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.3 mg $Na_2B_4O_7 \cdot 10H_2O$, 0.4 g $MgSO_4 \cdot 7H_2O$, 40 mg $FeSO_4 \cdot 7H_2O$, 84 mg $CaCl_2$, 500 μg thiamin hydrochloride, 0.1 g ethylenediaminetetraacetic acid, and 10 μg biotin, per liter of deionized water (pH 7.2). To avoid the decrease in pH throughout culture using Sakaguchi flask, 25 g/l of $CaCO_3$ was added to the culture broth. The composition of the medium for the main culture was the same as that for the preculture except for the concentration of biotin (20 μg/l). When necessary, kanamycin (20 μg/ml for the *E. coli* strains and 10 μg/ml for the *C. glutamicum* strains) or ampicillin (50 μg/ml for *E. coli*) was added to the medium.

Table 3.1   Bacterial strains and plasmids used in this study.

Strain/plasmid	Relevant characteristics	Reference/source
Strains		
E. coli		
JM109	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*)/F′ (*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15)	Yanisch-Perron et al. 1985
SCS110	*rpsL* (*Str*^{r}) *thr leu endA thi-1 lacY galK galT ara tonA tsx dam dcm supE44* Δ(*lac-proAB*)/F′(*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15)	Stratagene, La Jolla, CA, USA; Westmoreland et al. 1997
C. glutamicum		
ATCC 13032	Wild- type	National Institute of Technology and Evaluation, Biological Resource Center, Japan
Plasmids		
pECt	*E. coli–C. glutamicum* shuttle expression plasmid	Sato et al. 2008
pECt-odhA	*C. glutamicum odhA* gene on pECt	Kim et al. 2009
pECt-Anti-odhA	*odhA* antisense RNA (3837 bp) on pECt	Kim et al. 2009

Table 3.2   Primers for PCR of *odhA* gene fragments to construct the *odhA* overexpressing and *odhA* antisense RNA expressing plasmids.

Plasmid carrying the amplified *odhA* gene fragment	Primer sequence	Restriction enzymes for cloning
pECt-odhA	5′-CAAAT<u>ACATGT</u>TACAACTGGGGCTTAGG-3′	*Pci*I
	5′-CCATC<u>GGATCC</u>CTCGAGCTGCGTTGTGG-3′	*Bam*HI
pECt-Anti-odhA	5′-AA<u>GTCGAC</u>TACGCCCGCATGTG-3′	*Sal*I
	5′-GGG<u>ACATGT</u>TTAAGCCTCGAAAGCCTCG-3′	*Pci*I

Restriction sites artificially added for cloning are underlined.
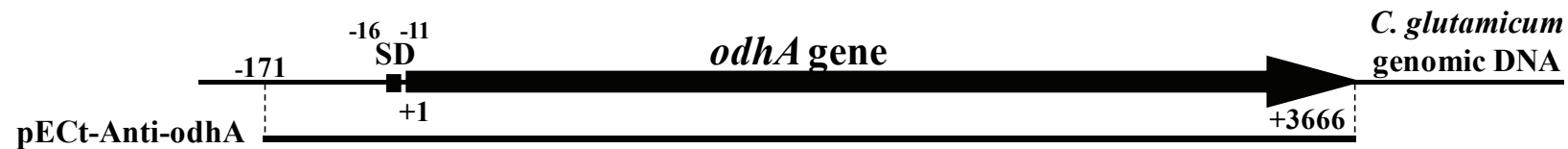
Figure 3.1   The target region of *odhA* antisense RNA on pECt-Anti-odhA. *Numbers* indicate the position of the nucleotides when the position of the first 'irst tthe initiation codon GTG for the *odhA* gene was defined as +1, as determined by Asakura et al. (2007). *SD*, Shine-Dalgarno sequence encompassing -11 to -16 regions in the upstream region of the *odhA* gene.

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3.2.2   Construction of *odhA* gene and *odhA* antisense RNA expressing strains, respectively

The target regions for *odhA* overexpression and *odhA* antisense RNA expression for metabolic pathway control were, respectively, amplified by polymerase chain reaction (PCR) from the genomic DNA of *C. glutamicum* ATCC 13032 using *Z-Taq* DNA polymerase (Takara Bio, Shiga, Japan) and the primers shown in Table 3.2. The PCR products were cloned in a pGEM-T easy vector (Promega, Madison, WI, USA), and the sequences of the cloned PCR products were confirmed using a BigDye terminator cycle sequencing kit v1.1 (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 310 genetic analyzer (Applied Biosystems).

For the construction of the *odhA* overexpression plasmid pECt-odhA, the PCR product corresponding to the *odhA* gene on the pGEM-T easy vector was digested with *Pci*I and *Bam*HI, and then cloned into the *Nco*I and *Bam*HI sites of the *E. coli–C. glutamicum* shuttle expression plasmid pECt (Sato et al. 2008).

For the construction of the *odhA* antisense RNA expression plasmid, the PCR product on the pGEM-T easy vector was digested using appropriate restriction enzymes, as shown in Table 3.2, and then inserted into pECt in an antisense orientation relative to the open reading frame of the *odhA* gene including the coding regions and ribosome binding site, as shown in Figure 3.1.

The resulting plasmids, pECt-odhA for *odhA* overexpression and pECt-Anti-odhA for *odhA* antisense RNA expression, were introduced into the *C. glutamicum* wild-type strain ATCC 13032, respectively. In the *odhA* overexpressing and *odhA* antisense RNA expressing strain, the expression of the *odhA* gene and *odhA* antisense RNA cloned under the *trc* promoter on pECt were induced by addition of

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isopropyl-β-ᴅ-thiogalactoside (IPTG).

3.2.3   Culture conditions for glutamate production assay

In this study, Sakaguchi flask culture was used for preliminary evaluation of glutamate production because of its easy operation. However, since *C. glutamicum* requires a lot of oxygen supply for its growth and glutamate production, Sakaguchi flask culture is not suitable for detailed evaluation of glutamate production by *C. glutamicum* because of the disadvantage in supplying oxygen into the culture broth. Moreover, a large amount of *C. glutamicum* cells was necessary for measurement of ODHC specific activity. Therefore, for further evaluation of effects of *odhA* overexpression and *odhA* antisense RNA expression on glutamate production and ODHC specific activity, we used a jar bioreactor, which enables to supply enough amount of oxygen to the culture by changing the agitation speed and to obtain a large amount of *C. glutamicum* cells.

The *C. glutamicum* strains carrying pECt, pECt-odhA and pECt-Anti-odhA, respectively, were grown at 30°C for 24 h on a CM2B plate for seed preparation. For the preculture, cells on the CM2B plate were collected and inoculated into 40 ml of the synthetic medium in a Sakaguchi flask and then incubated aerobically at 31.5°C. Seventeen hours after incubation, 1 ml of this preculture was inoculated into 40 ml of the synthetic medium in a Sakaguchi flask for glutamate production assay, and the cells were cultivated for 38 h at 31.5°C.

In jar bioreactor culture, 80 ml of the preculture was inoculated into 2 l of the synthetic medium in a 5-l jar bioreactor (KMJ-5B; Mitsuwa Rikagaku, Osaka, Japan), and the cells were aerobically cultivated at 31.5°C. The agitation speed was

changed to allow enough oxygenation, and the airflow rate was maintained at 2 l/min. The pH was maintained at 7.2 by automatic addition of 14% (v/v) ammonia solution, which was also supplied as a nitrogen source. Foaming was controlled by the addition of an antifoaming agent (Disfoam GD-113K; NOF, Japan). After cell growth reached the early exponential phase (OD_{660} = 12), Tween 40 was added to the medium in order to achieve the final concentrations of 4 mg/ml for triggering glutamate production in both flask and jar bioreactor. Expression of the *odhA* gene and *odhA* antisense RNA was induced by IPTG addition into the culture media, at the appropriate time, at a final concentration of 0.1 mM for *odhA* overexpression and 1 mM for *odhA* antisense RNA expression for the purposeful alteration of metabolic pathway, respectively.

3.2.4   Measurements of cell growth and glucose and glutamate concentrations

Cell growth was monitored by measuring the optical density of the culture at 660 nm (OD_{660}) using a spectrophotometer (U-2000; Hitachi High-Technologies, Tokyo, Japan) after dilution of the culture with 0.2 M HCl to dissolve $CaCO_3$. Glucose and glutamate in the supernatant were measured using a biochemical analyzer (2700; YSI, Yellow Springs, OH, USA) and F-kit glutamate (R-Biopharm AG, Darmstadt, Germany), respectively.

3.2.5   Measurement of ODHC specific activity

ODHC specific activity was measured according to the method described by Shiio and Ujigawa-Takeda (1980). *C. glutamicum* cells were harvested, washed twice with 0.2% KCl, and suspended in 3 ml of 0.1 M *N*-tris(hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES)·NaOH buffer (pH 7.7) containing 30% (v/v)

glycerol. Cells were disrupted by sonication and then centrifuged to remove the cell debris. One milliliter of the supernatant, applied to the gel filtration column (PD-10 Desalting Column; GE Healthcare UK, Buckinghamshire, UK) to remove low-molecular-weight compounds, was used for the enzyme assay.

The reaction mixture was prepared as follows: 100 mM TES·NaOH (pH 7.7), 5 mM $MgCl_2$, 3 mM cysteine, 0.3 mM thiamine pyrophosphate, 0.2 mM coenzyme A, 1 mM 3-acetylpyridine adenine dinucleotide ($APAD^+$) instead of oxidized form of nicotinamide adenine dinucleotide (NAD^+), and 150 µl of the cell extract. After addition of 1 mM 2-oxoglutarate to the reaction mixture, the initial increase in the absorbance of APADH at 365 nm was measured at 31.5°C for 3 min with 15-s intervals. ODHC specific activity was defined as the amount of enzyme required for the conversion of an equivalent of 1 µmol of NAD^+ into reduced form of nicotinamide adenine dinucleotide (NADH) in 1 min. The concentration of total protein in the crude extract was determined by Bradford's method (Bradford 1976) using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

3.3   Results

3.3.1   Confirmation of OdhA protein overexpression in the *odhA* overexpressing strain of *C. glutamicum*

Overexpression of the OdhA protein in *C. glutamicum* ATCC 13032 carrying pECt-odhA was verified by 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Cells of the ATCC 13032 strains carrying pECt-odhA and pECt, respectively, were cultured at 30°C and harvested 2, 4, and 6 h after IPTG addition. Proteins were obtained from the harvested cells by sonication and then separated by SDS-polyacrylamide gel.
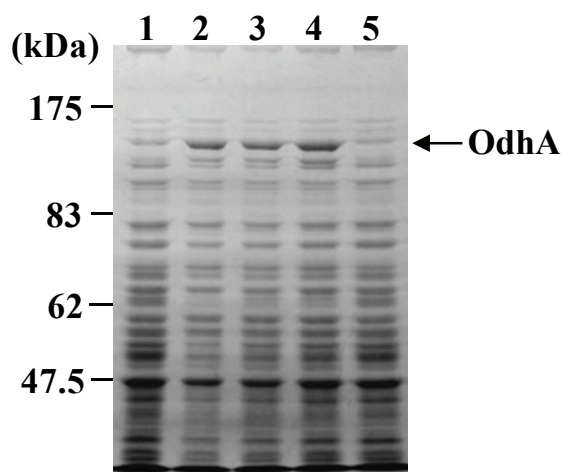
Figure 3.2   Confirmation of OdhA overexpression in the *odhA* overexpressing strain. All the cellular proteins from the ATCC 13032/pECt-odhA and ATCC 13032/pECt strains separated by 7.5% SDS-polyacrylamide gel electrophoresis is shown. *Lane 1* proteins from the cells of ATCC 13032/pECt-odhA before IPTG addition; *lane 2-4* proteins obtained from the cells of ATCC 13032/pECt-odhA 2 h, 4 h, and 6 h after 0.1 mM IPTG addition, respectively; *lane 5* proteins obtained from the cells of ATCC 13032/pECt 6 h after IPTG addition; *arrow* the position corresponding to the OdhA protein (138.8 kDa)

As shown in Figure 3.2, the protein band with a molecular mass of about 140 kDa, corresponding to the OdhA protein, was detected in ATCC 13032/pECt-odhA but not in ATCC 13032/pECt after visualization with Coomassie Brilliant Blue staining; therefore, OdhA overexpression was successfully exhibited in the *odhA* overexpressing strain.

3.3.2   Effect of *odhA* overexpression on glutamate production triggered by Tween 40 addition

To investigate the effect of *odhA* overexpression on glutamate production, jar bioreactor cultures of ATCC 13032/pECt-odhA and ATCC 13032/pECt were carried out under Tween 40-triggered glutamate production. When cell growth reached the early exponential phase ($OD_{660} = 12$), Tween 40 for triggering glutamate production and IPTG for *odhA* overexpression were simultaneously added to the media.

As shown in Figure 3.3, cell growth was reduced and glutamate was produced in both ATCC 13032/pECt-odhA and ATCC 13032/pECt by Tween 40 addition. However, in the presence of IPTG, the ATCC 13032/pECt-odhA strain showed significantly low glutamate production (less than 4 g/l) and the increase in ODHC specific activity in spite of Tween 40 addition; on the other hand, the control strain ATCC 13032/ pECt showed the decreased ODHC specific activity and glutamate production (about 18 g/l). These results indicate that the increase in ODHC specific activity by *odhA* overexpression resulted in the decreased glutamate production, and supports the view (see Shingu and Terui 1971; Kawahara et al. 1997; Shimizu et al. 2003; Shirai et al. 2005) that decrease in ODHC specific activity is an important factor for glutamate overproduction by *C. glutamicum*.
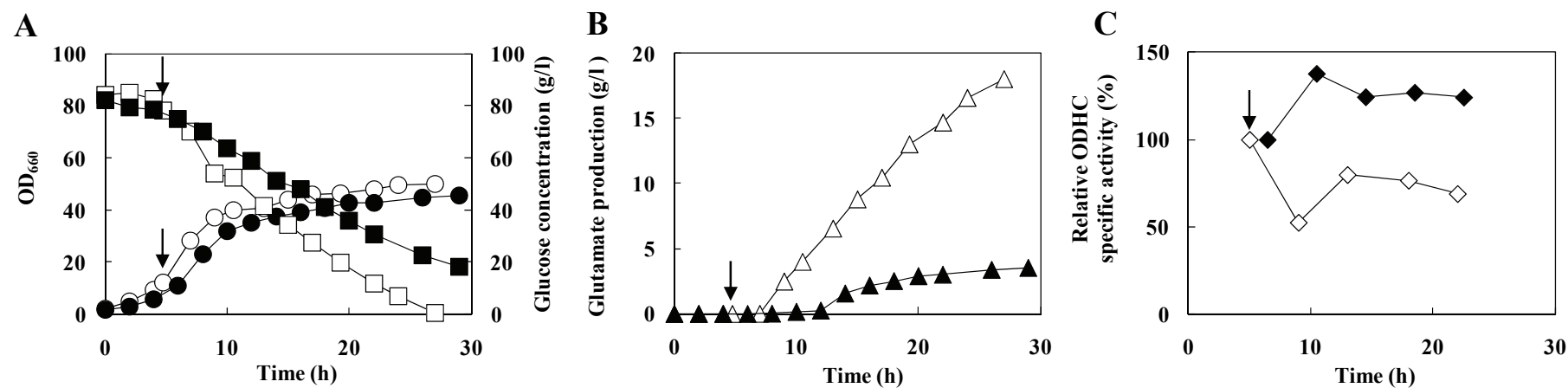
Figure 3.3 Cell growth (A, *circles*), glucose consumption (A, *squares*), glutamate production (B, *triangles*), and relative ODHC specific

activity (C, *diamonds*) of the *odhA* overexpressing strain ATCC 13032/pECt-odhA (*closed symbols*) and the control strain ATCC 13032/pECt (*open*

symbols) under Tween 40-triggered glutamate production in jar bioreactor culture. *Arrows* indicate both IPTG and Tween 40 addition for *odhA*

overexpression and triggering glutamate overproduction, respectively

3.3.3 Effect of *odhA* antisense RNA expression on glutamate production in flask culture

It was assumed that controlling the expression level of the OdhA protein by *odhA* antisense RNA expression as a metabolic engineering approach leads to the change of glutamate production in *C. glutamicum*. Therefore, we developed *C. glutamicum* strain carrying the *odhA* antisense RNA expression plasmid pECt-Anti-odhA and examined glutamate production by this strain. The target region for the antisense RNA on the pECt-Anti-odhA includes the ribosome binding site and complete coding region of *odhA* gene. The *odhA* antisense RNA expression was induced by 1 mM IPTG addition at the early exponential growth phase.

In the flask culture, neither was cell growth and glucose consumption of *C. glutamicum* affected, nor was there any significant increase in glutamate production by *odhA* antisense RNA expression only in the ATCC 13032/pECt-Anti-odhA strain (Figure 3.4). Under Tween 40 addition condition, cell growth of the *odhA* antisense RNA expressing strain with IPTG addition was similar to that without IPTG addition; exponential growth was initially observed, and then the cell growth was reduced after Tween 40 addition (Figure 3.4A). As expected, Tween 40 addition induced glutamate production in the *odhA* antisense RNA expressing strain (Figure 3.4C). Moreover, the level of Tween 40-triggered glutamate production with IPTG addition was higher than that with no IPTG added (Figure 3.4C). These results indicate that the expression of *odhA* antisense RNA as a metabolic engineering approach can further enhance Tween 40-triggered glutamate production by *C. glutamicum*.
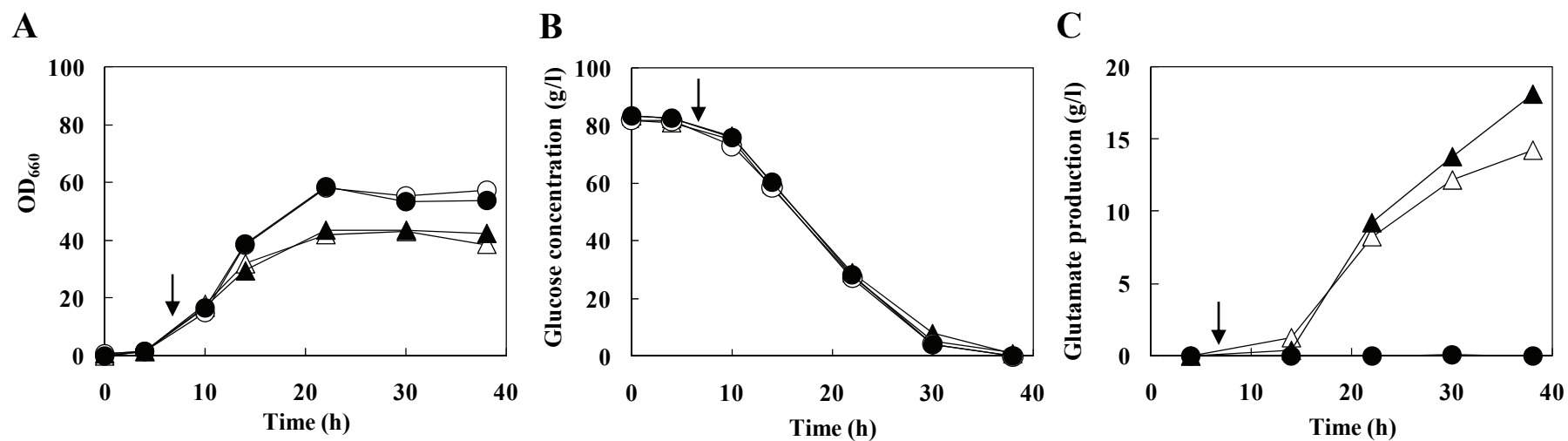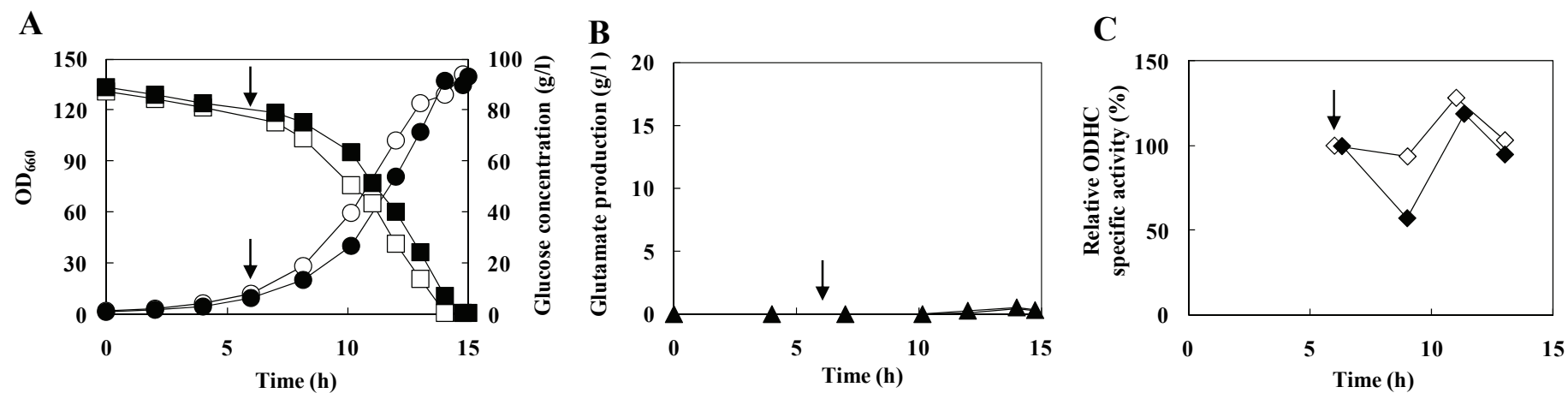
Figure 3.4 Cell growth (A), glucose consumption (B), and glutamate production (C) of the *odhA* antisense RNA expressing strain ATCC 13032/pECt-Anti-odhA in Sakaguchi flask culture. The representative results of three independent experiments under each culture condition are shown. *Arrows* Tween 40 and IPTG addition; *open circles* neither IPTG nor Tween 40 addition; *closed circles* IPTG addition; *open triangles* Tween 40 addition; *closed triangles* both Tween 40 and IPTG addition

Figure 3.5 Cell growth (A, *circles*), glucose consumption (A, *squares*), glutamate production (B, *triangles*), and relative ODHC specific

activity (C, *diamonds*) of the *odhA* antisense RNA expressing strain ATCC 13032/pECt-Anti-odhA by *odhA* antisense RNA expression without any

triggers for glutamate overproduction in jar bioreactor culture. *Arrows* IPTG addition for *odhA* antisense RNA expression; *open symbols* without

IPTG addition; *closed symbols* with IPTG addition

3.3.4   Effect of *odhA* antisense RNA expression on glutamate production without any triggers in jar bioreactor cultures

Decrease in ODHC specific activity in the TCA cycle is one of the important factors for producing glutamate (Shingu and Terui 1971; Kawahara et al. 1997). To further investigate the effect of *odhA* antisense RNA expression on glutamate production and ODHC specific activity in *C. glutamicum* without any triggers, the *odhA* antisense RNA expressing strain was cultivated in a jar bioreactor.

As shown in Figure 3.5, ODHC specific activity in this strain in the presence of IPTG (i.e., *odhA* antisense RNA expressing condition) was lower than that in the absence of IPTG, suggesting that *odhA* antisense RNA expression can indeed lead to decrease in ODHC specific activity. However, no significant glutamate production was achieved by expression of *odhA* antisense RNA only (less than 1 g/l) as shown in Figure 3.5B. In addition, in the *odhA* antisense RNA expressing strain, cell density became significantly high (OD_{660} = over 140) in a short time and glucose consumption was also high in both the absence and presence of IPTG (Figure 3.5A). From the mid-exponential to the early stationary phases, cell density and glucose consumption in the presence of IPTG were slightly lower than those in the absence of IPTG. It was assumed that the decreased cell density and glucose consumption by IPTG addition results from the malfunction of TCA cycle due to the decrease in ODHC specific activity, leading to the decreased cell growth by expression of *odhA* antisense RNA. These results suggest that the expression of *odhA* antisense RNA constructed in this study alone is able to decrease ODHC specific activity, leading to slight decrease in cell growth and substrate consumption; however, it does not directly lead to glutamate production by *C. glutamicum*. This means that decrease in ODHC specific activity might not be the only

factor for glutamate overproduction by *C. glutamicum*.

3.3.5 Effect of *odhA* antisense RNA expression on glutamate production triggered by Tween 40 addition

For further investigation on the effect of *odhA* antisense RNA expression under Tween 40-triggered glutamate production, jar bioreactor cultures of the *odhA* antisense RNA expressing strain was also carried out.

Figure 3.6 shows the time course for cell growth, glucose consumption, glutamate production, and ODHC specific activity of the *odhA* antisense RNA expressing strain under Tween 40-triggered glutamate production. Similar to results obtained by the Sakaguchi flask culture experiments (Figure 3.4), Tween 40 addition resulted in reduced cell growth and glutamate overproduction; Tween 40-triggered glutamate production was enhanced about 10% at 34 h in the ATCC 13032/pECt-Anti-odhA strain by IPTG-induced *odhA* antisense RNA expression as shown in Figure 3.6B. Moreover, ODHC specific activity in the ATCC 13032/pECt-Anti-odhA was further lowered by *odhA* antisense RNA expression under Tween 40-triggered glutamate production, leading to enhanced glutamate production in jar bioreactor culture (Figure 3.6B and C). Our results shown in this chapter suggest that the metabolic engineering approach by the *odhA* antisense RNA expression is effective for the enhancement of glutamate production triggered by Tween 40 addition.
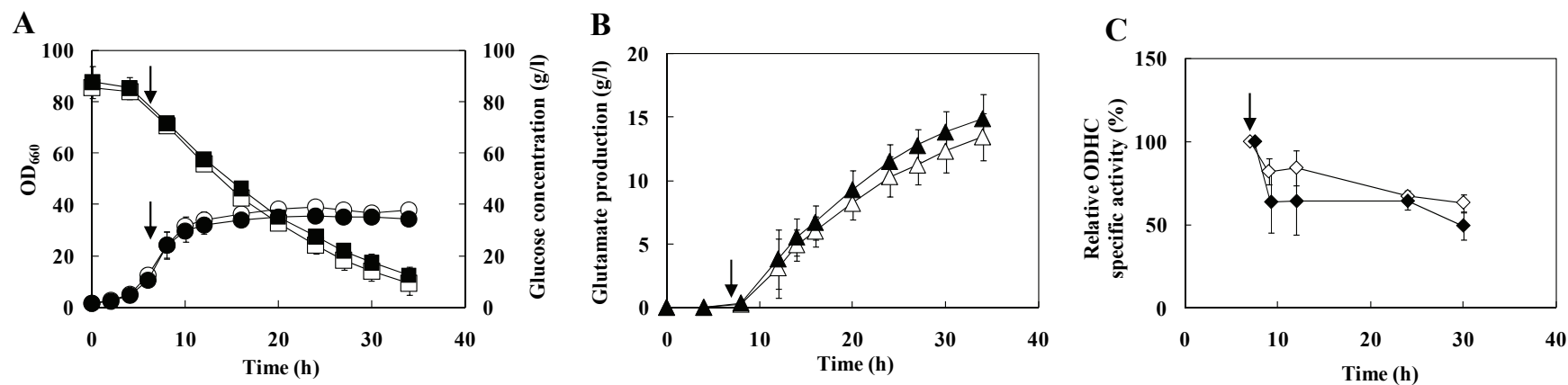
Figure 3.6 Cell growth (A, *circles*), glucose consumption (A, *squares*), glutamate production (B, *triangles*), and relative ODHC specific activity (C, *diamonds*) of the *odhA* antisense RNA expressing strain ATCC 13032/pECt-Anti-odhA under Tween 40-triggered glutamate production in jar bioreactor culture. Average with standard deviation in three independent experiments is shown. *Arrows* Tween 40 and/or IPTG addition; *open symbols* with Tween 40 addition only; *closed symbols* with both Tween 40 and IPTG addition

3.4 Discussion

It has been already demonstrated that decrease in ODHC specific activity is one of the key factors in glutamate overproduction by *C. glutamicum*, which is triggered by biotin depletion, penicillin addition, or Tween 40 addition (Shingu and Terui 1971; Kawahara et al. 1997). Therefore, we focused on the relationship between change in ODHC specific activity and glutamate production triggered by Tween 40 addition. In this study, the effects of *odhA* overexpression and the *odhA* antisense RNA expression were analyzed to further investigate the role of the *odhA* gene and its product in glutamate production by *C. glutamicum*.

First, the *odhA* overexpressing strain was constructed by introducing a plasmid pECt-odhA carrying the *C. glutamicum odhA* gene cloned under *trc* promoter on *E. coli*–*C. glutamicum* shuttle expression plasmid pECt (Sato et al. 2008). As expected, ODHC specific activity was increased, and glutamate production was concomitantly decreased by the *odhA* overexpression under Tween 40-triggered glutamate production condition (Figure 3.3). This result supports the view that the decrease in ODHC specific activity is important for glutamate production by *C. glutamicum* as described in "Introduction" section. In spite of the increase in ODHC specific activity and low glutamate production, the cell growth and glucose consumption in the *odhA*-overexpressing strain was lower than those in Tween 40-treated ATCC 13032/pECt strain. Since ODHC catalyzes the formation of NADH as well as succinyl-CoA, ODHC activity by the overexpression of *odhA* may increase the level of these metabolites. The relationship among changes in the balance of metabolites level, redox and cell growth should be carefully examined in the future.

Asakura et al. (2007) reported that the *odhA* deletion strain could produce

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glutamate without any triggers; however, this strain showed a severe growth defect under the aerobic growth conditions, implying the necessity of the *odhA* gene in *C. glutamicum*. To improve cell growth, the mutant strains carrying the missense mutation in the *odhA* gene were constructed using PCR-based *in vitro* mutagenesis (Nakamura et al. 2006). Although these mutants could produce glutamate with less than half of ODHC specific activity of the wild-type strain in the presence of excess biotin, they also showed a moderate growth defect. In these respect, the *odhA* deletion strain and the *odhA* mutant strains might not be suitable for industrial glutamate production. As an alternative method for down-regulation of the expression of the *odhA* gene and its product, antisense RNA technology as a metabolic engineering approach was considered for this study. This technology shows promise for application in industrial production processes. Among several antisense RNA methods such as antisense oligonucleotides, antisense RNA expression, and small interfering RNA (Lee and Roth 2003), the antisense RNA expression system was used because of its advantages: it is possible to control antisense RNA expression in *trans* at a certain time according to the requirements**.**

In this chapter, we attempted to decrease the ODHC specific activity through *odhA* antisense RNA expression as a metabolic engineering approach. It was expected that the expressed *odhA* antisense RNA hybridizes to the complementary sequence of the target mRNA and leads to translation stoppage from the *odhA* mRNA, resulting in decrease in ODHC specific activity. To evaluate the effect of *odhA* antisense RNA expression on glutamate production, the *C. glutamicum* strain carrying the *odhA* antisense RNA expression plasmid was constructed and cultivated by both Sakaguchi flask and jar bioreactor methods. It has been assumed that decrease in ODHC specific

activity leads to malfunction of TCA cycle, and as a result, cell growth and substrate uptake decrease and probably glutamate production is induced. As shown in Figures 3.5C and 3.6C, we could confirm that *odhA* antisense RNA expression could decrease ODHC specific activity. Although the level of decrease in ODHC specific activity in the *odhA* antisense RNA-expressing strain was similar to those in the ATCC 13032/pECt treated with Tween 40, cell growth and glucose consumption in the *odhA* antisense RNA-expressing strain became higher than those in the Tween 40-treated ATCC 13032/pECt strain (Figures 3.3 and 3.5). As is well known, Tween 40 affects the biosynthesis of fatty acids, particularly mycolic acid that is one of the components in cell wall of *C. glutamicum*. Therefore, the cell growth and glucose consumption might be decreased in the Tween 40-treated *C. glutamicum* strain due to the malfunction of TCA cycle and fatty acid biosynthesis.

Moreover, *odhA* antisense RNA expression alone could not achieve glutamate production (Figure 3.5B), but it could enhance glutamate production triggered by Tween 40 addition (Figure 3.6B). No glutamate production in the *odhA* antisense RNA-expressing strain without triggers suggests that not only decrease in ODHC specific activity but other factors as well might contribute to glutamate production by *C. glutamicum*. It can be thought that other factor(s) triggered by Tween 40 addition is important for glutamate overproduction in the *odhA* antisense RNA-expressing strain. Therefore, other factors have to be considered to explain the precise mechanism of glutamate overproduction by *C. glutamicum*. Niebisch et al. (2006) identified the OdhI protein that directly binds to the OdhA protein (one of the subunits of ODHC) and inhibits ODHC activity. The phosphorylation status of OdhI is controlled by serine/threonine protein kinase G (PknG) and the phosphatase (Ppp; Schultz et al. 2007).

The unphosphorylated OdhI can bind to the OdhA protein and thus inhibit ODHC activity. Moreover, it was shown that the deletion strain of the *odhI* gene impaired glutamate production under biotin depletion, penicillin addition, and Tween 40 addition, indicating that inhibition of ODHC activity by the OdhI protein is essential for glutamate production (Schultz et al. 2007).

Chapter 4

Expression changes of OdhA and OdhI proteins during glutamate overproduction in *Corynebacterium glutamicum*

4. 1   Introduction

The mechanism of glutamate overproduction in *C. glutamicum* is not completely understood at present. However, it has been demonstrated that glutamate production is accompanied by a decrease in 2-oxoglutarate dehydrogenase complex (ODHC) activity, triggered by biotin depletion, penicillin addition, or Tween 40 addition (Shingu and Terui 1971; Kawahara et al. 1997). Inhibition of ODHC activity is caused by the complex between the E1o subunit OdhA of ODHC and unphosphorylated OdhI (Niebisch et al. 2006). Using copurification and surface plasmon resonance experiments with the different OdhI and OdhA length variants, Krawczyk et al. (2010) revealed that the entire forkhead-associated (FHA) domain of OdhI and the C-terminal dehydrogenase domain of OdhA are required for interaction. Moreover, the FHA domain was also sufficient for inhibition of ODHC activity. However, phosphorylated OdhI was binding-incompetent to the C-terminal dehydrogenase domain of OdhA, relieving the inhibition of ODHC activity (Krawczyk et al. 2010). Moreover, OdhI can also be phosphorylated, not only by PknG, but also by other serine/threonine protein kinases (STPKs) present in *C. glutamicum*, such as PknA, PknB, and PknL (Schultz et al. 2009). This phosphorylation of OdhI by STPKs relieves the inhibition of ODHC activity. Dephosphorylation of OdhI is catalyzed by the phosphatase domain of the phospho-serine/threonine protein phosphatase Ppp *in vitro*, confirming that OdhI is a

substrate of Ppp (Krawczyk et al. 2010). In these respects, the phosphorylation status of the OdhI protein is also of key importance for glutamate production in *C. glutamicum* (Schultz et al. 2007).

Protein expression and its phosphorylation status changes play important roles in prokaryotic signaling and regulation. However, the dynamics of protein expression and its phosphorylation status during glutamate overproduction in *C. glutamicum* is still not reported. In these respects, the expression change of some proteins, such as OdhA and OdhI, would also be one of the important factors for understanding the cellular state of glutamate production in *C. glutamicum*. In this chapter, it was assumed that glutamate production accompanied by inhibition of ODHC activity might be affected by the expression change of some proteins, such as OdhA and OdhI. Therefore, the correlation between glutamate production and the expression change of OdhA and OdhI proteins were investigated by Western blotting under glutamate production conditions in *C. glutamicum*.

4.2 Materials and methods

4.2.1 Bacterial strains, media, and culture conditions

All strains and plasmids used in this study are listed in Table 4.1. The strain used for cloning and overexpression of OdhA and OdhI proteins was *Escherichia coli* BL21 (DE3) containing pLysS (Studier and Moffatt, 1986). All *E. coli* strains in a test tube were grown and maintained aerobically in Lennox (L) medium (1% polypeptone, 0.5% Bacto yeast extract, 0.5% NaCl, and 0.1% glucose; pH 7.2).

In this study, Sakaguchi flask culture was used for investigation of protein expression and glutamate production assays. Two culture media were used for *C. glutamicum*. A CM2B plate (1% polypeptone, 1% Bacto yeast extract, 0.5% NaCl, 10 µg/l D-biotin, and 1.5% agar; pH 7.2; Miwa et al. 1985) was used for the main culture. For the preculture, the synthetic medium (Shirai et al. 2006) used was 80 g glucose, 30 g $(NH_4)_2SO_4$, 3 g Na_2HPO_4, 6 g KH_2PO_4, 2 g NaCl, 3.9 mg $FeCl_3$, 0.9 mg $ZnSO_4 \cdot 7H_2O$, 0.3 mg $CuCl_2 \cdot 2H_2O$, 5.56 mg $MnSO_4 \cdot 5H_2O$, 0.1 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.3 mg $Na_2B_4O_7 \cdot 10H_2O$, 0.4 g $MgSO_4 \cdot 7H_2O$, 40 mg $FeSO_4 \cdot 7H_2O$, 84 mg $CaCl_2$, 500 µg thiamin hydrochloride, 0.1 g ethylenediaminetetraacetic acid, and 20 µg biotin, per liter of deionized water (pH 7.2). Using a Sakaguchi flask, 25 g/l of $CaCO_3$ was added to the culture medium in order to avoid a decrease in pH throughout the culture. When necessary, media were supplemented with 34 µg/ml chloramphenicol, 20 µg/ml kanamycin (*E. coli*), or 10 µg/ml kanamycin (*C. glutamicum*).

Table 4.1　　　　Bacterial strains, plasmids, and primers used in this study.

Strain/plasmid	Relevant characteristics	Reference/source
Strains		
E. coli		
BL21(DE3)	F- *ompT hsdS*$_B$ (r$_B$-m$_B$-) *gal dcm* (DE3)	Studier and Moffatt 1986
BL21(DE3)/ pLysS	F- *ompT hsdS*$_B$ (r$_B$-m$_B$-) *gal dcm* (DE3) pLysS (Cam^R)	Studier and Moffatt 1986
BL21(DE3)/ pLysS/pET-28b(+)-odhA	*C. glutamicum odhA* gene on pET-28b(+) in BL21(DE3)/pLysS	
BL21(DE3)/ pLysS/pET-28b(+)-odhI	*C. glutamicum odhI* gene on pET-28b(+) in BL21(DE3)/pLysS	
C. glutamicum		
ATCC 31831	Wild-type	National Institute of Technology and Evaluation, Biological Resource Center, Japan
ATCC 13032	Wild-type	National Institute of Technology and Evaluation, Biological Resource Center, Japan
Plasmids		
pHT1	*E. coli–C. glutamicum* shuttle vector	Hirasawa et al. 2003
pHT1-odhI	*C. glutamicum odhI* gene on pHT1	Kim et al. 2010

Table 4.2   Primers for PCR of the *odhA* and *odhI* genes for construction of OdhA and OdhI overexpression plasmids in *E. coli*, respectively.

	Primer sequence	Restriction enzymes for cloning
OdhA overexpression	5′-CCC<u>GCTAGC</u>GTGAGCAGCGCTAGTAC-3′	*Nhe*I
	5′-CC<u>GGATCC</u>TTAAGCCTCGAAAGCC-3′	*Bam*HI
OdhI overexpression	5′-GGG<u>GCTAGC</u>ATGAGCGACAACAACGG-3′	*Nhe*I
	5′-GG<u>GGATCC</u>TTACTCAGCAGGGCCTG-3′	*Bam*HI

Restriction sites artificially added for cloning are underlined.

After dilution of the culture with 0.2 N HCl to dissolve $CaCO_3$ in case of the *C. glutamicum* strains, cell growth was monitored by measurement of optical density at 600 nm (OD_{600}) for *E. coli* strains and at 660 nm for *C. glutamicum* strains using a spectrophotometer (U-2000; Hitachi High-Technologies, Tokyo, Japan). Glutamate concentrations in the supernatant were measured by an F-kit glutamate (R-Biopharm AG, Darmstadt, Germany).

4.2.2   Overexpression of OdhA and OdhI proteins in *E. coli*

DNA fragments encoding OdhA and OdhI were respectively amplified by PCR with *Z-Taq* DNA polymerase using *C. glutamicum* ATCC 13032 genomic DNA as a template and the oligonucleotide pairs (Table 4.2). The resulting DNA fragments, confirmed by DNA sequence using the dideoxy method, as previously described, were digested with both *Bam*HI and *Nhe*I, and then cloned into the *Bam*HI/*Nhe*I sites of the expression plasmids pET-28b(+), resulting in pET-28b(+)-odhA and pET-28b(+)-odhI, respectively. These resultant plasmids were transferred into *E. coli* BL21(DE3)/pLysS (Studier and Moffatt 1986), resulting in *E. coli* BL21(DE3)/pLysS/pET-28b(+)-odhA and *E. coli* BL21(DE3)/pLysS/pET-28b(+)-odhI, respectively.

The strains, *E. coli* BL21(DE3)/pLysS/pET-28b(+)-odhA and *E. coli* BL21(DE3)/pLysS/pET-28b(+)-odhI, were aerobically cultivated at 30°C in 100 ml of L medium (1% polypeptone, 0.5% Bacto yeast extract, 0.5% NaCl, and 0.1% glucose; pH 7.2) containing 20 μg/ml kanamycin and 34μg/ml chloramphenicol. When cell growth reached an early exponential phase (OD_{600}=around 0.5) after inoculation of the preculture to be the initial 0.1 OD_{600} into new fresh L medium, 100 μM final concentration of isopropyl-β-ᴅ-thiogalactopyranoside (IPTG) was added to the culture

medium for overexpression of the target OdhA and OdhI proteins, respectively. Followed by another 12 h incubation period at 20°C, each cell pellet was harvested by centrifugation at 9,500 × g for 5 min at 4°C.

4.2.3 Purification of OdhA and OdhI proteins from *E. coli*

Cell pellets were washed twice, re-suspended in 100 ml of a binding buffer containing 20 mM imidazole, treated by enzymatic lysis buffer (0.2 mg/ml lysozyme, 1 mM $MgCl_2$, 1 mM PMSF, and 20 μg/ml DNase) supplemented with protease inhibitor cocktail (Sigma, Saint Louis, MO, USA), and then disrupted by ultrasonic homogenizer (UH-50, SMT Co. Ltd., Tokyo, Japan) at 50 W for 30 s with 30 s intervals for twenty times on ice. Lysates were centrifuged at 9,500 × g for 10 min at 4°C for removal of cellular debris; the supernatant was then loaded onto a 5 ml HisTrap FF crude kit (GE Healthcare) equilibrated in the lysis buffer for OdhA and OdhI protein purification, respectively. After washing the column with the binding buffer, the His_6-tagged bound OdhA and OdhI proteins were eluted by application of 1x phosphate buffer containing 100 mM imidazole, respectively. The elution fractions of the purified His_6-tagged OdhA and OdhI proteins, confirmed by 12.5% SDS-PAGE, were concentrated and exchanged against 10 mM Tris buffer by application of Amicon Ultra-15 Centrifugal Filter Devices (Millipore Corporation USA) with a molecular weight cut-off of 100 kDa for the OdhA protein and Amicon Ultra-4 Centrifugal Filter Devices (Millipore Corporation USA) with a molecular weight cut-off of 10 kDa for the OdhI protein, respectively. Purified His_6-tagged OdhA and OdhI proteins were used for production of rabbit anti-OdhI polyclonal antibodies (Gene Design, Japan) with six times of boosting for two months, respectively.

4.2.4   Protein preparation and protein determination

C. glutamicum cells in Sakaguchi flasks were harvested by centrifugation at 9,500 × *g* for 5 min at 4°C, washed twice, and suspended in 500 μl of 50 mM sodium phosphate buffer (pH 7.0) containing 12.5% protease inhibitor cocktail for use with bacterial cell extracts (Sigma, Saint Louis, MO, USA). The cells were then disrupted by the BIORUPTOR^R (UCD-250HSA, Cosmo Bio Co., LTD, Japan) at 250 W for 10 s with 20 s intervals for fifteen times and centrifuged at 9,500 × *g* for 5 min at 4°C for removal of cellular debris. Supernatant containing 20 μg of total proteins was used for SDS-PAGE. Concentrations of protein samples were determined by Bradford's method (Bradford 1976) using a Bio-Rad protein assay kit and bovine serum albumin as standard (Bio-Rad Laboratories, Hercules, CA, USA).

4.2.5   Analysis of protein expression by Western blotting

For analysis of protein expression of OdhA and OdhI during glutamate production in *C. glutamicum* by Western blotting analysis using OdhA and OdhI antibodies, SDS-PAGE was performed in 7.5% or 12.5% separating gels for OdhA and OdhI proteins, respectively. Prior to loading on a gel, 20 μg of each total protein aliquot was combined with 3× loading buffer [125 mM Tris-HCl, pH 7.0, 4% (*w/v*) SDS, 20% (*v/v*) glycerol, 10% (*v/v*) 2-mercaptoethanol, and 0.01% (*w/v*) bromophenol blue] and heated at 98°C for 5 min. Electrophoresis was performed at constant voltage (50 V) for 30 min in stacking gels and subsequently at 100 V for about 2 h in separating gels. Proteins were fixed in the gels by gentle shaking with 25 mM Tris, 192 mM glycine, and 25% methanol. Separated and fixed proteins on SDS-PAGE were then electroblotted onto nitrocellulose membranes (Hybond-P membrane, Amersham

Bioscineces) using a Mini Semi-Dry Transfer Cell (BE-310, Bio Craft, Japan) at 45 mA for 45 min in buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol (Towbin et al. 1979).

Blots were blocked at room temperature with 5% (*w/v*) membrane blocking reagent in phosphate buffered saline (PBS; 80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, pH 7.5) containing 0.1% (*v/v*) Tween20 for 1 h, incubated, respectively, with rabbit anti-OdhI antibodies in a 1:3,000 dilution or rabbit anti-OdhA antibodies in a 1:6,000 dilution at room temperature for 1 h, and subsequently with Horseradish Peroxidase-linked species-specific whole antibody (from donkey; GE Healthcare UK) in a 1:6,000 dilution. Detection was performed using Amersham ECL Plus Western Blotting Detection Reagents (GE Healthcare UK) and an ImageQuant Imager 350/350Lumi (GE Healthcare UK).

The amount of expression of each protein was measured by densitometric analysis using the software Image-J 1.43r6 (National Institutes of Health, USA). Quantification of the relative abundance of expression of each protein was calculated by normalization of the densitometry using the standard protein expression.

4.2.6 Comparison of the DNA sequences of *odhI* genes and amino acid sequences of OdhI proteins between ATCC 13032 and ATCC 31831

It was hypothesized that the genome sequence of the *C. glutamicum* ATCC 31831 strain is generally similar to that of the ATCC 13032 strain, whose genome sequence has already been determined by three independent research groups (Ikeda and Nakagawa 2003; Kalinowski et al. 2003; Yukawa et al. 2007). Therefore, the genome

sequence database and the protein database in the ATCC 13032 strain whose databases have already been determined were used.

Part of the *odhI* gene was amplified by polymerase chain reaction (PCR) from *C. glutamicum* ATCC 31831 genomic DNA using *Z-Taq* polymerase (Takara Bio Inc., Shiga, Japan) and the set of primers 5′-GAATTCAACCCACTTGCGGGTAGTGG-3′ and 5′-GAATTCTTAGGCATTCTATACACAAAACG-3′, which were designed using the sequence data of the ATCC 13032 genomic DNA. PCR fragments independently cloned on a pGEM-T Easy Vector (Promega Co., Madison, WI, USA) were determined using the BigDye Terminator Cycle Sequencing Kit ver. 3.1 (Applied Biosystems, Foster City, CA, USA) and an ABI Prism genetic analyzer 310NT (Applied Biosystems). The sequences of five independently cloned PCR fragments on a pGEM-T Easy Vector from the ATCC 31831 strain were determined, compared with each other, and, thus, the sequence of the *odhI* gene in the ATCC 31831 strain was obtained.

Conversion of DNA sequence to amino acid sequence in the *odhI* gene in the ATCC 31831 strain was performed using the web site (http://people.alfred.edu/~bde1/dna.php).

DNA sequence and amino acid sequence alignments of the *odhI* gene and OdhI proteins between ATCC 13032 and ATCC 31831 were respectively compared using CLUSTAL X (1.83) multiple sequence alignment.

4.3   Results and discussion

4.3.1   Confirmation and purification of OdhA and OdhI proteins from *E. coli*

Overexpression of OdhA and OdhI proteins in *E. coli* BL21(DE3)/pLysS/pET-28b(+)-odhA and *E. coli* BL21(DE3)/pLysS/pET-28b(+)-odhI by addition of 100 μM IPTG was confirmed by 12.5% SDS-polyacrylamide gel electrophoresis, respectively (data not shown). Moreover, the His_6-tagged purified protein bands, corresponding to the OdhA and OdhI proteins, were successfully detected, respectively (data not shown). As a result, the purified His_6-tagged OdhA and OdhI proteins were used for production of rabbit anti-OdhA and anti-OdhI polyclonal antibodies, respectively.

4.3.2   Changes in protein expression during glutamate production in the *odhI*-overexpressing strain

Western blotting was performed for analysis of changes in protein expression during glutamate production in the *odhI*-overexpressing strain. Cells of *C. glutamicum* carrying ATCC 31831/pHT1 or ATCC 31831/pHT1-odhI were cultivated in Sakaguchi flasks and harvested by centrifugation at the point of 12, 20, 28, and 36 h. Total protein samples were extracted by sonication and 20 μg of total protein were applied on 12.5% SDS-PAGE for Western blot analysis using rabbit anti-OdhI polyclonal antibodies, which were successively confirmed for use as anti-OdhI antibodies for detection of OdhI protein (data not shown). Moreover, the quantitative relative abundance of protein expression level was analyzed.
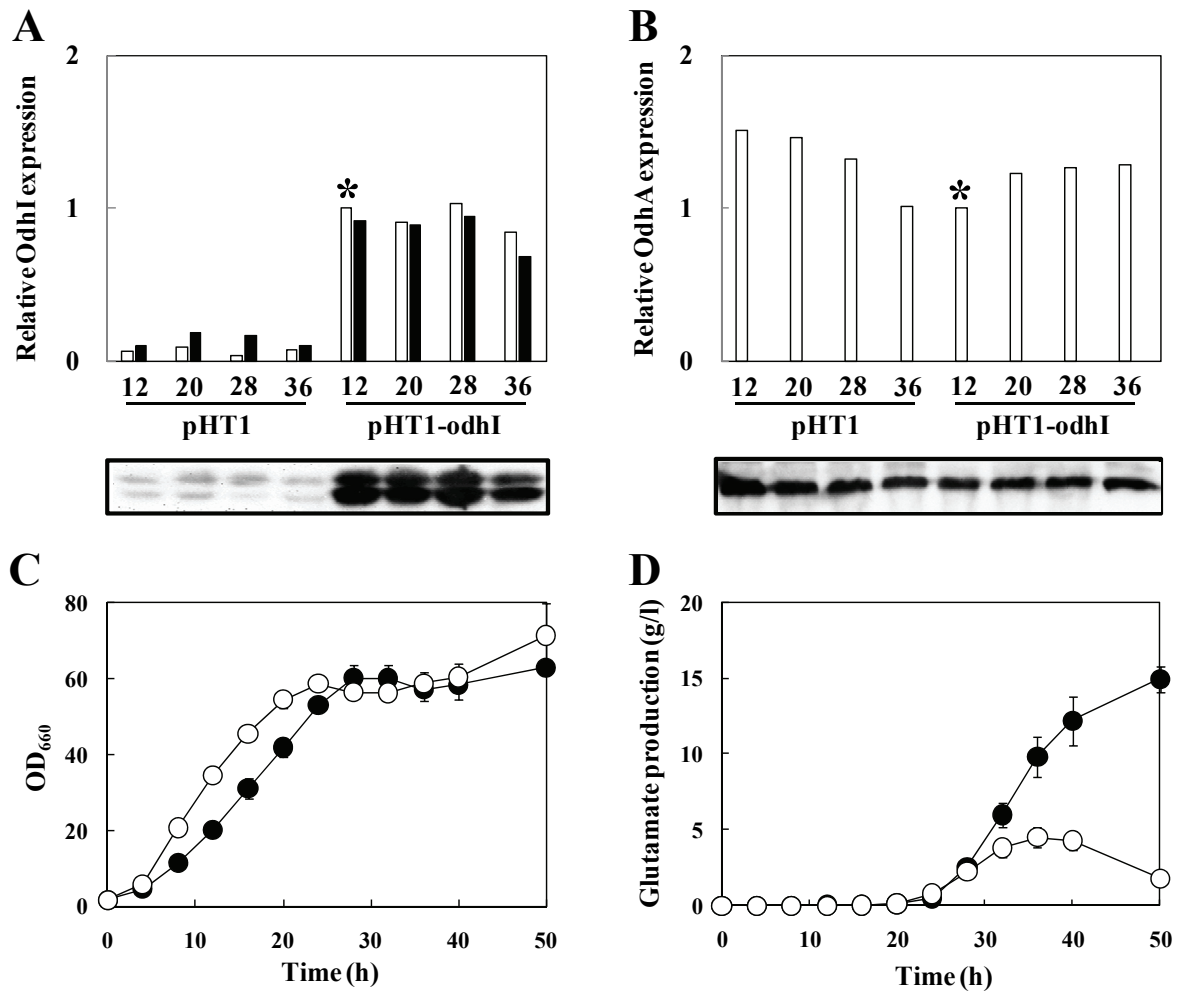
Figure 4.1 Protein expression of OdhI (A) and OdhA (B) proteins, cell growth (C), and glutamate production (D) in *C. glutamicum* ATCC 31831 carrying either the pHT1 or the pHT1-odhI strain, respectively. Cells were cultivated in Sakaguchi flasks and harvested at the point of 12, 20, 28, and 36 h. Protein samples (20 μg) were applied on SDS-PAGE for Western blot analysis. Relative protein expression was calculated by densitometry using the software Image-J 1.43r6. Normalization was performed using the protein expression labeled by the *asterisk* (*). *White bars* represent expression of unphosphorylated forms; *black bars* represent expression of phosphorylated forms; c*losed circles* represent the *odhI* overexpressing strain ATCC 31831/pHT1-odhI; *open circles* represent the control strain ATCC 31831/pHT1.

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As shown Figure 4.1A, unphosphorylated (lower) and phosphorylated (upper) OdhI bands in the ATCC 31831/pHT1-odhI (*odhI*-overexpressing) strain were strongly detected, whereas unphosphorylated and phosphorylated OdhI bands in ATCC 31831/pHT1 (control) strains were slightly detected in Western blotting during the time course of cultivation, indicating that *odhI*-overexpression resulted in enhanced OdhI protein expression in both unphosphorylated and phosphorylated forms. Phosphorylated form of the OdhI protein showed a slower migration than unphosphorylated OdhI on SDS-PAGE, which made it possible to distinguish the phosphorylation status of OdhI by Western blotting (Niebisch et al. 2006). Presence of STPKs, such as PknA, PknB, PknG, and PknL and phospho-serine/threonine protein phosphatase Ppp encoded by *ppp* were already reported by the genome sequence of *C. glutamicum* (Ikeda and Nakagawa, 2003; Kalinowski et al. 2003). It could be considered that phosphorylated forms of the OdhI protein come from the highly expressed unphosphorylated OdhI in *C. glutamicum* cells by STPKs, including PknA, PknB, PknG, and PknL. Schultz et al. (2009) reported that all four STPKs can contribute to OdhI phosphorylation, with PknG being the most important one, and Fiuza et al. (2008) also reported that the purified kinase domains of PknA and PknB were shown to phosphorylate OdhI *in vitro*. Moreover, the phosphatase domain of Ppp catalyzed the dephosphorylation of OdhI *in vitro*, confirming that OdhI is a substrate of Ppp (Krawczyk et al. 2010). Furthermore, unphosphorylated OdhI bands were strongly expressed during the time course of cultivation in the *odhI*-overexpression strain. In these respects, high glutamate production in the *odhI*-overexpressing strain (Figure 4.1D) resulted from a high level of unphosphorylated OdhI expression, which is caused by a high level of unphosphorylated OdhI binding to the OdhA protein. These results are consistent with

those of the previous report showing that binding of unphosphorylated OdhI protein to OdhA resulted in reduction of ODHC activity, and, thus, glutamate production (Niebisch et al. 2006). As a result, the higher unphosphorylated OdhI was expressed, the higher glutamate was produced in the *odhI*-overexpression strain. However, changes in protein expression during the time course of cultivation in the same strain were not significantly detected. In this respect, it is possible to make the following conclusion with regard to changes in OdhI protein expression between the ATCC 31831/pHT1 and ATCC 31831/pHT1-odhI strains; high expression of phosphorylated and unphosphorylated OdhI in ATCC 31831/pHT1-odhI, and low expression in the ATCC 31831/pHT1 strain. Therefore, high glutamate production in the *odhI*-overexpressing strain by metabolic engineering might come from strongly expressed OdhI expression.

To investigate change of OdhA expression, 20 μg of total proteins were applied on 7.5% SDS-PAGE for Western blotting using rabbit anti-OdhA polyclonal antibodies; quantitative relative abundance was then analyzed. Generally, compared to the change of OdhI expression, the change of OdhA expression was not significant during the time course of cultivation, as shown in Figure 4.1. Moreover, the level of OdhA expression between the ATCC 31831/pHT1 and ATCC 31831/pHT1-odhI strains was almost the same; however, the level of OdhA expression in the ATCC 31831/pHT1-odhI strains was lower than that in the ATCC 31831/pHT1 strains. As shown in Figure 4.1B, expression of OdhA in the ATCC 31831/pHT1 strain was slightly decreased during the time course of cultivation; however, expression of OdhA in the ATCC 31831/pHT1-odhI strain did not change significantly. Kataoka et al. (2006) reported on transcriptome analysis of *C. glutamicum* during glutamate overproduction, in which expression of *odhA* and *sucB*, encoding the E1o and E2o subunits of ODHC,

respectively, were decreased by triggering agents during conditions of glutamate overproduction. In these respects, expression of the *odhA* gene might be directly related to protein expression of OdhA for glutamate production.

Because the strain *C. glutamicum* carrying ATCC 31831/pHT1 produced a small amount of glutamate (about 5 g/l) without any triggers, the strain was changed to *C. glutamicum* ATCC 13032. The pHT1 and pHT1-odhI plasmids, which were purified from *C. glutamicum* ATCC 31831, were introduced into *C. glutamicum* ATCC 13032, respectively. Constructed *C. glutamicum* ATCC 13032/pHT1-odhI and ATCC 13032/pHT1 cells were cultivated in Sakaguchi flasks, respectively. Expression of the OdhI protein was confirmed by 12.5% SDS-PAGE during the time of cultivation (data not shown). Western blotting was conducted in the same manner described in the previous section.

As expected, glutamate production was increased by expression of the *odhI* gene in the ATCC 13032/pHT1-odhI strain, compared to control strains. However, the amount of glutamate production in the ATCC 13032/pHT1-odhI strain was very small (about 1.5 g/l as shown in Figure 4.2D) compared to the ATCC 31831/pHT1-odhI strain (more than 15 g/l shown in Figure 4.1D). As expected, protein expression patterns of the OdhI protein in ATCC 13032 were almost consistent with those in ATCC 31831, as shown in Figure 4.2A; unphosphorylated and phosphorylated OdhI bands in the pHT1-odhI strain were strongly detected. Moreover, unphosphorylated OdhI bands, which are highly related to glutamate production by inhibition of ODHC activity, appeared to be of higher abundance than phosphorylated OdhI bands in the pHT1-odhI strain. Unphosphorylated OdhI bands only slightly appeared in the pHT1 strain. From the data presented here, it can be concluded that the increased amount of

unphosphorylated OdhI expression is functionally involved in glutamate production in
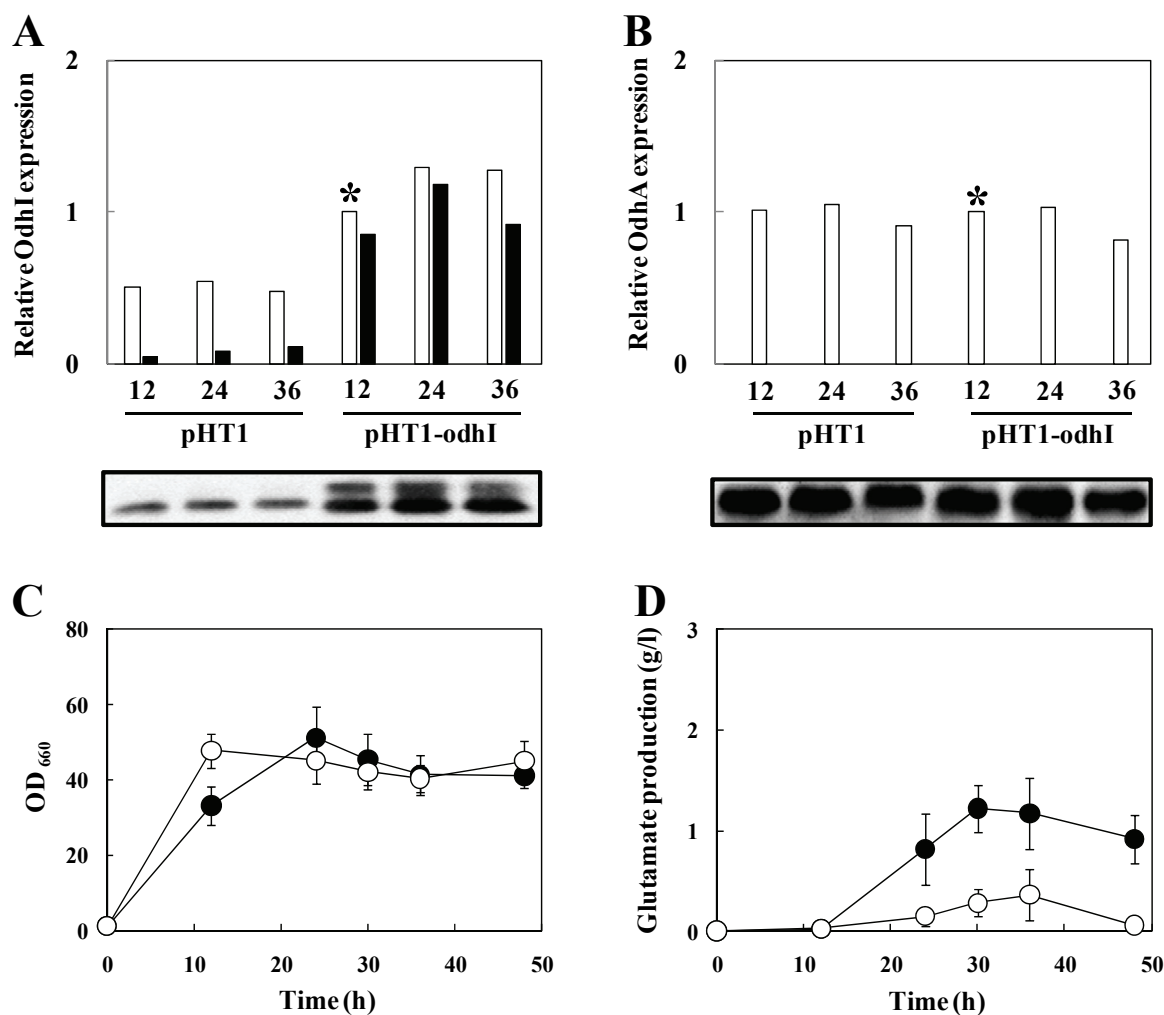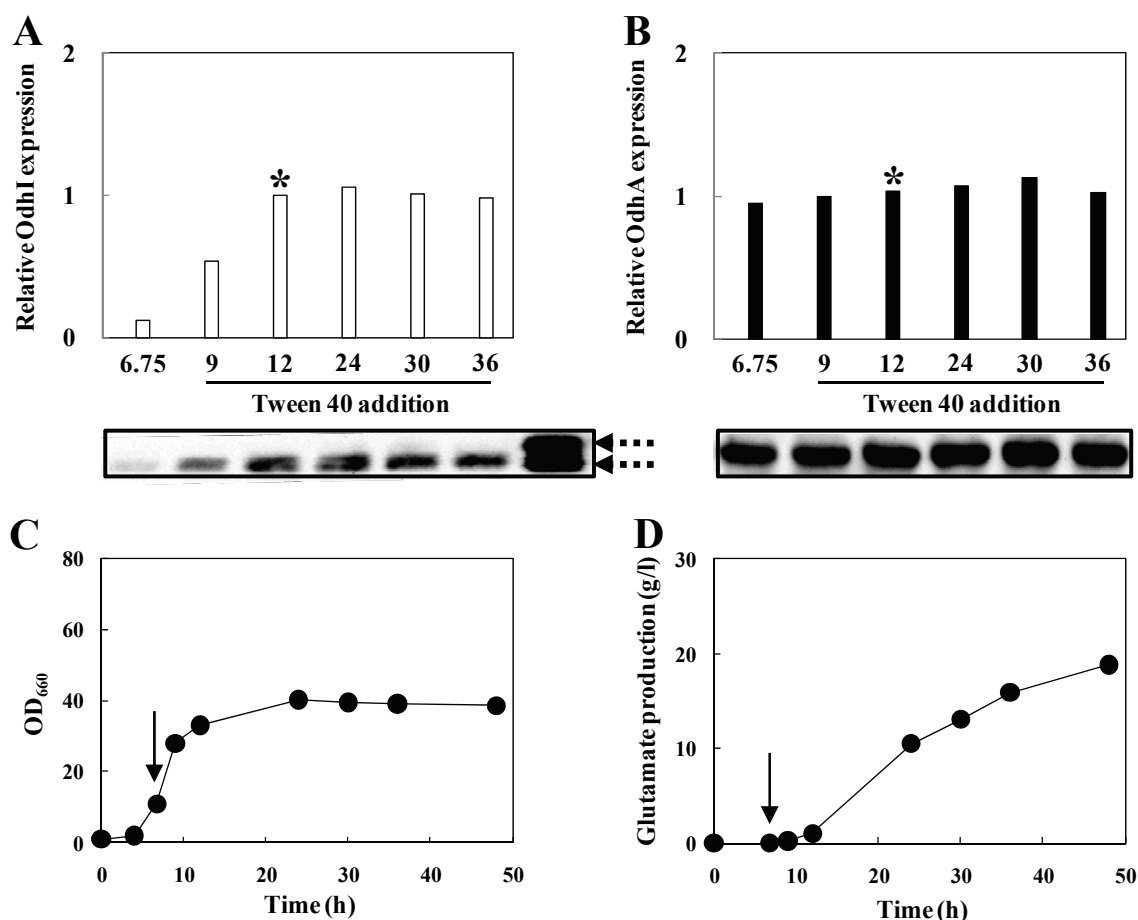
C. glutamicum.

Figure 4.2 Protein expression of OdhI (A) and OdhA (B) proteins, cell growth (C), and glutamate production (D) in *C. glutamicum* ATCC 13032 carrying either the pHT1 or the pHT1-odhI strain, respectively. Cells were cultivated in Sakaguchi flasks and harvested at the point of 12, 20, 28, and 36 h. Protein samples (20 μg) were applied on SDS-PAGE for Western blot analysis. Relative protein expression was calculated by densitometry using the software Image-J 1.43r6. Normalization was performed using the protein expression labeled by the *asterisk* (*). *White bars* represent expression of unphosphorylated forms; *black bars* represent expression of phosphorylated forms; c*losed circles* represent the *odhI* overexpressing strain ATCC 13032 /pHT1-odhI; *open circles* represent the control strain ATCC 13032 /pHT1.

4.3.3 Changes in protein expression during Tween 40-trrigered glutamate production

The *C. glutamicum* ATCC 13032 wild-type strain was cultivated for investigation of changes in protein expression on Tween 40-triggered glutamate production. After cell growth reached the early exponential phase (OD_{660} = 12 at 6. 75 h), Tween 40 was added to the medium with a final concentration of 4 mg/ml for triggering glutamate production. As expected, glutamate was produced by addition of Tween 40 (Figure 4.3D).

Protein samples at the point of 6.75, 9, 12, 24, 30, and 36 h were used to investigate changes of protein expression during Tween 40-triggered glutamate production conditions. As shown in Figure 4.3A, the only unphosphorylated OdhI bands, which was surely confirmed by comparison between unphosphorylated (lower) and phosphorylated (upper) OdhI proteins in the *odhI*-overexpressing strain (data not shown), were detected during the time course of cultivation in Tween 40-triggered glutamate production conditions. Interestingly, unphosphorylated OdhI protein expression was gradually increased after addition of Tween 40 (at the point of 6.75 h) and its expression was not changed after 12 h. Strikingly, the phosphorylated OdhI bands did not apparently appear during the time course. In the case of OdhA expression, it was present in the same level during the time course in Tween 40-triggered glutamate production (Figure 4.3B).

Figure 4.3 Expression of OdhI (A) and OdhA (B) proteins, cell growth (C), and glutamate production (D) in *C. glutamicum* ATCC 13032 under Tween 40-triggered glutamate production. Cells were cultivated in Sakaguchi flasks and harvested at the point of 6.75, 9, 12, 24, 30, and 36 h. *Arrows* indicate the point of Tween 40 addition at 6.75 h. Protein samples (20 μg) were applied on SDS-PAGE for Western blot analysis. Relative protein expression was calculated by densitometry using the software Image-J 1.43r6. Normalization was performed using the protein expression labeled by the *asterisk* (*). *White bars* represent expression of unphosphorylated OdhI; *black bars* represent expression of OdhA. *Arrows with dashed lines* represent the position of unphosphorylated (*lower*) and phosphorylated (*upper*) OdhI expression to distinguish the phosphorylation status of the OdhI protein.

4.3.4   Comparison of DNA sequences of *odhI* genes and amino acid sequences of OdhI proteins between ATCC 13032 and ATCC 31831

As mentioned in the "Results" section (Figure 4. 1D and 4.2D), the reason for the similar response to the protein expression patterns by the *odhI*-overexpressing strain, despite having totally different glutamate production phenomena between ATCC 13032 and ATCC 31831, remains unclear. Even though the protein expression patterns in ATCC 31831 are in accordance with those in ATCC 13032, glutamate production patterns in the *odhI*-overexpressing strains is totally different, as described above (Figure 4.1D and 4.2D). Expression of the *odhI* gene resulted in differing amounts of glutamate production between ATCC 13032 (about 1.5 g/l at 30 h in Figure 4.2D) and ATCC 31831 (about 15 g/l at 50 h in Figure 4.1D) using the same *odhI*-overexpressing plasmid, pHT1-odhI. The biological reason for these different observations between the ATCC 13032 and ATCC 31831 strains is currently obscure. Therefore, the DNA sequence of the *odhI* gene and amino acid sequence of the OdhI protein were, respectively, compared between ATCC 13032 and ATCC 31831.

The DNA sequence and amino acid sequence alignments of the *odhI* gene and OdhI proteins between ATCC 13032 and ATCC 31831 were respectively compared using CLUSTAL X (1.83) multiple sequence alignment. As a result, there were seven different DNA sequences between ATCC 13032 and ATCC 31831, as shown in Figure 4.4; the *odhI* gene shared 98.4% (425 among 432) identity. Intriguingly, most DNA sequence substitutions resulted in the same amino acids, except for one amino acid at the position of the 44 residue, as shown in Figure 4.5. Only one amino acid residue (A) from 143 amino acids in ATCC 13032 is different from an amino acid (V) present in sequences of the OdhI protein in ATCC 31831, sharing 99.3% (142 among 143) identity.

Moreover, there was no change in the phosphorylation sites of the OdhI protein. Phosphorylation sites of OdhI proteins for two threonine residues are involved in phosphorylation by STPKs, such as PknG, PknA, PknB, and PknL (Schultz et al. 2009).

Figure 4.4 Alignment of DNA sequences of OdhI proteins between ATCC 13032 (upper line) and ATCC 31831 (lower line). There were seven different DNA sequences in the *odhI* gene between ATCC 13032 and ATCC 31831, as indicated by the *gray boxes*. The sequences of *odhI* genes in the *a box*, condoning for two threonine residues, represent the encoding regions for phosphorylation sites by STPKs, such as PknG, PknA, PknB, and PknL (Schultz et al. 2009).

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ATCC 13032    MSDNNGTPEPQVE TT SVFRADLLKEMESSTGTAPASTGAENLP A GSALLVVKRGPNAGARFLLDQPTTTAGRHPESDIFL
ATCC 31831    MSDNNGTPEPQVE TT SVFRADLLKEMESSTGTAPASTGAENLP V GSALLVVKRGPNAGARFLLDQPTTTAGRHPESDIFL


ATCC 13032    DDVTVSRRHAEFRINEGEFEVVDVGSLNGTYVNREPRNAQVMQTGDEIQIGKFRLVFLAGPAE
ATCC 31831    DDVTVSRRHAEFRINEGEFEVVDVGSLNGTYVNREPRNAQVMQTGDEIQIGKFRLVFLAGPAE
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Figure 4.5 Alignment of amino acid sequences of OdhI proteins between ATCC 13032 (upper line) and ATCC 31831 (lower line). As shown in Figure 4.4, most DNA sequence substitutions resulted in the same amino acids, except for one amino acid at the position of the 44th residue from an N-terminal (alanine form ATCC 13032 to valine ATCC 31831). Residues of OdhI proteins in *the box* represent important phosphorylation sites by STPKs, such as PknG, PknA, PknB, and PknL (Schultz et al. 2009).

4.4   Conclusion

In this chapter, it was assumed that glutamate production accompanied by decreased ODHC activity might be affected by the expression change of some proteins. Niebisch et al. (2006) identified the novel protein OdhI, a 15-kDa protein, comparing 143 amino acid residues, of *C. glutamicum*. The importance of the OdhI protein for glutamate production in *C. glutamicum* was reported in Chapter 2 and by Schultz et al. (2007). Moreover, the unphosphorylated form of OdhI has a direct interaction with the E1o subunit of ODHC (OdhA) and specifically binds to OdhA, resulting in the inhibition of ODHC activity. In these respects, the relationship between glutamate production and the protein expression change of OdhA and OdhI proteins was investigated by Western blotting under glutamate production conditions in *C. glutamicum* in this chapter.

As expected, the *odhI*-overexpressing strains in both ATCC 31831 and ATCC 13032 showed strong unphosphorylated and phosphorylated OdhI expression with almost constant OdhA expression, resulting in enhanced glutamate production without any triggers (Figure 4.1 and 4.2). In these respects, it is consistent with the fact that high glutamate production in the *odhI*-overexpressing strain resulted from a high level of OdhI expression, which is caused by reduction of ODHC activity from high binding affinity to OdhA (Niebisch et al. 2006). Addition of Tween 40 leading to glutamate production, also resulted in gradually increased expression of unphosphorylated OdhI; however, phosphorylated OdhI expression did not appear with constant OdhA expression (Figure 4.3). In these respects, the increased amount of unphosphorylated OdhI expression is functionally involved in glutamate overproduction in *C. glutamicum*.

Chapter 5

General conclusion

The discovery of a glutamate-producing bacterium, *Corynebacterium glutamicum,* opens a new era for the microbial fermentation industry. It has been widely used in industrial fermentation as an amino acid producer. Therefore, extensive studies have focused on improvement of glutamate production due to the usefulness of glutamate in our daily life, such as a flavor enhancer, therapeutic, feed additive, cosmetics, and so on. Even though *C. glutamicum* is used as a glutamate producer that is induced by triggers such as biotin limitation and addition of Tween 40 and penicillin (Shiio et al. 1962; Takinami et al. 1965; Nara et al. 1964), the regulatory mechanism of glutamate overproduction by *C. glutamicum* is still not completely understood. Shingu and Terui (1971) and Kawahara et al. (1997) found that activity of the 2-oxoglutarate dehydrogenase complex (ODHC) is dramatically reduced during glutamate overproduction. Kawahara et al. (1997) reported that glutamate overproduction by *C. glutamicum* is highly associated with change in metabolic flow from the TCA cycle to glutamate production by decreased ODHC activity. Moreover, Niebisch et al. (2006) determined that unphosphorylated OdhI is responsible for inhibition of ODHC specific activity by specifically binding to the E1o subunit of ODHC (OdhA). Moreover, Nakamura et al. (2007) identified a possible glutamate exporter of *C. glutamicum* coded by NCgl1221. Disruption of NCgl1221 abolished glutamate production, and specific mutations in the NCgl1221 gene induced constitutive glutamate secretion in *C. glutamicum* without any triggers. Based on these results, comprehensive analysis and

integrated metabolic engineering approaches were applied in order to understand the mechanism of glutamate overproduction in *C. glutamicum*.

The background and significance of this study were described in Chapter 1. The general introduction focused mainly on the regulatory mechanisms of glutamate overproduction by *C. glutamicum*. Finally, the objective of this study was also presented in this chapter.

In Chapter 2, the requirement for *de novo* synthesis of the OdhI protein in penicillin-induced glutamate overproduction by *C. glutamicum* was investigated in order to understand the cellular condition by analysis of protein production changes of *C. glutamicum* during penicillin-induced glutamate production. It was revealed that *de novo* protein synthesis after addition of penicillin was absolutely required for glutamate overproduction in *C. glutamicum* by examination of the effect of addition of chloramphenicol, an inhibitor of *de novo* protein synthesis. To identify the *de novo* synthesized proteins responsible for penicillin-induced glutamate overproduction in *C. glutamicum*, proteomic analysis was performed using two-dimensional gel electrophoresis under penicillin addition. Of more than 500 proteins detected, 13 proteins, including OdhI (an inhibitory protein for ODHC), significantly increased by addition of penicillin. To determine whether *de novo* OdhI synthesis is necessary for penicillin-induced glutamate overproduction in *C. glutamicum*, the *odhI*-overexpressing strain in which amplification of the *odhI* gene was used as a metabolic engineering approach was constructed and examined, resulting in glutamate overproduction without any triggers with the decreased ODHC activity. Moreover, continuous glutamate overproduction was also achieved by the *odhI*-overexpressing strain without any triggers. In these respects, amplification of the *odhI* gene in the *odhI*-overexpressing

strain of *C. glutamicum* in metabolic engineering can be useful for efficient glutamate overproduction.

In Chapter 3, the effect of *odhA* overexpression and *odhA* antisense RNA expression on Tween-40-triggered glutamate production by *C. glutamicum* was analyzed for further investigation of the role of the *odhA* gene and its product in glutamate overproduction by *C. glutamicum*. Recombinant strains of *C. glutamicum*, in which expression of the *odhA* gene and its product can be controlled by *odhA* overexpression or *odhA* antisense RNA expression as a method of metabolic pathway control, were constructed to examine the effect of modulation of ODHC activity on Tween 40-triggered glutamate overproduction in *C. glutamicum*. As a result, ODHC specific activity was increased by metabolic engineering using *odhA* overexpression, resulting in dramatically reduced glutamate production, despite addition of Tween 40, indicating that a decrease in the specific activity of ODHC is required for glutamate production induced by addition of Tween 40. *odhA* antisense RNA expression alone did not result in glutamate overproduction in spite of the decrease in ODHC specific activity. Rather, it enhanced glutamate production triggered by addition of Tween 40 due to the additional decrease in ODHC specific activity, suggesting that metabolic engineering by *odhA* antisense RNA expression is effective for achievement of a particular desired goal, enhancement of Tween 40-triggered glutamate overproduction.

In Chapter 4, expression changes of OdhA and OdhI proteins during glutamate production in *C. glutamicum* were investigated for correlation between glutamate overproduction and protein expression changes. Overexpression of the *odhI* gene in *odhI*-overexpressing strains in both ATCC 31831 and ATCC 13032 showed strong unphosphorylated and phosphorylated OdhI expression with almost constant OdhA

expression, resulting in enhanced glutamate production. Moreover, addition of Tween 40 resulted in increased unphosphorylated OdhI expression with constant OdhA expression, leading to glutamate production. In these respects, the increased amount of unphosphorylated OdhI expression is functionally involved in glutamate overproduction in *C. glutamicum*.

In conclusion, metabolic engineering techniques based on better understanding of the cellular state by comprehensive analysis of glutamate production in *C. glutamicum* were applied in order to understand the regulatory mechanism of glutamate overproduction in *C. glutamicum* and to improve glutamate production. *De novo* synthesis of unphosphorylated OdhI protein is an absolutely requirement and is functionally involved in glutamate overproduction in *C. glutamicum* for decrease in ODHC activity. Moreover, *odhI* overexpression alone as a metabolic engineering approach can produce glutamate without any triggers. Furthermore, the additional decrease of ODHC specific activity by *odhA* antisense RNA expression as a metabolic engineering approach is also effective for enhancement of glutamate production triggered by addition of Tween 40. As a result, glutamate production in *C. glutamicum* was successively improved by metabolic engineering approaches using *odhI* overexpression and *odhA* antisense RNA expression in this study, respectively (Figure 5.1).

Metabolic engineering approaches used in this study can be applied as a promising tool for production of targeted materials.
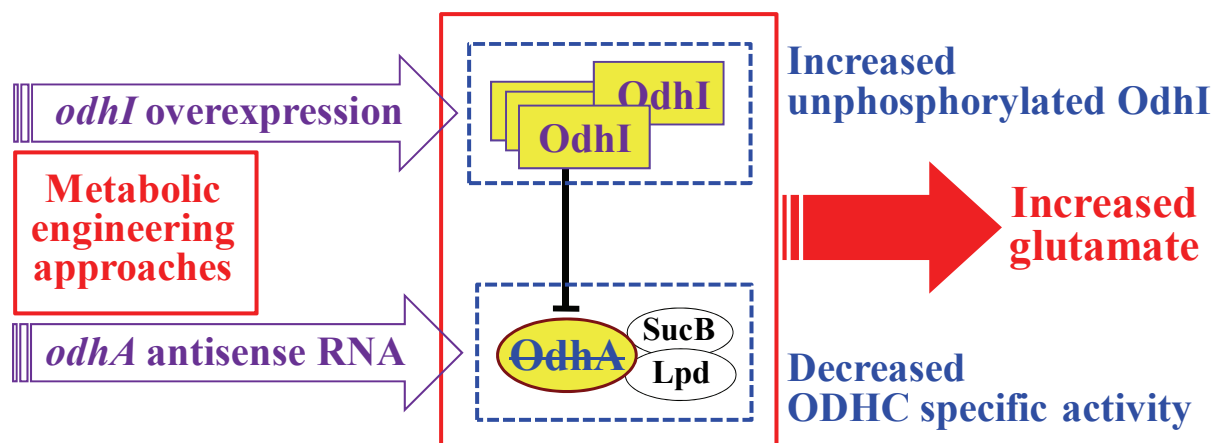
Figure 5.1 Increased glutamate production in *C. glutamicum* by metabolic engineering

approaches, such as *odhI* overexpression and *odhA* antisense RNA expression with increased

unphosphorylated OdhI expression and decreased ODHC specific activity, respectively.

References

Asakura Y, Kimura E, Usuda Y, Kawahara Y, Matsui K, Osumi T, Nakamatsu T (2007) Altered metabolic flux due to deletion of *odhA* causes L-glutamate overproduction in *Corynebacterium glutamicum*. Appl Environ Microbiol 73:1308-1319

Asakura Y, Kimura E, Abe C, Kawahara Y, Nakamatsu T, Usuda Y, Tsujimoto Y, Kurahashi O (1995) Japan Patent H08-501926 (International Patent WO95/34672)

Bailey JE (1991) Toward a science of metabolic engineering. Science 252:1668-1675

Barthe P, Roumestand C, Canova MJ, Kremer L, Hurard C, Molle V, Cohen-Gonsaud M (2009) Dynamic and structural characterization of a bacterial FHA protein reveals a new autoinhibition mechanism. Structure 17:568-578

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254

Bendt AK, Burkovski A, Schaffer S, Bott M, Farwick M, Hermann T (2003) Towards a phosphoproteome map of *Corynebacterium glutamicum*. Proteomics 3:1637-1646

Desai RP, Papoutsakis ET (1999) Antisense RNA strategies for the metabolic engineering of *Clostridium acetobutylicum*. Appl Environ Microbiol 65:936-945

Eggeling L, Sahm H (1999) Amino acid production: principles of metabolic engineering. In: Lee SY, Papoutsakis ET (eds) Metabolic engineering. Dekker, New York, pp 153-176

Fiuza M, Canova MJ, Zanella-Cléon I, Becchi M, Cozzone AJ, Mateos LM, Kremer L, Gil JA, Molle V (2008) From the characterization of the four serine/threonine

protein kinases (PknA/B/G/L) of *Corynebacterium glutamicum* toward the role of PknA and PknB in cell division. J Biol Chem 283:18099-18112

Gande R, Gibson KJ, Brown AK, Krumbach K, Dover LG, Sahm H, Shioyama S, Oikawa T, Besra GS, Eggeling L (2004) Acyl-CoA carboxylases (*accD2* and *accD3*), together with a unique polyketide synthase (*Cg-pks*), are key to mycolic acid biosynthesis in Corynebacterianeae such as *Corynebacterium glutamicum* and *Mycobacterium tuberculosis*. J Biol Chem 279:44847-44857

Gutmann M, Hoischen C, Krämer R (1992) Carrier-mediated glutamate secretion by *Corynebacterium glutamicum* under biotin limitation. Biochim Biophys Acta 1112:115-123

Heijnen JJ, Gulik WM, Shimizu H, Stephanopoulos G (2004) Metabolic flux control analysis of branch points: an improved approach to obtain flux control coefficients from large perturbation data. Metab Eng 6:391-400

Hermann T (2004) Using functional genomics to improve productivity in the manufacture of industrial biochemicals. Curr Opin Biotechnol 15:444-448

Hermann T, Pfefferle W, Baumann C, Busker E, Schaffer S, Bott M, Sahm H, Dusch N, Kalinowski J, Pühler A, Bendt AK, Krämer R, Burkovski A (2001) Proteome analysis of *Corynebacterium glutamicum*. Electrophoresis 22:1712-1723

Hirasawa T, Kumagai Y, Nagai K, Wachi M (2003) A *Corynebacterium glutamicum rnhA recG* double mutant showing lysozyme-sensitivity, temperature-sensitive growth, and UV-sensitivity. Biosci Biotechnol Biochem 67:2416-2424

Hirasawa T, Yamada K, Nagahisa K, Dinh TN, Furusawa C, Katakura Y, Shioya S, Shimizu H (2009) Proteomic analysis of responses to osmotic stress in

laboratory and sake-brewing strains of *Saccharomyces cerevisiae*. Process Biochem 44:647-653

Hoischen C, Krämer R (1989) Evidence for an efflux carrier system involved in the secretion of glutamate by *Corynebacterium glutamicum*. Arch Microbiol 151:342-347

Ikeda M, Nakagawa S (2003) The *Corynebacterium glutamicum* genome: features and impacts on biotechnological processes. Appl Microbiol Biotechnol 62:99-109

Jäger W, Peters-Wendisch PG, Kalinowski J, Pühler A (1996) A *Corynebacterium glutamicum* gene encoding a two-domain protein similar to biotin carboxylases and biotin-carboxyl-carrier proteins. Arch Microbiol 166:76-82

Kalinowski J, Bathe B, Bartels D, Bischoff N, Bott M, Burkovski A, Dusch N, Eggeling L, Eikmanns BJ, Gaigalat L, Goesmann A, Hartmann M, Huthmacher K, Krämer R, Linke B, McHardy AC, Meyer F, Möckel B, Pfefferle W, Pühler A, Rey DA, Rückert C, Rupp O, Sahm H, Wendisch VF, Wiegräbe I, Tauch A (2003) The complete *Corynebacterium glutamicum* ATCC 13032 genome sequence and its impact on the production of L-aspartate-derived amino acids and vitamins. J Biotechnol 104:5-25

Kataoka M, Hashimoto KI, Yoshida M, Nakamatsu T, Horinouchi S, Kawasaki H (2006) Gene expression of *Corynebacterium glutamicum* in response to the conditions inducing glutamate overproduction. Lett Appl Microbiol 42:471-476

Kawahara Y, Takahashi-Fuke K, Shimizu E, Nakamatsu T, Nakamori S (1997) Relationship between the glutamate production and the activity of 2-oxoglutarate dehydrogenase in *Brevibacterium lactofermentum*. Biosci Biotechnol Biochem 61:1109-1112

Kim J, Hirasawa T, Sato Y, Nagahisa K, Furusawa C, Shimizu H (2009) Effect of *odhA* overexpression and *odhA* antisense RNA expression on Tween-40-triggered glutamate production by *Corynebacterium glutamicum*. Appl Microbiol Biotechnol 81:1097-1106

Kim J, Fukuda H, Hirasawa T, Nagahisa K, Nagai K, Wachi M, Shimizu H (2010) Requirement of de novo synthesis of the OdhI protein in penicillin-induced glutamate production by *Corynebacterium glutamicum*. Appl Microbiol Biotechnol 86:911-920

Kinoshita S, Udaka S, Shimono M (1957) Studies on the amino acid fermentation. Part I. Production of L-glutamic acid by various microorganisms. J Gen Appl Microbiol 3:193-205

Kinoshita S (1999) Taxonomic position of glutamic acid producing bacteria. In: Flickinger MC, Drew SW (eds) Encyclopedia of bioprocess technology: fermentation, biocatalysis, and bioseparation. Wiley, New York, pp 1330-1336

Kimura E, Abe C, Kawahara Y, Nakamatsu T (1996) Molecular cloning of a novel gene, *dtsR*, which rescues the detergent sensitivity of a mutant derived from *Brevibacterium lactofermentum*. Biosci Biotechnol Biochem 60:1565-1570

Kimura E, Abe C, Kawahara Y, Nakamatsu T, Tokuda H (1997) A *dtsR* gene-disrupted mutant of *Brevibacterium lactofermentum* requires fatty acids for growth and efficiently produces L-glutamate in the presence of an excess of biotin. Biochem Biophys Res Commun 234:157-161

Kimura E, Yagoshi C, Kawahara Y, Ohsumi T, Nakamatsu T, Tokuda H (1999) Glutamate overproduction in *Corynebacterium glutamicum* triggered by a decrease

in the level of a complex comprising DtsR and a biotin-containing subunit. Biosci Biotechnol Biochem 63:1274-1278

Krawczyk S, Raasch K, Schultz C, Hoffelder M, Eggeling L, Bott M (2010) The FHA domain of OdhI interacts with the carboxyterminal 2-oxoglutarate dehydrogenase domain of OdhA in *Corynebacterium glutamicum*. FEBS Lett [Epub ahead of print]

Lee LK, Roth CM (2003) Antisense technology in molecular and cellular bioengineering. Curr Opin Biotechnol 14:505-511

Lessard P (1996) Metabolic engineering: the concept coalesces. Nat Biotechnol 14:1654-1655

Leuchtenberger W, Huthmacher K, Drauz K (2005) Biotechnological production of amino acids and derivatives: current status and prospects. Appl Microbiol Biotechnol 69:1-8

Liang XY, Van Doren SR (2008) Mechanistic insights into phosphoprotein-binding FHA domains. Acc Chem Res 41:991-999

Li L, Wada M, Yokota A (2007) A comparative proteomic approach to understand the adaptations of an H^+-ATPase-defective mutant of *Corynebacterium glutamicum* ATCC14067 to energy deficiencies. Proteomics 7:3348-3357

Liebl W, Ehrmann M, Ludwig W, Schleifer KH (1991) Transfer of *Brevibacterium divaricatum* DSM 20297[T], "*Brevibacterium flavum*" DSM 20411, "*Brevibacterium lactofermentum*" DSM 20412 and DSM 1412, and *Corynebacterium lilium* DSM 20137[T] to *Corynebacterium glutamicum* and their distinction by rRNA gene restriction patterns. Int J Syst Bacteriol 41:255–260

Meynial-Salles I, Cervin MA, Soucaille P (2005) New tool for metabolic pathway engineering in *Escherichia coli*: one-step method to modulate expression of chromosomal genes. Appl Environ Microbiol 71:2140-2144

Miwa K, Matsui K, Terabe M, Ito K, Ishida M, Takagi H, Nakamori S, Sano K (1985) Construction of novel shuttle vectors and a cosmid vector for the glutamic acid-producing bacteria *Brevibacterium lactofermentum* and *Corynebacterium glutamicum*. Gene 39:281-286

Nakamura J, Hirano S, Ito H, Wachi M (2007) Mutations of the *Corynebacterium glutamicum* NCgl1221 gene, encoding a mechanosensitive channel homolog, induce L-glutamic acid production. Appl Environ Microbiol 73:4491-4498

Nakamura J, Hirano S, Yamaguchi M, Ito H (2006) Japan Patent 2006-101875

Nakayama K, Kitada S, Kinoshita S (1961) Studies on lysine fermentation I. The control mechanism of lysine accumulation by homoserine and threonine. J Gen Appl Microbiol 7:145-154

Nakazawa E, Kawashima N, Koyama I, Ishii K, Kawahara Y (1994) Japan Patent H6-237779

Nara T, Samejima H, Kinoshita S (1964) Effect of penicillin on amino acid fermentation. Agric Biol Chem 28:120-124

Niebisch A, Kabus A, Schultz C, Weil B, Bott M (2006) Corynebacterial protein kinase G controls 2-oxoglutarate dehydrogenase activity via the phosphorylation status of the OdhI protein. J Biol Chem 281:12300-12307

Nielsen J (2001) Metabolic engineering. Appl Microbiol Biotechnol 55:263-283

Nunheimer TD, Birnbaum J, Ihnen ED, Demain AL (1970) Product inhibition of the fermentative formation of glutamic acid. Appl Microbiol 20:215-217

Ohnishi J, Mitsuhashi S, Hayashi M, Ando S, Yokoi H, Ochiai K, Ikeda M (2002) A novel methodology employing *Corynebacterium glutamicum* genome information to generate a new L-lysine-producing mutant. Appl Microbiol Biotechnol 58:217-223

Pallen M, Chuadhuri R, Khan A (2002) Bacterial FHA domains: neglected players in the phospho-threonine signalling game? Trends Microbiol 10:556-563

Portevin D, de Sousa-D'Auria C, Montrozier H, Houssin C, Stella A, Lanéelle MA, Bardou F, Guilhot C, Daffé M (2005) The acyl-AMP ligase FadD32 and AccD4-containing acyl-CoA carboxylase are required for the synthesis of mycolic acids and essential for mycobacterial growth: identification of the carboxylation product and determination of the acyl-CoA carboxylase components. J Biol Chem 280:8862-8874

Radmacher E, Stansen KC, Besra GS, Alderwick LJ, Maughan WN, Hollweg G, Sahm H, Wendisch VF, Eggeling L (2005) Ethambutol, a cell wall inhibitor of *Mycobacterium tuberculosis*, elicits L-glutamate efflux of *Corynebacterium glutamicum*. Microbiology 151:1359-1368

Sahm H, Eggeling L, Graaf AA de (2000) Pathway analysis and metabolic engineering in *Corynebacterium glutamicum*. Biol Chem 381:899-910

Sano K, Shiio I (1970) Microbial production of L-lysine III. Production by mutants resistant to S-(2-aminoethyl)-L-cysteine. J Gen Appl Microbiol 16:373-391

Sato H, Orishimo K, Shirai T, Hirasawa T, Nagahisa K, Shimizu H, Wachi M (2008) Distinct roles of two anaplerotic pathways in glutamate production induced by biotin limitation in *Corynebacterium glutamicum*. J Biosci Bioeng 106:51-58

Schaffer S, Weil B, Nguyen VD, Dongmann G, Günther K, Nickolaus M, Hermann T, Bott M (2001) A high-resolution reference map for cytoplasmic and membrane-associated proteins of *Corynebacterium glutamicum*. Electrophoresis 22:4404-4422

Schleusener D, Fischer F, Kruip J, Rögner M, Poetsch A (2005) Mapping the membrane proteome of *Corynebacterium glutamicum*. Proteomics 5:1317-1330

Schultz C, Niebisch A, Gebel L, Bott M (2007) Glutamate production by *Corynebacterium glutamicum*: dependence on the oxoglutarate dehydrogenase inhibitor protein OdhI and protein kinase PknG. Appl Microbiol Biotechnol 76:691-700

Schultz C, Niebisch A, Schwaiger A, Viets U, Metzger S, Bramkamp M, Bott M (2009) Genetic and biochemical analysis of the serine/threonine protein kinases PknA, PknB, PknG and PknL of *Corynebacterium glutamicum*: evidence for non-essentiality and for phosphorylation of OdhI and FtsZ by multiple kinases. Mol Microbiol 74:724-741

Schwinde JW, Hertz PF, Sahm H, Eikmanns BJ, Guyonvarch A (2001) Lipoamide dehydrogenase from *Corynebacterium glutamicum*: molecular and physiological analysis of the *lpd* gene and characterization of the enzyme. Microbiology 147:2223-2231

Shibukawa M, Kurima M, Ohuchi S (1970) L-Glutamic acid fermentation with molasses. Part XII. Relationship between the kind of phospholipids and their fatty acid composition in the mechanism of extracellular accumulation of L-glutamate. Agr Biol Chem 34:1136-1141

Shibukawa M, Ohsawa T (1966) L-Glutamic acid fermentation with molasses. Part VI. Effect of the saturated-unsaturated fatty acid ratio in the cell membrane fraction on the extracellular accumulation of L-glutamate. Agr Biol Chem 30:75-758

Shiio I, Nakamori S (1970) Microbial production of L-threonine. Part II. Production by α-amino-β-hydroxyvaleric acid resistant mutants of glutamate producing bacteria. Agric Biol Chem 34:448-456

Shiio I, Otsuka S, Katsuya N (1963) Cellular permeability and extracellular formation of glutamic acid in *Brevibacterium flavum.* J Biochem 53: 333-340

Shiio I, Otsuka SI, Takahashi M (1962) Effect of biotin on the bacterial formation of glutamic acid. Part I. Glutamate formation and cellular permeability of amino acids. J Biochem 51:56-62

Shiio I, Ujigawa-Takeda K (1980) Presence and regulation of ioketoglutarate dehydrogenase complex in a glutamate producing bacterium, *Brevibacterium flavum*. Agric Biol Chem 44:1897-1904

Shimizu H (2002) Metabolic engineering Integrating methodologies of molecular breeding and bioprocess systems engineering. J Biosci Bioeng 94:563-573

Shimizu H, Tanaka H, Nakato A, Nagahisa K, Kimura E, Shioya S (2003) Effects of the changes in enzyme activities on metabolic flux redistribution around the 2-oxoglutarate branch in glutamate production by *Corynebacterium glutamicum*. Bioprocess Biosyst Eng 25:291-298

Shimizu H, Hirasawa T (2007) Production of glutamate and glutamate-related amino acids: Molecular mechanism analysis and metabolic engineering. In: Wendisch VF (ed), Microbiology monograph. Volume 5. Amino acid

biosynthesis-pathways, regulation and metabolic engineering. Springer, Belgium, pp 1-36

Shingu H, Terui G (1971) Studies on process of glutamic acid fermentation at the enzyme level. Part I. On the change of α-ketoglutaric acid dehydrogenase in the course of culture. J Ferment Technol 49:400-405

Shirai T, Matsuzaki K, Kuzumoto M, Nagahisa K, Furusawa C, Shioya S, Shimizu H (2006) Precise metabolic flux analysis of coryneform bacteria by gas chromatography-mass spectrometry and verification by nuclear magnetic resonance. *J Biosci Bioeng* 102:413-424

Shirai T, Nakato A, Izutani N, Nagahisa K, Shioya S, Kimura E, Kawarabayasi Y, Yamagishi A, Gojobori T, Shimizu H (2005) Comparative study of flux redistribution of metabolic pathway in glutamate production by two coryneform bacteria. Metab Eng 7:59-69

Stephanopoulos G (1999) Metabolic fluxes and metabolic engineering. Metab Eng 1:1-11

Stephanopoulos G, Vallino JJ (1991) Network rigidity and metabolic engineering in metabolite overproduction. Science 252:1675-1681

Studier FW, Moffatt BA (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J Mol Biol 189: 113-130

Takinami K, Yoshii H, Tsuri H, Okada H (1965) Biochemical effects of fatty acid and its derivatives on L-glutamic acid fermentation. Part III. Biotin-Tween 60 relationship in the accumulation of L-glutamic acid and the growth of *Brevibacterium lactofermentum*. Agric Biol Chem 29:351-359

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76:4350-4354

Tummala SB, Junne SG, Papoutsakis ET (2003a) Antisense RNA downregulation of coenzyme A transferase combined with alcohol-aldehyde dehydrogenase overexpression leads to predominantly alcohologenic *Clostridium acetobutylicum* fermentations. J Bacteriol 185:3644-3653

Tummala SB, Welker NE, Papoutsakis ET (2003b) Design of antisense RNA constructs for downregulation of the acetone formation pathway of *Clostridium acetobutylicum*. J Bacteriol 185:1923-1934

Udaka S (1960) Screening method for microorganisms accumulating metabolites and its use in the isolation of *Micrococcus glutamicus*. J Bacteriol 79:754-755

Usuda Y, Tujimoto N, Abe C, Asakura Y, Kimura E, Kawahara Y, Kurahashi O, Matsui H (1996) Molecular cloning of the *Corynebacterium glutamicum* ('*Brevibacterium lactofermentum*' AJ12036) *odhA* gene encoding a novel type of 2-oxoglutarate dehydrogenase. Microbiology 142:3347-3354

Vertès AA, Inui M, Kobayashi M, Kurusu Y, Yukawa H (1993) Presence of *mrr*- and *mcr*-like restriction systems in coryneform bacteria. Res Microbiol 144:181-185

Vertès AA, Inui M, Kobayashi M, Yukawa H (2005) Manipulating Corynebacteria, from Individual Genes to Chromosomes. Appl Environ Microbiol 71:7633-7642

Wagner EG, Simons RW (1994) Antisense RNA control in bacteria, phages, and plasmids. Annu Rev Microbiol 48:713-742

Wendisch VF, Bott M, Eikmanns BJ (2006) Metabolic engineering of *Escherichia coli* and *Corynebacterium glutamicum* for biotechnological production of organic acids

and amino acids. Curr Opin Microbiol 9:268-274

Wendisch VF, Bott M, Kalinowski J, Oldiges M, Wiechert W (2006) Emerging *Corynebacterium glutamicum* systems biology. J Biotechnol 124:74-92

Westmoreland J, Porter G, Radman M, Resnick MA (1997) Highly mismatched molecules resembling recombination intermediates efficiently transform mismatch repair proficient *E. coli*. Genetics 145:29-38

Yanisch-Perron C, Vieira J, Messing J (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33:103-119

Yang YT, Bennett GN, San KY (1998) Genetic and metabolic engineering. Elec J Biotech 3:134-141

Yao W, Deng X, Liu M, Zheng P, Sun Z, Zhang Y (2009) Expression and localization of the Corynebacterium glutamicum NCgl1221 protein encoding an L-glutamic acid exporter. Microbiol Res. 164:680-687

Yukawa H, Omumasaba CA, Nonaka H, Kós P, Okai N, Suzuki N, Suda M, Tsuge Y, Watanabe J, Ikeda Y, Vèrtes AA, Inui M (2007) Comparative analysis of the *Corynebacterium glutamicum* group and complete genome sequence of strain R. Microbiology 153:1042-1058

List of publications

Publications related to this study

1. Jongpill Kim, Takashi Hirasawa, Yoshiyasu Sato, Keisuke Nagahisa, Chikara Furusawa, Hiroshi Shimizu (2009) "Effect of *odhA* overexpression and *odhA* antisense RNA expression on Tween-40-triggered glutamate production by *Corynebacterium glutamicum*." Applied Microbiology and Biotechnology 81: 1097-1106

2. Jongpill Kim, Hirohisa Fukuda, Takashi Hirasawa, Keisuke Nagahisa, Kazuo Nagai, Masaaki Wachi, Hiroshi Shimizu (2010) "Requirement of de novo synthesis of the OdhI in penicillin-induced glutamate production by *Corynebacterium glutamicum*." Applied Microbiology and Biotechnology 86:911-920

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