Chapter 13
Molecular Mechanisms and Metabolic Engineering of Glutamate Overproduction in *Corynebacterium glutamicum*

Takashi Hirasawa, Jongpill Kim, Tomokazu Shirai, Chikara Furusawa, and Hiroshi Shimizu

Abstract Glutamate is a commercially important chemical. It is used as a flavor enhancer and is a major raw material for producing industrially useful chemicals. A coryneform bacterium, *Corynebacterium glutamicum*, was isolated in 1956 by Japanese researchers as a glutamate-overproducing bacterium and since then, remarkable progress in glutamate production has been made using this microorganism. Currently, the global market for glutamate is over 2.5 million tons per year. Glutamate overproduction by *C. glutamicum* is induced by specific treatments—biotin limitation, addition of fatty acid ester surfactants such as Tween 40, and addition of β-lactam antibiotics such as penicillin. Molecular biology and metabolic engineering studies on glutamate overproduction have revealed that metabolic flow is significantly altered by these treatments. These studies have also provided insight into the molecular mechanisms underlying these changes. In this chapter, we review our current understanding of the molecular mechanisms of glutamate overproduction in *C. glutamicum*, and we discuss the advances made by metabolic engineering of this microorganism.

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Abbreviations

^{13}C-MFA	^{13}C metabolic flux analysis
CoA	coenzyme A
FBA	flux balance analysis
FHA	forkhead-associated
GC/MS	gas chromatography/mass spectrometry
GDH	glutamate dehydrogenase
ICDH	isocitrate dehydrogenase
K_m	Michaelis-Menten constant
MFA	metabolic flux analysis
MSG	mono sodium glutamate
NMR	nuclear magnetic resonance
ODHC	2-oxoglutarate dehydrogenase complex
PC	pyruvate carboxylase
PEPC	phosphoenolpyruvate carboxylase
TCA	tricarboxylic acid

13.1 Introduction

In 1908, Dr. Kikunae Ikeda identified monosodium glutamate (MSG) as the compound imparting the taste of Umami to numerous foods. Umami is distinct from other tastes such as bitter, sour, salty and sweet. Ikeda and his colleagues began to industrially produce MSG by subjecting wheat protein gluten to acid hydrolysis. Ajinomoto Co. Inc. was the first company to produce MSG on an industrial scale.

In 1956, Japanese researchers at Kyowa Hakko Kogyo Co. Ltd. (currently, Kyowa Hakko Bio Co. Ltd.) isolated a microorganism secreting high amounts of glutamate (Kinoshita et al. 1957; Udaka 1960). This microorganism was initially named *Micrococcus glutamicus* but was renamed *Corynebacterium glutamicum*. Using *C. glutamicum*, a fermentation process to produce glutamate directly from sugar and ammonia was developed. At present, *C. glutamicum* is widely used as a host species for producing glutamate and other amino acids such as lysine, arginine, threonine, and valine (Nakayama et al. 1961; Nakayama and Yoshida 1972; Sano and Shiio 1970; Shiio and Nakamori 1970; Tsuchida et al. 1975).

C. glutamicum is a facultatively anaerobic, rod-shaped, high G+C Gram-positive bacterium (Fig. 13.1). Recently, this microorganism has attracted attention as a host for producing useful compounds such as lactate and succinate as well as amino

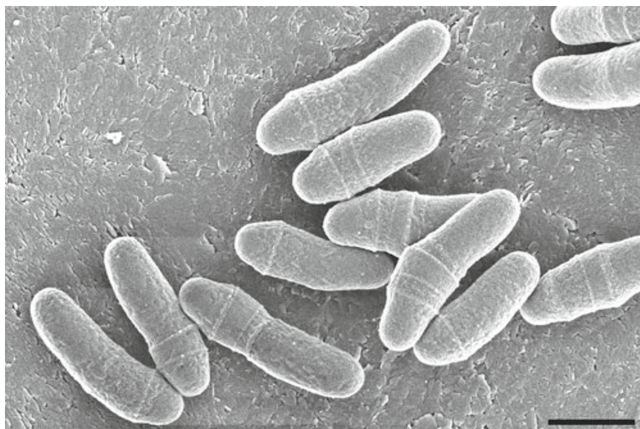

Fig. 13.1 Scanning electron micrograph of *C. glutamicum*. The *bar* represents 1 μm

acids (Okino et al. 2005; Wendisch et al. 2006). It has also been employed for secreted protein production (Kikuchi et al. 2003; Umakoshi et al. 2011). The complete genomic DNA sequence of *C. glutamicum* has been determined (Ikeda and Nakagawa 2003; Kalinowski et al. 2003; Yukawa et al. 2007). A related species *Corynebacterium efficiens* was also isolated as a glutamate producing bacterium under high temperature growth conditions (Fudou et al. 2002). The complete genome sequence of this species is also available (Nishio et al. 2003).

Glutamate has been used as a flavor enhancer and a raw material for producing useful chemicals for many years. The global market of glutamate is the largest among the amino acids (Shimizu and Hirasawa 2007) and is over 2.5 million tons per year (Becker and Wittmann 2012).

Following the discovery of *C. glutamicum*, numerous molecular biology and metabolic engineering studies for glutamate overproduction have been conducted. In this section, we review recent progress in the metabolic engineering of glutamate overproduction by *C. glutamicum* and discuss our current understanding of the underlying molecular mechanisms.

13.2 Glutamate Production by *C. glutamicum*

Glutamate is synthesized from 2-oxoglutarate in the tricarboxylic acid (TCA) cycle by glutamate dehydrogenase (GDH) reaction, which is the main pathway for glutamate biosynthesis when nitrogen supply is sufficient.

C. glutamicum requires biotin (vitamin B7) for growth. Wild-type *C. glutamicum* does not secrete glutamate when excess biotin is present in the culture medium. However, when biotin is limiting, *C. glutamicum* can produce large amounts of glutamate (Shiio et al. 1962). Even in the presence of excess biotin,

certain treatments can induce glutamate overproduction by *C. glutamicum*—addition of fatty acid ester surfactants such as polyoxyethylene sorbitan monopalmitate (Tween 40) and polyoxyethylene sorbitan monostearate (Tween 60) (Takinami et al. 1965), addition of β-lactam antibiotics such as penicillin (Nara et al. 1964), and addition of the antimycobacterial agent ethambutol (Radmacher et al. 2005). These treatments affect cell surface structures including the cytoplasmic membrane, cell wall peptidoglycan layer and outer mycolic acid-containing layer of *C. glutamicum*; biotin is a cofactor for the enzyme for fatty acid biosynthesis. Similarly, additions of fatty acid ester surfactants, β-lactam antibiotics and ethambutol affect fatty acid biosynthesis, peptidoglycan biosynthesis and formation of mycolic acid-containing layer, respectively. Therefore, it was thought that overproduced glutamate passively leaked out of *C. glutamicum* due to the increased cell surface permeability. However, this leak model of glutamate production cannot account for the high glutamate production observed (more than 60–80 g/L), based on the differences between intracellular and extracellular glutamate levels.

Shigu and Terui (1971) reported a change in the enzyme activity of the 2-oxoglutarate dehydrogenase complex (ODHC), which is located at the branch point between the TCA cycle and the glutamate biosynthesis pathway, during glutamate production by *C. glutamicum*. Kinoshita (1985) proposed that glutamate production by *C. glutamicum* is regulated by the ODHC activity. Later, Kawahara and colleagues found that ODHC activity decreases during glutamate overproduction induced by biotin limitation, Tween 40 addition and penicillin addition (Kawahara et al. 1997). Moreover, the importance of a balanced supply of acetyl-CoA and oxaloacetate has been emphasized (Hasegawa et al. 2008), particularly for oxaloacetate-supplying anaplerotic reactions, which are important for glutamate production (Sato et al. 2008; Shirai et al. 2007). Very recently, a novel protein, OdhI, was identified as a protein contributing to the decrease in ODHC activity by interacting with OdhA, a subunit of the ODHC. In addition, phosphorylation status of OdhI was found to influence its ability to decrease in ODHC activity and affect glutamate production (Kim et al. 2010, 2011; Niebisch et al. 2006; Schultz et al. 2007). Furthermore, a mechanosensitive channel, NCgl1221, was found to be important for glutamate overproduction by *C. glutamicum* (Nakamura et al. 2007).

13.3 Molecular Mechanisms of Glutamate Overproduction in *C. glutamicum*

Molecular biology studies of glutamate overproduction by *C. glutamicum* have been focused on cell surface structures and metabolic flux changes and have revealed some important factors for glutamate overproduction (Fig. 13.2). Particularly, studies on DtsR1, NCgl1221, and OdhI have contributed to our understanding of the mechanism of glutamate overproduction. In this section, the roles of these important proteins in glutamate overproduction are reviewed. Moreover, we discuss the

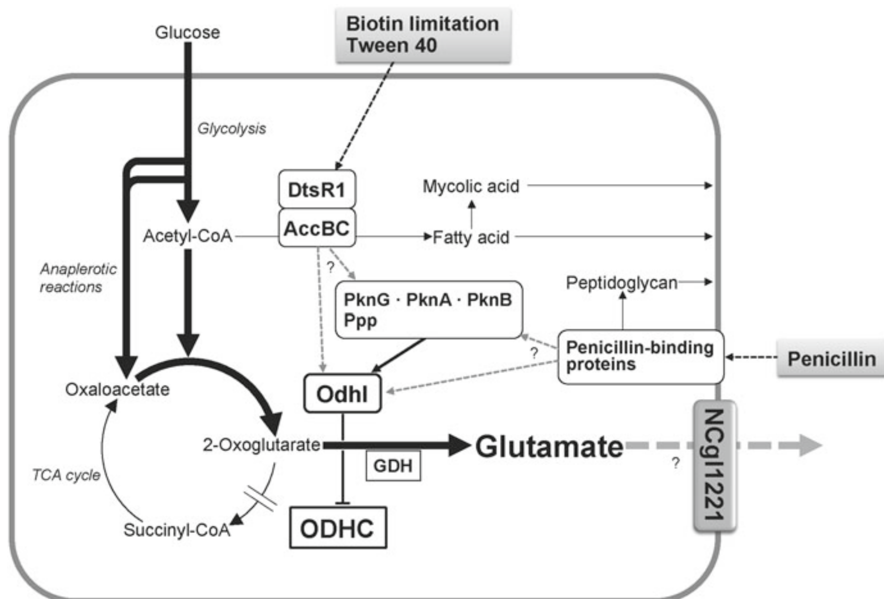

Fig. 13.2 Possible molecular mechanisms of glutamate overproduction by *C. glutamicum* involving DtsR1, OdhI and NCgl1221

relationship between cell surface structure and glutamate production, as well as genome-wide analyses of glutamate overproduction.

13.3.1 Role of DtsR1 in Glutamate Overproduction

As described above, glutamate overproduction in *C. glutamicum* is achieved by addition of Tween 40 to the culture medium. Kimura et al. (1996) analyzed the Tween 40-sensitive mutant of *C. glutamicum* and cloned a gene, *dtsR1*, which rescued the Tween 40-sensitivity. The *dtsR1* gene product shows high homology with the β subunit of propionyl-coenzyme A (CoA) carboxylase from other microorganisms, suggesting that it is a subunit of biotin-containing fatty acid biosynthesis enzyme complex, i.e. the acyl-CoA carboxylase complex (AccBC-DtsR1).

The *dtsR1*-disrupted strain shows auxotrophy for oleic acid and its ester (Tween 80). It can produce glutamate in the presence of excess biotin (Kimura et al. 1997) and exhibits decreased ODHC activity compared with the wild-type strain. Moreover, the intracellular level of DtsR1 is decreased by biotin limitation and Tween 40 addition, but is not changed by penicillin addition (Kimura et al. 1999). These results suggest that the target of biotin limitation and Tween 40 treatment is the acyl-CoA carboxylase complex, including DtsR1, and these treatments seem to inhibit fatty acid biosynthesis in *C. glutamicum*.

13.3.2 Role of the Mechanosensitive Channel Encoded by the NCgl1221 Gene in Glutamate Overproduction and Secretion

Many researchers have sought to understand the mechanisms of glutamate overproduction and its secretion. Until the early 1990s, it was thought that passive leakage of glutamate through the membrane and cell wall was the main mode of glutamate secretion induced by triggers mentioned above. In 2007, however, possible glutamate exporter protein encoded by NCgl1221 gene was identified.

It was reported that disruption of the *odhA* gene, encoding the catalytic subunit of ODHC, enhances glutamate production without the need for induction treatments (Asakura et al. 2007). However, some *odhA* disruptants do not produce glutamate, probably due to genetic instability of the disruptants. Nakamura et al. (2007) found that glutamate production in some *odhA* disruptants is not induced by *odhA* disruption, but is caused by other mutation in the gene NCgl1221. The NCgl1221 gene product shows high homology with mechanosensitive channels, such as the *Escherichia coli* YggB protein (Levina et al. 1999). Mechanosensitive channels sense changes in membrane tension and mediate adaptation to changes in osmotic pressure (Berrier et al. 1992). Electrophysiological analyses using NCgl1221-expressing bacteria revealed that NCgl1221 protein is, in fact, a mechanosensitive channel (Börngen et al. 2010; Hashimoto et al. 2010).

The specific mutations in NCgl1221, which probably involve in the conformational changes induced by various triggers, lead to glutamate overproduction without requiring *odhA* disruption. Moreover, disruption of NCgl1221 abolishes glutamate secretion due to the increase in intracellular glutamate level and NCgl1221 overexpression increases glutamate production with the need for triggers, i.e. biotin limitation, Tween 40 addition, or penicillin treatment. These results suggest that the activation of the NCgl1221 due to the changes in membrane tension induced by triggers is important for glutamate overproduction in *C. glutamicum*, and that NCgl1221 protein is a possible glutamate exporter.

13.3.3 OdhI and Decreased ODHC Activity During Glutamate Overproduction

As described in Sect. 13.2, the activity of the ODHC is decreased during glutamate overproduction induced by biotin limitation, Tween 40 addition, and penicillin treatment (Kawahara et al. 1997). However, the molecular mechanism responsible for the decrease in ODHC activity during glutamate production was unknown until 2006, when a crucial protein was identified.

Niebisch et al. (2006) identified the protein OdhI based on an analysis of a *C. glutamicum* mutant for *pknG*, encoding a serine/threonine protein kinase. OdhI is a 15-kDa protein and is a substrate for PknG serine/threonine kinase. The OdhI protein

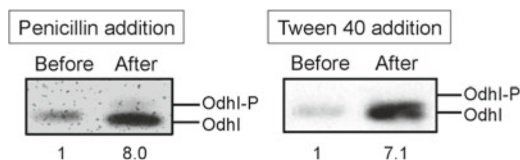

Fig. 13.3 Phosphorylation status of OdhI protein during penicillin- and Tween 40-induced glutamate overproduction in *C. glutamicum*. Phosphorylation status of OdhI protein before and after penicillin and Tween 40 addition was analyzed by western blotting with rabbit polyclonal OdhI antiserum. Values bellows the OdhI protein bands indicate the amount of unphosphorylated OdhI relative to that before penicillin and Tween 40 addition

has a forkhead-associated (FHA) domain, which binds to phosphothreonine epitopes on proteins and mediates interactions with other proteins. Two threonine residues Thr14 and Thr15, in OdhI are phosphorylated by PknG. Other protein kinases—PknA, PknB and PknL—can also phosphorylate the OdhI protein (Schultz et al. 2009). Phosphorylated OdhI is dephosphorylated by the Ppp phosphatase (Schultz et al. 2007, 2009). OdhI interacts with the OdhI protein, which is a catalytic subunit of ODHC, and unphosphorylated OdhI can inhibit ODHC activity by this direct interaction.

Barthe et al. (2009) solved the structure of the phosphorylated and unphosphorylated forms of OdhI. The phosphothreonine residue in OdhI interacts with its own FHA domain, and as a result, phosphorylated OdhI cannot interact with OdhA to inhibit ODHC activity. In contrast, the FHA domain in unphosphorylated OdhI can freely interact with OdhA, and thereby inhibit ODHC activity. Biochemical analyses revealed that the FHA domain of unphosphorylated OdhI interacts with the C-terminal dehydrogenase domain of OdhA (Krawczyk et al. 2010).

The deletion of *odhI* abolishes glutamate production in *C. glutamicum* triggered by biotin limitation, Tween 40 addition and penicillin addition (Schultz et al. 2007). Moreover, the phosphorylation status of OdhI was analyzed using western blotting. Boulahya et al. (2010) reported that OdhI is dephosphorylated under biotin limitation condition. Kim et al. (2011) analyzed Tween 40- and penicillin-induced glutamate overproduction in *C. glutamicum*, and suggested that the unphosphorylated OdhI increases substantially following both triggers (Fig. 13.3). These results suggest that OdhI has an important role in glutamate production by *C. glutamicum* induced by biotin limitation, Tween 40 addition and penicillin treatment, and that unphosphorylated form of OdhI is abundant during glutamate overproduction.

13.3.4 Relationship Between Cell Surface Structure and Glutamate Production by C. glutamicum

As described above, glutamate overproduction by *C. glutamicum* is induced by biotin limitation, fatty acid ester surfactant addition, and penicillin treatment. Biotin limitation and addition of fatty acid esters affect fatty acid biosynthesis in

C. glutamicum. Penicillin is a β-lactam antibiotic that binds to penicillin-binding proteins and inhibits the peptidoglycan biosynthesis. These treatments affect the cell surface integrity of *C. glutamicum*. Thus, it was thought that glutamate leaked passively through the membrane. Consequently, some relationship between cell surface state changes and glutamate overproduction were investigated.

In *C. glutamicum*, the cytoplasmic membrane is covered with a thick peptidoglycan layer, which is, in turn, surrounded by an arabinogalactan layer. The peptidoglycan of *C. glutamicum* consists of an L-alanyl-D-glutamyl-*meso*-diaminopimelyl-D-alanine peptide with β-1,4 linked *N*-acetylglucosamine and *N*-acetylmuramic acid containing side chains, as in *E. coli*. It should be noted that the *C. glutamicum* cell wall contains long chain fatty acids, called mycolic acids, similar to Mycobacteria. Mycolic acids are α-alkyl-β-hydroxylated fatty acids, i.e. R_1–CH(OH)–CH(R_2)–COOH (R_1 and R_2 represent alkyl chains). The alkyl chain lengths of mycolic acids in *C. glutamicum* (32–36) are shorter than that in Mycobacteria (50–56).

Hoischen and Kramer (1990) analyzed the relationship between membrane structure and glutamate production. The total amount of lipids including phospholipids is reduced, and the ratio of saturated to unsaturated fatty acids is changed under biotin limitation. Nampoothiri et al. (2002) examined the impact of overexpression or deletion of the genes for lipid or fatty acid biosyntheses genes on glutamate production by *C. glutamicum*, but no significant correlation between phospholipid composition and glutamate efflux was found.

Analyses of lysozyme-sensitive mutants of *C. glutamicum* have also been performed. Since *C. glutamicum* exhibits tolerance to the lytic enzyme lysozyme, due to the presence of mycolic acid-containing layer, lysozyme-sensitive mutants of *C. glutamicum* are expected to have defects in cell surface structure. Hirasawa et al. (2000) cloned the *ltsA* gene, which complements the temperature-sensitive growth and lysozyme-sensitivity of the *C. glutamicum* mutant strain KY9714. The *ltsA* gene product exhibits high homology with *purF*-type glutamine-dependent asparagine synthetases, which belongs to the glutamine-dependent amidotransferases, of various organisms. However, *ltsA* cannot complement asparagine auxotrophy of *E. coli asnA asnB* double mutant, suggesting that the LtsA protein is a novel glutamine-dependent amidotransferase involved in formation of cell surface structure, particularly the mycolic acid-containing layer. Moreover, the *ltsA* mutant strains can produce glutamate by elevating culture temperature, suggesting that the defects in cell surface structure by *ltsA* mutations can elicit glutamate overproduction (Hirasawa et al. 2000, 2001).

Hashimoto et al. (2006) examined the relationship between mycolic acid layer formation and glutamate overproduction in *C. glutamicum*. The cellular content of mycolic acid decreased following treatment with the various triggers, and the content of short chain mycolic acids increased under biotin limitation, indicating that formation of the mycolic acid layer is perturbed by treatments that induce glutamate overproduction. It is thought that the mycolic acid layer functions as a permeability barrier to glutamate secretion. Thus, changes in the mycolic acid layer during glutamate production might alter membrane tension, which is sensed by the mechanosensitive channel NCgl1221.

13.3.5 *Genome-Wide Analyses of Glutamate Overproduction Mechanism in C. glutamicum*

Genome-wide analyses including transcriptomic and proteomic analyses during glutamate overproduction have been conducted by a number of research groups. Kataoka et al. (2006) reported the transcriptomic analysis of *C. glutamicum* during glutamate overproduction by biotin limitation, Tween 40 addition, and penicillin treatment, using DNA microarray. The results of DNA microarray analysis indicated that the genes related to glycolysis, the pentose phosphate pathway, and the TCA cycle are downregulated. In particular, the expression of *odhA* and *sucB* encoding subunits of the ODHC is reduced. In contrast, the genes NCgl2944, NCgl2945, NCgl2946, and NCgl2975 are upregulated during glutamate overproduction, but the functions of proteins encoded by these genes are unknown.

Proteomic analysis during glutamate production by *C. glutamicum* was performed by Kim et al. (2010). They examined the effect of chloramphenicol, an antibiotic that inhibit *de novo* protein synthesis, on penicillin-induced glutamate overproduction by *C. glutamicum*, and found that protein synthesis within 4 h after penicillin addition is required for glutamate production. To identify the proteins whose synthesis was induced by penicillin addition, proteomic analysis (2-dimensional gel electrophoresis) was performed. The analysis indicated that penicillin increased the expression of 13 proteins including the OdhI protein. Moreover, *odhI* overexpression induced glutamate overproduction without requiring triggers. These results indicate that OdhI synthesis is required for penicillin-induced glutamate overproduction.

13.4 Metabolic Engineering of Glutamate Overproduction in *C. glutamicum*

Metabolic engineering can be defined as the directed improvement of product formation or cellular properties through the modification of intracellular biochemical reaction(s) in complex metabolic networks (Stephanopoulos et al. 1998). To improve production of target product in bioprocesses using cells, both genetic modification of metabolic pathways and process operation strategies are important. In metabolic engineering, a system for evaluating cellular metabolism after genetic manipulation is crucial, and is called metabolic flux analysis (MFA). Metabolic flux is defined as the rate of intracellular biochemical reaction per unit cell. MFA is conducted based on measurements of extracellular metabolic reaction rates or based on ^{13}C isotope labeling experiments and measurement of ^{13}C enrichment in metabolites using nuclear magnetic resonance (NMR) spectroscopy, gas chromatography/mass spectrometry (GC/MS), etc. Based on measurement of ^{13}C enrichment in metabolites (^{13}C-MFA), metabolic flux can be precisely determined. For example, the metabolic fluxes for forward and reverse reactions of reversible reactions can be

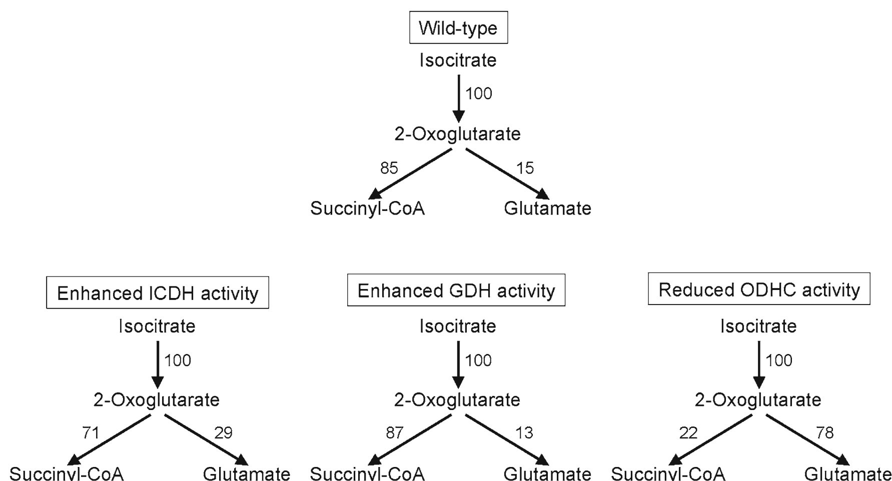

Fig. 13.4 Impact of change in activities of ICDH, GDH and ODHC on metabolic flux redistribution at the 2-oxoglutarate branch in *C. glutamicum*. Glucose was used as a carbon source in this experiment. Input flux to 2-oxoglutarate from isocitrate was normalized to 100, and redistributed fluxes to succinyl-CoA and glutamate from 2-oxoglutarate are shown

individually determined. In this section, we review *in vivo* and *in silico* MFA of glutamate production by *C. glutamicum*. We also discuss metabolic engineering of glutamate overproduction in *C. glutamicum*.

13.4.1 Metabolic Flux Analysis at the 2-Oxoglutarate Branch in the TCA Cycle During Glutamate Production

MFA has been performed on *C. glutamicum* during glutamate overproduction under biotin limitation. Here, MFA was performed based on the extracellular reaction rates including sugar consumption, glutamate production, and CO_2 evolution, and the flux redistribution around the 2-oxoglutarate branch in the TCA cycle during glutamate production was examined.

The impact of changes in enzyme activity at the 2-oxoglutarate branch on glutamate production by *C. glutamicum* was assessed based on MFA (Shimizu et al. 2003) (Fig. 13.4). Metabolic flux distributions in the *icd*- and *gdh*-overexpressing *C. glutamicum* recombinant strains under sufficient biotin supply were determined. The *icd* gene encodes isocitrate dehydrogenase (ICDH), which converts isocitrate to 2-oxoglutarate and *gdh* encodes GDH, which converts 2-oxoglutarate to glutamate (Börmann et al. 1992; Eikmanns et al. 1995). A 3.0-fold enhancement of ICDH activity was observed in the *icd*-overexpressing strain, and a 3.2-fold increase in GDH activity was achieved in the *gdh*-overexpressing strain. In addition, the impact of decreased ODHC activity under biotin limitation on metabolic flux redistribution at the 2-oxoglutarate branch

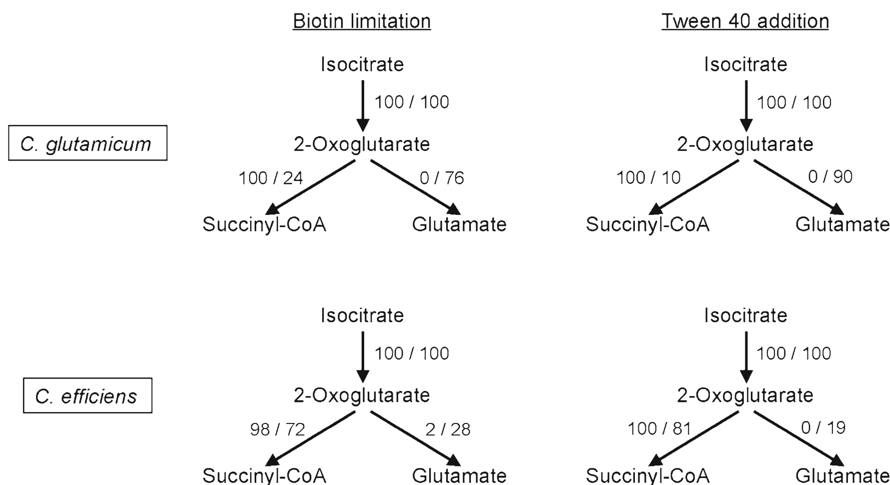

Fig. 13.5 Comparison of metabolic flux redistributions at the 2-oxoglutarate branch between *C. glutamicum* and *C. efficiens* in glutamate overproduction under biotin limitation and Tween 40 addition. Glucose was used as a carbon source in this experiment. Input flux to 2-oxoglutarate from isocitrate was normalized to 100, and redistributed fluxes to succinyl-CoA and glutamate from 2-oxoglutarate are shown

was also analyzed. When grown with sufficient biotin, the elevated ICDH and GDH activities had little impact on glutamate production by *C. glutamicum*—approximately 70% of carbon flux still flowed to the TCA cycle. In contrast, significant flux changes for GDH and ODHC were observed when the ODHC activity was decreased by biotin limitation—78% of carbon was utilized for glutamate formation. These results indicate that the ODHC activity has a great impact on glutamate production by *C. glutamicum*.

MFA of another coryneform bacterium, *C. efficiens*, was also performed (Shirai et al. 2005). *C. efficiens* was isolated by Ajinomoto Co., Ltd. and can grow at higher temperatures than *C. glutamicum*. Therefore, cooling during fermentation process is not necessary, and as a result, the costs of production can be decreased by using this bacterium. *C. efficiens* can produce glutamate following biotin limitation and Tween 40 addition. Thus, MFA of *C. efficiens* was performed and flux redistribution at the 2-oxoglutarate branch was compared with that of *C. glutamicum* during glutamate production (Fig. 13.5). Glutamate production in *C. glutamicum* was larger than that in *C. efficiens* under biotin limitation and Tween 40 addition. In both species, activities of ICDH and GDH were not affected by biotin limitation or Tween 40 addition, whereas ODHC activity was significantly decreased. Flux redistribution occurred after ODHC activity was reduced by biotin limitation and Tween 40 addition in both species. However, the carbon flow toward glutamate production depended on the magnitude of the decrease in ODHC activity—the carbon flow to glutamate production was larger in *C. glutamicum* than in *C. efficiens*, and was consistent with the difference in glutamate production between the two species. Moreover, the

Michaelis-Menten constants (K_m) for ICDH and ODHC to 2-oxoglutarate were lower than that of GDH, suggesting that the affinity of 2-oxoglutarate for GDH was lower than for ICDH and ODHC. Thus, the accumulation of 2-oxoglutarate mediated by decreased ODHC activity is important for glutamate overproduction in both species. In addition, the K_m of GDH was lower in *C. glutamicum* than in *C. efficiens*, and this phenomenon may underlie the difference in glutamate production levels between the two species.

13.4.2 Analyses of Importance of Anaplerotic Reactions During Glutamate Overproduction Based on ^{13}C Metabolic Flux Analysis

For glutamate production, a balanced supply of acetyl-CoA and oxaloacetate is important to proceed TCA cycle reactions toward 2-oxoglutarate, because it is a substrate for glutamate biosynthesis catalyzed by GDH. In particular, anaplerotic reactions, which supply oxaloacetate from the glycolytic intermediates, are crucial. *C. glutamicum* possesses two enzymes for anaplerotic reactions; phosphoenolpyruvate carboxylase (PEPC) encoded by *ppc* and pyruvate carboxylase (PC) encoded by *pyc* (Börmann et al. 1992; Eikmanns et al. 1995; O'Regan et al. 1989; Peters-Wendisch et al. 1998). PC requires biotin as a cofactor for its activity. MFA was conducted to understand the role of these anaplerotic reactions in glutamate production.

Sato et al. (2008) genetically and metabolically analyzed the roles of PEPC and PC on glutamate production by *C. glutamicum* under biotin limitation using the *ppc* and *pyc* disruptants. The *pyc* disruptant produced high amounts of glutamate under biotin limitation, but lactate was simultaneously produced. In contrast, the *ppc* disruptant could not produce glutamate under biotin limitation. MFA, based on the ^{13}C-labeling experiment and measurement of ^{13}C enrichment in glutamate using NMR spectroscopy, revealed that the flux for anaplerotic reactions in the *pyc* disruptant was lower than in the wild-type under biotin limitation, whereas the flux for lactate production was higher. Disruption of *ldh* encoding lactate dehydrogenase, which is involved in lactate production, enhanced glutamate production in the *pyc* disruptant of *C. glutamicum*. These results indicate that the PEPC reaction is important for glutamate production under biotin limitation, because PC is a biotin-containing enzyme that does not function under biotin limitation.

Shirai et al. developed an analysis system for precise ^{13}C-MFA for coryneform bacteria, and examined the role of anaplerotic reactions in Tween 40-induced glutamate overproduction by *C. glutamicum* (Shirai et al. 2006, 2007) (Fig. 13.6). Metabolic reaction model of *C. glutamicum* considering the reversibility of the reversible reaction and two anaplerotic reactions was constructed, and ^{13}C-MFA was performed based on time course data of ^{13}C enrichment of proteinogenic amino acids measured by GC/MS during cell growth and glutamate production phases. In this ^{13}C-MFA, metabolic flux distribution is tuned as the measured GC/MS data agreed with the GC/

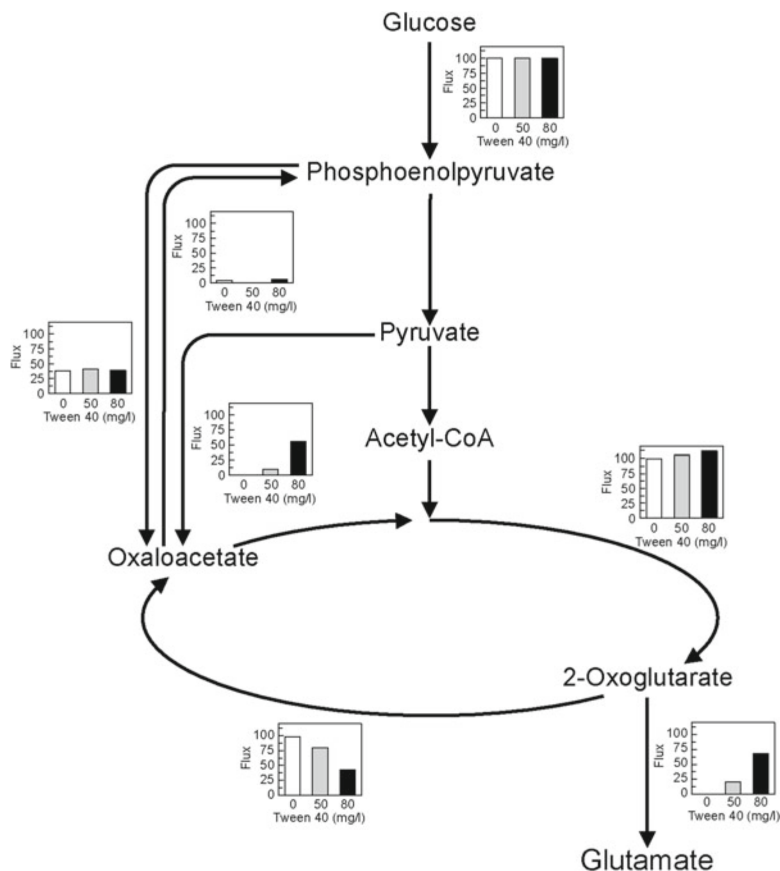

Fig. 13.6 ^{13}C-MFA of *C. glutamicum* during Tween 40-induced glutamate overproduction. Metabolic flux distribution of *C. glutamicum* focused on anaplerotic reactions and the 2-oxoglutarate branch in the absence and presence of Tween 40 is shown. The flux for glucose uptake is normalized to 100

MS data calculated from the tuned metabolic flux distribution. The results indicated that PEPC rather than PC is active during the growth phase, whereas PC is active during Tween 40-induced glutamate production phase. These ^{13}C-MFA results are consistent with reported enzyme activity measurement (Hasegawa et al. 2008).

13.4.3 In Silico Prediction of Metabolic Flux Profiles Using a Genome-Scale Metabolic Model of C. glutamicum

Recently, genome-scale cellular metabolic networks have been reconstructed and metabolic flux balance analysis (FBA) has been conducted using reconstructed

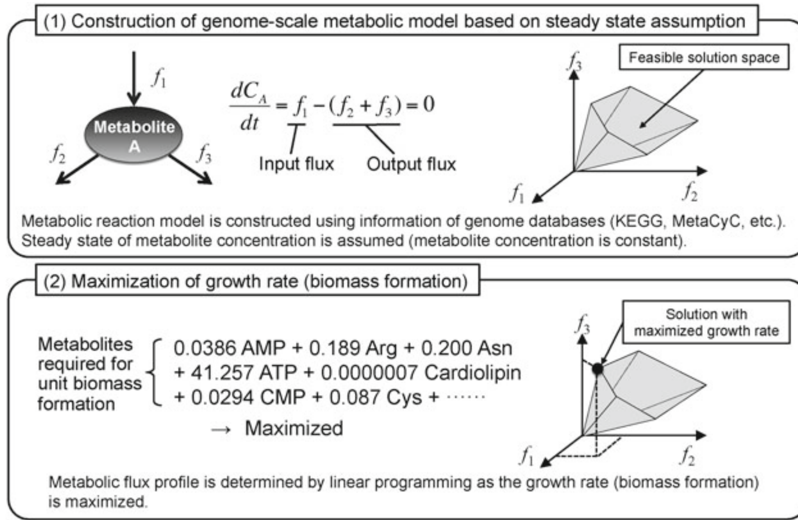

Fig. 13.7 Concept of prediction of metabolic flux profiles using genome-scale metabolic model based on flux balance analysis

genome-scale metabolic networks for bacteria, archaea and eukarya (Oberhardt et al. 2009). FBA is the analysis of metabolic flux profiles in which steady state of metabolic flux is assumed, and metabolic flux profiles are calculated by optimizing an objective function (Fig. 13.7). In FBA, the biomass production rate is generally used as the objective function to be optimized. Since it can be assumed that organisms maximize their growth rate by adaptation and evolution, prediction of metabolic profiles by FBA using genome-scale metabolic models is performed by maximizing the biomass production as an optimization process (Edwards and Palsson 2000; Edwards et al. 2001).

The genome-scale metabolic models of *C. glutamicum* were constructed and the metabolic flux profiles have been successfully predicted (Kjeldsen and Nielsen 2009; Shinfuku et al. 2009). Using genome-scale metabolic model of *C. glutamicum*, metabolic flux profiles under various oxygen supply conditions were simulated, and the predicted yield of CO_2 and organic acids agreed well with experimental data (Shinfuku et al. 2009).

Using genome-scale model of *C. glutamicum*, amino acid production can be also simulated as recently performed for lysine production (Shinfuku et al. 2009). In this simulation, lysine production was used as the objective function and was maximized with fixed glucose uptake and biomass production rates by Vallino and Stephanopoulos (1993). The simulated metabolic flux profile in central metabolism during maximized lysine production agreed with that determined experimentally. However, it is difficult to simulate metabolic flux during glutamate production in *C. glutamicum* using genome-scale metabolic models. As described above, various factors regulate glutamate production in *C. glutamicum*, including ODHC activity,

activities of anaplerotic reactions and conformational changes in mechanosensitive channel. Therefore, other modeling strategies that incorporate these regulatory mechanisms are necessary to simulate metabolic flux profiles during glutamate overproduction in *C. glutamicum*.

13.4.4 Enhancement of Glutamate Production Using Metabolic Engineering

Several studies have reported efforts to enhance glutamate production by *C. glutamicum*. An important target for improvement of glutamate production is the 2-oxoglutarate branch in the TCA cycle. As described above, deletion of the *odhA* gene encoding a catalytic subunit of the ODHC resulted in glutamate overproduction without the need for a trigger (i.e. biotin limitation, Tween 40 addition, or penicillin addition) (Asakura et al. 2007). Expression of *odhA* antisense RNA, which is expected to abolish translation of OdhA, enhanced Tween 40-induced glutamate production, whereas overexpression of *odhA* reduced Tween 40-induced glutamate overproduction (Kim et al. 2009). In contrast, overexpression of the *odhI* whose product inhibits ODHC activity resulted in continuous glutamate production without the need for a trigger (Kim et al. 2010) (Fig. 13.8).

Another target for enhancement of glutamate production is anaplerotic reactions. Peters-Wendisch et al. (2001) examined glutamate production by *pyc*-disrupted and *pyc*-overexpressing strains of *C. glutamicum* treated with Tween 60. The *pyc* gene encodes PC, and Tween 60 induces glutamate production similarly to Tween 40. Glutamate production in the *pyc*-overexpressing strains was higher than that in the wild-type strain. In addition, glutamate production could be decreased by disrupting *pyc*. Yao et al. (2009b) reported that *pyc* disruption enhanced glutamate production in the *dtsR1*-disruptant without requiring a trigger, but glutamate production in this strain could be reduced by disrupting *ppc*, which encodes PEPC.

13.4.5 Metabolic Engineering for Glutamate Production from Renewable Resources

Recently, a number of different groups have attempted to produce useful chemicals including amino acids from renewable resources, using microbial cells. Metabolic engineering has been applied to *C. glutamicum* to enhance its ability and to utilize biomass resources and waste byproducts obtained during industrial production processes for producing chemical compounds. In this section, we discuss metabolic engineering of glutamate production from renewable resources in *C. glutamicum*.

Utilization of pentose sugars such as xylose and arabinose, which are obtained by hydrolyzing lignocellulosic biomass, has been attempted in *C. glutamicum*.

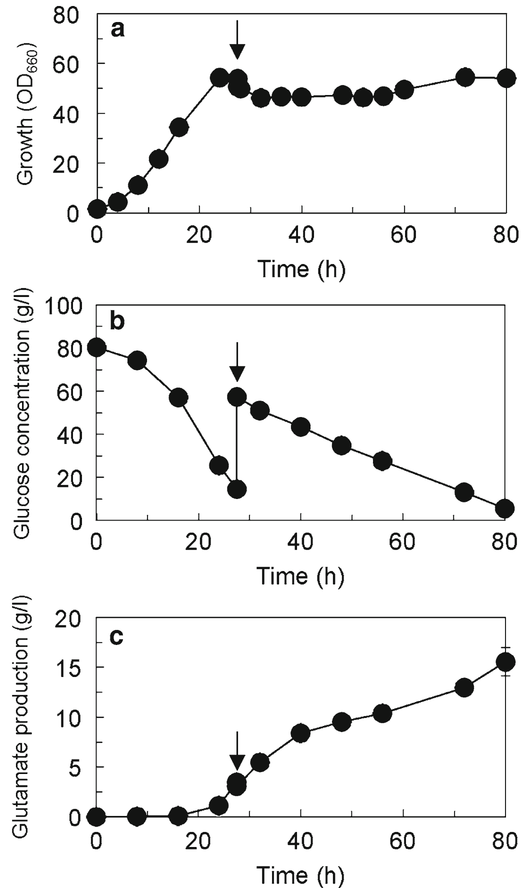

Fig. 13.8 Continuous glutamate production by the *odhI*-overexpressing *C. glutamicum* recombinant strain. The strain was cultivated aerobically and glucose was supplemented prior to glucose depletion (i.e. 27.5 h after starting cultivation, indicated by arrows). Time course of cell growth (**a**), glucose concentration in culture supernatant (**b**), and glutamate concentration in the culture supernatant (**c**) are shown

A *C. glutamicum* recombinant strain carrying *E. coli xylA* and/or *xylB* genes encoding xylose isomerase and xylulokinase, respectively, can grow on xylose as the sole carbon source (Kawaguchi et al. 2006). Moreover, Kawaguchi et al. (2008) indicated that a strain expressing the *E. coli araBAD* genes is able to grow on arabinose as the sole carbon source. Schneider et al. (2011) successfully achieved ethambutol-induced glutamate production from arabinose in a strain carrying this *E. coli araBAD* genes. Gopinath et al. (2011) constructed a strain expressing *E. coli xylA* and *araBAD* genes, which was able to grow on both xylose and arabinose, and this strain produced glutamate, which is induced by ethambutol addition, from a mixture of xylose, arabinose, rice straw and wheat bran hydrolysates.

Glycerol is known as a major byproduct of biodiesel production. Therefore, utilization of glycerol for useful chemical production is an important objective of metabolic engineering. However, *C. glutamicum* cannot grow well on glycerol as the carbon source. Rittmann et al. (2008) reported that the *C. glutamicum* recombinant strain harboring the glycerol utilization pathway from *E. coli* (i.e. expressing

the *E. coli glpP*, *glpK*, and *glpD* genes, which encode glycerol facilitator, glycerol kinase and glycerol-3-phosphate dehydrogenase, respectively) can grow on glycerol and produce glutamate from glycerol.

An alternative strategy to produce useful chemicals from biomass resources is to use the recombinant strains secreting carbohydrate-degrading enzymes. Tsuchidate et al. (2011) constructed a recombinant *C. glutamicum* strain secreting endoglucanases derived from various microorganisms, and succeeded in Tween 40-induced glutamate production from β-glucan by this recombinant. Yao et al. (2009a) constructed a recombinant *C. glutamicum* strain expressing α-amylase from *Streptococcus bovis* on the cell surface. This strain was able to produce glutamate from starch.

13.5 Conclusion

Although the molecular mechanisms of glutamate overproduction are increasingly well known and much progress has been made in the metabolic engineering of glutamate production in *C. glutamicum*, there are a number of unknown factors that regulate glutamate overproduction and secretion. For example, it is not known how *odhI* expression is induced by the various triggers—biotin limitation, Tween 40 addition, and penicillin treatment. Moreover, the molecular mechanisms of glutamate secretion are still unclear.

Glutamate production by *C. glutamicum* can be achieved without cell growth, i.e. cells can be switched completely from the growth phase to the glutamate production phase. If this switching mechanism can be applied more generally to other production process of useful chemicals using microorganisms, efficient and cost-effective production processes could be developed for numerous compounds. Genome DNA sequencing of the *C. glutamicum* strains used in industrial glutamate production is ongoing (Lv et al. 2011, 2012). Once complete, this sequence information will help uncover the molecular mechanisms of glutamate production mechanism in *C. glutamicum* and facilitate the metabolic engineering of this microorganism.

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