論文 / 著書情報
Article / Book Information

題目(和文)	
Title(English)	Studies on the Regulatory Mechanism of Glutamate Metabolism in Corynebacterium glutamicum
著者(和文)	Tanyanut Supkulsutra
Author(English)	Tanyanut Supkulsutra
出典(和文)	学位:博士(工学), 学位授与機関:東京工業大学, 報告番号:甲第9298号, 授与年月日:2013年9月25日, 学位の種別:課程博士, 審査員:和地 正明,中村 聡,丹治 保典,福居 俊昭,小倉 俊一郎
Citation(English)	Degree:Doctor (Engineering), Conferring organization: Tokyo Institute of Technology, Report number:甲第9298号, Conferred date:2013/9/25, Degree Type:Course doctor, Examiner:,,,,
学位種別(和文)	博士論文
Type(English)	Doctoral Thesis

Doctoral Thesis (2013)

Studies on the Regulatory Mechanism of Glutamate Metabolism in *Corynebacterium glutamicum*

Tanyanut Supkulsutra

10D52091

Department of Bioengineering,

Faculty of Bioscience and Biotechnology,

Tokyo Institute of Technology

Contents

Abbreviations

AccBC	:	Acetyl-CoA carboxylase, biotin carboxyl carrier protein
AP	:	Alkaline phosphatase
CBB	:	Coomasie Brilliant Blue
Ct	:	Cycle threshold
DDW	:	Double-distilled water
DIG	:	Digoxigenin
DtsR1	:	Acetyl -CoA carboxylase subunit β
EF-Tu	:	Elongation factor Tu
FA gel	:	Formaldehyde agarose gel
FHA domain	:	Forkhead-associated domain
GDH	:	L-Glutamate dehydrogenase
HTH	:	Helix-turn-helix
IPTG	:	Isopropyl β-D-1-thiogalactopyranoside
IS	:	Isertion sequence
Km	:	Kanamycin
L medium	:	Lennox medium

LdhA	:	L-Lactate dehydrogenase
LldD	:	L-Lactate dehydrogenase [cytochrome]
LldP	:	L-Lactate permease
LldR	:	L-Lactate dehydrogenase regulator
MCS	:	Multiple cloning site
MscS	:	Small conductance mechanosentitive channel
MSG	:	Monosodium glutamate
OAA	:	Oxaloacetate
OD	:	Optical density
OdhA	:	2-oxoglutarate dehydrogenase E1 component (E1o)
ODHC	:	2-oxoglutarate dehydrogenase complex
OdhI	:	2-oxoglutarate dehydrogenase inhibitor
OP	:	Operon
PBP	:	Penicillin binding protein
PC	:	Pyruvate carboxylase
PCR	:	Polymerase chain reaction
PDH	:	Pyruvate dehydrogenase

PEP	:	Phosphoenolpyruvate
PEPC	:	Phosphoenolpyruvate carboxylase
PEPCK	:	Phosphoenolpyruvate carboxykinase
PK	:	Pyruvate kinase
PTS	:	Phosphotransferase system
qRT-PCR	:	Quantitative real-time polymerase chain reaction
RM system	:	Restriction modification system
RT-PCR	:	Reverse transcription polymerase chain reaction
SD sequence	:	Shine-Dalgarno sequence
SDS	:	Sodium dodecyl sulfate
SD	:	Standard deviation
SDS-PAGE	:	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	:	Scanning electron microscopy
TCA cycle	:	Tricarboxylic acid cycle
TRN	:	Transcriptional regulatory network
WT	:	Wild type

Chapter 1 Introduction

1-1 *C. glutamicum;* physiology and morphology

Corynebacterium glutamicum is a Gram-positive, aerobic bacterium, which was isolated by Japanese researchers in 1956 as an L-glutamate-producing bacterium (Kinoshita et al., 1957; Udaka, 1960). The natural habitats of *C. glutamicum* strains reported so far are soil, soils contaminated with bird feces, sewage and manure, vegetables, and fruits. Most strains of *C. glutamicum* form pale yellow or yellow colonies as shown in Fig. 1-1, but spontaneous color variants are easily found. *C. glutamicum* has ellipsoidal spheres to short rod-shaped cells, which are often arranged in V-formations. Their cells can be characterized by a unique mode of cell division called snapping division, which produces cells in angular and palisade arrangements as shown in Fig. 1-1. Of the structures *C. glutamicum* possesses, its cell wall is probably one of the most unique parts. Besides the peptidoglycan layer, the cell wall consists of short-chain mycolic acids, along with arabinogalactan polymers. *C. glutamicum* is non-motile, non-pathogenic and non-sporulating strain. It has been used as a producer of L-glutamate, which is used as a flavor enhancer in food industries for several decades. Several other amino acids such as lysine and threonine are also produced by fermentation using this bacterium (Hermann, 2003; Ikeda, 2003; Kimura, 2005; Kinoshita, 2005).

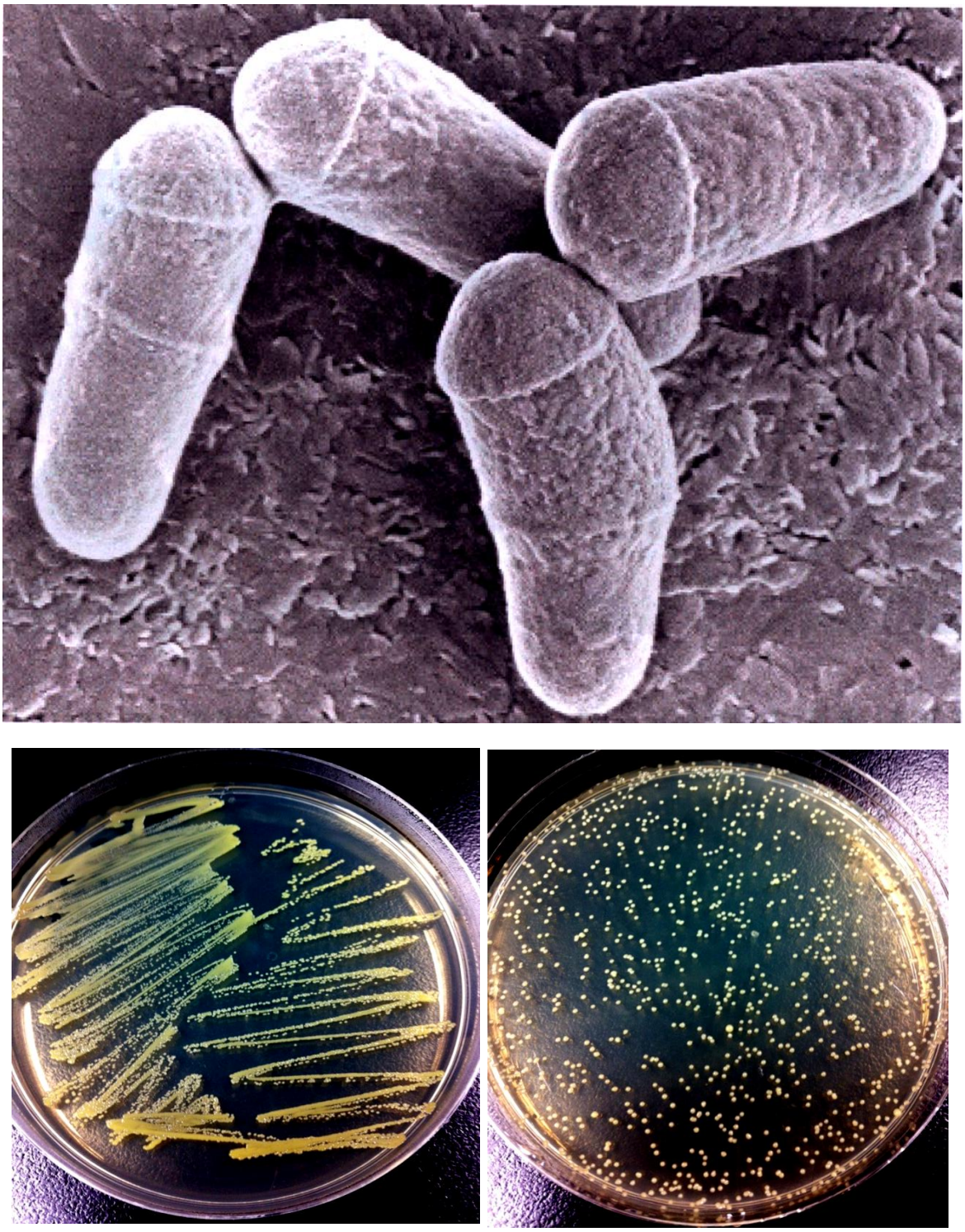


Fig. 1-1 ***C. glutamicum*** **cells morphology (SEM) and colony characteristic**

1-2 L-Glutamate biosynthesis in *C. glutamicum*; production, pathway and regulations

L-Glutamate was identified as a compound with distinctive taste known as “umami”, which is one of the five basic tastes aside from sweet, sour, salty, and bitter (Ikeda, 1908; O'Mahony and Ishii, 1986; Sano, 2009). More than 2.6 million tons of monosodium glutamate (MSG) is produced annually worldwide by fermentation using *C. glutamicum* or closely related species and its demand is still steadily increasing every year (Ajinomoto fact sheet: food product business, 2012). As shown in the L-glutamate metabolic pathway (Fig. 1-2), glucose is metabolized through glycolysis pathway and TCA cycle to the 2-oxoglutarate and is metabolized into L-glutamate in one-step conversion via glutamate pathway.

Wild-type *C. glutamicum* releases more than 80 g/L of L-glutamate under appropriate culture conditions. *C. glutamicum* has a unique mechanism of L-glutamate secretion, in which special treatments or growth conditions such as biotin limitation are required (Fig. 1-2). *C. glutamicum* is a biotin auxotroph, but the presence of biotin inhibits L-glutamate production (Shiio et al., 1962). Biotin is a cofactor of acetyl Co-A carboxylase, which forms a complex with a protein homolog of β subunit of acetyl-CoA carboxylase encoded by *dtsR1* gene (Kimura, 2002, 2003, 2005; Kimura et al., 1996; Kimura et al., 1999). Thus, the biotin limitation may affect fatty acid synthesis. Besides from biotin limitation, L-glutamate production is also induced in response to fatty acid ester surfactants treatment such as tween40 or tween60 (Shiio et al., 1963; Takinami et al., 1965; Takinami et al., 1968). Penicillin treatment can induce the L-glutamate production by binding to the

penicillin binding proteins (PBPs), which catalyzes the peptidoglycan synthesis (Eggeling et al., 2001; Nara et al., 1964; Nunheimer et al., 1970; Shiio et al., 1963). Ethambutol treatment also triggers L-glutamate production, which inhibits the mycolic acid formation in cell wall (Hashimoto et al., 2006; Radmacher et al., 2005). Moreover, the 2-oxoglutarate dehydrogenase complex (ODHC) activity has been shown to be decreased during L-glutamate production induced by biotin limitation, fatty acid ester surfactants, or penicillin treatment (Kawahara et al., 1997; Shingu and Terui, 1971). Since ODHC is located at the branched point between TCA cycle and L-glutamate biosynthesis pathway (Shiio et al., 1961; Shiio and Ujigawa-Takahashi, 1980), a decrease in ODHC activity was suggested to shift the metabolic flux from energy production via TCA cycle to L-glutamate production (Hasegawa et al., 2008; Shimizu et al., 2003). It was reported that disruption of *odhA*, which encodes the E1o subunit of ODHC, resulted in L-glutamate secretion without inducible treatment (Asakura et al., 2007). Metabolic linkage between acetyl-CoA carboxylase and ODHC is considered to be an important key for triggering L-glutamate secretion, as a disruption of *dtsR1* caused production of significant amount of L-glutamate and reduction in ODHC activity (Kimura et al., 1997). Recently, the unphosphorylated form of OdhI has been found to act as a strong inhibitor of the ODHC by binding to OdhA subunit (Niebisch et al., 2006). *odhI* disruption greatly diminished the glutamate production induced by biotin limitation, tween 40, penicillin, or ethambutol addition. Therefore, OdhI is essential for efficient L-glutamate production (Schultz et al., 2007). Moreover, it is also reported that OdhI synthesis is induced during the L-glutamate production triggered by tween 40 or penicillin treatment (Kim et al., 2010; Kim et al., 2011).

1-3 *C. glutamicum* glutamate exporter, NCgl1221; physiology and roles

It was recently shown that the NCgl1221 gene encoding a protein homolog of the small conductance mechanosensitive channel (MscS) is involved in the mechanism of L-glutamate secretion (Borngen et al., 2010; Hashimoto et al., 2010; Nakamura et al., 2007; Yamashita et al., 2013). Disruption of NCgl1221 greatly diminished L-glutamic acid secretion under biotin limited-conditions. Very recently, it has been demonstrated that the NCgl1221 channel is indeed served as a path for glutamate efflux (Hashimoto et al., 2012). Therefore NCgl1221 is necessary for efficient glutamate production. The mechanism of L-glutamate production by *C. glutamicum* was proposed. The inducible treatments lead to the alternation in membrane tension. NCgl1221 structure is then altered and activated, which leads to L-glutamate excretion (Fig. 1-2) (Demain and Birnbaum, 1968; Hashimoto et al., 2012; Nakamura et al., 2007; Wachi, 2013). NCgl1221 (V419::IS1207) mutation (Fig. 1-3), which results in a truncation of the C-terminal extracytoplasmic domain (420-533aa), induces constitutive L-glutamate production without any inducible treatments (Nakamura et al., 2007; Nakayama et al., 2012). This C-terminal domain may have a negative regulatory role in channel opening. The mutation of NCgl1221 also caused a 2-fold decrease of ODHC activity (Nakamura et al., 2007). Thus, NCgl1221 and ODHC should be connected by some unknown factor(s), which may be transcriptional regulators.

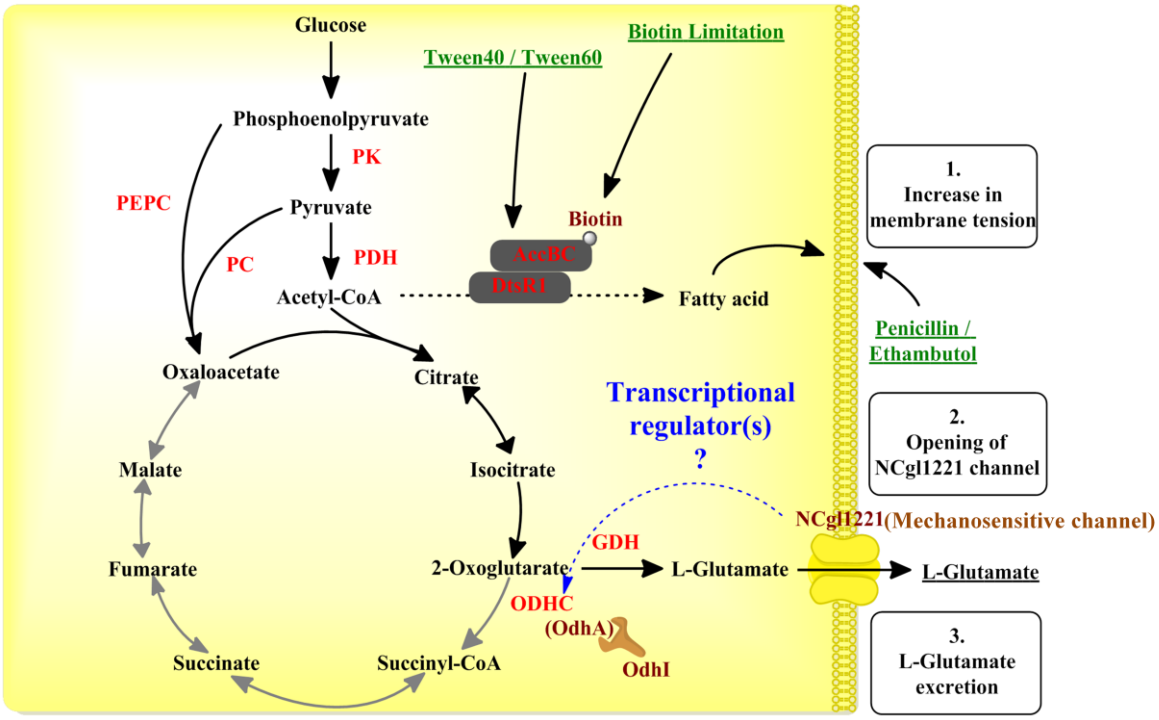


Fig. 1-2 **Metabolic pathway and a possible mechanism of L-glutamate production by *C. glutamicum*.**

(1) Inducing treatments leading to L-glutamate production alter the membrane tension by inhibiting lipid or cell wall synthesis.

(2) NCgl1221 structure is altered by sensing a change in membrane tension, which opens the mechanosensitive channel.

(3) Activated NCgl12211 exports L-glutamate as a compatible solute to prevent cell from bursting.

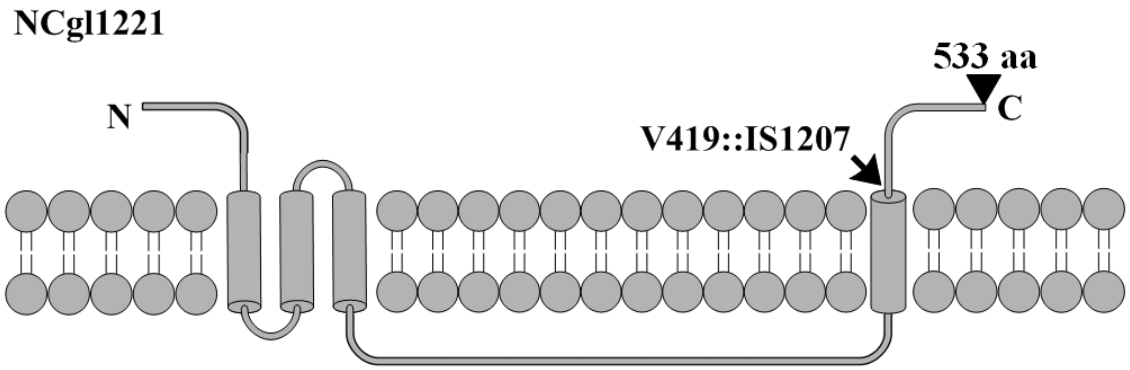


Fig. 1-3 **Membrane topology of NCgl1221 predicted by the PhD.htm program (http://www.predictprotein.org). An arrow indicates the mutation inducing constitutive L-glutamate production.**

1-4 Objectives and research contributions

The present research aims to identify the transcriptional regulator(s) in *C. glutamicum*, which connects NCgl1221 with glutamate metabolism. In this study, I mainly focused on the glutamate metabolism under biotin-limited conditions. Biotin limitation is a physiological treatment for glutamate production, which contains unclarified metabolic linkages between the treatment and metabolic profile under glutamate-producing conditions. It would be interesting to be able to understand more about the regulation and control of metabolic profile under glutamate-producing conditions, in which the treatment by biotin limitation could offer. As of the screening for candidate transcriptional regulator(s) by microarray analysis, two transcriptional regulators, NCgl2814 (LldR) and NCgl1386 were chosen for gene manipulation to study their roles in the glutamate metabolism under biotin-limited conditions.

In this study, I could show that NCgl2814 (LldR) is a repressor to the lactate metabolism which mainly regulates the lactate assimilation for glutamate production. Moreover, I also elucidated that lactate metabolism is required for efficient glutamate production under biotin-limited conditions, which prevents the accumulation of pyruvate under biotin-limited conditions, then lactate is later re-used for glutamate production. Since L-lactate has been long imaged as a waste by-product simultaneously formed during glutamate production, in this study, I would like to emphasize that L-lactate formation under biotin-limited, glutamate-producing conditions, is important and necessary for efficient glutamate production. This study would be advantaged to guide the future researches, which attempt to optimize metabolic flow for glutamate production, to

recognize lactate metabolism for candidate adjustment of metabolic flux for optimal highest glutamate yield.

The second transcriptional regulators, NCgl1386, in this study, was proven to form an operon with NCgl1385 (OdhI, ODHC inhibitor) and NCgl1387 (predicted function as RNase). NCgl1386 is shown to be an activator to this operon. Overproduction of NCgl1386 caused the inhibition of growth, which greatly affected glucose consumption and glutamate production. Additionally, NCgl1387 was also shown to inhibit glucose consumption and glutamate production by controlling the expression of this operon, negatively. NCgl1387 may function as an RNase. Regulation by RNase is unique; therefore, finding another regulatory mechanism of gene expression in *C. glutamicum* is expected by studying the function of NCgl1387. Although the regulatory mechanisms of NCg1386 and NCgl1387 are not yet clarified, it still would be interesting to be able to understand the roles of those two genes in the glutamate metabolism. Since NCgl1385 (OdhI) is a novel inhibitory protein directly control the glutamate metabolism, study of this operon would definitely open a path to a better understanding of glutamate metabolism.

Chapter 2 Screening for candidate transcriptional regulators involved in glutamate metabolism

2-1 Introduction to *C. glutamicum* transcriptional regulators in glutamate metabolism

Regulation of gene expression at the transcriptional level is a fundamental biological process to control the flow of information from a gene to its product and to the specific cellular function. Transcriptional regulation is typically mediated by DNA-binding proteins that interact with cognate target sites in the genome sequence and thereby regulate the expression of one or more target genes. The sum of such direct regulatory interactions at the transcriptional level represents the transcriptional regulatory network (TRN) of bacteria (Brinkrolf et al., 2010; Schroder and Tauch, 2010). In conjunction with a diverse metabolism, the TRN enables a rapid adaptation of microorganisms to changing environmental conditions. The main components of a TRN are regulatory proteins termed transcriptional regulators that sense diverse stimuli and bind to specific DNA sequences to control gene expression by altering transcriptional rates. Transcriptional regulators are often needed to be bound to a regulatory binding site to switch a gene on (activator) or to shut off a gene (repressor) (Brinkrolf et al., 2010).

TRN of *C. glutamicum* that is involved in the conversion of the carbon sources, such as sucrose, glucose and fructose, into the biotechnologically relevant products, L-lysine and L-glutamate, is shown in Fig. 2-1 (Brinkrolf et al., 2010). This partial network reconstruction revealed a high complexity of regulatory interactions in the central

metabolism of *C. glutamicum* exerted by 19 transcription regulators, including ten repressors, six dual regulators, one activator, and two sigma factors (in addition to the housekeeping sigma factor SigA) (Brinkrolf et al., 2010).

My research aims to identify transcriptional regulator(s) that is involved in the glutamate metabolism. *C. glutamicum* ATCC 13869 wild-type (WT) strain and NCgl1221 active mutant strain (BL1) carrying V419::IS1207 mutation were grown in the glutamate production medium containing 300 μg/L of biotin. WT cannot produce glutamate in the presence of biotin, while mutant BL1 can still produce glutamate in this condition. Total cellular RNA was isolated for microarray analysis. Genes encoding transcriptional regulators showing high fold-change between WT and BL1 were select as candidate gene(s) for further study.

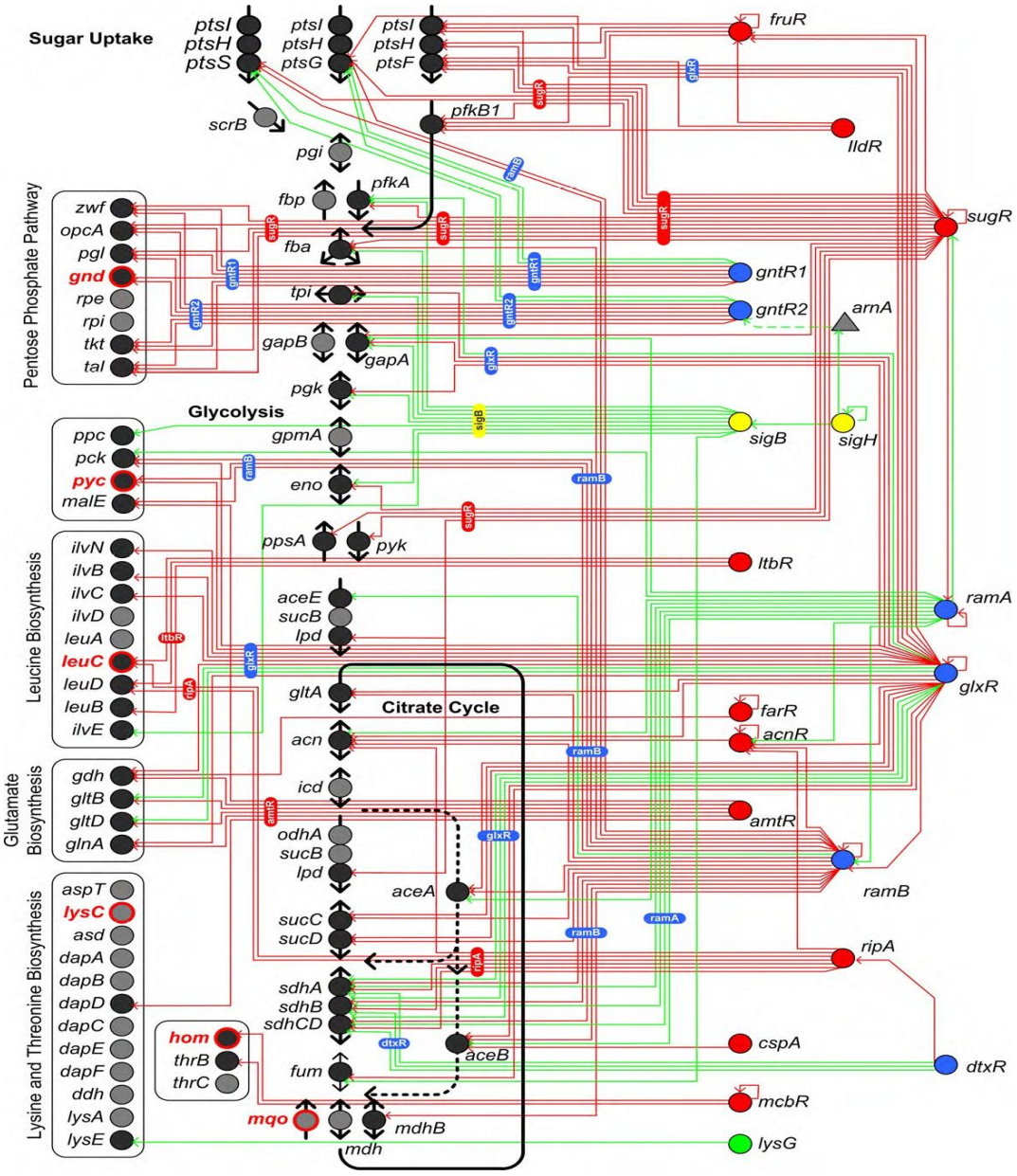


Fig. 2-1 **Transcription regulators involved in controlling the conversion of the sugar substrates into the amino acids L-lysine and L-glutamate in *C. glutamicum*. Reactions involved in glycolysis and the citrate cycle are shown by black arrows and are labeled by gene names. Colour code: red node, repressor; green node, activator; blue node, dual regulator; yellow node, sigma factor; dark-gray node, target gene; light-gray node, gene without regulation; red line; repression; green line, activation; gray triangle node, antisense RNA; green dashed line, positive control by antisense RNA (Brinkrolf et al., 2010).**

2-2 Materials and Methods

2-2-1 Bacterial strains and media

Strains used in the experiments are listed in Table 2-1. *C. glutamicum* and *E. coli* cells were cultured in Lennox (L) medium (1% polypeptone, 0.5% yeast extract, 0.5% NaCl, 0.1% glucose, pH 7.0). For the glutamate production by biotin limitation experiment, the preculture medium contained (per liter): 80 g glucose, 30 g $(NH_4)_2SO_4$, 1.0 g KH_2PO_4, 0.01 g $MnSO_4 \cdot 5H_2O$, 0.4 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 200 μg vitamin B_1 HCl, 13.72 ml soybean hydrolysate (total nitrogen, 0.48 g) and 5 μg biotin (pH 8.3). For the main culture, biotin was excluded from the preculture medium and 0.25 g of $CaCO_3$ was added to the 5 ml culture to maintain pH. *C. glutamicum* strain cultured in L medium for 24 h was directly inoculated into the preculture medium at 1% volume. After 24 h cultivation, the preculture was directly inoculated into the main culture medium at 5% volume for L-glutamate fermentation. All media were cultivated at 30°C. Kanamycin (20 μg/ml) was added to the medium at the start culture if needed.

2-2-2 Competent cells and transformation

Plasmid pECt (Sato et al., 2008) (Table 2-1) isolated from *E.coli* JM109 and JM110; *C. glutamicum* ATCC 13869, ATCC 13032, and ATCC 31831 by FastGene™ Plasmid Mini Kit (Nippon Genetics, Japan) were used. Concentrations of isolated plasmids were determined by NanoDrop **(**Thermo Fisher Scientific, Wilmington, DE, USA). To prepare competent cells of *C. glutamicum* ATCC 13869, ATCC 13032 and ATCC 31831, cells grown in L broth were harvested when the optical density of the culture at 660 nm

(OD_{660}) reached 1.0. Cells were washed with DDW and 15% glycerol solution, and then re-suspended again with 15% glycerol solution. 100 μl of re-suspended cells were transformed with 5 μl of pECt plasmids (Sato et al., 2008) by electroporation system ECM399 (BTX Harvard Apparatus, MA, USA) at 1.9 kV. Transformed cells were cultered with 1 ml of fresh L medium in water bath at 30°C, 180 rpm for 2 h. Transformed cells were then centrifuged at low speed and the supernatants were discarded. Cell pellets were suspended in 100 μl of L medium and spread on the 1.5% L agar plates containing 20 μg/ml of kanamycin. Plates were incubated at 30°C for 24 h. Transformation efficiency is measured in transformants per μg DNA used.

2-2-3 Growth and glutamate production assay

For growth measurement, overnight cultures of each strain were diluted (1:50) with fresh L broth and incubated at 30°C with shaking. Cell growth was monitored by measuring OD_{660}. To measure glucose consumption and glutamate production, the main glutamate production cultures were sampled every 24 h. The supernatant of cultures were separated from cell pellet by centrifugation. Glucose and glutamate concentrations in the culture supernatant were measured using Biotech-analyzer AS-210 (Sakura Seiki, Japan) with a glucose oxidase sensor, or glutamate oxidase sensor respectively

2-2-4 Microarray analysis

WT and BL1 cells grown for 3 h in L medium and for 5 h in main culture medium for glutamate production were used for total RNA isolation. Two volume of RNAprotect Bacterial Reagent (Qiagen, Valencia, CA, USA) were added directly to cultures to stabilize

cellular RNAs. Cells were harvested by centrifugation and total cellular RNA was isolated using an RNeasy Midi Kit (Qiagen). To eliminate residual genomic DNA, RNA preparations were treated with RNase-Free DNase I (Qiagen). Quality and concentration of RNA samples were determined by NanoDrop (Thermo Fisher Scientific). *C. glutamicum*-specific microarray was carried out by Roche Diagnostic K.K., Japan.

2-2-5 Northern blot analysis

Total RNA isolation of WT and BL1 strain from production medium were carried out as mentioned in section 2-2-4. RNA samples (Fig. 2-7a) were run in the formaldehyde-agarose (FA) gel and then blotted to 0.45 μm Nylon membranes (positively charged, Roche, Germany). Probes against *odhA, odhI, dtsR1* or *accBC* genes were prepared from the PCR product amplified with PrimeSTAR® HS DNA polymerase (Takara, Japan) by the primer s(Operon, Japan) listed in Table 2-2 from the chromosomal DNA of *C. glutamicum* ATCC 13869 isolated with High Pure PCR Template Preparation Kit (Roche, Germany). PCR products were purified by PCR Product Purification Kit (Qiagen, CA, US) and labeled with DIG-High Prime (Roche). Probe and membrane were mixed in the preheated hybridization buffer (DIG Easy Hub, Roche) in the hybrization tube. The tube is then incubated in the hybridization incubator, HB-80 (TAITEC, Japan) at 65°C, 45 rpm for 30 min, followed by the post hybridization washes as stated in the DIG Easy Hub (Roche) protocol. Membrane was washed and blocked by DIG Wash and Block Buffer Set (Roche). Anti-Digoxigenin-AP, Fab fragments (Roche) and CDP-Star Solution (Roche) were used for the immunological detection on film. The film was developed and fixed by Rendol and Renfix solutions (Fuji Photo Film, Japan).

Table 2-1 Bacterial strains and plasmid

Strain	Relevant characteristics	Source or reference
E. coli		
JM109	*recA1 endA1 gyrA96 thi hsdR17* e14$^-$ (*mcrA*$^-$) *supE44 relA1* Δ(*lac-proAB*)/F' (*traD36 proAB*$^+$ *lacI*^{q} *lacZΔM15*)	**Yanisch-Perron et al., 1985**
JM110	*dam dcm supE44 hsdR17 thi leu rpsL1 lacY galK galT ara tonA thr tsx Δ*(*lac-proAB*)*/*F′(*traD36 proAB*$^+$ *lac I*^{q} *lacZΔM15*)	**Yanisch-Perron et al., 1985**
C. glutamicum		
ATCC13869	Wild type (WT)	**Ajinomoto Co., Ltd**
ATCC13869	BL1; NCgl1221 active mutant containing V419::IS1207 mutation	**Nakamura et. al, 2007**
ATCC13032	Wild type (WT)	**American Type Culture Collection**
ATCC31831	Wild type (WT)	**American Type Culture Collection**
Plasmid	**Relevant characteristics**	**Source or reference**
pECt	Km^r ; *E.coli – C. glutamicum* shuttle vector, containing *trc* promoter and *lacI*^{q}	**Sato et al., 2008**

Table 2-2 PCR primers

Primer name	Sequence 5′ → 3′
OdhA-F	GTAGACGAGATGTTCCAGCAGTTCC
OdhA-R	CATACCGATGACAACTTCGTCGAGG
AccBC-F	TCACCAAGGTTCTTGTCGCTAACC
AccBC-R	GAATGCTGGGTTTTCCACGATGTG
DtsR1-F	CCCATTGATCGGTCTTTACGAAGG
DtsR1-R	GACTTAGCCAGAGCTACGGTATCG
OdhI-F	ATGAGCGACAACAACGGCACCCC
OdhI-R	CTCAGCAGGGCCTGCGAGGAAAACC

2-3 Results

2-3-1 Restriction profiles of *C. glutamicum* strains

C. glutamicum ATCC 13032 was isolated by Kyowa Hakko Co. Ltd. ATCC 13032 is a type culture of *C. glutamicum.* ATCC 13869 was isolated by Ajinomoto Co. Ltd. ATCC 31831 is a laboratory strain and has been used for genetic analysis in our laboratory. Each bacterial strain contains different types of restriction modification system (RM system), which could affect the transformation efficiency during the process of strain constuction. The restriction profiles of *C. glutamicum* strains were examined to achieve the optimal transformation efficiency of *C. glutamicum* strains using plasmid obtained from various strains of *E. coli* and *C. glutamicum*. The result as shown in Table 2-3 indicated that each *C. glutamicum* strain harbors different types of RM system. Highest transformation efficiency was achieved by plasmids isolated from homologous strains. Plasmid from *E. coli* JM109 (r^-, dam^+/dcm^+) (Yanisch-Perron et al., 1985) showed the highest efficiency for *C. glutamicum* ATCC 13869, while plasmid from *E. coli* JM110 (r^+, dam^-/dcm^-) (Yanisch-Perron et al., 1985) showed the highest efficiency for *C. glutamicum* ATCC 31831. However, plasmid from both *E. coli* strains showed lower transformation efficiency for *C. glutamicum* ATCC 13032. Since *C. glutamicum* ATCC 31831 showed relatively higher transformation efficiencies compared with ATCC 13032 and ATCC 13869, ATCC 31831 strains was used for further genetic manipulations such as gene disruption and overproduction.

2-3-2 Growth and glutamate production assay

C. glutamicum ATCC 13869 WT and BL1 strains were used to compare growth and glutamate production. BL1 strain is the strain, in which NCgl1221 gene has been replaced with the NCgl1221 V419::IS1207 mutation, resulting in truncation of the C-terminal extracellular domain. V419::IS1207 mutation induces constitutive L-glutamate production without any inducible treatments. In this study, BL1 was used just only for the screening, since its metabolic profiles were easy to investigate (flux toward L-glutamate, constitutively). Firstly, both strains were cultured in L medium and glutamate production medium as described in section 2-2-1 and 2-2-3. BL1 strain showed slower growth than WT (Fig. 2-2), which may be resulted from the mutation that causes constitutive glutamate production (Nakamura et al., 2007).

From these results, the condition to distinguish growth and glutamate production between WT and BL1 was set up. Overnight culture from L medium was directly inoculated into main production medium without preculture. Main glutamate production medium (per liter) containing 10 g of glucose and 300 μg of biotin was used. The medium did not contain $CaCO_3$. This condition should distinguish WT, which cannot produce L-glutamate in the presence of biotin, from the mutant BL1, which can still produce the L-glutamate. To measure growth, pH, glucose consumption and glutamate production, the main glutamate production cultures were sampled every 2 h from 0 h to 10 h and 24 h. As expected, BL1 still produced much higher glutamate while WT did not produce glutamate (Fig. 2-3a, c). In BL1 strain, after 6 h, the glutamate production stopped due to the accumulation of the L-glutamate, which acidified the medium because the medium did not

contain $CaCO_3$ (Fig. 2-3b). At the 3 h of growth in L medium (indicated in Fig. 2-2 by rectangle) and 5 h of growth in production medium (indicated in Fig. 2-3a by rectangle), were chosen for RNA isolation and protein extraction for further analysis.

2-3-3 Gene expression profiles of the wild-type and NCgl1221 active mutant strains

The result of the microarray data analysis is summarized in Table 2-4 and in Fig. 2-4. From the result of microarray analysis in L medium, 13 genes were up-regulated and 53 genes were down-regulated more than 2-fold in BL1 mutant cells compared with WT. In production medium, numbers of genes up-regulated were 18, that were almost similar to that in L medium, but numbers of genes down-regulated were greatly increased. This is probably because of very slow growth of BL1 mutant in production medium where metabolic flux shifted toward L-glutamate production instead of growth. In Table 2-5 to 2-7, genes involved in glycolysis, TCA cycle, anaplerosis, fatty acid, L-lactate and L-glutamate biosynthesis are listed. Genes, which showed high fold-changes in only one medium, are shown by blue and genes, which showed high fold-changes in both medium, are shown by green. Focusing on the central metabolic pathways, four genes as shown in Fig. 2-5, *pck*, which encodes the anaplerotic enzyme, phosphoenolpyruvate carboxykinase, involved in the back flux in anaplerosis, *accBC*, which is a biotin binding protein in fatty acid biosynthesis, *odhA*, which encodes E1o of the ODHC, and its inhibitor, *odhI*, were down-regulated in both medium.

Moreover, genes encoding for transcriptional regulators, which showed high fold-changes, are listed in Table 2-8. NCgl1386 and NCgl2814 were highly down-regulated in

both medium. NCgl1386 is a transcriptional regulator of the MerR family. MerR family is known to be an activator involved in heavy metal resistance. NCgl2814 (LldR) is a transcriptional repressor that belongs to a FadR subfamily of GntR family. It has been characterized as an L-lactate-responsive transcriptional regulator of L-lactate utilization genes (Gao et al., 2008; Georgi et al., 2008; Teramoto et al., 2011). These genes were manipulated for overexpression and deletion strain for further analysis.

To confirm the microarray analysis at the transcriptional level of gene expression, northern blot analysis of several genes were performed. From the northern blot analysis result (Fig. 2-6b), transcriptional level of *odhA*, *odhI*, *dtsR1* and *accBC* genes showed consistency with the microarray result (on the right panel). All genes were down-regulated in the production medium.

Table 2-3 Transformation efficiency of *C. glutamicum* strains

Strain (plasmid recipient)	Strain (plasmid donor)	Colony (transformants)	Transformation efficiency
ATCC13869 (Ajinomoto)	JM109	150	1.50×10^3
	JM110	16	1.60×10^2
	ATCC13869	289	2.89×10^3
	ATCC31831	8	8.00×10^1
	ATCC13032	21	2.10×10^2
ATCC31831 (Lab. strain)	JM109	15	1.50×10^2
	JM110	128	1.28×10^3
	ATCC13869	176	1.76×10^3
	ATCC31831	>1000	$>1.00 \times 10^4$
	ATCC13032	78	7.80×10^2
ATCC13032 (Kyowa Hakko) (Type strain)	JM109	1	1.00×10^1
	JM110	0	0
	ATCC13869	4	4.00×10^1
	ATCC31831	0	0
	ATCC13032	134	1.34×10^3

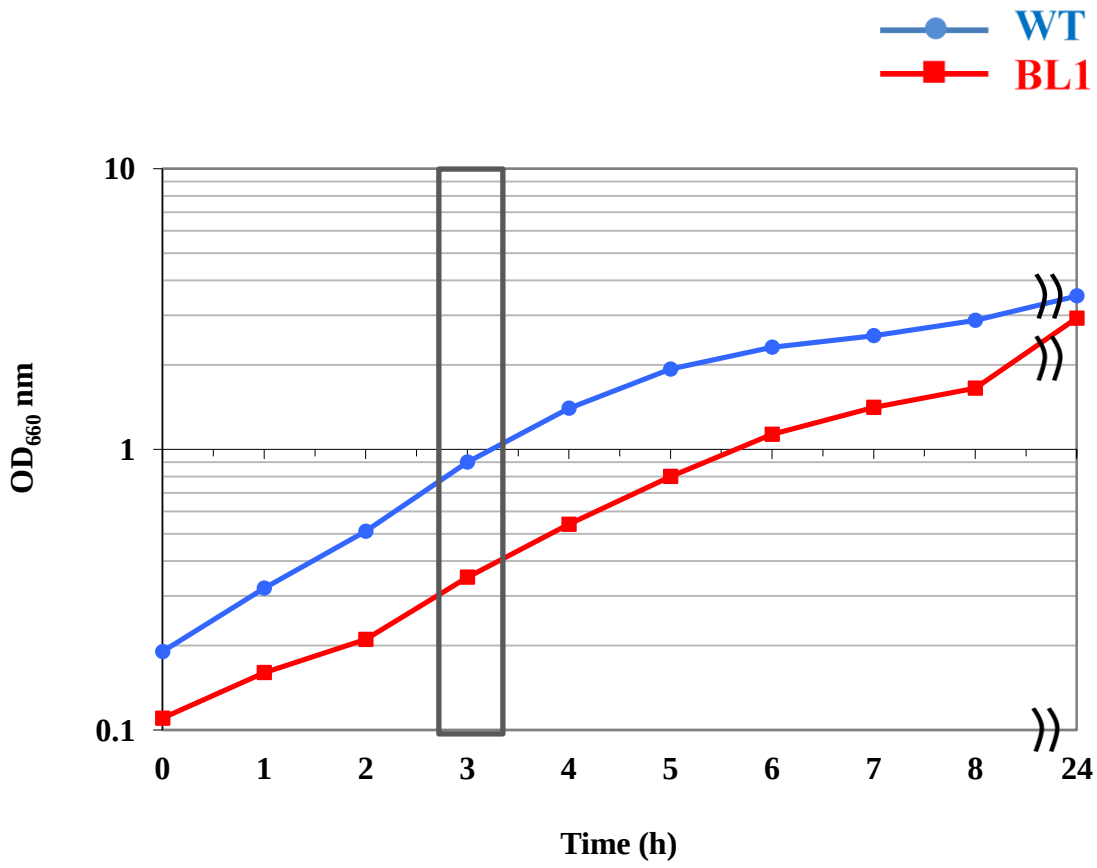


Fig. 2-2 **Comparison of growth between WT and BL1 strain in L medium. Growth was monitored by measuring OD_{660} in L medium. Rectangle indicate the time chosen for RNA isolation or protein extraction for further analysis**

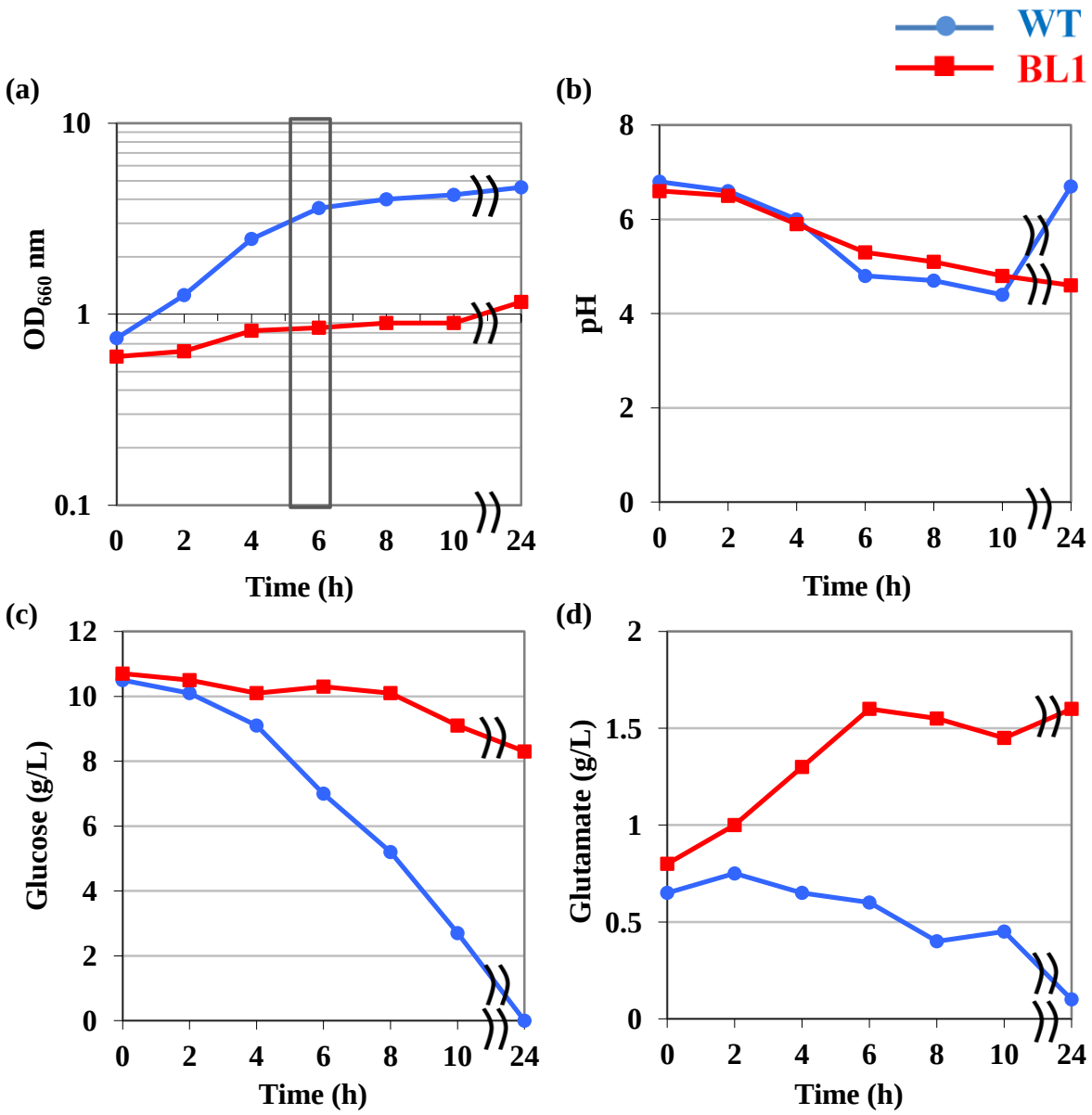


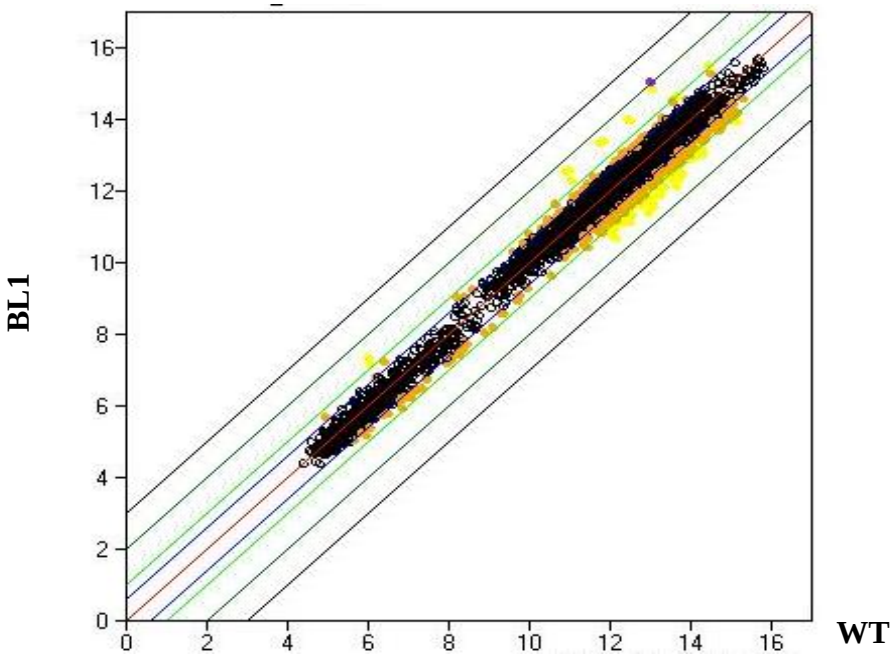
Fig. 2-3 **Comparison of cell growth (a), culture pH (b), glucose consumption (c), and glutamate production (d) of WT and BL1 grown in production medium with biotin supplement. Rectangle indicate the time chosen for RNA isolation or protein extraction for further analysis**

Table 2-4 Summary of microarray analysis.

Sample	Fold change (BL1/WT)	Number of genes (Total 6,040 genes)
L medium	8-fold up	0
	4-fold up	1
	2-fold up	12
	2-fold down	53
	4-fold down	0
	8-fold down	0
Production medium	8-fold up	0
	4-fold up	2
	2-fold up	16
	2-fold down	249
	4-fold down	35
	8-fold down	17

Remark: colors represent genes in scatter plot (Fig. 2-5)

(a) L medium



(b) Production medium

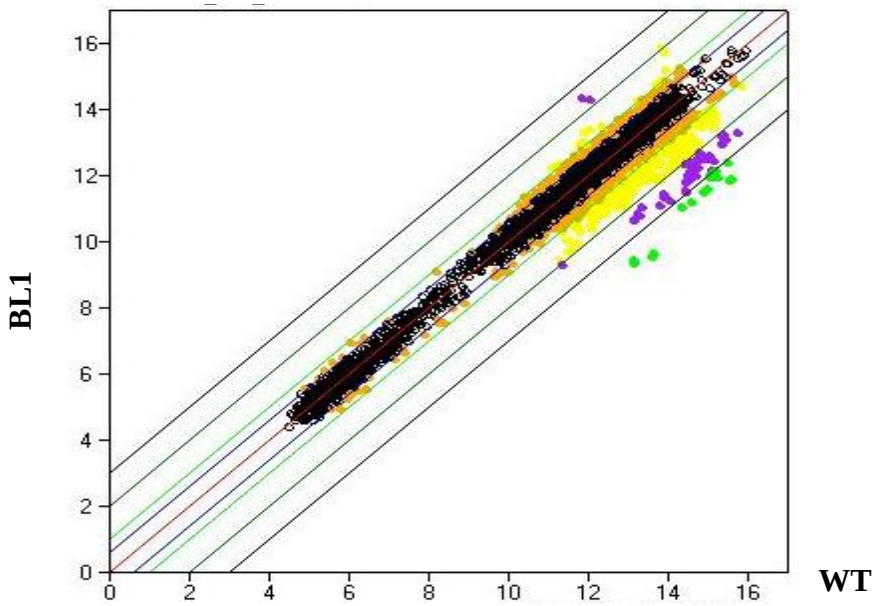


Fig. 2-4 Scatter plot of BL1/WT fold change in (a) L medium (b) production medium

Table 2-5 Microarray analysis of glycolytic pathway genes

Gene Name	Locus Tag	Description	Fold change (BL1/WT)	
			L medium	Production medium
pgi	**NCgl0817**	Glucose-6-phosphate isomerase	**1.23 down**	**1.69 down**
pfkB	**NCgl1202**	6-Phosphofructokinase	**1.13 up**	**1.01 down**
fbp	**NCgl0976**	Fructose-1,6-bisphosphatase	**1.26 down**	**2.22 down**
fda	**NCgl2673**	Fructose bisphosphate aldolase	**1.20 down**	**1.30 down**
tpi	**NCgl1524**	Triosephosphate isomerase	**1.19 down**	**1.06 down**
gapA	**NCgl1526**	Glyceraldehyde-3-phosphate dehydrogenase	**1.74 up**	**1.12 up**
gapB	**NCgl0900**		**1.46 down**	**1.84 down**
pgk	**NCgl1525**	3-Phosphoglycerate kinase	**1.20 up**	**1.47 down**
pgm	**NCgl0390**	Phosphoglycerate mutase	**1.59 down**	**2.39 down**
eno	**NCgl0935**	Enolase	**1.11 up**	**1.47 down**
pyk	**NCgl2008**	Pyruvate kinase	**1.15 down**	**2.29 down**
pps	**NCgl0528**	Phosphoenolpyruvate synthetase	**1.08 up**	**1.02 up**
ptsI	**NCgl1858**	Phophoenolpyruvate sugar phosphotransferase	**2.43 up**	**1.04 down**
aceE	**NCgl2167**	Pyruvate dehydrogenase	**1.09 down**	**1.06 down**

Table 2-6 Microarray analysis of TCA cycle genes

Gene Name	Locus Tag	Description	Fold change (BL1/WT)	
			L medium	Production medium
gltA	**NCgl0795**	Citrate synthase	**1.03 up**	**2.05 down**
acn	**NCgl1482**	Aconitase	**1.07 down**	**2.01 down**
icd	**NCgl0634**	Isocitrate dehydrogenase	**1.39 down**	**2.94 down**
odhA	**NCgl1084**	Alpha-ketoglutarate decarboxylase	**1.66 down**	**2.24 down**
lpdA	**NCgl0658**	Dihydrolipoamide dehydrogenase	**1.07 down**	**1.42 down**
sucB	**NCgl2161**	Dihydrolipoamide acetyltransferase	**1.05 up**	**2.68 down**
sucC	**NCgl2477**	Succinyl-CoA synthetase	**1.02 up**	**1.20 up**
sucD	**NCgl2476**		**1.12 up**	**1.21 up**
sdhC	**NCgl0359**	Succinate: menaquinone oxidoreductase	**1.12 down**	**1.24 down**
sdhA	**NCgl0360**		**1.25 down**	**2.61 down**
sdhB	**NCgl0361**		**1.11 down**	**3.07 down**
fum	**NCgl0976**	Fumarase	**1.26 down**	**2.22 down**
mqo	**NCgl1926**	Malate: quinone oxidoreductase	**1.13 down**	**2.41 down**
odhI	**NCgl1385**	Putatitive signal transduction protein, FHA domain	**1.67 down**	**2.02 down**

Table 2-7 Microarray analysis of anaperotic pathway, fatty acid, L-lactate and L-glutamate biosynthesis pathway genes

Gene Name	Locus Tag	Description	Fold change (BL1/WT)	
			L medium	Production medium
ppc	**NCgl1523**	Phosphoenolpyruvate carboxylase	**1.22 up**	**1.07 up**
pck	**NCgl2765**	Phosphoenolpyruvate carboxykinase	**1.69 down**	**3.10 down**
pyc	**NCgl0659**	Pyruvate carboxylase	**1.09 up**	**1.64 down**
accBC	**NCgl0670**	Biotin carboxylase (acyl-CoA carboxylase)	**1.67 down**	**2.81 down**
dtsR1	**NCgl0678**	Acetyl/propionyl-CoA carboxylase subunit beta	**1.10 down**	**2.02 down**
ldhA	**NCgl2810**	NAD-dependent L-lactate dehydrogenase	**1.25 up**	**1.53 up**
lldP	**NCgl2816**	Integral membrane transport protein, lactate permease	**1.22 up**	**1.40 down**
lldD	**NCgl2817**	Quinone-dependent L-lactate dehydrogenase	**1.07 down**	**1.18 dowm**
gdh	**NCgl1999**	Glutamate dehydrogenase	**1.36 up**	**1.04 up**
gluE	**NCgl1221**	Small-conductance mechanosensitive channel	**1.10 down**	**1.24 up**

Table 2-8 Microarray analysis of transcriptional regulator genes

Locus Tag	Gene	Protein family	Description	Fold change (BL1/WT)	
				L medium	Production medium
NCgl1386	**-**	**MerR**	Predicted transcriptional regulator	**1.709 down**	**2.167 down**
NCgl1504	***surfR***	**ArsR**	Repressor of iron-sulfur cluster biogenesis genes	1.045 up	**2.401 up**
NCgl1859	***fruR***	**DeoR**	Repressor of fructose PTS system genes	**2.924 up**	1.157 up
NCgl2794	***farR***	**GntR**	Repressor of arginine and glutamate biosynthesis genes	1.441 down	**1.776 down**
NCgl2814	***lldR***	**GntR**	Repressor involved in carbon metabolism	**2.335 down**	**3.381 down**
NCgl2877	**-**	**PadR**	Transcriptional regulator PadR-like family	1.038 down	**4.629 down**

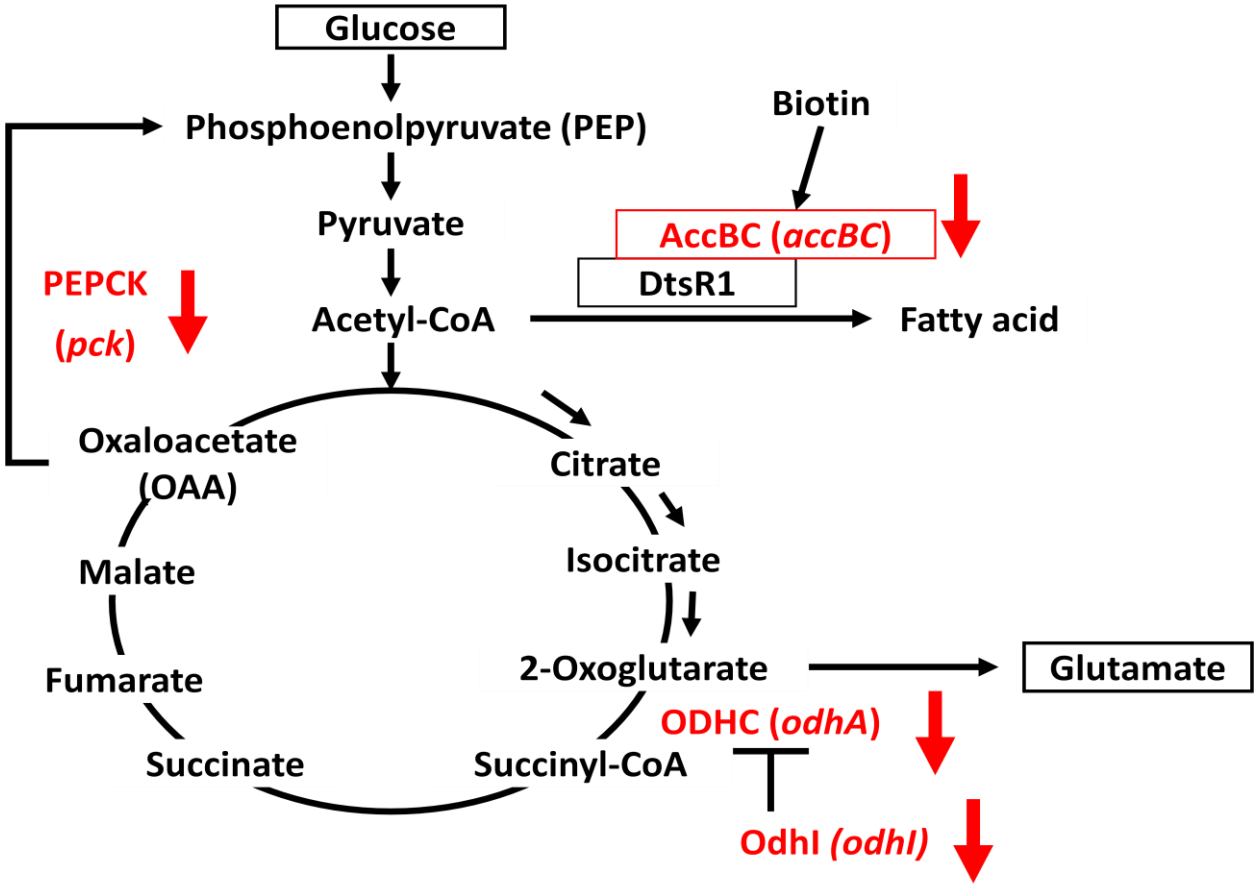
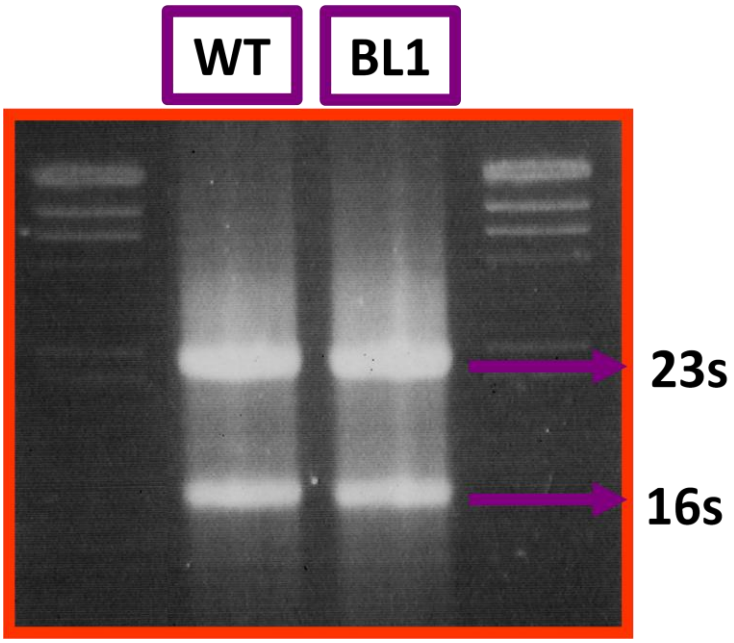


Fig. 2-5 **Schematic of genes in central metabolism which show highly down-regulated by microarray analysis in BL1 mutant**

(a)



(b)

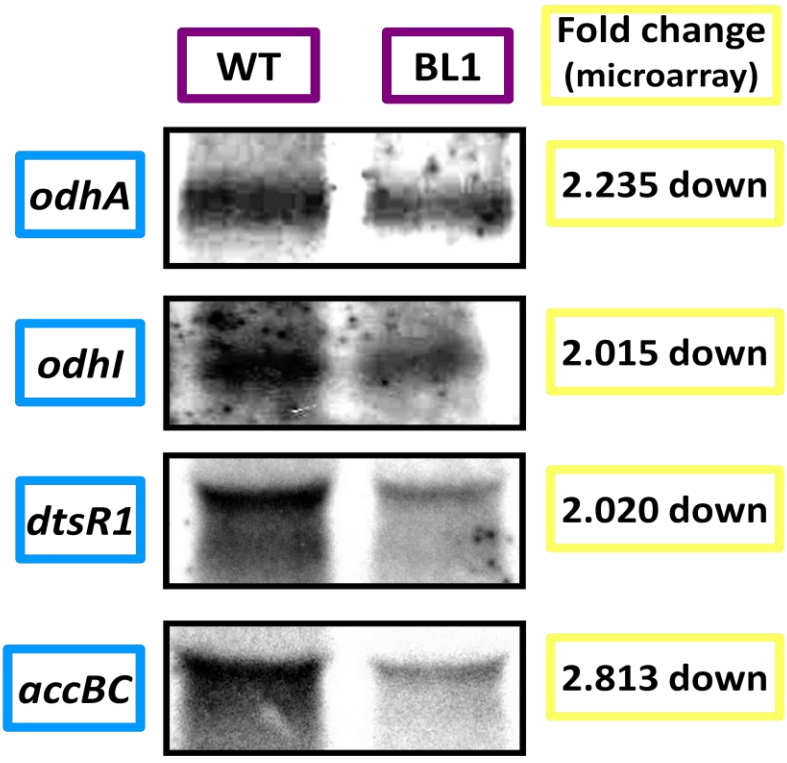


Fig. 2-6 **Comparison of (a) Total RNA isolation (b) Northern blot analysis between WT and BL1 in production medium**

2-4 Discussion

C. glutamicum NCgl1221 gene had been found to encode an L-glutamate exporter transmembrane protein, which is activated upon treatments causing the alternations in membrane tension such as biotin limitation, fatty acid surfactant or penicillin treatment. Disruption of this gene greatly diminished L-glutamate secretion. Specific mutations of this gene lead to a constitutive L-glutamate secretion without any treatments or inductions. From the previous research, the mutations of NCgl1221 also caused the 2-fold decrease of ODHC (2-Oxoglutarate dehydrogenase complex) activity, the rate-determining branchpoint enzyme for L-glutamate production. Thus, the mutations of NCgl1221 gene should result in reduction of ODHC. From this concept, the present research topic aims to identify the transcriptional regulator(s) in *C. glutamicum*, which connects NCgl1221 with glutamate metabolism.

The growth and glutamate production were compared between the *C. glutamicum* ATCC 13869 WT strain and NCgl1221 active mutant strain (BL1) (Fig. 2-2 and 2-3). L medium is used for normal growth. In L medium, both WT and BL1 did not produce glutamate. Production medium with excess biotin was used for the glutamate production. WT did not produce glutamate in the presence of biotin, while mutant BL1 could still produce the glutamate in this condition. Total cellular RNA was isolated for microarray analysis and northern blot analysis.

From the result of microarray analysis (Table 2-4), in L medium, 13 genes were up-regulated and 53 genes were down-regulated more than 2-fold in BL1 mutant cells. In

production medium, numbers of genes up-regulated were 18, that were almost similar to that in L medium, but numbers of genes down-regulated were greatly increased. This is probably because of very slow growth of BL1 mutant in production medium where metabolic flow is shifted from growth to glutamate production. Focusing on the central metabolic pathways, four genes as shown in Fig. 2-5, *pck*, which encodes the anaplerotic enzyme, phosphoenolpyruvate carboxykinase, involved in the back flux in anaplerosis, *accBC*, which is a biotin binding protein in fatty acid biosynthesis, *odhA* of the ODHC, and its inhibitor, *odhI*, were down-regulated in both medium. Some of these genes were chosen for the confirmation of microarray analysis by northern blot analysis. The northern blot analysis, as shown in Fig. 2-6b, the expression of *odhA*, *odhI*, *dtsR1* and *accBC* were down-regulated in production medium.

Genes encoding transcriptional regulators, NCgl1386 and NCgl2814, have been found to be highly down-regulated in the BL1 mutant cells (Table 2-8). NCgl1386 is a transcriptional regulator of the MerR family, while NCgl2814 (LldR) is a transcriptional regulator which belongs to a FadR subfamily of GntR family. In order to investigate the role of these transcriptional regulators in glutamate metabolism, deletion and overexpression strains of these genes have been constructed.

Chapter 3 Roles of transcritional regulators LldR (NCgl2814)

3-1 Introduction to LldR (NCgl2814)

L-Lactate is usually produced as a by-product during glutamate production. The excreted L-lactate is re-consumed, which may contribute to glutamate production (Sato et al., 2008; Stansen et al., 2005; Uy et al., 2003). *C. glutamicum* catalyzes the production of L-lactate from pyruvate with a NAD-dependent L-lactate dehydrogenase (EC 1.1.1.27) encoded by *ldhA* (Bott and Niebisch, 2003; Inui et al., 2004), while the quinone-dependent L-lactate dehydrogenase (EC 1.1.2.3) encoded by *lldD* is responsible for oxidation of L-lactate to pyruvate (Bott and Niebisch, 2003; Stansen et al., 2005) (Fig. 3-1). In *C. glutamicum*, *lldD* forms an operon with NCgl2816 encoding a permease for L-lactate utilization. The function of the permease gene NCgl2816 is dispensable, while LldD is essential for growth on L-lactate (Stansen et al., 2005). Recent study reported that overexpression of *ldhA* gene could restore the growth defect of *ΔlldD* strain when cells were grown on L-lactate as sole carbon source. This indicates LdhA can function *in vivo* to convert L-lactate to pyruvate (Sharkey et al., 2011). *lldR* (NCgl2814) as shown in Fig. 3-1, encodes a GntR-type transcriptional repressor of the NCgl2816-*lldD* operon and the repression by LldR is relieved in the presence of L-lactate. L-Lactate binds to LldR, preventing repression of NCgl2816-*lldD* by LldR (Gao et al., 2008; Georgi et al., 2008). LldR also acts as a repressor of *ldhA* in the absence of L-lactate, but *ldhA* expression is primarily repressed by the DeoR-type transcription regulator, SugR, in the absence of sugar (Engels et al., 2008; Toyoda et al., 2009a, b). Moreover, LldR also represses the *fruR-fruk-ptsF* operon responsible for fructose utilization (Gao et al., 2008). It was reported that

overexpression of *lldR* resulted in a decrease in expression of *ldhA* and NCgl2816-*lldD* operon, while disruption of *lldR* showed a significant increase in expression of NCgl2816-*lldD* operon (Gao et al., 2008; Georgi et al., 2008). Disruption of *lldR* showed a significant increase in *ldhA* expression in the absence of sugar (Gao et al., 2008) and showed no significant change in *ldhA* expression in the presence of sugar (Georgi et al., 2008).

In this study, I investigated the role of the transcriptional regulator LldR in the glutamate metabolism under biotin-limited conditions in *C. glutamicum*. By disruption and overexpression of *lldR*, it is suggested that L-lactate metabolism, which is controlled by LldR, has a buffering function of pyruvate pool for glutamate production.

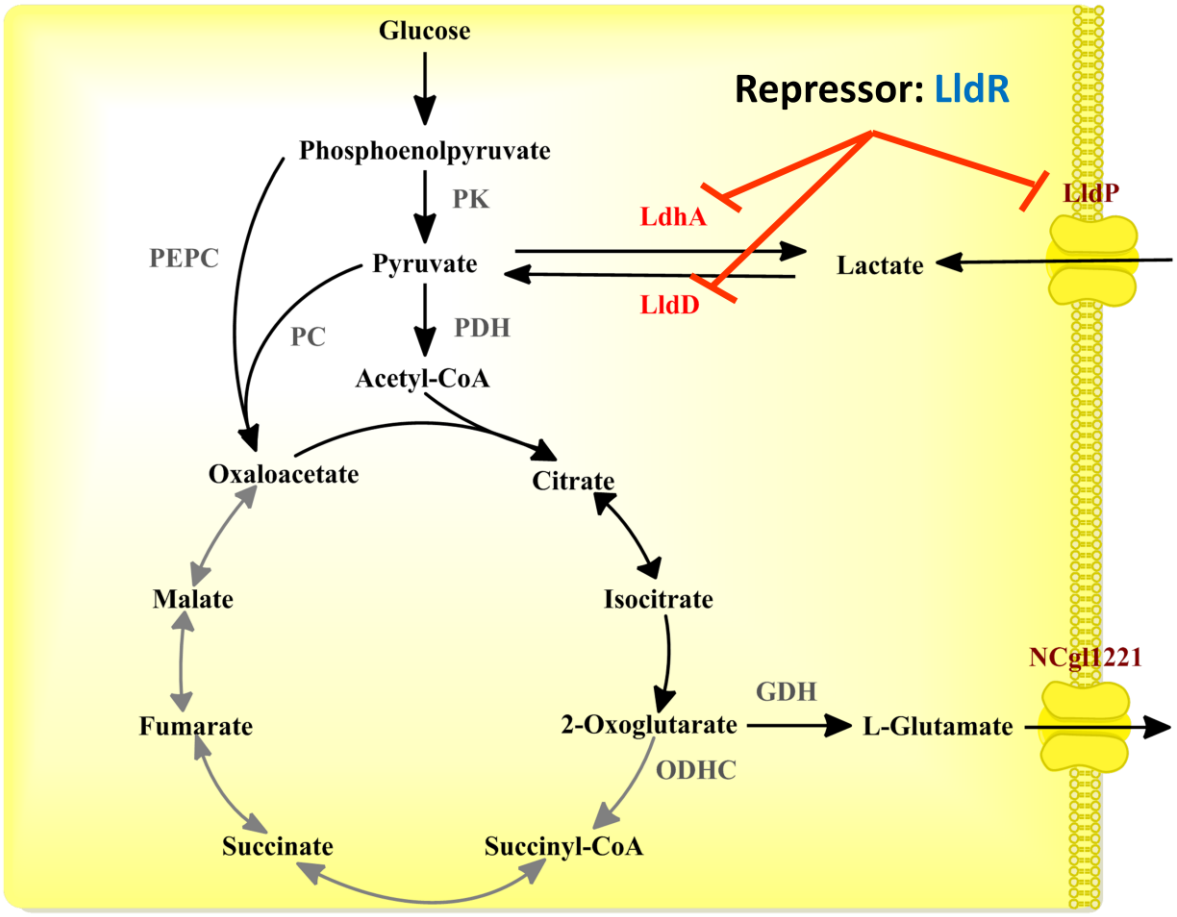


Fig. 3-1 **L-Glutamate metabolic pathway by *C. glutamicum* and a role of LldR repressor in the lactate metabolism**

3-2 Materials and Methods

3-2-1 Bacterial strains and media

Strains used in the experiments are listed in Table 3-1. *C. glutamicum* and *E. coli* cells were cultured in L medium (1% polypeptone, 0.5% yeast extract, 0.5% NaCl, 0.1% glucose, pH 7.0). For the glutamate production by biotin limitation, the preculture medium contained (per liter): 80 g glucose, 30 g $(NH_4)_2SO_4$, 1.0 g KH_2PO_4, 0.01 g $MnSO_4 \cdot 5H_2O$, 0.4 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 200 μg vitamin B_1 HCl, 13.72 ml soybean hydrolysate (total nitrogen, 0.48 g) and 15 μg biotin (pH 8.3). For the main culture, biotin was excluded from the preculture medium and 0.25 g of $CaCO_3$ was added to the 5 ml culture to maintain pH. *C. glutamicum* strain cultured in L medium for 24 h was directly inoculated into the preculture medium at 1% volume. After 24 h cultivation, the preculture was directly inoculated into the main culture medium at 5% volume for L-glutamate fermentation. Kanamycin (20 μg/ml) and 1mM IPTG was added to the medium at the start culture if needed. For the glutamate production by penicillin G addition, *C. glutamicum* strain cultured in L medium for 24 h was directly inoculated into the main culture medium at 2% volume. For the main culture, biotin was supply at 300 μg and the media was cultured until OD_{660} reached 2.0. 10 μM Penicillin G and 0.25 g of $CaCO_3$ were added to the medium. Kanamycin (20 μg/ml) and 1mM IPTG was also added at the if needed. All media were cultivated at 30°C.

3-3-2 Overexpression of *lldR*

The DNA fragment spanning the *lldR* gene but not its promoter region was amplified from chromosomal DNA of *C. glutamicum* ATCC 31831 (Fig.3-2) (isolated with High Pure PCR Template Preparation Kit (Roche, Germany)) by PCR (PrimeSTAR® HS DNA polymerase, Takara, Japan) with the primer pair LldR-F and LldR-R (Operon, Japan) as indicated in Table 3-2 and purified by PCR Product Purification Kit (Qiagen, CA, US). The putative Shine-Dalgarno (SD) sequence of *C. glutamicum pyc* gene was also introduced just upstream of the *lldR* gene for efficient translation. Plasmid pECt-Ptuf (Fig.3-2, Table 3-1) isolated by High Pure Plasmid Isolation Kit (Roche) was used. The DNA fragment was digested with *Nco*I and *Sal*I (Takara) and then ligated (Ligation Mighty Mix, Takara) with the vector pladmid pECt-Ptuf digested with *Nco*I and *Sal*I. Plasmid pECt-Ptuf-*lldR* was isolated from *E. coli* JM110 by High Pure Plasmid Isolation Kit (Roche) and the insertion of *lldR* was confirmed with restriction digest and sequencing.

To prepare competent cells of *C. glutamicum* ATCC 31831, cells grown in L broth were harvested when OD_{660} reached 1.0. Cells were washed with DDW and 15% glycerol solution, and then re-suspended again with 15% glycerol solution. 100 μl of re-suspended cells were transformed with 5 μl of ligated mixture by electroporation system ECM399 (BTX Harvard Apparatus) at 1.9 kV. Transformed cells were cultered with 1 ml of fresh L medium in water bath at 30°C, 180 rpm for 2 h. Transformed cells were then centrifuged at low speed and the supernatants were discarded. Cell pellets were suspended in 100 μl of L medium and spread on the 1.5% L agar plates contains 20 μg/ml of kanamycin. Plates were

incubated at 30°C for 24 h. Transformant of *C. glutamicum* ATCC 31831 *lldR*-overexpressing strain (WT/p*lldR*) was selected and used for further analysis.

3-2-3 Disruption of *lldR*

C. glutamicum ATCC 31831 *lldR* disruptant strain constructed previously was used (Gao et al., 2008). Deletion of *lldR* was confirmed by colony PCR (illustra puReTaq Ready-To-Go PCR Beads, Amersham Biosciences) as shown on Fig. 3-3. Deletion of the *lldR* gene was also confirmed by qRT-PCR assay as described later (Fig. 3-11a). Plasmid pECt-Ptuf-*lldR* isolated from *E. coli* JM110 was also introduced into ATCC31831 *lldR* disruptant, resulting in *lldR*-complemented strain (*ΔlldR*/p*lldR*).

3-2-4 SDS-PAGE

Cells grown in L medium for 24 h were harvested by centrifugation, then suspended in sodium phosphate buffer (50 mM, pH 7.0) and disrupted by sonication. Cell debris was removed by centrifugation. Quality and concentration of protein extracts were determined by NanoDrop (Thermo Fisher Scientific). Protein extracts were then separated in 10% polyarylamide gel by SDS-PAGE using the tris-glycine-SDS running buffer. The gel was stained with Coomassie Brilliant Blue (CBB).

3-2-5 Growth and glutamate production assay

For growth measurement in L medium, overnight cultures of each strain were diluted (1:50) with fresh L broth and incubated at 30°C with shaking. Cell growth was monitored by measuring OD_{660}. For growth measurement in production medium containing

$CaCO_3$, samples were diluted with 0.1 M HCl to dissolve $CaCO_3$ prior to measurement. To measure glucose consumption, glutamate production or lactate formation, the main cultures were sampled every 24 h. The supernatant of cultures were separated from cell pellet by centrifugation. Glucose, glutamate and lactate concentrations in the culture supernatant were measured using Biotech-analyzer AS-210 (Sakura Seiki, Tokyo) with a glucose oxidase sensor, glutamate oxidase sensor or lactate oxidase sensor, respectively.

3-2-6 Quantitative real time RT-PCR (qRT-PCR) analysis

Cells grown for 24 h in the main culture medium for glutamate production under biotin-limited conditions were used for total RNA isolation. $CaCO_3$ was removed by low speed centrifugation before use. Two volume of RNAprotect Bacterial Reagent (Qiagen) were added directly to the culture to stabilize cellular RNAs. Cells were harvested by centrifugation and total cellular RNA was isolated using an RNeasy Mini Kit (Qiagen). To eliminate residual genomic DNA, RNA preparations were treated with RNase-Free DNase I (Qiagen). Quality and concentration of RNA samples were determined by NanoDrop (Thermo Fisher Scientific). qRT-PCR assays were performed using the QuantiFast SYBR Green RT-PCR kit (Qiagen) and Eco^{TM} Real-Time PCR Systems (Illumina, San Diego, CA, USA). The quality and specificity of the amplification process was verified by melting curve analysis. The target gene transcripts were normalized to the reference gene transcript (16S rRNA) from the same RNA sample. Each gene was analyzed using RNA isolated from three independent samples. The cycle threshold (Ct) for each sample was generated according to the procedures described in the Eco ^{TM} Real-Time PCR System user guide. Primers (Operon) are listed in Table 3-3.

Table 3-1 Bacterial strains and plasmid

Strain	Relevant characteristics	Source or reference
E. coli		
JM110	*dam dcm supE44 hsdR17 thi leu rpsL1 lacY galK galT ara tonA thr tsx Δ*(*lac-proAB*)/F′(*traD36 proAB*$^{+}$ *lac I*^{q} *lacZΔM15*)	**Yanisch-Perron et al., 1985**
C. glutamicum		
ATCC31831	Wild type (WT)	**American Type Culture Collection**
ATCC31831	*ΔlldR*	**Gao et al., 2008**
ATCC31831	*ΔldhA*	**Sato et al., 2008**
Plasmid	**Relevant characteristics**	**Source or reference**
pECt	Km^{r} ; *E.coli – C. glutamicum* shuttle vector, containing *trc* promoter and *lacI*^{q}	**Sato et al., 2008**
pECt-Ptuf	Km^{r} ; pECt inserted the promoter of elongation factor Tu (*tuf*) gene into the upstream of the trc promoter	**Takeuchi et al., unpublished**
pECt-Ptuf-*lldR*	Km^{r} ; pECt-Ptuf containing the whole *lldR* gene	**This work**

Table 3-2 PCR primers

Primer name	Sequence 5′ → 3′	Generated restriction site
LldR-F	CGCCATGGGCGAGCTCGATCTAATGAG TGTCTAATGAGTGTGAAAGCACATGA	*Nco*I
LldR-R	TGAGGTCGACAACGGTGTTTTGG	*Sal*I
LldR-FW	AATGATCGGTACCCCGGTGAAC	*Kpn*I
LldR-RW	TGAGGTCGACAACGGTGTTTTGG	*Sal*I

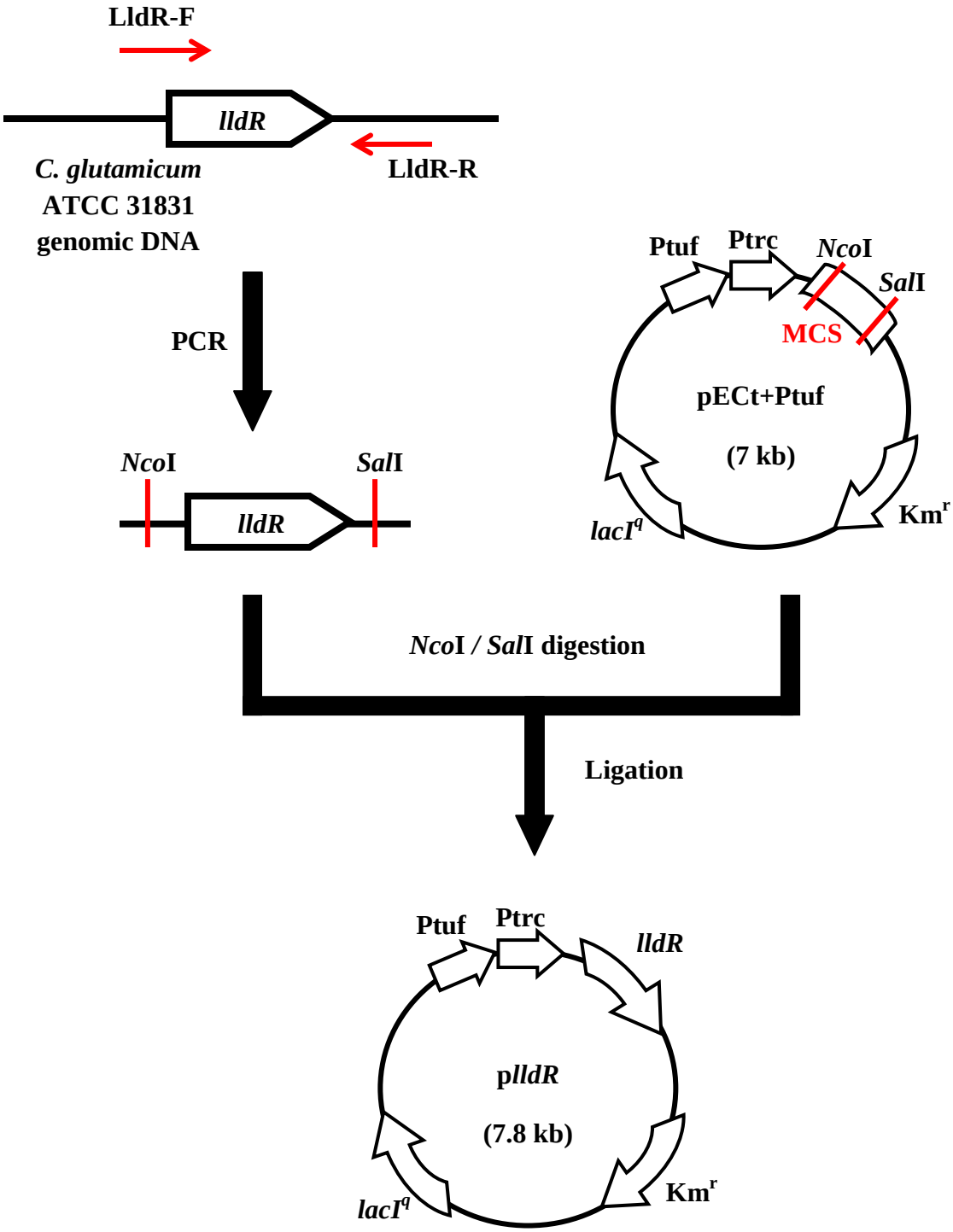


Fig. 3-2 **Construction of *lldR* overexpressing vector**

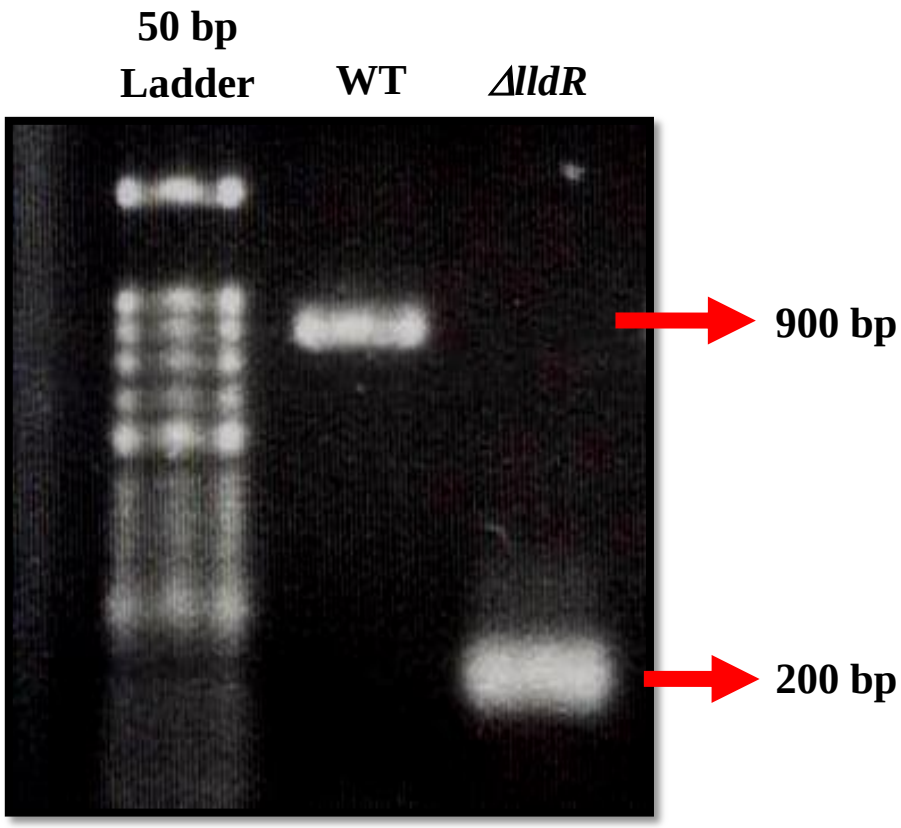


Fig. 3-3 **Confirmation of *LldR* disruption by colony PCR**

Table 3-3 qRT-PCR primers

Primer name	Sequence 5′ → 3′
lldR-qFW	GATTGGGTCACCGAGGAGCT
lldR-qRW	AAATGGTGCCGAGGGCTTCGA
lldD-qFW	GGTGAAACGTCAACTGCCCAAC
lldD-qRW	CTGGGGTGCGTCGTTTAGCA
ldhA-qFW	GTCCTCATTGGCGCAGGAGATG
ldhA-qRW	TCTTCGCAGTCAGCGTAGGTGC
16S-rRNA-F5	AGAGTTTGATCCTGGCTCA
16S-rRNA-F3	ACGTGTTACTCACCCGTTCG
16S-rRNA-R5	ACGTTCCCGGGCCTTGTACA
16S-rRNA-R3	CGGCTACCTTGTTACGAC

3-3 Results

3-3-1 Analysis of LldR overexpression by SDS-PAGE

Overexpression of LldR was confirmed by SDS-PAGE. Synthesis of a protein of about 26 kDa, which corresponds to LldR, was induced by IPTG induction in wild-type and *lldR*-disruptant cells transformed with pECt-*lldR* (Fig. 3-4). Overepression of the *lldR* gene was also confirmed by qRT-PCR assay as described later (Fig. 3-11a).

3-3-2 Effects of overexpression/deletion of *lldR* on growth and glutamate production under biotin-limited conditions

Firstly, I examined effects of overexpression and deletion of *lldR* on growth in L broth. Cell growth was monitored by measuring OD_{660}. There were no significant differences in growth rate between *C. glutamicum* strains WT, WT/p*lldR* without and with 1mM IPTG, and *ΔlldR* (Fig. 3-5). Overproduction and deletion of *lldR* also had no effects on the growth under biotin-limited conditions (Fig. 3-6a). These results indicate that overexpression and deletion of *lldR* have no effects on growth rate.

To clarify the role of LldR in glutamate production under biotin-limited conditions, we examined the effect of overexpression and deletion of *lldR* on glutamate production induced by biotin limitation as described in section 3-2-1 and 3-2-5 (Fig. 3-6, Table 3-4). After 120 h of cultivation, WT reached its maximal L-glutamate production at about 25 g/L, where glucose was almost depleted (Fig. 3-6b, c). By disrupting the *lldR* gene, glutamate production was slightly increased: this was observed reproducibly, although it was not significant statistically (Table 3-4). Glucose consumption was also slightly increased. *lldR*

disruptant reached its maximal L-glutamate production at about 26 g/L after 120 h cultivation, which was 3% increase compared with that of WT (Fig. 3-6c, Table 3-4). By overexpressing the *lldR* gene, the slightly decreased glucose consumption was observed (Fig. 3-6b, Table 3-4). Overexpression of *lldR* showed negative effect on L-glutamate production. At the 120 h culture, the LldR overproducer with the addition of IPTG (WT/p*lldR*+IPTG) reached its maximal L-glutamate production at about 21 g/L, which was decreased 16% compared with that of WT (Fig. 3-6c, Table 3-4). The yield of glutamate production was also decreased (Table 3-4). The LldR overproducer without IPTG (WT/p*lldR*−IPTG) also showed slightly decreased glutamate production at about 23 g/L, which was decreased 8% compared with that of WT, although it was not significant statistically (Fig. 3-6c, Table 3-4).

L-Lactate was simultaneously formed during glutamate production under biotin-limited conditions (Fig. 3-6d). In the wild-type cells, L-lactate was produced in the first 24 h and it was re-consumed thereafter. On the other hand, in the LldR overproducer with the addition of IPTG (WT/p*lldR*+IPTG), L-lactate was produced like WT for the first 24 h and it further increased for the next 24 h. The produced lactate was, however, not re-consumed afterward. L-Lactate reached its maximum at about 7 g/L after 120 h cultivation (Fig. 3-6d). WT/p*lldR*–IPTG showed a delay in re-consumption of L-lactate compared with WT (Fig. 3-6d). These indicate that overproduction of the LldR suppresses the lactate utilization. *lldR* disruptant formed less L-lactate than WT, although it was not significant statistically (Fig. 3-6d, Table 3-4).

3-3-3 Effects of deletion of *ldhA* on growth and glutamate production induced by biotin limitation and penicillin treatment

To examine the importance of lactate metabolism during glutamate production, *⊿ldhA* mutant strain (Sato et al., 2008) was used to compare with WT. *ldhA* disruption had no effect on growth in both L medium (Fig. 3-7) and production medium (Fig. 3-8a). In production medium, *ldhA* disruptant showed no lactate formation (Fig. 3-8d) as expected. Glucose consumption and glutamate production were significantly decreased compared with WT (Fig. 3-8b, c, Table 3-5). This suggests that lactate metabolism has positive effect on glutamate production under biotin-limited conditions.

It is well known that pyruvate carboxylase (PC) is a biotin containing enzyme, which converts pyruvate to oxaloacetate (Fig. 3-1). Therefore, biotin limitation causes the decreased intracellular level of biotin-bound PC and results in the intracellular accumulation of pyruvate (Fig. 3-9c). Under these conditions, glutamate production solely depends on the anaplerotic reaction catalyzed by phosphoenolpyruvate carboxylase (PEPC), which converts phosphoenolpyruvate to oxaloacetate (Fig. 3-1) (Sato et al., 2008). Enzymatic activities of pyruvate kinase (PK) and pyruvate dehydrogenase (PDH) decrease during glutamate production under biotin-limited conditions. Moreover, the enzymatic activity of PEPC and carbon flux from phosphoenolpyruvate to oxaloacetate increase under biotin-limited conditions, while the enzymatic activity of PC significantly decrease (Fig. 3-9) (Hasegawa et al., 2008). Disruption of *pyc*, which encodes PC, showed increased L-lactate production in addition to glutamate production under biotin-limited conditions, possibly because of pyruvate accumulation. The increased glutamate production suggested

that the produced lactate was reused by the cells to produce glutamate (Sato et al., 2008). In the *pyc ldhA* double disruptant, lactate formation was decreased but enhancement of glutamate production was not observed when compared with the *pyc* single disruptant. Glucose consumption decreased slightly compared with the *pyc* single disruptant (Sato et al., 2008).

To estimate the effect of biotin on pyruvate accumulation and glutamate production, the glutamate production induced by penicillin G addition was examined in WT and *ΔldhA* mutant as described on section 3-2-1 and 3-2-5. Under penicillin addition conditions where biotin was supplemented, the increase in PC activity was observed (Fig. 3-9b) (Hasegawa et al., 2008). After 120 h of cultivation, WT showed lower growth than in biotin-limited conditions (Fig. 3-8a), probably because penicillin inhibit cell wall synthesis. However, the glutamate production of WT was higher compared with that under biotin limitation, even though glucose was not yet depleted (Fig. 3-8b, c, 3-10b, c). *ΔldhA* showed increased growth rate compared with WT (Fig. 3-10a) and slightly increased glucose consumption and glutamate production compared with WT (Fig. 3-10b,c). L-Lactate was not produced in *ldhA* disruptant and rarely observed in WT (Fig. 3-10d), indicating that under penicillin addition conditions, carbon flux toward L-glutamate was higher than that in biotin-limited conditions. It seems that pyruvate was not accumulated, which resulted in higher L-glutamate production and less L-lactate formation (Fig. 3-8d, 3-10d). Moreover, *ΔldhA*, which eradicated the branched flux toward L-lactate, resulted in increase of metabolic flux toward growth and L-glutamate compared with WT under penicillin addition conditions.

3-3-4 Effects of overexpression/deletion of *lldR* on gene expression

To confirm the role of LldR that controls the gene expression in lactate metabolism under biotin-limited conditions, we performed the quantitative real-time RT-PCR (qRT-PCR) assays to compare the gene expression involved in lactate metabolism, *lldD* responsible for oxidation of L-lactate to pyruvate and *ldhA* responsible for the formation of L-lactate from pyruvate. qRT-PCR assay confirmed overexpression and deletion of *lldR*: levels of the *lldR* mRNA were about 5-fold and 16-fold in the WT/p*lldR* cells without and with IPTG, respectively, compared with that of WT, while it was negligible in the *⊿lldR* cells (Fig. 3-11a). Overexpression of *lldR* with the addition of IPTG caused a significant decrease in *lldD* expression, which was about 2.4-fold decrease compared with WT under biotin-limited conditions (Fig. 3-11b). Deletion of *lldR* caused a significant increase in *lldD* expression up to 3.2-fold compared with WT (Fig. 3-11b). On the other hand, both overexpression and deletion of *lldR* have no significant effect on *ldhA* expression (Fig. 3-11b). These results sugest that LldR mainly controls the expression of *lldD* gene but not *ldhA* gene, at least, under biotin-limited conditions.

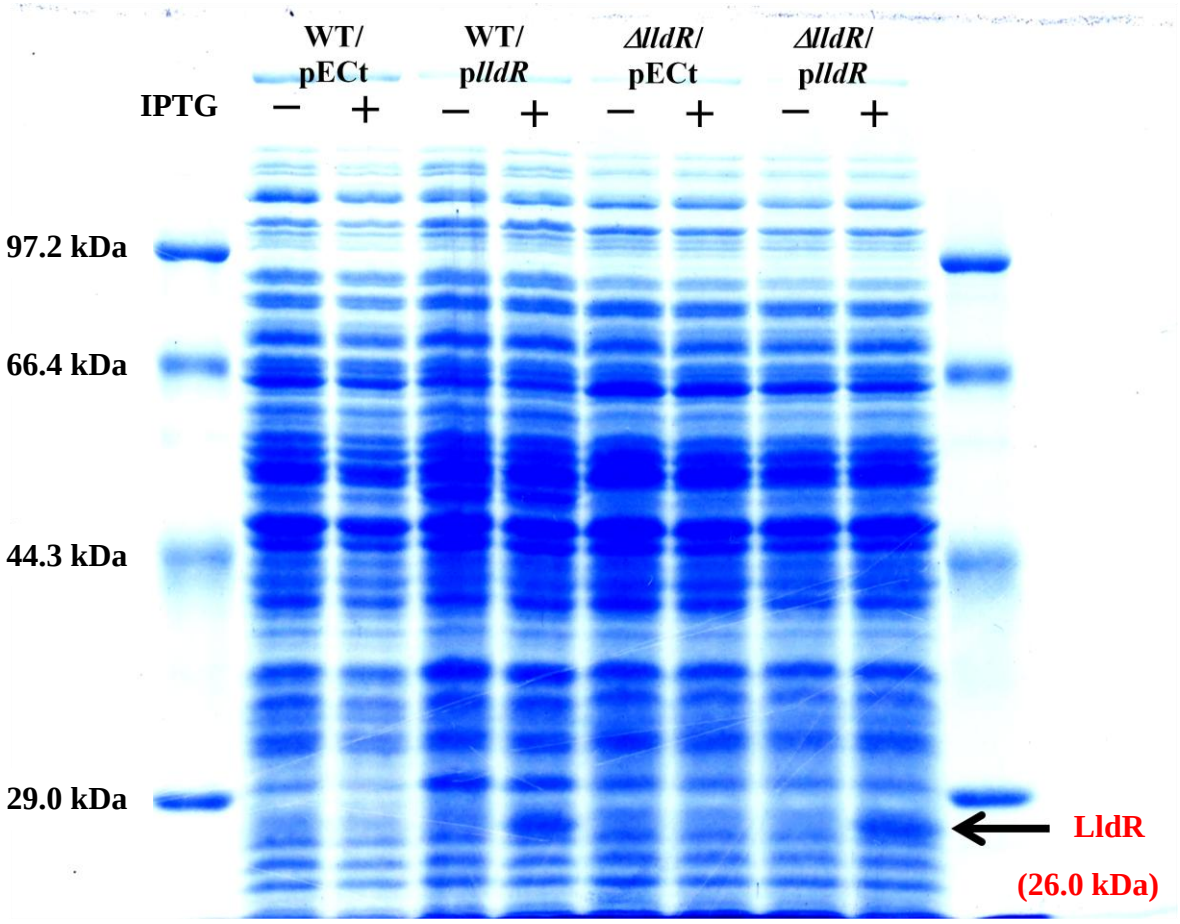


Fig. 3-4 **Confirmation of LldR overexpression by 10% polyarylamide gel SDS-PAGE. The signs "–" and "+" represent without and with 1mM IPTG addition, respectively. LldR overexpression indicates as arrows (26 kDa). WT and *ΔlldR* carried an empty vector were used as a negative control (WT/pECt and *ΔlldR*/pECt, respectively). Numbers shown to the left of gel are molecular weights (kDa).**

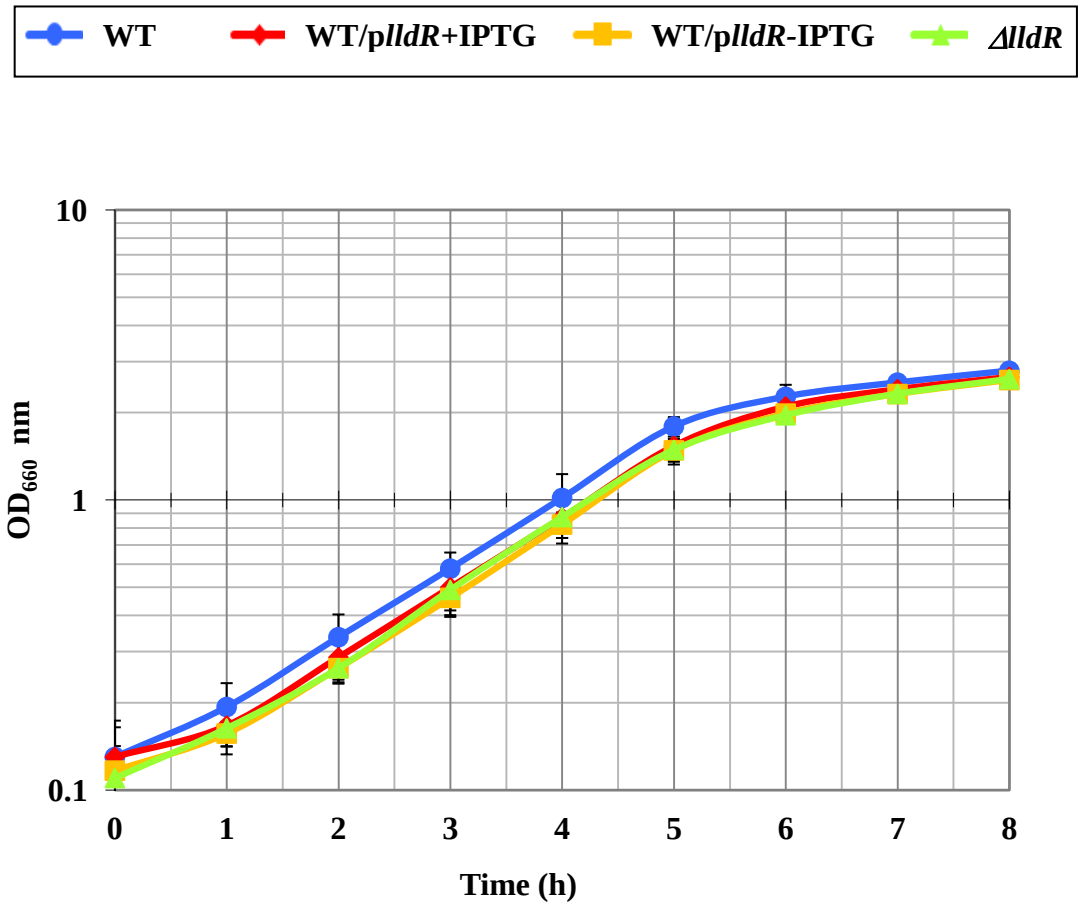


Fig. 3-5 **Effects of overproduction and deletion of LldR on growth in L medium. Growth of WT, LldR overproducers without or with 1mM IPTG addition (WT/p*lldR*-IPTG and WT/p*lldR*+IPTG, respectively), and *ΔlldR* in L broth are shown. Growth was monitored by measuring OD_{660}. The mean values of three independent experiments are presented with standard deviations.**

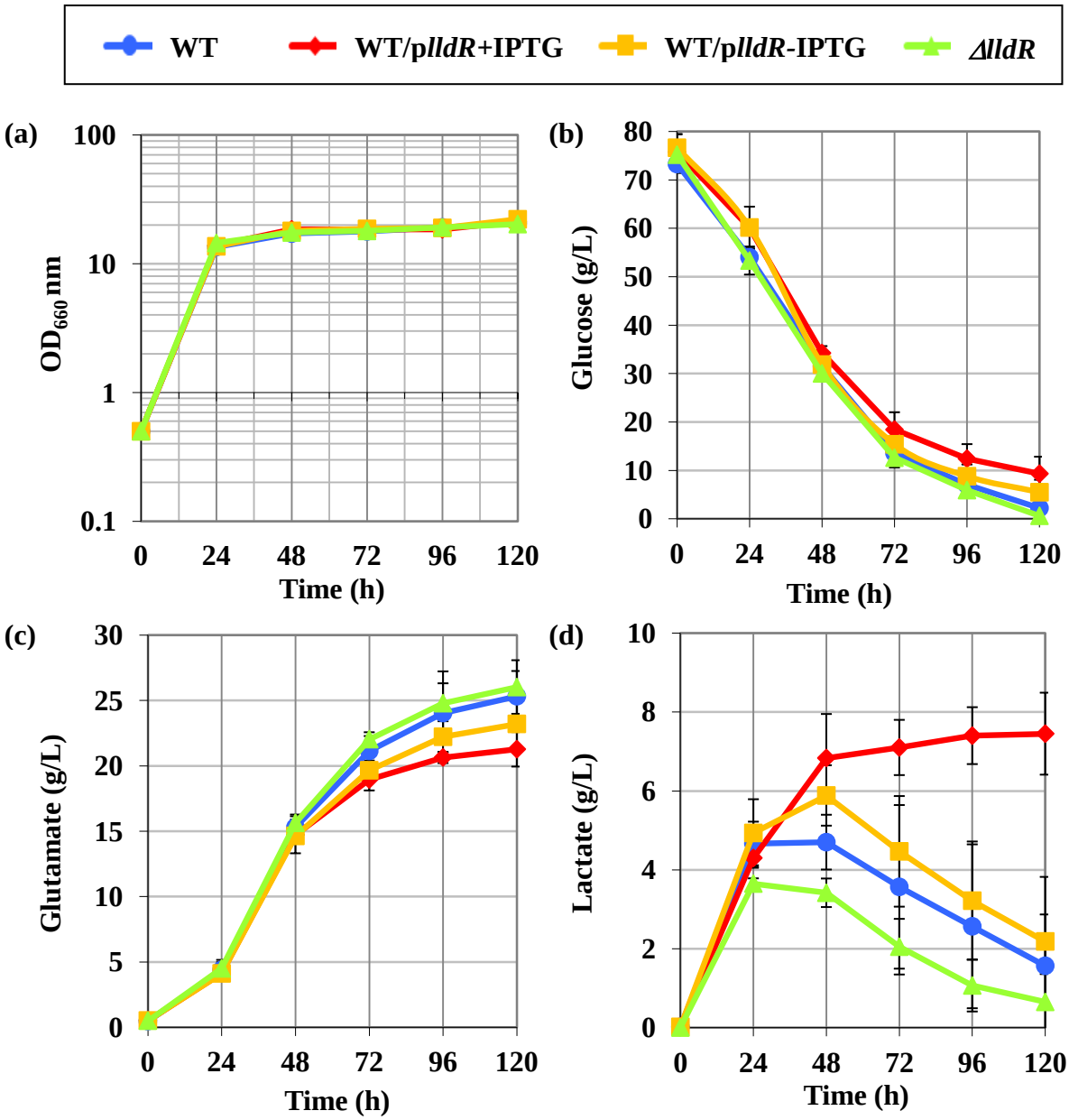


Fig. 3-6 **Effects of overproduction and deletion of LldR on glutamate production induced by biotin limitation. Cell growth (a), glucose consumption (b), glutamate production (c), and lactate formation (d) of WT, LldR overproducers without or with 1mM IPTG addition (WT/p*lldR*-IPTG and WT/p*lldR*+IPTG, respectively), and *ΔlldR* grown in production medium. The mean values of three independent experiments are presented with standard deviations.**

Table 3-4. Effects of overexpression or deletion of *lldR* on lactate and glutamate production under biotin-limited conditions

Strains	Glucose consumption (g/L)	Lactate formation (g/L)	Glutamate production (g/L)	Production yield [a] (%)	Specific productivity (g/ L•OD•day)
WT	71.0 ± 2.5	1.5 ± 1.3	25.3 ± 1.9	43.6 ± 1.9	0.25 ± 0.03
WT/p*lldR* −IPTG	71.1 ± 4.9	2.2 ± 1.6	23.2 ± 0.6	40.0 ± 2.4	0.21 ± 0.02
WT/p*lldR* +IPTG	66.4 ± 0.4 [c]	7.5 ± 1.0 [b]	21.3 ± 1.3 [c]	39.2 ± 2.6 [d]	0.20 ± 0.01 [d]
⊿lldR	74.6 ± 0.4 [d]	0.7 ± 0.7	26.0 ± 2.0	42.7 ± 3.5	0.26 ± 0.02

Glucose consumption, lactate formation, glutamate production and specific productivity under biotin-limited conditions at 120 h (5 days) are shown. Data represent means ± SDs for three independent experiments.

a) Production yield was calculated as;

$$\frac{\text{Glutamate production (g/L)} / 147}{\text{Glucose consumption (g/L)} / 180} \times 100$$

Student's t-test: b) $p < 0.01$, c) $0.01 \leq p < 0.05$, d) $0.05 \leq p < 0.1$

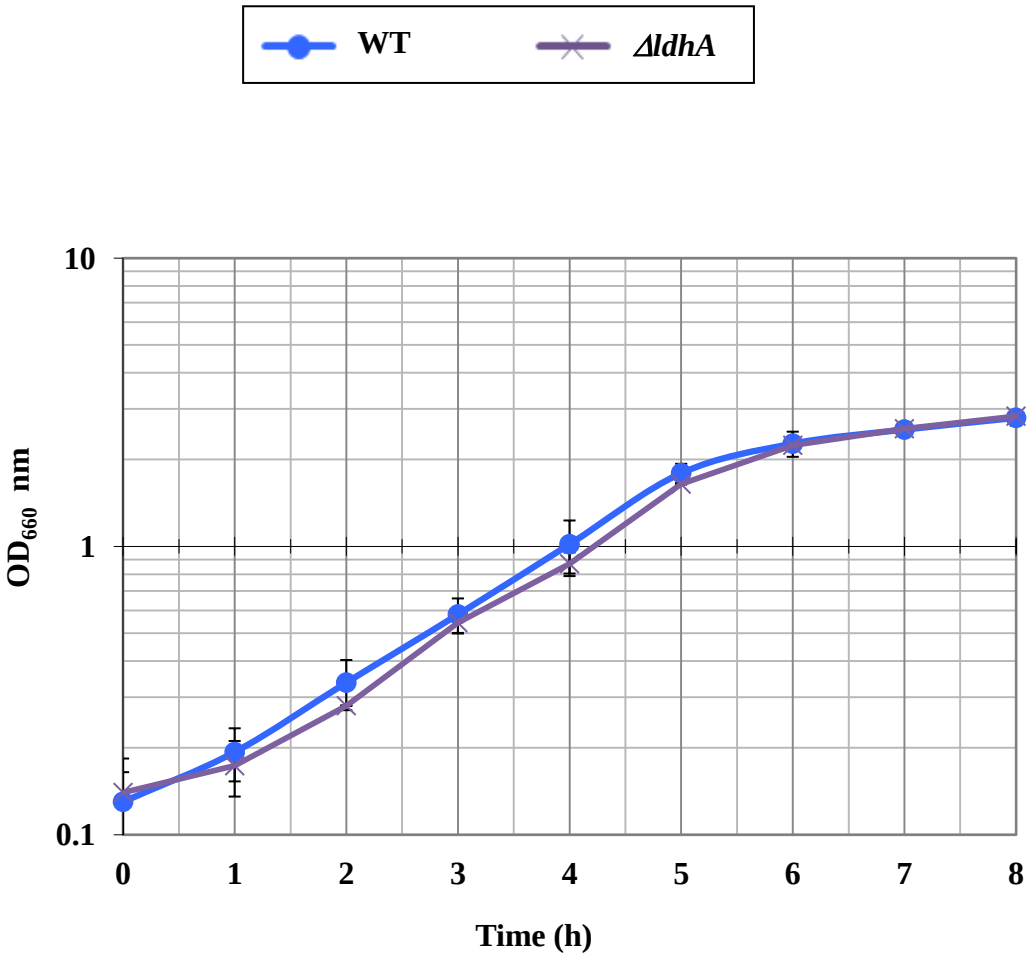


Fig. 3-7 **Effects of overproduction and deletion of LldR on growth. Growth of WT and *ΔldhA* in L broth are shown. Growth was monitored by measuring OD_{660}. The mean values of three independent experiments are presented with standard deviations.**

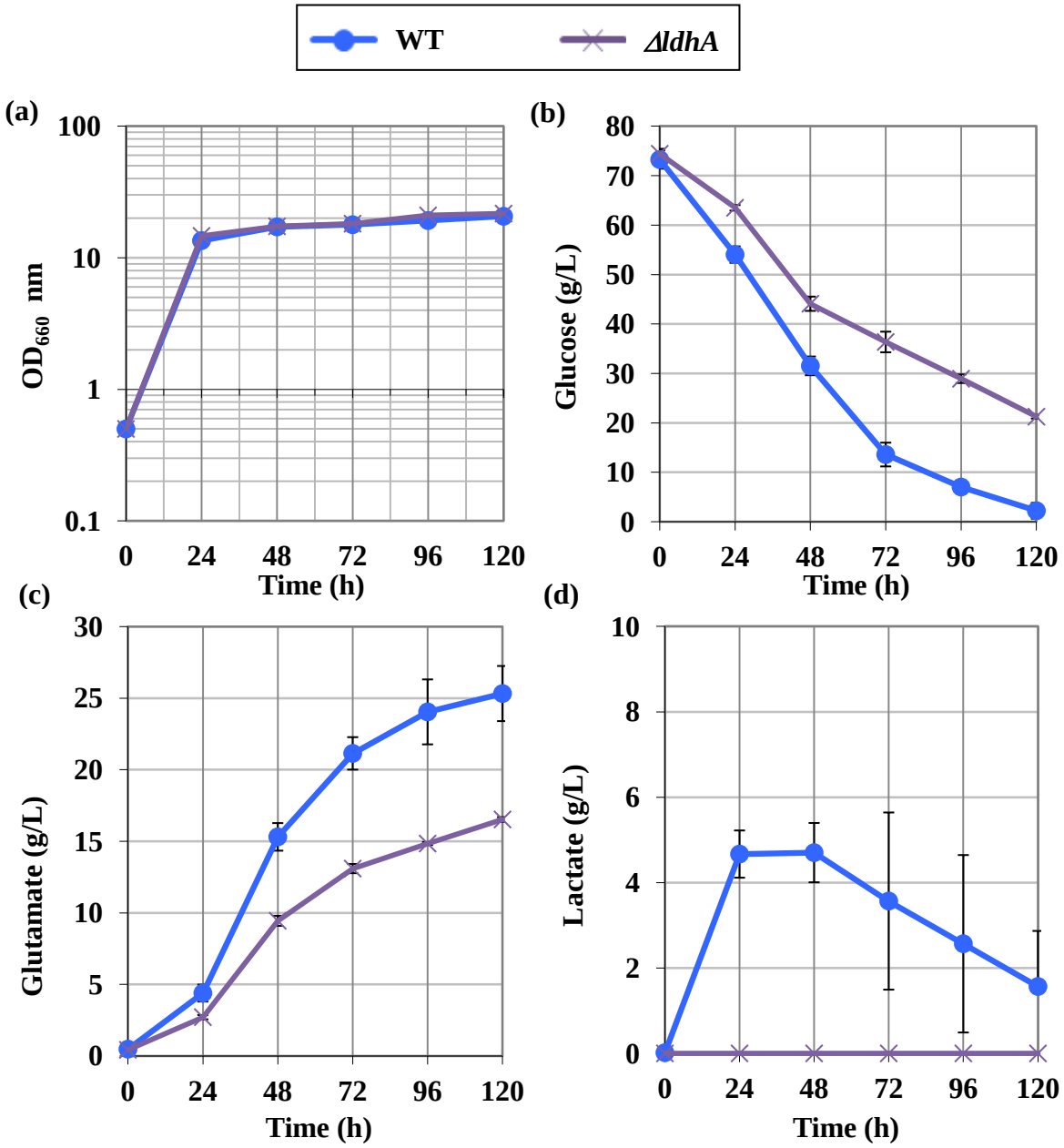


Fig. 3-8 **Effects of deletion of *ldhA* on glutamate production induced by biotin limitation. Cell growth (a), glucose consumption (b), glutamate production (c), and lactate formation (d) of WT and *ΔldhA* grown in production medium. The mean values of three independent experiments are presented with standard deviations.**

Table 3-5. Effects of deletion of *ldhA* on lactate and glutamate production under biotin-limited conditions

Strains	Glucose consumption (g/L)	Lactate production (g/L)	Glutamate production (g/L)	Production yield [a] (%)	Specific productivity (g/ L•OD•day)
WT	**71.0 ± 2.5**	**1.5 ± 1.3**	**25.3 ± 1.9**	**43.6 ± 1.9**	**0.25 ± 0.03**
ΔldhA	**53.2 ± 0.8 [b]**	**0.0 ± 0.0**	**16.5 ± 0.2 [c]**	**38.0 ± 0.5 [c]**	**0.21 ± 0.02 [c]**

Glucose consumption, lactate production and glutamate production under biotin-limited conditions at 120 h (5 days) are shown. Data represent means ± SDs for three independent experiments.

a) Production yield was calculated as;

$$\frac{\text{Glutamate production (g/L)} / 147}{\text{Glucose consumption (g/L)} / 180} \times 100$$

Student's t-test: b) $p < 0.01$, c) $0.01 \leq p < 0.05$

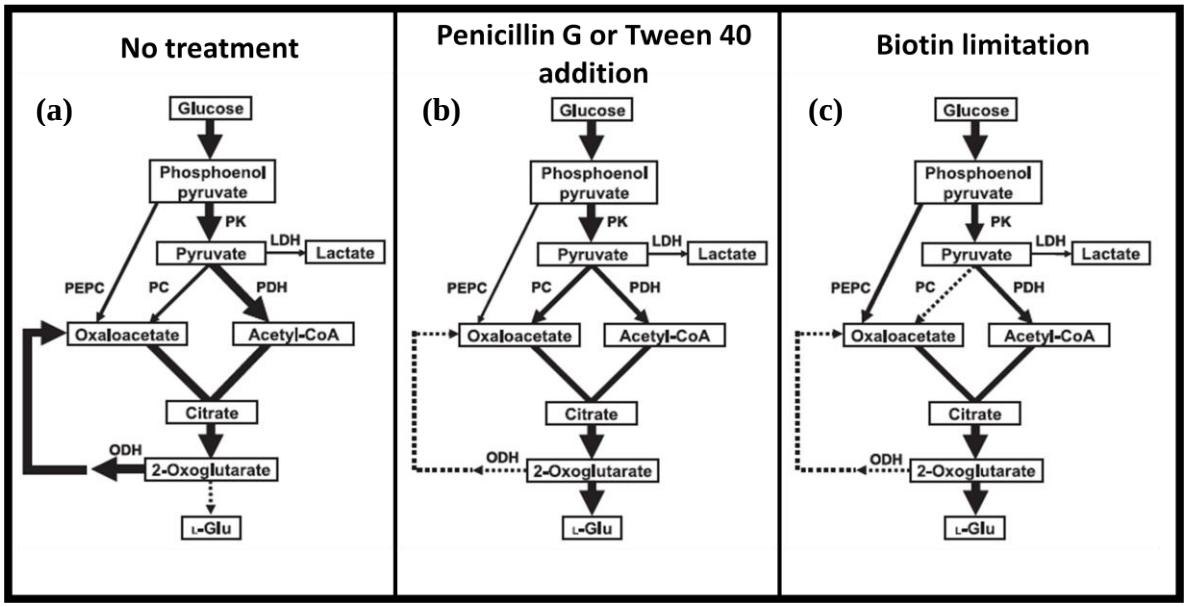


Fig. 3-9 **Schematic diagrams of metabolic changes in L-glutamate overproducing *C. glutamicum*. Presumed metabolic directions and fluxes are represented by the width of arrows and dotted arrows. (a) In nonproducer, (b) in producer in the presence of Tween 40 and penicillin, and (c) in producer under biotin-limited conditions (Hasegawa et al., 2008).**

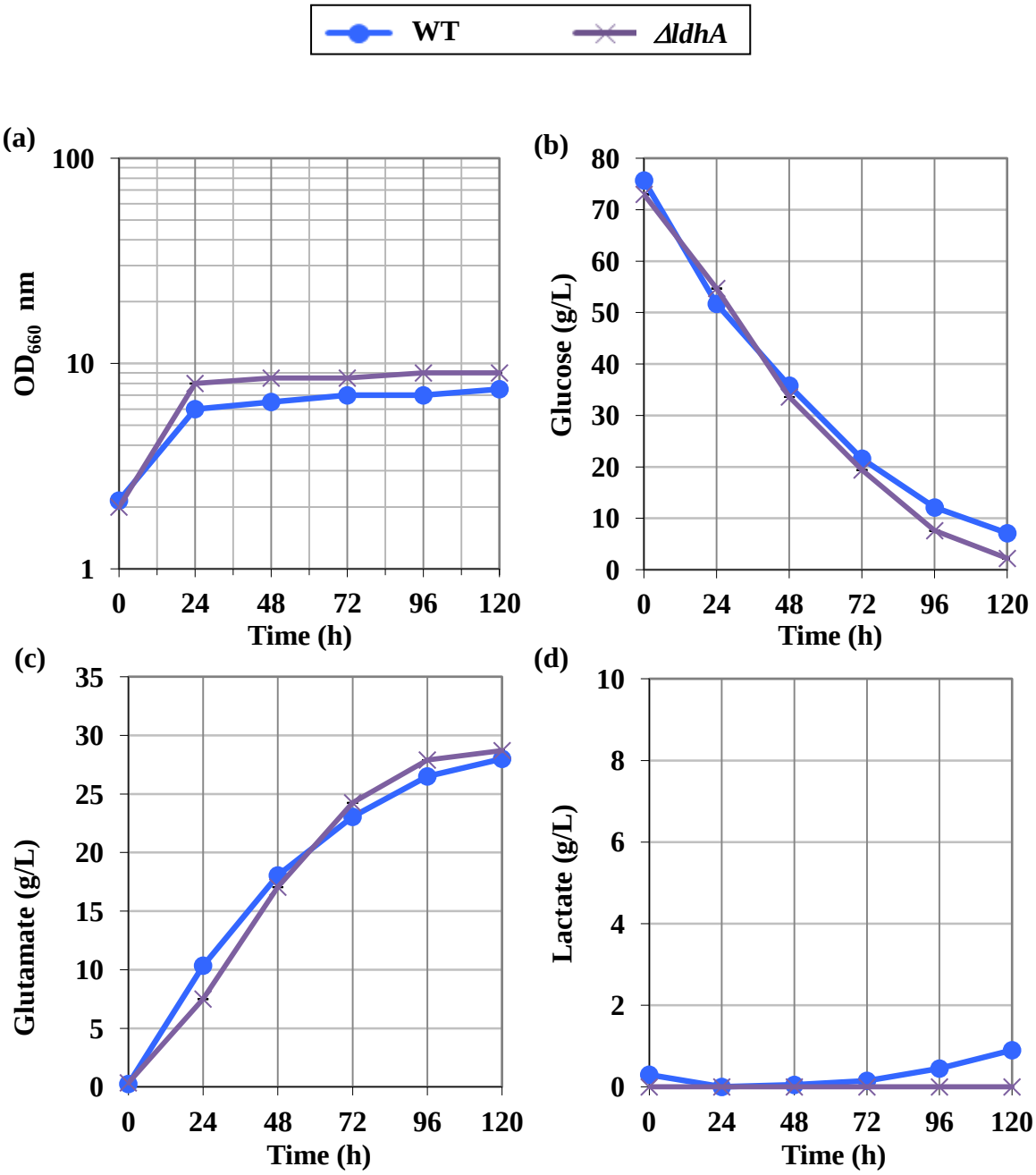


Fig. 3-10 **Effects of deletion of *ldhA* on glutamate production induced by penicillin G treatment. Cell growth (a), glucose consumption (b), glutamate production (c), and lactate formation (d) of WT and *ΔldhA* grown in production medium.**

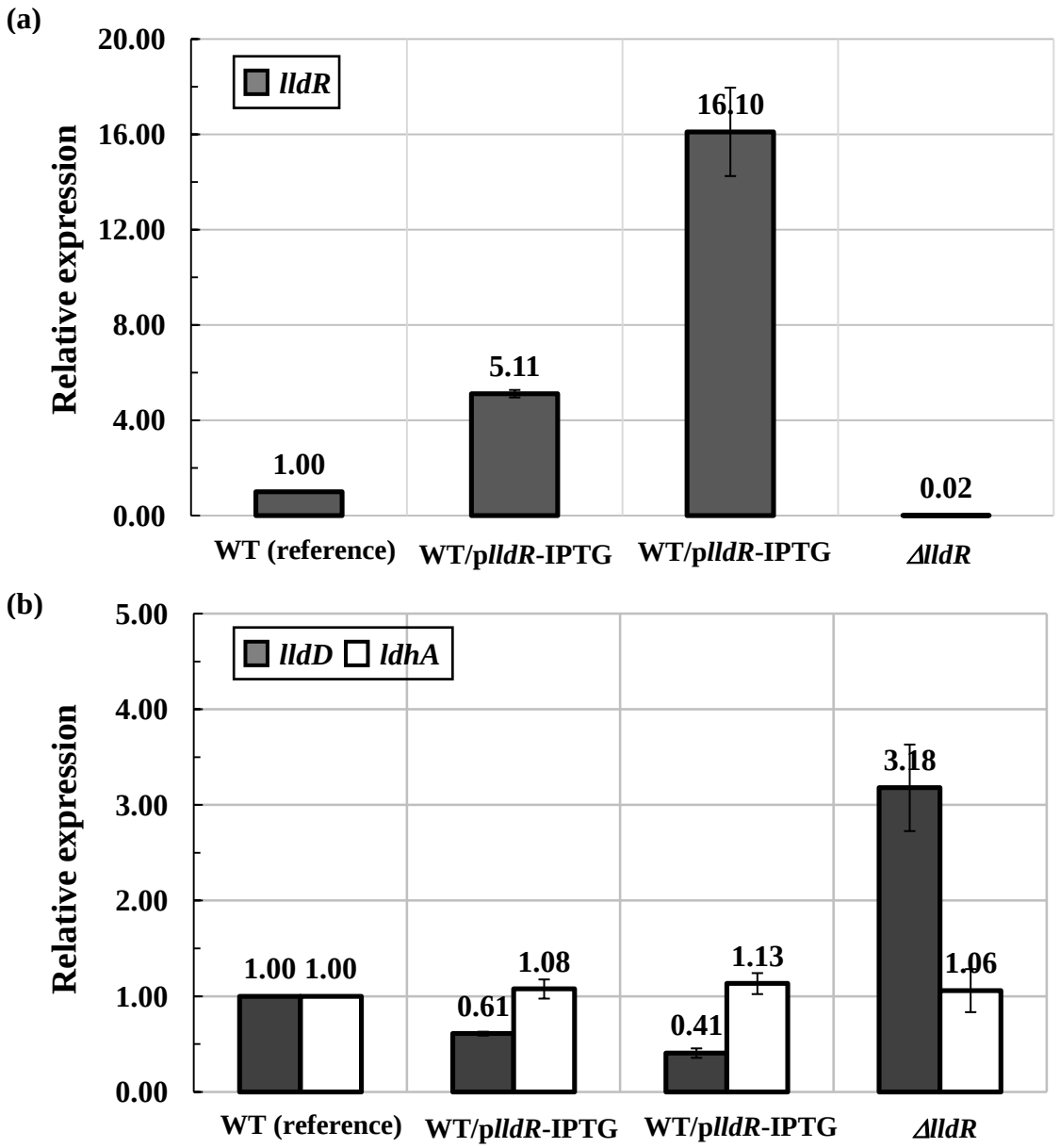


Fig. 3-11 **Effects of overproduction and deletion of LldR on gene expression. Comparative expression profiling of WT, LldR overproducers without or with 1mM IPTG addition (WT/p*lldR*-IPTG and WT/p*lldR*+IPTG, respectively), and *ΔlldR* in production medium. Relative expression levels of LldR (a), LldD (L-lactate dehydrogenase responsible for oxidation of L-lactate to pyruvate) and LdhA (L-lactate dehydrogenase catalyzes the formation of L-lactate from pyruvate) (b) were measured by qRT-PCR. The mean values of three independent experiments are presented with standard deviations.**

3-4 Discussion

L-Lactate is usually formed as a by-product during glutamate production and re-consumed afterward, which may contribute to the glutamate formation (Sato et al., 2008; Stansen et al., 2005; Uy et al., 2003). *ldhA* and *lldD* encode lactate dehydrogenases, which are involved in lactate metabolism. LdhA is responsible for production of lactate, while LldD is for its assimilation (Bott and Niebisch, 2003; Inui et al., 2004; Stansen et al., 2005). LldR is a transcription repressor of both genes, which is controlled by L-lactate availability (Gao et al., 2008; Georgi et al., 2008).

In this study, we investigated the role of LldR in the glutamate production under biotin-limited conditions by overexpression and disruption of *lldR* gene. The overexpression and deletion of *lldR* have no effect on growth rate in L medium (Fig. 3-5) nor under biotin-limited conditions (Fig. 3-6a). It was also reported that overexpression and deletion of *lldR* have no significant effects on growth rate and biomass formation compared with WT in minimal medium containing glucose as a sole carbon source (Georgi et al., 2008). In the *lldR* disruptant strain, glutamate production was slightly increased reproducibly, although it was not significant statistically. *lldR* disruptant produced 3% higher glutamate compared with WT (Fig. 3-6c, Table 3-4). *lldR* disruptant formed less L-lactate than WT (Fig. 3-6d, Table 3-4), probably because L-lactate re-utilization is more efficient without the repression of the *lldD* gene by LldR. On the other hand, LldR overproducer without or with IPTG produced less L-glutamate, 8% and 16% decrease respectively, compared with WT (Fig. 3-6c, Table 3-4). WT produced L-Lactate for the first 24 h, and it was re-consumed thereafter. On the other hand, in the LldR overproducer

with IPTG addition L-lactate production increased for 48 h but it was not re-consumed throughout the culture (Fig. 3-6d). The increase of L-lactate production was roughly comparable to the decrease of glutamate production in LldR overproducer with the addition of IPTG compared with WT (Fig. 3-6c, d, Table 3-4). The LldR overproducer without IPTG showed a delay in a re-consumption of L-lactate compared with WT (Fig. 3-6d). These results suggest that LldR controls L-lactate utilization by regulating *lldD* expression and L-lactate formed during glutamate production is re-consumed for glutamate production. It was reported that repression by LldR is relieved in the presence of L-lactate at the extent of 40 mM (3.6 g/L) (Georgi et al., 2008), which is comparable to the levels in my experimental conditions (Fig. 3-6d). However, LldR was extensively overproduced in my experiments, i.e. about 16-fold higher compared with WT (Fig. 3-11a), and therefore it seems that higher lactate levels were required for derepression of *lldD* by LldR.

ldhA disruption showed significantly decreased in glucose consumption and glutamate production under biotin-limited conditions (Fig. 3-8b,c). Accumulation of pyruvate may significantly increase, which might lead to a decrease in glycolytic flow. This problem was overcome under penicillin treatment conditions where pyruvate accumulation may be abolished due to higher activity of PC compared with that of biotin limitation (Fig. 3-9, 3-10) (Hasegawa et al., 2008). These results suggest that the decrease in metabolic flow to lactate formation is not effective for the enhancement of glutamate production under biotin-limited conditions since the produced L-lactate is reused by the cells to produce L-glutamate.

qRT-PCR revealed that overexpression of *lldR* with the addition of IPTG decreased *lldD* expression significantly, which was about 2.4-fold decrease under biotin-limited conditions compared with WT (Fig. 3-11b). On the contrary, deletion of *lldR* resulted in an increase in *lldD* expression up to 3.2-fold increase compared with WT (Fig. 3-11b). However, overexpression and deletion of *lldR* have no significant effects on *ldhA* expression compared with WT under biotin-limited conditions (Fig. 3-11b). The previous studies suggest that LldR represses the *ldhA* gene depending on the culture conditions, i.e., sugar and L-lactate availability. Gao et al. (2008) used a culture medium containing a trace amount of glucose. In this case, SugR is a major repressor of *ldhA* in the absence of sugar. Moreover, the absence of sugar results in lower lactate; therefore LldR also represses *ldhA*. Georgi et al. (2008) and Toyoda et al. (2009a, b) used culture media containing higher amounts of glucose. In this case, lactate is formed in higher levels and therefore repression by LldR is relieved. Under these conditions, deletion of *lldR* did not cause a change in expression levels of *ldhA*. Moreover, it is likely that repression of SugR is relieved, at least partially, under biotin-limited conditions because glutamate production medium contains high concentrations of glucose. My result is consistent with those of Georgi et al. (2008) and Toyoda et al. (2009a, b).

In this study, we concluded that LldR mainly controls the expression of *lldD* but not of *ldhA*, at least under biotin-limited conditions (Fig. 3-12). The produced lactate is reused by the cells to produce glutamate. L-Lactate metabolism, which is controlled by LldR, has a buffering function of pyruvate pool for glutamate production under biotin-limited conditions.

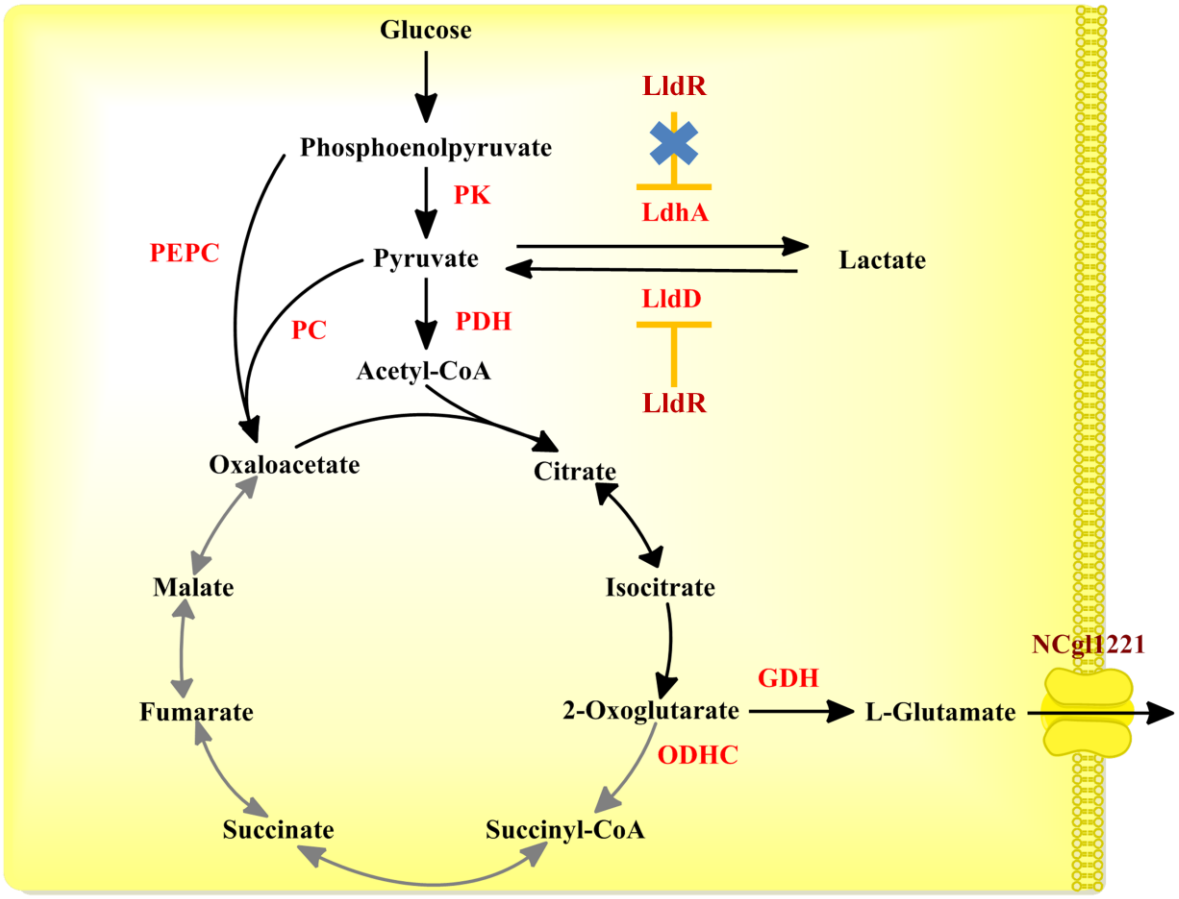


Fig. 3-12 **Proposed schematic of the role of LldR to lactate metabolism during glutamate production under biotin–limited conditions. LldR only represses LldD but not LdhA.**

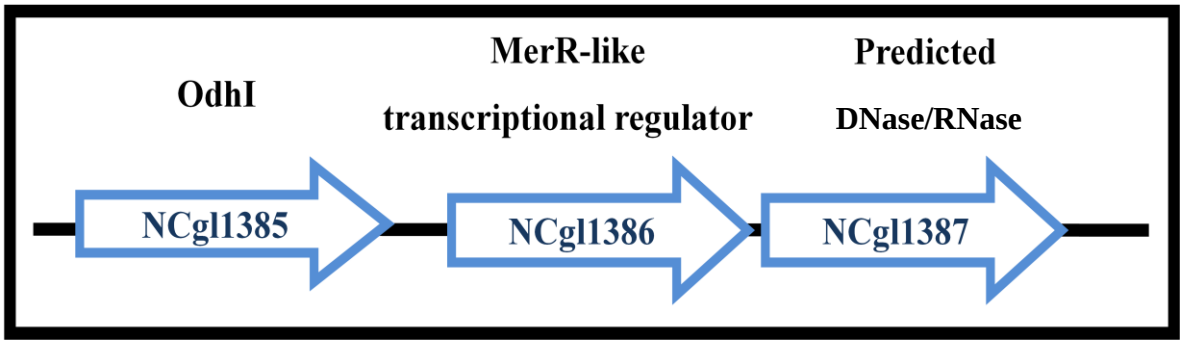
Chapter 4 Roles of transcritional regulator NCgl1386

4-1 Introduction to NCgl1386, MerR-like transcriptional regulator

NCgl1386 is a predicted MerR-like regulator. The MerR family transcriptional regulators have been shown to mediate responses to stress including exposure to heavy metals, drugs, or oxygen radicals in bacterial and some archaeal species. They regulate transcription of multidrug/metal ion transporter genes and oxidative stress regulons by reconfiguring the spacer between the -35 and -10 promoter elements. A typical MerR regulator is comprised of two distinct domains that consist of the regulatory (effector-binding) domain and the active (DNA-binding) domain. Their N-terminal domains contain a DNA-binding winged HTH motif, while the C-terminal domains are often dissimilar and bind specific coactivator molecules such as metal ions, drugs, and organic substrates. The activities of this family of proteins are regulated by the specific ligand molecules that bind the proteins (Brown et al., 2003). From the database (Fig.4-1), it is predicted that NCgl1386 forms an operon with NCgl1385 (OdhI, an inhibitor of ODHC, as its importance has been described in section 1-2) and NCgl1387, which is a predicted protein containing DNase/RNase activity.

In this study, I investigated the roles of the transcriptional regulator NCgl1386 in the glutamate metabolism under biotin-limited conditions in *C. glutamicum*. By disruption and overexpression of NCgl1386, it is shown that NCgl1386 has negative effect on growth and the glutamate production. However, the mechanism and role of NCgl1386 in the glutamate metabolism remains unclear.

(a) Predicted operon



(b) Putative functional domains

Fig. 4-1 Gene alignment around the NCgl1386

(a) Predicted operon (CoryneRegNet 6.0e) (b) Putative functional domains of NCgl1385 (OdhI), NCgl1386 and NCgl1387

4-2 Materials and Methods

4-2-1 Bacterial strains and media

Strains used in the experiments are listed in Table 4-1. *C. glutamicum* and *E. coli* cells were cultured in L medium (1% polypeptone, 0.5% yeast extract, 0.5% NaCl, 0.1% glucose, pH 7.0). For the glutamate production by biotin limitation, the preculture medium contained (per liter): 80 g glucose, 30 g $(NH_4)_2SO_4$, 1.0 g KH_2PO_4, 0.01 g $MnSO_4 \cdot 5H_2O$, 0.4 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 200 μg vitamin B_1 HCl, 13.72 ml soybean hydrolysate (total nitrogen, 0.48 g) and 15 μg biotin (pH 8.3). For the main culture, biotin was excluded from the preculture medium and 0.25 g of $CaCO_3$ was added to the 5 ml culture to maintain pH. *C. glutamicum* strain cultured in L medium for 24 h was directly inoculated into the preculture medium at 1% volume. After 24 h cultivation, the preculture was directly inoculated into the main culture medium at 5% volume for L-glutamate fermentation. Kanamycin (20 μg/ml) and 1mM IPTG was added to the medium at the start culture if needed. All media were cultivated at 30°C.

4-2-2 Gene overexpression

The DNA fragment spanning the NCgl1386 gene but not its promoter region was amplified from chromosomal DNA of *C. glutamicum* ATCC 31831 (Fig.4-2) (isolated with High Pure PCR Template Preparation Kit (Roche, Germany)) by PCR (PrimeSTAR® HS DNA polymerase, Takara, Japan) with the primer pair (Operon, Japan) NCgl1386-F and NCgl1386-R as indicated in Table 4-3 and purified by PCR Product Purification Kit (Qiagen, CA, US). The putative Shine-Dalgarno (SD) sequence of *C. glutamicum pyc* gene

was also introduced just upstream of the NCgl1386 gene for efficient translation. Plasmid pECt-Ptuf (Table 4-2) isolated by High Pure Plasmid Isolation Kit (Roche) was used. The DNA fragment was digested with *Sac*I and *Pst*I and then ligated (Ligation Mighty Mix, Takara) with the vector pladmid pECt-Ptuf digested with *Sac*I and *Pst*I (Takara). Plasmid pECt-Ptuf-NCgl1386 was isolated from *E. coli* JM110 by High Pure Plasmid Isolation Kit (Roche) and the insertion of NCgl1386 was confirmed with restriction digest and sequencing.

To prepare competent cells of *C. glutamicum* ATCC 31831, cells grown in L broth were harvested when OD_{660} reached 1.0. Cells were washed with DDW and 15% glycerol solution, and then re-suspended again with 15% glycerol solution. 100 μl of re-suspended cells were transformed with 5 μl of ligated mixture by electroporation system ECM399 (BTX Harvard Apparatus) at 1.9 kV. Transformed cells were cultered with 1 ml of fresh L medium in water bath at 30°C, 180 rpm for 2 h. Transformed cells were then centrifuged at low speed and the supernatants were discared. Cell pellets were suspended in 100 μl of L medium and spread on the 1.5% L agar plates containing 20 μg/ml of kanamycin. Plates were incubated at 30°C for 24 h. Transformant of *C. glutamicum* ATCC 31831 NCgl1386 overexpressing strain (WT/p1386) was selected and used for further analysis.

The DNA fragment spanning the NCgl1387 gene, and NCgl1386-1387 gene were amplified and ligated to plasmid vector pECt-Ptuf (Fig. 4-3, 4-4) as a similar manner to NCgl1386. The transformation process in *E. coli* JM110, seletion, plasmid isolation, insert confirmation by restriction digestion and sequencing were also carried out as mentioned

above. The ligated vectors were used to transform *C. glutamicum* ATCC 31831, resulted in NCgl1387 overexpressing strain (WT/p1387) and NCgl1386-1387 overexpressing strain (WT/p1386-1387). Moreover, an in-frame deletion of NCgl1386 and intact NCgl1387 overexpressing strain in *C. glutamicum* ATCC 31831 was also constructed (Fig. 4-5) from the pECt-Ptuf-NCgl1386-1387 (Table 4-2), which resulted in WT/pΔ1386-1387 strain. Transformants of these strains were selected and used for further analysis.

4-2-3 Gene disruption

The DNA fragment spanning the upstream and downstream flanking region of NCgl1386 (Fig. 4-6) and NCgl1387 (Fig. 4-7) gene were amplified from chromosomal DNA of *C. glutamicum* ATCC 31831 by PCR (PrimeSTAR® HS DNA polymerase, Takara) with the primer pair (Opreon) listed in Table 4-3 (U for upstream and D for downstream). The DNA fragments of upstream and downstream were digested and then ligated (Ligation Mighty Mix, Takara) into one linear fragment. Then they were digested again and ligated with the pK18mob*sacB* vector (Table 4-2) (Schafer et al., 1994) digested with the same enzymes. The transformation process in *E. coli* JM110, seletion, plasmid isolation, insert confirmation by restriction digestion and sequencing were also carried out in similar manner as mentioned in section 4-2-2.

To prepare competent cells of *C. glutamicum* ATCC 31831, cells grown in L broth were harvested when OD_{660} reached 1.0. Cells (total 15 ml) were washed with DDW and 15% glycerol solution, and then re-suspended again with 15% glycerol solution. 100 μl of re-suspended cells were transformed with 5 μl of ligated mixture by electroporation system

ECM399 (BTX Harvard Apparatus) at 1.9 kV. Transformed cells were cultured with 1 ml of fresh L medium in water bath at 30°C, 180 rpm for 3 h. Transformed cells were then centrifuged at low speed and the supernatants were discarded. Cell pellets were suspended in 100 μl of L broth and spread on the 1.5% L agar plates containing 20 μg/ml of kanamycin. Plates were incubated at 30°C for 24 h. A key feature to this method is that the constructed plasmid (pK18mob*sacB*+NCgl1386-U+D and pK18mob*sacB*+NCgl1387-U+D, Table 4-2) cannot replicate in *C. glutamicum*. Only cells, in which homologous recombination occurred between the chromosomal DNA and plasmid DNA (Fig. 4-8), could grow on plates containing kanamycin. Transformants were selected and cultured in fresh L medium containing 20 μg/ml of kanamycin. Overnight cultures from L medium were inoculated into L medium containing no kanamycin. The overnight cells were diluted 100 to 1000 times with 0.85% NaCl then spread on L agar plates supplemented with 20% sucrose. As cells carrying the *sacB* gene encoding levansucrase by the plasmid DNA that had been integrated into the chromosome were killed in the presence of sucrose, only those cells, in which the *sacB* gene had been excised from the chromosome by a second homologous recombination between the intact and deleted gene (Fig. 4-8), could grow on the plate supplemented with sucrose. From the sucrose-tolerant recombinants, the desired gene knockout strains were selected and confirmed by colony PCR (illustra puReTaq Ready-To-Go PCR Beads, Amersham Biosciences) as shown in Fig. 4-9. Deletion of the NCgl1386 and NCgl1387 gene was also confirmed by qRT-PCR assay as described later (Fig. 4-16).

Plasmid pECt-Ptuf-NCgl1386 and pECt-Ptuf-NCgl1387 isolated from *E. coli* JM110 were also introduced into *C. glutamicum* ATCC 31831 NCgl1386 and NCgl1387 disruptants, resulting in NCgl1386-complemented strain (*ΔNCgl1386*/p1386) and NCgl1387-complemented strain (*ΔNCgl1387*/p1387), respectively. Moreover, plasmid pECt-Ptuf-NCgl1386 was also introduced to *ΔNCgl1387* strain and plasmid pECt-Ptuf-NCgl1387 was introduced to *ΔNCgl1386* strain, resulting in *ΔNCgl1387*/p1386 and *ΔNCgl1386*/p1387, respectively.

4-2-4 SDS-PAGE

Cells grown in L medium for 24 h were harvested by centrifugation, then suspended in sodium phosphate buffer (50 mM, pH 7.0) and disrupted by sonication. Cell debris was removed by centrifugation. Quality and concentration of protein extracts were determined by NanoDrop (Thermo Fisher Scientific). Protein extracts were then separated in 10% polyarylamide gel by sodium SDS-PAGE using the tris-glycine-SDS running buffer. The gel was stained with CBB.

4-2-5 Growth and glutamate production assay

For growth measurement in L medium, overnight cultures of each strain were diluted (1:50) with fresh L broth and incubated at 30°C with shaking. Cell growth was monitored by measuring OD_{660}. For growth measurement in production medium containing $CaCo_3$, samples were diluted with 0.1 M HCl to dissolve $CaCO_3$ prior to measurement. To measure glucose consumption or glutamate production, the main cultures were sampled every 24 h. The supernatant of cultures were separated from cell pellet by centrifugation.

Glucose and glutamate concentrations in the culture supernatant were measured using Biotech-analyzer AS-210 (Sakura Seiki, Tokyo) with a glucose oxidase sensor or glutamate oxidase sensor, respectively.

4-2-6 RT-PCR analysis

Cells of *C. glutamicum* ATCC 31831 WT grown in L medium until OD_{660} reached 1.0 were used for total RNA isolation. Two volume of RNAprotect Bacterial Reagent (Qiagen) were added directly to the culture to stabilize cellular RNAs. Cells were harvested by centrifugation and total cellular RNA was isolated using an RNeasy Mini Kit (Qiagen). To eliminate residual genomic DNA, RNA preparations were treated with RNase-Free DNase I (Qiagen). Quality and concentration of RNA samples were determined by NanoDrop (Thermo Fisher Scientific). RT-PCR was performed (Fig. 4-11a) using the PrimeScript™ One Step RT-PCR kit ver 2.0 (Takara). Primers (Operon) are listed in Table 4-4. mRNA templates amplified by PCR (PrimeSTAR® HS DNA polymerase, Takara) without reverse transcriptase were used as negative controls using the same primer.

4-2-7 Quantitative real time RT-PCR (qRT-PCR) analysis

Cells grown in L medium until OD_{660} reached 1.0 were used for total RNA isolation. Two volume of RNAprotect Bacterial Reagent (Qiagen) were added directly to the culture to stabilize cellular RNAs. Cells were harvested by centrifugation and total cellular RNA was isolated using an RNeasy Mini Kit (Qiagen). To eliminate residual genomic DNA, RNA preparations were treated with RNase-Free DNase I (Qiagen). Quality and concentration of RNA samples were determined by NanoDrop (Thermo Fisher Scientific).

qRT-PCR assays were performed using the QuantiFast SYBR Green RT-PCR kit (Qiagen) and Eco™ Real-Time PCR Systems (Illumina, San Diego, CA, USA). The quality and specificity of the amplification process was verified by melting curve analysis. The target gene transcripts were normalized to the reference gene transcript (16S rRNA) from the same RNA sample. Each gene was analyzed using RNA isolated from three independent samples. The cycle threshold (Ct) for each sample was generated according to the procedures described in the Eco ™ Real-Time PCR System user guide. Primers (Operon) are listed in Table 4-4.

Table 4-1 Bacterial strains

Strain	Relevant characteristics	Source or reference
E. coli		
JM109	*recA1 endA1 gyrA96 thi hsdR17* e14$^-$ (*mcrA*$^-$) *supE44 relA1* Δ(*lac-proAB*)/F' (*traD36 proAB*$^+$ *lacI*^{q} *lacZΔM15*)	**Yanisch-Perron et al., 1985**
JM110	*dam dcm supE44 hsdR17 thi leu rpsL1 lacY galK 82al Tara tonA thr tsx Δ*(*lac-proAB*)/F′(*traD36 proAB*$^+$ *lac I*^{q} *lacZΔM15*)	**Yanisch-Perron et al., 1985**
C. glutamicum		
ATCC31831	Wild type (WT)	**American Type Culture Collection**
ATCC31831	*ΔNCgl1386*	**This work**
ATCC31831	*ΔNCgl1387*	**This work**

Table 4-2 Plasmids

Plasmid	Relevant characteristics	Source or reference
pECt	Km^r ; *E.coli – C. glutamicum* shuttle vector, containing *trc* promoter and *lacI*^{q}	**Sato et al., 2008**
pECt-Ptuf	Km^r ; pECt inserted the promoter of elongation factor Tu (*tuf*) gene into the upstream of the trc promoter	**Takeuchi et al., unpublished**
pECt-Ptuf-NCgl1386	Km^r ; pECt-Ptuf containing the whole NCgl1386 gene	**This work**
pECt-Ptuf-NCgl1387	Km^r ; pECt-Ptuf containing the whole NCgl1387 gene	**This work**
pECt-Ptuf-NCgl1386-1387	Km^r ; pECt-Ptuf containing the whole NCgl1386 and NCgl1387 gene	**This work**
pECt-Ptuf-⊿NCgl1386-NCgl1387	Km^r ; pECt-Ptuf-NCgl1386-1387 containing an in frame deletion of NCgl1386 and intact whole NCgl1387 gene	**This work**
pK18*mobsacB*	Km^r ; mobile vector, α-*lac* multi cloning site, *sacB*	**Schafer et al., 1994**
pK18*mobsacB* +NCgl1386-U+D	Km^r,*sacB*; pk18*mobsacB* containing ligated upstream and downstream flanking region of NCgl1386 gene	**This work**
pK18*mobsacB* +NCgl1387-U+D	Km^r,*sacB*; pk18*mobsacB* containing ligated upstream and downstream flanking region of NCgl1387 gene	**This work**

Table 4-3 PCR primers

Primer name	Sequence 5′ → 3′	Generated restriction site
NCgl1386-F	GCGAGCTCGAAAGGAATAATTACTCTA ATGAGTGCACTCCGTAAAAC	*Sac*I
NCgl1386-R	CATCCTGCAGGCGCGCAGGTAAAG	*Pst*I
NCgl1386-FW	AGTGGTACCAGTCCCTGTCCGC	*Kpn*I
NCgl1386-RW	CATCGGATCCGCGCGCAGGTAAAG	*Bam*HI
NCgl1387-F	GCGAGCTCGAAAGGAATAATTACTCTA ATGAGTTTTGTTGAACTTGA	*Sac*I
NCgl1387-R	GTTGAAACCTGCAGGTGTGATTAGTC	*Sse*8387I
NCgl1387-FW	GCACGCATGCTTGGTGAAAAACGC	*Sph*I
NCgl1387-RW	GCTATAAGCTTGGTCTCTACCCTAGACC	*Hind*III
NCgl1387+N86-F	CACGCGGCCGCGGTGAAAAACGC	*Not*I
NCgl1387+N86-R	GATGCGGCCGCTTGGGGATGTTTTACGG	*Not*I
DEL1386-UF	TGATCAGTCCCGGGGGGGAACAGGC	*Sma*I
DEL1386-UR	GATGCATGCGTTTGGGGATGTTTTACGG	*Sph*I
DEL1386-DF	GCACGCATGCTTGGTGAAAAACGC	*Sph*I
DEL1396-DR	GCTATAAGCTTGGTCTCTACCCTAGACC	*Hind*III
DEL1387-UF	TTCCCGGGAAAGTTCTTTGCAGTG	*Sma*I
DEL1387-UR	GCATCTAGAGCCATCTGGTTCCATGG	*Xba*I
DEL1387-DF	GATTCTAGAGGCGACGGACTTGATGGC	*Xba*I
DEL1387-DR	TTGAAAGCTTATGGTCTCTGCCTG	*Hind*III

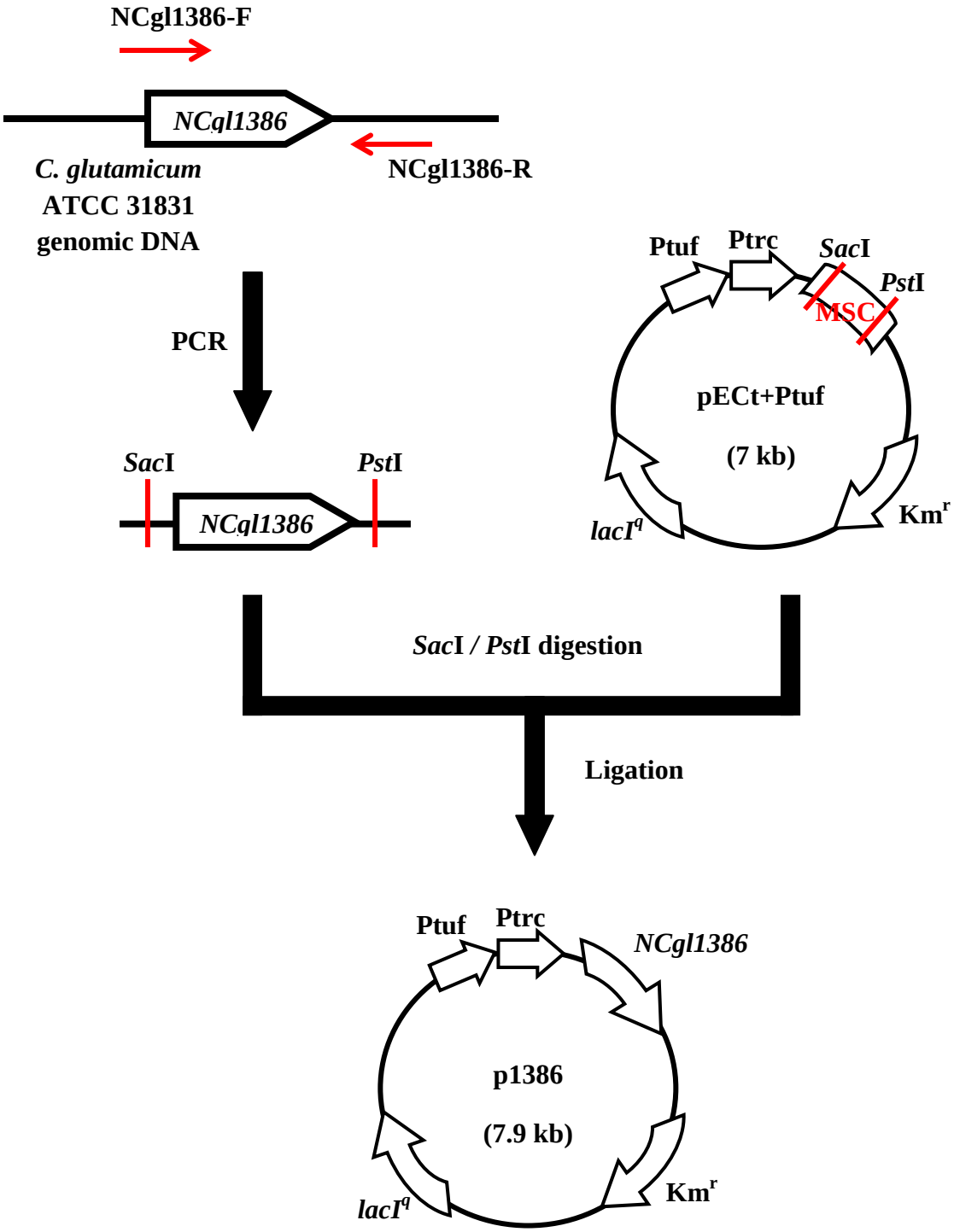


Fig. 4-2 Construction of NCgl1386 overexpressing vector

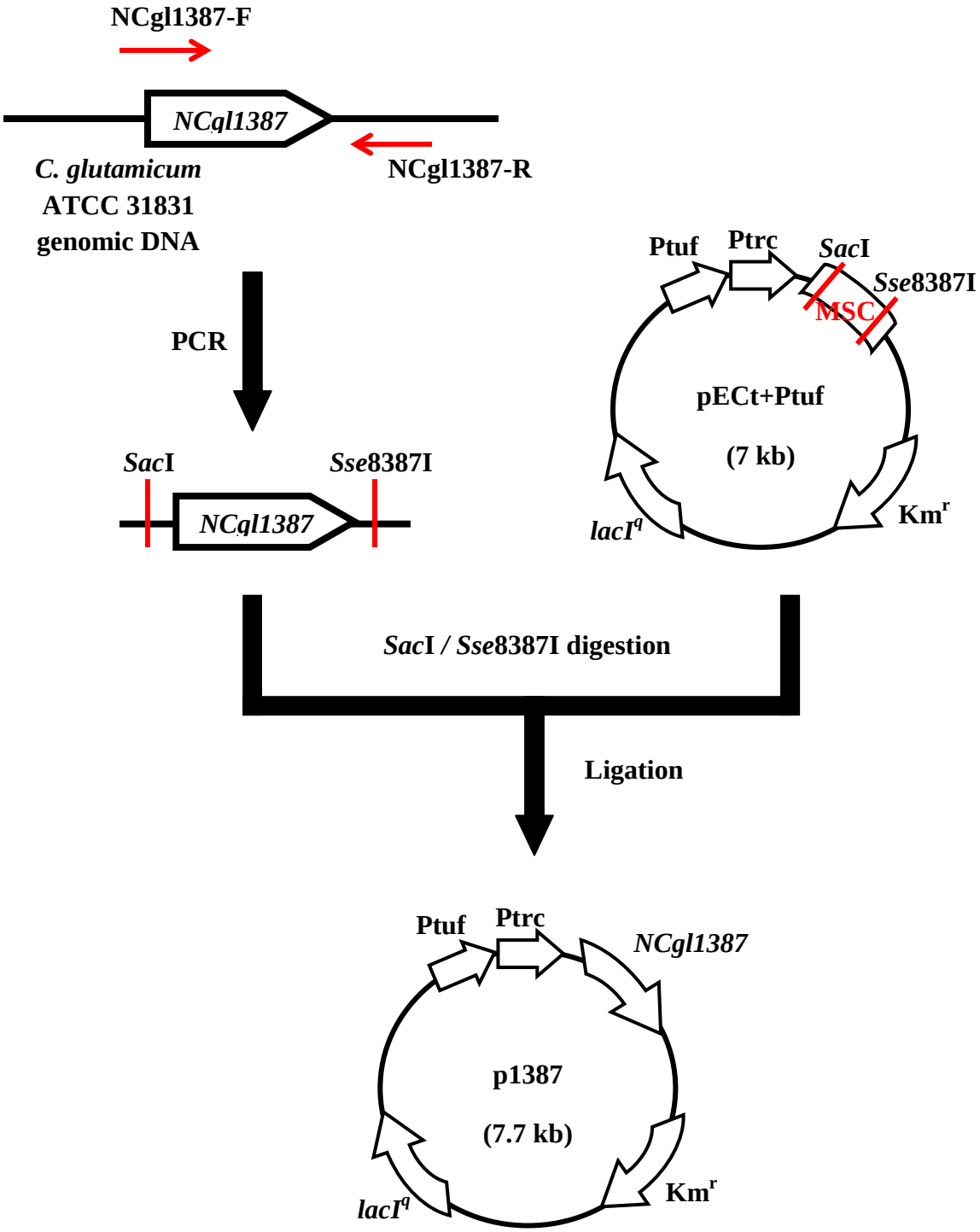


Fig. 4-3 Construction of NCgl1387 overexpressing vector

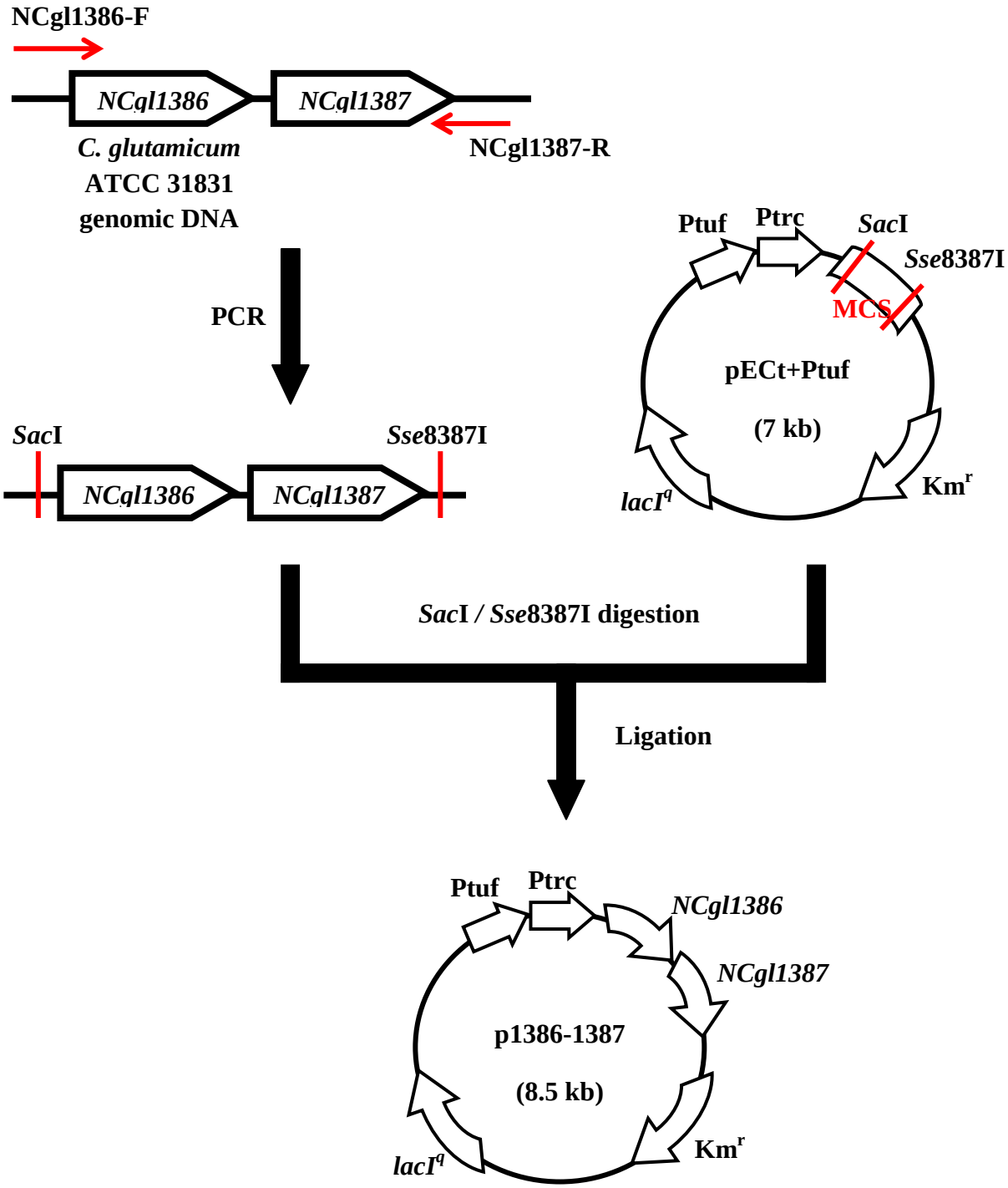


Fig. 4-4 Construction of NCgl1386-87 overexpressing vector

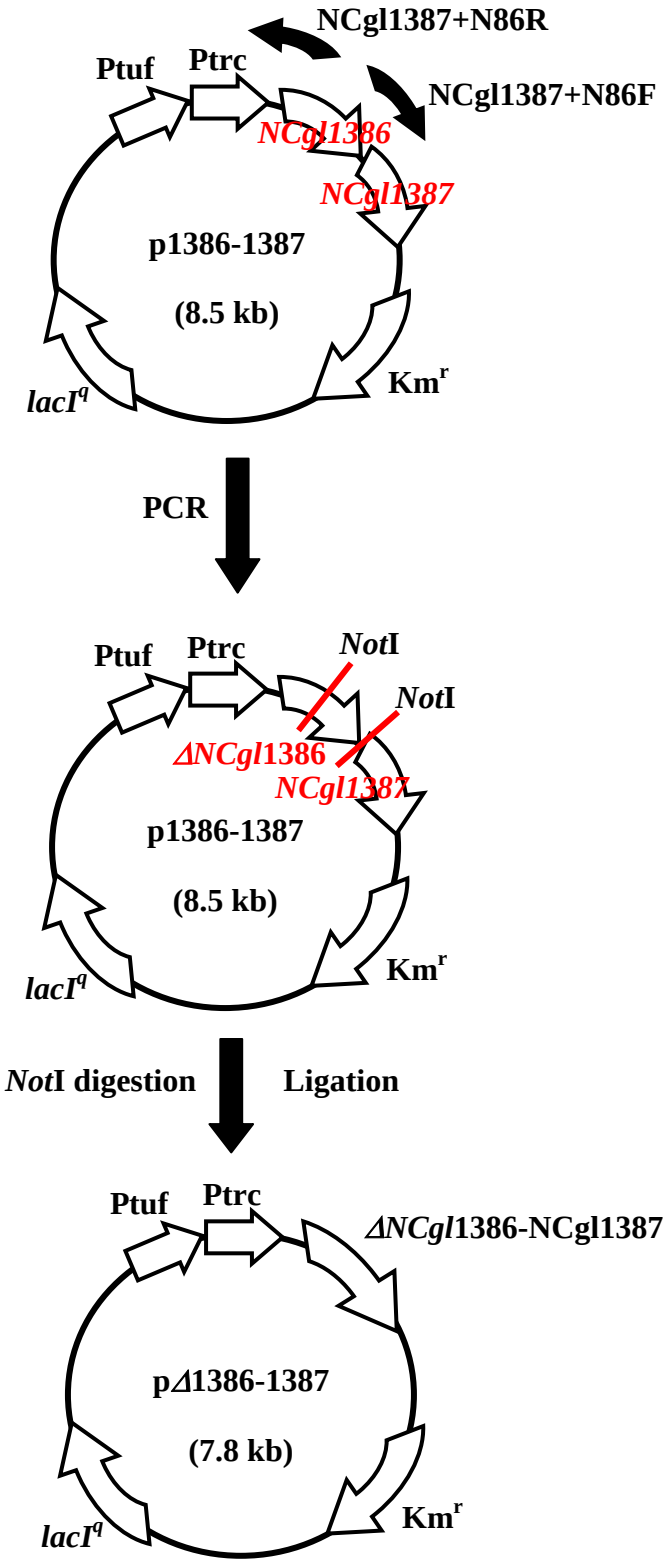


Fig. 4-5 Construction of NCgl1387-N86 overexpressing vector

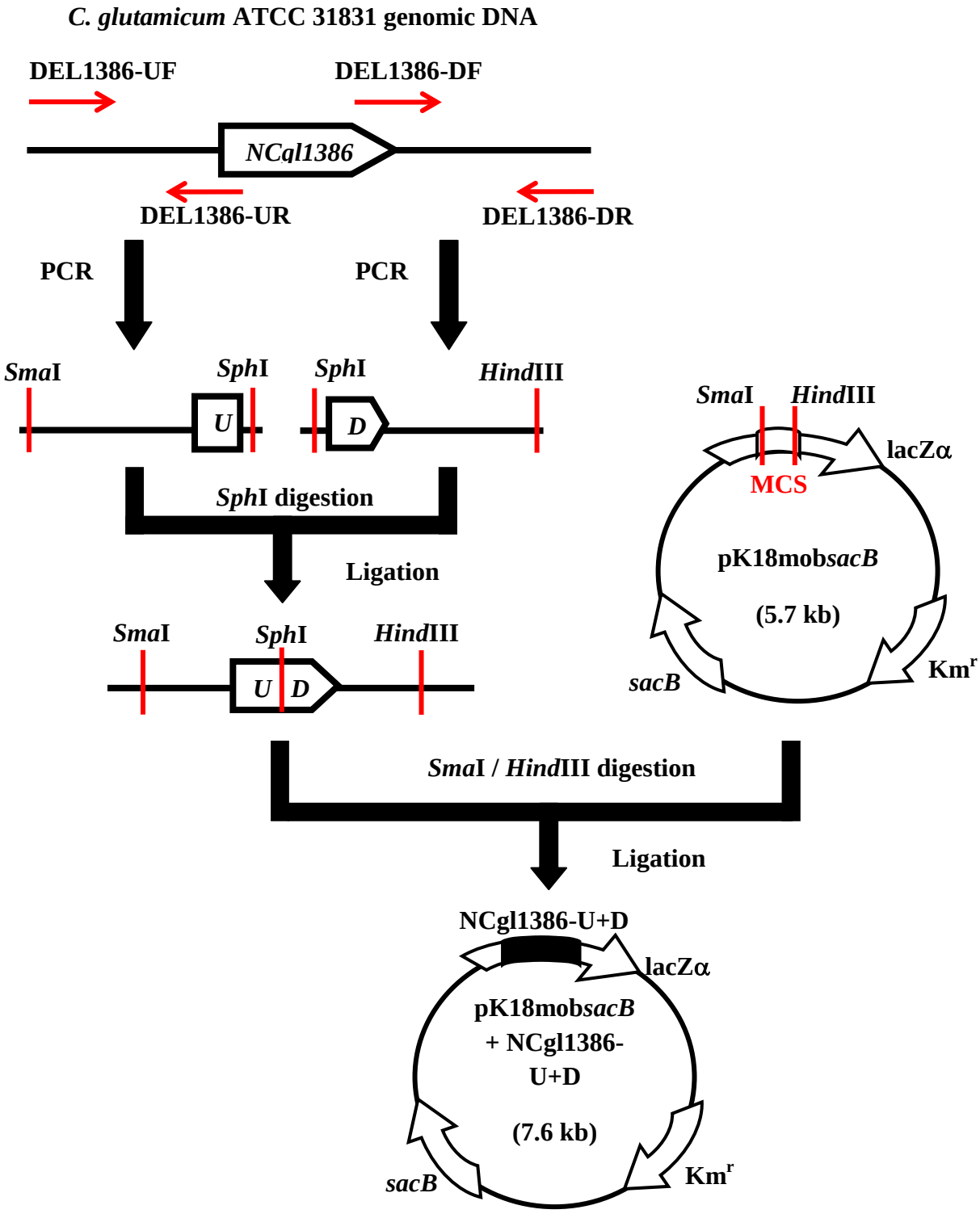


Fig. 4-6 Construction of NCgl1386 deletion vector

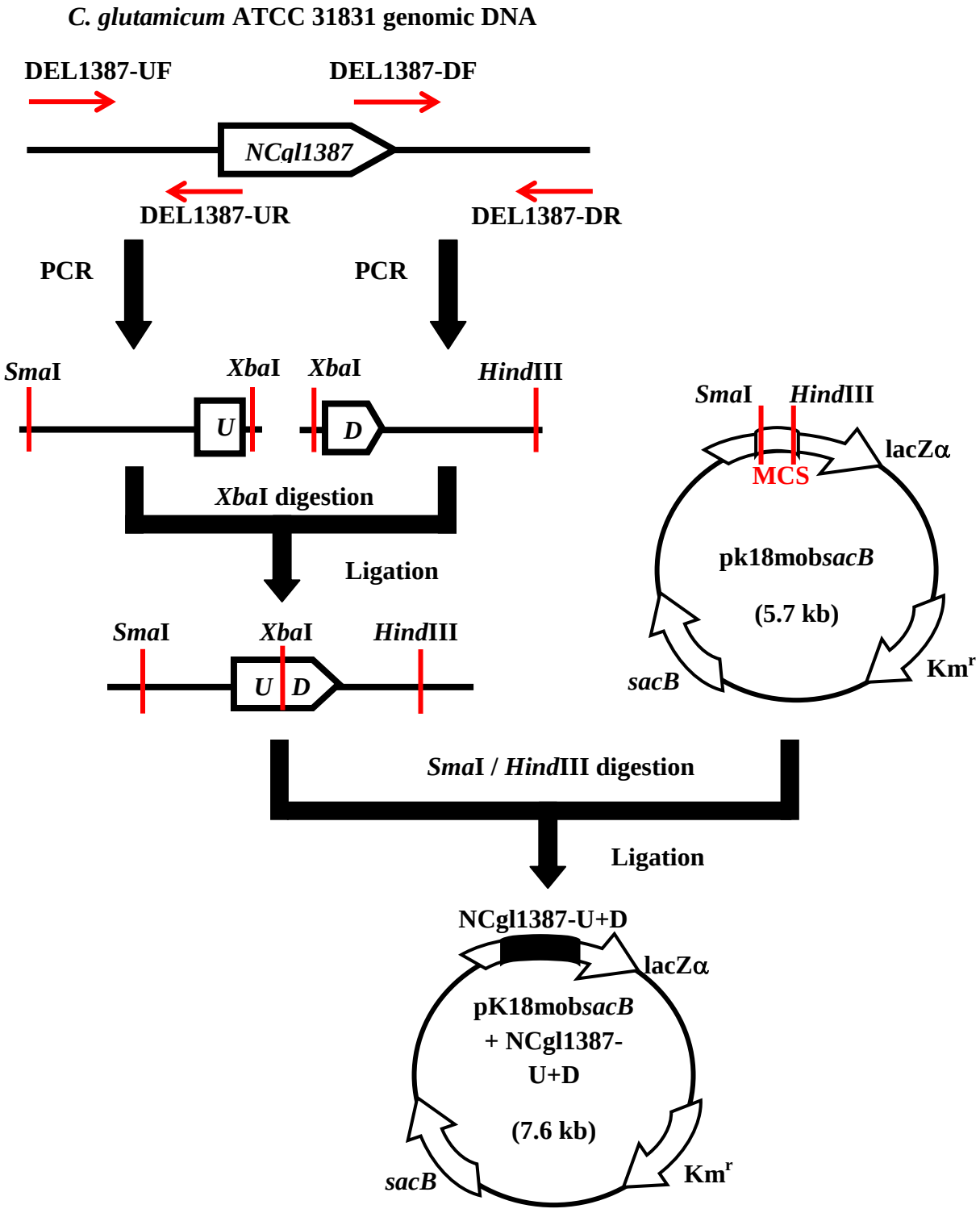


Fig. 4-7 Construction of NCgl1387 deletion vector

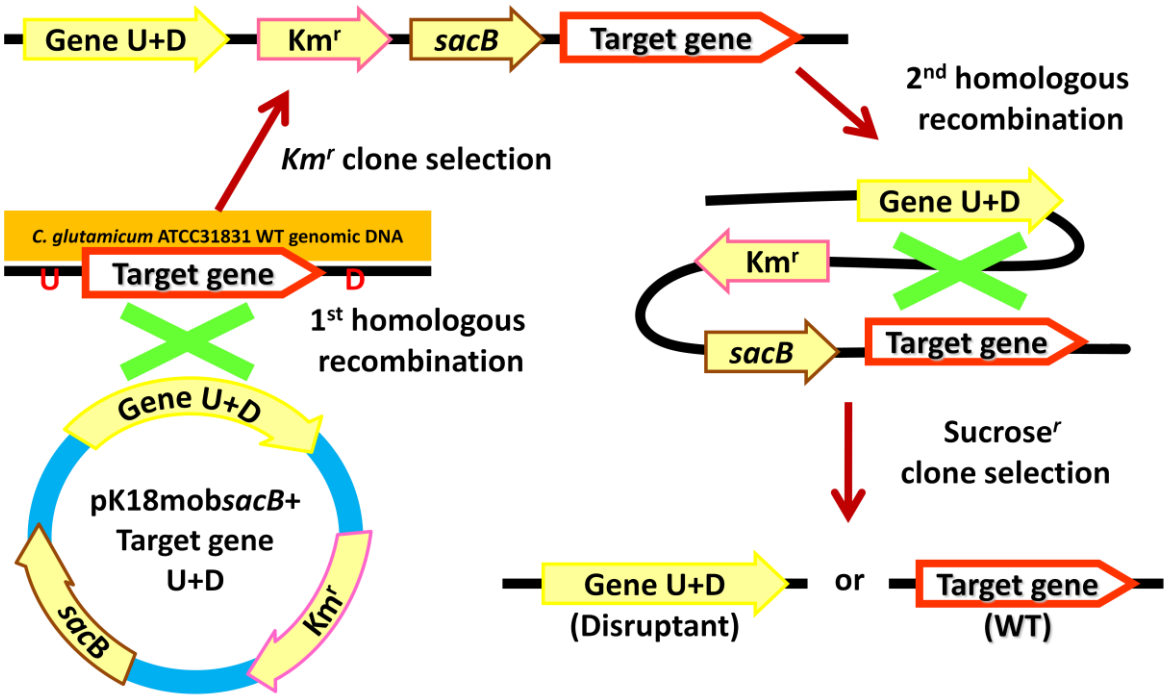


Fig. 4-8 Strategy of gene disruption by pK18mob*sacB* vector

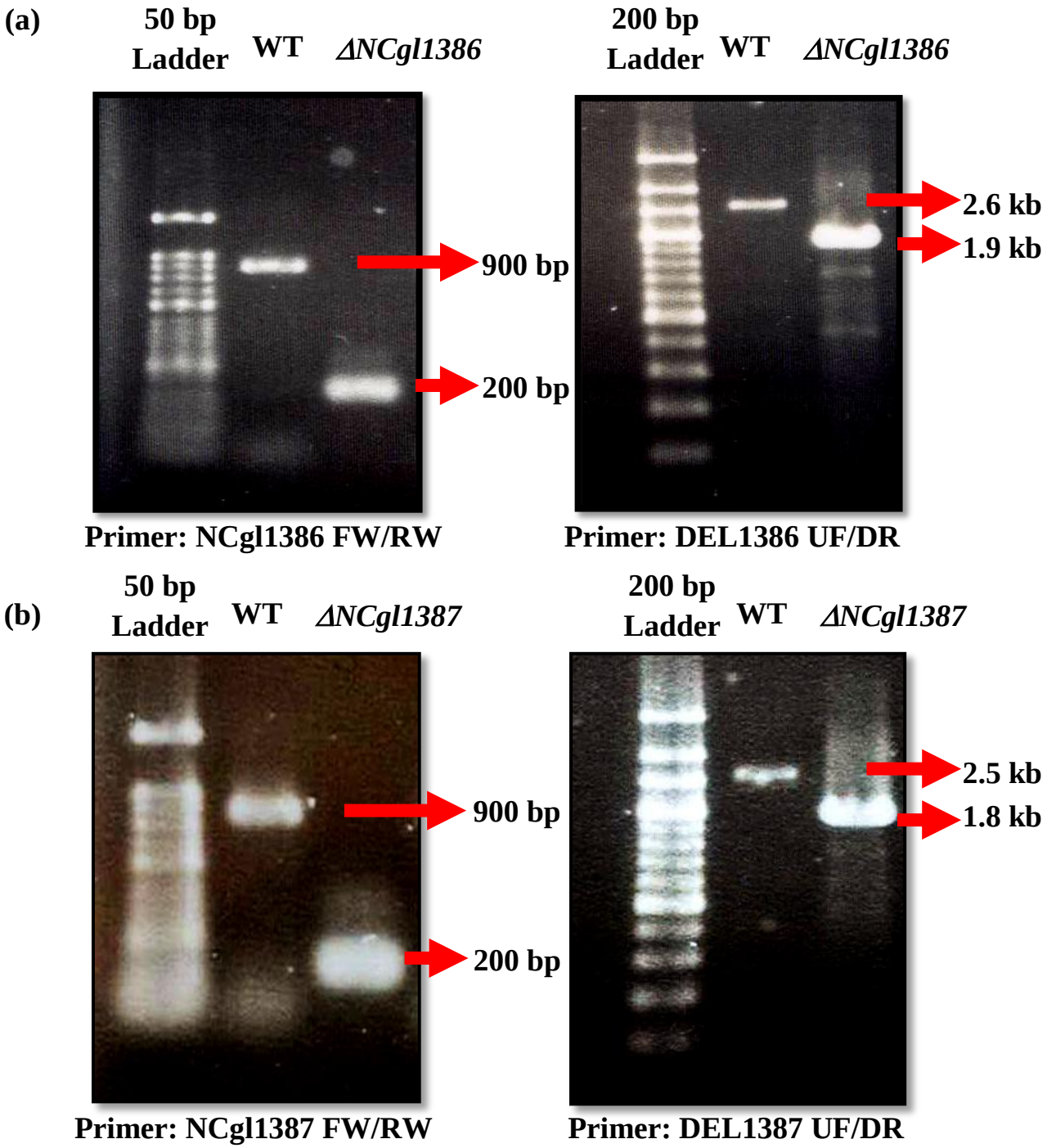


Fig. 4-9 **Confirmation of (a) NCgl1386 gene disruption (b) NCgl1387 gene disruption by colony PCR**

Table 4-4 RT-PCR and qRT-PCR primers

Primer name	Sequence 5′ → 3′
OP85-86-F	GCGTGGACCAAATGCAGGCGCTC
OP85-86-R	CCAGGATGTAGCGGAGGCGTTCC
OP86-87-F	CCAACGATGACGTGGCGATC
OP86-87-R	CGCCTTCAAAGTGGGAGACGATC
NCgl1385-qFW	TCGAGACCACCTCAGTATTC
NCgl1385-qRW	GTCCACGCTTGACTACAAGA
NCgl1386-qFW	GAACCGTTCCGGTGAAACCA
NCgl1386-qRW	TAATCAGCCCCTCTGATTCG
NCgl1387-qFW	TGGCATTCACACCATGGAAC
NCgl1387-qRW	CAGCGAGATATGCCTGGATT
16S-rRNA-F5	AGAGTTTGATCCTGGCTCA
16S-rRNA-F3	ACGTGTTACTCACCCGTTCG
16S-rRNA-R5	ACGTTCCCGGGCCTTGTACA
16S-rRNA-R3	CGGCTACCTTGTTACGAC

4-3 Results

4-3-1 Effects of overexpression/deletion of NCgl1386-1387 on protein expression

First, effects of overexpression/deletion of NCgl1386 on protein expression were examined. Synthesis of a protein of about 28 kDa, which corresponds to NCgl1386, was induced by IPTG induction in wild-type and NCgl1386-disruptant cells transformed with pECt-Ptuf-NCgl1386 (Fig. 4-10a). Interestingly, overproduction of a protein of about 24 kDa was also observed. This 24 kDa protein was revealed as NCgl1387 gene product by qRT-PCR analysis as shown later (Fig. 4-12a). These results suggested that NCgl1386 has a positive effect on expression of NCgl1386-1387. Then, effects of overexpression/deletion of NCgl1387 on protein expression were examined. Overexpression of NCgl1387 showed a decreased in whole protein profile in wild-type and NCgl1387 disruptant cells transformed with pECt-Ptuf-NCgl1387 compared with WT (Fig. 4-10b). Interestingly, NCgl1387 disruptant also showed the overexpressed band of about 28 kDa (Fig. 4-10b). This 28 kDa protein was revealed as NCgl1386 gene product by qRT-PCR analysis as shown later (Fig. 4-12b). These results suggest that NCgl1387 has a negative effect on expression of NCgl1386-1387.

4-3-2 Determination of transcriptional units of NCgl1385-1386-1387

Gene alignment around the NCgl1386 suggested that NCgl1386 forms an operon with NCgl1385 and NCgl1387 (Fig. 4-1). To determine whether NCgl1385, NCgl1386 and NCgl1387 form an operon, RT-PCR was carried out as described in section 4-2-6. Amplified cDNA from transcription units spanning NCgl1385-1387, NCgl1385-1386 and

NCgl1386-1387 clearly indicate that NCgl1385, NCgl1386 and NCgl1387 form an operon (Fig. 4-11b).

4-3-3 Effects of overexpression/deletion of NCgl1386-1387 on gene expression of NCgl1385-1386-1387

I performed the quantitative real-time RT-PCR (qRT-PCR) assays to compare the gene expression of the NCgl1385-1386-1387 operon in the overexpressing and disrupting strains of NCgl1386 and NCgl1387. NCgl1386 was overexpressed 7.52-fold higher than WT in the NCgl1386 overexpressing strain with IPTG addition (WT/p1386+IPTG). Expression of NCgl1386 was negligible in disruptant (*ΔNCgl1386*) (Fig. 4-12a). Overexpression of NCgl1386 caused the overexpression of NCgl1387. This indicates that the 24 kDa protein in Fig. 4-10a was the NCgl1387 gene product. Very curiously, deletion of NCgl1386 also caused the overexpression of NCgl1387. Overexpression of NCgl1387 in WT/p1386–IPTG was comparable with that in *ΔNCgl1386*. Expression of NCgl1385 (*odhI*) was not affected in NCgl1386 disruptant but 2-fold decrease was observed in NCgl1386 overexpressing strain. These results suggest that NCgl1386 controls the expression of NCgl1386-1387 operon positively. However, why overexpression of NCgl1387 was also observed in NCgl1386 disruptant remains to be elucidated. Probably, NCgl1386 controls the expression of NCgl1386-1387 directly as an activator and also indirectly via unknown regulator(s), which is under control of NCgl1386.

NCgl1387 was overexpressed 20.30-fold higher than WT in the NCgl1387 overexpressing strain with IPTG addition (WT/p1387+IPTG). Expression of NCgl1387

was negligible in disruptant (*ΔNCgl1387*) (Fig. 4-16b). Both NCgl1385 (*odhI*) and NCgl1386 showed about 5-fold decrease in expression in WT/p1387+IPTG compared with WT. On the contrary, about 7-fold and 6-fold increase in expression of NCgl1385 and NCgl1386, respectively, were observed in *ΔNCgl1387*. These results suggest that NCgl1387 acts as a negative regulator to this operon. Probably NCgl1387 functions as an RNase. This was also suggested from the protein expression profiles, in which whole protein expression was decreased in the NCgl1387 overexpressing strains (Fig. 4-10b).

Expression of NCgl1385 (*odhI*) was ambiguous (roughly 2-fold decrease in WT/p1386-IPTG and 1.5-fold decrease in WT/p1386+IPTG). In these strains, *odhI* expression may be overwhelmed by the negative control of co-overexpressed NCgl1387 (Fig. 4-12a). In order to avoid the negative effect by NCgl1387, qRT-PCR was carried out in NCgl1387 disruptant carrying p1386 (*ΔNCgl1387*/p1386). Without the negative interference from NCgl1387, it was shown that NCgl1386 also positively controlled the expression of *odhI*, in which the overexpression of NCgl1386 (19-fold higher than WT) consequently increased the expression of *odhI* (13-fold higher than WT) in *ΔNCgl1387*/p1386+IPTG compared with *ΔNCgl1387* (Fig. 4-13) These results suggest that NCgl1386 controls the expression of NCgl1386-1386-1387 as an activator.

4-3-4 Effects of overexpression/deletion of NCgl1386-1387 on growth and glutamate production under biotin-limited conditions

I examined effects of all constructed strains on growth in L broth and production medium. I also examined the effect of all constructed strains on glutamate production induced by biotin limitation as described on section 4-2-1 and 4-2-5. Overexpression of NCgl1386 with IPTG addition (WT/p1386+IPTG) significantly decreased growth in both L medium and production medium (Fig. 4-14a, b) compared with WT. Growth retardation consequently resulted in a significant decrease of glucose consumption and glutamate production (Fig. 4-14c, d, Table 4-5). NCgl1386 disruptant *(ΔNCgl1386*) showed a decrease in growth in both media (Fig. 4-14a, b), and glucose consumption (Fig. 4-14c, Table 4-5) but glutamate production was not affected (Fig, 4-14d, Table 4-5). As described above, overexpression of NCgl1386 resulted in co-overexpression of NCgl1387. NCgl1386 disruptant also showed overexpression of NCgl1387, which was comparable to that of NCgl1386 overexpressing strain without IPTG addition (Fig. 4-12a). From these results, it is suggested that NCgl1386 controls the expression of NCgl1387, which might be responsible for growth retardation.

Overexpression of NCgl1387 with IPTG addition (WT/p1387+IPTG) showed only a slight decrease of growth in L medium (Fig. 4-15a), while NCgl1387 disruptant (*ΔNCgl1387*) showed no significant changes of growth in L medium compared with WT (Fig. 4-15a). Both strains showed no significant differences in growth in production medium (Fig. 4-12b). Overexpression of NCgl1387 with IPTG addition showed a decrease in glucose consumption and glutamate production (Fig. 4-15, Table 4-5). NCgl1387

disruptant, on the other hand, showed a slight decrease in glucose consumption and glutamate production (Fig. 4-15c, d, Table 4-5). From these results, it is suggested that NCgl1387 is partly responsible for the growth retardation. There might be another gene(s) that is responsible for growth inhibition, probably NCgl1386 itself. As showed in qRT-PCR, overexpression of NCgl1387 resulted in a decrease in NCgl1386 expression. Furthermore, NCgl1387 disruptant showed an increase in NCgl1386 expression (Fig. 4-12b). These suggest that NCgl1387 may act as a negative regulator of NCgl1386. Overexpression of NCgl1387 alone did not induce severe growth inhibition. Somehow, both NCgl1386 and NCgl1387 genes may be required for growth inhibition as qRT-PCR showed co-overexpression of NCgl1386 and NCgl1387 in NCgl1386 overexpressing strain (Fig. 4-12a).

A strain overexpressing both NCgl1386 and NCgl1387 was also constructed. NCgl1386-1387 overexpressing strain with or without the addition of IPTG (WT/p1386-1387+IPTG, WT/p1386-1387-IPTG) showed comparable results to NCgl1386 overexpressing strain alone with or without the addition of IPTG (Fig. 4-14, 4-16, Table 4-5). This means that overexpression of NCgl1386 alone is sufficient to cause a decrease in growth as well as glutamate production. I also constructed a plasmid carrying an in-frame deletion of NCgl1386 and intact NCgl1387 (pΔ1386+1387). This plasmid may express more NCgl1387 than p1387. Because the transcription from the intact NCgl1386 start site is read through to NCgl1387. As expected, WT/pΔ1386+1387+IPTG showed a strong growth decrease in L medium (Fig. 4-16a). Glucose consumption and glutamate production were highly decreased in production medium (Fig. 4-16c, d, Table 4-5). Interestingly,

WT/p*Δ*1386+1387+IPTG showed similar growth compared with WT in production medium (Fig. 4-16b). Similar phenomenon was also observed in NCgl1387 overexpressing strain with IPTG addition (WT/p1387+IPTG) (Fig. 4-15b). It is suggested that in the presence of glucose, growth inhibition by NCgl1387 overexpression may be suppressed since the strain showed decrease in both NCgl1385 (*odhI*) and NCgl1386 expression in qRT-PCR (Fig.4-12b). OdhI functions as an inhibitor of ODHC. Decrease of OdhI might consequently cause an increase of the flux toward TCA cycle rather than the flux toward L-glutamate production (Fig. 4-15c, d). NCgl1387 itself may partly affect the pathway for growth somehow as the glucose consumption is decreased (Fig. 4-15c), which is probably why the growth of NCgl1387 overexpressing strain, where OdhI is diminished, is comparable with WT in production medium (Fig. 4-15b). However, *ΔNCgl1387*, where OdhI and NCgl1386 are highly increased (Fig. 4-12b), did not show growth decrease in both medium (Fig. 4-15c, d). These suggest that there might be some unknown requirements for growth inhibition by NCgl1386.

To observe the connection and relation between NCgl1386 and NCgl1387, overexpression of NCgl1387 in NCgl1386 disruptant (*ΔNCgl1386*/p1387) and overexpression of NCgl1386 in NCgl1387 disruptant (*ΔNCgl1387*/p1386) strains were performed. *ΔNCgl1386*/p1387+IPTG showed similar results to NCgl1387 overexpressing strain with IPTG addition (Fig. 4-14, 4-17). In L medium, the growth inhibition was also higher compared with WT/p1387+IPTG (Fig. 4-14a, 4-17a). In production medium, it showed growth restoration compared with *ΔNCgl1386* (Fig. 4-14b, 4-17b). This may be due to the overexpression of NCgl1387, as NCgl1386 disruptant overexpressed NCgl1387

(Fig. 4-12a). As a result, overexpression of NCgl1387 in *ΔNCgl1386* might be higher than that of NCgl1387 overexpressing strain (WT/p1387). The glucose consumption and glutamate production of *ΔNCgl1386*/p1387+IPTG (Fig. 4-17c, d, Table 4-5) also showed higher decrease than the NCgl1387 overexpression alone (Fig. 4-15c, d, Table 4-5). *ΔNCgl1387*/p1386+IPTG showed similar results to NCgl1386 overexpressing strain with IPTG addition (Fig. 4-14, 4-17); however, the level of inhibition was higher. Growth in both L and production media, glucose consumption and glutamate production of *ΔNCgl1386*/p1387+IPTG (Fig. 4-17c, d, Table 4-5) showed higher decrease than the NCgl1386 overexpressing strain alone (Fig. 4-14c, d, Table 4-5). Without the negative control of NCgl1387, overexpression of NCgl1386 in *Δ*NCgl1387 showed much higher expression than that of NCgl1386 overexpressing strain (WT/p1386) (Fig. 4-13). These results suggest that NCgl1386 and NCgl1387 function independently from each other and NCgl1387 is not needed for severe growth inhibition by NCgl1386. Only NCgl1386 is enough to induce growth retardation, however, the mechanism is still unknown.

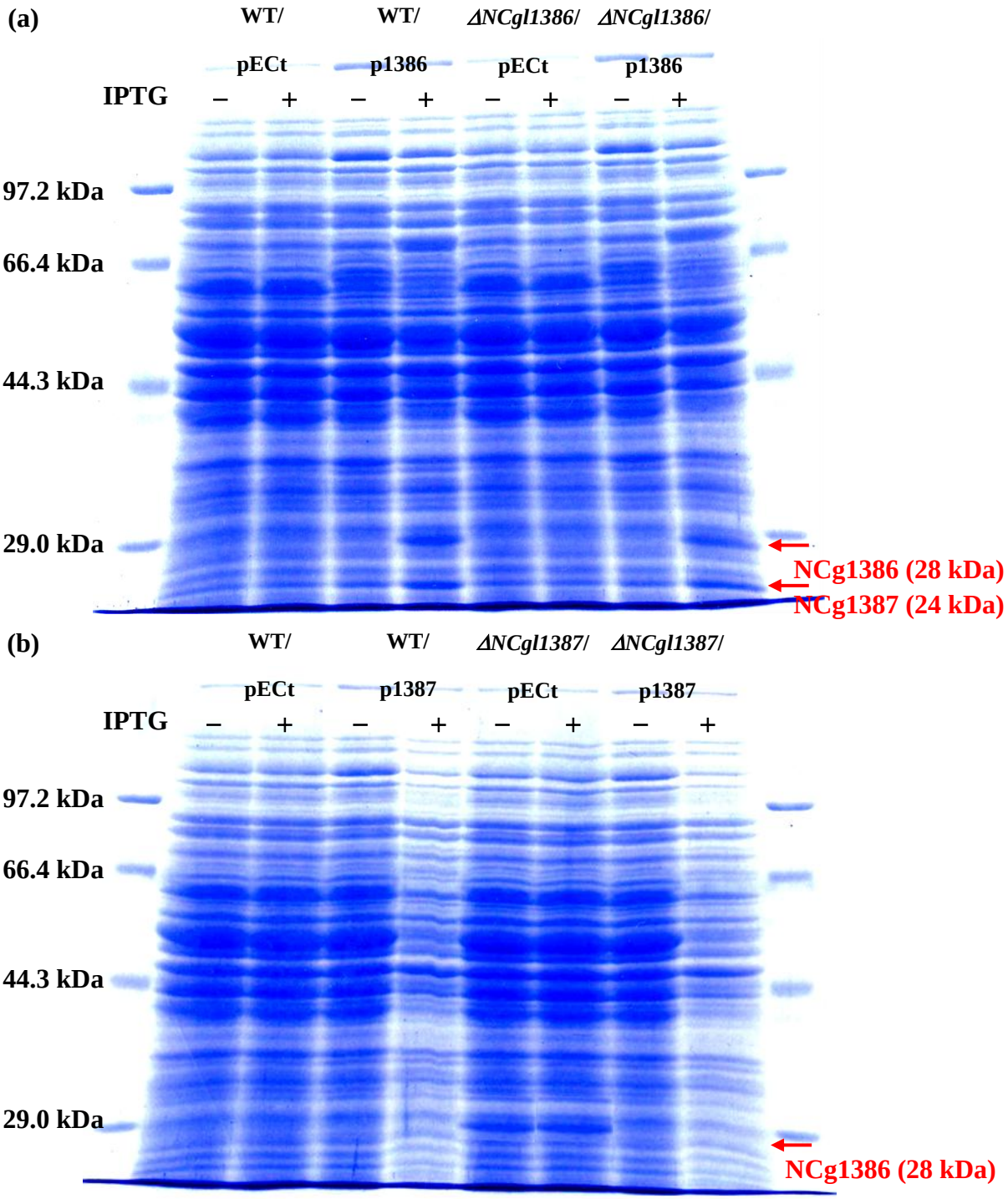


Fig. 4-10 **Confirmation of NCgl1386 (a) and NCgl1387 (b) overexpression by 10% polyarylamide gel SDS-PAGE. The signs "–" and "+" represent without and with 1mM IPTG addition, respectively. WT and disruptant carried an empty vector pECt were used as a negative control. Numbers shown to the left of gel are molecular weights (kDa).**

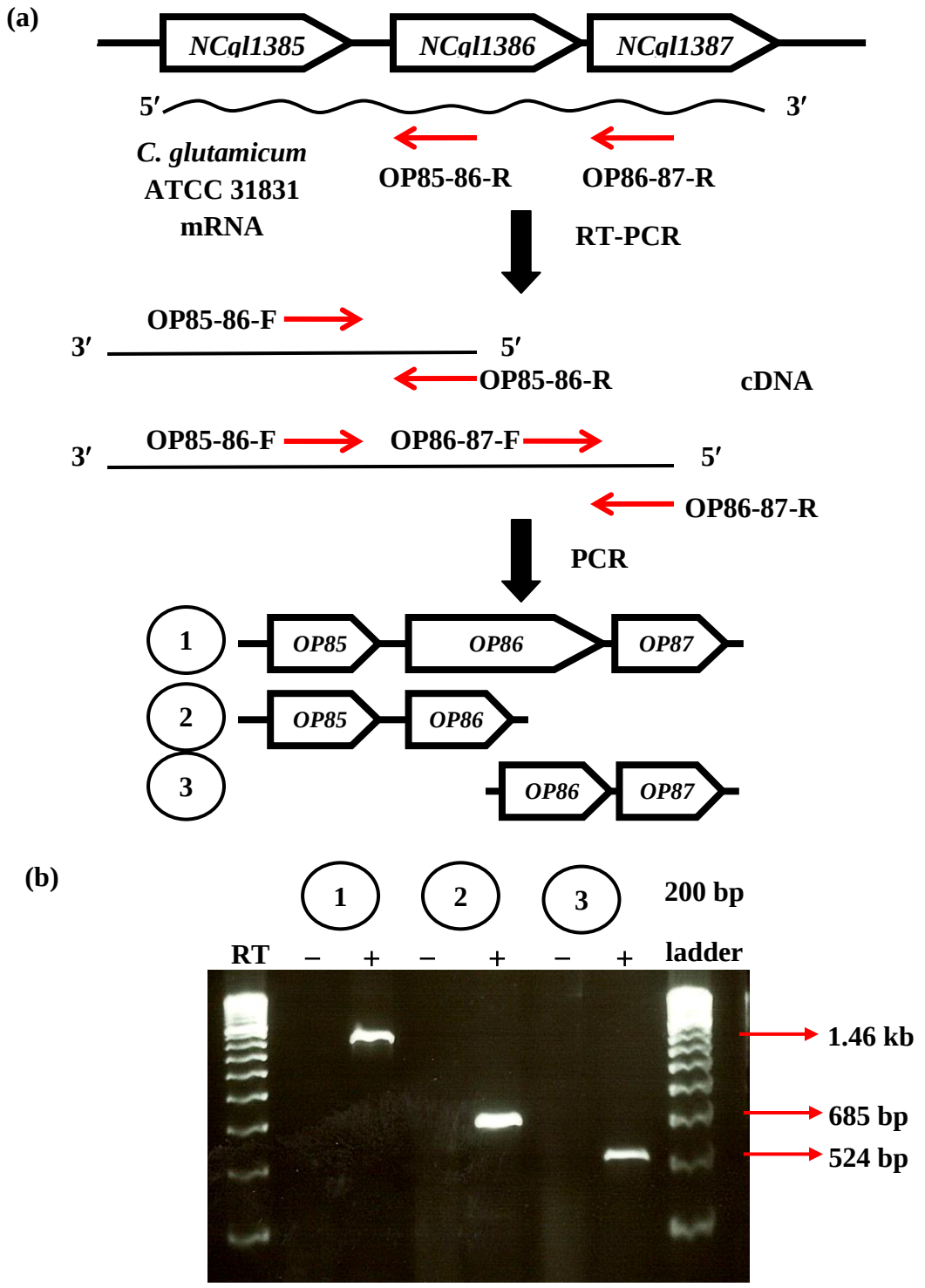


Fig. 4-11 **RT-PCR process for NCgl1385-87 operon confirmation (a) and cornfirmation of an operon by RT-PCR (b). The signs "–" and "+" represent without and with reverse transcriptase (RT)**

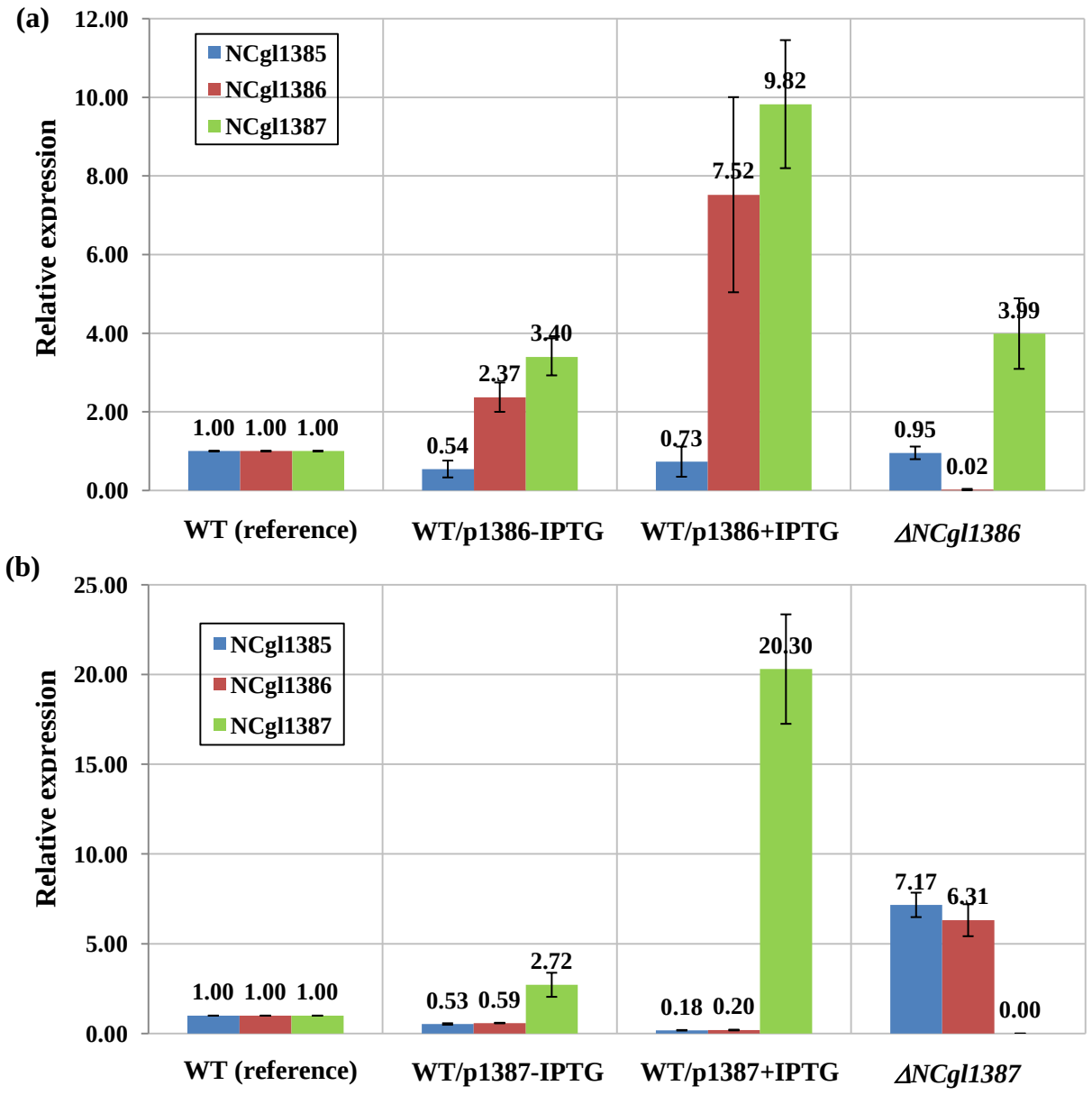


Fig. 4-12 **Comparative expression profiling of (a) WT, NCgl1386 overproducers without or with 1mM IPTG addition (WT/p1386-IPTG and WT/p1386+IPTG, respectively), and *ΔNCgl1386* in L medium and (b) WT, NCgl1387 overproducers without or with 1mM IPTG addition (WT/p1387-IPTG and WT/p1387+IPTG, respectively), and *ΔNCgl1387* in L medium. Relative expression levels of Ncgl1385, NCgl1386, and NCgl1387 were measured by real time-RT-PCR. The mean values of three independent experiments are presented with standard deviations.**

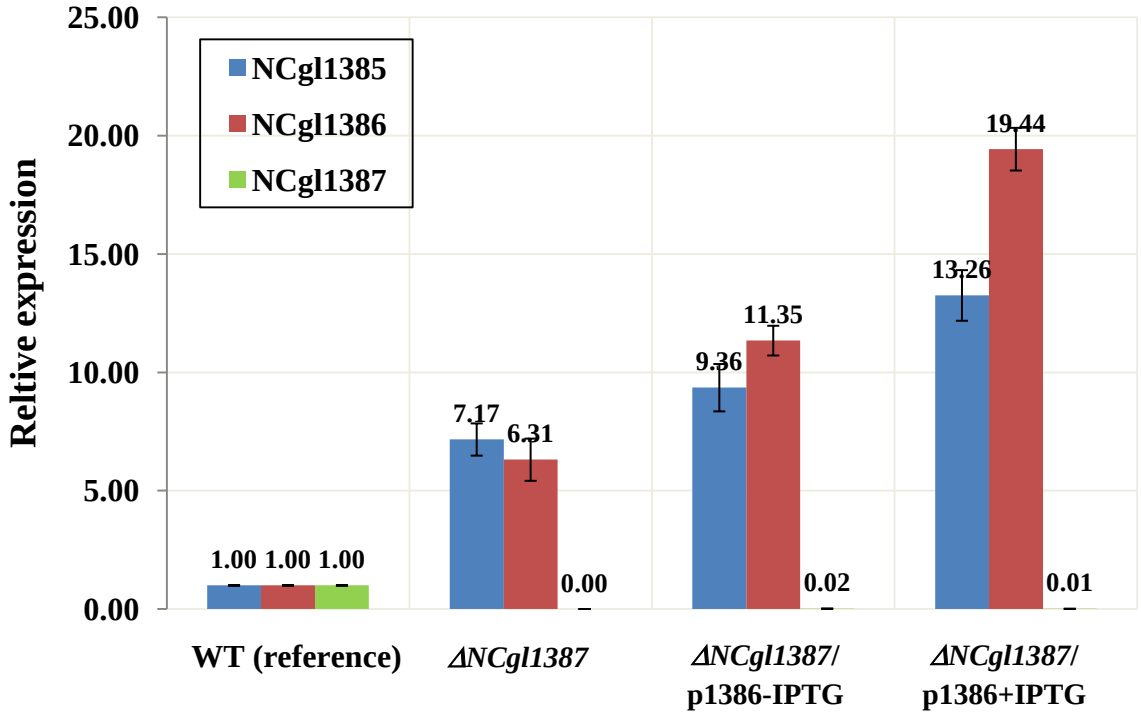


Fig. 4-13 **Comparative expression profiling of WT, *ΔNCgl1387*, and *ΔNCgl1387* with NCgl1386 overproducers without or with 1mM IPTG addition (*ΔNCgl1386*/p1386-IPTG and *ΔNCgl1386*/p1386+IPTG, respectively), in L medium. Relative expression levels of Ncgl1385, NCgl1386, and NCgl1387 were measured by real time-RT-PCR. The mean values of three independent experiments are presented with standard deviations.**

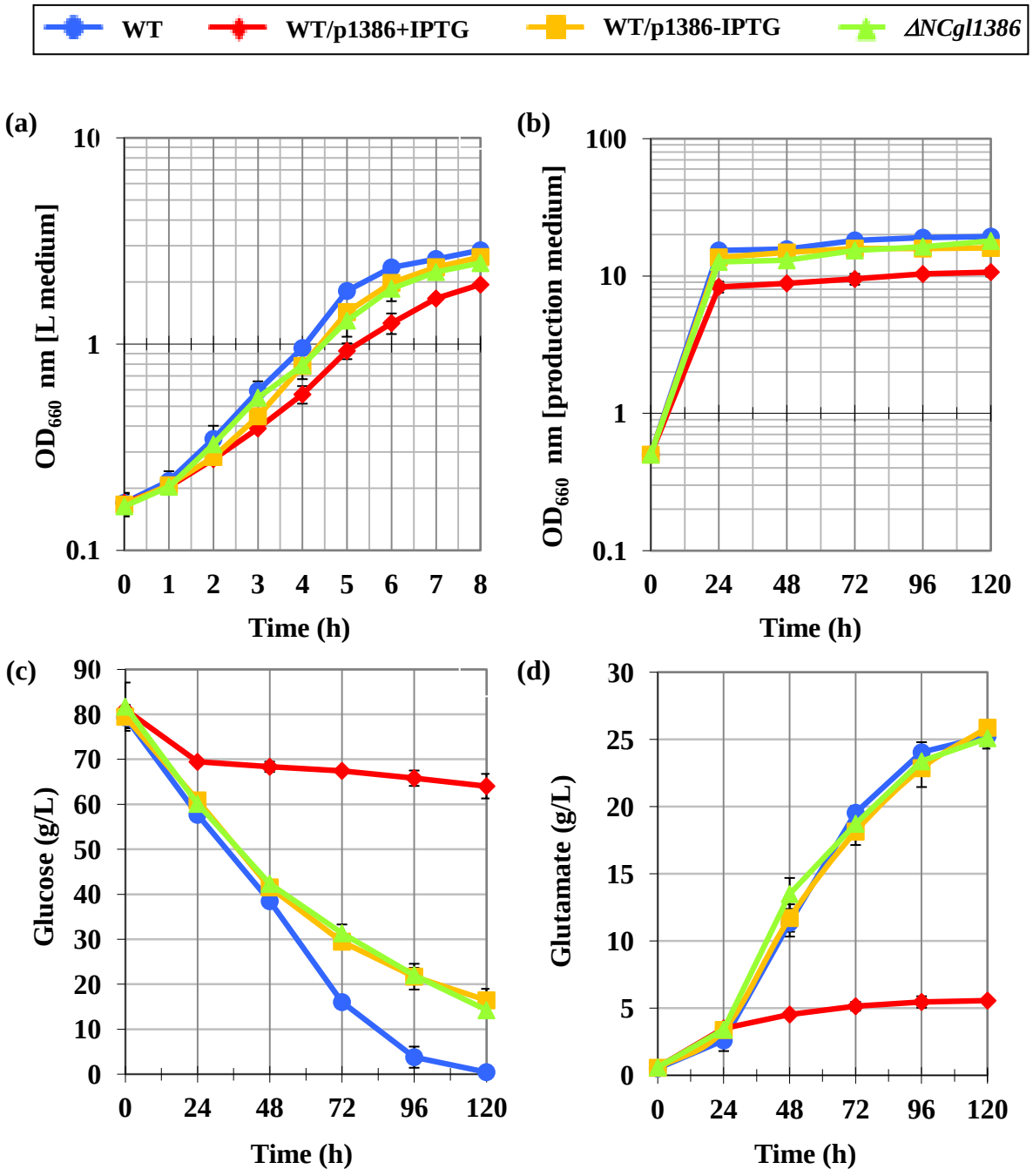


Fig. 4-14 **Glutamate production induced by biotin limitation. Cell growth in L medium (a), cell growth in production medium (b), glucose consumption (c), and glutamate production (d) are shown. Plasmid vector is induced by 1mM IPTG addition. The mean values of three independent experiments are presented with standard deviations.**

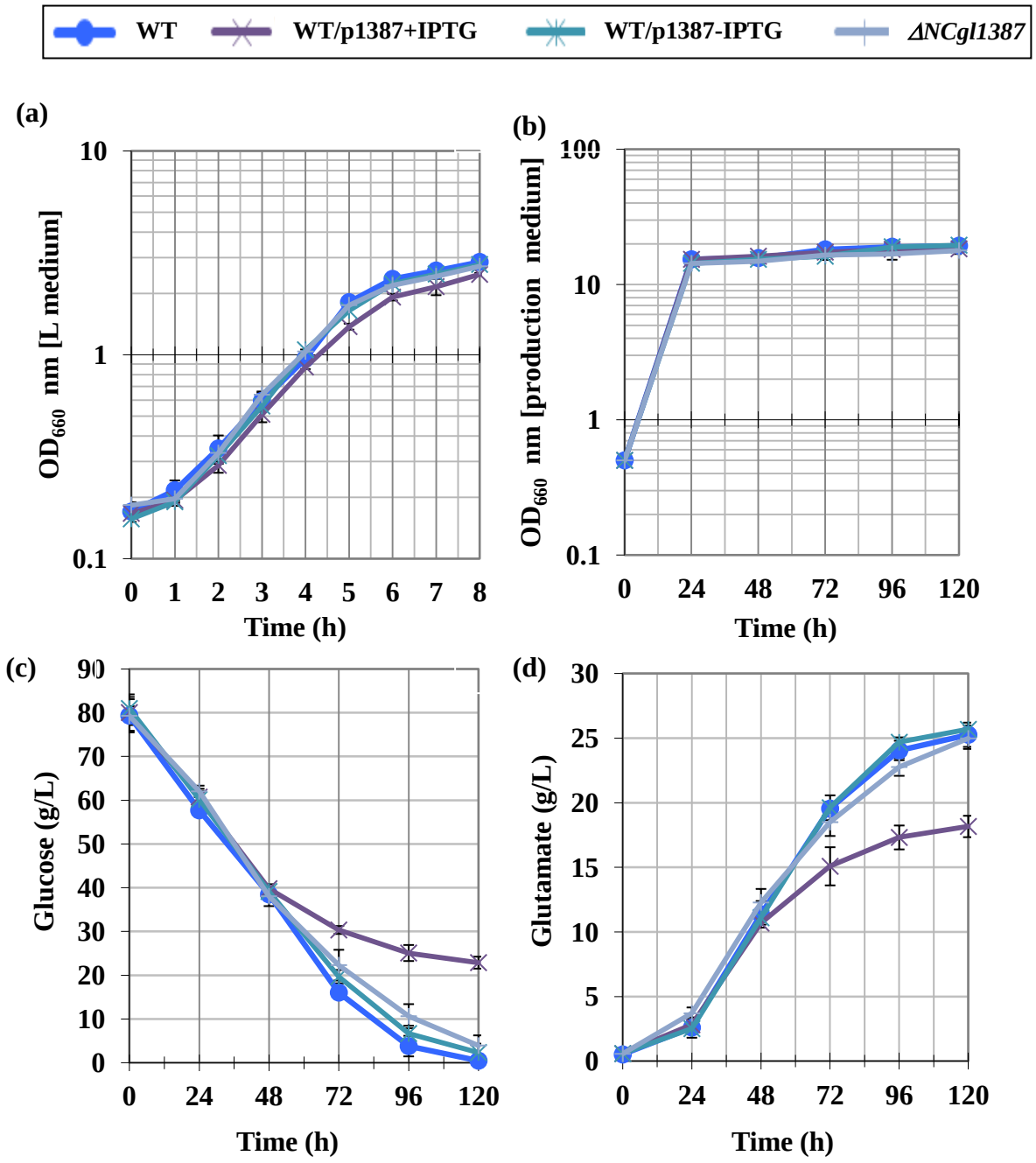


Fig. 4-15 **Glutamate production induced by biotin limitation. Cell growth in L medium (a), cell growth in production medium (b), glucose consumption (c), and glutamate production (d) are shown. Plasmid vector is induced by 1mM IPTG addition. The mean values of three independent experiments are presented with standard deviations.**

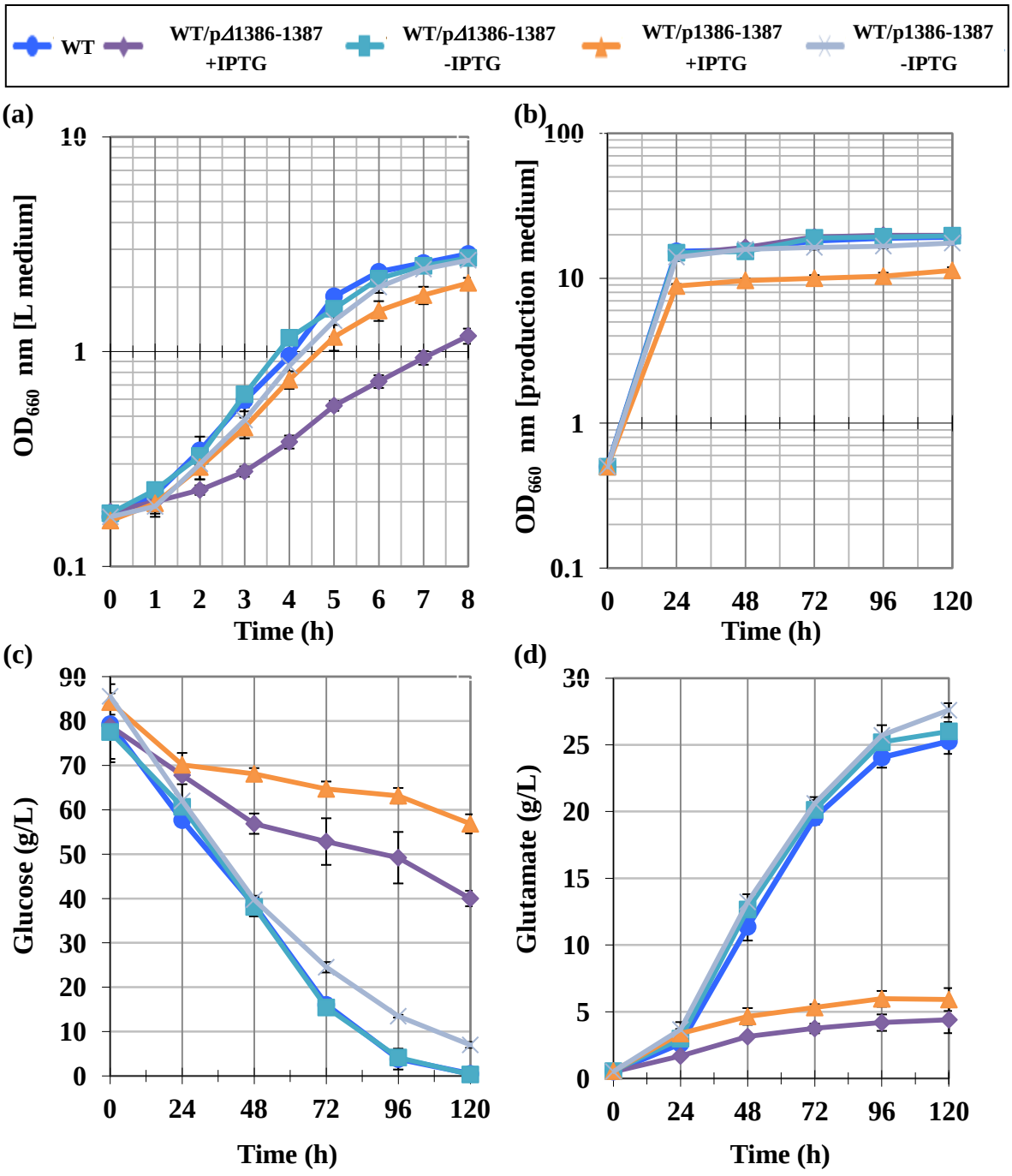


Fig. 4-16 **Glutamate production induced by biotin limitation. Cell growth in L medium (a), cell growth in production medium (b), glucose consumption (c), and glutamate production (d) are shown. Plasmid vector is induced by 1mM IPTG addition. The mean values of three independent experiments are presented with standard deviations.**

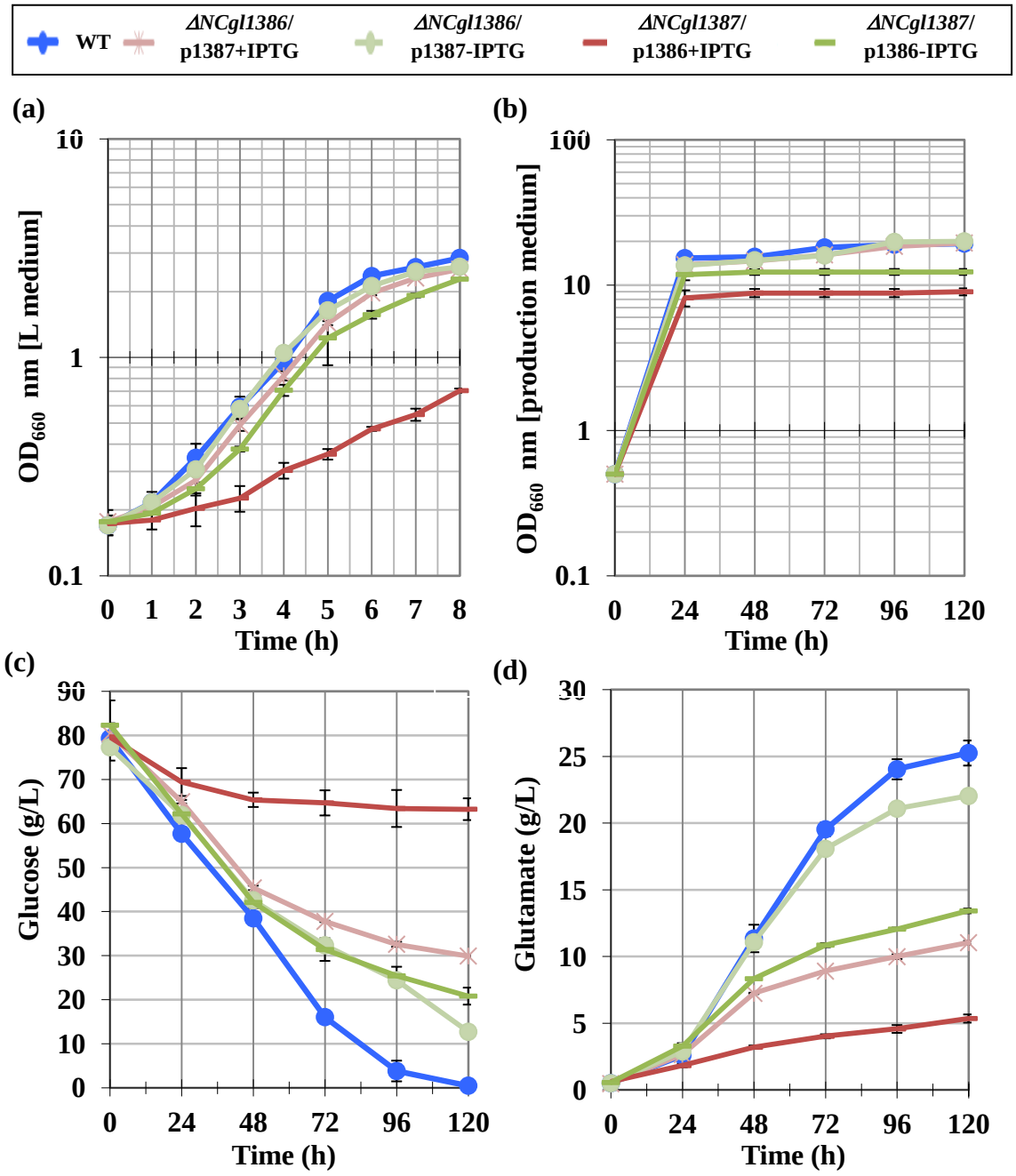


Fig. 4-17 **Glutamate production induced by biotin limitation. Cell growth in L medium (a), cell growth in production medium (b), glucose consumption (c), and glutamate production (d) are shown. Plasmid vector is induced by 1mM IPTG addition. The mean values of three independent experiments are presented with standard deviations.**

Table 4-5 Glutamate production under biotin-limited conditions

Strains	Glucose consumption (g/L)	Glutamate production (g/L)	Production yield [a] (%)	Specific productivity (g/L•OD•day)
WT	78.8 ± 2.8	25.3 ± 1.0	39.2 ± 0.4	0.26 ± 0.01
WT/p1386−IPTG	63.0 ± 3.8 [c]	25.9 ± 0.4	50.4 ± 3.7 [c]	0.32 ± 0.01 [c]
WT/p1386+IPTG	17.0 ± 2.5 [b]	5.6 ± 0.1 [b]	40.7 ± 6.3	0.10 ± 0.01 [b]
ΔNCgl1386	67.5 ± 4.7 [c]	25.1 ± 0.6	45.7 ± 3.5 [d]	0.28 ± 0.02
WT/p1387−IPTG	78.6 ± 0.7	25.7 ± 0.3	40.0 ± 0.8	0.26 ± 0.01
WT/p1387+IPTG	57.1 ± 2.8 [b]	18.2 ± 0.8 [b]	39.1 ± 3.7	0.20 ± 0.01 [c]
ΔNCgl1387	78.8 ± 2.8	25.3 ± 1.0	39.2 ± 0.4	0.28 ± 0.01 [c]
WT/pΔ1386-1387 −IPTG	77.2 ± 6.6	26.0 ± 0.7	41.4 ± 2.4	0.04 ± 0.01 [b]
WT/pΔ1386-1387+IPTG	38.9 ± 6.2 [b]	4.4 ± 1.0 [b]	14.2 ± 4.3 [c]	0.26 ± 0.00 [d]
WT/p1386-1387−IPTG	78.6 ± 6.0	27.6 ± 0.5 [c]	43.1 ± 2.5	0.32 ± 0.01 [b]
WT/p1386-1387+IPTG	27.4 ± 5.5 [b]	5.9 ± 0.9 [b]	26.6 ± 2.6 [c]	0.11 ± 0.02 [b]
ΔNCgl1386/ p1387−IPTG	73.3 ± 0.2 [d]	22.1 ± 0.3 [c]	36.9 ± 0.4 [b]	0.22 ± 0.00 [b]
ΔNCgl1386/ p1387+IPTG	47.0 ± 0.6 [b]	11.1 ± 0.1 [b]	28.8 ± 0.6 [b]	0.11 ± 0.00 [b]
ΔNCgl1387/ p1386-IPTG	61.5 ± 7.5 [c]	20.1 ± 0.8 [b]	40.3 ± 3.2	0.22 ± 0.01
ΔNCgl1387/ p1386+IPTG	16.4 ± 4.8 [b]	5.6 ± 0.5 [b]	44.2 ± 11.4	0.12 ± 0.01 [b]

Glucose consumption and glutamate production under biotin-limited conditions at 120 h (5 days) are shown. Data represent means ± SDs for three independent experiments.

a) Production yield was calculated as;

$$\frac{\text{Glutamate production (g/L)} / 147}{\text{Glucose consumption (g/L)} / 180} \times 100$$

Student's t-test: b) $p < 0.01$, c) $0.01 \leq p < 0.05$, d) $0.05 \leq p < 0.1$

4-4 Discussion

In this study, I aim to clarify the role of MerR-like transcriptional regulator, NCgl1386. All known members of the MerR family regulators are activators (Brown et al., 2003). To study the role of NCgl1386 in glutamate metabolism under biotin-limited conditions, strains with NCgl1386 overexpression and disruption had been constructed.

Overexpression of NCgl1386 induced with IPTG showed a marked decrease in growth as well as glutamate production and glucose consumption (Fig. 4-14, Table 4-5). Disruption of NCgl1386 showed a slight decrease in growth, glucose consumption and glutamate production (Fig. 4-14, Table 4-5). As NCgl1385 (*odhI*), NCgl1386 and NCgl1387 form an operon (Fig. 4-11), effects of overexpression and deletion of NCgl1386 to this operon were studied by qRT-PCR (Fig. 4-12a). The results revealed that both overexpression and deletion of NCgl1386 led to overexpression of NCgl1387, but effect on expression of NCgl1385 was ambiguous. Therefore, effects of overexpression and disruption of NCgl1387 were examined. Overexpression of NCgl1387 induced with IPTG showed only a slight decrease in growth in L medium (Fig. 4-15a). However, in production medium, it showed growth similar to WT, while glucose consumption and glutamate production decreased (Fig. 4-15, Table 4-5). Later, qRT-PCR revealed that NCgl1387 negatively control the NCgl1385-1386-1387 operon (Fig. 4-12b). Not only the expressions of NCgl1385 and NCgl1386 were highly decreased in NCgl1387 overexpressing strain with IPTG addition, while both genes were also highly overexpressed in NCgl1387 disruptant. Since high overexpression of NCgl1387 in NCgl1387 overexpressing strain with IPTG addition did not result in growth inhibition as severe as NCgl1386 overexpressing strain

with IPTG addition, suggesting that NCgl1386 has an important key to trigger the growth inhibition. As overexpressing strain of NCgl1386-1387 and overexpression of NCgl1386 in NCgl1387 disruptant with IPTG addition (*ΔNCgl1387*/p1386+IPTG) showed growth inhibition similar to NCgl1386 overexpressing strain (Fig. 4-14, 4-16, 4-17, Table 4-5), indicating that NCgl1386 alone could cause growth inhibition and NCgl1387 is essentially not required to trigger such inhibition but the mechanism of growth inhibition remains to be elucidated. qRT-PCR revealed that NCgl1386 has a positive effect on the NCgl1386-1387 operon (Fig. 4-12a) and it was shown that overexpressed NCgl1386 upregulated NCgl1385 (*odhI*) expression, that is, NCgl1386 positively controls the NCgl1385-1386-1387 operon. Moreover, overexpression of NCgl1387 in NCgl1386 disruptant with IPTG addition (*ΔNCgl1386*/p1387+IPTG) caused a similar result to that of overexpressing strain of NCgl1387 with IPTG addition (Fig. 4-15, 4-17, Table 4-5). It is suggested that NCgl1386 and NCgl1387 function independently. Furthermore, NCgl1387 displayed a slight growth decrease in L medium but restored growth in production medium (in the presence of glucose), which is probably because *odhI* is diminished by the overexpression of NCgl1387 (Fig 4-12b). As OdhI is ODHC inhibitor, decrease of OdhI consequently resulted in the higher metabolic flow toward TCA cycle for growth than the flux toward glutamate production. However, the reasons why NCgl1386 disruptant also showed overexpression of NCgl1387, (this overexpressed NCgl1387 did not negatively control the expression of OdhI), and why NCgl1387 disruptant, where OdhI and NCgl1386 are overexpressed, did not inhibit growth remain to be answered.

Predicted roles of NCgl1385-1387 operon are summarized in Fig. 4-17, NCgl1385 encodes OdhI protein, which in its unphosphorylated form inhibits OdhA subunit in ODHC, resulting in decrease of the metabolic flux toward TCA cycle and increase in the metabolic flux toward glutamate production. NCgl1386 encodes a Mer-like activator, which controls the expression of NCgl1385-1386-1387 and NCgl1386-1387 operon positively. NCgl1386 is also an activator to an unknown gene(s) or operon, which also cause growth inhibition. NCgl1387 encodes a negative regulator, which may act as an RNase not only to the NCgl1385-1386-1387 and NCgl1386-1387 mRNA transcripts but also an unknown mRNA transcript(s) required for metabolic flow of glucose utilization for growth.

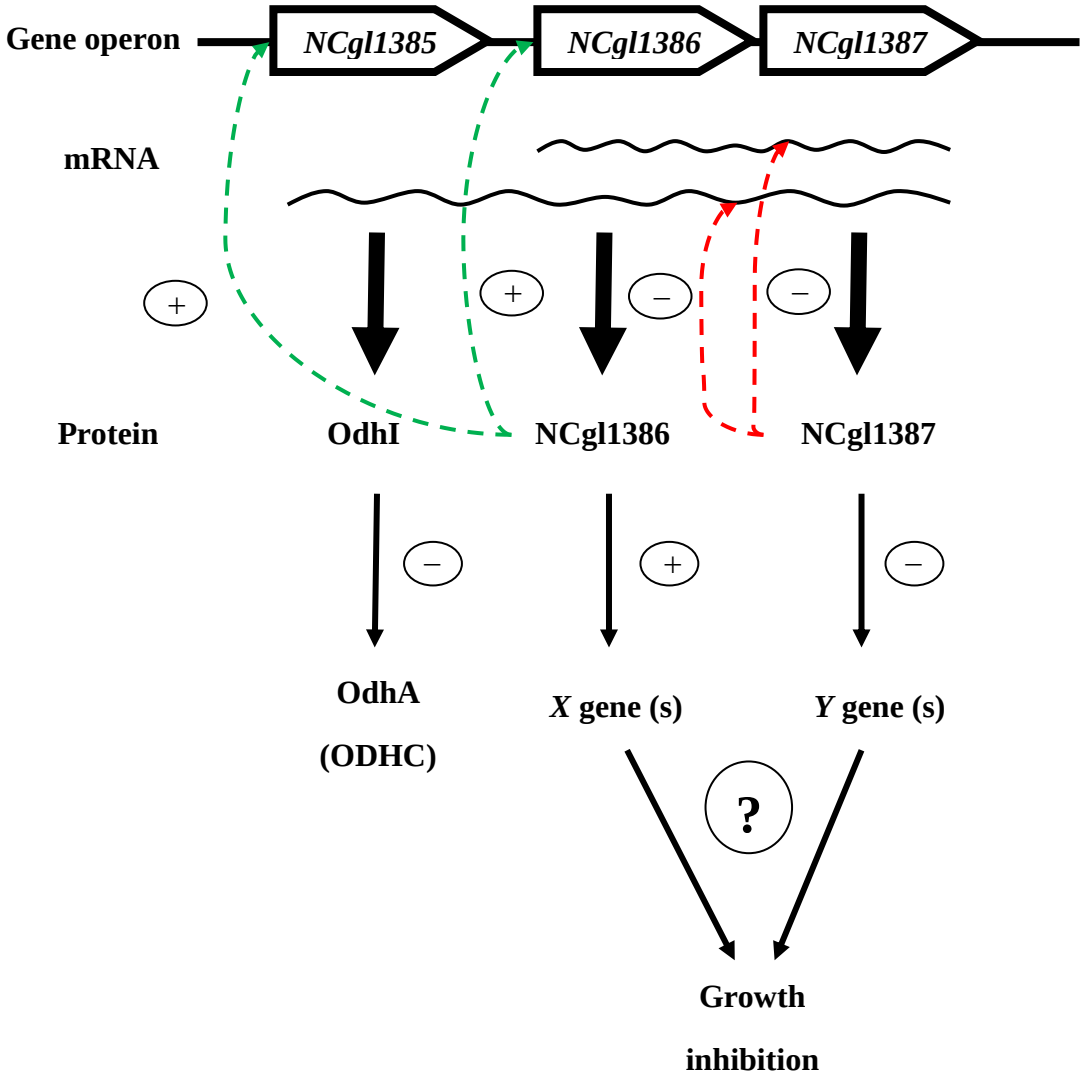


Fig. 4-17 **Schematic of predicted regulatory roles of NCgl1385-1386-1387 proteins. NCgl1385, NCgl1386 and NCgl1387 form an operon. NCgl1386 functions as an activator of NCgl1385-1385-1387 and NCgl1386-1387 as well as of unknown gene *X*. NCgl1387 degrades NCgl1385-1386-1387 mRNA and NCgl1386-1387 mRNA as well as mRNA of unknown gene *Y*.**

Chapter 5 Conclusion and future prospects

Corynebacterium glutamicum is a gram positive, rod-shaped aerobic bacterium used for the fermentative production of L-glutamate and several other amino acids. L-Glutamate production by *C. glutamicum* is triggered under biotin-limited conditions as well as by the addition of fatty acid esters and β-lactam antibiotics (Nara et al., 1964; Nunheimer et al., 1970; Radmacher et al., 2005; Shiio et al., 1963; Shiio et al., 1962; Takinami et al., 1965; Takinami et al., 1968). *C. glutamicum* NCgl1221 gene had been found to encode an L-glutamate exporter transmembrane protein, which is activated upon treatments causing the alternations in membrane tension such as biotin limitation. Disruption of this gene greatly diminished L-glutamate secretion. Specific mutations of this gene lead to a constitutive L-glutamate secretion without any treatments or inductions. The mutation of NCgl1221 also caused a decrease of 2-Oxoglutarate dehydrogenase complex (ODHC) activity, a key enzyme of L-glutamate metabolism. Thus, mutation of NCgl1221 gene should cause a reduction of ODHC activity, which may be controlled by a regulator. The present research aims to identify the regulator(s) in *C. glutamicum*, which connects NCgl1221 with glutamate metabolism.

C. glutamicum ATCC 13869 WT strain and NCgl1221 active mutant strain (BL1) were grown in the glutamate production medium containing excess biotin. WT cannot produce L-glutamate in the presence of biotin, while mutant BL1 could produce L-glutamate in this condition. Total cellular RNA was isolated for microarray analysis. Genes encoding transcriptional regulators, NCgl1386 and NCgl2814, have been found to be highly down-regulated in the mutant cells. In order to investigate the role of these

transcriptional regulators in glutamate metabolism, deletion and overexpression strains of these genes have been constructed.

It was found that glutamate production decreased by overproduction of NCgl2814 (LldR). LldR is known as a repressor for *ldhA* and *lldD* encoding lactate dehydrogenases. LdhA is responsible for production of lactate, while LldD is for its assimilation. In the wild-type cells, lactate was produced in the first 24 h and it was re-consumed thereafter. On the other hand, in the overproduced cells, lactate was produced like WT, but it was not re-consumed. This means that lactate assimilation, which is catalyzed by LldD, was suppressed by the overproduction of LldR, but lactate production, which is catalyzed by LdhA, was not affected, indicating that LldD functions as a repressor for *lldD* but not for *ldhA* under glutamate-producing conditions (Fig. 5-1). Repression of *lldD* gene was also confirmed by qRT-PCR in the LldR overexpressing strain, while expression of *ldhA* was not affected, indicating that LldR only represses *lldD* during the glutamate production under biotin-limited conditions. From these results, it is suggested that lactate metabolism, which is controlled by LldR, has a buffering function of pyruvate pool for glutamate production.

Furthermore, lactate metabolism during the glutamate production under biotin-limited conditions and penicillin addition conditions were also studied by the *ldhA* disruption compared with WT. Glucose consumption and glutamate production were significantly decreased under biotin-limited conditions in *ldhA* disruptant compared with WT. On the other hand, glucose consumption and glutamate production of *ldhA* disruptant increased to a comparable amount or even higher compared with WT under penicillin

addition conditions. Higher L-glutamate was produced under the penicillin addition conditions compared with the biotin-limited conditions in the WT. These results suggest that accumulation of pyruvate may significantly increase under biotin-limited conditions due to the limit of pyruvate carboxylase (PC), which is a biotin-containing enzyme, which consequently leads to a decrease in glycolytic flow. This problem was overcome under penicillin addition conditions where pyruvate accumulation may be abolished due to higher activity of PC compared with that of biotin limitation. Therefore, under penicillin addition conditions, a buffering function of pyruvate pool for glutamate production by lactate metabolism is not needed and *ldhA* disruptant is more efficient for glutamate production under this condition rather than biotin-limited conditions. From these results, it is suggested that the decrease in metabolic flow to lactate formation is not effective for the enhancement of glutamate production under biotin-limited conditions since the produced L-lactate is reused by the cells to produce L-glutamate. Glutamate production assay with overproducer/deletion strains of *ldhA* and *lldD* as well as flux analysis with ^{13}C-labeled substates and promoter activity assay will be necessary to confirm this conclusion. Disruption of *lldR* together with the manipulation of genes involved in the flux toward glutamate production and co-overexpression of *ldhA* and *lldD* might give a new aspect to improve the glutamate production under biotin-limited conditions.

Overproduction of NCgl1386 caused severe growth retardation and a decrease in glutamate production. It is confirmed by RT-PCR that NCgl1386 forms an operon with NCgl1385 (*odhI*), encoding an inhibitor of ODHC, and NCgl1387, which has been found to act as a negative regulator to this operon. Overexpression of NCgl1387 also caused a slight

growth inhibition, which is independent from the inhibition caused by NCgl1386. Therefore, NCgl1386 and NCgl1387 separately function for growth inhibition via the control of some unknown gene(s). NCgl1386 functions as an activator of NCgl1385-1386-1387 and NCgl1387 functions as a negative regulator of NCgl1385-1386-1387 (Fig. 5-1). Moreover, NCgl1386 and NCgl1387 control the expression of unknown genes responsible for growth inhibition. Microarray analysis with overproducing strains and deletion strains may be needed to find out genes under control of NCgl1386 and NCgl1387. However, the regulatory mechanism for growth inhibition by NCgl1386 and NCgl1387 still need to be elucidated. NCgl1387 may function as an RNase. qRT-PCR analysis suggested that NCgl1387 degrades NCgl1385-1386-1387 mRNA as well as NCgl1386-1387. Regulation of gene expression by RNase is very unique. *in vitro* assay for RNase activity is required to elucidate the function of NCgl1387 RNase. To clarify the role of NCgl1386, DNaseI footprint may be useful to identify binding site of this regulator. Overproducer/deletion strains of *odhI* are necessary for studying the function of this operon. Moreover, overexpression of OdhI in NCgl1386 disruptant, where *odhI* expression was unaffected by the overexpression of NCgl1387, as well as in NCgl1387 disruptant, where *odhI* is overexpressed, could offer an answer to several remaining unsolved questions and might give an interesting outcome to glutamate production.

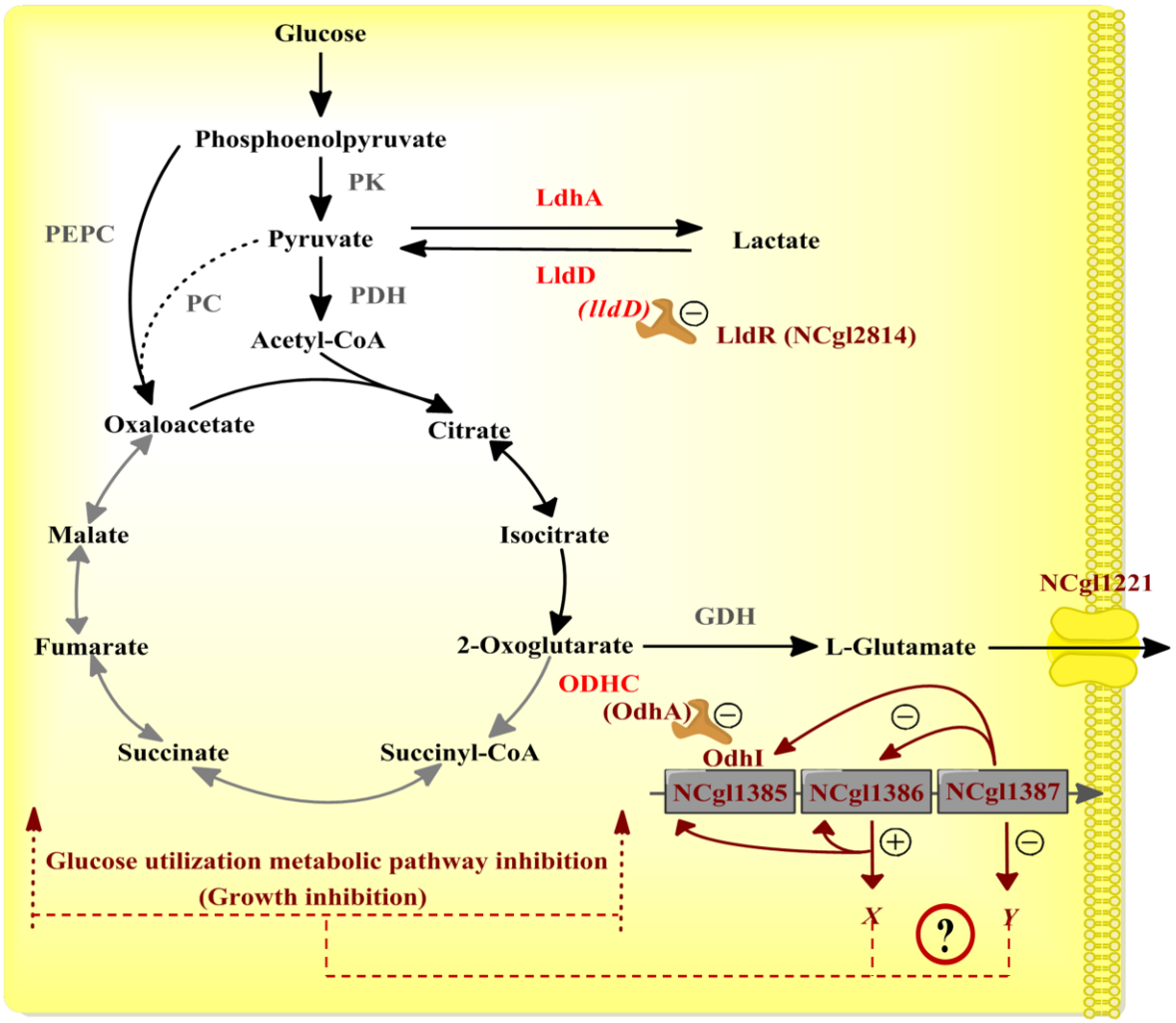


Fig. 5-1 **Summarized schematic of transcriptional regulators involved in L-glutamate production under biotin-limited conditions in *C. glutamicum***

Acknowledgements

I would like to express my deepest gratitude and appreciation to Professor Masaaki Wachi, Department of Bioengineering, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, for giving me the opportunity to study in his laboratory and for all of his enthusiastic support, careful guidance, and continuous encouragement in my research.

I am very grateful to the members of my committee, Professor Satoshi Nakamura, Professor Yasunoru Tanji, Associate Professor Toshiaki Fukui, and Associate Professor Syun-ichiro Ogura, Department of Bioengineering, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, for their insightful comments and constructive suggestions.

I would like to gratefully acknowledge Associate Professor Kenji Ueda, Life Science Reasearch Center, College of Bioresource Sciences, Nihon University, for his helpful suggestions and valuable comments throughout my research during my internship.

I would like to express my gratitude to the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan for the Japanese government (Monbukagakusho) scholarship, which has financially supported me during this study.

Furthermore, this work was supported in part by a grant-in-aid for Scientific Research (B) (20380047 to M. W.) from the Japan Society for Promotion of Science, a grant from the Ministry of Economy, Trade and Industry of Japan entrusted by the New

Energy and Industrial Technology Development Organization, and the supporting program by the Skylark Food Science Institute.

I would like to thank all members in Wachi lab, especially, Yusuke Inoue, Takahiro Yamanouchi, Tomoya Maeda, Kousuke Kumagai, Kazutaka Ito, Akina Horikawa, Chikako Yamashita, Saho Takeuchi, Motoyuki Sagara, Yuki Kitahara, Akira Kumagai, Jo Yongil, and Natalia Maria Theresia for all the help, support and encouragement during my research and study.

Lastly, I would like to thank all of my friends and family who always be there to support me and gave me so much inspiration to keep me moving forward, to fight and overcome the hardships, to relieve and comfort me from stressful situations, and continue to encourage me to try my best and make an effort and eagerness throughout my study until graduation.

Tanyanut Supkulsutra

Bibliography

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

Borngen, K., Battle, A.R., Moker, N., Morbach, S., Marin, K., Martinac, B., and Kramer, R. (2010) The properties and contribution of the *Corynebacterium glutamicum* MscS variant to fine-tuning of osmotic adaptation. *Biochim. Biophys. Acta.*, **1798**, 2141-2149.

Bott, M., and Niebisch, A. (2003) The respiratory chain of *Corynebacterium glutamicum*. *J. Biotechnol.*, **104**, 129-153.

Brinkrolf, K., Schroder, J., Puhler, A., and Tauch, A. (2010) The transcriptional regulatory repertoire of Corynebacterium glutamicum: reconstruction of the network controlling pathways involved in lysine and glutamate production. *J Biotechnol*, **149**, 173-182.

Brown, N.L., Stoyanov, J.V., Kidd, S.P., and Hobman, J.L. (2003) The MerR family of transcriptional regulators. *FEMS Microbiol Rev*, **27**, 145-163.

Demain, A.L., and Birnbaum, J. (1968) Alteration of permeability for the release of metabolites from the microbial cell. *Curr. Top. Microbiol. Immunol.*, **46**, 1-25.

Eggeling, L., Krumbach, K., and Sahm, H. (2001) L-glutamate efflux with Corynebacterium glutamicum: why is penicillin treatment or Tween addition doing the same? *J. Mol. Microbiol. Biotechnol.*, **3**, 67-68.

Engels, V., Lindner, S.N., and Wendisch, V.F. (2008) The global repressor SugR controls expression of genes of glycolysis and of the L-lactate dehydrogenase LdhA in *Corynebacterium glutamicum*. *J. Bacteriol.*, **190**, 8033-8044.

Gao, Y.G., Suzuki, H., Itou, H., Zhou, Y., Tanaka, Y., Wachi, M., Watanabe, N., Tanaka, I., and Yao, M. (2008) Structural and functional characterization of the LldR from *Corynebacterium glutamicum*: a transcriptional repressor involved in L-lactate and sugar utilization. *Nucleic Acids Res.*, **36**, 7110-7123.

Georgi, T., Engels, V., and Wendisch, V.F. (2008) Regulation of L-lactate utilization by the FadR-type regulator LldR of *Corynebacterium glutamicum*. *J. Bacteriol.*, **190**, 963-971.

Hasegawa, T., Hashimoto, K., Kawasaki, H., and Nakamatsu, T. (2008) Changes in enzyme activities at the pyruvate node in glutamate-overproducing *Corynebacterium glutamicum*. *J. Biosci. Bioeng.*, **105**, 12-19.

Hashimoto, K., Kawasaki, H., Akazawa, K., Nakamura, J., Asakura, Y., Kudo, T., Sakuradani, E., Shimizu, S., and Nakamatsu, T. (2006) Changes in composition and content of mycolic acids in glutamate-overproducing Corynebacterium glutamicum. *Biosci Biotechnol Biochem*, **70**, 22-30.

Hashimoto, K., Murata, J., Konishi, T., Yabe, I., Nakamatsu, T., and Kawasaki, H. (2012) Glutamate is excreted across the cytoplasmic membrane through the NCgl1221 channel of *Corynebacterium glutamicum* by passive diffusion. *Biosci. Biotechnol. Biochem.*, **76**, 1422-1424.

Hashimoto, K., Nakamura, K., Kuroda, T., Yabe, I., Nakamatsu, T., and Kawasaki, H. (2010) The protein encoded by NCgl1221 in *Corynebacterium glutamicum*

functions as a mechanosensitive channel. *Biosci. Biotechnol. Biochem.*, **74**, 2546-2549.

Hermann, T. (2003) Industrial production of amino acids by coryneform bacteria. *J Biotechnol*, **104**, 155-172.

Ikeda, K. (1908) A production method of seasoning mainly consists of salt of L-glutamic acid. *Japanese patent 14805.*

Ikeda, M. (2003) Amino Acid Production Processes. In Microbial Production of L-Amino Acids, ed. by Faurie, R., Thommel, J., Bathe, B., Debabov, V.G., Huebner, S., Ikeda, M., Kimura, E., Marx, A., Möckel, B., Mueller, U. and Pfefferle, W., Springer, Berlin, Heidelberg, pp. 1-35.

Inui, M., Murakami, S., Okino, S., Kawaguchi, H., Vertes, A.A., and Yukawa, H. (2004) Metabolic analysis of *Corynebacterium glutamicum* during lactate and succinate productions under oxygen deprivation conditions. *J. Mol. Microbiol. Biotechnol.*, **7**, 182-196.

Kawahara, Y., Takahashi-Fuke, K., Shimizu, E., Nakamatsu, T., and 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., Fukuda, H., Hirasawa, T., Nagahisa, K., Nagai, K., Wachi, M., and 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.

Kim, J., Hirasawa, T., Saito, M., Furusawa, C., and Shimizu, H. (2011) Investigation of phosphorylation status of OdhI protein during penicillin- and Tween 40-triggered glutamate overproduction by *Corynebacterium glutamicum*. *Appl. Microbiol. Biotechnol.*, **91**, 143-151.

Kimura, E. (2002) Triggering mechanism of L-glutamate overproduction by DtsR1 in coryneform bacteria. *J. Biosci. Bioeng.*, **94**, 545-551.

Kimura, E. (2003) Metabolic Engineering of Glutamate Production. In Microbial Production of l-Amino Acids, ed. by Faurie, R., Thommel, J., Bathe, B., Debabov, V.G., Huebner, S., Ikeda, M., Kimura, E., Marx, A., Möckel, B., Mueller, U. and Pfefferle, W., Springer, Berlin, Heidelberg, pp. 37-57.

Kimura, E. (2005) L-Glutamate Production. In Handbook of *Corynebacterium glutamicum*, ed. by Eggeling, L. and Bott, M., CRC Press, Taylor & Francis Group, Boca Raton, pp. 439-463.

Kimura, E., Abe, C., Kawahara, Y., and 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., and 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., and Tokuda, H. (1999) Glutamate overproduction in *Corynebacterium glutamicum* triggred by a decrease

in the level of a complex comprising DtsR and a biotin-containing subunit. *Biosc. Biotech. Biochem.*, **63**, 1274-1278.

Kinoshita, S. (2005) A Short History of the Birth of the Amino Acid Industry in Japan. In Handbook of *Corynebacterium glutamicum*, ed. by Eggeling, L. and Bott, M., CRC Press, Taylor & Francis Group, Boca Raton, pp. 3-5.

Kinoshita, S., Udaka, S., and Shimono, M. (1957) Studies on the amino acid fermentation. Part 1. Production of L-glutamic acid by various microorganisms. *J. Gen. Appl. Microbiol.*, **3**, 193-205.

Nakamura, J., Hirano, S., Ito, H., and 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.

Nakayama, Y., Yoshimura, K., and Iida, H. (2012) A gain-of-function mutation in gating of *Corynebacterium glutamicum* NCgl1221 causes constitutive glutamate secretion. *Appl. Environ. Microbiol.*, **78**, 5432-5434.

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

Niebisch, A., Kabus, A., Schultz, C., Weil, B., and 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.

Nunheimer, T.D., Birnbaum, J., Ihnen, E.D., and Demain, A.L. (1970) Product inhibition of the fermentative formation of glutamic acid. *Appl. Microbiol.*, **20**, 215-217.

O'Mahony, M., and Ishii, R. (1986) A comparison of English and Japanese taste languages: taste descriptive methodology, codability and the umami taste. *Br. J. Psychol.*, **77 (Pt 2)**, 161-174.

Radmacher, E., Stansen, K.C., Besra, G.S., Alderwick, L.J., Maughan, W.N., Hollweg, G., Sahm, H., Wendisch, V.F., and Eggeling, L. (2005) Ethambutol, a cell wall inhibitor of Mycobacterium tuberculosis, elicits L-glutamate efflux of *Corynebacterium glutamicum*. *Microbiology*, **151**, 1359-1368.

Sano, C. (2009) History of glutamate production. *Am J Clin Nutr*, **90**, 728S-732S.

Sato, H., Orishimo, K., Shirai, T., Hirasawa, T., Nagahisa, K., Shimizu, H., and 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.

Schafer, A., Tauch, A., Jager, W., Kalinowski, J., Thierbach, G., and Puhler, A. (1994) Small mobilizable multi-purpose cloning vectors derived from the Escherichia coli plasmids pK18 and pK19: selection of defined deletions in the chromosome of Corynebacterium glutamicum. *Gene*, **145**, 69-73.

Schroder, J., and Tauch, A. (2010) Transcriptional regulation of gene expression in Corynebacterium glutamicum: the role of global, master and local regulators in the modular and hierarchical gene regulatory network. *FEMS Microbiol Rev*, **34**, 685-737.

Schultz, C., Niebisch, A., Gebel, L., and 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.

Sharkey, M.A., Maher, M.A., Guyonvarch, A., and Engel, P.C. (2011) Kinetic characterisation of recombinant Corynebacterium glutamicum NAD+-dependent LDH over-expressed in E. coli and its rescue of an lldD- phenotype in C. glutamicum: the issue of reversibility re-examined. *Arch Microbiol*, **193**, 731-740.

Shiio, I., Ohtska, S., and Takahashi, M. (1961) Significance of α-ketoglutarate dehydrogenase on the glutamic acid formation in *Brevibacterium flavum*. *J. Biochem.*, **50**, 164-165.

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

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

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

Shimizu, H., Tanaka, H., Nakato, A., Nagahisa, K., Kimura, E., and 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.

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

Stansen, C., Uy, D., Delaunay, S., Eggeling, L., Goergen, J.L., and Wendisch, V.F. (2005) Characterization of a *Corynebacterium glutamicum* lactate utilization operon induced during temperature-triggered glutamate production. *Appl. Environ. Microbiol.*, **71**, 5920-5928.

Takinami, K., Yoshii, H., Tsuji, H., and 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.

Takinami, T., Yoshii, H., Yamada, Y., Okada, H., and Kinoshita, K. (1968) Control of L-glutamic acid fermentation by biotin and fatty acid. *Amino Acid Nucleic Acid*, **18**, 120-160.

Teramoto, H., Inui, M., and Yukawa, H. (2011) Transcriptional regulators of multiple genes involved in carbon metabolism in Corynebacterium glutamicum. *J Biotechnol*, **154**, 114-125.

Toyoda, K., Teramoto, H., Inui, M., and Yukawa, H. (2009a) The ldhA gene, encoding fermentative L-lactate dehydrogenase of *Corynebacterium glutamicum*, is under the control of positive feedback regulation mediated by LldR. *J. Bacteriol.*, **191**, 4251-4258.

Toyoda, K., Teramoto, H., Inui, M., and Yukawa, H. (2009b) Molecular mechanism of SugR-mediated sugar-dependent expression of the ldhA gene encoding L-lactate dehydrogenase in *Corynebacterium glutamicum*. *Appl. Microbiol. Biotechnol.*, **83**, 315-327.

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

Uy, D., Delaunay, S., Germain, P., Engasser, J.M., and Goergen, J.L. (2003) Instability of glutamate production by *Corynebacterium glutamicum* 2262 in continuous culture using the temperature-triggered process. *J. Biotechnol.*, **104**, 173-184.

Wachi, M. (2013) Amino Acid Exporters in *Corynebacterium glutamicum*. In *Corynebacterium glutamicum*, ed. by Yukawa, H. and Inui, M., Springer, Berlin, Heidelberg, pp. 335-349.

Yamashita, C., Hashimoto, K., Kumagai, K., Maeda, T., Takada, A., Yabe, I., Kawasaki, H., and Wachi, M. (2013) L-Glutamate Secretion by the N-Terminal Domain of the Corynebacterium glutamicum NCgl1221 Mechanosensitive Channel. *Biosci Biotechnol Biochem*, **77**, 1008-1013.

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