A Thesis for the Degree of Master of Science

Stable production of 2'-fucosyllactose by enhancing lactose uptake and expressing biosynthetic genes in chromosome of *Corynebacterium glutamicum*

대사공학적으로 설계된 코리네박테리움 글루타미쿰에서 유당이용 증대와 염색체에서의 유전자 발현을 통한 2'-푸코실락토오스의 안정적 생산

By

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Department of Agricultural Biotechnology
Seoul National University
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Advisor : Professor Jin-Ho Seo

Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science

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農 學 碩 士 學 位 論 文

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指導敎授 徐鎭浩

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朴素姬

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ABSTRACT

The ingredient that differentiates human milk with mammalian milk is oligosaccharides. 50 ~ 80% of human milk oligosaccharides (HMOs) are fucosylated. 2'-Fucoyllactose (2'-FL) is the most abundant in fucosyl oligosaccharides. 2'-FL has prebiotic effects on promoting the growth of beneficial bacteria in the intestines, inhibits the growth of pathogenic bacteria, and improves immunity and brain development. Therefore, 2'-FL is getting attention as a key material for infant formula, functional foods, medicines and cosmetics. *Corynebacterium glutamicum*, which was used in this study, is a Gram-positive bacterium approved as GRAS (Generally Recognized As Safe) and has already been widely used for amino acid production industrially.

Previous studies have produced 2'-FL using metabolically engineered *C. glutamicum*. 2'-FL is synthesized by α-1,2 fucosylation of lactose and GDP-$_L$-fucose. The GDP-$_L$-fucose biosynthetic pathway was introduced into *C. glutamicum* and lactose was transported into the cells by introducing the *lacYA* operon. α-1,2 Fucosylation was achieved by expressing codon optimized α-1,2 fucosyltransferase (CO*fucT2*) derived from *Helicobacter pylori*. Batch fermentation and fed-batch fermentation were carried out, resulting in production of 2'-FL of 0.6 g/L in batch fermentation and 11.5 g/L in fed-batch fermentation.

In this study, *C. glutamicum* was further engineered to enhance the production of 2'-FL. The following three strategies were employed. First, some genes were removed so that only necessary genes can be expressed for 2'-FL production in order to minimize a metabolic burden

on the cell. Among the genes used previously, phosphomannomutase (*manB*) and GTP-mannose-1-phosphate guanylyltransferase (*manC*) already exist on the chromosome of *C. glutamicum*. The two genes were removed from the expression vector and as a result, 0.62 g/L of 2’-FL was produced in batch fermentation. Second, in the *lacYA* operon, only *lacY* was expressed to improve the utilization of lactose, and 0.93 g/L of 2’-FL was produced in batch fermentation. Third, the lactose permease gene (*lacY*) was expressed with the RBS (Ribosome Binding Site) and *tac* promoter, resulting in more lactose transport into the cell. As a result, 2'-FL was able to be produced at 1.94 g/L in batch fermentation and this is 3.3 times higher compared with the control strain which is developed in previous studies. Furthermore, the constructed strain was grown in fed-batch fermentation to improve the performance of 2’-FL production. 25.5 g/L of 2’-FL is produced in fed-batch fermentation, a 2.2 fold enhancement relative to the control strain.

Next, the use of antibiotics should be avoided for industrial fermentations. Since 2'-FL is used for foods and medicines, antibiotics-free production of 2’-FL could improve consumers' perception and can save the cost of separation and purification of antibiotics. Therefore, to construct a fermentation system that stably produces 2’-FL without using antibiotics, the genes necessary for producing 2'-FL were inserted into the chromosome of *C. glutamicum*. For the chromosomal integration, a double crossover method using the pK19mobsacB vector was used and the IS (Insertion Sequence) element was selected as a site for insertion. It was possible to introduce the genes on the chromosome of *C. glutamicum* by inserting CO*fucT2* into the site where IS*Cg2b* is

deleted. Then, IS*Cg2f* and IS*Cg1a* were further disrupted to provide a site for the insertion of the 2'-FL biosynthesis related genes, such as *gmd* and *wcaG*. As a result, 0.84 g/L of 2'-FL was produced in the batch fermentation without using kanamycin. When the genes (CO*fucT2*, *gmd*, *wcaG*) are expressed simultaneously on plasmids and chromosomes, 3.01 g/L of 2'-FL could be produced in flask fermentation without using antibiotics.

A fermentation system for producing high concentration of 2'-FL without using antibiotics was constructed. This study is expected to provide a technical basis for industrial production of 2'-FL by engineered *C. glutamicum*.

Keywords: Metabolic engineering, 2'-fucosyllactose, GDP-L-fucose, lactose permease, pK19mobsacB, double crossover method, fed-batch fermentation, *Corynebacterium glutamicum*

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I. INTRODUCTION

1. Human milk

Breastfeeding was thought to be done by the uneducated and those of lower classes in 1950s. While those who could not afford to buy infant formula considered breast milk as outdated, they thought it was superior to feed infant formula. (Nathoo and Ostry 2009). However, by the 1960s, as the function of human milk was reestablished, breastfeeding has resumed in Canada and the US, especially among more educated, affluent women (Nathoo and Ostry 2009). Nowadays, the World Health Organization (WHO) recommends exclusive breastfeeding for six months after birth.

Human milk is considered the best diet for newborn nutrition. In addition to providing the baby with all the nutrients needed for growth and development, breast milk contains a variety of physiologically active factors that promote healthy colonization of the neonatal intestine, prevent infection and help the immune system mature (Jantscher-Krenn and Bode 2012, Richards, Patel et al. 2013).

Breast milk consists of 3 to 5% fat, 0.8 to 0.9% protein, 6.9 to 7.2% carbohydrates, 0.2% inorganic salts and other ingredients (Jenness, 1979). These roughly classified ingredients are subdivided into many useful ingredients that provide health benefits as well as key nutrients. These health benefits include prebiotic effects, prevention of pathogen infection, modulation of immune responses, reduction of inflammatory processes, neurological development, and improved vaccine response (Lanting, Huisman et al. 1994, Severin and Wenshui 2005, Boehm and

Stahl 2007, Hahn-Zoric, Fulconis et al. 2008, Jantscher-Krenn and Bode 2012).

The composition of human milk and bovine milk shown in Table 1 shows a considerable difference. The oligosaccharide content of breast milk is much higher than that of bovine milk. High concentrations of oligosaccharides are the most unique feature of human milk. Oligosaccharides in breast milk are involved in several physiological functions.

Table 1. Composition of human and bovine milk (Jenness, 1979)

Contents	Human milk	Bovine milk
Fat (g/L)		
Total (g/L)	**42**	**38**
Fatty acids-length ≤8C (%)	trace	6
Polyunsaturated fatty acids (%)	14	3
Protein (g/L)		
Total	**11**	**33**
Casein 0.4	3	25
a-lactalbumin	3	1
Lactoferrin	2	Trace
IgA	1	0.03
IgG	0.01	0.6
Lysozyme	0.5	Trace
Serum albumin	0.5	0.3
β-lactoglobulin	-	3
Carbohydrate (g/L)		
Lactose	70	48
Oligosaccharides	**5 - 15**	**0.05**
Minerals (g/L)		
Calcium	0.3	1.25
Phosphorus	0.14	0.93
Sodium	0.15	0.47
Potassium	0.55	1.55
Chlorine	0.43	1.03

2. Human milk oligosaccharides (HMOs)

Oligosaccharides contained in breast milk is called Human Milk Oligosaccharides (HMOs). These are the third most abundant in human milk, followed by lactose and fat. Numerous studies have found that this major component is present in approximately 5-15 g/L in mature milk and approximately 22 g/L in colostrum (Newburg 1997, Coppa, Pierani et al. 1999, Kunz, Rudloff et al. 2000, Rivero-Urgell and Santamaria-Orleans 2001, Bode 2012).

To date, more than 200 types of HMOs have been found and their structure has been revealed. In fact, about 200 HMOs were found in breast milk (Ninonuevo, Park et al. 2006, Bode 2012, Jantscher-Krenn and Bode 2012). The composition of HMOs are not only very complex, but their physiological functions are closely related to their structures. Because HMOs are not digested in the small intestine of infant, the structures are maintained. (Miller and McVeagh 2007). Basically, HMOs are composed of the five monosaccharides; D-glucose (Glc), D-galactose (Gal), *N*-acetylglucosamine (GlcNAc), L-fucose (Fuc), and sialic acid [*N*-acetylneuraminic acid (NeuAc)] with lactose (Lac) core at the reducing end (Bode 2012, Jantscher-Krenn and Bode 2012). Biosynthesis of HMOs begins with a lactose molecule. Lactose can be elongated by an enzymatic attachment of GlcNAc residues linked in β1-3 or β1-6 linkage to the Gal residue followed by further addition of Gal in the β1-3 (lacto-*N*-biose) or β1-4 bond (*N*-acetyllactosamine) (Fig. 1A). Additional modifications are made by attachments of lactosamine, fucose, and/or NeuAc residues at different positions of the core region and the core elongation chain (Kunz, Rudloff et al. 2000, McVeagh and

Miller 2008, Bode 2012). Elongation with lacto-*N*-biose terminates the chain, in the mean time *N*-acetyllactosamine can be continuously extended by the addition of one of the two disaccharides. A chain branch is introduced by the β1-6 linkage between two disaccharide units. Branched structures are defined as *iso*-HMO; linear structures without branches as *para*-HMO (Fig. 1B). Lactose or the elongated oligosaccharide chain can be fucosylated at α1-2, α1-3 or α1-4 linkage and/or sialylated at α2-3 or α2-6 linkage (Fig. 1C–E). In addition, some HMOs have several isomeric forms, such as lacto-*N*-fucopentaose (LNFP, Fig. 1D) or sialyllacto-*N*-tetraose (LST, Fig. 1E).

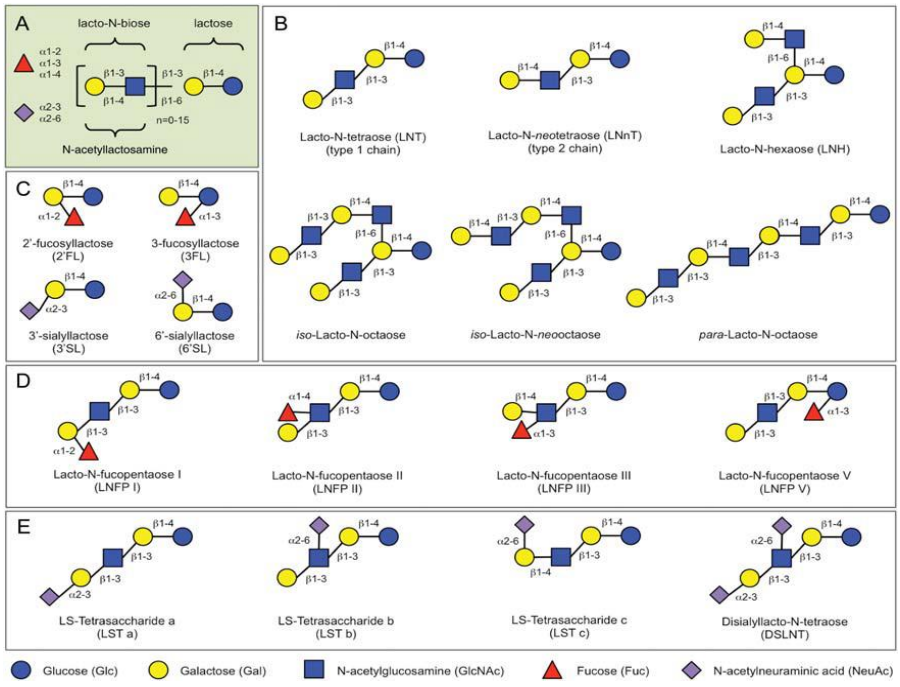


Figure 1. Typical HMO structures. (A) HMOs follow a basic structural blueprint. (Monosaccharide key is shown at the bottom of the figure.) (B) Lactose can be fucosylated or sialylated in different linkages to generate trisaccharides. (C) Lactose can be elongated by addition of either lacto-*N*-biose (type I) or *N*-acetyllactosamine (type II) disaccharides. Addition of disaccharides to each other in the β1-3 linkage leads to linear chain elongation (*para*-HMO); a β1-6 linkage between two disaccharides introduces chain branching (*iso*-HMO). (D) Elongated type I or II chains can be fucosylated in different linkages to form a variety of structural isomers, some of which have Le blood group specificity. (E) The elongated chains can also be sialylated in different linkages to form structural isomers. Disialylated lacto-*N*-tetraose (bottom right) prevents NEC in neonatal rats (Bode 2012).

3. 2’-Fucosyllactose (2’-FL)

3.1. Structure and functions of 2’-FL

About 200 HMOs have been found in breast milk, the majority of which are present in the fucosylated form, which is referred as fucosyloligosaccharides. About 50-80% of the HMOs are fucosylated and 10-20% are sialylated (Kunz, Rudloff et al. 2000, Ninonuevo, Park et al. 2006, Bode 2012). Fucosyloligosaccharide is sufficient to attract attention because it has various functions. They are used not only as growth factors for *Bifidobacterium* or *Lactobacillus*, but also as receptors for cell surface receptors, so infants prevent infection of the enteric pathogens and binding of toxins (Morrow, Ruiz-Palacios et al. 2004, Newburg, Ruiz-Palacios et al. 2005).

As shown in Table 2, 2'-fucosyllactose (2'-FL) is the most abundant component of fucosylated HMOs and has similar physiological properties to that of HMOs. (Chaturvedi, Warren et al. 2001, Castanys-Muñoz, Martin et al. 2013, Smilowitz, O’Sullivan et al. 2013).

2’-FL is a trisaccharide composed of lactose and fucose (Fig. 2). Fucose is bound to the galactose moiety of lactose through α1-2 linkage. Therefore, 2’-FL is referred as L-fucopyranosyl-(1→2)-D-galactopyranosyl-(1→ 4)-D-glucose. 2'-FL has various functions in infants. First, it is degraded by fucosidase of *Bifidobacterium* and acts as a soluble prebiotic fiber. In addition, by balancing Th1 and Th2 cells, the immune response can be controlled and the infant can be protected from infection by pathogens. 2'-FL inhibits adhesion of intestinal pathogens such as *Campylobacter jejuni*, *Pseudomonas aeruginosa*, *Escherichia coli* enterotoxins and *Calcivirus*. It is also known to inhibit

leukocyte adhesion, thereby reducing the inflammatory process by preventing the extravasation of endothelial cells (Castanys- Muñoz, Martin et al. 2013). 2'-FL is a key component of HMOs because of these useful functionalities. However, all women around the world can not synthesize 2'-FL. About 20% of women do not produce 2'-FL due to their genetic defects. (Castanys- Muñoz, Martin et al. 2013). For this reason, 2'-FL is attracting attention as a material for functional foods, medicines, and cosmetics. Thus, the necessity of consuming more 2'-FL is emerging (Han, Kim et al. 2012).

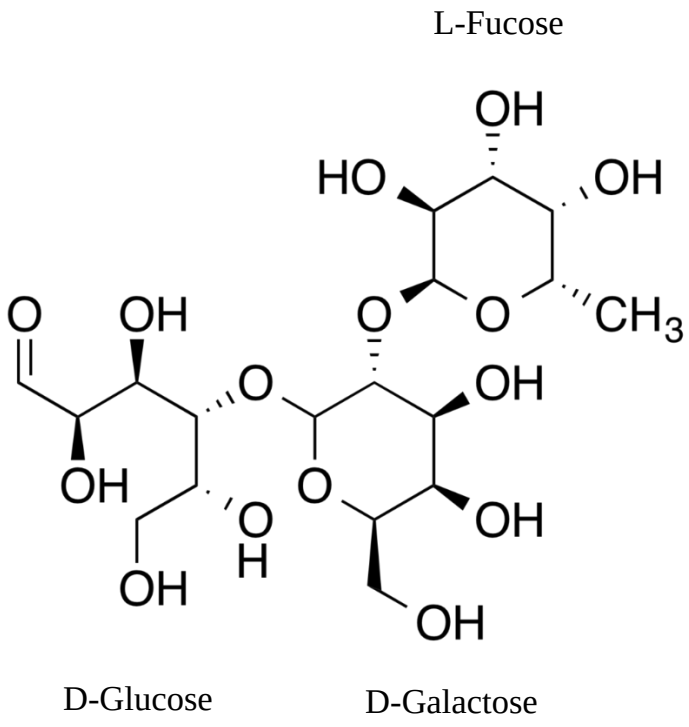


Figure 2. Structure of 2’-fucosyllactose (2’-FL)

Table 2. Contents of major carbohydrates in human milk (Smilowitz, O'Sullivan et al. 2013)

Metabolite	Contents (μmole/L)
2'-Fucosyllactose (2'-FL)	**$2.50 \times 10^3 \pm 1.70 \times 10^3$**
3-Fucosyllactose (3-FL)	$2.10 \times 10^3 \pm 1.20 \times 10^3$
3'-Sialyllactose (3'-SL)	144 ± 43.7
6'-Sialyllactose (6'-SL)	119 ± 54.9
Fucose	182 ± 135
Galactose	92.3 ± 49.1
Glucose	$1.50 \times 10^3 \pm 530$
Lactodifucotetraose (LDFT)	266 ± 199
Lacto-N-neotetraose (LNnT)	121 ± 67.5
Lacto-N-fucopentaose (LNFP Ⅰ)	189 ± 159
Lacto-N-fucopentaose (LNFP Ⅱ)	210 ± 168
Lacto-N-fucopentaose (LNFP Ⅲ)	233 ± 74.0
Lacto-N-tetraose (LNT)	506 ± 284
Lactose	$170 \times 10^3 \pm 7.30 \times 10^3$

3.2. 2’-FL production method

Methods for producing 2'-FL on an industrial scale include chemical synthesis, enzyme synthesis and whole cell synthesis. First, chemical synthesis has been performed for a long time (Gokhale, Hindsgaul et al. 1990, Kameyama, Ishida et al. 1991, Kretzschmar and Stahl 1998). However, the method of chemically producing 2'-FL is not only uneconomical, but it also takes a lot of time. Multiple protection and deprotection steps are also required (Gokhale, Hindsgaul et al. 1990, Kameyama, Ishida et al. 1991, Kretzschmar and Stahl 1998). These problems are the main disadvantages of this method in industrial applications.

Another method for 2'-FL production is enzymatic synthesis (Albermann, Piepersberg et al. 2001). α-1,2-Fucosyltransferase, an enzyme used in the production of 2'-FL, has high stereoselectivity and this method can be efficient. In addition, there is an advantage that by-products are hardly produced. However, since the cost of guanosine 5'-diphospho-β-L-fucose (GDP-L-fucose) used as fucose donor is very high. Also, the costs of enzyme purification and cofactors are expensive. Therefore, this method also has a disadvantage in producing on a large scale.

Finally, the method for producing 2'-FL is whole cell synthesis using microorganisms. This method does not require the preparation of costly substrates, GDP-L-fucose and cofactors involved in GDP-L-fucose biosynthesis such as nicotinamide dinucleotide phosphate (NADPH) and guanosine triphosphate (GTP). In addition, enzyme isolation is not required. (Lee, Pathanibul et al. 2012). For these reasons, this method is

suitable for producing 2'-FL on a large scale. Therefore, in this paper, a study was conducted to produce 2'-FL using a microbial fermentation method.

3.3. Biosynthesis of GDP-$_L$-fucose

GDP-$_L$-fucose which is an activated sugar nucleotide is a key material used as a fucose donor in order to produce 2'-FL (Fig. 3). GDP-L-fucose is produced via two metabolic pathways; the salvage pathway and *de novo* pathway. In the salvage pathway, L-fucose kinase (EC 2.7.1.52) phosphorylates L-fucose with consumption of ATP. Then, GDP-$_L$-fucose is synthesized by the action of L-fucose-1-phosphate guanylyltransferase (EC 2.7.7.30) which combines L-fucose-2-phosphate with GTP (Becker and Lowe 2003).

In the *de novo* pathway, GDP-$_L$-fucose is synthesized through the metabolic pathway shown in Fig. 4. Fructose-6-phosphate produced during glycolysis is converted into mannose-1-phosphate by mannose-6-phosphate isomerase (ManA, E.C. 5.3.1.8) and phosphomannomutase (ManB, E.C. 5.4.2.8). Then, mannose-1-phosphate guanyltransferase (ManC, E.C. 2.7.7.22) combines mannose-1-phosphate with GTP to produce GDP-$_D$-mannose. By the two enzymes, GDP-$_D$-mannose-4,6-dehydratase (Gmd, E.C. 4.2.1.27) and GDP-4-keto-6-deoxymannose 3,5-epimerase 4-reductase (WcaG, EC 1.1.1.271), GDP-$_D$-mannose is converted to GDP-$_L$-fucose through the following steps. First, GMD removes a water molecule from GDP-$_D$-mannose. Then, WcaG engages in the reaction, the reduction of the keto group at the C_4 position of GDP-4-keto-6-deoxymannose to produce GDP-$_L$-fucose. In this reaction, NADPH acts as a cofactor offering reducing power (Albermann, Distler et al. 2000, Becker and Lowe 2003, Jang, Lee et al. 2010).

Although the method of synthesizing GDP-$_L$-fucose through the

salvage pathway is simpler, fucose used as a precursor of GDP-$_L$-fucose is expensive. Because of the high price of fucose, this method is not suitable for large-scale production of 2'-FL. On the other hand, in the *de novo* pathway, 2'-FL is produced through several steps. However, since the starting material is economical, previous studies have constructed a system that produces 2'-FL from *C. glutamicum* via the de novo pathway. (Fig. 4) (Chin, Park et al. 2013).

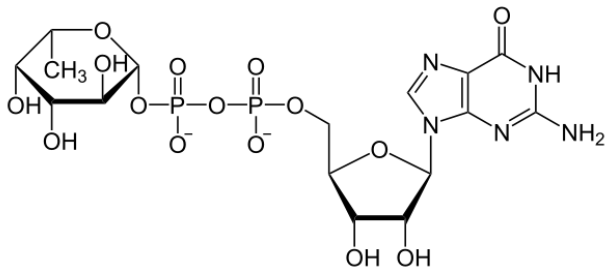


Figure 3. Structure of guanosine 5'- diphospho-β-$_L$-fucose (GDP-L-fucose)

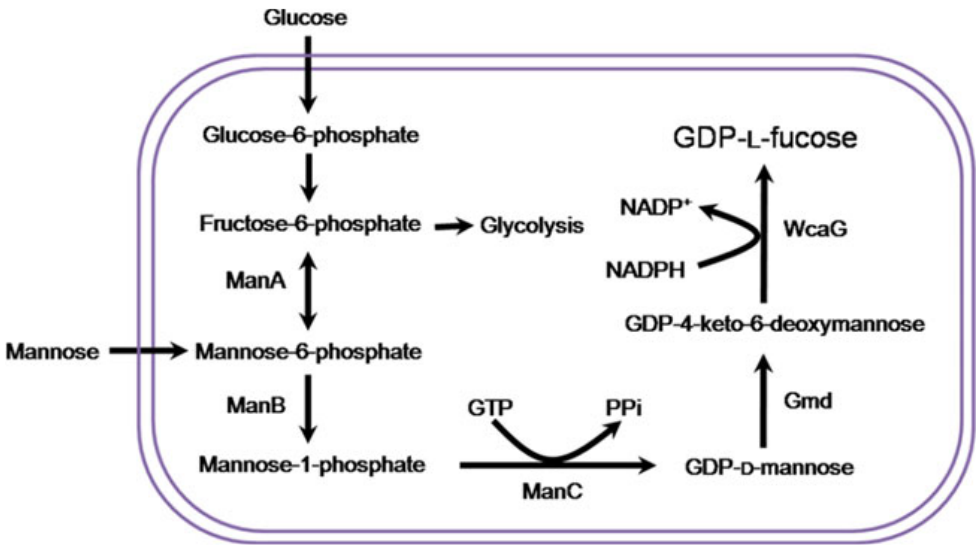


Figure 4. *De novo* biosynthetic pathway of GDP-$_{L}$-fucose.

ManA, mannose-6-phosphate isomerase; ManB, phosphomannomutase; ManC, GTP-mannose-1-phosphate guanylyltransferase; Gmd, GDP-$_{D}$-mannose-4,6-dehydratase; WcaG, GDP-4-keto-6-deoxymannose-3,5-epimerase-4-reductase (Chin, Park et al. 2013).

3.4. α-1, 2-fucosyltransferase

Fucosyltransferases are enzymes that transfer L-fucose of GDP-$_L$-fucose to various oligosaccharide acceptors (Breton, Oriol et al. 1998). Fucosyltransferases are kind of glycosyltransferases because α-fucosylated products are formed from a β-fucosylated sugar nucleotide, GDP-$_L$-fucose (Zhang, Lau et al. 2010). Fucosyltransferases are classified as α-1, 2-, α-1, 3 and/or α-1, 4-, α-1, 6- and O-fucosyltrnasferases based on the types of acceptors and the regional specificity during the fucosyltransferase-catalyzed reaction (Ma, Simala-Grant et al. 2006).

2'-FL is formed through fucosylation of lactose by α-1, 2-fucosyltransferase. This enzyme transfers fucose from GDP-$_L$-fucose to the galactose of lactose. α-1, 2-fucosyltransferases exist in eukaryotes and prokaryotes. Fucosyltransferase is known to be involved in tissue development, angiogenesis, fertilization, cell adhesion, inflammation and tumor metastasis in eukaryotes (Ma, Simala-Grant et al. 2006, Miyoshi 2008). In the case of prokaryotes, fucosyltransferases have been implicated in the synthesis of lipopolysaccharide (LPS) and exopolysaccharide (EPS), which are involved in molecular mimicry, adhesion, and the regulation of host immune response. (Ma, Simala-Grant et al. 2006).

α-1,2-Fucosyltransferase is an important enzyme for 2'-FL production because it participates in the binding of fucose of GDP-$_L$-fucose and galactose of lactose by the α-1,2 glycosidic linkage. α-1,2-Fucosyltransferase from *Helicobacter pylori* has been mainly used to produce 2'-FL (Albermann, Piepersberg et al. 2001, Drouillard, Driguez

et al. 2006, Lee, Pathanibul et al. 2012, Baumgärtner, Seitz et al. 2013). In previous studies, α-1,2-fucosyltransferase of *H. pylori* was introduced for the production of 2'-FL through *C. glutamicum* (Jo, Thesis. 2016).

4. *Corynebacterium glutamicum*

4.1. What is *Corynebacterium glutamicum*?

In the mid-1950s, bacteria accumulating L-glutamic acid were isolated. This bacterium was originally named *Micrococcus glutamicus* (Kinoshita, Udaka et al. 1957). Since its discovery decades ago, *C. glutamicum* has played an important role in producing amino acids and nucleotides on an industrial scale. Amino acids such as L-valine, L-histidine, L-phenylalanine, L-tryptophan, L-glutamate and L-lysine (Ikeda 2003) and nucleotides such as 5'-inosinic acid (IMP), 5'-guanylic acid (GMP), 5'-xanthylic acid (XMP) and others have been produced in an industrial scale or have been attempted to produce.

C. glutamicum is an aerobic or facultative anaerobic, Gram-positive, non-spore forming bacterium. It is usually a rod-shape, somewhat irregular ("coryneform") morphology (Fig .5) (Eggeling and Bott 2005). Initially, to make superior strains, many random mutations and screening tests were performed. These methods have disadvantages in that it takes a lot of time and gives no reasons for improvements. Fortunately, however, many genetic engineering tools have recently been developed for *C. glutamicum*. In the 1980s, host-vector systems for coryneform bacteria were developed and this allows the development of strains in a more rational manner (Katsumata, Ozaki et al. 1984, Santamaria, Gil et al. 1984, Kiyoshi, Kazuhiko et al. 1985, Yoshihama, Higashiro et al. 1985). In the 1990s, various genetic engineering tools for coryneform bacteria were developed (Haynes and Britz 1989, Schäfer, Kalinowski et al. 1990, Schwarzer and Pühler 1991, Ikeda and Katsumata 1998). Furthermore, the complete genome of *C.*

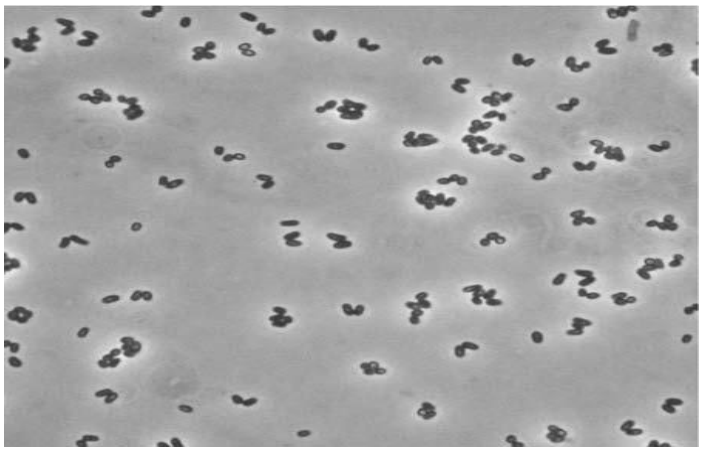
glutamicum ATCC 13032 has been revealed by two independent research teams: the Japanese Kyowa Hakko Co. & Kitasato Univ. team and German Degussa Co. & Bielefeld Univ. team identified 3,309,401 and 3,282,708 base pairs, respectively.

4.2. 2’-FL production in *C. glutamicum*

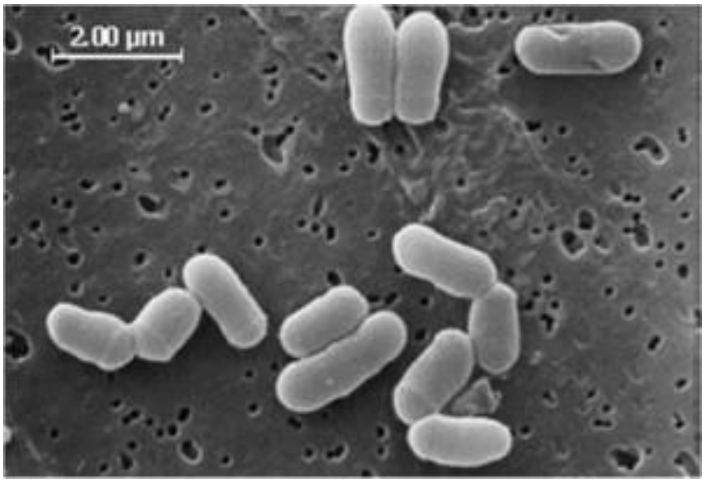
C. glutamicum has a high ability to regenerate NADPH. When glucose is used as the sole carbon source, the proportion of carbon flux to the pentose phosphate pathway (PPP) is higher in *C. glutamicum* than in other microorganisms (Marx, de Graaf et al. 1996, Eggeling and Bott 2005). A wild-type strain *C. glutamicum* ATCC 13032 has a greater NADPH potential over 80% during growth. This is a key feature for efficient amino acid production in mutants derived from this parent strain during the past decades (Eggeling and Bott 2005). In addition, the carbon flux ratio to PPP is significantly increased by the increased cell requirement of NADPH. *C. glutamicum* is also used in fermentative production of nucleotides of interest as a flavor enhancer for food products (Komata 1976). In fact, mutants of *C. glutamicum* that secrete IMP, XMP and GMP have been developed (Aharonowitz and Demain 1978). Above all, *C. glutamicum* is classified as "Generally Recognized As Safe" (GRAS) microorganisms. It is therefore believed that *C. glutamicum* has sufficient potential to be an ideal host for production of amino acids or nucleotides as well as production of food additives or therapeutic agents such as 2’-FL.

GDP-$_{L}$-fucose and lactose are required in order to produce 2’-FL in microbial cells. However, the GDP-$_{L}$-fucose biosynthetic pathway does not exist in wild-type *C. glutamicum*, so it cannot biosynthesize GDP-$_{L}$-fucose. Thus, in previous studies, the strain capable of synthesizing GDP-$_{L}$-fucose was developed (Chin, Park et al. 2013). In addition, a wild-type *C. glutamicum* does not have lactose-permeable enzymes. Therefore, it cannot utilize lactose. However, since it is necessary to

transport lactose into cells to produce 2'-FL, the lactorse permease gene derived from *Escherichia coli* K-12 was introduced as a *lacYA* operon which *lacZ*, a gene for β-galactosidase, was removed. (Chin, Seo et al. 2016). In addition, the codon-optimized α-1, 2-fucosyltransferase gene (CO*fucT2*) derived from Helicobacter pylori was introduced for fucosylation (Fig. 6) (Jo, Thesis. 2016).



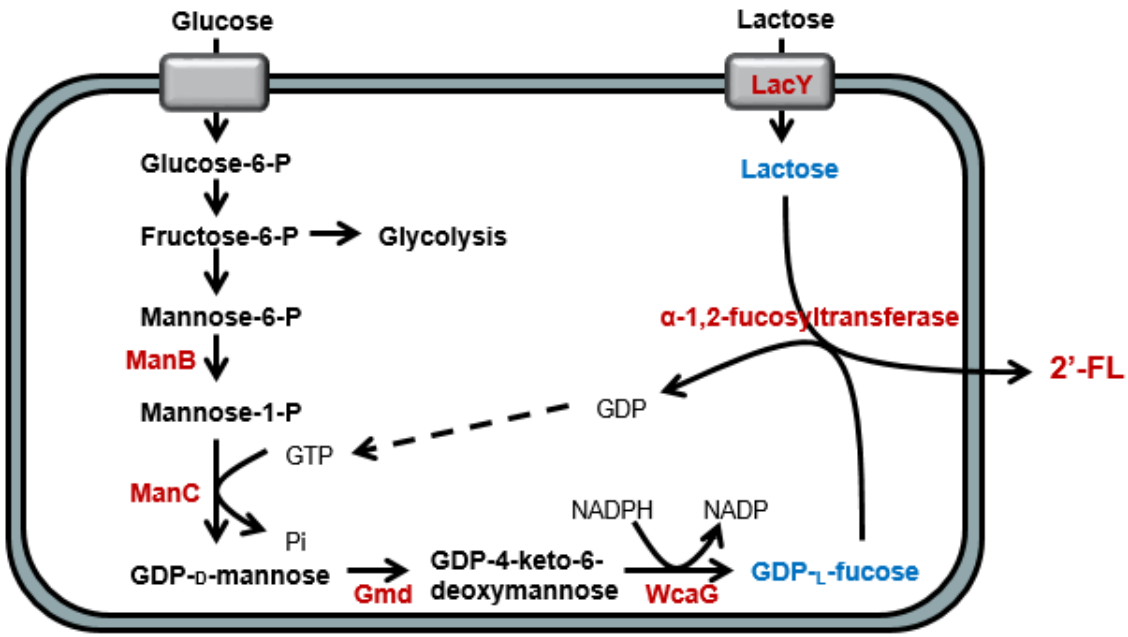
(A)



(B)

Figure 5. *Corynebacterium glutamicum*. (A) Phase-contrast micrograph of *C. glutamicum* cells grown on complex medium. Note frequent V-type arrangement of cell pairs, due to "snapping division." (B) Same cells placed on a nucleopore membrane and viewed by scanning electron microscopy (Eggeling and Bott 2005).

Figure 6. Biosynthesis pathway of 2'-FL from glucose and lactose in engineered *C. glutamicum* (Jo, Thesis. 2016).



ManB : Phosphomannomutase
ManC : GTP-mannose-1-phosphate guanylyltransferase
Gmd : GDP-D-mannose-4,6-dehydratase from *E. coli* K-12

WcaG : GDP-4-keto-6-deoxymannose-3,5-epimerase-4-reductase from *E. coli* K-12
LacY : Lactose permease from *E. coli* K-12

5. Research objectives

This research was focused on developments of strains to produce 2'-FL from *C. glutamicum* through metabolic engineering design and chromosomal integration. The specific objectives of this research were described as follows.

(1) To develop a strain with improved production of 2'-FL by enhancing lactose utilization

(2) To construct a 2'-FL production system without using antibiotics

(3) To reduce trehalose, a by-product produced during the production of 2'-FL

Ⅱ. MATERIALS AND METHODS

1. Reagents and Enzymes

All experiments were carried out using chemicals of reagent grade. Lactose, ethidium bromide, isoniazid, protocatechuic acid, biotin, cupric sulfate, sulfuric acid and antifoam 204 were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Glucose, ammonium sulfate, urea, potassium phosphate monobasic, potassium phosphate dibasic, magnesium sulfate heptahydrate, ferrous sulfate, sodium chloride, sodium hydroxide, ammonia water and hydrochloric acid were purchased from Duksan (Ansan, Korea). Kanamycin monosulfate, IPTG and MOPS were purchased from Duchefa (Haarlem, The Netherlands). Fructose, calcium chloride, zinc sulfate, manganese(II) sulfate and Nickel(II) chloride were purchased from Junsei Chemical (Tokyo, Japan). Brain heart infusion, bacto-tryptone, yeast extract and bacto-agar were purchased from Difco (Detroit, MI., USA).

Restriction enzymes and calf intestinal alkaline phosphatase (CIP) were purchased from New England Biolabs (Beverly, MA, USA). T4 ligation mix and In-Fusion® HD cloning kit were purchased from Takara (Otsu, Japan).

2. Strains and Plasmids

2.1. Strains

E. coli Top10 (Invitrogen, Carlsbad, CA, USA) was used for construction of plasmid DNA. *C. glutamicum* ATCC 13032 (KACC,

Suwon, Korea) was used as host strain to produce 2'-FL.

The site where the IS element was removed was selected to integrate the genes on the chromosome. Among the IS elements, IS*Cg2b* and IS*Cg2f* belonging to the IS30 family were deleted and IS*Cg1a* belonging to the ISL3 family was deleted. To construct IS*Cg2b*, IS*Cg2f*, IS*Cg1a* knock-out strain (ΔISCg2b),(ΔISCg2f) and (ΔISCg1a), all these genes were removed on the chromosome of *C. glutamicum*. Codon-optimized α-1,2-fucosyltransferase gene (CO*fucT2*) was integrated in the site where IS*Cg2b* was deleted. Then, GDP-D-mannose-4,6-dehydratase (*gmd*) and GDP-4-keto-6-deoxymannose-3,5-epimerase-4-reductase (*wcaG*) were integrated in the site where IS*Cg2f* was deleted. All the genetic manipulations were done by a double crossover method using a pK19mobsacB vector (Schäfer, Tauch et al. 1994).

In order to reduce trehalose, *otsA* and *treY* gene, which are involved in the production of trehalose, were separately deleted. To construct ΔotsA and ΔtreY, double croessover method using a pK19mobsacB vector was also used.

The wild type and recombinant strains were incubated on Brain-heart infusion (BHI, Difco) containing appropriate antibiotics and stocked in a deep freezer at -80°C suspended in 15% glycerol.

2.2. Plasmids

Plasmids pVWEx2 and pEKEx2 were donated kindly by Prof. J. B. Park at Ewha Womans University. They were used as the backbone vectors for the expression of heterologous genes or overexpression of

innate genes.

Plasmid pVTY harbors the lactose permease (*lacY)* gene from *E. coli* K-12 under the *tac* promoter with ribosome binding site (RBS). Plasmid pEGWTT(CO) was already constructed in the previous studies. It harbors the *gmd-wcaG* genes from *E. coli* under *tac* promoter and the codon-optimized α-1,2-fucosyltransferase gene (CO*fucT2*) from *H. pylori* under *tac* promoter. CO*fucT2* is transcribed monocistronically by addition of the *tac* promoter (Jo, Thesis. 2016).

Plasmid pK19mobsacB was donated kindly by Prof. K. J. Jeong at Korea Advanced Institute of Science and Technology (KAIST). It was used as a vector to delete or integrate target genes on the chromosome.

All deletion vectors used in this study, pK19-ΔIS*Cg2b*, pK19-Δ IS*Cg2f*, pK19-Δ IS*Cg2b*, pK19-Δ*otsA* and pK19-Δ*treY*, were constructed to delete IS*Cg2b,* IS*Cg2f,* IS*Cg1a, otsA* and *treY* existed on chromosome of *C. glutamicum* respectively. These plasmids carry flanking region of the target genes. Then, to construct integration vectors, genes involved in the production of 2'-FL were inserted internally with flanking fragments of deleted genes. As a result, pK19-ΔIS*Cg2b*::*fucT2*(CO) and pK19-Δ IS*Cg2f* ::*GW* could be constructed.

Plasmid pEGW was previously constructed for overexpression of the genes for GDP-$_{L}$-fucose biosynthesis (Chin, Park et al. 2013). All constructs were confirmed by restriction enzyme digestion and DNA sequencing.

Table 3. List of strains and plasmids used in this study

Strains/Plasmids	Relevant description	Reference
Strains		
E. coli TOP10	F$^-$, *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC*) φ80lacZ*Δ*M15* Δ*lacX74 recA1 araD139* Δ(*ara-leu*)7697 *galU galK rpsL* (Str^R) *endA1 nupG*	Invitrogen (Carlsbad, CA, USA)
C. glutamicum	Wild-type strain, ATCC 13032	(ABE, TAKAYAMA et al. 1967)
BCGWTTL(CO)	*C. glutamicum* ATCC 13032 harboring pVBCL and pEGWTT(CO)	(Jo, Thesis. 2016)
GWTTL(CO)	*C. glutamicum* ATCC 13032 harboring pVL and pEGWTT(CO)	This study
GWTTLY(CO)	*C. glutamicum* ATCC 13032 harboring pVLY and pEGWTT(CO)	This study
GWTTY(CO)	*C. glutamicum* ATCC 13032 harboring pVTY and pEGWTT(CO)	This study
ΔISCg2b	*C. glutamicum* ATCC 13032 Δ*ISCg2b*	This study
ΔISCg2b::fucT2(CO)	*C. glutamicum* ATCC 13032 Δ*ISCg2b*::CO*fucT2*	This study
ΔISCg2bΔISCg2f ::fucT2(CO)	*C. glutamicum* ATCC 13032 Δ*ISCg2b*Δ*ISCg2f*::CO*fucT2*	This study
ΔISCg2bΔISCg2fΔISCg1a ::fucT2(CO)	*C. glutamicum* ATCC 13032 Δ*ISCg2b*Δ*ISCg2f*Δ*ISCg1a*::CO*fucT2*	This study
ΔISCg2bΔISCg2fΔISCg1a ::fucT2(CO)::GW	*C. glutamicum* ATCC 13032 Δ*ISCg2b*Δ*ISCg2f*Δ*ISCg1a*::CO*fucT2*::*GW*	This study

ΔotsA	*C. glutamicum* ATCC 13032 ΔotsA	This study
ΔtreY	*C. glutamicum* ATCC 13032 Δ*treY*	This study
ΔISCg2b BCGWTTL(CO)	*C. glutamicum* ATCC 13032 Δ*ISCg2b*::CO*fucT2*, Δ*ISCg2f::GW*, Δ*ISCg1a* harboring pVBCL and pEGW	This study
ΔISCg2b::fucT2(CO) BCGWL	*C. glutamicum* ATCC 13032 Δ*ISCg2b*::CO*fucT2*, Δ*ISCg2f::GW*, Δ*ISCg1a* harboring pVBCL and pEGW	This study
ΔISCg2bΔISCg2f ::fucT2(CO) BCGWL	*C. glutamicum* ATCC 13032 Δ*ISCg2b*::CO*fucT2*, Δ*ISCg2f::GW*, Δ*ISCg1a* harboring pVBCL and pEGW	This study
ΔISCg2bΔISCg2fΔISCg1a ::fucT2(CO) GWY	*C. glutamicum* ATCC 13032 Δ*ISCg2b*::CO*fucT2*, Δ*ISCg2f::GW*, Δ*ISCg1a* harboring pVTY and pEGW	This study
ΔISCg2bΔISCg2fΔISCg1a ::fucT2(CO)::GW Y	*C. glutamicum* ATCC 13032 Δ*ISCg2b*::CO*fucT2*, Δ*ISCg2f::GW*, Δ*ISCg1a* harboring pVTY	This study
ΔISCg2bΔISCg2fΔISCg1a ::fucT2(CO)::GW GWTTY(CO)	*C. glutamicum* ATCC 13032 Δ*ISCg2b*::CO*fucT2*, Δ*ISCg2f::GW*, Δ*ISCg1a* harboring pVTY and pEGWTT(CO)	This study
ΔotsA GWTTY(CO)	*C. glutamicum* ATCC 13032 Δ*otsA* harboring pVTY and pEGWTT(CO)	This study
ΔtreY GWTTY(CO)	*C. glutamicum* ATCC 13032 Δ*treY* harboring pVTY and pEGWTT(CO)	This study

Plasmids		
pEKEx2	Km^R; *C. glutamicum/E. coli* shuttle vector for regulated gene expression (P_{tac}, *lacIq*, pBL1, *oriVC.g.*, *oriVE.c.*)	(Eikmanns, Kleinertz et al. 1991)
pVWEx2	Tc^R; *C. glutamicum/E. coli* shuttle vector for regulated gene expression (P_{tac}, *lacIq*, pHM1519, *oriVC.g.*, *oriVE.c.*)	(Wendisch and Jülich 1997)
pK19mobsacB	Mobilizable vector, Km^R	(Schäfer, Tauch et al. 1994)
pVL	pVWEx2 + *manB* + *manC*	This study
pVLY	pVWEx2 + *lac* promoter + *lacY*	This study
pVTY	pVWEx2 + *tac* promoter + *lacY*	This study
pVBCL	pVWEx2 + *manB* + *manC* + *lacYA*	(Jo, Thesis. 2016)
pEGW	pEKEx2 + *gmd-wcaG*	(Chin, Park et al. 2013)
pEGWTT(CO)	pEGW + *tac* promoter + CO*fucT2*	(Jo, Thesis. 2016)
pK19-ΔIS*Cg2b*	pK19mobsacB + flanking region of IS*Cg2b*	This study
pK19-ΔIS*Cg2b*::*fucT2*(CO)	pK19mobsacB + flanking region of IS*Cg2b* with internally inserted P*tac*-CO*fucT2*-terminator	This study
pK19-ΔIS*Cg2f*	pK19mobsacB + flanking region of IS*Cg2f*	This study
pK19-ΔIS*Cg2f*::*GW*	pK19mobsacB + flanking region of IS*Cg2f* with internally inserted P*tac*-*gmd*-wcaG-terminator	This study
pK19-ΔIS*Cg1a*	pK19mobsacB + flanking region of IS*Cg1a*	This study
pK19-Δ*otsA*	pK19mobsacB + flanking region of *otsA*	This study
pK19-Δ*treY*	pK19mobsacB + flanking region of *treY*	This study

Table 4. List of primers used in this study

Name	Sequence
F_inf_*Asi*SI_lacY	GAGACGAAATAC **GCGATCGC** ACCATCGAATGGCGCAAAAC
R_ovl_lacA_del	TATCAGGCAATTTTTATAAT TGCGATCACTCCGTTATGATATGTTG
R_inf_*Asi*SI_lacOY	GTCCTTTTAACA **GCGATCGC** CGGTAAATAGCTTGCCTGCTC
F1_*Pst*I_lacY	AA**CTGCAG** *AAGGAGATATACA* CACACAGGAAACAGCTATGTACTATTTA
R1_*Bam*HI_lacY	CG**GGATCC** GACATTGATTGCTTAAGCGACTTC
F1_*Sal*I_ISCg2b(L)	ACGC**GTCGAC** TCATGGTTCAGGGCACTG
R1_*Xho*I_*Spe*I_ ISCg2b(L)	TACAATCTCCTAGGCGAAT **CTCGAG ACTAGT** ACCTTGATTGATCATGTCGAGG
F2_*Spe*I_FucT2(CO)	G**ACTAGT** GAGAATCAAGACCGCTTTCGG
R2_*Xho*I_FucT2(CO)	CCG**CTCGAG** CAGGGTTATTGTCTCATGAGCG
F3_*Spe*I_*Xho*I_ ISCg2b(R)	CGACATGATCAATCAAGGT **ACTAGT CTCGAG** ATTCGCCTAGGAGATTGTACGA
R3_*Eco*RI_ ISCg2b(R)	CG**GAATTC** CTGCTCATGATTTCCCGCA
F1_seq_fucT2(CO)	CCTCGCAGGAAGCTTTC
F2_seq_fucT2(CO)	ATGCAACTGGAACTTTTTCCG
F3_seq_fucT2(CO)	GGCACGAAAACATCCTGTG
F1_*Hind*Ⅲ_ ISCg2f(L)	CCC**AAGCTT** ACTGCCCCCTCTGGAAATG

R1_*Nde*I_*Not*I_ ISCg2f(L)	CATCCAACCTAGGGCGA **CATATG GCGGCCGC** ATACCTTGATTGATCATGTCGAGG
F1_inf_*Nde*1_GW	TATGCGGCCGC **CATATG** GCAAGCTGATCCGGGC
R1_inf_*Nde*1_GW	ACCTAGGGCGA **CATATG** CAGGGTTATTGTCTCATGAGCGG
F3_*Not*I_*Nde*I_ ISCg2f(R)	CCTCGACATGATCAATCAAGGTAT **GCGGCCGC CATATG** TCGCCCTAGGTTGGATG
R3_*Sal*I_ ISCg2f(R)	ACGC**GTCGAC** CGATGGAATAATCAGACTCTGGAAC
F2_seq_ISCg2f_GW	GGAGATATACAATGTCAAAAGTCGC
F3_seq_ISCg2f_GW	TTACCCGCAAAATCACCC
F4_seq_ISCg2f_GW	GCTCGAACAGCGCG
F5_seq_ISCg2f_GW	TCATGTCATGGAGCTGGC
F6_seq_ISCg2f_GW	CGGTGAACGCTCTCC
F1_*Sal*I_ ISCg1a(L)	ACGC**GTCGAC** CACTTCCAACTGGCACGTT
R1_*Xho*I_*Asi*SI_ IS*Cg1a*(L)	GGTTTACGGGCTCTTCCTGTT **CTCGAG GCGATCGC** GGGTAGAGCCTTTTGTTGGTGT
F2_*Asi*SI _*Xho*I_ IS*Cg1a*(R)	ACACCAACAAAAGGCTCTACCC **GCGATCGC CTCGAG** AACAGGAAGAGCCCGTAAACC
R2_*Xba*I_ ISCg1a(R)	GC**TCTAGA** TGGTCAAAGCTTCCCCTGG
F1_inf_*Hind*Ⅲ_otsA(L)	ATGATTACGCC **AAGCTT** CCAGGAGGAAGCTGAGCAG
R1_ovl_otsA(L)	CGATTCGTGCGCGGT ATAAGATCCGGCTTAAGACTTCTTTGTG
F2_ovl_otsA(R)	CACAAAGAAGTCTTAAGCCGGATCTTAT ACCGCGCACGAATCG
R2_inf_*Pts*I_otsA(R)	CTCTAGAGTCGACC **CTGCAG** CATCTTAAGGTGCCAGGGCTTTA
F1_BamHI_treY_dis	CG**GGATCC** ATGGCACGTCCAATTTCCG
R1_treY_dis.ovl	CACGGTTGATGTGGGAGAC TTCCAGCTTGTCTTCATCGCC

F2_treY_dis.ovl	GCGATGAAGACAAGCTGGAA GTCTCCCACATCAACCGTGG
R2_EcoRI_treY_dis	CG**GAATTC** TCAAAACTCACTATCGGGTACTAAAA

The italic sequences present the RBS (ribosome binding site) and spacer.
The bold sequences present the recognition sites of specific restriction enzymes.
The underlined sequences are overlapped regions to construct the defected IS element fragment for construction of deletion vector.

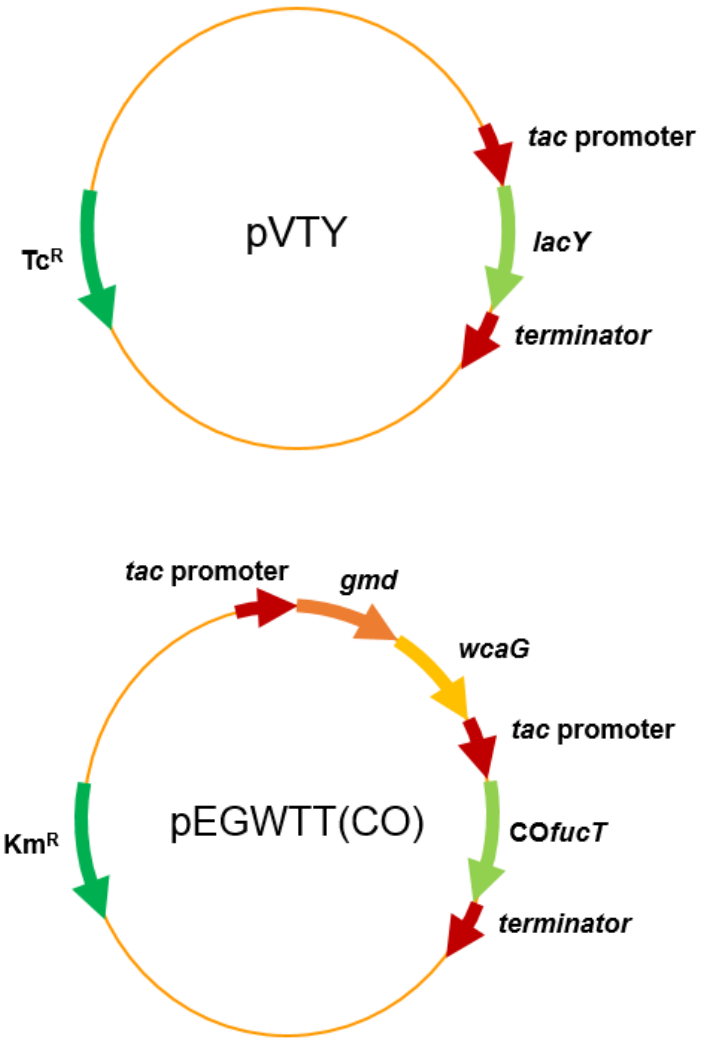


Figure 7. Genetic maps of plasmids pVTY and pEGWTT(CO)

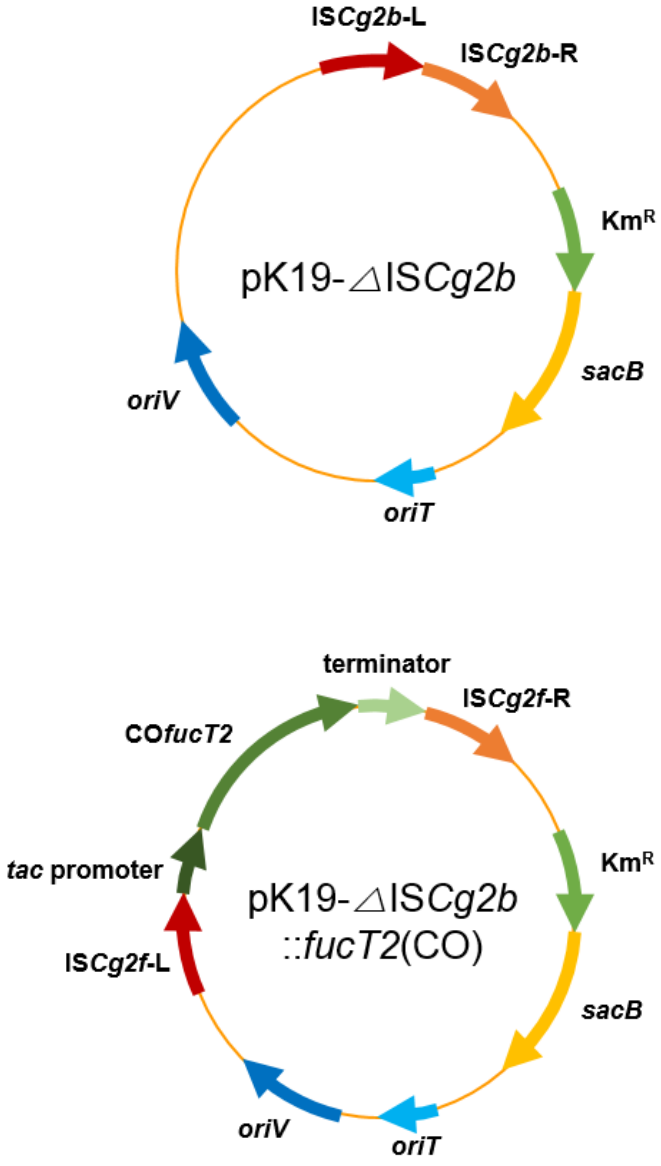


Figure 8. Genetic maps of plasmids pK19-ΔIS*Cg2b* and pK19-ΔIS*Cg2b*::*fucT2*(CO)

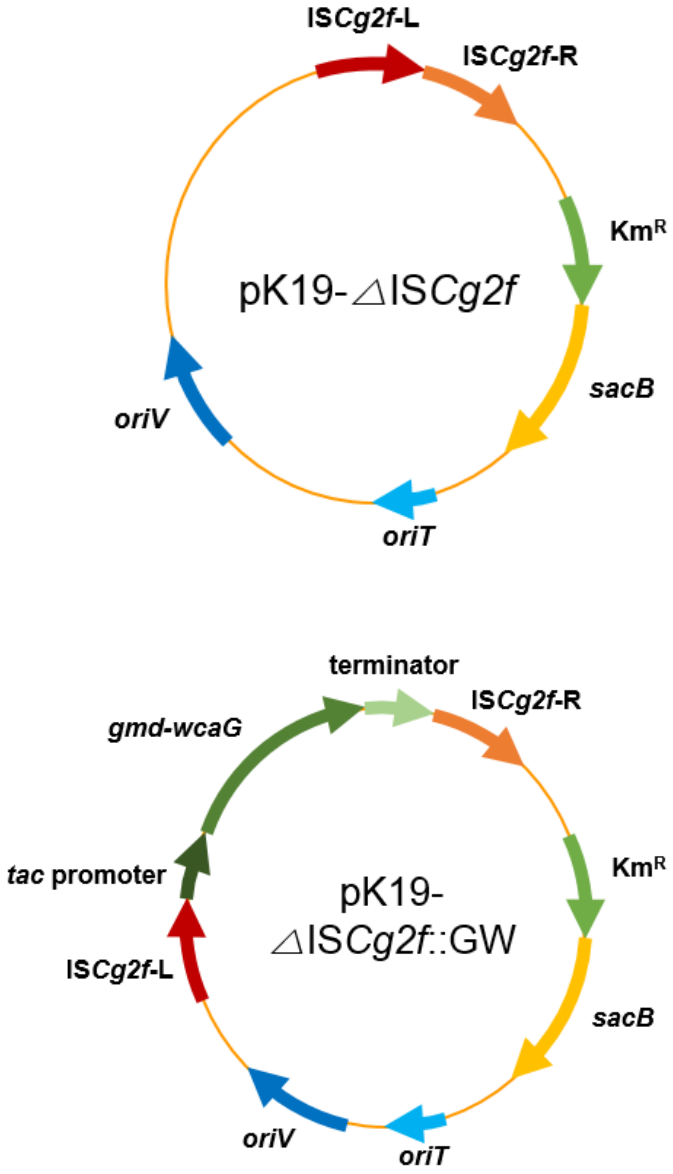


Figure 9. Genetic maps of plasmids pK19-ΔIS*Cg2f* and pK19-ΔIS*Cg2f*::GW

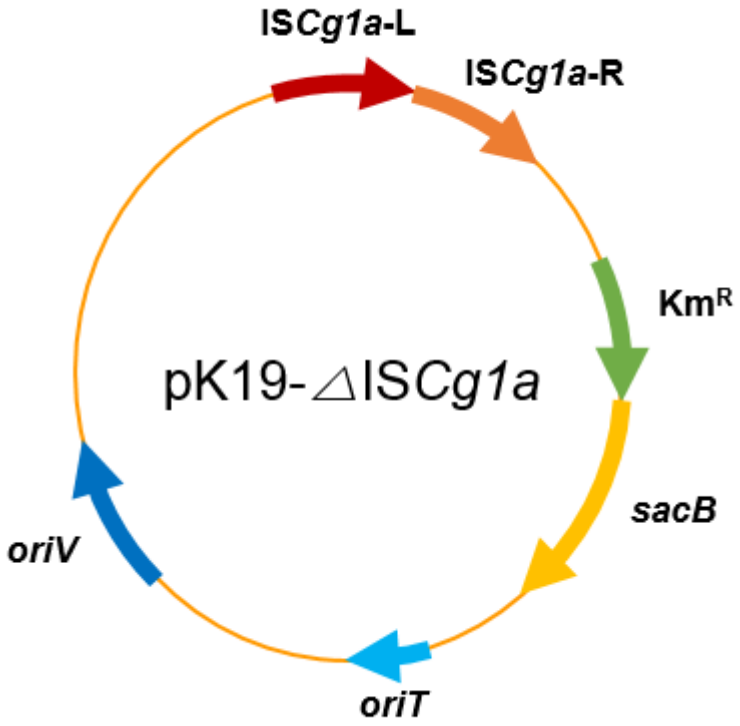


Figure 10. Genetic map of plasmidspK19-ΔIS*Cg1a*

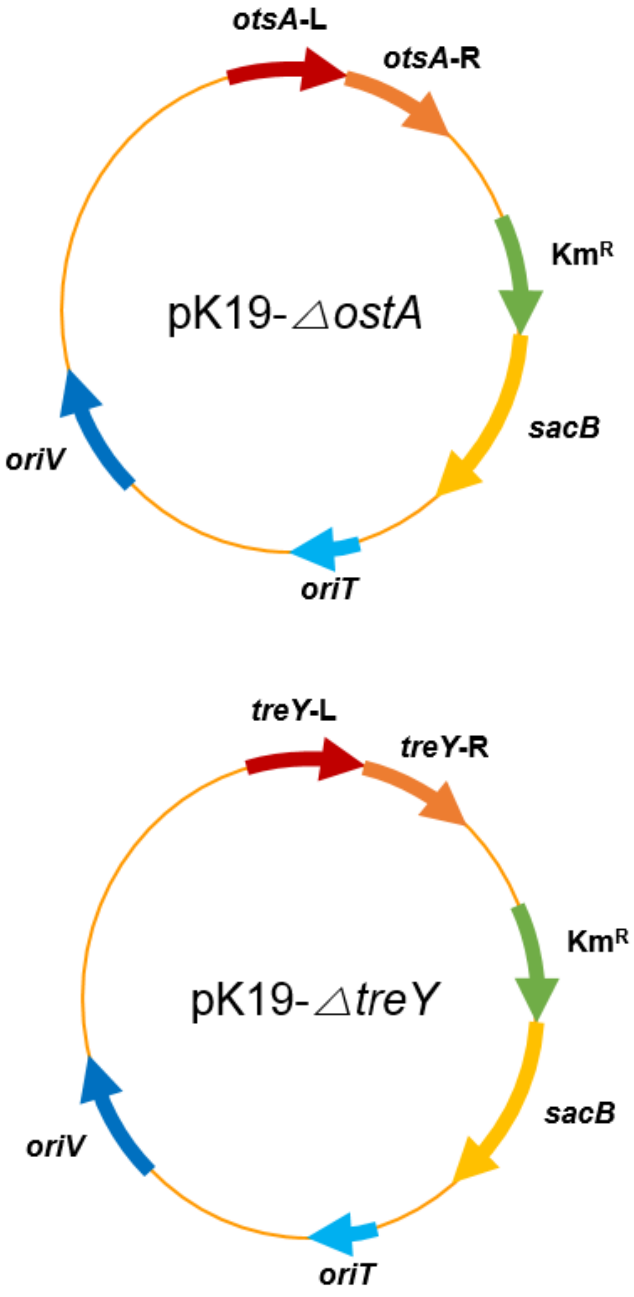


Figure 11. Genetic maps of plasmids pK19-Δ*ostA* and pK19-Δ*treY*.

3. DNA Manipulation and Transformation

3.1. Preparation of DNA

Mini-scale preparation of plasmid DNA was conducted by using DNA-spin™ Plasmid DNA Purification Kit from iNtRON (Sungnam, Korea). Preparation of *C. glutamicum* chromosomal DNAs for PCR template was performed by using DNeasy Blood & Tissue Kit from QIAGEN (Düsseldorf, Germany). Buffer for enzymatic lysis composed of 20 mM Tris·HCl (pH 8.0), 2 mM EDTA, 1.2% Triton X-100, 20 mg/mL lysozyme was used because *C. glutamicum* is Gram-positive bacteria. PCR amplified or enzyme treated DNA was purified by using the QIAquick® Gel Extraction / PCR purification Kit from QIAGEN (Düsseldorf, Germany) respectively.

3.2. Polymerase Chain Reaction (PCR)

PCRs were carried out with an Applied Biosystems Veriti 96 well Thermal Cycler (Lincoln, CA, USA). PCRs for cloning of genes were performed in 50 μL of PrimeStar™ dyemix solution from Takara (Otsu, Japan) containing 20 pM each of forward and reverse primers (Table 4), and 1 μL of the genomic DNA which is a template of cloning. After heating the reaction tubes for 5 min at 95℃, 30 cycles of PCR amplification were carried out as follows: 10 sec at 98℃, 5 sec at 55℃ and 1 min per 1 kb DNA at 72℃, followed by 7 min at 72℃ during the last cycle.

3.3. Digestion and ligation of DNA

Restriction enzymes *AsiS*I, *Pst*I, *BamH*I, *Sal*I, *Xho*I, *Spe*I, *Eco*RI,

*Hind*Ⅲ, *Nde*I and *Xba*I, and calf intestinal alkaline phosphatase (CIP) were purchased from New England Biolabs (Beverly, USA). Plasmid pVWEx2 was digested with *AsiS*I, *Pst*I and *BamH*I. Plasmid pK19mobsacB was digested with *AsiS*I, *Pst*I, *BamH*I, *Sal*I, *Xho*I, *Spe*I, *Eco*RI, *Hind*Ⅲ, *Nde*I and *Xba*I. The Ligation Mix and In-Fusion® HD cloning kit obtained from Takara (Otsu, Japan) were used for ligation of PCR products and plasmids.

3.4. Transformation of *E. coli*

Transformation of *E. coli* was performed as described by Sambrook (Sambrook and Russell, 1989). *E. coli* Top10 was cultured in 5 mL LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) for 12 hours. 0.5 mL of the culture was transferred to fresh 50 mL of LB medium and cultured until OD_{600} reached 0.5. Cells harvested by centrifugation at 6,000 rpm for 5 min at 4℃ were resuspended in 5 mL of cold 100 mM $CaCl_2$ solution containing 15% (v/v) glycerol. Resuspended cells were aliquoted to 100 μL, mixed with DNA, and kept on ice for 30 min. They were subjected to heat-shock at 42℃ for 45 sec, and 1 mL of LB medium was added to the test tubes and incubated at 37℃ for 1 hour to allow the bacteria to express the antibiotic resistance. Transformed cells were spread on LB agar plates with an appropriate concentration of antibiotics, kanamycin or tetracycline.

3.5. Electroporation of *C. glutamicum*

The modified protocol for preparation of electrocompetent *C. glutamicum* referred to Handbook of *Corynebacterium glutamicum* and

van der Rest *et al*. (van der Rest, Lange et al. 1999, Eggeling and Bott 2005). Briefly, incubated at 30℃, overnight cultures of *C. glutamicum* was inoculated in 100 mL of BHIS (37 g/L BHI, 91 g/L sorbitol) medium in a 500 mL baffled flask containing isoniazid, glycine and tween80. Then, incubated at 30℃, 250 rpm cultured until OD_{600} reached 1.75. The culture dispensed into 50 mL falcon tubes and harvested by centrifugation at 3,000 rpm for 20 min. After removing the supernatant, cell pellet was resuspended with 20 mL TG buffer (1 mM Tris·HCl (pH 7.5), 104.4 g/L glycerol) and centrifuged again. After repeating this step about three times, cell pellet was resuspended with 20 mL of 10% (v/v) glycerol as done before. Finally the cells were resuspended in 1 mL of 10% (v/v) glycerol and dispensed 150 μL of aliquots in cooled Eppendorf tubes and stored at -70°C. 10 μL of plasmid DNA was added into an electrocompetent cell and transferred the mixture into a pre-chilled electroporation cuvette (Bio-Rad, Hercules, CA, USA) with a gap width of 2 mm. The electroporation is performed at 2,500 V, 25 μF and 200 Ω in MicroPulser™ Electroporation apparatus (Bio-Rad, Hercules, CA, USA). After the electric shock, the transformant was transferred immediately into 1 mL of BHIS medium pre-warmed at 46°C and incubated for 6 min at 46°C without shaking to perform the heat-shock process. Then, the transformant was incubated for 1 hour at 30°C, 250rpm to regenerate cells. An appropriate volume of the transformants were spread on a BHIS agar plates containing appropriate antibiotics such as kanamycin or tetracycline and incubated the plates at 30℃ for 2 days.

4. Genetic manipulation methods

4.1. Construction of gene deletion vectors

To construct the target gene knock-out vector pK19mobsacB was used. About 500 bp of left and right flanking regions were respectively amplified with primer pairs as shown in Table 4. A total of five deletion vectors were constructed in this study. Among deletion vectors, for the plamids, which were to be used to make integration vectors, restriction enzyme recognition sites were added between the two flanking regions when constructing the plasmids. This sequence was created at the same time when the two fragments were joined together in a subsequent overlap PCR.

To construct pK19-ΔIS*Cg2b*, F1_*Sal*I_IS*Cg2b*(L) and R1_*Xho*I_*Spe*I _IS*Cg2b*(L) / F3_*Spe*I_*Xho*I_IS*Cg2b*(R) and R3_*Eco*RI_ IS*Cg2b*(R) were used. The PCR products were used as the templates for overlapping PCR and the second PCR was performed by using primers, F1_*Sal*I_IS*Cg2b*(L) and R3_*Eco*RI_IS*Cg2b*(R). The obtained PCR products were digested with *Sal*I and *Eco*RI, and plasmid pK19mobsacB was also digested with the same restriction enzymes. Then, the PCR products were cloned into pK19mobsacB to construct pK19-ΔIS*Cg2b*.

To construct pK19-ΔIS*Cg2f*, F1_*Hind*Ⅲ_IS*Cg2f*(L) and R1_*Nde*I_ *Not*I_ IS*Cg2f*(L) / F3_*Not*I_*Nde*I_IS*Cg2f*(R) and R3_*Sal*I_IS*Cg2f*(R) were used. The PCR products were used as the templates for overlapping PCR and the second PCR was performed by using primers, F1_*Hind*Ⅲ_IS*Cg2f*(L) and R3_*Sal*I_IS*Cg2f*(R). The obtained PCR

products were digested with *Sal*I and *Hind*Ⅲ, and plasmid pK19mobsacB was also digested with the same restriction enzymes. Then, the PCR products were cloned into pK19mobsacB to construct pK19-ΔIS*Cg2f*.

To construct pK19-ΔIS*Cg1a*, F1_*Sal*I_IS*Cg1a*(L) and R1_*Xho*I_*Asi*SI_IS*Cg1a*(L) / F2_*Asi*SI_*Xho*I_IS*Cg1a*(R) and R2_*Xba*I_IS*Cg1a*(R) were used. The PCR products were used as the templates for overlapping PCR and the second PCR was performed by using primers, F1_*Sal*I_IS*Cg1a*(L) and R2_*Xba*I_IS*Cg1a*(R). The obtained PCR products were digested with *Sal*I and *Xba*I, and plasmid pK19mobsacB was also digested with the same restriction enzymes. Then, the PCR products were cloned into pK19mobsacB to construct pK19-ΔIS*Cg1a*.

To construct pK19-Δ*otsA*, F1_inf_*Hind*Ⅲ_*otsA*(L) and R1_ovl_*otsA*(L) / F2_ovl_*otsA*(R) and R2_inf_*Pts*I_*otsA*(R) were used. The PCR products were used as the templates for overlapping PCR and the second PCR was performed by using primers, F1_inf_*Hind*Ⅲ_*otsA*(L) and R2_inf_*Pts*I_*otsA*(R). The plasmid pK19mobsacB were digested with *Hind*Ⅲ and *Pts*I. Then, the PCR products were cloned into pK19mobsacB to construct pK19-ΔIS*Cg1a*.

To construct pK19-Δ*treY*, F1_*Bam*HI_treY_dis and R1_treY_dis.ovl / F2_treY_dis.ovl and R2_*Eco*RI_treY_dis were used. The PCR products were used as the templates for overlapping PCR and the second PCR was performed by using primers, F1_*Bam*HI_treY_dis and R2_*Eco*RI_treY_dis. The obtained PCR products were digested with *Bam*HI and *Eco*RI, and plasmid pK19mobsacB was also digested with the same restriction enzymes. Then, the PCR products were cloned into

pK19mobsacB to construct pK19-Δ *treY*.

4.2. Construction of gene insertion vectors

To construct integration vectors, target genes were inserted between the flanking fragments in the deletion vectors. Two integration vectors were constructed in this study. To construct pK19-ΔIS*Cg2b*::*fucT2*(CO), CO*fucT2* including *tac* promoter and terminator was amplified with F2_*Spe*I_FucT2(CO) and R2_*Xho*I_FucT2(CO). Then, the obtained PCR products were digested with *Spe*I and *Xho*I, and plasmid pK19mobsacB was also digested with the same restriction enzymes. Then, the PCR products were cloned into pK19mobsacB to construct pK19-ΔIS*Cg2b*::*fucT2*(CO).

To construct pK19-ΔIS*Cg2f*::*GW*, *gmd-wcaG* inclusing *tac* promoter and terminator was amplified with F1_inf_*Nde*1_GW and R1_inf_*Nde*1_GW. Then, the obtained PCR products were digested with *Nde*1 and plasmid pK19mobsacB was also digested with the same restriction enzyme. Then, the PCR products were cloned into pK19mobsacB to construct pK19-ΔIS*Cg2f*::*GW*.

4.3. Screening of genetically manipulated strains

All genetic manipulations was carried out by the double crossover method (Schäfer, Tauch et al. 1994). To delete or integrate target genes, constructed plasmids were introduced into *C. glutamicum* by electroporation and the transformants spread on a BHIS agar plate with 25 μg/mL kanamycin were incubated for 2-3 days at 30℃. The cells

formed colonies in medium containing kanamycin had Km-resistance, and thus they were the plasmid-integrated clones. Then, the Km-resistant cells were cultured in LB medium overnight, and they were appropriately diluted and spread on a 10% (w/v) sucrose LB (LB, 0.5% sodium acetate) agar plate to pop out the integrated plasmid. After incubation for about 2 days, the cells formed colonies in sucrose medium were found, and they did not have *sacB* gene in their chromosome. Thus, they were the cells without the integrated plasmid. With the isolated clones, the desired strains in which target genes deleted or inserted, were checked by colony PCR with the primer pairs used when second PCR was carried out during constructing deletion vectors for overlapping PCR.

5. Media and Culture conditions

5.1. Media

Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 1% NaCl) which contains appropriate antibiotics (50 μg/mL of kanamycin, 15 μg/mL of tetracycline) was used to cultivate *E. coli* strains. Brain heart infusion (BHI) (Difco, USA) which contains appropriate antibiotics (25 μg/mL of kanamycin, 5 μg/mL of tetracycline) was used to incubate *C. glutamicum.*

The minimal medium used for *C. glutamicum* was CGXII, consisting of (per liter) 20 g of $(NH4)_2SO_4$, 5 g of urea, 1 g of KH_2PO_4, 1 g of K_2HPO_4, 0.25 g of $MgSO_4 \cdot 7H_2O$, 42 g of 3-morpholinopropanesulfo nic acid, 10 mg of $CaCl_2$, 10 mg of $FeSO_4 \cdot 7H_2O$, 10 mg of $MnSO_4 \cdot H_2O$, 1 mg of $ZnSO_4 \cdot 7H_2O$, 0.2 mg of $CuSO_4$, 0.02 mg of $NiCl_2 \cdot 6H_2O$, 0.2 mg of biotin (pH 7.0), and 0.03 mg of protocatechuic acid (Eggeling and Bott 2005).

5.2. Culture conditions

For the inoculation of recombinant *C. glutamicum*, a frozen stock was transferred to a test-tube containing 5 mL of BHI medium and incubated overnight at 30℃ and 250 rpm in a shaking incubator (Vision, Korea). For recombinant *C. glutamicum* which contains a single vector, 25 μg/mL of kanamycin was added when using pEKEx2 derived plasmid and 5 μg/mL of tetracycline were added when using pVWEx2 derived plasmid. For the dual vector system (pEKEx2 and pVWEx2 derived plasmids) 25 μg/ml of kanamycin and 5 μg/mL of tetracycline were added simultaneously.

The case of batch fermentation, 1 mL of cell culture broth grown overnight was inoculated in a 500 mL of baffled flask (NALGENE, USA) with 100 mL of CGXII media containing 40 g/L of glucose and grown at 30℃ and 250 rpm. The appropriate antibiotics were supplemented. When an optical density reached OD_{600} of 0.8, isopropyl-β-$_D$-l-thiogalactopyranoside (IPTG) was added to a final concentration 1.0 mM and lactose was added to a final concentration 10 g/L for induction of gene expression to produce 2'-FL.

The case of fed-batch fermentation was carried out in a bioreactor of 2.5 L jar (Kobiotech, Korea) with a 1 L initial working volume of CGXII medium containing 40 g/L of glucose and antibiotics of the same concentration as batch culture. The 100 ml pre-culture was prepared with the method like flask cultivations. Then, the culture solution was transferred to the bioreactor, giving an initial OD_{600} of approximately 1 or 2. Aeration rate and agitation speed were in between 2 ~ 2.5 vvm of air supply and 1,000 rpm, respectively. The pH was automatically controlled at 6.98 ~ 7.02 by addition of 28% ammonia water and 2N HCl. To keep the cell growth and a basal level of glucose after depletion of 4% glucose initially added, feeding solution was fed at a continuous feeding rate of 5.7 g/L/hr on average. The feeding solution was composed of 800 g/L of glucose. When initial glucose was consumed completely, 1.0 mM of IPTG was added for induction of the gene expression regulated by the *tac* promoter. Also, 20 g/L of lactose was added as a substrate for α-1,2-fucosyltransferase.

6. Analysis

6.1. Dry cell weight

By measuring the optical density of culture broth, cell growth was monitored. Absorbance at 600 nm was measured using a spectrophotometer (OPTIZEN POP, MECASYS, Korea) after culture broth samples were appropriately diluted to keep optical density between 0.1 and 0.5. Optical density was converted to dry cell weight by using the following conversion equation:

$$\text{Dry cell mass (g/L)} = 0.30 \times OD_{600}$$

6.2. Analysis of fermentation metabolites

Concentrations of glucose, lactose, lactate and 2'-FL were measured by a high performance liquid chromatography (1200 series, Agilent, Santa Clara, CA, USA) with a Rezex ROA-organic acid H^+ Column (Phenomenex, USA) heated at 60°C. A mobile phase of 5 mM H_2SO_4 was used at a flow rate of 0.6 mL/min. Detection was made with a reflective index detector.

III. RESULTS AND DISCUSSIONS

1. Development of strain with high 2'-FL productivity

1.1. Finding unnecessarily overexpressed genes

In the previous research, 2'-FL production system through *C. glutamicum* was constructed. According the research, six genes are involved in the biosynthesis of 2'-FL in *C. glutamicum*. To synthesize GDP-$_L$-fucose, *manB*, *manC*, *gmd* and *wcaG* genes were introduced. Next, to import lactose into the cell, the *lacYA* operon from *E. coli* K-12 was introduced. Then, CO*fucT2* derived from *H. pylori* was introduced to fucosylate lactose and GDP-$_L$-fucose. All genes were expressed in plasmids. Finally, the strain BCGWTTL(CO) harboring plasmids pVBCL and pEGWTT(CO) was constructed. As a result, 0.547 g/L of 2'-FL was produced in batchfermentation (Jo, Thesis. 2016).

Some genes were removed from plasmid to minimized metabolic burden for enhancement of 2'-FL production. If the gene is overexpressed unnecessarily, it may be necessary to remove it to improve the productivity. Therefore, in this study, unnecessary overexpressed sequences on the plasmid were removed.

Of the six genes introduced to produce 2'-FL, the genes already present in *C. glutamicum* chromosome are *manB* and *manC*. So these two genes were removed from plasmid pVBCL by restriction enzymes, *Pst*I and *Bam*HI. Thus, pVL which contains only *lacYA* operon was obtained. Then, pVL was transformed into *C. glutamicum* together with pEGWTT(CO) to conduct flask fermentation. Finally, the GWTTL(CO) strain was constructed. Batch culture was performed with this strain,

resulting in 0.62 g/L of 2'-FL(Fig. 12, Table 5). This is an increase of about 13% compared to the BCGWTTL (CO) strain. The results show that it is not necessary to express the genes for *manB* and *manC* in the plasmid. This means that the endogenous genes already present in chromosome of *C. glutamicum* is sufficient to produce 2'-FL.

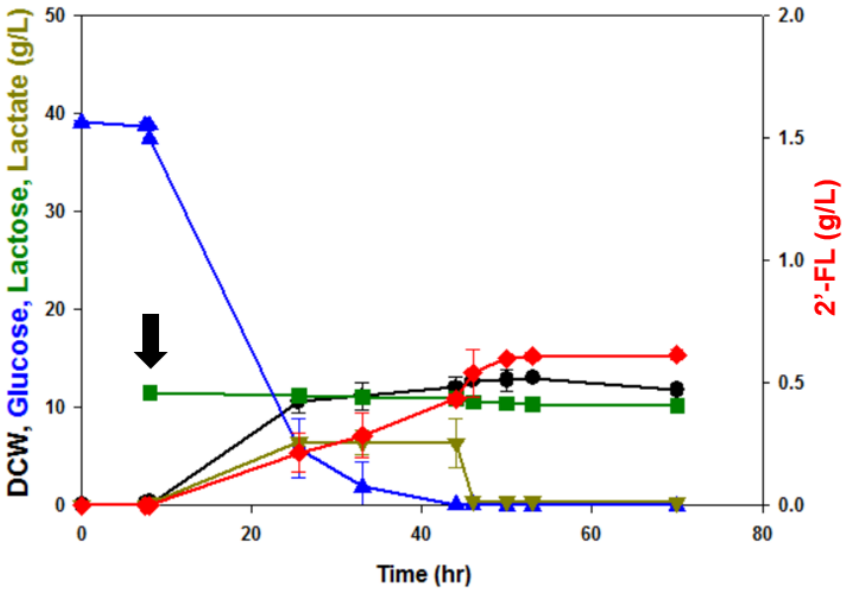


Figure 12. Flask fermentation of GWTTL(CO). As OD_{600} reached 0.8, IPTG and lactose were added (thick arrow).

Symbols: ●, DCW; ▲, Glucose; ■, Lactose; ▼, Lactate; ◆, 2'-FL

1.2. Enhancement of lactose utilization

C. glutamicum does not consume lactose because it lacks the genes that can utilize lactose. The *lacYA* operon from *E. coli* K-12 has been introduced to import lactose but still the amount of lactose consumption was low. Therefore, it was necessary to increase the utilization of lactose in *C. glutamicum.*

1.2.1. Construction of strain expressing *lacY*

The *lacYA* operon in which the β-galactosidase gene (*lacZ*) was removed to import lactose into the *C. glutamicum*(Chin, Seo et al. 2016). Since β-galactosidase gene cleaves lactose into glucose and galactose, this gene had to be removed to consume lactose. However, the *lacA* gene is not considered yet. The role of the *lacA* gene has not been clearly elucidated (Roderick 2005), and when introduced in the form of the *lacYA* operon, 2'-FL was produced to a certain extent. Therefore, the existence of *lacA* has not been greatly rethought. However, in this study, the effect of *lacA* gene on the production of 2'-FL is investigated by removing *lacA* from *lacYA* operon.

GWTTLY (CO), a strain lacking *lacA*, introduces the *lacY* operon in which only *lacA* is absent from *lacYA* operon. Batch fermentation with this strain resulted in the production of 0.93 g/L of 2'-FL (Fig. 13, Table 5). This result is 70% higher than BCGWTTL (CO) strain.

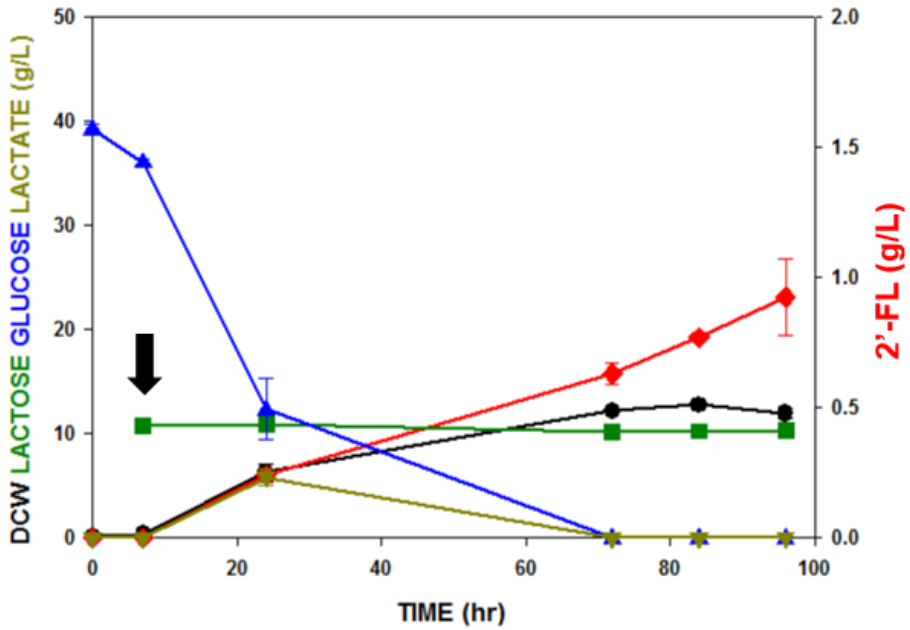


Figure 13. Flask fermentation of GWTTLY(CO). As OD_{600} reached 0.8, IPTG and lactose were added (thick arrow).

Symbols: ●, DCW; ▲, Glucose; ■, Lactose; ▼, Lactate; ◆, 2'-FL

1.2.2. Replacement of *lacY* promoter into strong promoter with Ribosome-binding site (RBS)

So far, all genes involved in 2'-FL production have been expressed under the *tac* promoter except for the *lacY* gene. In addition, all but the *lacY* gene were expressed in the presence of RBS. Instead, the *lacY* gene was expressed under the *lac* promoter without RBS. Therefore, in this study, the promoter of the *lacY* gene was replaced with the *tac* promoter, which is generally known stronger than the *lac* promoter and at the same time, RBS was added for more expression of lactose permease. Thus, pVTY which has the *lacY* gene under *tac* promoter with RBS is constructed. This plasmid is introduced into *C. glutamicum* with pEGWTT(CO).

Finally, the GWTTY(CO) strain was constructed(Table 3). Batch fermentation was carried out with this strain produced 1.94 g/L of 2'-FL(Fig. 14). This is an improvement of 255% over BCGWTTL(CO) strains. As can be seen in Table 5, the finally constructed strain, GWTTY(CO), produced 3.5 times 2'-FL compared to BCGWTTL(CO) and productivity increased about 3.1 times (Table 5).

Furthermore, fed-batch fermentation proceeded using GWTTY(CO) strain. As a result, a total of 25.5 g/L of 2'-FL was produced, which is about 2.2 times higher than BCGWTTL(CO) (Fig. 15, Table 6). This is because GWTTY(CO) strain express only endogenous *manB* and *manC* gene. Thus, metabolic burden could be minimized. Since *lacA* gene was deleted in plasmid and *lacY* gene was expressed in *tac* promoter with RBS, lactose utilization was enhanced. In conclusion, a strain producing more amount of 2'-FL efficiently was obtained by

constructing the GWTTY(CO) strain.

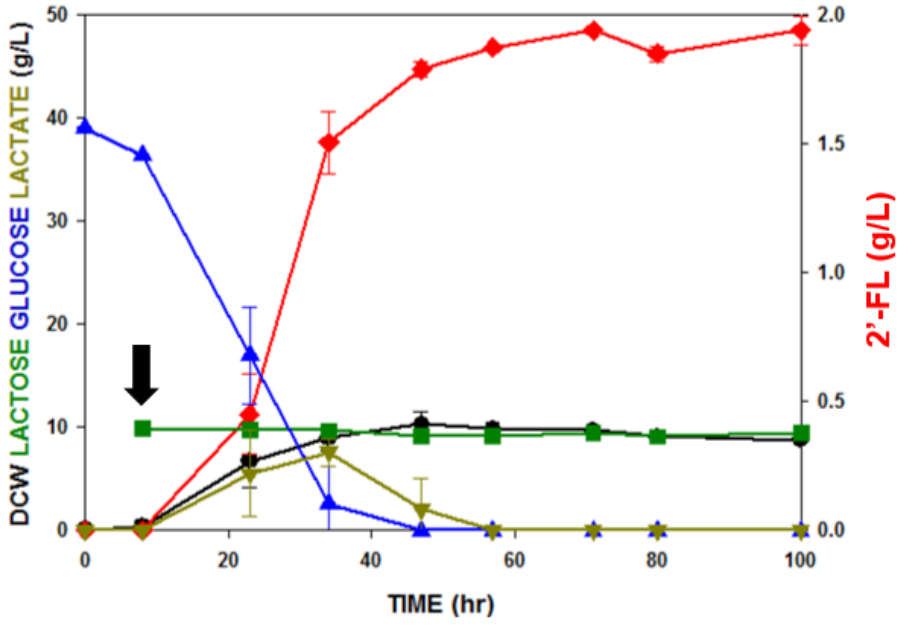


Figure 14. Flask fermentation of GWTTY(CO). As OD_{600} reached 0.8, IPTG and lactose were added (thick arrow).

Symbols: ●, DCW; ▲, Glucose; ■, Lactose; ▼, Lactate; ◆, 2'-FL

Table 5. Summary of flask fermentation of BCGWTTL(CO), GWTTL(CO), GWTTLY(CO) and GWTTY(CO)

Strains	Maximum dry cell weight (g/L)	Maximum 2'-FL concentration (g/L)	*Productivity (mg/L/h)
BCGWTTL(CO)	13.0	0.55	6.6
GWTTL(CO)	13.4	0.62	6.5
GWTTLY(CO)	12.8	0.93	9.6
GWTTY(CO)	11.7	1.94	20.2

*2’-FL yield and productivity were calculated based on total fermentation time

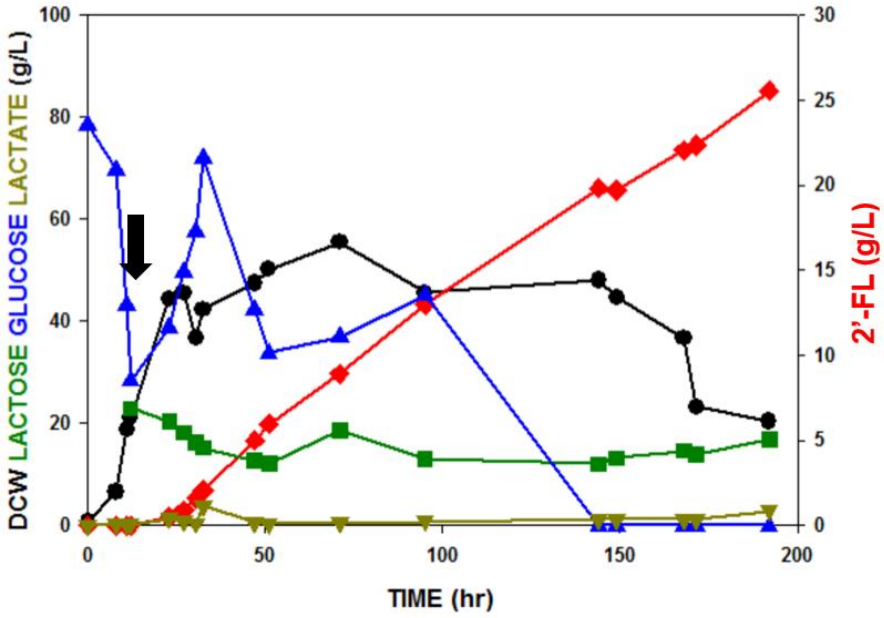


Figure 15. Fed- batch fermentation of GWTTY(CO). IPTG and lactose were added (thick arrow).

Symbols: ●, DCW; ▲, Glucose; ■, Lactose; ▼, Lactate; ◆, 2'-FL

Table 6. Summary of fed-batch fermentation of GWTTY(CO)

Strains	Maximum dry cell weight (g/L)	Maximum 2'-FL concentration (g/L)	*Productivity (mg/L/h)
GWTTY(CO)	55.5	25.5	0.13

*2'-FL productivity was calculated based on total fermentation time

2. Development of 2’-FL producing gene-inserted strains

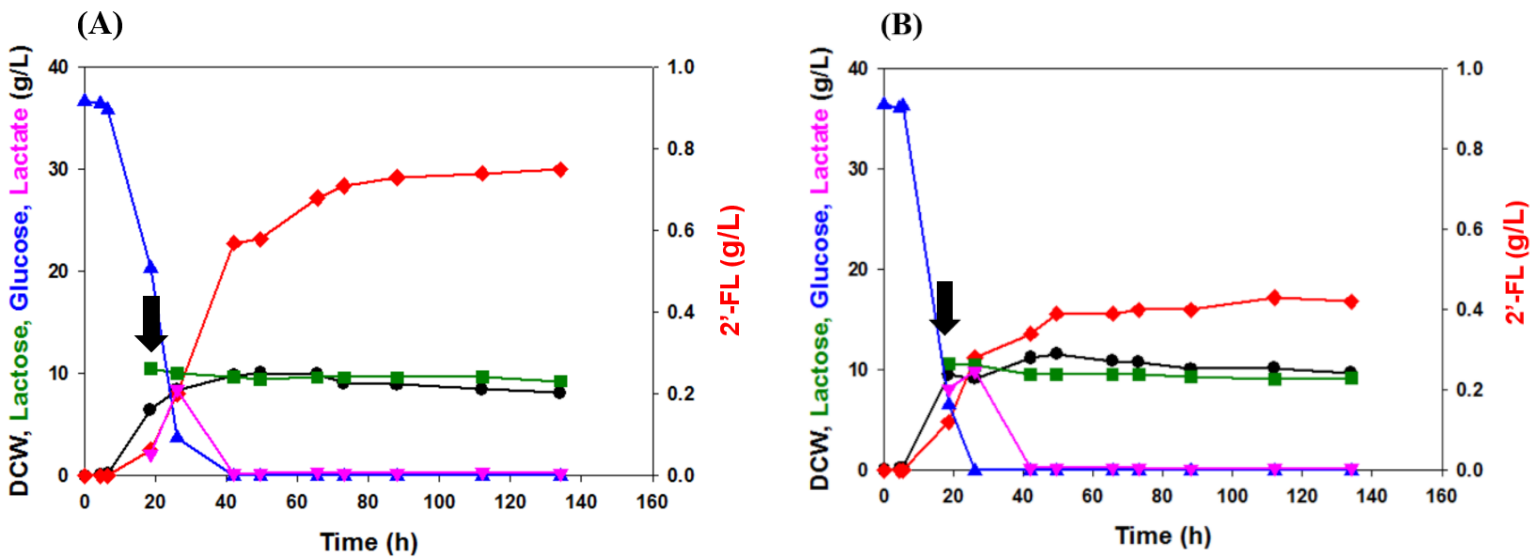
So far, to produce 2’-FL, two antibiotics were used. However, there are various disadvantages in producing materials with antibiotics in industry. First, since antibiotics are expensive materials, they can be costly to produce and ultimately can result in higher final product prices. Second, antibiotics need to be removed completely, which adds to the cost of producing 2’-FL. Lastly, it is difficult to obtain permission of products. In addition, the consumer's perception is also bad when using antibiotics. It is because that 2’-FL can be directly contacted to the skin or can be ingested by babies as a component of cosmetics and foods.

For these reasons, it is important to produce 2'-FL without the use of antibiotics. However, two antibiotics, kanamycin and tetracycline were used to produce 2'-FL. Thus, to see how much 2'-FL is produced without using antibiotics, 2'-FL production was observed under the following antibiotic conditions. First, batch fermentation was carried out under conditions of using both antibiotics, secondly using only one antibiotic, and finally using no antibiotics.

As can be seen from the Figure 16, 2’-FL production was maximized when using both antibiotics and reduced by half when using no antibiotics . When both antibiotics were used, 0.67 g/L of 2'-FL is produced and when no antibiotics were used, 0.40 g / L of 2'-FL is produced (Fig. 16, Table 7). Antibiotics were used to maintain expression vector stably. Thus, 2'-FL production genes which was in the expression vectors need to be integrated on the chromosome so that *C. glutamicum* produce this substance without using antibiotics.

Figure 16. Flask fermentation of BCGWTTL(CO) under various antibiotic conditions. (A) using kanamycin and tetracycline (B) using only kanamycin (C) using only tetracycline (D) No antibiotics are used. As OD_{600} reached 0.8, IPTG and lactose were added (thick arrow).

Symbols: ●, DCW; ▲, Glucose; ■, Lactose; ▼, Lactate; ◆, 2'-FL



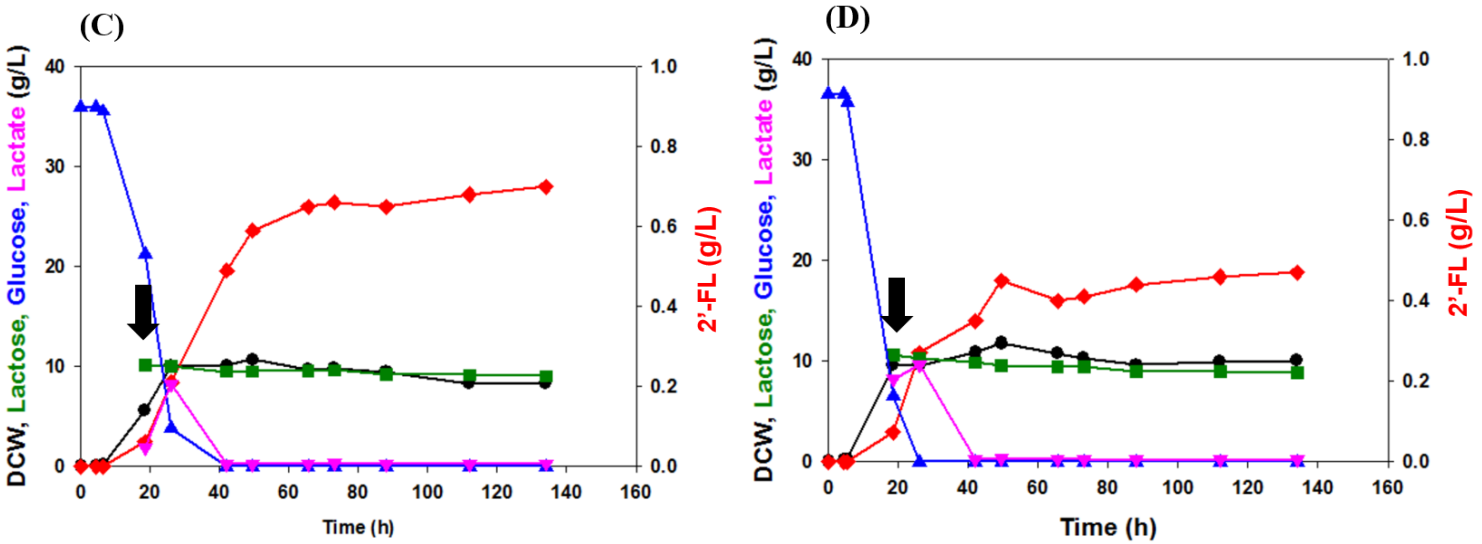

(C)
DCW, Lactose, Glucose, Lactate (g/L)
2'-FL (g/L)
Time (h)
(D)
DCW, Lactose, Glucose, Lactate (g/L)
2'-FL (g/L)
Time (h)

Table 7. Summary of flask fermentation of BCGWTTL(CO) under various antibiotic conditions. (A) using kanamycin and tetracycline (B) using only kanamycin (C) using only tetracycline (D) No antibiotics are used

Antibiotic conditions	Maximum dry cell weight (g/L)	Maximum 2'-FL concentration (g/L)
A	10.4	0.67
B	11.8	0.39
C	10.81	0.62
D	11.71	0.40

*2’-FL yield and productivity were calculated based on total fermentation time.

2.1. Determination of chromosomally integration site

To integrate target genes on the chromosome of *C. glutamicum*, the integration site should be determined first. When a gene is integrated on the chromosome, the amount of fermentation products decreased in most cases. Therefore, in order to overcome this problem, usually 16S rRNA or IS (Insertion Sequence) elements were selected to insert genes in the case of *C. glutamicum* (Eggeling and Bott 2005). Because these genes are present in multiple copies in the chromosome, they can be integrated into multiple copies, which may not cause the problem of decreased fermentation products(Amador, Martín et al. 2000).

In the case of 16S rRNA, deletion of this gene or integration at this site leads to the loss of the gene sequence when it is deleted. This can adversely affect cell growth. Therefore, in this study, the IS element sites were determined to integrate target genes.

Another reason for selecting the IS element is it is a mobile element that leads to DNA rearrangement(Choi, Yim et al. 2015). These rearrangements eventually lead to a reduction in enzyme production involved in producing 2'-FL.

Therefore, removal of the IS element can prevent DNA rearrangement from occurring. Therefore, the plasmid stability and the amount of 2'-FL production enzyme will ultimately increase. Eventually, the production of 2'-FL will also be improved.

2.2. Construction of IS*Cg2b* deleted strain

Among IS elemtents, the IS*Cg2b* gene deleted site was seletecd to integrate the CO*fucT2* gene. Because the effect of deletion IS*Cg2* family genes was greater than that of IS*Cg1a* family genes(Choi, Yim et al. 2015).

To integrate a gene into a gene deleted site, it is necessary to examine whether the deletion affects production of 2'-FL before proceeding with the integration of genes. Thus, the IS*Cg2b*-deleted strain, ΔISCg2b was constructed (Fig. 19). Then, pVBCL and pEGWTT(CO) were introduced in *C. glutamicum* by electroporation. Thus, ΔISCg2b BCGWTTL(CO) was constructed and batch culture was performed with this strain. As a result, 0.79 g/L of 2'-FL was produced(Fig. 17, Table 8). The production of 2'-FL was increased by 1.4 times as compared with the BCGWTTL (CO) strain. As IS element was removed from the chromosome of *C. glutamicum*, the 2'-FL was increased. Therefore, the deletion of the IS*Cg2b* gene has a good effect on the production of 2'-FL and integration into this site is possible.

The IS*Cg2b*-deleted DNA sequences of *C. glutamicum* are as follows. Flanking regions of the IS*Cg2b* gene are marked with shading. The bold sequences present the recognition sites of restriction enzymes which were added when constructing deletion vector to insert CO*fucT2* gene for construction of integration vector.

TCATGGTTCAGGGCACTGGCTTCAACTGGCCACATCACGAT
CACTTCCGAGTGGTCACCCTGCCATGGGCATCCCAGTTGGA
AAACGCAATTGAGCGCCTGGGTAACTTCCTGTCCACTTACA
AGCAGTAGTAGTTGTTAGGATTCACCACGAATCTCAGGATTT

TTGAGATTCGTGGTGAATTTTTGCGTTTTCCAGTCAGGCTCC
TGCAACTTTCGGACCGATTTCAGAGGGGCGGAGCTGGTTTG
TGGTGGATCCTTGAAATGGAACCTCGCAGGAAGCTTTCAGG
AAGACCAAGTTGGGCCTAGGGGTGGCGGGATTGCAAAAATC
CGTCCCCGGTTCGCCATGAAATGCTGATTTTGATCGAATCTTT
GCGCTAACTGTAGGGCGGGTTCAGGGGGTGAATGCACCACG
AGCAACCCGAAGGGTGCGAAGTGGGCATTCGTAGAACAATC
CCAGAGGAAAGCCGTACGGCTTTCCTCGACATGATCAATCA
AGGT**ACTAGTCTCGAG**ATTCGCCTAGGAGATTGTACGAAAA
TTCGTTCGGCTTTCGGATTTCCTGGCGATCTGAGACGAGAAG
TTGAACAGCTAACCTGCAGAAACCTTGCAAGAATCACAACA
GCCCCAATGGCCTCAAAAGTCACGCCCTCAGAATCGCTGCC
AGGCGTCTAAATCCCCTAAAACGGGACAATAGGTCACTGGG
CGATCCCAAGCCCTTAAAACGTGATCCTTAAATACCCACTGT
CCTCTATTCTGGGTTAGGCTTCACTGGGTAAAAGTGCCTGCC
TATGCCTGAAACTTGAGCATGGCAACAGCAAGGAGACACCG
TGGGAAAACATGCAGCTGAAACATCGGAACCGAAGAAAAA
TTCACCGTGGCGCATTGGTTTGTTGACGTTTTTGATTTCTTCA
GTTGTCGTGACGCTGGTGGGCATGGTGATGCTGTGGCCGGA
TTCTGATGATGTGGTGTTGGCGGATAACTTTTCGCAGACGTT
TGCGGGAAATCATGAGCAG

IS*Cg2b* sequences (1515 bp)

ATGTCAGGTCTTGCTGCGTCTACAGCGGTCGGGGTCAGTGA
ATTCACCGGGCGAAAGTGGGCGAAGGCCGCCGGGGTGAAA
CTGACCCGCGGCCCGCGAGGTGGCAATGCTTTTGACACCGC

CGAGAAACTTGAGATTGCAGCCAGCATGCTAGAGAAAGGAT
GCCTACCCCGAGAAATCGGCGAGTATGTCGGCATGACTCGG
GCCAATATATCCCTATGGCGCAAACAAGGCCCAGACAAGCTT
CGCCAACGCGCAGCCACCTTGCGCACCGGCAAGCGAGCAG
CTGAATTCATCCACGCCCCGGTGATGGGCCCTTATTATGGGC
CACGCACACTCCATCAAGTGTTGCGTGAGGACTACACAACA
CTGTTTGACGAGTTATCTGCGTTGGGGTTGCCAGCACAGGT
GTGTGGGGCCTTACTTCATCTTGCTCCACCACCATCATTACG
CTTTTCTTATATGTCGTGTGTAGTGCCGTTATTTGCTGATGAA
ATCAAAGTCGTAGGACAAGGCACACGATTATCGTTAGAAGA
GAAAATGATGATCCAACGTTTCCATGACACCGGGGTCAGTG
CAGCAGAAATCGGTCGACGCCTGGGTCGGTGTCGGCAAACA
ATTTCCAGGGAACTTCGACGTGGTCAAGATGATGATGGACG
TTATCGTGCACGCGACTCCTATGAAGGTGCGATCAGGAAACT
AGCGCGTCCGAAAACACCGAAACTTGATGCCAATCGTAGGC
TTCGGGCTGTGGTGGTCGAGGCGTTGAATAATAAATTATCTC
CGGAGCAGATTTCTGGTCTTTTAGCCACCGAGCATGCTAACG
ATAGCTCTATGCAGATTAGTCATGAAACTATTTACCAGGCGTT
ATATGTTCAAGGTAAAGGGGCGTTGCGTGATGAATTGAAGGT
GGAGAAATTTCTTCGTACCGGTCGGAAGGGACGTAAACCGC
AGTCGAAGTTGCCATCGAGAGGTAAGCCGTGGGTGGAGGGT
GCGTTGATTAGTCAACGCCCAGCAGAAGTTGCTGATCGTGC
TGTGCCTGGGCACTGGGAGGGCGATTTAGTAATTGGTGGTG
AAAACCAAGCGACAGCGTTGGTGACGTTGGTGGAGCGCAC
GAGCCGGTTGACGTTGATTAAGCGGTTGGGGGTTAATCATGA
GGCGTCGACTGTGACGGATGCGTTGGTGGAGATGATGGGTG

ATTTGCCGCAGGCGTTGCGTCGGAGTTTGACGTGGGATCAG
GGTGTGGAGATGGCAGAGCATGCGCGGTTTAGCGTGGTGAC
CAAGTGTCCGGTGTTTTTCTGTGATCCTCATTCGCCGTGGCA
GCGTGGGTCGAATGAGAATACGAATGGATTGGTCAGGGATT
TTTTCCCGAAGGGCACTAATTTTGCTAAAGTAAGTGACGAA
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AAATCGTAGTTGGTGCATCCACCGATTGA

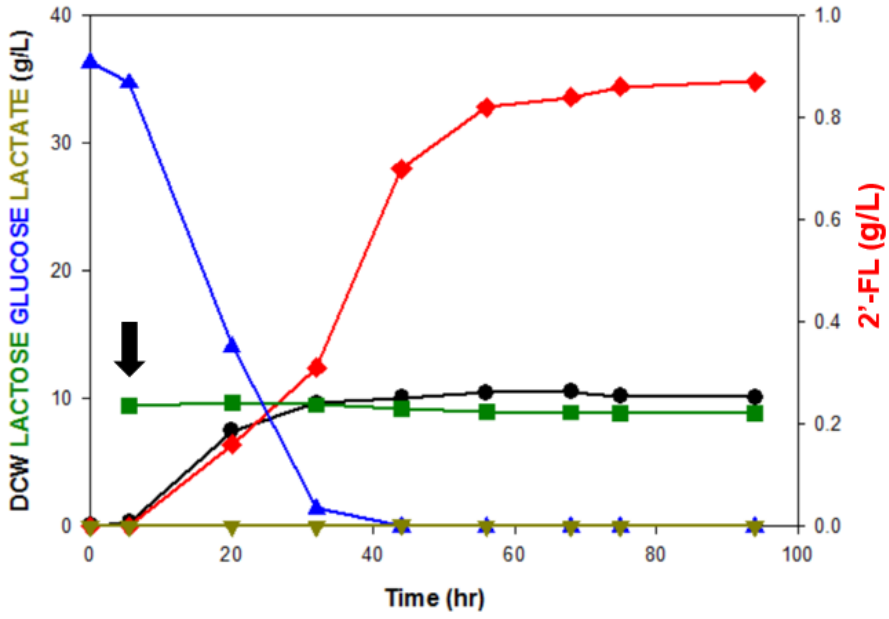


Figure 17. Flask fermentation of ΔISCg2b BCGWTTL(CO). As OD_{600} reached 0.8, IPTG and lactose were added (thick arrow).

Symbols: ●, DCW; ▲, Glucose; ■, Lactose; ▼, Lactate; ◆, 2'-FL

2.3. Construction of CO*fucT2* inserted strain

Since deletion of the IS*Cg2b* gene did not affect the production of 2'-FL as observed in a flask fermentation, this site was selected for integration and CO*fucT2* was selected as the gene to integrate in IS*Cg2b* deleted site.

The *tac* promoter, CO*fucT2*, and terminator were inserted together in the chromosome. As a result, ΔISCg2b::FucT2(CO) strain is constructed (Figure 19). When the DNA sequence was analyzed, it was found that three base sequences changed from G to A(AAG → AAA)(Figure 18). However, since the amino acid encoded was the same as lysine, there was no problem in expressing the protein.

CO*fucT2* is present on the chromosome so it was no longer expressed episomally. Therefore, pEGW which has *gmd* and *wcaG* and pVBCL which has *manB*, *manC* and *lacYA* operon were transformed into *C. glutamicum* and ΔISCg2b::fucT2(CO) BCGWL is constructed. Then, batch fermentation was carried out in this strain. Batch fermentation resulted in the production of 0.35 g/L of 2'-FL (Figure 20). This is because the CO*fucT2* gene was expressed only 1 copy in the chromosome after chromosomal insertion.

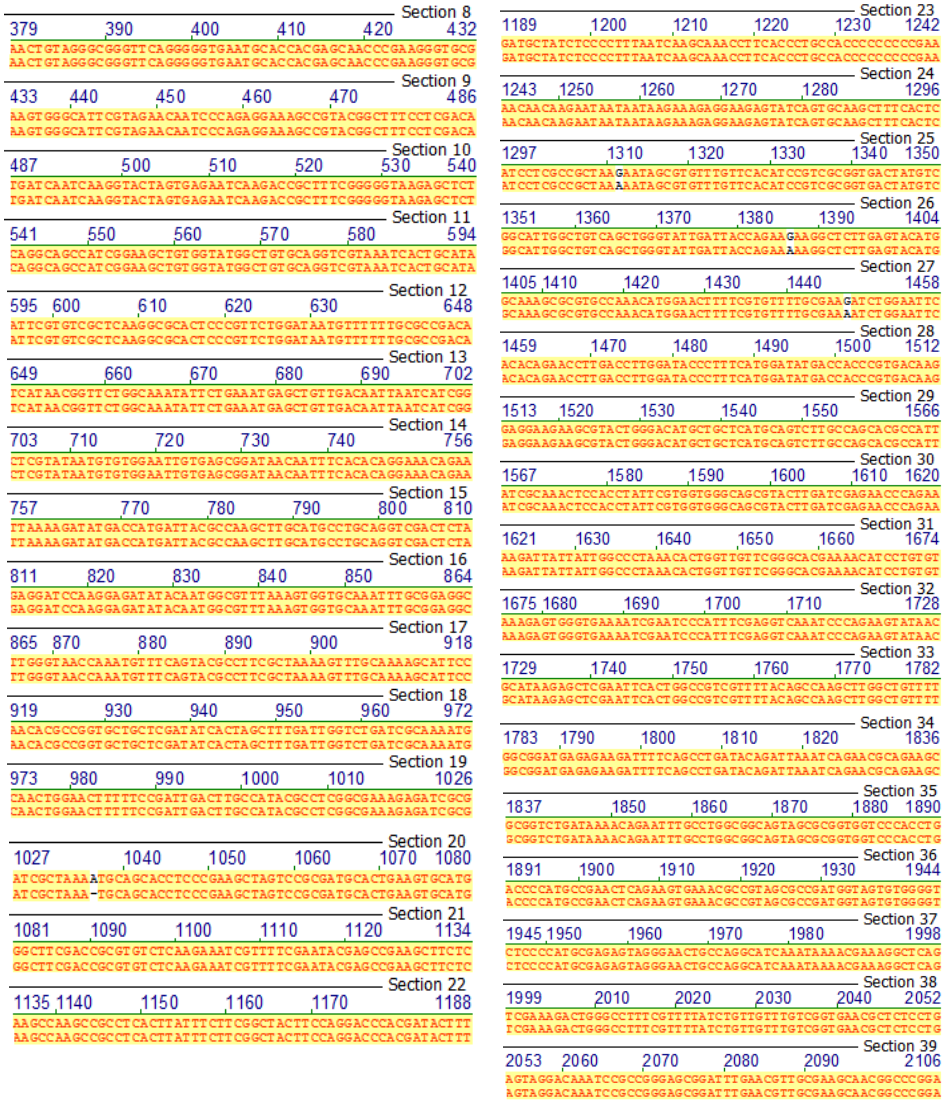
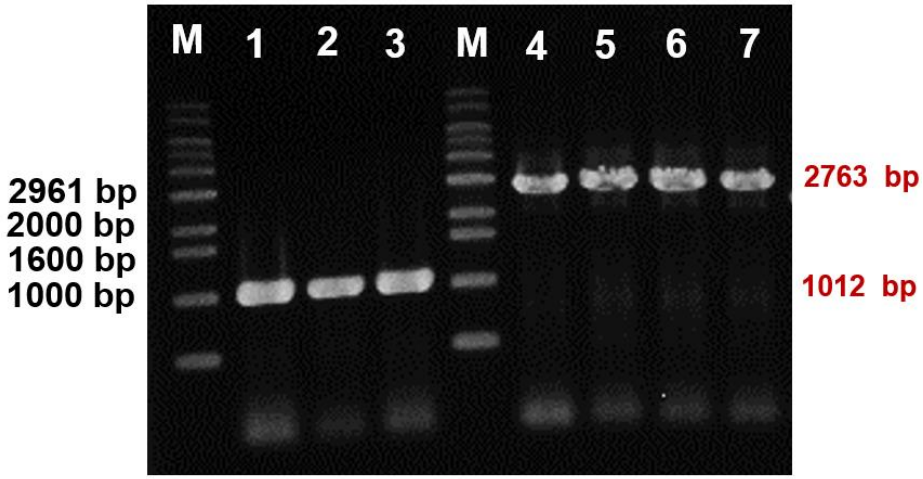


Figure 18. The sequences of inserted CO*fucT2*.



M: 1 kb ladder

1, 2, 3: deleted IS*Cg2b*

4, 5, 6, 7: inserted CO*fucT2*

Figure 19. Confirmation of ΔISCg2b and ΔISCg2b::FucT2(CO) strain construction by colony PCR with primer pairs, F1_*Sal*I_ISCg2b(L) and R3_*Eco*RI_ ISCg2b(R) (Table 4).

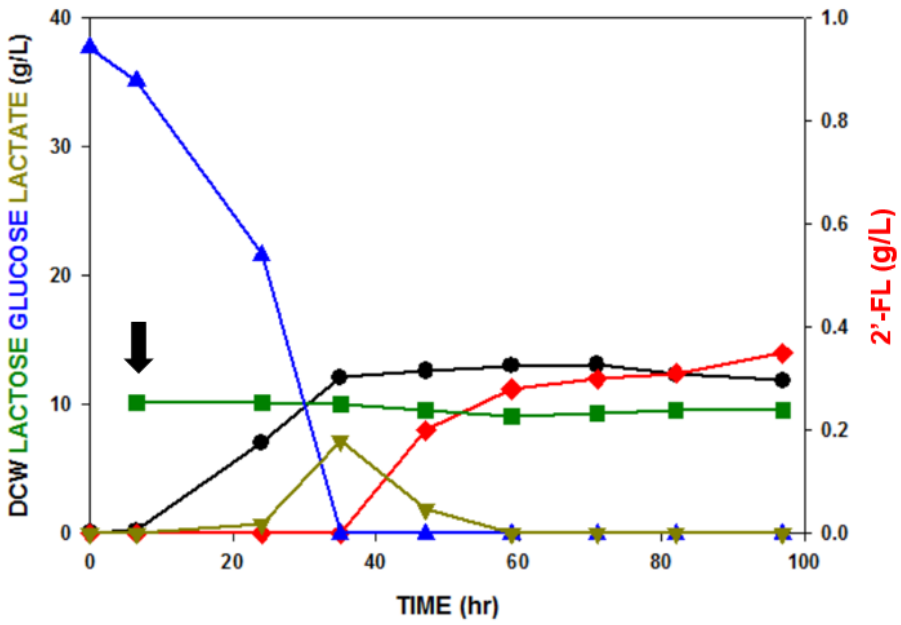


Figure 20. Flask fermentation of ΔISCg2b::fucT2(CO) BCGWL. As OD_{600} reached 0.8, IPTG and lactose were added (thick arrow).

Symbols: ●, DCW; ▲, Glucose; ■, Lactose; ▼, Lactate; ◆, 2'-FL

2.4. Construction of IS*Cg2f* deleted strain

The IS*Cg2f* gene belonging to the IS*Cg2* family was selected as another site for gene integration and this gene was deleted. 1515 bp of IS*Cg2f* gene of ΔISCg2b::fucT2(CO) strain was successfully deleted and ΔISCg2bΔISCg2f::fucT2(CO) strain was constructed (Figure 21). pEGW which has *gmd* and *wcaG* genes and pVBCL which has *manB, manC* and *lacYA* operon genes were transformed into this strain and ΔISCg2bΔISCg2f::fucT2(CO) BCGWL is constructed. Batch fermentation is performed by this strain and 0.42 g/L of 2'-FL was produced (Figure 22). As the IS element was removed, there was an increase of 1.2 times in 2'-FL production.

The IS*Cg2f*-deleted DNA sequences of *C. glutamicum* are as follows. Flanking regions of the IS*Cg2f* gene are marked with shading. The bold sequences present the recognition sites of restriction enzymes which were added when constructing deletion vector to insert *gmd* and *wcaG* genes for construction of integration vector.

ACTGCCCCCTCTGGAAATGTTGGAGCATGGGAGTTTCTGGA
TAAGGGTGCGGGGGTGGAGGGGGTAGTGATTGGGGCTGTTT
GACACCTTTGTTAGGTGACATTGTCACCTCATTCACTGCTTA
AAGATCCTAATGTCATTACAAAAAGCGTGCGAGGAGCGGAC
ATCGGGAATGGTGGGGCGATAATCGCCCGTTTAAGCGGCTCG
AGACGTCGATCGACCACTTGTGTCAAAGTGGGCACAAATTG
CCGTCAGGCGGCGCTCTGGAACGTCATCATTTTGGGACTGG
TGGGGCGAATGTGAAACCTGGGTGTTTGCTGATCAATTTTTC
ACCACAGGCGTGAGGTCTGGCCATAAAGTCGAACGAATTTT

TGCATCAAACCCTAGGCGGGTTCAGGGGGTGAATGCACCAC
GAGCAACCCGAAGGGTGCGAAGTGGGCATTCGTAGAACAAT
CCCAGAGGAAAGCCGTACGGCTTTCCTCGACATGATCAATC
AAGGTAT**GCGGCCGCCATATG**TCGCCCTAGGTTGGATGCAA
AAATCCACCACAAACATGTGGTCAGTCCACATGAATTGAAC
GAATTTTTGCATTTGGAACCGGCATGTCCCGATACCATCAAA
AAACCTCACCGCGCACCCAAGAATCTCCTTAATGGCCCTCG
ATTTGGCGTTCCAACTCTTTGATTCGGATCCGCAGGGCCTTT
TTCTCATCCCAATTGGCGGTGACATCGCGGAGAAAAGCTGC
AACGCCTTCGATTTTTCCGGAATCGTCCTTCAGGATGGTGAT
GGAGAATTCCAAAGACATTTTGGATCCATCGGCACGAATGCC
TGGAACGTTAAGCGGTTCGGAGCCATAGCGAGTTTCGCCGG
ATTCCATGACGCGATCCCATCCGTCCCAGTGGGCCTTGCGGT
GTTTTTCGGGAATGATGATGTCGAGTGATTTTCCAAGGGCTT
CGCCGGCCGTGTATCCAAAGAGTTTCTCGGAGCCGCCGTTC
CAGAGTCTGATTATTCCATCG

IS*Cg2f* sequences (1515 bp)

GTCAGGTCTTGCTGCGTCTACAGCGGTCGGGGTCAGTGAAT
TCACCGGGCGAAAGTGGGCGAAGGCCGCCGGGGTGAAACT
GACCCGCGGCCCGCGAGGTGGCAATGCTTTTGACACCGCCG
AGAAACTTGAGATTGCAGCCAGCATGCTAGAGAAAGGATGC
CTACCCCGAGAAATCGGCGAGTATGTCGGCATGACTCGGGC
CAATATATCCCTATGGCGCAAACAAGGCCCAGACAAGCTTCG
CCAACGCGCAGCCACCTTGCGCACCGGCAAGCGAGCAGCT
GAATTCATCCACGCCCCGGTGATGGGCCCTTATTATGGGCCA

CGCACACTCCATCAAGTGTTGCGTGAGGACTACACAACACT
GTTTGACGAGTTATCTGCGTTGGGGTTGCCAGCACAGGTGT
GTGGGGCCTTACTTCATCTTGCTCCACCACCATCATTACGCTT
TTCTTATATGTCGTGTGTAGTGCCGTTATTTGCTGATGAAATC
AAAGTCGTAGGACAAGGCACACGATTATCGTTAGAAGAGAA
AATGATGATCCAACGTTTCCATGACACCGGGGTCAGTGCAG
CAGAAATCGGTCGACGCCTGGGTCGGTGTCGGCAAACAATT
TCCAGGGAACTTCGACGTGGTCAAGATGATGATGGACGTTAT
CGTGCACGCGACTCCTATGAAGGTGCGATCAGGAAACTAGC
GCGTCCGAAAACACCGAAACTTGATGCCAATCGTAGGCTTC
GGGCTGTGGTGGTCGAGGCGTTGAATAATAAATTATCTCCGG
AGCAGATTTCTGGTCTTTTAGCCACCGAGCATGCTAACGATA
GCTCTATGCAGATTAGTCATGAAACTATTTACCAGGCGTTATA
TGTTCAAGGTAAAGGGGCGTTGCGTGATGAATTGAAGGTGG
AGAAATTTCTTCGTACCGGTCGGAAGGGACGTAAACCGCAG
TCGAAGTTGCCATCGAGAGGTAAGCCGTGGGTGGAGGGTGC
GTTGATTAGTCAACGCCCAGCAGAAGTTGCTGATCGTGCTGT
GCCTGGGCACTGGGAGGGCGATTTAGTAATTGGTGGTGAAA
ACCAAGCGACAGCGTTGGTGACGTTGGTGGAGCGCACGAG
CCGGTTGACGTTGATTAAGCGGTTGGGGGTTAATCATGAGGC
GTCGACTGTGACGGATGCGTTGGTGGAGATGATGGGTGATTT
GCCGCAGGCGTTGCGTCGGAGTTTGACGTGGGATCAGGGTG
TGGAGATGGCAGAGCATGCGCGGTTTAGCGTGGTGACCAAG
TGTCCGGTGTTTTTCTGTGATCCTCATTCGCCGTGGCAGCGT
GGGTCGAATGAGAATACGAATGGATTGGTCAGGGATTTTTTC
CCGAAGGGCACTAATTTTGCTAAAGTAAGTGACGAAGAAGT

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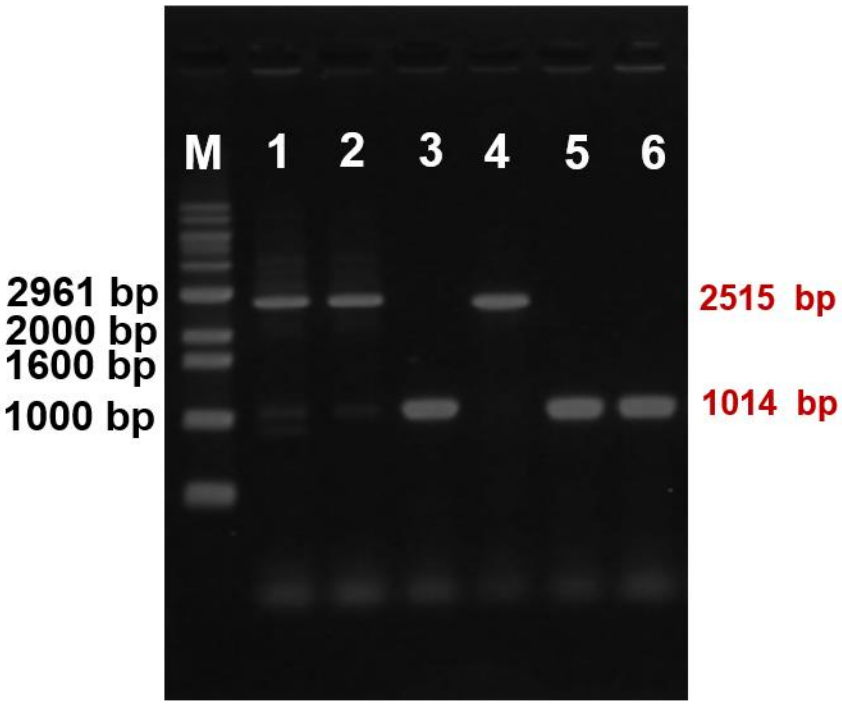
TGCATGGTTTTAAAAGCGCGACGCAGGTATATGAAAAAATCG
TAGTTGGTGCATCCACCGATTGAAT



M: 1 kb ladder

1, 2, 4: wild-type IS*Cg2f*

3, 5, 6: deleted IS*Cg2f*

Figure 21. Confirmation of ΔISCg2bΔISCg2f::fucT2(CO) strain construction by colony PCR with primer pairs, F1_*Hind*Ⅲ_ ISCg2f(L) and R3_*Sal*I_ ISCg2f(R) (Table 4).

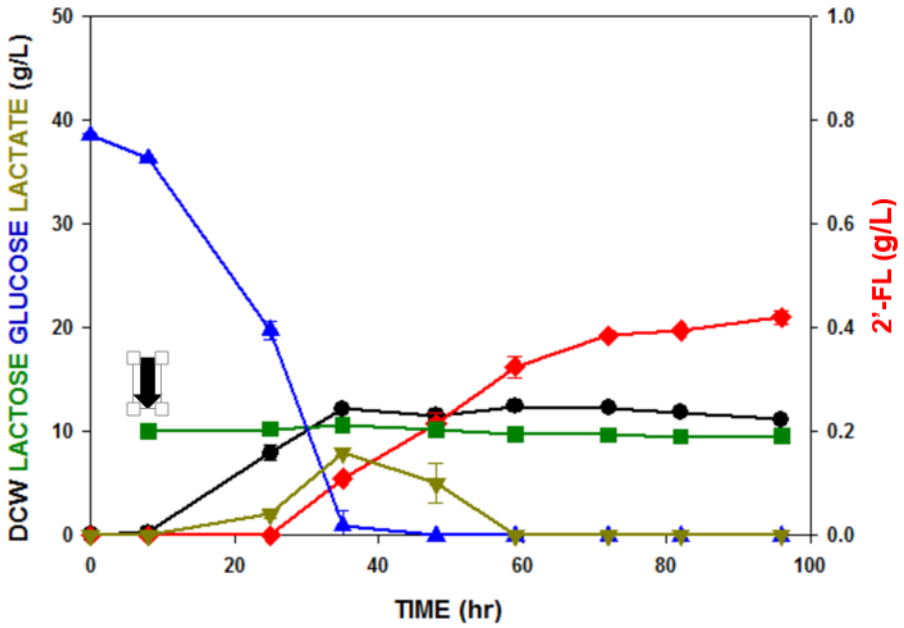


Figure 22. Flask fermentation of ΔISCg2bΔISCg2f::fucT2(CO) BCGWL. As OD_{600} reached 0.8, IPTG and lactose were added (thick arrow).

Symbols: ●, DCW; ▲, Glucose; ■, Lactose; ▼, Lactate; ◆, 2'-FL

2.5. Construction of IS*Cg1a* deleted strain

IS*Cg1a* gene deleted site was selected as the site for integration, and IS*Cg1a* was knock-out for this purpose. IS*Cg1a* gene from ΔISCg2bΔISCg2f::fucT2(CO) strain was deleted and ΔISCg2bΔISCg2fΔISCg1a::fucT2(CO) was constructed (Figure 23). pEGW which has *gmd* and *wcaG* genes and pVTY which has *lacY* gene were transformed into this strain and ΔISCg2bΔISCg2f::fucT2(CO) GWY is constructed. Batch fermentation is carried out by this strain and 0.60 g/L of 2'-FL was produced (Figure 24). Since the IS element was deleted, there was an increase of about 1.4 times in 2'-FL production.

The IS*Cg1a*-deleted DNA sequences of *C. glutamicum* are as follows. Flanking regions of the IS*Cg1a* gene are marked with shading. The bold sequences present the recognition sites of restriction enzymes which were added when constructing deletion vector to insert *lacY* gene for construction of integration vector.

CACTTCCAACTGGCACGTTGTGTTGTCCGGAGTCATTGCAG
GTCTGGGATACGGCACTTTGATGCCAGCAGTGCAGTCCATCG
CTGTTGGTGTAGTAGACAAAACCGAATTCGGTACGGCCTTCT
CCACTTTGTTCCTGTTTGTGGACTTAGGTTTTGGCTTTGGAC
CTATTATCCTGGGAGCAGTTTCTGCGGCAATTGGTTTCGGAC
CTATGTATGCAGCACTGGCAGGTGTGGGTGTGATTGCCGGAA
TCTTCTACCTGTTCACACACGCTCGCACCGATCGAGCTAAGA
ATGGCTTTGTTAAACACCCAGAGCCTGTCGCTTTAGTTAGCT
AGTTCTTTCAGCTTTCCCTCCCGATCAGCGTAAACCGGCCCT

TCCGGTTTTGGGGTACATCACAGAACCTGGGCTAGCGGTGT
AGACCCGAAAATAAACGAGCCTTTTGTCAGGGTTAAGGTTT
AGGTATCTAAGCTAACCAAACACCAACAAAAGGCTCTACCC
GCGATCGCCTCGAGAACAGGAAGAGCCCGTAAACCTCTGA
CTAGCGTCACCCTCTGATTAAGGCGACCGCGGATTTAAGAGC
AGAGGCTGCCACGAGCGCATCTTCACGGCTGTGTGTTGTAC
TAAAAGTACAGCGCACAGCCGTTCGTGCTTGATCCTCCTCA
AGCCCCAACGCCAGCAACACATGGGATACCTCTCCGGAACC
ACAGGCAGAACCAGGGGAGCACACAATGCCTTGGCGTTCC
AATTCCAGAAGAACAGTTTCAGATCCTATGCTGTCGAAGAG
AAAAGATGCGTGTCCATCAATGCGCATCCTAGGATGTCCAGT
CAGGTGTGCTCCCGGGATAGTGAGAACTTCCTCGATGAATTC
GCCAAGATCTGGATAGGATTCCGCCCTGGCCAATTCCAAGGC
AGTGGCAAAGGCGATAGCCCCCGCAACGTTTTCCGTGCCAC
TACGCCGCCCTTTTTCCTGGCCGCCGCCATGGATTACCGGCT
CCAGGGGAAGCTTTGACCA

IS*Cg1a* sequences (1311 bp)

ATGAAGTCTACCGGCAACATCATCGCTGACACCATCTGCCG
CACTGCGGAACTAGGACTCACCATCACCGGCGCTTCCGATG
CAGGTGATTACACCCTGATCGAAGCAGACGCACTCGACTAC
ACCTCCACCTGCCCAGAATGCTCCCAACCTGGGGTGTTTCGT
CATCACACCCACCGGATGCTCATTGATTTACCCATCGTCGGG
TTTCCCACCAAACTGTTTATCCGTCTACCTCGCTACCGCTGC
ACCAACCCCACATGTAAGCAAAAGTATTTCCAAGCAGAACT
AAGCTGCGCTGACCACGGTAAAAAGGTCACCCACCGGGTC

ACCCGCTGGATTTTACAACGCCTTGCTATTGACCGGATGAGT
GTTCACGCAACCGCGAAAGCACTTGGGCTAGGGTGGGATTT
AACCTGCCAACTAGCCCTCGATATGTGCCGTGAGCTGGTCTA
TAACGATCCTCACCATCTTGATGGAGTGTATGTCATTGGGGT
GGATGAGCATAAGTGGTCACATAATAGGGCTAAGCATGGTGA
TGGGTTTGTCACCGTGATTGTCGATATGACCGGGCATCGGTA
TGACTCACGGTGTCCTGCCCGGTTATTAGATGTCGTCCCAGG
TCGTAGTGCTGATGCTTTACGGTCCTGGCTTGGCTCCCGCGG
TGAACAGTTCCGCAATCAGATACGGATCGTGTCCATGGATGG
ATTCCAAGGCTACGCCACAGCAAGTAAAGAACTCATTCCTT
CTGCTCGTCGCGTGATGGATCCATTCCATGTTGTGCGGCTTG
CTGGTGACAAGCTCACCGCCTGCCGGCAACGCCTCCAGCGG
GAGAAATACCAGCGTCGTGGTTTAAGCCAGGATCCGTTGTAT
AAAAACCGGAAGACCTTGTTGACCACGCACAAGTGGTTGA
GTCCTCGTCAGCAAGAAAGCTTGGAGCAGTTGTGGGCGTAT
GACAAAGACTACGGGGCGTTAAAGCTTGCGTGGCTTGCGTA
TCAGGCGATTATTGATTGTTATCAGATGGGTAATAAGCGTGA
AGCGAAGAAGAAAATGCGGACCATTATTGATCAGCTTCGGG
TGTTGAAGGGGCCGAATAAGGAACTCGCGCAGTTGGGTCGT
AGTTTGTTTAAACGACTTGGTGATGTGTTGGCGTATTTCGAT
GTTGGTGTCTCCAACGGTCCGGTCGAAGCGATCAACGGACG
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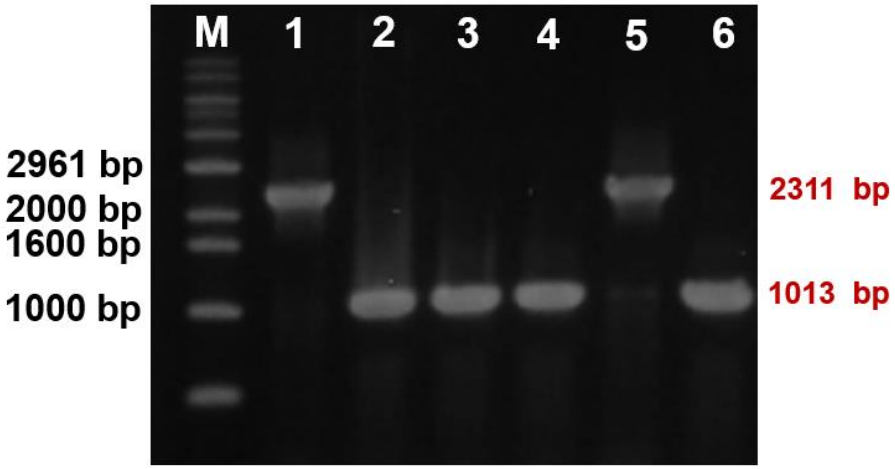
GGTCCATAAGATCAATGCACTCTAA



M: 1 kb ladder

1, 6: wild-type IS*Cg1a*

2, 3, 4, 6: deleted IS*Cg1a*

Figure 23. Confirmation of ΔISCg2bΔISCg2fΔISCg1a::fucT2(CO) strain construction by colony PCR with primer pairs, F1_SalI_ ISCg1a(L) and R2_XbaI_ ISCg1a(R) (Table 4).

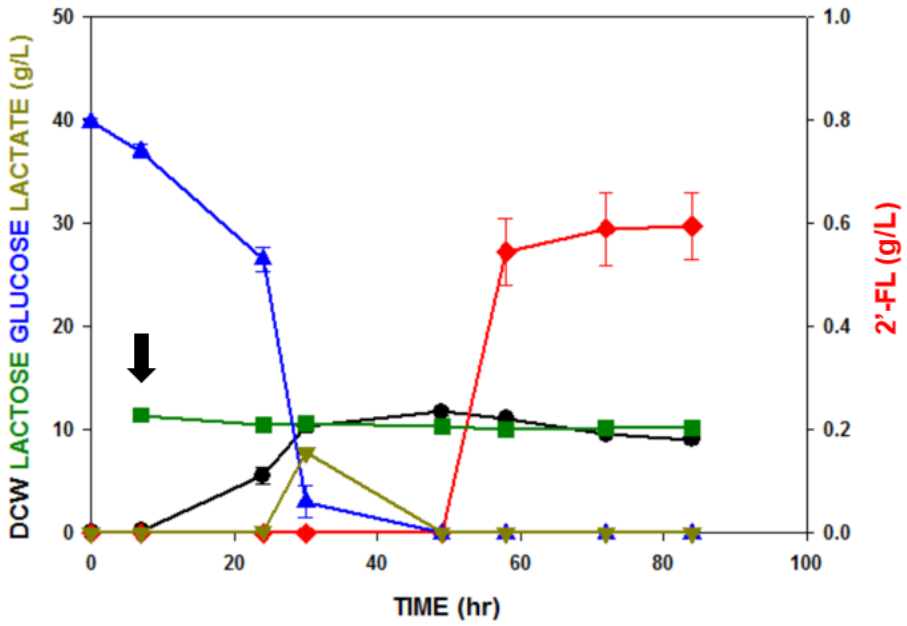


Figure 24. Flask fermentation of ΔISCg2bΔISCg2fΔISCg1a ::fucT2(CO) GWY. As OD_{600} reached 0.8, IPTG and lactose were added (thick arrow).

Symbols: ●, DCW; ▲, Glucose; ■, Lactose; ▼, Lactate; ◆, 2'-FL

2.6. Construction of *gmd-wcaG* inserted strain

Gmd and *wcaG* were selected as the next target genes to be integrated on the chromosome of *C. glutamicum* after integration of CO*fucT2*. These two genes contained in a vector called pEGWTT(CO) along with the CO*fucT2* gene. The pEGWTT (CO) vector contains only three genes: the *gmd*, *wcaG*, and CO*fucT2*. CO*fucT2* has already been integrated into chromosome of *C. glutamicum*. Therefore, if the integration of *gmd* and *wcaG* is successful, the use of pEGWTT(CO) plasmid is not required.

So far, two vectors, pVTL which has tetracycline as a resistance marker and pEGWTT(CO) which has kanamycin as a resistance marker, were used to produce 2'-FL using two antibiotics. However, all the genes in pEGWTT(CO) are inserted on the chromosome, so that it is no longer necessary to introduce this vector and there is no need to use kanamycin if the integration is done.

As shown in Figure 25 and 26, *gmd* and *wcaG* were inserted to ΔISCg2bΔISCg2fΔISCg1a::fucT2(CO) strain. The *tac* promoter and terminator were also inserted with these two gene. As a result, ΔISCg2bΔISCg2fΔISCg1a::fucT2(CO)::GW was constructed. Then, only pVTL plasmid was transformed into this strain and ΔISCg2bΔISCg2fΔISCg1a::fucT2(CO)::GW Y is constructed. Batch fermentation is performed without using kanamycin and 0.84 g/L of 2'-FL was produced (Figure 27). Unlike expectation that production would be reduced if genes are integrated into the chromosome, the production of 2'-FL increased 1.4 times.

The problem of production system by expressing genes through

plasmids is segregatoinal stability. Introduction of a plasmid into a cell caused a metabolic burden for microorganisms (Bentley, Mirjalili et al. 1990). So, when a plasmid-free strain starts to grow during fermentation, it grows faster because it has no burden. Thus, strains not introduced with plasmids become dominant species. For this reason, production system through gene expression episomally is unstable rather than production system by inserting producing genes into the chromosomes. Therefore, by the introduction of 2'-FL producing genes into *C. glutamicum,* 2'-FL production increased.

If the genes which are present in the chromosome and the genes which are present in the plasmid are expressed simultaneously, they may be more stable and more productive. This is because 2'-FL producing enzymes increased by expressing genes in the chromosome and plasmids. Thus, pYTY which has *lacY* gene and pEGWTT(CO) which has *gmd*, *wcaG* and CO*fucT2* genes were transformed into the ΔISCg2bΔISCg2fΔISCg1a::fucT2(CO)::GW strain to construct ΔISCg2bΔISCg2fΔISCg1a::fucT2(CO)::GW GWTTY(CO) strain. Then, the batch fermentation of this strain was carried out without using antibiotics. As a result, 3.01 g / L of 2'-FL was produced (Figure 28). It has been found that simultaneous expression of the genes in plasmids and chromosomes is more effective since the 2'-FL producing enzymes are increased. In conclusion, high amounts of 2'-FL can be produced without the use of antibiotics.

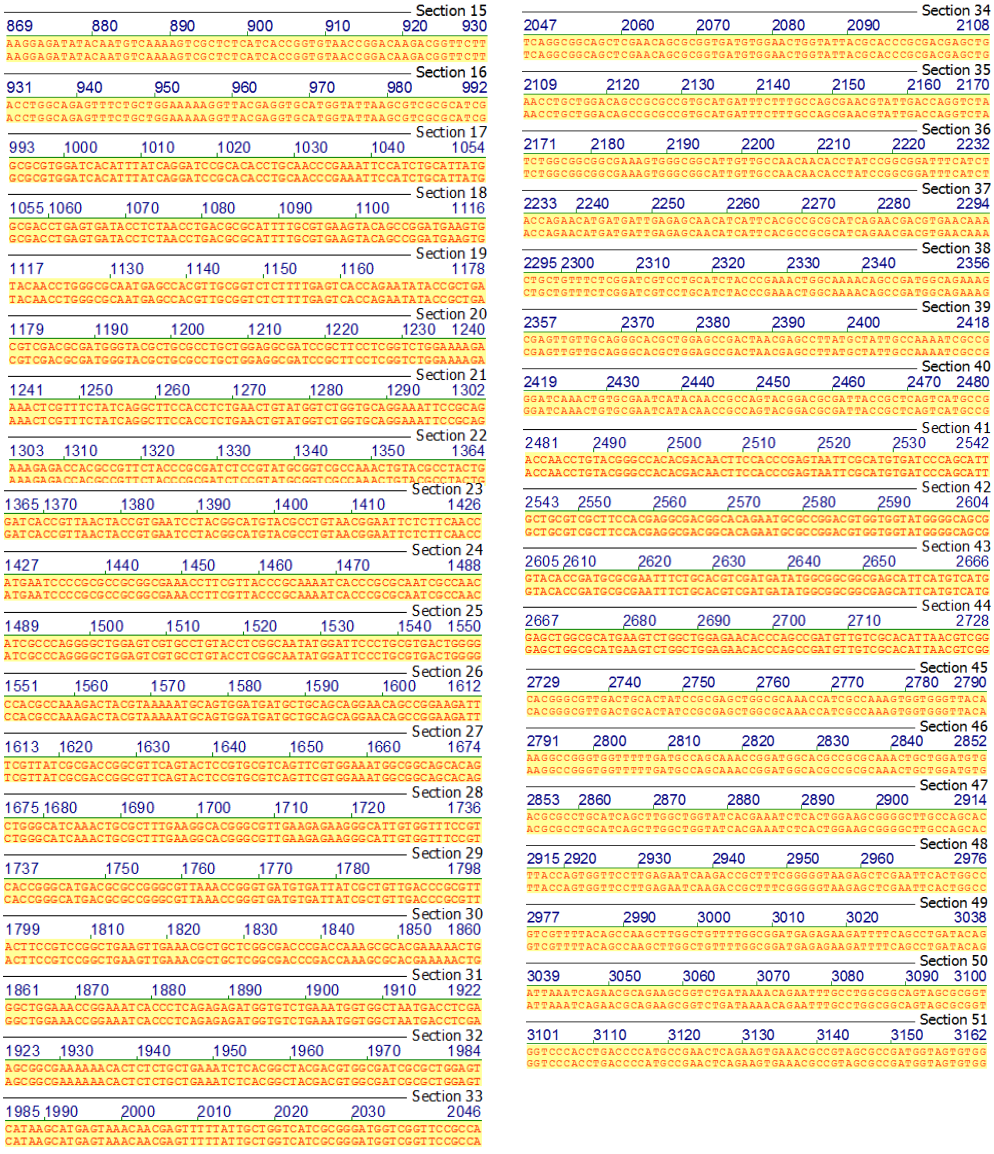
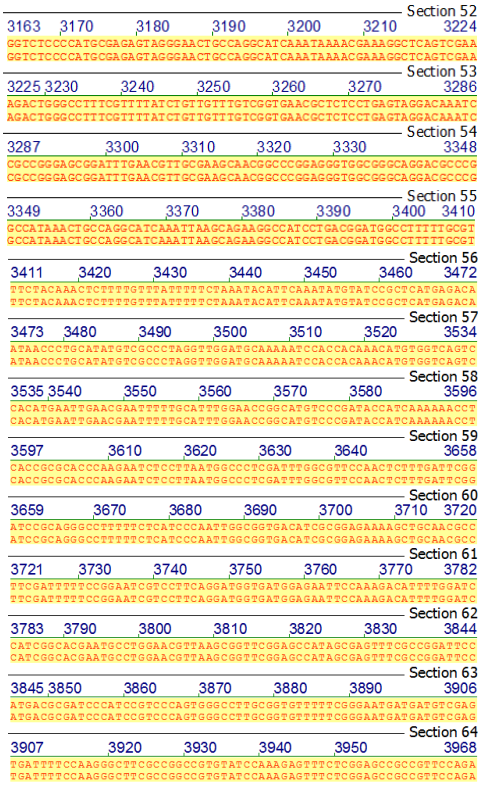


Figure 25. The sequences of inserted *gmd-wcaG*.


Section 52
3163 3170 3180 3190 3200 3210 3224
GGTCTCCCCATGCGAGAGTAGGGAACTGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAA
GGTCTCCCCATGCGAGAGTAGGGAACTGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAA
Section 53
3225 3230 3240 3250 3260 3270 3286
AGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCCTGAGTAGGACAAATC
AGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCCTGAGTAGGACAAATC
Section 54
3287 3300 3310 3320 3330 3348
CGCCGGGAGCGGATTTGAACGTTGCGAAGCAACGGCCCGGAGGGTGGCGGGCAGGACGCCCG
CGCCGGGAGCGGATTTGAACGTTGCGAAGCAACGGCCCGGAGGGTGGCGGGCAGGACGCCCG
Section 55
3349 3360 3370 3380 3390 3400 3410
GCCATAAACTGCCAGGCATCAAATTAAGCAGAAGGCCATCCTGACGGATGGCCTTTTTGCGT
GCCATAAACTGCCAGGCATCAAATTAAGCAGAAGGCCATCCTGACGGATGGCCTTTTTGCGT
Section 56
3411 3420 3430 3440 3450 3460 3472
TTCTACAAACTCTTTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACA
TTCTACAAACTCTTTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACA
Section 57
3473 3480 3490 3500 3510 3520 3534
ATAACCCTGCATATGTCGCCCTAGGTTGGATGCAAAAATCCACCACAAACATGTGGTCAGTC
ATAACCCTGCATATGTCGCCCTAGGTTGGATGCAAAAATCCACCACAAACATGTGGTCAGTC
Section 58
3535 3540 3550 3560 3570 3580 3596
CACATGAATTGAACGAATTTTTGCATTTGGAACCGGCATGTCCCGATACCATCAAAAAACCT
CACATGAATTGAACGAATTTTTGCATTTGGAACCGGCATGTCCCGATACCATCAAAAAACCT
Section 59
3597 3610 3620 3630 3640 3658
CACCGCGCACCCAAGAATCTCCTTAATGGCCCTCGATTTGGCGTTCCAACTCTTTGATTCGG
CACCGGCACCCAAGAATCTCCTTAATGGCCCTCGATTTGGCGTTCCAACTCTTTGATTCGG
Section 60
3659 3670 3680 3690 3700 3710 3720
ATCCGCAGGGCCTTTTTCTCATCCCAATTGGCGGTGACATCGCGGAGAAAAGCTGCAACGCC
ATCCGCAGGGCCTTTTTCTCATCCCAATTGGCGGTGACATCGCGGAGAAAAGCTGCAACGCC
Section 61
3721 3730 3740 3750 3760 3770 3782
TTCGATTTTTCCGGAATCGTCCTTCAGGATGGTGATGGAGAATTCCAAAGACATTTTGGATC
TTCGATTTTTCCGGAATCGTCCTTCAGGATGGTGATGGAGAATTCCAAAGACATTTTGGATC
Section 62
3783 3790 3800 3810 3820 3830 3844
CATCGGCACGAATGCCTGGAACGTTAAGCGGTTCGGAGCCATAGCGAGTTTCGCCGGATTCC
CATCGGCACGAATGCCTGGAACGTTAAGCGGTTCGGAGCCATAGCGAGTTTCGCCGGATTCC
Section 63
3845 3850 3860 3870 3880 3890 3906
ATGACGCGATCCCATCCGTCCCAGTGGGCCTTGCGGTGTTTTTCGGGAATGATGATGTCGAG
ATGACGCGATCCCATCCGTCCCAGTGGGCCTTGCGGTGTTTTTCGGGAATGATGATGTCGAG
Section 64
3907 3920 3930 3940 3950 3968
TGATTTTCCAAGGGCTTCGCCGGCCGTGTATCCAAAGAGTTTCTCGGAGCCGCCGTTCCAGA
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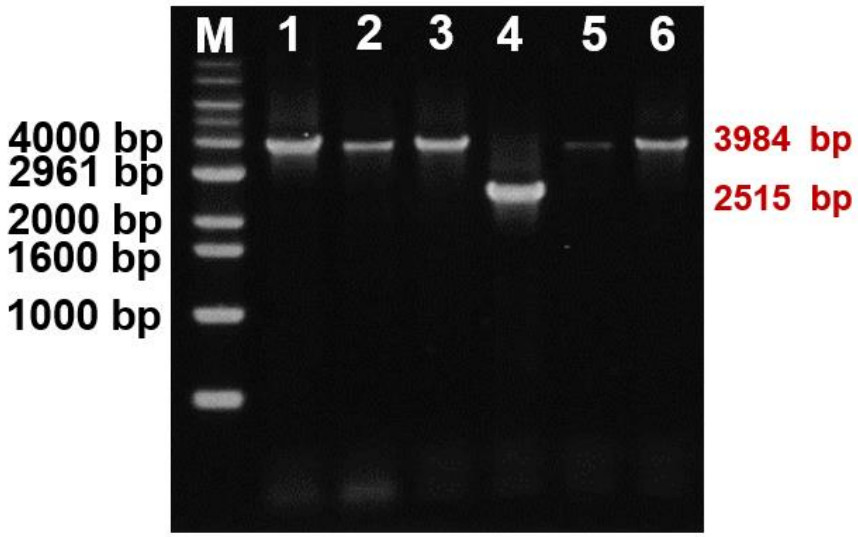

M: 1 kb ladder

1, 2, 3, 5, 6: inserted *gmd-wcaG*

4: wild-type

Figure 26. Confirmation of ΔISCg2bΔISCg2fΔISCg1a::fucT2(CO)::GW strain construction by colony PCR with primer pairs, F1_*Hind*Ⅲ_ ISCg2f(L) and R3_*Sal*I_ ISCg2f(R) (Table 4).

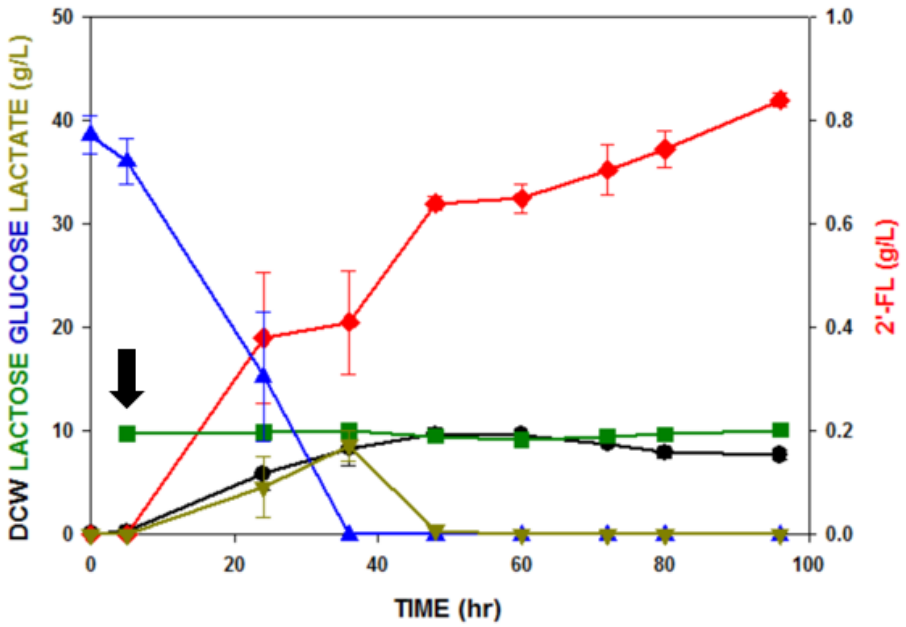

Figure 27. Flask fermentation of ΔISCg2bΔISCg2fΔISCg1a ::fucT2(CO)::GW Y. As OD_{600} reached 0.8, IPTG and lactose were added (thick arrow).

Symbols: ●, DCW; ▲, Glucose; ■, Lactose; ▼, Lactate; ◆, 2'-FL

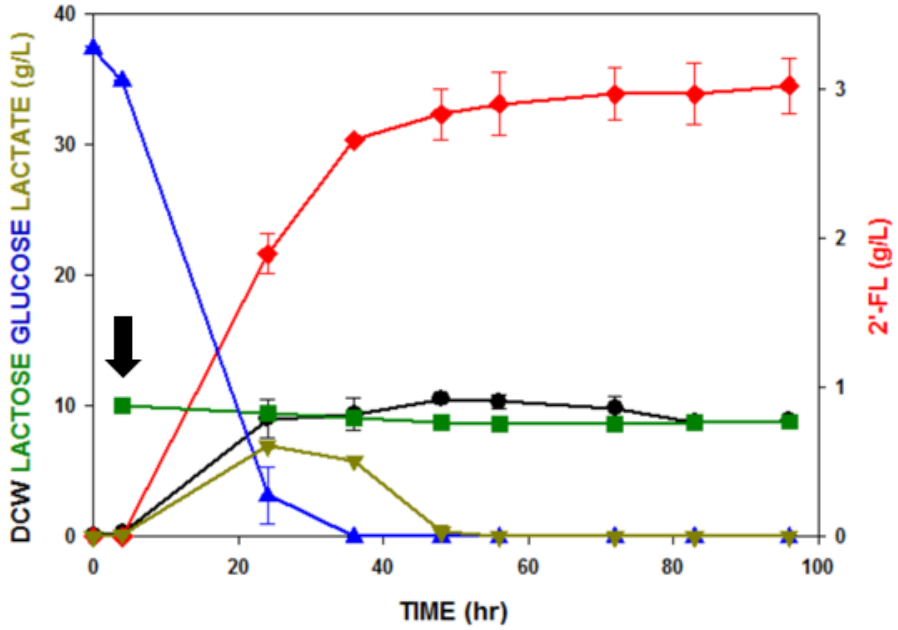

Figure 28. Flask fermentation of ΔISCg2bΔISCg2fΔISCg1a ::fucT2(CO)::GW GWTTY(CO) without using any antibiotics. As OD_{600} reached 0.8, IPTG and lactose were added (thick arrow).

Symbols: ●, DCW; ▲, Glucose; ■, Lactose; ▼, Lactate; ◆, 2'-FL

Table 8. Summary of flask fermentation of chromosomally engineered strains

Strains	Maximum dry cell weight (g/L)	Maximum 2'-FL concentration (g/L)	*Productivity (mg/L/h)
BCGWTTL(CO)	13.0	0.55	6.6
ΔISCg2b BCGWTTL(CO)	11.7	0.79	8.2
ΔISCg2b::fucT2(CO) BCGWL	13.1	0.35	3.6
ΔISCg2bΔISCg2f::fucT2(CO) BCGWL	12.4	0.42	4.4
ΔISCg2bΔISCg2fΔISCg1a::fucT2(CO) GWY	11.7	0.6	6.3
ΔISCg2bΔISCg2fΔISCg1a::fucT2(CO)::GW Y	9.9	0.84	8.8
ΔISCg2bΔISCg2fΔISCg1a::fucT2(CO)::GW GWTTY(CO) – No antibiotics	10.7	3.01	31.4

*2'-FL yield and productivity were calculated based on total fermentation time

3. Development of strains for trehalose reduction

C. glutamicum produces a variety of fermentation products, which in turn increases osmotic stress. In response to this stress, *C. glutamicum* produces a substance called trehalose(Eggeling and Bott 2005). Trehalose and lactose are the same in molecular weight, and the measurement is more difficult because two peaks are overlapped on HPLC measurement.

In fed-batch culture, trehalose is produced in a considerable amount. When the amount of trehalose was similar to that of lactose, two peaks is separated through HPCL (Figure 29). The amount is expected to be about 10 to 15 g/L. There is a need to reduce the amount of the substance. When a large amount of this substance is produced, the flux to 2'-FL is reduced. This is because glucose-6-phosphate which is used to produce 2'-FL can be used to produce trehalose. As shown in Figure 30, glucose-6-phosphate converts into α(1-4)glucans and trehalose is synthesized by TreYZ pathway. In addition, by condensation of glucose-6-phosphate and UDP-glucose, trehalose-6-phosphate is formed by OtsA. Then, trehalose is synthesized by the action of OtsB. Therefore, in order to reduce this substance, several genes involved in the production of trehalose were deleted.

Trehalose is known to be produced by the following three pathways: treYZ pathway, otsAB pathway and treS pathway(Tzvetkov, Klopprogge et al. 2003). The treS pathway is a pathway producing trehalose when malotose is used as a carbon source. However, since glucose is used as a carbon source in this study, trehalose production to this pathway could be neglected. Instead, *otsA* and *treY* genes were

deleted to inhibit the production of trehalose through otsAB pathway and treYZ pathway (Figure 30).

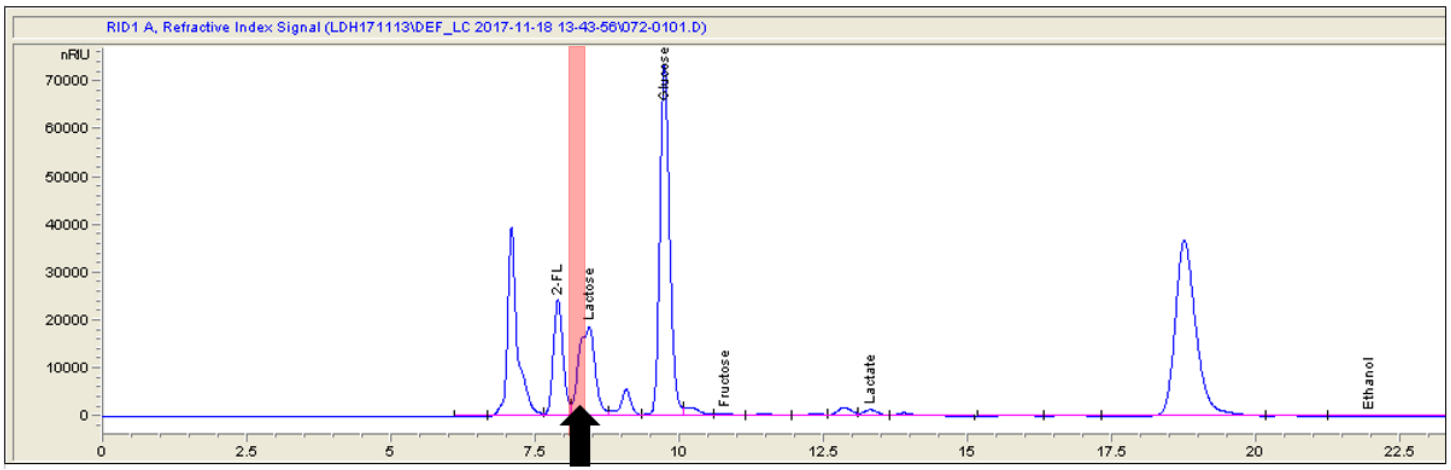

Figure 29. Fed-batch fermentation HPLC profile of GWTTY(CO) strain at 100-hours.

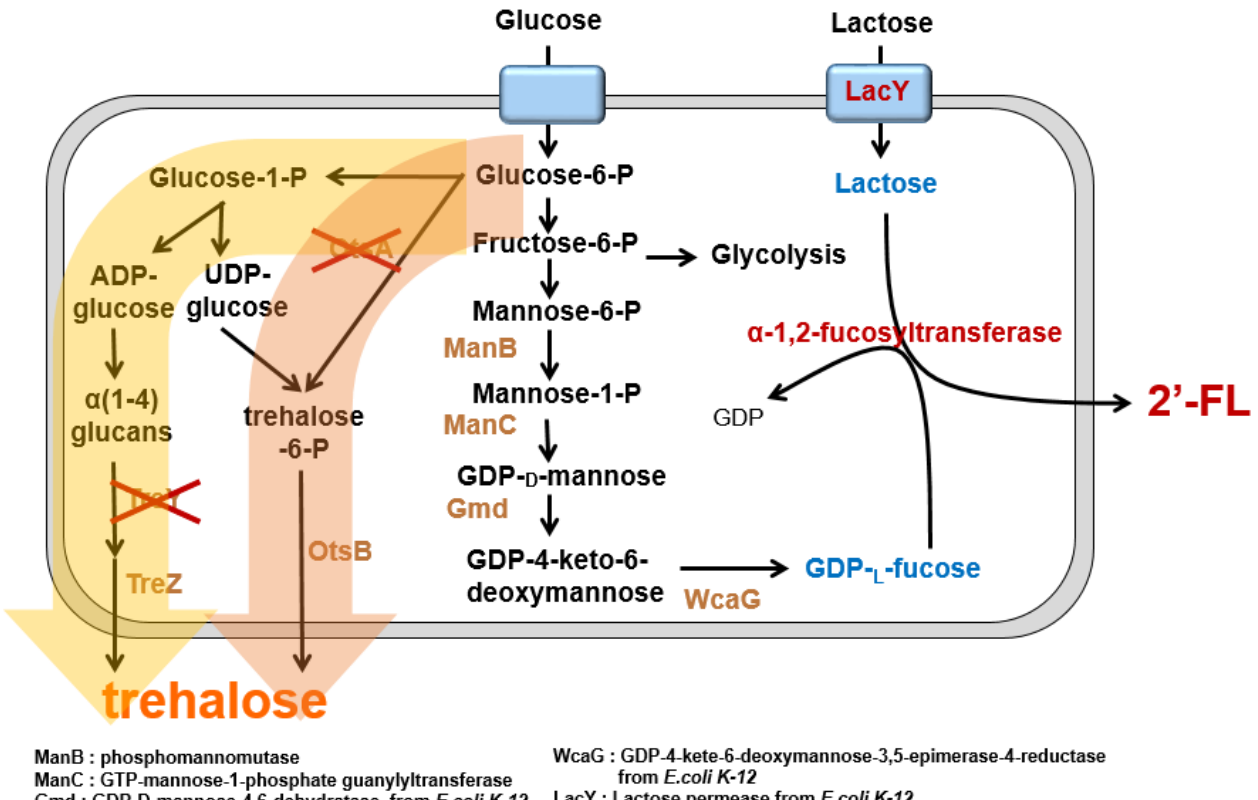

Figure 30. Trehalose synthesizing pathway and strategy for trehalose reduction.

3.1. Construction of *otsA* knock-out strain

The *otsA* gene is involved in the formation of trehalose by the otsAB pathway. The OtsA enzyme converts glucose-6-phosphate into trehalose-6-phosphte(Padilla, Krämer et al. 2004). This gene was removed from chromosome of *C. glutmicum* to construct Δ*otsA* strain by double crossover method (Figure 31). Then, pVTY and pEGWTT (CO) were transformed into this strain and Δ*otsA* GWTTY(CO) was constructed. Batch fermentation is carried out by this strain and 1.93 g/L of 2'-FL was produced (Fig. 32, Table 9).

3.2. Construction of *treY* knock-out strain

The *treY* gene is involved in the formation of trehalose by the treYZ pathway. The enzyme TreY leads to glycosyl-trehalose formation from glycogen-like molecules(Padilla, Krämer et al. 2004). This gene was removed from chromosome of *C. glutmicum* to construct Δ*treY* strain by double crossover method (Figure 31). Then, pVTY and pEGWTT (CO) were transformed into this strain and Δ*treY* GWTTY(CO) was constructed. Batch fermentation is performed by this strain and 1.81 g/L of 2'-FL was produced (Fig. 32, Table 9).

The effect of reducing trehalose by removing *otsA* and *treY* gene can not be judged by these results. The amount of trehalose should be measured at the fermenter level or measured through the trehalose measurement kit. In conclusion, strains without these two genes were constructed separately, and the results of each strain show that the

deletion of two genes does not significantly change the amount of 2'-FL produced. After this study, deletion of both genes or deletion of another gene involved in trehalose production could be attempted.

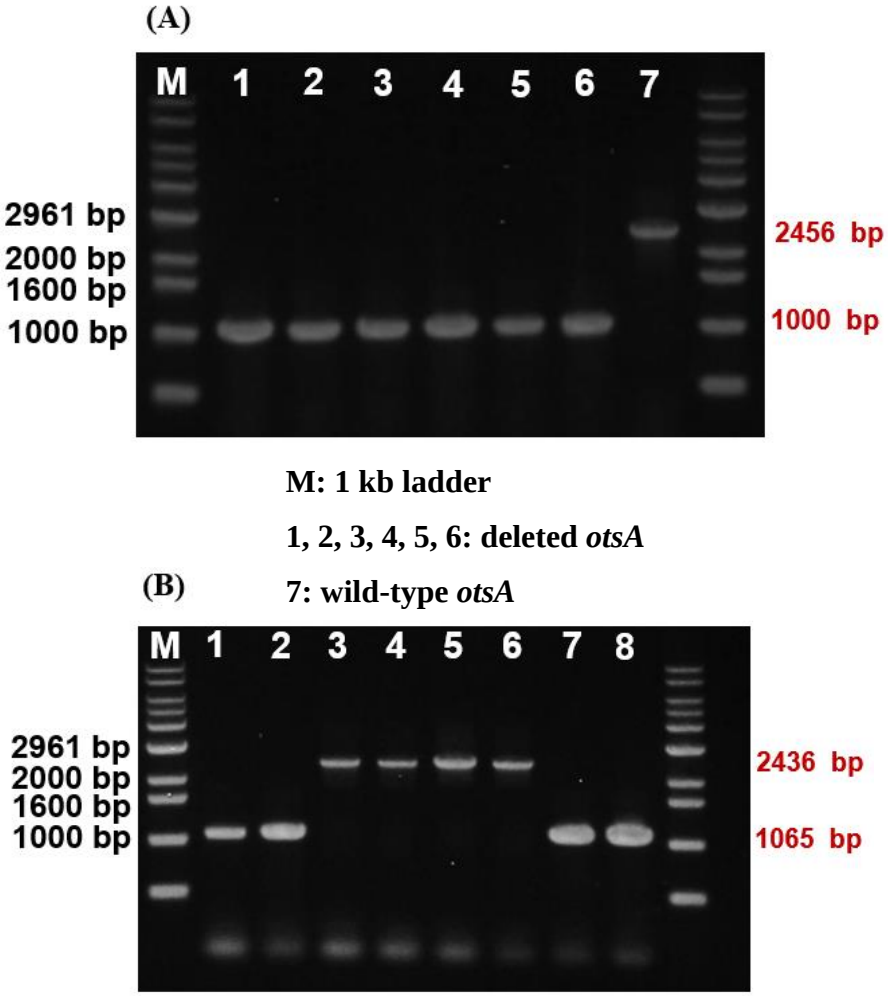

Figure 31. Confirmation of (A) Δ*otsA* and (B) Δ*treY* strain construction by colony PCR with primer pairs, (A) F1_inf_*Hind*Ⅲ_otsA(L)/R2_inf_*Pts*I_otsA(R) and (B) F1_*Bam*HI_treY_dis/R2_*Eco*RI_treY_dis (Table 4).

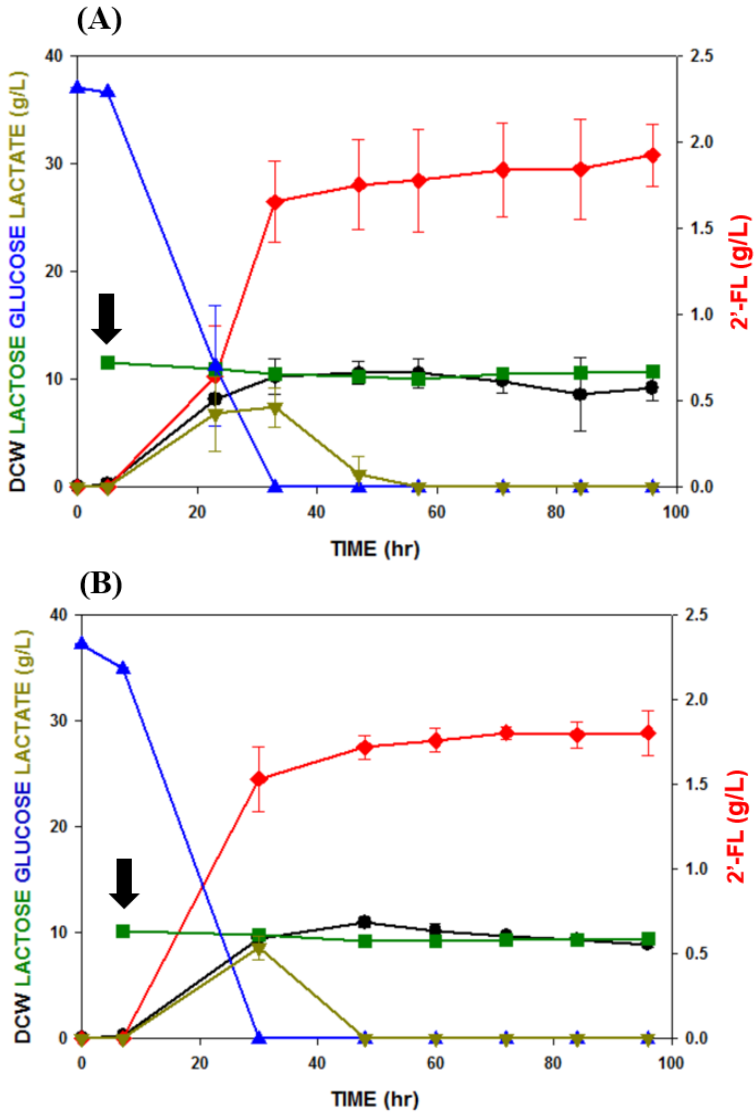

Figure 32. Flask fermentation of (A) Δ*otsA* GWTTY(CO) and (B) Δ*treY* GWTTY(CO). As OD_{600} reached 0.8, IPTG and lactose were added (thick arrow).

Symbols: ●, DCW; ▲, Glucose; ■, Lactose; ▼, Lactate; ◆, 2'-FL

Table 9. Summary of flask fermentation of GWTTY(CO), Δ*otsA* GWTTY(CO) and Δ*treY* GWTTY(CO)

Strains	Maximum dry cell weight (g/L)	Maximum 2'-FL concentration (g/L)	*Productivity (mg/L/h)
GWTTY(CO)	11.7	1.94	20.2
Δ*otsA* GWTTY(CO)	10.7	1.93	20.1
Δ*treY* GWTTY(CO)	11.0	1.81	18.9

*2'-FL yield and productivity were calculated based on total fermentation time.

IV. CONCLUSIONS

This thesis can draw the following conclusions:

(1) Corynebacterium glutamicum engineered to express the lactose permease gene under the tac promoter with RBS was able to produce 1.94 g/L of 2'-FL in batch fermentation, and 25.5 g/L in fed-batch fermentation, corresponding to 3.5 times improvement in batch fermentation and 2.2 times enhancement in fed-batch fermentation compared to the control strain BCGWTTL(CO).

(2) The engineered C. glutamicum strain by integrating COfucT2, gmd and wcaG into the IS element site of chromosome produced 0.84 g/L of 2'-FL in batch fermentation. By combining the chromosomally-integrated and vector expression systems, 3.01 g/L of 2'-FL was produced in batch fermentation, corresponding to 5.5 times improvement compared to the control strain BCGWTTL(CO).

(3) The genes involved in trehalose production were deleted. Thus, the Δ*otsA* GWTTY(CO) and Δ*treY* GWTTY(CO) was obtained. These strains produced 1.93 g / L and 1.81 g / L of 2'-FL,

respectively. Thus, it has been found that the deletion of these two genes separately does not have a critical effect on the production of 2'-FL.

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국 문 초 록

우유와 비교하였을 때 모유를 차별화하는 성분은 올리고당이다. 모유올리고당은 50~ 80%가 푸코실화 되어있다. 푸코실올리고당 중 2'-fucoyllactose (2'-FL)는 가장 많은 함량을 차지한다. 2'-FL 은 장내 유익균의 생육을 촉진하는 프리바이오틱 효과, 병원성 균의 성장 억제 및 면역력 증대, 두뇌 발달 등의 우수한 기능성을 가졌기 때문에 유아용 분유, 건강식품, 의약품 및 화장품 소재로 각광받고 있다.

선행연구에서는 GRAS (Generally Recognized As Safe)라는 장점을 가진 *Corynebacterium glutamicum* (*C. glutamicum*)을 이용하여 2'-FL 을 생산하였다. 2'-FL 은 lactose 와 GDP-L-fucose 가 α-1,2 결합으로 푸코실화 됨으로써 생성된다. 하지만 *C. glutamicum* 은 GDP-L-fucose 를 생합성하는 유전자가 존재하지 않고, lactose 를 소모하지 못한다. 따라서 *C. glutamicum* 에 GDP-L-fucose 생합성 경로를 도입하였고, *lacYA* 오페론을 도입하여 lactose 를 세포 내로 수송하고자 하였으며, 헬리코박터 파일로리 유래의 α-1,2 fucosyltransferase (*fucT2*)를 코돈 최적화하여 도입함으로써 α-1,2 fucosylation 을 도모하였다. 구축된 균주로 회분식 발효와 유가식 발효를 진행한 결과 각각 0.6 g/L 와 11.5 g/L 의 2'-FL 을 생산할 수 있었다.

본 연구에서는 대사공학적인 설계와 염색체 조작을 통해 코리네박테리움 글루타미쿰을 구축함으로써 2'-

푸코실락토오스의 생산성을 높이고자 하였다. 먼저, 다음의 세가지 전략을 통하여 2'-FL 생산성을 증대시키고자 하였다. 첫째, 불필요하게 유전자가 과발현되지 않도록 하였다. 2'-FL 생산하는데 사용되는 유전자들 중 *C. glutamicum* 의 염색체(chromosome)에 존재하는 유전자인 Phosphomannomutase (*manB*)와 GTP-mannose-1-phosphate guanylyltransferase (*manC*)를 발현벡터에서 삭제하여 회분식 발효를 진행하였고, 그 결과 0.62 g/L 의 2'-FL 이 생산됨을 확인하였다. 둘째, *lacYA* 오페론에서 *lacA* 를 발현벡터 상에서 제거하여, lactose 의 이용효율을 향상시키고자 하였으며, 이를 회분식 발효한 결과 0.93 g/L 의 2-FL 이 생산되었다. 셋째, lactose permease 를 RBS(Ribosome Binding Site)와 강력한 promoter 하에서 발현되게 함으로써 세포 내로 더 많은 lactose 가 수송되게 하였다. 위 세가지 전략이 모두 도입된 최종 균주로 발효를 진행한 결과 회분식 발효에서는 1.94 g/L, 유가식 발효에서는 25.5 g/L 의 2'-FL 을 생산할 수 있었고, 이는 이전 선행연구에서의 균주 대비 회분식 발효에서 3.3 배, 유가식 발효에서 약 2 배 증대된 결과이다.

다음으로, 항생제를 사용하지 않고 2'-FL 을 생산하는 시스템을 구축하였다. 2'-FL 은 식품이나 의약품 등 인체에 직접적인 영향을 미치는 분야에 사용되기 때문에 항생제가 없는 조건에서 생산된다면 소비자들의 인식을 향상 시킬 수 있을 뿐만 아니라 항생제의 분리·정제비용을 절약할 수 있다.

따라서, 2'-FL 을 생산하는데 필요한 유전자를 염색체상에 삽입함으로써 항생제를 사용하지 않고도 안정적으로 발효산물을 생산하는 시스템을 구축하고자 하였다. 염색체 조작은 pK19mobsacB 벡터를 이용한 double crossover 방법을 사용하였고, 유전자를 삽입할 자리로 IS (Insertion Sequence) element 를 선정하였다. 이에 따라 IS element 중 하나인 IS*Cg2b* 가 제거된 자리에 CO*fucT2* 를 삽입하여 *C. glutamicum* 의 염색체상에 유전자가 도입될 수 있다는 것을 밝혔다. 이어서 추가적으로 IS*Cg2f* 와 IS*Cg1a* 를 더 파쇄하여 2'-FL 생산 관련 유전자를 계속해서 도입할 수 있는 자리를 마련하였고, 그 중 한 자리인 IS*Cg2f* 에 *gmd* 와 *wcaG* 를 삽입하였다. 그 결과 회분식 발효에서 0.84 g/L 의 2'-FL 이 생성되었으며 kanamycin 항생제를 사용하지 않고도 2'-FL 이 안정적으로 생산되는 시스템이 구축되었다. 더 나아가 염색체상에 유전자가 도입된 최종 균주에 플라스미드를 도입하여 동시에 유전자를 발현시켰고 그 결과 항생제가 없는 조건에서도 3.01 g/L 의 2'-FL 을 생산하였다. 이로써 항생제를 사용하지 않고도 2'-FL 을 생산하는 시스템을 구축하였다.

본 연구에서는 코리네박테리움 글루타미쿰을 통하여 고농도의 2'-FL 을 생산하는 시스템과 항생제를 저감화하여 생산하는 시스템을 구축하였다. 이는 향후 코리네박테리움 글루타미쿰을 통하여 산업적으로 2'-FL 을 생산할 때 이점을 가지기 위한 밑바탕이 될 것으로 기대된다.

주요어: 대사공학, 2'-푸코실락토오스, GDP-L-fucose, lactose permease, pK19mobsacB, double crossover method, 유가식 발효, 코리네박테리움 글루타미쿰

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