Appl Microbiol Biotechnol (2012) 93:2327–2334
DOI 10.1007/s00253-011-3776-3

Modulation of guanosine nucleotides biosynthetic pathways enhanced GDP-L-fucose production in recombinant *Escherichia coli*

Won-Heong Lee · So-Yeon Shin · Myoung-Dong Kim · Nam Soo Han · Jin-Ho Seo

Received: 9 September 2011 / Revised: 9 November 2011 / Accepted: 21 November 2011 / Published online: 13 December 2011

Abstract Guanosine 5′-triphosphate (GTP) is the key substrate for biosynthesis of guanosine 5′-diphosphate (GDP)-L-fucose. In this study, improvement of GDP-L-fucose production was attempted by manipulating the biosynthetic pathway for guanosine nucleotides in recombinant *Escherichia coli*-producing GDP-L-fucose. The effects of overexpression of inosine 5′-monophosphate (IMP) dehydrogenase, guanosine 5′-monophosphate (GMP) synthetase (GuaB and GuaA), GMP reductase (GuaC) and guanosine–inosine kinase (Gsk) on GDP-L-fucose production were investigated in a series of fed-batch fermentations. Among the enzymes tested, overexpression of Gsk led to a significant improvement of GDP-L-fucose production. Maximum GDP-L-fucose concentration of 305.5±5.3 mg l^{-1} was obtained in the pH-stat fed-batch fermentation of recombinant *E. coli*-overexpressing Gsk, which corresponds to a 58% enhancement in the GDP-L-fucose production compared with the control strain overexpressing GDP-L-fucose biosynthetic enzymes. Such an enhancement of GDP-L-fucose production could be due to the increase in the intracellular level of GMP.

Keyword Recombinant *Escherichia coli* · GDP-L-fucose · Guanosine nucleotides · Guanosine–inosine kinase · pH-stat fed-batch fermentation

W.-H. Lee · S.-Y. Shin · J.-H. Seo (✉)
Department of Agricultural Biotechnology and Center for Agricultural Biomaterials, Seoul National University, Seoul 151-921, South Korea
e-mail: jhseo94@snu.ac.kr

M.-D. Kim
School of Biotechnology and Bioengineering, Kangwon National University, Chuncheon 200-701, South Korea

M.-D. Kim
Institute of Bioscience and Biotechnology, Kangwon National University, Chuncheon 200-701, South Korea

N. S. Han (✉)
Department of Food Science and Technology, Chungbuk National University, Cheongju 361-763, South Korea
e-mail: namsoo@cbnu.ac.kr

Introduction

GDP-L-fucose is an essential substrate for the biosynthesis of fucosyloligosaccharide, which is a common human milk oligosaccharide and acts as a bioactive agent involved in a wide range of biological functions such as growth promotion of probiotic bacteria, inhibition of pathogens infection and brain development (Bode 2006). GDP-L-fucose can be synthesized in *Escherichia coli* as it is used as a substrate for biosynthesis of extracellular polysaccharide (EPS) (Stevenson et al. 1996). The metabolic pathway for biosynthesis of GDP-L-fucose in *E. coli* is illustrated in Fig. 1. Overexpression of GDP-L-fucose pathway from mannose 6-phosphate is required for efficient production of GDP-L-fucose, indicating that sufficient supply of guanosine 5′-triphosphate (GTP) as well as the enhanced carbon flux from fructose 6-phosphate to GDP-D-mannose should be achieved. Guanosine nucloetides such as guanosine 5′-monophosphate (GMP), guanosine 5′-diphosphate (GDP) and GTP are indispensable materials for cell growth and biosynthesis of DNA and RNA. Guanosine nucleotides are end-products of purine nucleotide biosynthesis and they interconvert with each other through several reactions inside the cell (Mantsala and Zalkin 1992; Teshiba and Furuya 1989). Especially, GMP acts

as a precursor for synthesis of GDP and GTP. For efficient GDP-L-fucose production, it might be necessary to increase the intracellular levels of guanosine nucleotides including GMP.

Mutant strains of *Corynebacterium* have been mainly used for industrial-scale production of purine nucleotides and their derivatives. These strains have been derived via several chemical–UV mutagenesis and selection of a mutant showing higher resistance to the analogs of purines such as 8-azaguanine (Teshiba and Furuya 1989). These mutant strains are also known to be powerful in producing guanosine nucleotides (Furuya et al. 1973; Teshiba and Furuya 1989). Hence, *Corynebacterium* mutants have been used for GDP-L-fucose production as a source of GTP supply. Production of GDP-L-fucose by the combination of the recombinant *E. coli* strains and *C. ammoniagenes* mutant was reported elsewhere (Koizumi et al. 2000). However, this method requires complicated processes such as separated cultivation and mixing of the two bacteria and additional permeabilization of cell membrane.

In *E. coli*, guanosine nucleotides can be synthesized from phosphoribosylpyrophosphate (PRPP), which is called the de novo pathway, by several steps. They can also be synthesized from exogenous guanosine or guanine, which is called the salvage pathway (Fig. 1) (Nijkamp and De Haan 1967; Petersen 1999). In the de novo pathway, PRPP (from ribose 5-phosphate) is converted to inosine 5′-monophosphate (IMP) through 11 serial reactions. Then, IMP is converted to GMP by IMP dehydrogenase (GuaB, E.C. 1.1.1.205) and GMP synthetase (GuaA, E.C. 6.3.5.2). GMP is further phosphorylated to GDP and GTP by other enzymes. GMP is converted to IMP by GMP reductase (GuaC, E.C. 1.7.1.7). In the salvage pathway, guanosine is directly phosphorylated to GMP by guanosine–inosine kinase (Gsk, E.C. 2.7.1.73) or indirectly converted to GMP through serial phosphorolysis and phosphorylation catalyzed by purine nucleoside phosphorylase (DeoD, E.C. 2.4.2.1) and guanine phosphoribosyltransferase (Gpt, E.C. 2.4.2.22), respectively.

As shown in Fig. 1, sufficient supply of GMP can be expected to increase the levels of intracellular guanosine nucleotides, which may lead to the efficient GDP-L-fucose production. For sufficient supply of GMP, amplification of metabolic flux from guanosine to GMP as well as flux from IMP to GMP should be achieved.

Hence, this study was undertaken to improve the production of GDP-L-fucose in recombinant *E. coli* by increasing the intracellular levels of guanosine nucleotides including GMP. Several metabolic enzymes such as GuaA and GuaB, GuaC and Gsk involved in the biosynthesis of GMP were overexpressed in recombinant *E. coli* able to produce GDP-L-fucose. The effects of overexpression of those enzymes on GDP-L-fucose production in recombinant *E. coli* were investigated in a series of pH-stat fed-batch fermentations.

Materials and methods

Strains and plasmids

E. coli TOP10 [F-*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G] and BL21star (DE3) [F^-, *omp*T, *hsd*SB($r_B^-m_B^-$), *gal*, *dcm rne131*(DE3)] (Invitrogen, Carlsbad, CA, USA) were used for genetic manipulation and GDP-L-fucose production, respectively. Plasmid pmBCGW for overexpression of ManB, ManC, Gmd and WcaG was previously constructed using plasmid pETDuet-1 (Lee et al. 2009). The genes encoding GuaA,

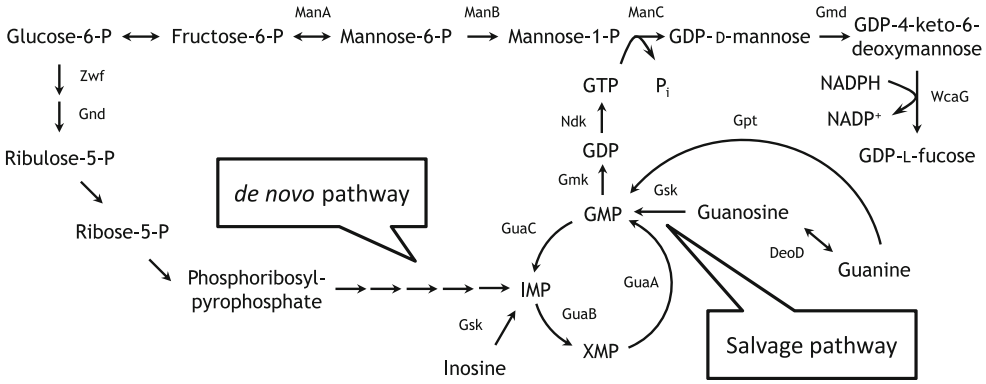

Fig. 1 The metabolic pathway for GDP-L-fucose and guanosine nucleotides biosynthesis in *E. coli*. The names of enzymes are abbreviated as follows: ManA mannose-6-phosphate isomerase, ManB phosphomannomutase, ManC mannose-1-phosphate guanylyltransferase, Gmd GDP-D-mannose-4,6-dehydratase, WcaG GDP-4-keto-6-deoxymannose 3,5-epimerase 4-reductase, Zwf glucose-6-phosphate dehydrogenase, Gnd 6-phosphogluconate dehydrogenase, GuaB IMP dehydrogenase, GuaA GMP synthetase, GuaC GMP reductase, Gsk guanosine–inosine kinase, DeoD purine nucleotide phosphorylase, Gpt guanine phosphoribosyltransferase, Gmk guanylate kinase, Ndk nucleotide diphosphate kinase. P_i and XMP denote phosphate and xanthosine 5′-monophosphate

GuaB, GuaC and Gsk were obtained by polymerase chain reactions (PCR) from the genomic DNA of the *E. coli* K12 strain (ATCC 10798). Two primers of guaBA_F and guaBA_R were used for the amplification of the *guaA–guaB* gene cluster. After digestion of the PCR fragment containing *guaA–guaB* gene cluster with *Nde*I and *Xho*I, the DNA fragment was cloned into pACYCDuet-1, and plasmid pHguaBA was constructed. Other primers, guaC_F and guaC_R, were used for amplifying the *guaC* gene, and gsk_F and gsk_R were used for *gsk* gene. The amplified *guaC* and *gsk* genes were digested with *Nco*I and *Pst*I, respectively, and cloned into plasmid pACYCDuet-1. The resulting plasmids were labeled as pHguaC and pHgsk, respectively. Plasmids and primers used in this work are listed in Table 1. All constructed plasmids were subjected to DNA sequencing. PCR reactions, general DNA manipulations and bacterial transformations were done as described previously (Byun et al. 2007).

Culture conditions

Luria-Bertain (LB) medium (0.5% (w/v) yeast extract, 1% (w/v) tryptone, and 1% (w/v) NaCl) with appropriate concentrations of antibiotics was used for plasmid preparation and seed cultivation. Fed-batch fermentation was performed in a 2.5-l bioreactor containing 1.0 l of a defined medium at 25 °C (Lee et al. 2011). After complete depletion of 2% (w/v) glucose added initially, concentrated solution containing 800 $g l^{-1}$ glucose and 20 $g l^{-1}$ $MgSO_4 7H_2O$ was fed by the pH-stat feeding mode. For pH-stat feeding, appropriate amount of the concentrated solution was fed automatically into the bioreactor when the pH rose to a value higher than its set-point due to the depletion of glucose. When dry cell mass reached approximately 35 $g l^{-1}$, 0.1 mM IPTG was added to induce the *T7* promoter-mediated gene expression. Agitation speed was increased to a maximum value of 1,400 rpm in order to prevent the limitation of dissolved oxygen, and air flow rate was maintained at 1 vvm throughout the cultivation. The pH was controlled at 6.8 by 28% NH_4OH.

Analytical methods

Dry cell mass was determined from measuring optical density at 600 nm (Ultrospec 2000, Amersham Pharmacia Biotech, Piscataway, NJ, USA) using a predetermined curve. Concentrations of glucose and acetate were determined by using an M930 high performance liquid chromatography (HPLC) system (Younglin, Seoul, Korea) equipped with an Aminex HPX-87H cation exchange column (Bio-Rad, Richmond, CA, USA) and a RI detector (Knauer, Germany) as described previously (Byun et al. 2007).

In order to measure intracellular concentration of GDP-L-fucose, cells resuspended in extraction buffer (containing 50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol and 5 mM EDTA) were disrupted by an ultrasonic processor (Cole-Parmer, Vernon Hills, IL, USA). After boiling for 1 min and subsequent centrifugation at 15,000×*g* for 20 min, the supernatant was collected and used for determination of intracellular GDP-L-fucose concentration. The intracellular concentration of GDP-L-fucose was determined using an HPLC system equipped with a CAPCELL PAK C18 MG column (250×4.6 mm, Shiseido, Tokyo, Japan) according to the method described previously (Lee et al. 2011).

Overexpression of GDP-L-fucose biosynthetic enzymes and guanosine nucleotide biosynthetic enzymes inside the cell

Table 1 List of primers and plasmids used in this study

Name	Sequence[a] of PCR primers and description for plasmids	Source
PCR primers		
guaBA_F (*Nde*I)	5′-GGAATTCCATATGCTACGTATCGCTAAAGAA-3′	
guaBA_R (*Xho*I)	5′-CCGCTCGAGTCATTCCCACTCAATGGTAGC-3′	
guaC_F (*Nco*I)	5′-ACATGCCATGGGCATGCGTATTGAAGAAGATCTG-3′	
guaC_R (*Pst*I)	5′-AAAACTGCAGTTACAGGTTGTTGAAGATGCG-3′	
gsk_F (*Nco*I)	5′-ACATGCCATGGGCATGAAATTTCCCGGTAAACGT-3′	
gsk_R (*Pst*I)	5′-AAAACTGCAGTTAACGATCCCAGTAAGACTC-3′	
Plasmids		
pETDuet-1	Two *T7* promoters with two MCS, pBR322 replicon (copy number ~40), Amp^r	Novagen
pACYCDuet-1	Two *T7* promoters with two MCS, p15A replicon (copy number 10–12), Cm^r	Novagen
pmBCGW	pETDuet-1 + *gmd–wcaG* (*Nde*I/*Xho*I) + *manB–manC* (*Nco*I/*Sac*I), Amp^r	Lee et al. (2009)
pHguaBA	pACYCDuet-1 + *guaA–guaB* (*Nde*I/*Xho*I), Cm^r	This study
pHguaC	pACYCDuet-1 + *guaC* (*Nco*I/*Pst*I), Cm^r	This study
pHgsk	pACYCDuet-1 + *gsk* (*Nco*I/*Pst*I), Cm^r	This study

[a] The underlined sequences indicate the recognition sites of the restriction enzymes

was analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12% polyacrylamide) according to the same method previously described (Lee et al. 2009). The procedure for measuring intracellular concentrations of guanosine nucleotides was as follows. Cells were grown to 0.35 gl^{-1} in a LB medium and expression of guanosine nucleotide biosynthetic enzyme was induced by 0.1 mM IPTG. After 9 h, the cells were harvested and the supernatant was obtained by the same method for extraction of GDP-L-fucose. To quantify the intracellular concentrations of guanosine nucleotides, the supernatant was injected into HPLC equipped with the Partisil 10 SAX anion exchange column (250×4.6 mm, Whatman, Clifton, NJ, USA) according to the method described elsewhere (Sigal et al. 2003). A column was eluted at a flow rate of 1.5 ml min^{-1} by the following gradient program: 100% (v/v) eluent A (7 mM KH_2PO_4 at pH 4.0) and 0% eluent B (250 mM KH_2PO_4 and 500 mM KCl at pH 4.5) for 15 min; 0% to 100% eluent B over 45 min; 100% eluent B over 40 min. Guanosine nucleotides were monitored by absorbance at 254 nm.

Results

pH-stat fed-batch fermentations of recombinant *E. coli* overexpressing guanosine nucleotides biosynthetic enzymes

Previously, an improvement of GDP-L-fucose production in recombinant *E. coli*-expressing Gmd and WcaG proteins was obtained by overexpression of GDP-D-mannose biosynthetic enzymes, ManB and ManC. For efficient GDP-D-mannose supply (Lee et al. 2009), however, sufficient GTP supply as well as the enhanced carbon flux from glucose to GDP-D-mannose are needed.

To increase intracellular GTP supply, systems for overexpression of the enzymes involved in guanosine nucleotides biosynthesis were constructed and the effects of additional overexpression of those enzymes were investigated in the fed-batch fermentations. Fed-batch fermentations of two recombinant *E. coli* strains overexpressing GuaB–GuaA and GuaC involved in the de novo guanosine nucleotide biosynthesis were carried out in synthetic medium using glucose as the sole carbon source. The results of the control strain, *E. coli* BL21star(DE3) overexpressing ManB, ManC, Gmd and WcaG only, were reputed in a previous study (Lee et al. 2011). Figure 2a shows the profile of fed-batch fermentation of *E. coli* BL21star(DE3) overexpressing GuaB and GuaA. After 0.1 mM IPTG induction, cell growth was maintained to reach 100.0±0.6 gl^{-1} at the end of fermentation, which was 20% higher than the control strain. Along with cell growth, GDP-L-fucose increased continuously. As a result, 261.7±0.6 mg l^{-1} of GDP-L-fucose was

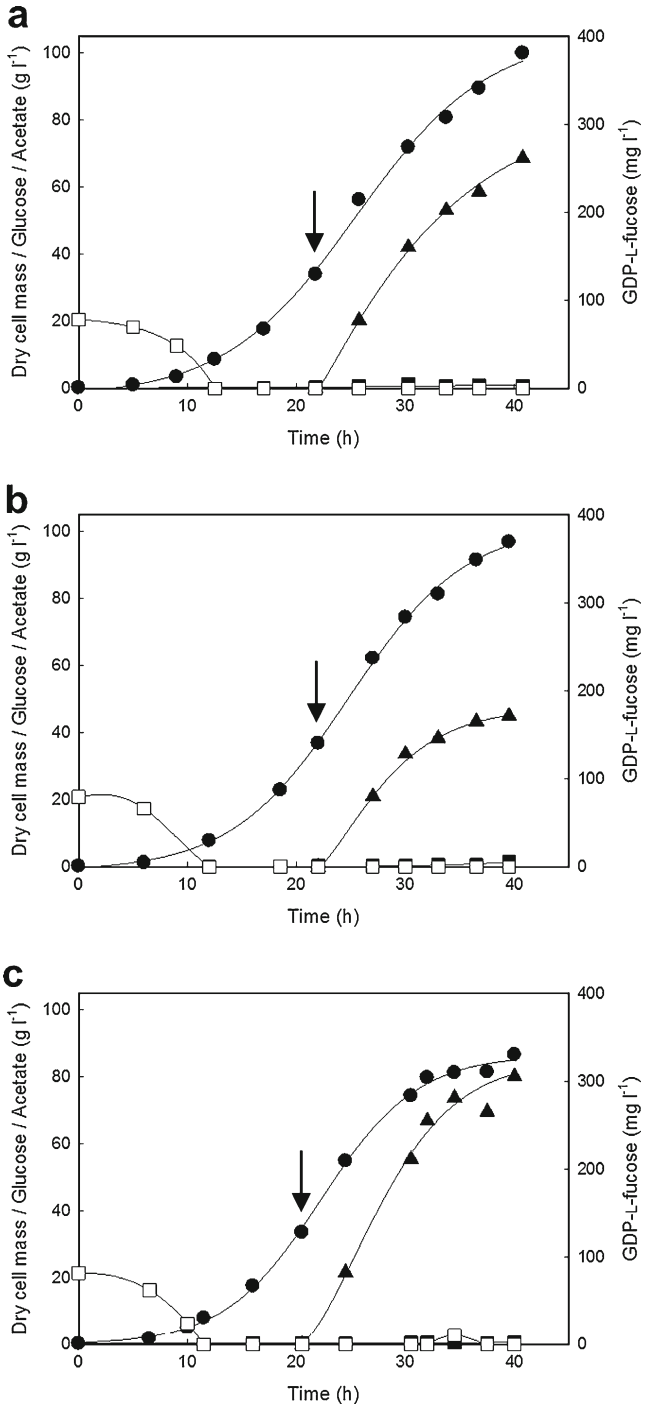

Fig. 2 Profiles of GDP-L-fucose production in the pH-stat fed-batch fermentations of recombinant *E. coli* BL21star(DE3) strains harboring plasmid pmBCGW + pHguaBA (**a**), pmBCGW + pHguaC (**b**) and pmBCGW + pHgsk (**c**). *Arrow* indicates the addition of 0.1 mM IPTG. *Filled circle* denotes dry cell mass, *filled triangle* denotes GDP-L-fucose concentration, *unfilled square* denotes glucose concentration, *filled square* denotes acetate concentration. All data in the figures are averages from three measurements of harvested samples

obtained with 2.6±0.01 mg g $cell^{-1}$ of specific GDP-L-fucose content, which corresponded to a 35% enhancement in GDP-L-fucose production compared with the control

strain. Contrary to the GuaB- and GauA-overexpressing strain, an enhancement of GDP-L-fucose production was not observed in the GuaC-overexpressing strain. Production of GDP-L-fucose slowed down after 8 h of IPTG induction, even though cell growth was sustained until the end of fermentation (Fig. 2b). Fed-batch fermentation of recombinant *E. coli* BL21star(DE3) overexpressing GuaC resulted in 171.9±1.8 mg l^{-1} of GDP-L-fucose concentration, which corresponded to a 11% reduction in GDP-L-fucose production compared with the control strain.

Guanosine nucleotides can be synthesized from the salvage pathway as depicted in Fig. 1. GMP can be derived from guanosine by guanosine–inosine kinase (Gsk) and further phosphorylated to GTP. Therefore, the effect of Gsk overexpression was also investigated in the fed-batch fermentation. Because glucose was used as the sole carbon source for GDP-L-fucose production, guanosine or guanine was not added during fed-batch fermentation. Profiles of cell growth and GDP-L-fucose production in *E. coli* BL21star(DE3) overexpressing Gsk were illustrated in Fig. 2c. A significant improvement of GDP-L-fucose production was obtained. After addition of IPTG at 20.5 h of culture, GDP-L-fucose concentration increased hyperbolically for 20 h. Unlike the strain overexpressing the enzymes involved in the de novo biosynthesis, cell growth slowed down after 11.5 h of IPTG induction and cell concentration reached just 86.7±1.7 gl^{-1} at the end of fermentation. Finally, 305.5±5.3 mg l^{-1} of GDP-L-fucose was obtained with 3.5±0.06 mg g $cell^{-1}$ of specific GDP-L-fucose content, which corresponded to a 58% enhancement in GDP-L-fucose production compared with the control strain. In addition, expression patterns of the GDP-L-fucose-producing enzymes and guanosine nucleotide biosynthetic enzymes were analyzed by SDS-PAGE. As shown in Fig. 3, GuaA, GuaB, GuaC and Gsk were expressed functionally until the end of fermentation. The SDS-PAGE result also suggested that the enzymes involved in GDP-L-fucose biosynthesis were expressed functionally. The results of fed-batch fermentations using the recombinant *E. coli* strains are summarized in Table 2.

To verify that such an enhancement of GDP-L-fucose production was related to an increase in the intracellular levels of guanosine nucleotides, intracellular concentrations of guanosine nucleotides were measured. The *E. coli* strain able to overexpress Gsk only was used to avoid consumption of intracellular guanosine nucleotides for GDP-L-fucose production. Culture conditions were the same as the conditions for GDP-L-fucose production in the batch fermentation because an enhancement of GDP-L-fucose production was also observed in a batch fermentation of the recombinant *E. coli*-overexpressing GDP-L-fucose biosynthetic enzymes and Gsk (data not shown). Figure 4 shows the HPLC analysis results of intracellular guanosine nucleotides

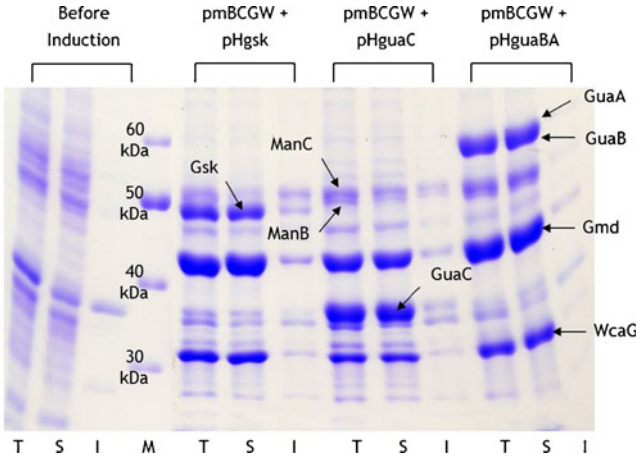

Fig. 3 SDS-PAGE analysis of overexpressed enzymes in the pH-stat fed-batch fermentations of recombinant *E. coli* BL21star(DE3) strains. Cells were harvested at the end of fermentations. *T*, *S* and *I* denote total, soluble and insoluble protein fractions, respectively. The *arrows* indicate the corresponding protein bands with the estimated molecular weight. Lane *M* indicates size marker. The *numbers in lane M* denote the molecular weight corresponding to each band

in the Gsk overexpressing strain. Specific GMP and IMP contents of the Gsk-overexpressing strain were 2.27± 0.14 mg g $cell^{-1}$ and 0.32±0.04 mg g $cell^{-1}$, which were about 50% and 120% higher than the control strain (1.49± 0.07 mg g $cell^{-1}$ and 0.14±0.03 mg g $cell^{-1}$), respectively. Interestingly, intracellular concentration of GDP was not changed (2.01±0.23 mg g $cell^{-1}$) and GTP was not detected by HPLC analysis. These results clearly confirmed that an increase in GMP concentration by Gsk overexpression led to the enhancement of GDP-L-fucose production.

Discussion

GDP-D-mannose is one of the most important substrate for GDP-L-fucose production. As ManC requires GTP as cosubstrate for GDP-D-mannose biosynthesis, efficient supply of GTP can be important for GDP-L-fucose production as much as the carbon flux from glucose to GDP-D-mannose.

Guanosine nucleotides, end-products in the purine nucleotide biosynthesis, are essential materials for cell growth since they are used as precursors for biosynthesis of several amino acid, vitamins and nucleic acids (Teshiba and Furuya 1989). Especially, GTP is known to be the starting material for biosynthesis of essential cofactors in living organisms such as riboflavin (vitamin B_2) and tetrahydrobiopterin (Jimenez et al. 2005; Lim et al. 2001; Stahmann et al. 2000; Yamamoto et al. 2003). Several strategies for manipulation of the purine nucleotide pathway have been attempted for efficient production of those compounds. Overexpression and evolution of the genes involved in the

Table 2 Summary of fed-batch fermentations of *E. coli* BL21star(DE3) strains producing GDP-L-fucose

Plasmid	Overall glucose feed rate[a] (g h^{-1})	Dry cell mass (g l^{-1})	GDP-L-fucose concentration (mg l^{-1})	GDP-L-fucose productivity[b] (mg l^{-1} h^{-1})	Specific GDP-L-fucose content (mg g $cell^{-1}$)	Yield[c] (mg GDP-L-fucose g consumed $glucose^{-1}$)
pmBCGW[d]	15.0	85.0±0.6	193.6±1.9	10.2±0.10	2.3±0.02	1.0±0.01
pmBCGW + pHguaBA	13.3	100.0±0.6	261.7±0.6	13.8±0.03	2.6±0.01	1.5±0.01
pmBCGW + pHguaC	17.5	96.9±1.1	171.9±1.8	9.8±0.10	1.8±0.02	0.8±0.01
pmBCGW + pHgsk	14.6	86.7±1.7	305.5±5.3	15.7±0.27	3.5±0.06	1.5±0.01

[a] After complete depletion of the initial 2% (w/v) glucose, feeding solution containing 800 $g l^{-1}$ glucose and 20 $g l^{-1}$ $MgSO_4 \cdot 7H_2O$ was added by pH-stat feeding mode

[b] GDP-L-fucose productivity estimated during the GDP-L-fucose production period after IPTG induction

[c] Yield were estimated during the GDP-L-fucose production period after IPTG induction

[d] The results of fed-batch fermentation of recombinant *E. coli* BL21star(DE3) overexpressing ManB, ManC, Gmd and WcaG were cited in previous study (Lee et al. 2011)

limiting steps of the purine nucleotide metabolism were attempted for efficient microbial riboflavin production (Lim et al. 2001; Stahmann et al. 2000). It was reported that production of riboflavin in recombinant *Ashbya gossypii* was improved by a 2.8-fold compared to the control strain by overexpression of the *ADE4* gene encoding PRPP amidotransferase (Ade4p), the first enzyme in the de novo purine nucleotides biosynthesis (Jimenez et al. 2005). Since PRPP amidotransferase is known to be directly regulated by AMP and GMP (Matsui et al. 2001a), Ade4p was evolved further to be insensitive to feedback inhibition by end-products, which enhanced riboflavin production by 10 times (Jimenez et al. 2005).

As mentioned in "Introduction", the sufficient flux of GMP from IMP might be able to increase the intracellular levels of GDP and GTP. Overexpression of GuaBA was attempted to increase GTP supply and hence, improved the tetrahydrobiopterin production in recombinant *E. coli* (Yamamoto et al. 2003).

In addition, inosine as well as GMP are synthesized from IMP (Fig. 1), which indicates that biosynthesis of GMP competes against biosynthesis of inosine. Therefore, it can be suggested that a strategy against the inosine production may be needed to improve intracellular guanosine nucleotide concentration. It was reported that increased GuaB activity was responsible for GMP biosynthesis in the guanosine-producing *B. subtilis* mutant (Matsui et al. 1977). It was also reported that improvement of inosine production was obtained by disruption of *guaA*, *guaB*, *gsk* and *xapA* genes in recombinant *E. coli* (Matsui et al. 2001a; Matsui et al. 2001b; Shimaoka et al. 2006). These results suggested that the genes including *guaB*, *guaA* and *gsk*, might be important for efficient production of guanosine nucleotides including GMP.

Based on the results reported elsewhere, GuaB, GuaA and GuaC (in the de novo pathway) and Gsk (in the salvage pathway) were overexpressed in recombinant *E. coli*-producing GDP-L-fucose. A series of pH-stat fed-batch fermentations were carried out in an effort to identify an enzyme responsible for GDP-L-fucose production.

As expected, overexpression of IMP dehydrogenase (GuaB) and GMP synthetase (GuaA) was effective in GDP-L-fucose production. GTP supply might be improved by an elevated flux from IMP to GMP. As a result, a 35% enhancement in GDP-L-fucose production was obtained compared with the control strain. The yield of GDP-L-fucose from glucose was also increased by 50%, indicating that glucose was utilized more efficiently than the control strain. Overexpression of GMP reductase (GuaC) was not effective in GDP-L-fucose production. Especially, a 22% reduction in specific GDP-L-fucose content was observed, which seemed to be due to the conversion of GMP to IMP.

It is interesting to note that overexpression of guanosine–inosine kinase (Gsk) was the most effective on GDP-L-fucose production among the enzymes tested. A 58% enhancement in GDP-L-fucose production was obtained relative to the control strain. It was reported that the Gsk mutant, which was released from feedback inhibition by GTP, increased the guanosine nucleotide pools dramatically (Petersen 1999). GMP might be increased by overexpression of Gsk, and further, might be used for production of GDP-L-fucose by further phosphorylation to GDP and GTP. The specific GMP content of recombinant *E. coli* overexpressing Gsk was 2.27±0.14 mg g $cell^{-1}$, which was 1.5-fold higher than that of the control strain without Gsk overexpression (Fig. 4).

Moreover, fed-batch fermentation of the *E. coli* overexpressing Gsk was carried out in a synthetic medium without addition of guanine or guanosine. Purine nucleosides and nucleotides are known to interconvert with each other (e.g., adenosine to inosine, GMP to IMP), and Gsk catalyzes the conversion of inosine to IMP as well as the conversion of guanosine to GMP (Mantsala and Zalkin 1992; Matsui et al. 2001b). An increase in the intracellular concentration of IMP was also observed by Gsk overexpression as illustrated

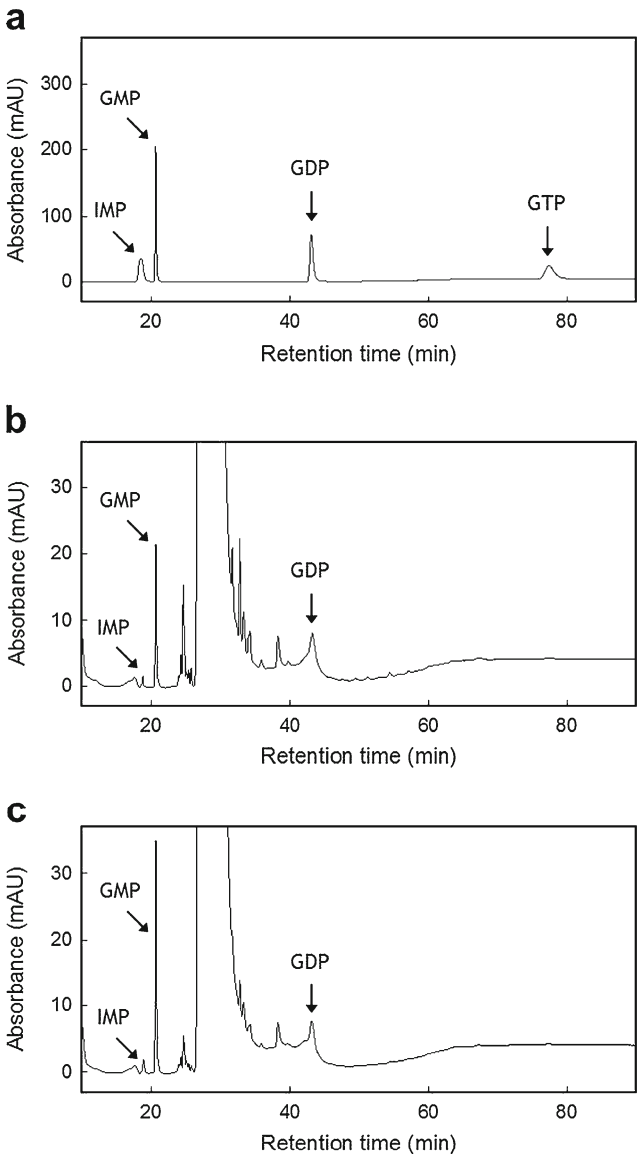

Fig. 4 HPLC analysis of intracellular concentrations of guanosine nucleotides in recombinant *E. coli* BL21star(DE3) strains. Cells were collected at 9 h after 0.1 mM IPTG induction in batch fermentations. Standard solution containing 50 mg l^{-1} of IMP, GMP, GDP and GTP (**a**) and cell crude extracts from recombinant *E. coli* BL21star(DE3) harboring pACYCDuet-1 (**b**) and pHgsk (**c**), respectively.

in Fig. 4. Gsk has been reported to play a key role in controlling the nucleotide-monophosphate level by recycling of guanosine (Usuda et al. 1997). Consequently, interconversion of purine nucleosides and IMP biosynthesis by Gsk overexpression may be a reason for enhancement of GDP-L-fucose production even without addition of exogenous guanosine. It was reported that extracellular purines inhibit the transcription of the genes involved in the de novo AMP and GMP biosynthesis (Escobar-Henriques and Daignan-Fornier 2001), which suggests that addition of guanine or guanosine may be able to exert undesirable effects on GDP-L-fucose production.

In this study, the effect of the amplification of the guanosine nucleotide biosynthesis on GDP-L-fucose production was investigated by additional overexpression of the enzymes involved in the de novo pathway and salvage pathway for guanosine nucleotides biosynthesis. More research is in progress to characterize the global network of the biosynthesis and metabolism of guanosine nucleotides for possible applications to GTP-dependent biosynthesis.

Acknowledgement This work was supported by a Korea Research Council of Fundamental Science and Technology (KRCF) grant. This work was also supported by the Advenced Biomass R&D Center (ABC) of Korea Grant funded by the Ministry of Education, Science and Technology (2010–0029799). MD Kim was supported by the Regional Technology Innovation Program of the Ministry of Knowledge Economy (RTI05-01-02).

References

Bode L (2006) Recent advances on structure, metabolism, and function of human milk oligosaccharides. J Nutr 136:2127–2130

Byun SG, Kim MD, Lee WH, Lee KJ, Han NS, Seo JH (2007) Production of GDP-L-fucose, L-fucose donor for fucosyloligosaccharide synthesis, in recombinant *Escherichia coli*. Appl Microbiol Biotechnol 74:768–775

Escobar-Henriques M, Daignan-Fornier B (2001) Transcriptional regulation of the yeast GMP synthesis pathway by its end products. J Biol Chem 276:1523–1530

Furuya A, Okachi R, Takayama K, Abe S (1973) Accumulation of 5′-guanine nucleotides by mutants of *Brevibacterium ammoniagenes*. Biotechnol Bioeng 15:795–803

Jimenez A, Santos MA, Pompejus M, Revuelta JL (2005) Metabolic engineering of the purine pathway for riboflavin production in *Ashbya gossypii*. Appl Environ Microbiol 71:5743–5751

Koizumi S, Endo T, Tabata K, Nagano H, Ohnishi J, Ozaki A (2000) Large-scale production of GDP-fucose and Lewis X by bacterial coupling. J Ind Microbiol Biotechnol 25:213–217

Lee WH, Han NS, Park YC, Seo JH (2009) Modulation of guanosine 5′-diphosphate-D-mannose metabolism in recombinant *Escherichia coli* for production of guanosine 5′-diphosphate-L-fucose. Bioresour technol 100:6143–6148

Lee WH, Chin YW, Han NS, Kim MD, Seo JH (2011) Enhanced production of GDP-L-fucose by overexpression of NADPH regenerator in recombinant *Escherichia coli*. Appl Microbiol Biotechnol 91:967–976

Lim SH, Choi JS, Park EY (2001) Microbial production of riboflavin using riboflavin overproducers, *Ashbya gossypii*, *Bacillus subtilis*, and *Candida famate*: an overview. Biotechnol Bioprocess Eng 6:75–88

Mantsala P, Zalkin H (1992) Cloning and sequence of *Bacillus subtilis purA* and *guaA*, involved in the conversion of IMP to AMP and GMP. J Bacteriol 174:1883–1890

Matsui H, Sato K, Enei H, Hirose Y (1977) Mutation of an inosine-producing strain of *Bacillus subtilis* to DL-methionine sulfoxide resistance for guanosine production. Appl Environ Microbiol 34:337–341

Matsui H, Kawasaki H, Shimaoka M, Kurahashi O (2001a) Investigation of various genotype characteristics for inosine accumulation in *Escherichia coli* W3110. Biosci Biotechnol Biochem 65:570–578

Matsui H, Shimaoka M, Takenaka Y, Kawasaki H, Kurahashi O (2001b) *gsk* disruption leads to guanosine accumulation in *Escherichia coli*. Biosci Biotechnol Biochem 65:1230–1235

Nijkamp HJ, De Haan PG (1967) Genetic and biochemical studies of the guanosine 5′-monophosphate pathway in *Escherichia coli*. Biochim Biophys Acta 145:31–40

Petersen C (1999) Inhibition of cellular growth by increased guanine nucleotide pools. Characterization of an Escherichia coli mutant with a guanosine kinase that is insensitive to feedback inhibition by GTP. J Biol Chem 274:5348–5356

Shimaoka M, Takenaka Y, Mihara Y, Kurahashi O, Kawasaki H, Matsui H (2006) Effects of *xapA* and *guaA* disruption on inosine accumulation in *Escherichia coli*. Biosci Biotechnol Biochem 70:3069–3072

Sigal N, Gorzalczany Y, Sarfstein R, Weinbaum C, Zheng Y, Pick E (2003) The guanine nucleotide exchange factor trio activates the phagocyte NADPH oxidase in the absence of GDP to GTP exchange on Rac. J Biol Chem 278:4854–4861

Stahmann KP, Revuelta J, Seulberger H (2000) Three biotechnical processes using *Ashbya gossypii*, *Candida famata*, or *Bacillus subtilis* compete with chemical riboflavin production. Appl Microbiol Biotechnol 53:509–516

Stevenson G, Andrianopoulos K, Hobbs M, Reeves PR (1996) Organization of the *Escherichia coli* K-12 gene cluster responsible for production of the extracellular polysaccharide colanic acid. J Bacteriol 178:4885–4893

Teshiba S, Furuya A (1989) Production of nucleotides and nucleosides by fermentation. Gordon and Breach Science Publisher, NY, USA

Usuda Y, Kawasaki H, Shimaoka M, Utagawa T (1997) Molecular cloning and transcriptional analysis of a guanosine kinase gene of *Brevibacterium acetylicum* ATCC 953. J Bacteriol 179:6959–6964

Yamamoto K, Kataoka E, Miyamoto N, Furukawa K, Ohsuye K, Yabuta M (2003) Genetic engineering of *Escherichia coli* for production of tetrahydrobiopterin. Metab Eng 5:246–254