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Modulation of guanosine 5′-diphosphate-D-mannose metabolism in recombinant *Escherichia coli* for production of guanosine 5′-diphosphate-L-fucose

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ABSTRACT

Guanosine 5'-diphosphate (GDP)-L-fucose, an activated form of a nucleotide sugar, plays an important role in a wide range of biological functions. In this study, the enhancement of GDP-L-fucose production was attempted by supplementation of mannose, which is a potentially better carbon source to be converted into GDP-L-fucose than glucose, and combinatorial overexpression of the genes involved in the biosynthesis of GDP-D-mannose, a precursor of GDP-L-fucose. Supply of a mannose and glucose led to a 1.3-fold-increase in GDP-L-fucose concentration (52.5 ± 0.8 mg l^{-1}) in a fed-batch fermentation of recombinant *E. coli* BL21star(DE3) overexpressing the *gmd* and *wcaG* genes, compared with the case using glucose as a sole carbon source. A maximum GDP-L-fucose concentration of 170.3 ± 2.3 mg l^{-1}, corresponding to a 4.4-fold enhancement compared with the control strain overexpressing *gmd* and *wcaG* genes only, was achieved in a glucose-limited fed-batch fermentation of a recombinant *E. coli* BL21star(DE3) strain overexpressing *manB*, *manC*, *gmd* and *wcaG* genes. Further improvement of GDP-L-fucose production was not obtained by additional overexpression of the *manA* gene.

1. Introduction

Human milk contains large amounts of complex oligosaccharides including lactose, d-galactose, *N*-acetylglucosamine, L-fucose and sialic acid (Boehm and Stahl, 2007; Kunz et al., 2000) and there has been increasing interest in the biological functions of human milk oligosaccharides, especially fucosyloligosaccharides that are able to defend infants against enteric pathogens (Kunz and Rudloff, 2006; Morrow et al., 2004; Newburg et al., 2004). The availability of large amounts of fucosylated oligosaccharides would make them useful as precursors for therapeutic and protective purposes such as the prevention of pathogen infection, improvement of immune system response and reduction of inflammatory processes (Newburg et al., 2005; Severin and Wenshui, 2005). Fucosylated oligosaccharides such as the Lewis blood group antigen can be chemically synthesized by several complicated procedures consisting of multiple protection and deprotection steps (Kretzschmar and Stahl, 1998). In contrast to the complexity of chemical synthesis, enzymatic methods using glycosyltransferases provide more efficient ways for fucosylated oligosaccharide synthesis. Enzymatic fucosylation of oligosaccharides requires guanosine 5'-diphosphate (GDP)-L-fucose as a donor of L-fucose (Albermann et al., 2000; Bulter and Elling, 1999). However, the high cost of GDP-L-fucose limits its application for large-scale production of fucosyloligosaccharides.

The metabolic pathway of GDP-L-fucose synthesis from glucose is found in bacteria, plants and human (Bulter and Elling, 1999). Fructose-6-phosphate, an intermediate of glycolysis, is converted to GDP-L-fucose by way of GDP-D-mannose in reactions catalyzed by five enzymes. For conversion of fructose-6-phosphate to GDP-D-mannose, mannose-6-phosphate isomerase (ManA, E.C. 5.3.1.8), phosphomannomutase (ManB, E.C. 5.4.2.8) and mannose-1-phosphate guanyltransferase (ManC, E.C. 2.7.7.22) are required. Further transformation of GDP-D-mannose to GDP-L-fucose is catalyzed by GDP-D-mannose-4, 6-dehydratase (Gmd, E.C. 4.2.1.47) and GDP-L-fucose synthase (WcaG, E.C. 1.1.1.271). The ManC and WcaG-mediated reactions require guanosine 5'-triphosphate (GTP) and NADPH as cofactors, respectively (Albermann et al., 2000; Sullivan et al., 1998). Biosynthesis of GDP-L-fucose has been developed using recombinant microorganisms. A recombinant *Saccharomyces cerevisiae* system expressing the *gmd* and *wcaG* genes from *Escherichia coli* K12 produced 0.2 mg l^{-1} GDP-L-fucose from galactose (Mattila et al., 2000). Although *S. cerevisiae* is known to have a high level of cytosolic GDP-D-mannose useful for protein mannosylation (Hashimoto et al., 1997; Kukuruzinska et al., 1987; Romanos et al., 1992), high concentration of GDP-L-fucose was not obtained. Another microbial system for GDP-L-fucose production was established by combination of *Corynebacterium ammoniagenes*

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producing GTP and recombinant *E. coli* overexpressing GDP-L-fucose biosynthetic enzymes (Koizumi et al., 2000). A batch type reaction with the resting cells of the two strains and utilization of GMP and mannose as two starting materials resulted in 18.4 g l^{-1} of GDP-L-fucose concentration in 22 h. However, this coupling method requires complicated procedures including cultivation and separation of two microbes and permeabilization of cell membrane. It also requires two expensive substrates, GMP and mannose. Therefore, it is desirable to develop a recombinant microbial system producing GDP-L-fucose from a relatively cheap carbon source such as glucose. In our previous report, a high titer of GDP-L-fucose was obtained in batch and fed-batch fermentations of a recombinant *E. coli* B strain overexpressing the *gmd* and *wcaG* genes (Byun et al., 2007). In addition, overexpression of glucose-6-phosphate dehydrogenase (G6PDH) improved the volumetric productivity of GDP-L-fucose, which indicated that NADPH supplementation would be a critical factor for GDP-L-fucose production.

In this study, the enhancement of GDP-L-fucose production was attempted by the supplementation of GDP-D-mannose via both supplying mannose as an alternative carbon source and overexpressing the genes related to the biosynthesis of GDP-D-mannose without addition of external GDP-D-mannose. Fed-batch fermentations of recombinant *E. coli* overexpressing the *gmd* and *wcaG* genes were carried out by using mannose or a mixture of mannose and glucose as carbon sources. Various recombinant *E. coli* systems were constructed for the combinatorial overexpression of the GDP-D-mannose biosynthetic enzymes such as ManA, ManB and ManC. The effects of the overexpression of ManA, ManB, and ManC were assessed in a glucose-limited fed-batch fermentation scheme.

2. Methods

2.1. Genetic manipulation

E. coli TOP10 and BL21star(DE3) were used as bacterial hosts for genetic manipulation and GDP-L-fucose production, respectively. Plasmid pETGW containing the dicistronic *gmd* and *wcaG* gene cluster was previously constructed using plasmid pETDuet-1 (Byun et al., 2007). The *manA*, *manB* and *manC* genes were obtained by the polymerase chain reactions (PCR) using genomic DNA of *E. coli* K12 (ATCC 10798) as template. To amplify the *manA* gene, two PCR primers, manA_F and manA_R, were designed to contain the 5'- and 3'-end regions of the *manA* gene, and the recognition sites of the *Kpn*I and *Xho*I restriction enzymes, respectively. The amplified *manA* gene digested with *Kpn*I and *Xho*I was cloned into plasmid pACYCDuet-1 cut with the same enzymes and plasmid pKJmanA was constructed. The *manB* gene was amplified by using the manB_F and manB_R primers containing the recognition sites of *Nco*I and *Eco*RI enzymes, respectively. By digestion of the *manB* gene and plasmid pETGW with *Nco*I and *Eco*RI, and ligation with each other, plasmid pmBGW was constructed. The *manC* gene was amplified by using the manC_F and manC_R primers containing the recognition sites of *Nco*I and *Sac*I enzymes, respectively. By digestion of the *manC* gene and plasmid pETGW with *Nco*I and *Sac*I, and ligation with each other, plasmid pmCGW was constructed. The polycistronic *manC-manB* gene cluster was obtained by PCR using the manC_F and manB_R. The amplified *manC-manB* gene was combined with plasmid pETDuet-1 after their digestion with *Nco*I and *Eco*RI. The resulting plasmid was cut again with *Kpn*I and *Xho*I, and then connected with the *manA* gene prepared previously, resulting in the construction of the pmABC plasmid. After the digestion of plasmid pmABC with *Nco*I and *Sac*I, the released *manC-manB* gene cluster was inserted into plasmid pETGW, and the resulting vector was called pmBCGW. Two PCR primers of T7 + manA_F and T7 + manA_R were used for the amplification of the 1361 bp DNA fragment containing the *T7* promoter and *manA* gene in plasmid pmABC. After digestion of plasmid pmBCGW and the 1,361 bp DNA fragment with *Hind*III and *Not*I, DNA fragments with the estimated sizes were ligated and the resulting plasmid was named pmABCGW. Plasmids and primers used and created in this work are listed in Tables 1 and 2, respectively. Expression of the *manA*, *manB*, *manC*, *gmd* and *wcaG* genes were controlled by the IPTG-inducible *T7* promoter. All constructed plasmids were subjected to DNA sequencing. PCR reactions, general DNA manipulation and bacterial transformation were carried out as described previously (Byun et al., 2007).

Table 2
DNA oligomers used in this study.

PCR primers	Sequence[a]
manA_F (*Kpn*I)	5′-AGGAATTCGGTACCATGCAAAAACTCATTAACTCAGTG-3́
manA_R (*Xho*I)	5́-AAGGCTCGAGTTACAGCTTGTTGTAAACACG-3́
manB_F (*Nco*I)	5′-ACATGCCATGGATGAAAAAATTAACCTGCTTT-3́
manB_R (*Eco*RI)	5′-ACCGGAATTCTTACTCGTTCAGCAACGTCAG-3́
manC_F (*Nco*I)	5́-ACATGCCATGGATGGCGCAGTCGAAACTCTAT-3́
manC_R (*Sac*I)	5́-AGTCCGAGCTCTTACACCCGTCCGTAGCGATC-3́
T7 + manA_F (*Hind*III)	5′-ATACCCAAGCTTTCGAACAGAAAGTAATCGTATTGT-3′
T7 + manA_R (*Not*I)	5′-ATAAGAATGCGGCCGCTTACAGCTTGTTGTAAACACGC-3′

[a] The underlined sequences indicate the corresponding recognition sites of the restriction enzymes as described in Section 2.

Table 1
Strains and plasmids used in this study.

Name	Description	Source
E. coli strains		
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	Invitrogen
BL21star(DE3)	F-*omp*T $hsdS_B(r_B^- m_B^-)$ *gal dcm rne131*(DE3)	Invitrogen
Plasmids		
pETDuet-1	Bi-directional *T7* promoter, pBR322 replicon (copy number ~40), Amp^r	Novagen
pACYCDuet-1	Bi-directional *T7* promoter, p15A replicon (copy number 10–12), Cm^r	Novagen
pETGW	pETDuet-1 + *gmd-wcaG* (*Nde*I/*Xho*I), Amp^r	Byun and others (2007)
pKJmanA	pACYCDuet-1 + *manA* (*Kpn*I/*Xho*I), Cm^r	This study
pmABC	pETDuet-1 + *manA* (*Kpn*I/*Xho*I) + *manB-manC* (*Nco*I/*Eco*RI), Amp^r	This study
pmBGW	pETGW + *manB* (*Nco*I/ *Eco*RI), Amp^r	This study
pmCGW	pETGW + *manC* (*Nco*I/*Sac*I), Amp^r	This study
pmBCGW	pETGW + *manB-manC* (*Nco*I/*Eco*RI), Amp^r	This study
pmABCGW	pmBCGW + *T7* promoter and *manA* (*Hind*III/*Not*I), Amp^r	This study