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Development and Characterization of Expression Vectors for *Corynebacterium glutamicum* S

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In an attempt to develop a variety of expression vector systems for *Corynebacterium glutamicum*, six types of promoters, including P_{tac}, P_{sod}, P_{sod} with a conserved Shine-Dalgarno (SD) sequence from *C. glutamicum*, P_{ilvC}, P_{ilvC} with a conserved SD-1 ($P_{ilvC\text{-}M1}$), and P_{ilvC} with a conserved SD-2 ($P_{ilvC\text{-}M2}$), were cloned into a modified shuttle vector, pCXM48. According to analysis of promoter strength by quantitative reverse transcription PCR, P_{sod} and $P_{sod\text{-}M}$ were superior to *tac* and *ilvC* promoters in terms of transcription activity in *C. glutamicum*. All of the promoters have promoter activities in *Escherichia coli*, and $P_{sod\text{-}M}$ displayed the highest level of transcriptional activity. The protein expression in constructed vectors was evaluated by measuring the fluorescence of green fluorescent protein (GFP) and SDS-PAGE. *C. glutamicum* harboring plasmids showed GFP fluorescence with an order of activity of $P_{ilvC} > P_{ilvC\text{-}M1} > P_{sod} > P_{ilvC\text{-}M2} > P_{sod\text{-}M}$, whereas all plasmids except pCSP30 with P_{sod} displayed fluorescence activities in *E. coli*. Of them, the strongest level of GFP was observed in *E. coli* with $P_{sod\text{-}M}$, and this seems to be due to the introduction of the conserved SD sequence in the translational initiation region. These results demonstrate that the expression vectors work well in both *C. glutamicum* and *E. coli* for the expression of target proteins. In addition, the vector systems harboring various promoters with different strengths, conserved SD sequences, and multiple cloning sites will provide a comfortable method for cloning and gene expression, and consequently contribute to the metabolic engineering of *C. glutamicum*.

Keywords: *Corynebacterium*, expression vector, P_{tac}, P_{sod}, P_{ilvC}

Introduction

Corynebacterium glutamicum is a gram-positive soil bacterium that has been widely used in the industrial production of many amino acids, including monosodium glutamate and lysine [10]. Recently, with the accumulation of rapidly increasing information and techniques regarding *C. glutamicum*, such as genetic manipulation tools [12, 22, 34, 35], whole genome information [11, 15], functional genomic techniques [38, 40], and integration of systems biology into metabolic engineering [3, 18], *C. glutamicum* is becoming regarded as one plausible microorganism for the large production of bio-based chemicals, materials, and fuels including D-ornithine, 2-ketoisovalerate, succinate, cadaverine, putrescine, 1,2-propanediol, ethanol, 1-butanol, and polygalacturonic acid [1]. Additionally, since *C. glutamicum* belongs to the GRAS (generally regarded as safe) microorganisms, it can be applicable to production of food- or pharmaceutical-grade proteins [7].

Bacterial promoters play a crucial role in the expression and regulation of genes regarding production of valuable metabolites or proteins in microorganisms [26, 41]. The well-known *Escherichia coli* promoters such as *tac*, *trc*, *lac*UV5, and P_R, P_L promoters have been used for gene expression in *C. glutamicum* [5, 24, 29, 36]. The expression by these promoters was inducible following the addition of lactose and its analog IPTG, arabinose [16], or acetic acid [6]. Although the *E. coli* promoters were active in *C. glutamicum*, they display very weak activities and the transcriptional regulation of gene expression was relatively inefficient when compared with *E. coli* [29]. For the purpose of efficient modulation of gene expression, many endogenous

promoters from *C. glutamicum* have been isolated and characterized based on the promoter sequences in the -30 and -10 regions and regulation mechanisms [17, 22, 26, 39]. The promoters of *sod* gene coding for superoxide dismutase [2, 23, 27], *eftu* encoding elongation factor tu [2], *dapA* coding for dihydrodipicolinate synthase [39], and *gdh* encoding glutamate dehydrogenase [9] were employed for metabolic engineering of *C. glutamicum* to produce several metabolites. However, to date, suitable expression vector systems with endogenous strong promoters were limited, and most were utilized for replacement of promoters of genes within the chromosome sequences. Besides this, the strength of promoters described above, along with *tac* promoter at the transcriptional level, was not compared and evaluated with each other in corynebacteria. On one side, many reports illustrate that protein expression levels are strongly influenced by the mRNA secondary structure and the short Shine-Dalgarno (SD) sequence in the 5′-untranslated region (5′-UTR) of bacterial mRNAs as well as the promoter strength [4, 8, 14, 25, 30, 33], and so, it is difficult to choose appropriate promoter(s) for optimal expression of each gene [41]. In this sense, it is necessary to construct several expression vector systems harboring a variety of promoters with different strengths, conserved SD sequence, and multiple cloning sites comfortable for gene cloning and expression.

In this study, I report the construction of expression vector systems for *C. glutamicum* with three types of promoters, P_{tac}, P_{sod}, and P_{ilvC}, which are known to function in *C. glutamicum*, and verified its capability through the analyses of quantitative reverse transcription PCR (qRT-PCR) and protein expression using green fluorescent protein (GFPuv). Furthermore, I developed novel expression vector systems in which the conserved SD sequence of *C. glutamicum* was introduced into promoters P_{ilvC} and P_{sod} and confirmed that these systems function in both *C. glutamicum* and *E. coli*.

Materials and Methods

Bacterial Strains, Plasmids, and Culture Conditions

Bacterial strains and plasmids used in this study are described

Table 1. The bacterial strains and plasmids used in this study.

Strain or plasmid	Characteristics	Source or reference
Strains		
E. coli Top10	F⁻*mcr*A Δ(*mrr-hsa*RMS-*mcr*BC) φ80*lac*ZΔM15 ΔlacX74 *recA1* *araD139* Δ*(ara-leu)*7679 *gal*U *gal*K *rps* (Str^R) endA1 *nup*G	Invitrogen, USA
C. glutamicum		
ATCC 13032	Wild-type strain	This lab.[a]
Plasmids		
pKK223-3	Expression vector; P_{tac}, Amp^R	This lab.[b]
pCES208	*E. coli*/*C. glutamicum* shuttle vector, 5.93 kb; Kan^R	[24]
pXMJ19	*E. coli*/*C. glutamicum* shuttle vector, 6.6 kb; Cm^R	[13, 22]
pGFPuv	GFPuv expression vector, 3.3 kb; Amp^R	This lab.
pCXM48	pXMJ19 derivative, 4.84 kb	This work
pCXT20	pCXM48 derivative, 5.36 kb; P_{tac}	This work
pCXS30	pCXM48 derivative, 5.51 kb; P_{sod}	This work
pCXS35	pCXM48 derivative, 5.51 kb; $P_{sod\text{-}M}$ with conserved SD	This work
pCXI40	pCXM48 derivative, 5.51 kb; P_{ilvC}	This work
pCXI43	pCXM48 derivative, 5.51 kb; $P_{ilvC\text{-}M1}$ with conserved SD	This work
pCXI45	pCXM48 derivative, 5.51 kb; $P_{ilvC\text{-}M2}$ with conserved SD	This work
pCTP20	pCXM48 derivative, 6.07 kb; P_{tac}GFPuv	This work
pCSP30	pCXM48 derivative, 6.22 kb; P_{sod}GFPuv	This work
pCSP35	pCXM48 derivative, 6.22 kb; $P_{sod\text{-}M}$GFPuv	This work
pCIP40	pCXM48 derivative, 6.22 kb; P_{ilvC}GFPuv	This work
pCIP43	pCXM48 derivative, 6.22 kb; $P_{ilvC\text{-}M1}$GFPuv	This work
pCIP45	pCXM48 derivative, 6.22 kb; $P_{ilvC\text{-}M2}$GFPuv	This work

[a]ATCC, American Type Culture Collection.

[b]Amp^R, ampicillin resistance; Kan^R, kanamycin resistance; Cm^R, chloramphenicol resistance.

in Table 1. *E. coli* Top 10 was employed as the host for general DNA manipulation. The DNA template of *sod* and *ilvC* promoters was obtained from *C. glutamicum* ATCC 13032, whereas pKK223-3 and pGFPuv were used as DNA templates for obtaining P_{tac}-multiple cloning sites-4 (MCS-4)-T_{rrnB} fragment and *gfp* gene, respectively. MCS-2 was obtained from an *E. coli*/*C. glutamicum* shuttle vector, pCES208 [24]. The *E. coli*/*C. glutamicum* shuttle vector pXMJ19 and its derivatives [13, 22] were used for the construction of expression vector systems with several promoters. *E. coli* and *C. glutamicum* were grown in LB medium (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl) at 37°C and 32°C, respectively. If necessary, 25 and 4.5 μg/ml of chloramphenicol were added to the culture media of *E. coli* and *C. glutamicum*, respectively. In the case of *E. coli* Top 10/pCTG20, when cell OD reached about 0.4, 1 mM of IPTG was added into the LB medium.

Recombinant DNA Techniques and Transformation

All the general recombinant DNA techniques were carried out according to Sambrook *et al*. [31]. Restriction enzymes, *pfu*-x DNA polymerase, plasmid mini-prep kit, and gel extraction kit were purchased from New England Biolab (USA), Solgent Corp. (Korea), Intron (Korea), and Macrogen (Korea), respectively. Primer sequences used in this study are listed in Table 2. All PCR constructs were verified by DNA sequencing. Plasmid DNA was transformed into *C. glutamicum* by electroporation [37].

Subcloning of pXMJ19

To remove P_{tac}, *rrn*B transcriptional terminator (T_{rrnB}), and the *lacI*^{q} gene in pXMJ19 and insert MCS-2 of pCES208 into modified pXMJ19 by polymerase chain reaction (PCR), a 4.7 kb fragment from pXMJ19 and 0.12 kb MCS-2 in pCES208 were amplified by using primer sets P1-P2 and P3-P4, respectively (Fig. 1A). Both fragments were gel-purified, digested with *Not*I and *Nhe*I, and ligated with each other. The resulting plasmid, pCXM48, was introduced into *E. coli* Top 10, and transformants were selected on chloramphenicol-containing LB agar plates (Fig. 1B).

Construction of Expression Vectors

A P_{tac}-T_{rrnB} from pKK223-3 was cloned into pCXM48 by PCR using primers P5 and P6. A 0.59 kb PCR product digested by *Xba*I and *Kpn*I was ligated with pCXM48/*Xba*I/*Kpn*I, and yielded pCXT20 (Fig. 1C). To construct P_{sod}- and P_{ilvC}-containing expression vectors, 0.3 kb of P_{sod} and P_{ilvC} fragments were amplified by using primer sets P7-P8 and P9-P10, respectively, and gel-purified. The digested products were then cut with *Xba*I and *Eco*RI, cloned into the same restriction sites of pCXT20 in which P_{tac} was removed, generating pCXS30 and pCXI40, respectively (Fig. 3)

Construction of Expression Vectors with Conserved SD Sequence of *C. glutamicum*

To introduce the conserved SD sequence of *C. glutamicum* in

Table 2. Primer sequences used in this study.

Primer	Sequence (5′-3′)[a]	Restriction enzyme site or comment
P1	CTGAATAAGAATGCGGCCGCATATGTATCCGCTCATGAGACAA	*Not*I
P2	CCGCTACTAGCTAGCACCACCCTGGCGCCGGGGAT	*Nhe*I
P3	ATTGGAGCTCCACCGCGGTG	
P4	CCGCTACTAGCTAGCCCTCACTAAAGGGAACAAAAGC	*Nhe*I
P5	CTAGGCTCTAGATCAAGGCGCACTCCCGTTCT	*Xba*I
P6	CGGGGTACCGCAAAAAGGCCATCCGTCAG	*Kpn*I
P7	CTAGGCTCTAGAAAGCGCCTCATCAGCGGTAACCAT	*Xba*I
P8	CCCGGAATTCAAAATCCTTTCGTAGGTTTCCGC	*Eco*RI
P9	CTAGGCTCTAGACCAGGCAAGCTCCGCGCACTGCTT	*Xba*I
P10	CCCGGAATTCAATCTCGCCTTTCGTAAAAATTTTGGTGAAAA	*Eco*RI
P11	CCCGGAATTCAACCTCCTTTCGTAGGTTTCCGC	*Eco*RI
P12	CCCGGAATTCAATCTCTCCTTTCGTAAAAATTTTGGTGAAAA	*Eco*RI
P13	CCCGGAATTCAATCCCTCCTTTCGTAAAAATTTTGGTGAAAA	*Eco*RI
P14	CCCGGAATTCATGAGTAAAGGAGAAGAACTTTTC	*Eco*RI
P15	CACCCAAGCTTTTATTTGTAGAGCTCATCCATGC	*Hin*dIII
P16	TGCCCGAAGGTTATGTACAG	GFP-F
P17	TCAGCACGCGTCTTGTAGTT	GFP-R
P18	GAGCGCAACCCTTGTCTTAT	*C. glutamicum* 16S rDNA-F
P19	AGTTAACCCCGGCAGTCTCT	*C. glutamicum* 16S rDNA-R
P20	TCAAGTCATCATGGCCCTTA	*E. coli* 16S rDNA-F
P21	GGTCGCTTCTCTTTGTATGC	*E. coli* 16S rDNA-R

[a]Restriction enzyme sites are underlined.

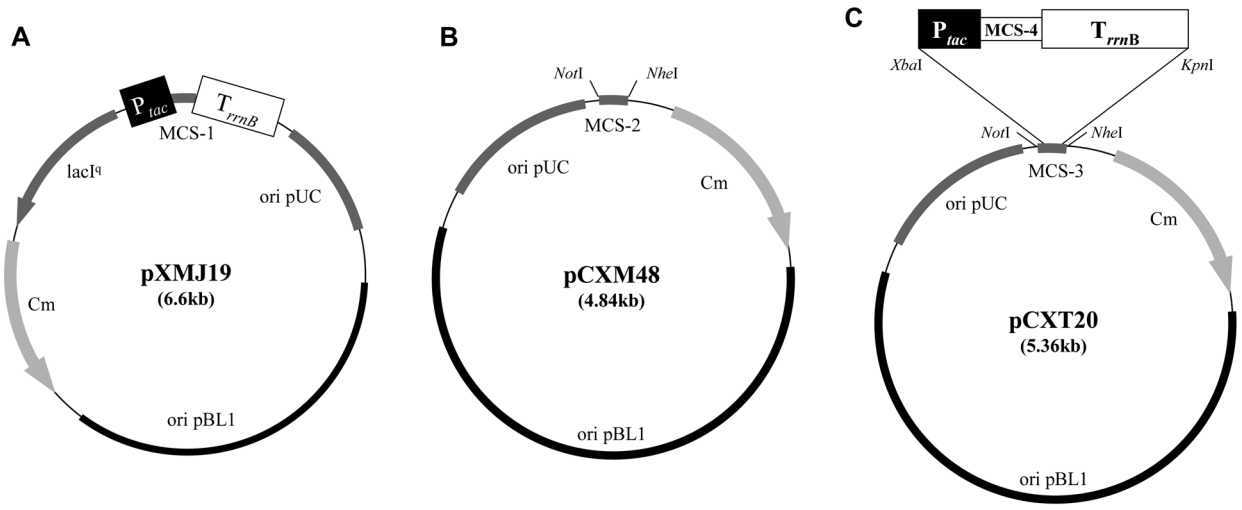

Fig. 1. Schematic representation of recombinant plasmids.

(**A**) Plasmid map of pXMJ19 with P_{tac}, MCS-1, and T_{rrnB}. The MCS-1 contains the following restriction sites (5′→3′ direction); *Hin*dIII, *Pst*I, *Sal*I, *Xba*I, *Bam*HI, *Sma*I, *Kpn*I, and *Eco*RI. (**B**) Plasmid map of pCXM48 with the MCS-2 site from pCES208. MCS-2 contains the following restriction sites; *Not*I, *Xba*I, *Bam*HI, *Pst*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Sal*I, *Kpn*I, and *Nhe*I. (**C**) Plasmid map of the P_{tac}-containing expression vector pCXT20. MCS-3 has *Not*I-*Xba*I-*Kpn*I-*Nhe*I restriction sites; MCS-4 has *Eco*RI, *Bam*HI, *Pst*I, and *Hin*dIII sites. T_{rrnB} means *rrn*B transcriptional terminator.

expression vectors with *sod* and *ilvC* promoters, fragments $P_{sod\text{-}M}$, $P_{ilvC\text{-}M1}$, and $P_{ilvC\text{-}M2}$ from the chromosomal DNA of *C. glutamicum* were amplified using primer sets P7-P11, P9-P12, and P9-P13, respectively (Fig. 2). The resulting purified fragments cut by *Xba*I and *Eco*RI were then cloned into the *Xba*I/*Eco*RI-cleaved pCXT20 in which P_{tac} was removed to produce pCXS35, pCXI43, and pCXI45, respectively (Fig. 3).

Construction of GFPuv-Containing Expression Vectors

A *gfp* gene was cloned using the constructed expression vectors. A 0.7 kb product amplified by using primers P14 and P15 was inserted into *Eco*RI/*Hin*dIII-digested pCXT20, pCXS30, pCXS35, pCXI40, pCXI43, and pCXI45, respectively, and designated pCTP20, pCSP30, pCSP35, pCIP40, pCIP43, and pCIP45.

Measurement of GFPuv Fluorescence

Overnight cultures using recombinant *E. coli* and *C. glutamicum* cells harboring expression vectors with gene *gfp* in LB medium were harvested by centrifugation at 5,000 ×*g* for 10 min, washed three times with PBS buffer (NaCl 4 g/l, KCl 0.1 g/l, Na_2HPO_4 0.72 g/l, KH_2PO_4 0.12 g/l, pH 7.4), and then resuspended in 1 ml of the same buffer. A bead beator (Biospec Product, Inc.) disrupted the cells, and cell debris was removed by centrifugation at 13,000 ×*g* for 30 min, yielding crude extracts, which were used for the

A P_{tac} (pCXT20) : GATAACAATTTCACAC*AGGA*AACAgaattc

B
wild P_{sod} : TGCGGAAACCTAC*GAAAGGATT*TTTTACCC
P_{sod} with *Eco*RI (pCXS30) : TGCGGAAACCTAC*GAAAGGATT*TTgaattc
P_{sod} with conserved SD and *Eco*RI (pCXS35) : TGCGGAAACCTAC*GAAAGGAGG*TTgaattc

C
wild P_{ilvC} : AAAATTTTTAC*GAAAGGCGA*GATTTTCTCC
P_{ilvC} with *Eco*RI (pCXI40) : AAAATTTTTAC*GAAAGGCGA*GATTgaattc
P_{ilvC} with conserved SD1 and *Eco*RI (pCXI43) : AAAATTTTTAC*GAAAGGAGA*GATTgaattc
P_{ilvC} with conserved SD2 and *Eco*RI (pCXI45) : AAAATTTTTAC*GAAAGGAGG*GATTgaattc

Fig. 2. Comparison of DNA sequences in 3′-regions of P_{tac}, P_{sod}, and P_{ilvC}.

Underlined italic characters are the putative SD sequence or conserved SD sequence introduced in the 3′-regions of each promoter. The underlined lower case letters are the *Eco*RI site introduced into the wild-type sequence.

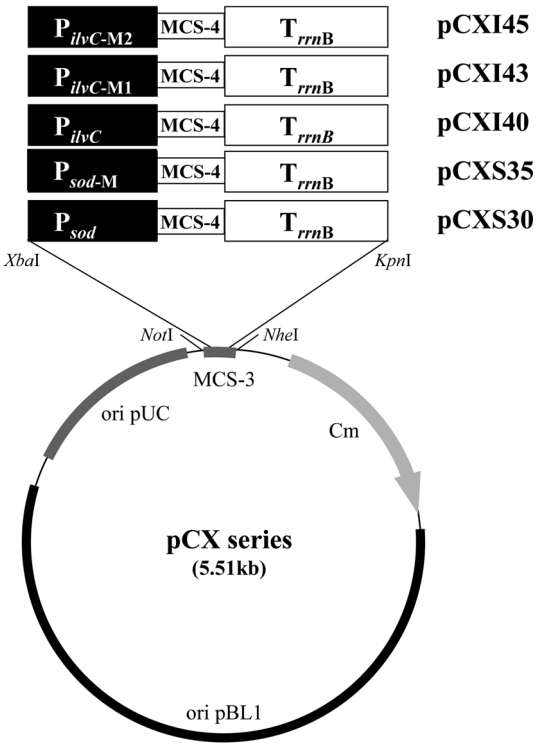

Fig. 3. Schematic diagram of expression vectors.
pCXS30, pCXS35, pCXI40, pCXI43, and pCXI45 contain promoters P_{sod}, P_{sod} with a conserved SD; P_{ilvC}, P_{ilvC} with a conserved SD-1; and P_{ilvC} with a conserved SD-2, respectively.

measurement of fluorescence. GFPuv fluorescence was measured by using a spectrofluorophotometer (Shimadzu, RF-5300PC) with excitation at 395 nm and emission at 508 nm. All of the measurements were performed using independent cultures three times.

Analysis of Protein Concentration and SDS-PAGE

Protein concentration in crude extracts was determined by using the Bio-Rad protein assay kit (Bio-Rad, USA) with bovine serum albumin as the standard. Protein expression was monitored using 10% SDS-PAGE. Native SDS-PAGE was performed as follows. Loading samples of native state were prepared by adding a loading dye (0.21 M Tris-HCl, pH 8.45, 11% glycerol, 0.8% SDS, 0.004% Coomassie blue G, 0.004% phenol red) into crude extracts and incubated for 2 h at 37°C. After running the SDS-PAGE, the gel was washed three times with 10 mM Tris-HCl buffer (pH 8.0) for 1 h, and then GFP fluorescence was monitored at 260 nm of UV light.

Quantitative Reverse Transcription PCR (qRT-PCR)

For analysis of transcriptional levels of *gfp* in expression vectors, cells were cultivated to the mid-exponential growth phase ($OD_{0.8}$ ~1.0), and total RNA was extracted from cells using the IQeasy RNA extraction Mini kit (Intronbio, Korea) according to the manufacturer's instructions and stored at –72°C. Reverse transcription was performed by using SuperScript II (Invitrogen). Following reverse transcription, cDNA was amplified by using primer pairs P16-P17 (GFP primer set), P18-P19 (*C. glutamicum* 16S rDNA primer set), and P20-P21 (*E. coli* 16S rDNA primer set), respectively. Real-time PCR was performed in a Step One Plus machine (Applied Biosystems (AB)) using the SYBR Green PCR kit (AB), according to the manufacturer's instructions in a total volume of 20 µl. Cycling conditions for amplification of GFP, *C. glutamicum* 16S rDNA (*C. glutamicum* internal control), and *E. coli* 16S rDNA (*E. coli* internal control) were 10 min at 95°C, 40 cycles of 15 sec at 95°C, and 30 sec at optimal Tm (59°C). Quantification was carried out with StepOne software v.2.2.2 (AB). The relative GFP expression levels were analyzed using the $2^{-(\Delta\Delta C_T)}$ method [20], normalized to 16S rDNA expression of *C. glutamicum* or *E. coli* and represented as x-fold increase in a recombinant cell harboring pCSP30, pCSP35, pCIP40, pCIP43, or pCIP45 (sample ΔC_T) compared with the corresponding cell with pCTP20 (positive control ΔC_T).

Results and Discussion

Development of Vector Systems from pBL1 Family

The typical autonomously replicating vectors for *C. glutamicum* are based on the small cryptic plasmids pBL1 and pCG1 from *C. glutamicum* [22, 32]. Both vector systems are compatible in corynebacteria, and so it enables the study of the genetics, physiology, and metabolic engineering of *C. glutamicum*. Since the widely utilizing restriction enzyme sites including *Eco*RI and *Hin*dIII are present in pCG1 family plasmids, it is preferable to use pBL1 family plasmids for the construction of expression vectors [13]. In this work, I constructed expression vector systems based on plasmid pXMJ19 (Fig. 1A), a pBL1 family, as follows. First, the arrangement of MCS-1 in pXMJ19 is different to that of pKK223-3, which carries the *tac* promoter that is widely used for gene expression in *E. coli*, which led researchers to clone genes inconveniently using both *E. coli* and *C. glutamicum*. Thus, I deleted P_{tac}-MCS-1-T_{rrnB} from pXMJ19 and cloned P_{tac}-MCS-4-T_{rrnB} into a newly constructed vector, pCXM48 (Fig. 1B). Second, because the constitutive expression systems are superior to the inducible systems in economical aspects, the *lacI*^{q} gene was deleted from pXMJ19. Third, to conveniently clone genes in both pBL1 and pCG1 families, the MCS-2 of pCES208, which has the same replication origin of pCG1, was introduced into the modified pXMJ19. To do this, I constructed pCXM48 by deleting P_{tac}-MCS-1-T_{rrnB} and *lacI*^{q} and introducing MCS-2 of pCES208 into the subcloned pXMJ19 (Fig. 1B). Finally, an expression vector, pCXT20, was developed by cloning of P_{tac}-MCS-4-T_{rrnB} from pKK223-3 into pCXM48 (Fig. 1C).

Development of Expression Vector Systems

Two promoters, P_{sod} and P_{ilvC}, together with P_{tac} described above were selected based on the following reasons. First, the promoter of the *sod* gene was extensively used for metabolic engineering of *C. glutamicum* by exchanging the native promoters of the *dld*, *pyc*, *malE*, *dapB*, *lysC*, and *tkt* genes for the *sod* promoter, which resulted in a marked increase of L-lysine production [23]. Second, the *ilvC* promoter had one of the highest CAT (chloramphenicol acetyltransferase) activities isolated from the chromosomal library of *C. glutamicum* using a promoter-probe vector [26]. To facilitate gene cloning and expression, six bases in the 3′-terminus of a 30 nucleotide (nt) sequence in each promoter were replaced by an *Eco*RI sequence (Fig. 2). As a result, pCXS30 and pCXI40 with P_{sod} and P_{ilvC}, respectively, were constructed (Fig. 3). Meanwhile, the efficiency of translation initiation is known to be crucial for high-level expression of proteins, and is greatly influenced by the accessibility of ribosome to the SD sequence around the translation-initiation region of bacterial mRNAs [25, 30]. The putative SD sequences in P_{sod} and P_{ilvC} were presumed to be 5′-GAAAGGATT-3′ and 5′-GAAAGGCGA-3′ (Fig. 2), respectively, whereas the consensus SD sequence in *C. glutamicum* was proposed to be 5′-GAAAGGAGG-3′ [21]. To enhance the efficiency of translational initiation of protein in the constructed expression vector systems, the putative SD sequence of P_{sod} was replaced with 5′-GAAAGGAGG-3′ to yield pCXS35. In addition, two types of SD sequences, 5′-GAAAGGAGA-3′ and 5′-GAAAGGAGG-3′, were introduced in the presumed SD sequence of P_{ilvC}, resulting in plasmids pCXI43 and pCXI45, respectively (Fig. 3).

Promoter Strength Analysis by qRT-PCR

To evaluate the promoter strength at the transcriptional level, mRNA transcripts of gene *gfp* in six plasmids were measured by qRT-PCR in *C. glutamicum* (Fig. 4 and Supplementary Table S1). The relative transcript level of GFP by P_{sod} was about 2.7 times higher than those for P_{tac} and P_{ilvC}, which indicates that the *sod* promoter is superior to *ilvC* and *tac* promoters with respect to mRNA biosynthesis at the transcriptional level. Besides this, the mRNA transcript level of P_{tac} was similar to that of P_{ilvC}, which was known to be a strong promoter in *C. glutamicum* [26]. This result demonstrates that P_{tac} in *C. glutamicum* functions as a strong promoter with a high transcriptional activity. The P_{sod} and $P_{sod\text{-}M}$ displayed the same average expression levels, whereas $P_{ilvC\text{-}M1}$ and $P_{ilvC\text{-}M2}$ exhibited increased levels through mutations of the SD region in P_{ilvC}. The GFP transcript levels for the six expression vectors were also evaluated by qRT-PCR in *E. coli* (Fig. 4 and Supplementary Table S1). The average transcript levels by P_{sod} and P_{ilvC} promoters were 1.3 times and 3.6 times lower than that by the strong *tac* promoter, respectively, which imply that P_{sod} and P_{ilvC} originating from *C. glutamicum* are functional and can synthesize mRNA transcript in *E. coli*. Interestingly, the transcript level under the control of $P_{sod\text{-}M}$ was 5.9 and 7.6 times higher relative to those under P_{tac} and P_{sod} promoters. Thus, according to analysis of the qRT-PCT, *sod* and *sod*-M promoters showed the strongest transcriptional activity in *C. glutamicum*. In particular, the *sod*-M promoter had the highest level of promoter activity in both *C. glutamicum* and *E. coli*, which may facilitate efficient cloning and characterization of interesting genes/proteins in both strains.

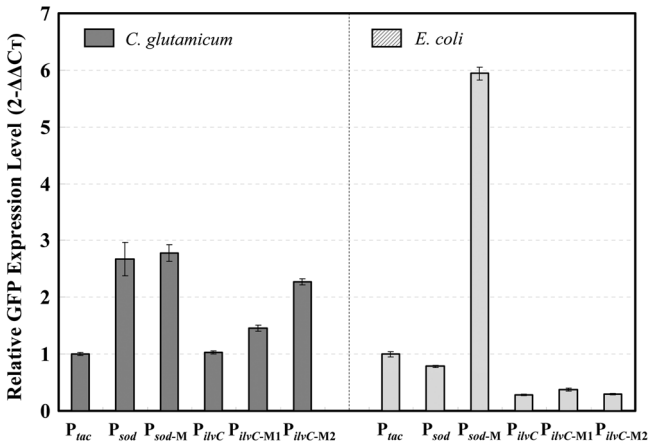

Fig. 4. Relative GFP expression level by quantitative reverse transcription PCR.

Relative GFP expression level means $2^{-(\Delta\Delta C_T)}$, which was calculated from the number of cycles required for the fluorescent signal to reach threshold (C_T). C_T values of 16S rDNAs from *C. glutamicum* and *E. coli* were used for normalization among samples. The relative GFP expression levels represent the x-fold increase in a recombinant cell harboring pCSP30, pCSP35, pCIP40, pCIP43, or pCIP45 (sample $\Delta\Delta C_T$) compared with the corresponding cell with pCTP20 (positive control $\Delta\Delta C_T$). All error bars represent the value of standard deviations, which were calculated from three experiments on the same sample in the same PCR reaction.

Indeed, I expected that the promoter strength at the transcription level is not influenced by variations in the ribosome binding sites of each promoter; however, the increases in transcription activity were observed in *C. glutamicum* with $P_{ilvC\text{-}M1}$ and $P_{ilvC\text{-}M2}$ as well as in *E. coli* harboring $P_{sod\text{-}M}$. By contrast, *C. glutamicum* having $P_{sod\text{-}M}$ along with *E. coli* containing $P_{ilvC\text{-}M1}$ and $P_{ilvC\text{-}M2}$ showed similar transcriptional promoter activities compared with

the activity of the corresponding wild-type promoter for each strain. It has been suggested that the expression of heterologous proteins in recombinant cells is affected by various factors: gene dosage, promoter strength, mRNA stability, and the efficiency of translation initiation [4, 14]. It seems that variations of transcriptional activity in mutant promoters result from the change of mRNA stability in mutant promoters to different sensitivity for endonucleases and exonucleases in each strain [14].

Expression Analyses by GFP Fluorescence Intensity and SDS-PAGE

To compare the developed expression vector systems at the translational level, GFPuv was expressed in the six vectors and then its expression strengths were analyzed using crude extracts of recombinant *C. glutamicum*. Cells harboring plasmids with promoters P_{sod}, $P_{sod\text{-}M}$, P_{ilvC}, $P_{ilvC\text{-}M1}$, and $P_{ilvC\text{-}M2}$ showed higher fluorescence intensities, with an order of activity of $P_{ilvC} > P_{ilvC\text{-}M1} > P_{sod} > P_{ilvC\text{-}M2} > P_{sod\text{-}M}$ (Fig. 5A). However, variants with more conserved SD sequences of *C. glutamicum*, including $P_{sod\text{-}M}$, $P_{ilvC\text{-}M1}$, and $P_{ilvC\text{-}M2}$, exhibited lower GFP fluorescence intensities than recombinant cells with the wild-type promoter P_{sod} or P_{ilvC}. Meanwhile, *C. glutamicum* with pCTP20 expressing GFP under the control of P_{tac} did not express GFP. The GFP expression of each clone in *C. glutamicum* was also confirmed by SDS-PAGE. Denatured crude extracts of *C. glutamicum* harboring plasmids did not show a distinct band corresponding to the molecular mass of about 27 kDa (Fig. 6A). When crude extracts were run on SDS-PAGE and refolded, the fluorescence could be detected in cells harboring pCSP30, pCSP35, pCIP40, pCIP43, and pCIP45 (Fig. 6B), and this result demonstrates that GFP is expressed in the developed expression vector systems. The expression levels (RFI/mg-protein) of all recombinant *C. glutamicum* with pCIP series in the LB medium were maintained consistently with culture time (data not shown), which means that the GFP expression by these promoters was constitutive. The expression strength was also analyzed in *E. coli*. Cells with $P_{sod\text{-}M}$, $P_{ilvC\text{-}M1}$, and $P_{ilvC\text{-}M2}$ revealed a large increase of GFP fluorescence over the corresponding strain with wild-type promoter (Fig. 5B). In particular, cells bearing pCSP35 with GFP attached to $P_{sod\text{-}M}$ displayed a 3.3-fold increase of fluorescence intensity compared with the positive control cells harboring pCTP20 in which GFP was linked to the *tac* promoter. The introduced SD sequences in pCSP35, pCIP43, and pCIP45 showed a high identity with the consensus SD sequence of *E. coli* (5′-AGGAGGT-3′) [14, 19] yielding a strong expression of GFP. A noticeable band

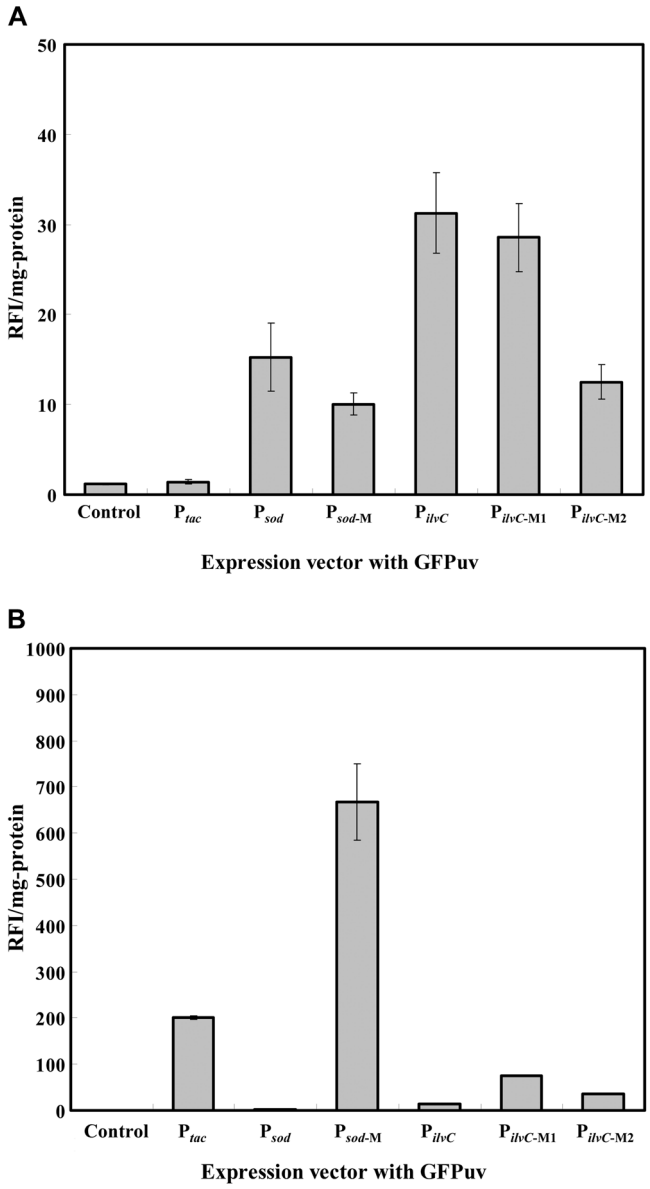

Fig. 5. GFP fluorescence intensities of expression vectors.
(**A**) Relative fluorescence intensity (RFI) of *C. glutamicum* containing vector expressing GFP. (**B**) Relative fluorescence intensity of *E. coli* containing vector expressing GFP. The fluorescence of GFP-harboring crude extracts was measured by using spectrofluorophotometry with excitation at 395 nm and emission at 508 nm. Control means cells harboring pCXM48.

with about 27 kDa appeared in the denatured state of crude extract from *E. coli* Top 10 with pCTP20, pCSP35, or pCIP43 (Fig. 6C). In addition, crude extracts from three types of cells displayed a bright fluorescence band on SDS-PAGE after refolding (Fig. 6D). These results coincided strongly with the result of GFP fluorescence intensity results.

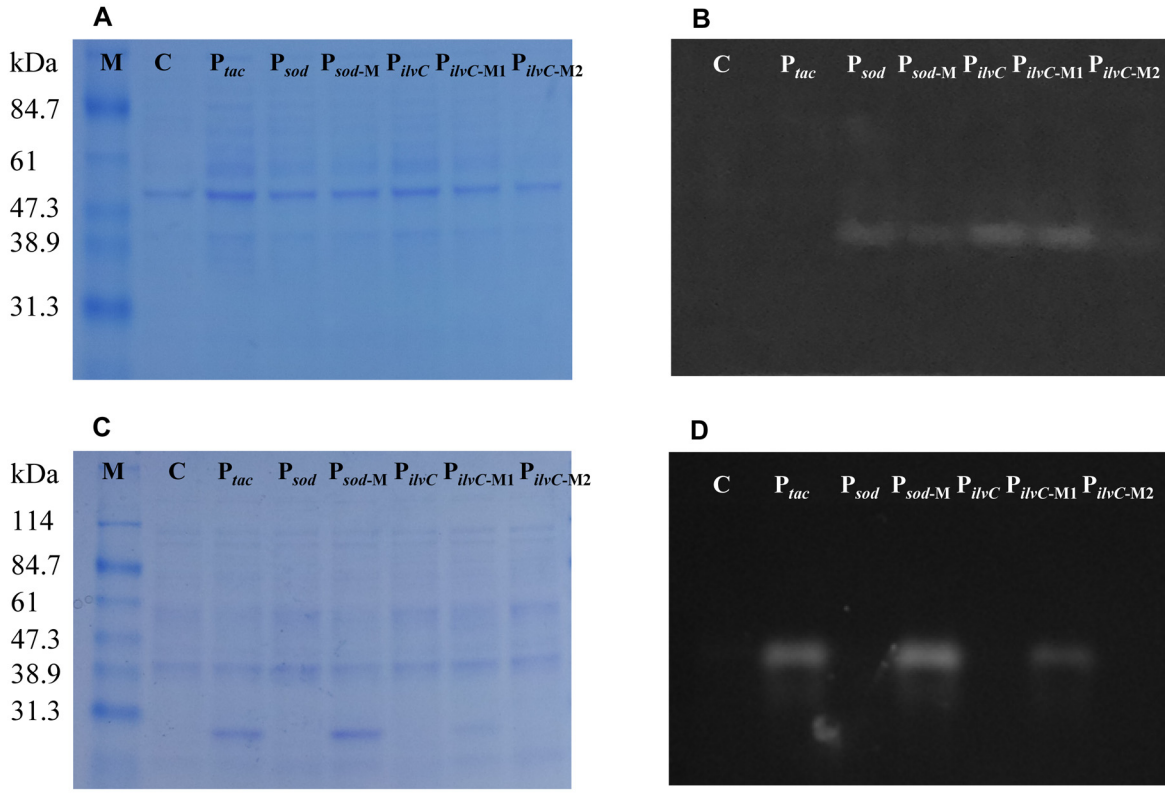

Fig. 6. SDS-PAGE of GFPuv expression in recombinant *C. glutamicum* and *E. coli*.
(**A**) SDS-PAGE of GFP expression with denatured crude extracts of *C. glutamicum*. (**B**) SDS-PAGE of GFP expression with crude extracts from *C. glutamicum*. After running on PAGE, proteins were refolded by washing with 10 mM Tris-HCl for 1 h. (**C**) SDS-PAGE of GFP expression with denatured crude extracts of *E. coli*. (**D**) SDS-PAGE of GFP expression with crude extracts from *E. coli*. After running on PAGE, proteins were refolded by washing with 10 mM Tris-HCl for 1 h. Proteins were separated by 10% SDS. Lanes: M, protein size marker; C, pCXM48; P_{tac}, pCTP20; P_{sod}, pCSP30; $P_{sod\text{-}M}$, pCSP35; P_{ilvC}, pCIP40; $P_{ilvC\text{-}M1}$, pCIP43; $P_{ilvC\text{-}M2}$, pCIP45. The protein loading amounts on gels in **A**, **B**, **C**, and **D** were 10, 20, 10, and 10 μg, respectively.

Considering that analysis of GFP expression, the constructed vectors were working in *C. glutamicum*, and especially $P_{sod\text{-}M}$ and $P_{ilvC\text{-}M1}$ mediated a strong expression of GFP in *E. coli* along with *C. glutamicum*. To conclude, plasmids pCXS-35 and pCXI43 with *sod*-M and *ilv*C-M1 promoter, respectively, provide substantive GFP expressions at both the transcription and translation levels in *C. glutamicum* and *E. coli*.

When target proteins were expressed in *C. glutamicum* for metabolic engineering, proteins were usually expressed and characterized in *E. coli* and then expressed in *C. glutamicum* using other expression vectors working in *C. glutamicum*. It is necessary to provide additional genetic manipulation for fine-tuning protein expression. In this sense, the expression vector systems, including those with *sod*-M and *ilv*C-M1 promoters, will afford efficient cloning and expression of interesting proteins in *C. glutamicum* without additional genetic work for metabolic engineering.

According to results regarding promoter strength analyses by qRT-PCR, GFP fluorescence, and SDS-PAGE, I found that GFP expression at the transcription level was not completely correlated with that at the translation level. Recently, many studies have demonstrated that protein expression levels are strongly influenced by the mRNA secondary structure and the accessibility of ribosome to the SD sequence around the translational-initiation region (TIR), as well as by the promoter strength [25, 30, 33]. Romasi and Lee [28] demonstrated that although the *tac* promoter has a strong transcriptional activity, IpdC was well expressed by P_{tac} but not AspC, whereas the *sod* promoter mediated the expression of AspC but not IpdC in *E. coli*. This suggests that the weak expression of AspC by P_{tac} is caused by a more stable mRNA secondary structure of TIR in P_{tac}-*aspC* than that in P_{sod} -*aspC*. Hence, the mismatch between transcription activity and protein

expression in this study was also caused by various factors such as promoter strength, mRNA stability, and the efficiency of translation initiation.

In conclusion, I developed expression vector systems for *C. glutamicum* with three types of promoters and their derivatives, which are known to function in *C. glutamicum*, and characterized each promoter's capability at the transcriptional and translational levels by analyses of qRT-PCR, GFP fluorescence, and SDS-PAGE. All the expression vectors work in both *C. glutamicum* and *E. coli*, which would facilitate efficient cloning and characterization of interesting genes/proteins in *E. coli*, at first, and then implement metabolic engineering of *C. glutamicum* without additional genetic works for fine-tuning of protein expression. In addition, the developed expression vectors and pCES208, another shuttle vector, have the same multiple cloning sites and different replication origins, which will be able to conveniently clone and express many target genes in *C. glutamicum* with two different vectors for over-production of valuable metabolites or proteins. I expect that the developed expression vector systems will apply to the study of genetics, physiology, and metabolic engineering of *C. glutamicum*.

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