Construction of a *Vitreoscilla* Hemoglobin Promoter-Based Tunable Expression System for *Corynebacterium glutamicum*

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Abstract: *Corynebacterium glutamicum* is an industrial strain used for the production of valuable chemicals such as L-lysine and L-glutamate. Although *C. glutamicum* has various industrial applications, a limited number of tunable systems are available to engineer it for efficient production of platform chemicals. Therefore, in this study, we developed a novel tunable promoter system based on repeats of the *Vitreoscilla* hemoglobin promoter (P_{vgb}). Tunable expression of green fluorescent protein (GFP) was investigated under one, four, and eight repeats of P_{vgb} (P_{vgb}, P_{vgb4}, and P_{vgb8}). The intensity of fluorescence in recombinant *C. glutamicum* strains increased as the number of P_{vgb} increased from single to eight (P_{vgb8}) repeats. Furthermore, we demonstrated the application of the new P_{vgb} promoter-based vector system as a platform for metabolic engineering of *C. glutamicum* by investigating 5-aminovaleric acid (5-AVA) and gamma-aminobutyric acid (GABA) production in several *C. glutamicum* strains. The profile of 5-AVA and GABA production by the recombinant strains were evaluated to investigate the tunable expression of key enzymes such as DavBA and $GadB_{mut}$. We observed that 5-AVA and GABA production by the recombinant strains increased as the number of P_{vgb} used for the expression of key proteins increased. The recombinant *C. glutamicum* strain expressing DavBA could produce higher amounts of 5-AVA under the control of P_{vgb8} (3.69 ± 0.07 g/L) than the one under the control of P_{vgb} (3.43 ± 0.10 g/L). The average gamma-aminobutyric acid production also increased in all the tested strains as the number of P_{vgb} used for $GadB_{mut}$ expression increased from single (4.81–5.31 g/L) to eight repeats (4.94–5.58 g/L).

Keywords: *Corynebacterium glutamicum*; P_{vgb}; tunable expression system; expression vectors; synthetic biology; *Vitreoscilla*; *vgb*

1. Introduction

Biorefinery processes have been developed to establish a sustainable alternative to produce chemicals, polymers, and fuels, and they currently rely on petroleum-based processes [1–17]. Development of recombinant microorganisms capable of converting a broader range of renewable biomass feedstock into bio-based chemicals, with properties comparable to those of conventional petrochemical products, have been extensively studied [18–27]. For example, processes for the production of bio-polymers, such as polylactic acid (PLA) and polybutylene succinate (PBS) in a biorefinery have been established [21–23]. Polymerization of microbial fermentation derived-monomers, such as diamines, dicarboxylic acids, and amino carboxylic acids, yields these bio-based polymers [4,5]. *Escherichia coli* is one of the most commonly used microorganisms for the production of monomers used to prepare bio-based polymers [1,4,5]. However, production of amino acid-derived products such as 5-aminovaleric acid (5-AVA) and gamma-aminobutyric acid (GABA) from glucose using recombinant *E. coli* is limited, due to its inability to accumulate important precursors such as L-lysine and L-glutamate [24,27]. In this regard, *Corynebacterium glutamicum*, which is a generally recognized as safe (GRAS) strain, is an excellent workhorse to develop because of its innate ability to produce large amounts of amino acids such as L-lysine and L-glutamate [1,4,5]. Several studies have demonstrated that *C. glutamicum* can produce high levels of bio-based nylon monomers such putrescine, succinic acid [4], GABA [27], cadaverine [28,29], 5-AVA and glutaric acid, [30]. Bio-based nylon-4, nylon-5 and nylon-6,5 can be produced from butyrolactam and Δ-valerolactam, which are derived from GABA and 5-AVA, respectively [4]. Biosynthetic production of both 5-AVA and GABA is more sustainable than chemical production because the fermentative production of 5-AVA and GABA is conducted under mild conditions with high catalytic efficiency [4,6,24–27,31]. In addition, 5-AVA and GABA have been successfully produced from biomass-derived sugars such as *Miscanthus* hydrolysate and empty fruit bunch biosugar solution [6,31].

To engineer *C. glutamicum* for biochemical production, its metabolic pathway has been manipulated using a plasmid-based expression system, which establishes the synthetic pathway for the production of biochemicals [5]. This is an important step for evaluating the success of the constructed pathway and for establishing the target metabolite production in recombinant strains. This also helps identify which key reactions can be improved in order to drive metabolic flux toward optimized chemical production [1,4,5]. For example, the production of GABA using *C. glutamicum* strains was established by using a synthetic promoter-based expression vector system ($P_{L26} < P_{I16} < P_{H36}$) capable of low, intermediate, and high-strength glutamate decarboxylase ($GadB_{mut}$) expression. It was demonstrated that the use of the high-strength promoter P_{H36} in the pHGmut strain (5.89 ± 0.35 g/L) enabled higher production of GABA than in pIGmut (5.32 ± 0.04 g/L) and pLGmut (4.87 ± 0.15 g/L) strains with expression of $GadB_{mut}$ under intermediate (P_{I16}) and low (P_{L26}) strength promoters, respectively [26]. Cadaverine production using a synthetic promoter-based expression system ($P_{L10} < P_{L26} < P_{I16} < P_{I64} < P_{H30} < P_{H36}$) was also evaluated, and it was observed that a high level of protein expression using the strongest promoter (P_{H36}) does not necessarily result in the highest level of metabolite production [28,29]. In case of batch fermentation for cadaverine production using recombinant *C. glutamicum* strains, the use of P_{H30} (23.8 g/L) for the expression of lysine decarboxylase produced a higher titer than that when the P_{H36} promoter was used (21.3 g/L) [28]. However, the repertoire of vector systems capable of different levels of protein expression in *C. glutamicum* is still limited [5]. Most promoters in plasmids derived from *E. coli* plasmids such as P_{tac}, P_{trc}, P_{lacUV5}, P_R, and P_L have been evaluated [4,5]. However, despite the adoption of the promoters from *E. coli* plasmids, the variety of genetic engineering tools applicable to *C. glutamicum* is still less compared to the tools available for *E. coli* [1,4,5]. Therefore, the discovery or creation of a new vector system and evaluation of its capability for tunable protein expression in a target host strain are important factors in developing recombinant strains for the production of biochemicals in biorefineries [4,5].

Recently, the use of five repeats of the P_{tac} promoter successfully enabled stable overexpression of *phaCAB* genes in recombinant *E. coli* and enhanced poly(R-3-hydroxybutyrate) (PHB) accumulation

5.6 times that of the control strain, which had a single copy of the P_{tac} promoter [32]. In another report, the use of the promoter from *Vitreoscilla* hemoglobin protein (P_{vgb}) was successfully demonstrated in *E. coli*. It was found that the expression vectors with increasing repeats of the P_{vgb} promoter enabled tunable expression of the PHB synthesis operon (*phaCAB*) and allowed enhanced accumulation of poly(hydroxybutyrate) (PHB) [33]. The highest accumulation of 90% PHB in 5.37 g/L CDW was achieved by the recombinant strain with eight repeats of the P_{vgb} promoter. The use of the P_{vgb} promoter system was also successfully demonstrated in recombinant *C. glutamicum*. It was used to investigate the effect of VHb protein expression on L-glutamate and L-glutamine production in the recombinant strains. However, it was observed that the expression of the VHb protein under the P_{tac} promoter was better than that with the use of a single repeat of P_{vgb} promoter (1.71 $\pm$ 0.08 nmol/g > 0.69 $\pm$ 0.10 nmol/g) [34]. Therefore, in this study, new tunable gene expression systems were developed based on repeats of P_{vgb}. The strength of the P_{vgb} promoter-based expression systems with increasing repeats of P_{vgb} were investigated by evaluating the expression level of GFP in recombinant strains. To demonstrate the use of the new tunable P_{vgb} promoter-based expression system as a novel platform for metabolic engineering of *C. glutamicum*, 5-AVA and GABA production were established in different strains of *C. glutamicum*. 5-AVA and GABA production were selected as model compounds, because they are derived from L-lysine and L-glutamate, respectively.

2. Results and Discussion

2.1. Construction of P_{vgb} Promoter-Based Tunable Expression Systems for C. glutamicum and Evaluation of Green Fluorescent Protein in the Recombinant Strains

To investigate the tunability of protein expression using P_{vgb} in recombinant *C. glutamicum*, several expression vector systems based on pCES208 plasmids were constructed. GFP was expressed in *C. glutamicum* under the control of increasing repeats of P_{vgb} (P_{vgb}, P_{vgb4}, P_{vgb8}). The strength of protein expression in the three constructs was investigated by measuring the intensity of fluorescence in the recombinant strains, by using the fluorescent activated cell sorting analysis (FACS) (Figure 1) [35]. The resulting recombinant *C. glutamicum* EGFPV1, EGFPV4, and EGPV8 strains harbored one (P_{vgb}), four (P_{vgb4}), and eight (P_{vgb8}) repeats of P_{vgb}, respectively. *C. glutamicum* KCTC 1857 was cultured and used as the negative control. Colonies were randomly picked and GFP expression in each cell was evaluated by using FACS. The measured fluorescence intensity obtained from colonies with P_{vgb}-promoter-based vectors ranged from 10^2–10^3, whereas the negative control cells did not show significant fluorescence. The fluorescence by clones with P_{vgb8} were more intense than the clones with P_{vgb4} and P_{vgb}, after 24 h of cultivation (Figure 2). However, after 48 h, no significant difference was observed in GFP expression between clones with P_{vgb4} and those with P_{vgb8} (data not shown). The increasing number of P_{vgb} in the constructed expression system was directly proportional to the intensity of the GFP fluorescence in the resulting recombinant strains (Figure 2). These observations were similar to the PHB production enhancement in recombinant *E. coli* strains harboring the PHB operon (*phaCAB*) under the control of P_{vgb8} compared to the use of P_{vgb}. Based on these results, we have demonstrated that the strength of protein expression using P_{vgb} was tunable by modulating the number of its copies in the constructed expression system. To further demonstrate tunability of the P_{vgb} promoter-based expression system in recombinant *C. glutamicum*, the developed constructs were used to produce 5-AVA and GABA from L-lysine and L-glutamate, respectively. 5-AVA and GABA were selected as model compounds to test the application of the P_{vgb} promoter-based expression system because their production in *C. glutamicum* has been extensively studied in recent times [6,25,26,29–31].
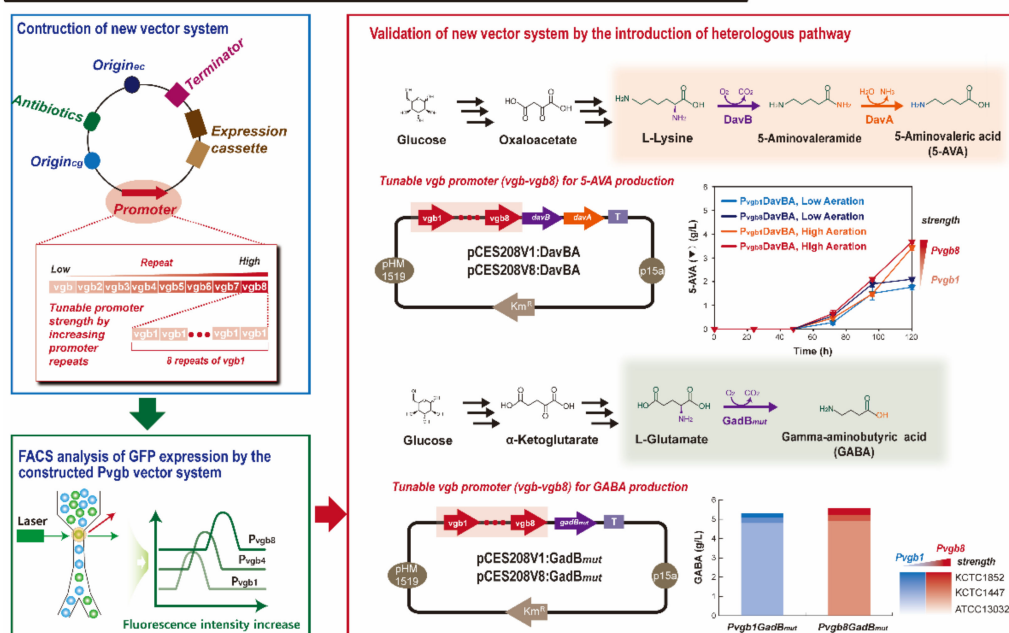
Figure 1. Schematic representation of the development of the *C. glutamicum* P$_{vgb}$ promoter-based vector system equipped with P$_{vgb}$ promoter.
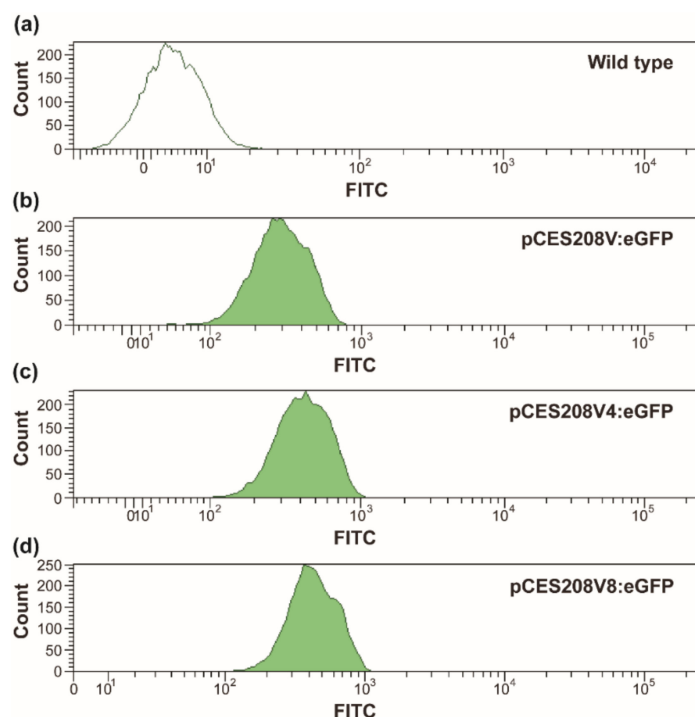

Figure 2. The fluorescence intensity of the recombinant EGFPV1 (**b**), EGFPV4 (**c**), and EGPV8 (**d**) strains after 24 h of cultivation. The wild type strain, *C. glutamicum* KCTC 1857 (**a**) was used as negative control.

2.2. 5-AVA Production Using Recombinant C. glutamicum with Tunable P$_{vgb}$ Promoter-Based Expression Systems

To demonstrate that the P$_{vgb}$ promoter-based expression system is a new platform for engineering better *C. glutamicum* strains, its feasibility to produce 5-AVA in *C. glutamicum* KCTC 1857 was evaluated [6,25,30]. Tunability of protein expression by P$_{vgb}$ was verified by developing *C. glutamicium* 5AVA1 and 5AVA8 strains for the expression of lysine 2-monooxygenase (DavB)

and delta-aminovaleramidase (DavA) under the control of single (P_{vgb}) and eight repeats (P_{vgb8}) of P_{vgb} [6,25,30]. 5-AVA production using the recombinant strains was evaluated in flask cultivation under low and high aeration conditions, as it was previously demonstrated that protein expression using P_{vgb} was enhanced under microaerobic conditions in recombinant *E. coli* strains [34]. The production of 5-AVA by the recombinant *C. glutamicum* 5AVA1 (3.43 ± 0.10 g/L) and 5AVA8 (3.68 ± 0.07 g/L) strains was better under high aeration conditions than at low aeration conditions (2.10–1.76 g/L) (Figure 3). Higher accumulation of L-lysine and glutarate was also detected after flask cultivation of 5AVA1 and 5AVA8 strains under high aeration conditions (4.41–4.46 g/L of L-lysine and 0.71–0.74 g/L of glutarate) than that at low aeration (3.99–4.58 g/L of L-lysine and 0.49–0.57 g/L of glutarate) (Figure 3c,d). 5-AVA production by all the recombinant strains were higher under high aeration conditions than that under flask cultivation with low aeration conditions. These results were similar to the trend of L-glutamate and L-glutamine production by recombinant *C. glutamicum* strains, which expressed the *Vitreoscilla* hemoglobin gene under P_{vgb} and P_{tac}, wherein chemical production was higher under high aeration than at low aeration [34]. This is because *C. glutamicum* is an obligate aerobic microorganism, and it requires high aeration for efficient amino acid production [1,4,5]. Additionally, because DavB requires oxygen as a co-substrate, higher aeration conditions are preferred for 5-AVA production [30].

Furthermore, the effect of eight repeats of P_{vgb} on 5-AVA production was evaluated to demonstrate the tunability of protein expression using the constructed P_{vgb} promoter-based vector system (Figure 3). Different levels of 5-AVA production were achieved when the number of P_{vgb} increased from single (P_{vgb}) to eight repeats (P_{vgb8}) (Figure 3). It was observed that 5-AVA production increased based on the number of repeats of P_{vgb}. 5-AVA production by the 5AVA8 strain (Figure 3d, 3.69 ± 0.07 g/L), which expressed DavBA under P_{vgb8}, was higher than that of the 5AVA1 strain (Figure 3c, 3.43 ± 0.10 g/L)), which harbored P_{vgb}. 5-AVA production by 5AVA1 and 5AVA8 strains (3.43–3.69 g/L) were comparably higher than that of the H30_AVA strain, which expressed DavBA under the strong synthetic promoter P_{H30} (1.4 ± 0.3 g/L g/L) (Table S1) [30]. This effect was similar to the observed trend of enhanced PHB accumulation in recombinant *E. coli* strains when the number of P_{tac} and P_{vgb} repeats increased to five and eight, respectively [32,33].
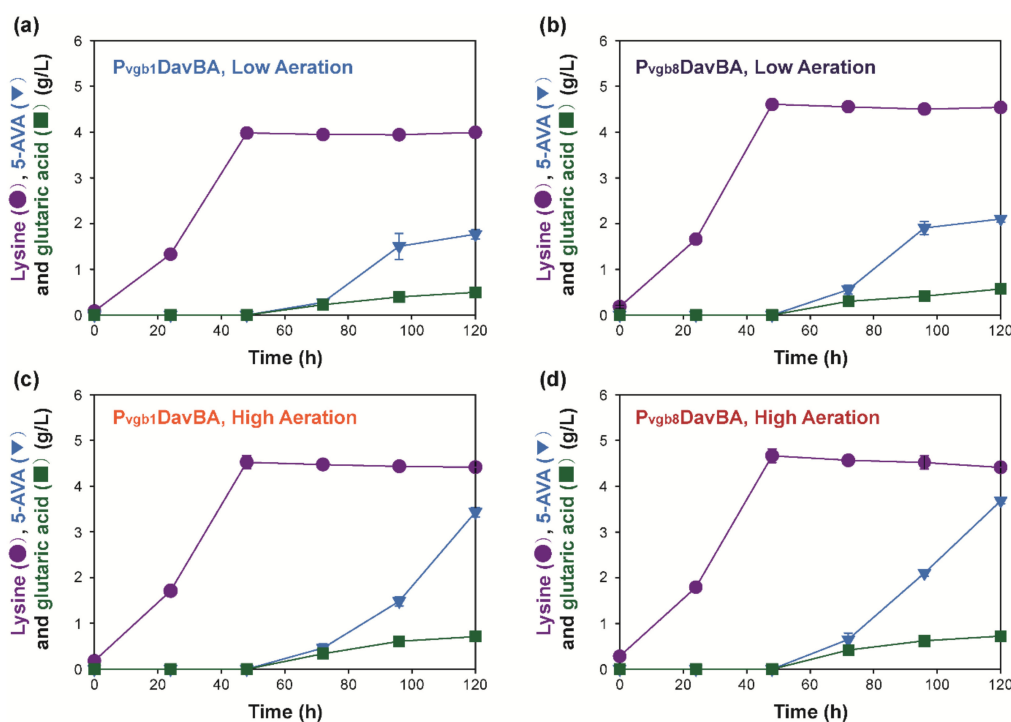

Figure 3. Time profile of 5-aminovaleric acid, L-lysine and glutaric acid production by recombinant *C. glutamicum* 5AVA1 (**a**, **c**) and 5AVA8 (**b**,**d**) strains under low (**a**,**b**) and high (**c**,**d**) aeration.

Finally, the effect of his-tagged DavB on 5-AVA production was also investigated, because we have previously reported that it enhanced 5-AVA production in *C. glutamcium* H30_AVA$_{His}$. In our previous study, we demonstrated enhanced 5AVA production by expressing his-tagged DavB along with DavA under the strong synthetic promoter P$_{H30}$ in H30_AVA$_{His}$ strain, which produced 4.2 ± 0.9 g/L of 5-AVA [30]. This significantly increased 5-AVA production compared to that by the H30_AVA strain, which expressed DavB without his-tag (4.2 ± 0.9 g/L >1. 4 ± 0.3 g/L) (Figure 4) [30]. Therefore, to investigate the effect of this additional his-tag on 5-AVA production under the control of the P$_{vgb}$ promoter system, recombinant strains 5AVA1$_{His}$ and 5AVA8$_{His}$ were constructed by inserting his-tagged DavB into the P$_{vgb}$ promoter system with single (P$_{vgb}$) and eight (P$_{vgb8}$) repeats, respectively. 5-AVA production by the resulting recombinant strains were only evaluated under high aeration conditions, as the low aeration condition did not increase 5-AVA production in 5AVA1 and 5AVA8 strains (Figure 3). Under high aeration conditions, the 5AVA8$_{His}$ (3.31 ± 0.08 g/L) strain produced higher concentrations of 5-AVA than the 5AVA1$_{His}$ (2.82 ± 0.03 g/L) strain (Figure 4, Table S1). As shown in Figure 4, 5-AVA production increased as the number of P$_{vgb}$ repeats increased from single to eight in 5AVA1$_{His}$ and 5AVA8$_{His}$ (3.31 ± 0.08 g/L > 2.82 ± 0.03 g/L). Glutaric acid production also increased in both 5AVA1$_{His}$ (0.64 ± 0.01 g/L) and 5AVA8$_{His}$ (0.67 ± 0.05 g/L). Interestingly, both L-lysine accumulation and 5-AVA production increased as the number of repeats of P$_{vgb}$ increased from a single repeat in 5AVA1$_{His}$ (4.00 ± 0.06 g/L) to eight repeats in the 5AVA8$_{His}$ (4.38 ± 0.03 g/L) strain. Based on these observations, the P$_{vgb}$ promoter system was successfully used as a new platform for 5-AVA production in *C. glutamicum*.
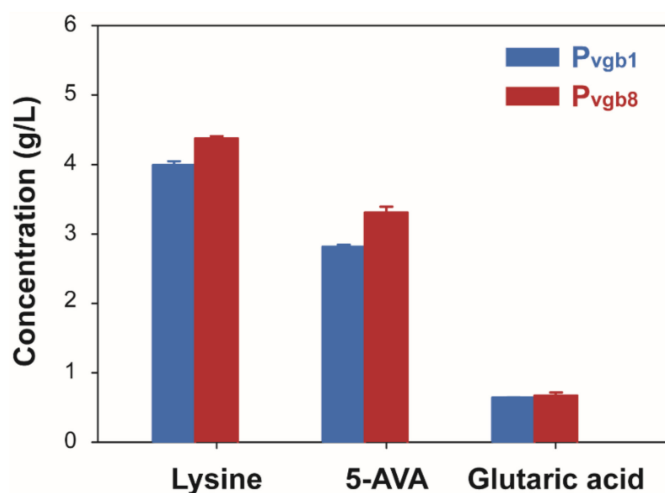

Figure 4. 5-Aminovaleric acid, L-lysine, and glutaric acid production by recombinant *C. glutamicum* 5AVA1$_{His}$ and 5AVA8$_{His}$ strains under high aeration conditions after 120 h flask cultivation.

2.3. Gamma-Aminobutyric Acid Production Using Recombinant C. glutamicum with Tunable P$_{vgb}$ Promoter-Based Expression Systems

The constructed P$_{vgb}$ promoter-based vector system was also used for the expression of mutated glutamate decarboxylase (GadB$_{mut}$) in order to investigate GABA production in the *C. glutamicum* strain ATCC 13032 and in high-L-glutamate producing strains *C. glutamicum* KCTC 1447 and *C. glutamicum* KCTC 1852 as host strains (Figure 5, Table S2) [26,31]. *C. glutamicum* KCTC 1447 and *C. glutamicum* KCTC 1852 were used because they are capable of high production of L-glutamate, an important precursor for GABA production [26,31]. *C. glutamicum* ATCC 13032 was also used as control host strain [30]. The effect of the eight repeats of P$_{vgb}$ on GABA production was evaluated under high aeration conditions because we have previously demonstrated that low aeration did not increase chemical production by recombinant strains expressing DavBA under single and eight repeats of P$_{vgb}$ during shake flask cultivation (Figure 3). It was observed that the average GABA production by *C. glutamicum* KCTC 1852-derived strains (5.31 g/L–5.58 g/L) was higher than that by recombinant

C. glutamicum ATCC 13032 and *C. glutamicum* KCTC 1447 strains (4.82–5.22 g/L) (Figure 5, Figure S3). This is because the host strain, *C. glutamicum* KCTC 1852, is naturally capable of higher L-glutamate production than *C. glutamicum* KCTC 1447 and *C. glutamicum* ATCC 13032 [30]. Higher GABA production was attributed to the efficient production of L-glutamate, an important pre-requisite for the development of strains for GABA production [26,31]. The level of GABA production in all the tested strains increased as the number of P_{vgb} repeats increased (Figure 5). For example, GABA production in V1GD1852 (5.31 ± 0.16 g/L) and V8GD1852 (5.58 ± 0.27 g/L) strains increased when GadB$_{mut}$ was expressed under P_{vgb} and P_{vgb8}, respectively. The same tendency was observed in V1GD13032 (4.82 ± 0.09 g/L) and V1GD1447 (5.09 ± 0.64 g/L) strains with respect to GABA production, under the control of the P_{vgb} promoter, and by V8GD13032 (4.94 ± 0.27 g/L) and V8GD1447 (5.22 ± 0.60 g/L) strains under the control of the P_{vgb8} promoter. Regarding GABA production using *C. glutamicum* ATCC 13032 strains (4.82–4.94 g/L), similar GABA production was achieved by pLGmut (4.87 ± 0.15 g/L), V1GD13032 (4.82 ± 0.09 g/L), and V8GD13032 (4.94 ± 0.27 g/L) strains, which expressed GadB$_{mut}$ under the synthetic promoters P_{L26}, P_{vgb}, and P_{vgb8}, respectively (Figure 4, Table S2) [26]. The level of GABA production using the constructed P_{vgb} promoter-based expression system (4.82–5.09 g/L) was comparable to the titers achieved by recombinant strains pHGmut (5.89 ± 0.35 g/L) and pIGmut (5.32 ± 0.04 g/L). The recombinant strains expressed GadB$_{mut}$ under synthetic promoters of high (P_{H36}) and intermediate (P_{I16}) strength [31]. The level of GABA production by the *C. glutamicum* KCTC 1852-derivative strains V1GD1852 (5.31 ± 0.16) and V8GD1852 (5.58 ± 0.27), expressing GadB$_{mut}$, were similar to the level of GABA production achieved when the strong synthetic promoter P_{H36} was used in the H36GM1852 strain (8.47 ± 0.06 g/L) (Table S2). This shows that the strength of GadB$_{mut}$ expression by the P_{vgb} promoter-based expression system is also tunable like previously used synthetic promoters (P_{L26}, P_{I16}, P_{H36}) (Table S2) [26,31]. Based on these results, we concur that the P_{vgb}-based expression system was capable of tunable expression of key proteins for 5-AVA (DavBA) and GABA (GadB$_{mut}$) production by increasing the number of P_{vgb} repeats. The addition of the newly constructed P_{vgb} promoter-based expression system into the repertoire of plasmids available for metabolic engineering of *C. glutamicum* provides an alternative method of fine-tuning protein expression levels for validating synthetic metabolic pathways and improving biochemical production in biorefineries.
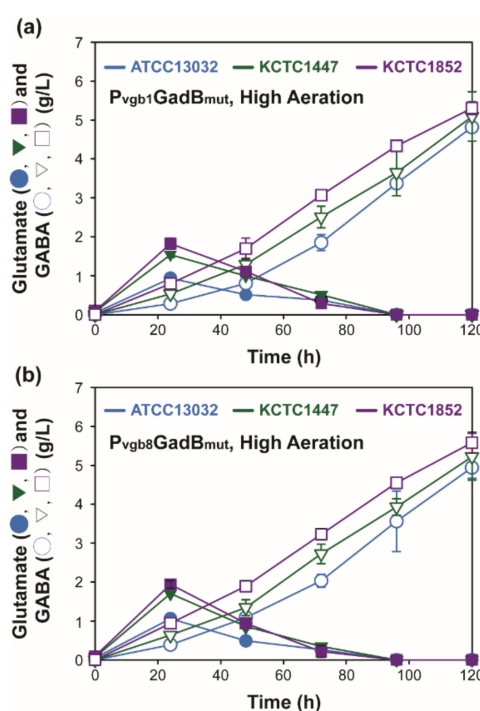

Figure 5. Time profile of Gamma-aminobutyric acid production by recombinant *C. glutamcium* strains expressing GadB$_{mut}$ under P_{vgb} (**a**) and P_{vgb8} (**b**) under high aeration condition.

3. Materials and Methods

3.1. Bacterial Strains and Plasmids

All the bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* XL1-Blue (Stratagene, La Jolla, CA, USA) was used for the general gene cloning studies. *C. glutamicum* ATCC 13032, *C. glutamicum* KCTC 1447, *C. glutamicum* KCTC 1852, and *C. glutamicum* KCTC 1857 strains were purchased from the Korean Collection for Type Cultures (KCTC, Daejeon, Korea). The pCES208-based plasmids: pCES208H30:DavBA, pCES208H30:DavB$_{His}$A and pHG$_{mut}$ were constructed as previously described [26,30].

3.2. Plasmid Construction

All DNA manipulations were performed following standard procedures [36]. Polymerase chain reaction (PCR) was performed with the C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA). The primers used in this study were synthesized at Bioneer (Daejeon, Korea). The pHG$_{mut}$ plasmid was cut at KpnI and BamHI sites to replace the synthetic promoter P$_{H36}$ with P$_{vgb}$ promoter. The subsequent repeats of the P$_{vgb}$ promoter were inserted into the pCES208V:eGFP vector at BamHI/BglII sites to obtain pCES208V4:eGFP and pCES208V8:eGFP plasmids. The pCES208V:eGFP, pCES208V4:eGFP, and pCES208V8:eGFP plasmids for the expression of enhanced GFP were constructed by inserting GFP at the BamHI/NotI sites of modified pCES208-based plasmids with one, four, and eight repeats of P$_{vgb}$, respectively. The plasmids for the expression of DavBA, DavB$_{His}$A, and GadB$_{mut}$ were also inserted at the BamHI/NotI sites of pCES208V:eGFP and pCES208V8:eGFP (Table 1).

Table 1. Strains and plasmids used in the present study.

Strains and Plasmids	Relevant Characteristics	Reference
Strains		
E. coli XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [FA1*proAB lacᵍZΔM15* Tn*10* (Tet^R)]	Stratagene
C. glutamicum KCTC 1447	L-Glutamate producer	KCTC
C. glutamicum KCTC 1852	L-Glutamate producer	KCTC
C. glutamicum KCTC 1857	L-Lysine producer	KCTC
C. glutamicum ATCC 13032	Wild type	ATCC
EGFPV1	*C. glutamicum* KCTC 1857 with pCES208V:eGFP	This study
EGFPV4	*C. glutamicum* KCTC 1857 with pCES208V4:eGFP	This study
EGFPV8	*C. glutamicum* KCTC 1857 with pCES208V8:eGFP	This study
5AVA1	*C. glutamicum* KCTC 1857 with pCES208V:DavBA	This study
5AVA8	*C. glutamicum* KCTC 1857 with pCES208V8:DavBA	This study
5AVA1$_{His}$	*C. glutamicum* KCTC 1857 with pCES208V:DavB$_{His}$DavA	This study
5AVA8$_{His}$	*C. glutamicum* KCTC 1857 with pCES208V8:DavB$_{His}$DavA	This study
H30_AVA	*C. glutamicum* KCTC 1857 with pCES208H30:DavBA	30
H30_AVA$_{His}$	*C. glutamicum* KCTC 1857 with pCES208H30:DavB$_{His}$A	30
H36GM13032	*C. glutamicum* ATCC 13032 with pCES208H36:GadB$_{mut}$	This study
H36GM1447	*C. glutamicum* KCTC 1447 with pCES208H36:GadB$_{mut}$	This study
H36GM1852	*C. glutamicum* KTC 1852 with pCES208H36:GadB$_{mut}$	This study
H30GM13032	*C. glutamicum* ATCC 13032 with pCES208H30:GadB$_{mut}$	This study
H30GM1447	*C. glutamicum* KCTC 1447 with pCES208H30:GadB$_{mut}$	This study
H30GM1852	*C. glutamicum* KTC 1852 with pCES208H30:GadB$_{mut}$	This study
V1GD133032	*C. glutamicum* ATCC 13032 with pCES208V:GadB$_{mut}$	This study
V1GD1447	*C. glutamicum* KCTC 1447 with pCES208V:GadB$_{mut}$	This study
V1GD1852	*C. glutamicum* KTC 1852 with pCES208V:GadB$_{mut}$	This study
V8GD133032	*C. glutamicum* ATCC 13032 with pCES208V8:GadB$_{mut}$	This study
V8GD1447	*C. glutamicum* KCTC 1447 with pCES208V8:GadB$_{mut}$	This study
V8GD1852	*C. glutamicum* KTC 1852 with pCES208V8:GadB$_{mut}$	This study
Plasmids		
pCES208V:eGFP	pCES208 derivative; P$_{vgb}$ promoter, eGFP, Km^R	This study
pCES208V4:eGFP	pCES208 derivative; P$_{vgb4}$ promoter, eGFP, Km^R	This study
pCES208V8:eGFP	pCES208 derivative; P$_{vgb8}$ promoter, eGFP, Km^R	This study

Table 1. *Cont.*

Strains and Plasmids	Relevant Characteristics	Reference
pCES208V:DavBA	pCES208 derivative; P_{vgb} promoter, *Pseudomonas putida* KT2440 *davBA* genes, Km^R	This study
pCES208V8:DavBA	pCES208 derivative; P_{vgb8} promoter, *Pseudomonas putida* KT2440 *davBA* genes, Km^R	This study
pCES208V:DavB$_{His}$A	pCES208 derivative; P_{vgb} promoter, *Pseudomonas putida* KT2440 *davB$_{His}$* and *davA* genes, Km^R	This study
pCES208V8:DavB$_{His}$A	pCES208 derivative; P_{vgb8} promoter, *Pseudomonas putida* KT2440 *davB$_{His}$* and *davA* genes, Km^R	This study
pCES208H30:DavBA	pCES208 derivative; P_{H30} promoter, *Pseudomonas putida* KT2440 *davBA* genes, Km^R	30
pCES208H30:DavB$_{His}$A	pCES208 derivative; P_{H30} promoter, *Pseudomonas putida* KT2440 *davB$_{His}$* and *davA* genes, Km^R	30
pCES208V:GadB$_{mut}$	pCES208 derivative; P_{vgb} promoter, *E. coli* mutated gadB (Glu89Gln/Δ452-466) gene, Km^R	This study
pCES208V8:GadB$_{mut}$	pCES208 derivative; P_{vgb8} promoter, *E. coli* mutated gadB (Glu89Gln/Δ452-466) gene, Km^R	This study
pHG$_{mut}$ / pCES208H36:GadB$_{mut}$	pCES208 derivative; P_{H36} promoter, *E. coli* mutated gadB (Glu89Gln/Δ452-466) gene, Km^R	26
pCES208H30:GadB$_{mut}$	pCES208 derivative; P_{H30} promoter, *E. coli* mutated gadB (Glu89Gln/Δ452-466) gene, Km^R	This study

3.3. Culture Conditions

E. coli XL1-Blue, used for general gene cloning experiments, was cultured at 37 °C in Luria-Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl). Flask cultures of the recombinant strains of *C. glutamicum* were obtained in triplicates by culturing at 30 °C and 250 rpm in a rotary shaker. High aeration conditions for flask cultivation were maintained by adding 20 mL of the appropriate culture medium into a 250-mL baffled flask. Low aeration conditions for flask cultivation were maintained by adding 100 mL of the culture medium into a 250-mL glass flask. Seed cultivation of *C. glutamicum* strains was carried out in 14-mL round-bottomed tubes containing 2 mL of Recovery Growth (RG) medium (10 g/L of glucose, 40 g/L of brain heart infusion, 10 g/L of beef extract, and 30 g/L of D-sorbitol) with incubation overnight at 30 °C and 250 rpm [29]. The main-flask cultures for 5-AVA production were grown in 250 mL baffled flasks containing 20 mL of CG50 medium for 120 h at 30 °C and 250 rpm. The CG50 medium for flask cultivation contained (per liter) 50 g glucose, 15 g yeast extract, 15 g $(NH4)_2SO_4 \cdot 7H_2O$, 0.5 g KH_2PO4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $MnSO_4 \cdot H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, and 20 μg/L of kanamycin (Km) for plasmid maintenance [30]. The GP1 medium optimized in our previous study [26] was used for GABA production. The GP1 medium for flask cultivation contained (per liter) 50 g $(NH_4)_2SO_4 \cdot 7H_2O$, 1 g K_2HPO_4, 3 g urea, 0.4 g $MgSO_4 \cdot 7H_2O$, 50 g peptone, 0.02 g $FeSO_4 \cdot 7H_2O$, 0.007 g $MnSO_4 \cdot H_2O$, 200 μg thiamine, and 1 mM of pyridoxal 5′-phosphate hydrate (PLP) [26]. PLP was added to the culture medium as it is a cofactor of glutamate decarboxylase. Moreover, 0.1 mM of PLP was the optimum concentration for prolonging GABA production using recombinant *C. glutamicum* strains [26]. Kanamycin and biotin were added to the GP1 culture medium at 25 and 50 μg/L, respectively. Only 50 μg/L of biotin was used in flask cultivation for GABA production because biotin-limited condition promotes L-glutamate accumulation [26]. $CaCO_3$ was added to the culture medium at 10 g/L to minimize the pH change during cultivation.

3.4. Analysis

The concentrations of glucose and organic acids were determined by high performance liquid chromatography (HPLC). The standard and sample concentrations of 5-AVA, GABA, L-lysine, and L-glutamate were determined by HPLC using an Optimapak C18 column (RStech, DaeJeon, Korea) as previously reported [37].

3.5. Fluorescence-Activated cell Sorting Analysis (FACS) for Measuring GFP Expression by Recombinant Strains

FACS analysis was used to investigate the GFP expression by using the constructed P_{vgb} system. The recombinant EGFPV1, EGFPV4 and EGFPV8 strains were grown in Brain Heart Infusion (BHI) media for 24 h at 30 °C. The cells were then collected and diluted using PBS buffer. FACS analysis (BD Biosciences, San Jose, CA, USA) was performed for 100,000 clones of each samples using argon ion laser (blue, 488 nm) and band-pass filter (530 nm $\pm$ 15 nm) [35].

4. Conclusions

The P_{vgb} promoter-based expression system constructed in this study was capable of tunable expression of green fluorescent protein in recombinant *C. glutamcium* strains, when the repeats of P_{vgb} promoter increased from one (P_{vgb}) to four (P_{vgb4}) to eight (P_{vgb8}). Furthermore, GABA and 5-AVA production by recombinant *C. glutamicum* strains also increased when the expression of DavBA and GadB$_{mut}$ in the P_{vgb} promoter-based expression system increased from single to eight repeats of the P_{vgb} promoter. This shows that the strength of protein expression using the P_{vgb} promoter-based tunable system was comparable to that of previously established synthetic promoters (P_{L26}, P_{I16}, P_{H30} P_{H36}) [26,28–30]. Based on the different levels of 5-AVA and GABA production by all the tested strains, the P_{vgb} promoter-based expression system was capable of tunable expression of DavBA and GadB$_{mut}$ by mere manipulation in the number of P_{vgb} repeats. The newly constructed P_{vgb} promoter-based expression system developed in this study expands the repertoire of plasmids available for metabolic engineering of *C. glutamicum* and provides another method for fine-tuning levels of protein expression for convenient and rapid validation of synthetic metabolic pathways, ultimately improving biochemical production in biorefineries.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4344/8/11/561/s1. Table S1: 5-AVA production using recombinant *C. glutamicum* strains expressing DavBA and DavB$_{His}$DavA under different synthetic promoters after 120 h of flask cultivation under high aeration. Table S2: GABA production by recombinant *C. glutamicum* strains expressing GadBmut under different synthetic promoters after 120 h of flask cultivation under high aeration.

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