Ribosomal binding site sequences and promoters for expressing glutamate decarboxylase and producing γ-aminobutyrate in Corynebacterium glutamicum

Feng Shi1,2,3*, Mingyue Luan1,2† and Yongfu Li4

Abstract
Glutamate decarboxylase (GAD) converts l-glutamate (Glu) into γ-aminobutyric acid (GABA). Corynebacterium glutamicum that expresses exogenous GAD gene, gadB2 or gadB1, can synthesize GABA from its own produced Glu. To enhance GABA production in C. glutamicum, ribosomal binding site (RBS) sequence and promoter were searched and optimized for increasing the expression efficiency of gadB2. R4 exhibited the highest strength among RBS sequences tested, with 6 nt the optimal aligned spacing (AS) between RBS and start codon. This combination of RBS sequence and AS contributed to gadB2 expression, increased GAD activity by 156% and GABA production by 82% compared to normal strong RBS and AS combination. Then, a series of native promoters were selected for transcribing gadB2 under optimal RBS and AS combination. PdnaK, PdtsR, PodhI and PclgR expressed gadB2 and produced GABA as effectively as widely applied Ptuf and PcspB promoters and more effectively than Psod promoter. However, each native promoter did not work as well as the synthetic strong promoter P_tacM which produced 20.2 ± 0.3 g/L GABA. Even with prolonged length and bicistronic architecture, the strength of PdnaK did not enhance. Finally, gadB2 and mutant gadB1 were co-expressed under the optimal promoter and RBS combination, thus converted Glu into GABA completely and improved GABA production to more than 25 g/L. This study provides useful promoters and RBS sequences for gene expression in C. glutamicum.

Keywords: Ribosomal binding site (RBS), Promoter, Glutamate decarboxylase, γ-Aminobutyric acid, Corynebacterium glutamicum, gadB2

Introduction
γ-Aminobutyric acid (GABA) is a non-protein amino acid widely synthesized by diverse microorganisms, plants and animals (Li and Cao 2010; Shi et al. 2016). It functions as the major inhibitory neurotransmitter for the mammalian central nervous system and has several therapeutic abilities including hypotension, anti-anxiety, anti-depression, anti-schizophrenia, diuresis and anti-obesity (Wong et al. 2003; Mohler 2012; Shi et al. 2016). Therefore, GABA has been used as a bioactive component in functional foods and feeds. In addition, GABA is a crucial building block for the synthesis of bio-plastics, such as the biodegradable polyamide nylon 4 (Park et al. 2013).

GABA is mainly converted from l-glutamate (Glu) by lactic acid bacteria (LAB) and Escherichia coli strains with high Glu decarboxylase (GAD) activity (Li and Cao 2010; Shi et al. 2016). Although production of GABA by LAB, such as Lactobacillus brevis (Shi et al. 2017b; Wu et al. 2017), is recognized as safe, the cost of production is high; whereas production of GABA by E. coli is high yield (Huang et al. 2016; Zhao et al. 2016a) but is not acceptable for food usage. Furthermore, Glu must be added as the precursor during GABA production by LAB.
and *E. coli*, making such bioconversion not cost-effective. *Corynebacterium glutamicum* is generally regarded as safe and is widely used for the industrial production of Glu, L-lysine (Lys) and other amino acids (Leuchtenberger et al. 2005; Schneider et al. 2011). Recently, two metabolic routes for de novo biosynthesis of GABA from glucose have been employed in *C. glutamicum*, both using its own produced Glu as precursor. One route is fulfilled through multi-step reactions via putrescine (Jorge et al. 2016, 2017), the other only involves one-step reaction catalyzed by GAD (Okai et al. 2014; Choi et al. 2015; Shi et al. 2017a). Therefore, the expression level and activity of GAD is the main determinant for high-yield production of GABA through the second route.

The expression level of a protein is determined by transcription, translation and degradation rates, among them the beginning of transcription and translation are usually the rate-limiting steps. The initiation of transcription is fundamentally controlled by the promoter elements, while the initiation of translation is mainly affected by the strength of ribosomal binding site (RBS). Therefore, optimization of promoters and RBS sequences has been applied for protein expression in *C. glutamicum*. For example, *aph* promoter combined with different RBS sequences were used for expression of reporter proteins in *C. glutamicum* (Zhang et al. 2017b). Combinatorial assembly of *tac*, *cspB* and *sod* promoters and *lacZ*, *cspB* and *sod* RBS elements conferred differential expression of two reporters, eGFP and mCherry in pTGR platform of *C. glutamicum* (Ravasi et al. 2012). RBS sequence is also recorded as Shine–Dalgarno (SD) sequence in bacteria. The expression of GFP was enhanced in *C. glutamicum* using *tac* promoter and *tpi* SD sequence in the secretion vector (Teramoto et al. 2011).

Optimization of RBS sequence has also been applied for metabolic engineering of *C. glutamicum*. Through constructing RBS libraries and regulating the pathway genes *aroG*, *aroB*, *aroD* and *aroE* by RBS of different strengths, 9 genetic modules were built up and shikimic acid synthesis was improved in *C. glutamicum* (Zhang et al. 2015a). After replacing the compressed RBS of *vio* genes with complete strong *C. glutamicum* RBS and altering gene order to form a novel *vio* operon, hyper production of violacein was achieved in *C. glutamicum* (Sun et al. 2016).

Because promoter is essential for gene transcription, series of promoters for gene expression in *C. glutamicum* have been studied. The chimeric *tac* promoter and its modified *tacM* promoter are the most commonly used promoters in expression plasmids of *C. glutamicum*. In addition, several native promoters of *C. glutamicum*, such as the strong constitutive promoters of *P*<sub>sod</sub>, *P*<sub>tuf</sub> and *P*<sub>cspB</sub> (Ravasi et al. 2012; Lee 2014; Man et al. 2016) and the inducible promoter of *P*<sub>prpD2</sub> (Pátek et al. 2013; Plassmeier et al. 2013), have been applied for modulating gene expression and for metabolic engineering of *C. glutamicum*. To obtain more promoters with different activities, synthetic promoters were recently isolated or constructed for gene expression in *C. glutamicum* (Yim et al. 2013; Rytter et al. 2014; Choi et al. 2015; Shen et al. 2017). Delightfully, the genetic elements including promoter regions and RBSs of nearly all the genes of *C. glutamicum* ATCC 13032 have been successfully identified based on an improved RNAseq technique (Pfeifer-Sancar et al. 2013), thus contribute to the analysis and usage of more and more native promoters.

It is reported that the initial translation efficiency and expression level of different proteins through the identical RBS sequence is dramatically different (Salis et al. 2009). To enhance the translation of GAD and production of GABA in recombinant *C. glutamicum*, a series of RBS sequence and aligned spacing (AS) were explored here for expressing a GAD gene, i.e. *gadB2* derived from *L. brevis* Lb85, by *tac* M promoter. Then, to improve the transcription of *gadB2* and yield of GABA, several types of native promoters and various architecture of the strong native promoter were explored. Considering the beneficial effects of *gadB1–gadB2* co-expression (Shi et al. 2013) as well as the mutant *gadB1<sup>T17I/D294G/E312S/Q346H</sup>* expression (Shi et al. 2014) on GABA production, co-expression of *gadB1<sup>mut</sup>* and *gadB2* was researched at last when the most effective RBS and promoter was verified.

**Materials and methods**

**Strains, media, and growth conditions**

Bacterial strains used in this study are listed in Table 1. *E. coli* JM 109 was employed as the host for constructing and propagating the plasmids. *E. coli* was grown in Luria–Bertani (LB) medium at 37 °C and 200 rpm. *C. glutamicum* SH, an L-glutamate-producing strain, was used for expressing *gadB2* and *gadB1* genes and producing GABA. SH was deposited in the China General Microbiological Culture Collection (CGMCC) center with accession number CGMCC 1.581. *C. glutamicum* was grown in LBG medium (LB supplemented with 5 g/L glucose) at 200 rpm and 30 °C. When necessary, 30 μg/mL kanamycin was added to the media.

**Construction of *gadB2* expression strains under different RBS sequence and promoter**

The nucleotide sequences of all primers are listed in Table 2. A series of RBS sequence derived from the consensus sequence AGGAG and an AS of 6–8 nt was designed and applied for expressing *gadB2*. For expressing with normal RBS sequence and AS of 7 nt, *gadB2* was amplified from the plasmid pJYW-4-*gadB1–gadB2* (Shi et al. 2014) on GABA production, co-expression of *gadB1<sup>mut</sup>* and *gadB2* was researched at last when the most effective RBS and promoter was verified.
et al. 2017a) using the primer pair of R-B2-F and gadB2-R. The PCR product was digested with HpaI and BamHI, and ligated into pJYW-5, a E. coli–C. glutamicum shuttle expression vector carrying a tacM promoter, resulting in the plasmid pJYW-5-gadB2. For expressing with the 12 designed RBS sequence (R1–R12) and AS of 7 nt as well as with RBS of R4 and AS of 6 nt (R4a) or 8 nt (R4b), gadB2 was amplified similarly using the 14 different

| Strains          | Characteristics                                      | Source |
|------------------|------------------------------------------------------|--------|
| E. coli JM 109   | E. coli gene cloning strain                          | Novagen|
| C. glutamicum SH | Wild type C. glutamicum                              | CGMCC  |
| R-B2             | C. glutamicum SH harbouring pJYW-5-gadB2             | This work|
| R1-B2            | C. glutamicum SH harbouring pJYW-5-R1-gadB2          | This work|
| R2-B2            | C. glutamicum SH harbouring pJYW-5-R2-gadB2          | This work|
| R3-B2            | C. glutamicum SH harbouring pJYW-5-R3-gadB2          | This work|
| R4-B2            | C. glutamicum SH harbouring pJYW-5-R4-gadB2          | This work|
| R5-B2            | C. glutamicum SH harbouring pJYW-5-R5-gadB2          | This work|
| R6-B2            | C. glutamicum SH harbouring pJYW-5-R6-gadB2          | This work|
| R7-B2            | C. glutamicum SH harbouring pJYW-5-R7-gadB2          | This work|
| R8-B2            | C. glutamicum SH harbouring pJYW-5-R8-gadB2          | This work|
| R9-B2            | C. glutamicum SH harbouring pJYW-5-R9-gadB2          | This work|
| R10-B2           | C. glutamicum SH harbouring pJYW-5-R10-gadB2         | This work|
| R11-B2           | C. glutamicum SH harbouring pJYW-5-R11-gadB2         | This work|
| R12-B2           | C. glutamicum SH harbouring pJYW-5-R12-gadB2         | This work|
| R4a-B2           | C. glutamicum SH harbouring pJYW-5-R4a-gadB2         | This work|
| R4b-B2           | C. glutamicum SH harbouring pJYW-5-R4b-gadB2         | This work|
| Psod-B2          | C. glutamicum SH harbouring pJYW-5-Psod-R4a-gadB2    | This work|
| PusxA-B2         | C. glutamicum SH harbouring pJYW-5-PusxA-R4a-gadB2   | This work|
| Pcsps-B2         | C. glutamicum SH harbouring pJYW-5-Pcsps-R4a-gadB2   | This work|
| PtuB-B2          | C. glutamicum SH harbouring pJYW-5-PtuB-R4a-gadB2    | This work|
| Pgdh-B2          | C. glutamicum SH harbouring pJYW-5-Pgdh-R4a-gadB2    | This work|
| Pdtr-B2          | C. glutamicum SH harbouring pJYW-5-Pdtr-R4a-gadB2    | This work|
| Podhi-B2         | C. glutamicum SH harbouring pJYW-5-Podhi-R4a-gadB2   | This work|
| Ppsx-B2          | C. glutamicum SH harbouring pJYW-5-Ppsx-R4a-gadB2    | This work|
| Psox-B2          | C. glutamicum SH harbouring pJYW-5-Psox-R4a-gadB2    | This work|
| Pcps-B2          | C. glutamicum SH harbouring pJYW-5-Pcps-R4a-gadB2    | This work|
| PgpB-B2          | C. glutamicum SH harbouring pJYW-5-PgpB-R4a-gadB2    | This work|
| Pdtrp-B2         | C. glutamicum SH harbouring pJYW-5-Pdtrp-R4a-gadB2   | This work|
| Pdtrp-B2         | C. glutamicum SH harbouring pJYW-5-Pdtrp-R4a-gadB2   | This work|
| Pdtrp-B2         | C. glutamicum SH harbouring pJYW-5-Pdtrp-R4a-gadB2   | This work|
| Pdtrp-B2         | C. glutamicum SH harbouring pJYW-5-Pdtrp-R4a-gadB2   | This work|
| Pdtrp-B2         | C. glutamicum SH harbouring pJYW-5-Pdtrp-R4a-gadB2   | This work|
| Pdtrp-B2         | C. glutamicum SH harbouring pJYW-5-Pdtrp-R4a-gadB2   | This work|
| Pdtrp-B2         | C. glutamicum SH harbouring pJYW-5-Pdtrp-R4a-gadB2   | This work|
| Pdtrp-B2         | C. glutamicum SH harbouring pJYW-5-Pdtrp-R4a-gadB2   | This work|
| Pdtrp-B2         | C. glutamicum SH harbouring pJYW-5-Pdtrp-R4a-gadB2   | This work|
| Pdtrp-B2         | C. glutamicum SH harbouring pJYW-5-Pdtrp-R4a-gadB2   | This work|
| Pdtrp-B2         | C. glutamicum SH harbouring pJYW-5-Pdtrp-R4a-gadB2   | This work|
| Pdtrp-B2         | C. glutamicum SH harbouring pJYW-5-Pdtrp-R4a-gadB2   | This work|
| Pdtrp-B2         | C. glutamicum SH harbouring pJYW-5-Pdtrp-R4a-gadB2   | This work|
| Pdtrp-B2         | C. glutamicum SH harbouring pJYW-5-Pdtrp-R4a-gadB2   | This work|
| Pdtrp-B2         | C. glutamicum SH harbouring pJYW-5-Pdtrp-R4a-gadB2   | This work|
Table 2 Primers used in this study

| Primers | Sequences (5′–3′) | Restriction sites |
|---------|-------------------|-------------------|
| R-B2-F  | GGGGTTAAGAGGGAGGGATTGCGATGAATAAAAACGATAGGA | HpaI |
| gadB2-R | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | BaeIII |
| R1-B2-F | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| R2-B2-F | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| R3-B2-F | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| R4-B2-F | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| R5-B2-F | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| R6-B2-F | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| R7-B2-F | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| R8-B2-F | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| R9-B2-F | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| R10-B2-F | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| R11-B2-F | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| R12-B2-F | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| R4a-B2-F | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| R4b-B2-F | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| R4a-B1mut-F | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| gadB1mut-R | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| M-R4a-B1mut-F | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| PsoD-F | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| PsoD-R | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| PuspA-F | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| PuspA-R | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| PcspB-F | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| PcspB-R | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| Ptuf-F | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| Ptuf-R | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| Pgdh-F | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| Pgdh-R | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| PdtsR-F | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| PdtsR-R | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| PodhI-F | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| PodhI-R | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| PsigB-F | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| PsigB-R | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| Phmp-F | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| Phmp-R | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| Ppqo-F | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| Ppqo-R | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| PgapA-F | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| PgapA-R | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| PilvE-F | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| PilvE-R | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| Pcgl1417-F | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| Pcgl1417-R | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| PdnaK-F | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| PdnaK-R | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| PclgR-F | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
| PclgR-R | CCGGGCTTAAAGAGGAAGGGATTGCAGTAATAAAAACGATAGGA | AflII |
forward primer Rn–B2-F and the same reverse primer gadB2–R, digested with AfII and BamHI, and ligated into plasmid pJYW-5 digested with the same enzymes, resulting in the 14 plasmids of pJYW-5–Rn-gadB2. Finally, these plasmids were transformed into *C. glutamicum* SH by the method described previously (Wang et al. 2015), generating 15 recombinant strains of R–B2, R1–B2 to R12–B2, R4a–B2 and R4b–B2.

Each promoter sequence was designed according to the predicted promoter (Pfeifer-Sancar et al. 2013) with its upstream 60 bp. For obtaining different native promoters, P_rna and P_raf were amplified from the genomic DNA of *C. glutamicum* ATCC 13032, while others were amplified from the genomic DNA of *C. glutamicum* SH using the corresponding primer pairs. To express gadB2 with these promoters, the *p_tacM* was deleted from pJYW-5–R4a-gadB2 at first; then, the PCR products of P_rna, P_raf, P_uapA, P_clpB, P_clpB, P_clpB, P_clpB, P_clpB, P_clpB, P_clpB, P_raf, and P_uapA were digested with XbaI and ligated into the *p_tacM*-deleted plasmid pJYW-5–R4a-gadB2, resulting in 19 plasmids of pJYW-5–P_rna–R4a-gadB2. Finally, these plasmids were transformed into *C. glutamicum* SH, generating 19 recombinant strains (from Psod–B2 to Ptrl–B2 in Table 1).

For expressing gadB2 with bicomtronic expression cassette (Zhao et al. 2016b), P_dnaK–2SD–gadB2 containing the first 41 bp of dnaK gene and the strong RBS (R4a) was amplified via overlap PCR using primers P_dnaK–F, P_dnaK–2SD–R, P_dnaK–2SD–F and gadB2–R, and then ligated into plasmid pJYW-5, resulting in the plasmid pJYW-5–P_dnaK–2SD–gadB2. To express gadB2 with prolonged P_dnaK, P_dnaK with the downstream 60 bp [P_dnaK(+1)] with the upstream 60 bp [P_dnaK(−1)], upstream 180 bp [P_dnaK(−2)] and upstream 240 bp [P_dnaK(−3)] were amplified with corresponding primers and then overlapped with R4a–gadB2 fragment that was amplified with primers P_dnaK–F and gadB2–R, generating plasmids pJYW-5–P_dnaK(+1)–R4a-gadB2, pJYW-5–P_dnaK(−1)–R4a-gadB2, pJYW–5–P_dnaK(−2)–R4a-gadB2 and pJYW-5–P_dnaK(−3)–R4a-gadB2. Finally, the 5 plasmids were transformed into *C. glutamicum* SH, yielding 5 recombinant strains, PdnaK2SD–B2, PdnaK(+1)–B2, PdnaK(−1)–B2, PdnaK(−2)–B2 and PdnaK(−3)–B2.

**Construction of gadB1mut expression strain and gadB2–gadB1mut co-expression strain**

*GadB1mut* gene with the strong RBS (R4a) was amplified from the plasmid pEC–gadB1T171/D294G/E1325/G346H (Shi et al. 2014) using the primer pair of R4a–B1mut–F and gadB1mut–R. The PCR product was digested with BglII and PstI, and ligated into pDXW-10, a shuttle expression vector between *E. coli* and *Corynebacteria* which carries a tacM promoter (Xu et al. 2010), resulting in the plasmid pDXW-10–R4a–gadB1mut. Then the DNA fragment containing P_tacM and R4a–gadB1mut was amplified.

---

**Table 2 continued**

| Primers         | Sequences (5′–3′) | Restriction sites |
|-----------------|-------------------|------------------|
| P_gadB2–F       | ATATCTAGATGGATTTTTGCTCGCCTCGCGTC | XbaI             |
| P_gadB2–R       | CGATTCTAGACGCAACACTACACAAATCGTG    | XbaI             |
| P_dnaK–F        | CATTCTAGATTTAGGTTCTCGCCGTTGTG     | XbaI             |
| P_dnaK–R        | CAGGCTTAGAGCTCTTATATGCGGTTAGAAT   | XbaI             |
| P_uapA–F        | CTATGCTAGACCCCTCTTGGTCCTGCTCTG    | XbaI             |
| P_uapA–R        | CTATTCTAGAGCTGTCCTTTCTGTTG       | XbaI             |
| P_gadB2–F       | CATGCTAGACGCCAAACATGCGCAGTTCTCA  | XbaI             |
| P_gadB2–R       | CGCGGTAGCTAGACGTTGGAAGTCTGA      | XbaI             |
| P_dnaK+1–F      | CATTAATCCCTCTCTTTCAGTGTGGTCCAAGGTC |                   |
| P_dnaK+1–R      | CCACCAACTCGAAGAGAGAGATTAGAATAAAAACGTACAGGAAACACAGC |                   |
| P_dnaK−1–F      | ATTACCTCTAGACGCTGAGACTTTGGTCTCACA  | XbaI             |
| P_dnaK−2–F      | ATTACCTCTAGACGATGTGCTCTTTTTCATC    | XbaI             |
| P_dnaK−3–F      | ATTACCTCTAGACCGGGTCCTCAAAATTGCTCTCA | XbaI             |
| P_dnaK−4–R      | CATCAACCTCTCTTCTTTGATTTTGAATCTGTCAC |                   |
| P_dnaK−5–F      | GAAAGGAGGAGGATGGATGA          |                   |
| P_dnaK−5–R      | ACACAGGCTCCGTCGTAGAT           |                   |
| P_dnaK−6–F      | TTAGCCGCCCAAAGCTTAAT           |                   |
| P_dnaK−6–R      | ACCGTTAGGAGAGAGACCGG           |                   |
| P_dnaK−7–F      | TCAAGTTATGCCGCTA              |                   |
| P_dnaK−7–R      | ATGAA GGATTG                  |                   |
| P_dnaK−8–F      | TCTCCTTTTCAGAATTTA            |                   |
| P_dnaK−8–R      | GAAAGGAGGAGGATGGATGA          |                   |
| P_dnaK−9–F      | ACACAGGCTCCGTCGTAGAT           |                   |
| P_dnaK−10–F     | TTAGCCGCCCAAAGCTTAAT          |                   |
| P_dnaK−10–R     | ACCGTTAGGAGAGAGACCGG          |                   |
| P_dnaK−11–F     | TCAAGTTATGCCGCTA              |                   |
| P_dnaK−11–R     | ATGAA GGATTG                  |                   |
| P_dnaK−12–F     | TCTCCTTTTCAGAATTTA            |                   |
| P_dnaK−12–R     | GAAAGGAGGAGGATGGATGA          |                   |
| P_dnaK−13–F     | ACACAGGCTCCGTCGTAGAT           |                   |
| P_dnaK−13–R     | TTAGCCGCCCAAAGCTTAAT          |                   |
| P_dnaK−14–F     | ACCGTTAGGAGAGAGACCGG          |                   |
| P_dnaK−14–R     | TCAAGTTATGCCGCTA              |                   |

The restriction sites are underlined. The RBSs are italicized. The ASs are in boldface.
from pDXW-10-R4a-gadB1mut using primer pair of M-R4a-B1mut-F and gadB1mut-R, digested with SalI and PstI, and ligated into pYW-5-R4a-gadB2 that was similarly digested, resulting in the plasmid pYW-5-R4a-gadB2-R4a-gadB1mut. The two plasmids were finally transformed into C. glutamicum SH, generating recombinant strains R4a-B1mut and R4a-B2B1mut.

GABA fermentation of recombinant C. glutamicum strains in shake flask
For GABA production in a shake flask, recombinant C. glutamicum cells were pre-cultured in seed medium at 30 °C and 200 rpm for 9 h, inoculated into a 500-mL baffled flask containing 25 mL of fermentation medium to a final optical density (OD562) of 1.9 and cultured at 30 °C and 200 rpm for 9 h, as described previously (Shi et al. 2013) by a cyclotron shaker. At 10, 11.5, 13, 14.5, 18 and 21.5 h of fermentation, 2 g/L urea was added to the culture to maintain the neutral conditions. After 24 h of fermentation, an appropriate volume of culture broth was harvested every 12 h, and the cell concentration, pH, residual glucose concentration and the Glu and GABA concentrations in the fermentation broth were measured by the method described previously (Shi et al. 2013).

Assay of GAD activity
The cells in the fermentation broth were harvested and washed twice with chilled phosphate buffer saline. However, no GAD activity could be detected after the crude enzyme was extracted from washed cells by method described previously (Shi et al. 2013). Therefore, the washed cells were re-suspended in equal volume of 0.02 M Na2HPO4–citric acid buffer (pH 4.8) containing 10% glycerol and 0.1% Triton, and applied directly as the crude enzyme of whole cell suspension. The GAD reaction was then performed at 37 °C for 1 h in a reaction mixture (1 mL) consisting of 0.4 M Na2HPO4–citric acid buffer (pH 5.0), 60 mM monosodium glutamate, 0.03 mM pyridoxal 5′-phosphate and appropriate volume of whole cell suspension. GAD activity was determined according to the formation of GABA in this reaction. One unit (U) of GAD activity is defined as 1.0 µmol GABA produced in 1 min in the initial reaction mixture. The specific activity is expressed as U/g of dry cell weight (DCW). The DCW per liter (g/L) was calculated according to an experimentally determined formula: DCW = 0.6495 × OD562 − 2.7925.

Real-time PCR analysis of gadB2 transcription
The mRNA transcription levels of gadB2 gene in recombinant C. glutamicum during fermentation were determined by real-time PCR (RT-PCR) combined with reverse transcription as described previously (Wang et al. 2015). Total RNA was extracted from cells that were harvested at 20 and 40 h. After disposed DNA with DNase I, the quality and amount of RNA were analyzed and quantified by electrophoresis. Then the mRNAs were reverse transcribed into cDNAs and the cDNAs were used for RT-PCR analysis. Primers for RT-PCR are listed in Table 2. The relative abundance of gadB2 mRNAs was quantified based on the cycle threshold (Ct) value and was calculated by the 2−ΔΔCt method (Livak and Schmittgen 2001). To standardize the transcription levels, the relative abundance of 16S rRNA was used as the internal standard.

Results
To improve GAD expression and GABA production in recombinant C. glutamicum SH, the RBS sequences and promoters for expressing GAD gene were explored here. As expression of gadB2 produced more GABA in C. glutamicum than expression of gadB1 (Shi and Li 2011), gadB2 was selected for engineering of RBS sequence and promoter.

Optimization of RBS sequence for gadB2 expression and GABA production
RBS is a pivotal region for controlling translation initiation and protein expression. For expressing target protein(s) in C. glutamicum, AGAAAGGAG was used as normal RBS sequence in our previous studies. However, some conserved RBS sequences, such as AAAAAAGGAGG (Amador et al. 1999) and GAAAGGGAGG (Martín et al. 2003), have been reported in C. glutamicum. In addition, several sequences such as GAAA-GGAG, GAAAGGCCG and GAAAGGA were used as strong RBS for expressing target genes in C. glutamicum (Kang et al. 2014; Zhang et al. 2015b). Thus these 5 RBS sequences were analyzed here for gadB2 expression. Recently, AGGag was detected as a conserved motif in about 92% of 5′-UTR sequences of the entire protein-coding genes in C. glutamicum (Pfeifer-Sancar et al. 2013). Therefore, a seeding sequence of (A/G)3AGGAG was used as strong RBS for expressing target genes in C. glutamicum (Shi and Li 2011), gadB2 was selected for engineering of RBS sequence and promoter.

In recombinant C. glutamicum, the optimal pH for cell growth and Glu biosynthesis is about 7.0, whereas that for GAD activity and conversion of Glu to GABA is 5.0–6.0. For effective production of GABA, the cultivated medium was initially maintained at neutral pH by adding urea during 10–21.5 h of fermentation, and then the pH was let to decline and change spontaneously thereafter. During the fermentation, all the strains that harbor different RBS sequences grew similarly and exhibited similar pH variation. The cell density increased fast before 24 h and nearly
maintained thereafter at OD_{562} of approximately 45–50. Glucose was consumed rapidly before 24 h, slowly thereafter and nearly exhausted at the end of fermentation. The pH value decreased to the lowest level of about 4.9–5.6 at 36 h, partially due to the exhaust of urea and accumulation of acidic Glu (Fig. 1a), and rose gradually to about 5.2–6.6 thereafter, partially due to the conversion of acidic Glu to neutral GABA (Fig. 1b). As pH decreased to the lowest level at 36 h, GAD became active and GABA began to synthesize quickly. Therefore, before 36 h was regarded as Glu fermentation stage and after 36 h was regarded as GABA conversion stage. It is worth mentioning that the yield of GABA was obviously different in C. glutamicum strains harboring different RBS sequences (Fig. 1b). Thus these recombinant strains were classified into three levels, high (H), medium (M) and low (L), according to their production capacity of GABA. In the H level, GABA production was higher than 10 g/L, with R4-B2 strain the highest (13.3 ± 0.5 g/L). The GABA production of R4-B2 was even higher than that of R-B2 under the normal RBS sequence. Meanwhile, less Glu was remained in these strains (Fig. 1a). However, GABA production decreased to about 6–10 g/L in the M level strains and even lower than 6 g/L in the L level strains. Furthermore, the GAD activity of H level strains was slightly higher than M level strains and obviously higher than L level strains, also with R4-B2 the highest (16.5 ± 0.2 U/g DCW) (Fig. 1c). Therefore, the translation of GadB2 was most efficient under the RBS sequence of R4.

Optimization of aligned spacing for gadB2 expression and GABA production

Even under the RBS sequence of R4, approximately 11 g/L of Glu was remained and not converted to GABA. Besides RBS sequence, aligned spacing between RBS and translational start codon is also important for translation efficiency. AS has been revealed to be 4–12 nt in C. glutamicum, with 6–8 nt as the most common (Pfeifer-Sancar et al. 2013). Therefore, the AS of 6–8 nt for RBS sequence of R4 was then analyzed for expressing gadB2 and producing GABA.

The three gadB2-expressing strains with different AS grew and consumed glucose similarly (Fig. 2a, b), but their pH value varied differently (Fig. 2c). The variation range of the pH of R4a-B2 with AS of 6 nt was less than that of R4-B2 with AS of 7 nt, whereas the pH range of R4b-B2 with AS of 8 nt was more than that of R4-B2. Most importantly, compared with R4-B2, significant more GABA (20.2 ± 0.3 g/L) was produced and less Glu (5.1 ± 0.6 g/L) was remained in R4a-B2, whereas in R4b-B2, same amount of GABA and Glu were produced (Fig. 2d, e). Meanwhile, the total amount of Glu and GABA was obviously high in R4a-B2 (Fig. 2f). In addition, the GAD activity of R4a-B2 (32.0 ± 2.5 U/g DCW) was significantly higher than R4-B2 (16.5 ± 0.2 U/g DCW) and R4b-B2 (21.4 ± 1.2 U/g DCW). Therefore, the AS of 6 nt with RBS of R4 (R4a) was more preferable than the AS of 7 nt and 8 nt for translation of GadB2 and production of GABA in C. glutamicum. Then R4a was selected as the most prominent combination of RBS sequence and AS thereafter.

Different promoters for gadB2 expression and GABA production

The translation efficiency of GadB2 was improved in R4a-B2, where gadB2 was expressed by P_{tacM} promoter. P_{tacM} was derived from P_{tac} (Xu et al. 2010). P_{tac} was confirmed to be a strong promoter for expressing lysE
and improving l-ornithine production (Rytter et al. 2014; Zhang et al. 2017a), and \( P_{\text{tacM}} \) was proven to be a stronger promoter than \( P_{\text{tac}} \) (Xu et al. 2010). Then the transcription of \( \text{gadB}2 \) under the RBS and AS of R4a was researched and three kinds of native promoters for the transcription of \( \text{gadB}2 \) were verified.

The first kind was widely used constitutive promoters (\( P_{\text{sod}}, P_{\text{tuf}}, P_{\text{cspB}} \) and \( P_{\text{uspA}} \)) and several native promoters of genes involved in carbon metabolism (\( \text{dtsR} \)) and Glu biosynthesis (\( \text{gdh} \) and \( \text{odh} \)). \( P_{\text{sod}} \) and \( P_{\text{tuf}} \) have been widely applied to enhance gene expression and optimize metabolic pathways for production of amino acids, such as l-ornithine (Kim et al. 2015), Lys (Becker et al. 2011; Shang et al. 2018) and l-araginine (Man et al. 2016) in \( C. \) glutamicum. \( P_{\text{cspB}} \) and \( P_{\text{uspA}} \) were used as strong promoters for expressing reporters in \( C. \) glutamicum (Ravasi et al. 2012; Zhao et al. 2016b). So these promoters were analyzed here for expressing \( \text{gadB}2 \).

The growth, glucose consumption and pH variation of recombinant \( C. \) glutamicum strains were not affected by the replacement of these 8 promoters, but the GABA production was quite different (Fig. 3b). R4a-B2, \( \text{P}dtsR-B2, \text{P}tuf-B2, \text{P}cspB-B2, \text{P}odhl-B2 \) and \( P_{\text{uspA}}-B2 \) with \( P_{\text{tacM}}, P_{\text{dtsR}}, P_{\text{tuf}}, P_{\text{cspB}}, P_{\text{odh}I} \) and \( P_{\text{uspA}} \) promoter, respectively produced GABA more than 10 g/L, whereas \( P_{\text{gdh}}-B2 \) with \( P_{\text{gdh}} \) promoter and \( P_{\text{sod}}-B2 \) with \( P_{\text{sod}} \) promoter only produced about 5 g/L GABA. Unexpectedly, although the GABA production of \( \text{P}dtsR-B2 \) (16.4 ± 0.1 g/L) was significantly higher than that of strains under other native promoters, it was obviously lower than that of R4a-B2 under \( P_{\text{tacM}} \) promoter (20.2 ± 0.3 g/L) and more Glu was remained (Fig. 3a). Meanwhile, the GAD activity of \( P_{\text{gdh}}-B2 \) and \( P_{\text{odh}I} \) (1.1–3.1 U/g DCW) was significantly lower than that of other strains (3.2–32.0 U/g DCW), especially R4a-B2 under \( P_{\text{tacM}} \) promoter (Fig. 3c), basically consistent with their GABA production. In addition, at Glu fermentation stage (20 h), only \( P_{\text{odh}I}-B2 \) showed higher transcription level of \( \text{gadB}2 \) than R4a-B2, whereas other strains showed significantly lower level than R4a-B2 (Fig. 3d). While at GABA conversion stage (40 h), \( PdtsR-B2 \) and \( Podhl-B2 \) showed slightly lower and other strains showed significantly lower transcription level of \( \text{gadB}2 \) than R4a-B2. Therefore, these constitutive

![Fig. 2](image-url) GABA fermentation by \( \text{gadB}2 \)-expressing \( C. \) glutamicum strains under R4 RBS sequence with AS of 6 nt (R4a), 7 nt (R4) and 8 nt (R4b). a Cell growth, b glucose consumption, c pH variation, d Glu production, e GABA production, f total amount of Glu and GABA. Circles R4a-B2, empty diamonds in dotted line R4-B2, empty squares in dotted line R4b-B2. Averages of three independent experiments are provided.
promoters and Glu synthesis-related promoters were not as effective as $P_{tacM}$ for transcription of $gadB2$.

The second kind was $sigB$ promoter and several $\sigma^B$-recognized promoters, considering that GAD mainly acts at stationary phase when pH is below 6.0. Some promoters, such as $P_{gmp}$, $P_{pqo}$, $P_{gapA}$, $P_{ilvE}$ and $P_{cg1417}$, had been proven to be $\sigma^B$-dependent (Larisch et al. 2007; Pátek and Nešvera 2011). Then these promoters were analyzed here for expressing $gadB2$. During fermentation, most $gadB2$-expressing strains under $\sigma^B$-related promoters grew and consumed glucose similarly with R4a-B2 under $P_{tacM}$ promoter, except $P_{gadA}$-B2 which grew and consumed glucose much slowly. Unfortunately, all the 5 $\sigma^B$-recognized promoters tested here were far less robust for producing GABA (less than 3 g/L) and expressing GAD activity (less than 5 U/g DCW), although most of them except $P_{gapA}$ were able to enhance the $gadB2$ transcription level at GABA conversion stage (Fig. 4). $P_{sigB}$-B2 showed comparable $gadB2$ transcription level and GAD activity with R4a-B2; meanwhile, its GAD activity increased significantly during 12–24 h, whereas that of R4a-B2 decreased continuously during the whole fermentation. However, although the GABA production of $P_{sigB}$-B2 was significantly higher than that of other 5 $\sigma^B$-controlled strains, it was only half of that R4a-B2. Therefore, these $\sigma^B$-related promoters were not effective for producing GABA.

The third kind was several promoters of genes involved in stress response, because on account of transcriptome analysis, the expression of several stress response genes, i.e. $dnaK$, $clgR$, $clpB$, $trxB1$, $dnaJ$ and $sufR$, up-regulated significantly during GABA conversion stage. Then, $P_{dnaK}$, $P_{clgR}$, $P_{clpB}$, $P_{trxB1}$, $P_{sufR}$ and $P_{dnaJ}$ were selected for

---

**Fig. 3** GABA production and expression level of $gadB2$-expressing *C. glutamicum* strains controlled by some constitutive promoters. a Glu concentration, b GABA concentration, c GAD activity, d $gadB2$ transcription level. White bars at 36 h, black bars at 72 h, grey bars at 20 h, dark grey bars at 40 h. Each point represents the average of three independent experiments.
expressing gadB2. The growth and glucose consumption of gadB2-expressing strains was not affected by the replacement of these stress response promoters, but the GABA production was quite different (Fig. 5b). Pdnak-B2 and PclgR-B2 accumulated GABA to more than 10 g/L, whereas PclpB-B2 accumulated to approximately 8.0 g/L, whereas PtrxB1-B2, Pdnal-B2 and PsufR-B2 accumulated only less than 3 g/L. It was regrettable that although the GABA production of Pdnak-B2 (15.8 ± 0.7 g/L) was significantly higher than that of strains under other stress response promoters, it was obviously lower than that of R4a-B2 and more Glu was remained (Fig. 5a). In addition, the GAD activity of all strains decreased continuously during the whole fermentation, with R4a-B2 always exhibiting the highest activity, followed by Pdnak-B2 and PclgR-B2, whereas Pdnal-B2 and PsufR-B2 nearly no activity (Fig. 5c). The GAD activity of these strains was basically consistent with their GABA production. Furthermore, at 20 h, PtrxB1-B2, PclgR-B2 and Pdnak-B2 showed similar transcription level of gadB2 to R4a-B2 and other 3 strains showed significantly lower level than R4a-B2 (Fig. 5d). While at 40 h, the gadB2 transcription level of Pdnak-B2, PclpB-B2, PtrxB1-B2, PclgR-B2 and PsufR-B2 was significantly higher than that of R4a-B2. Thus the transcription of gadB2 was actually enhanced by these stress responsive promoters during GABA conversion stage. However, the GAD activity and GABA production did not improve accordingly, perhaps due to the translation and stability of GadB2 in these strains. Among all the three kinds of native promoters, Pdnak seems to be most effective for gadB2 expression and GABA production.
Effect of length and architecture of P\textsubscript{dnaK} on gadB2 expression and GABA production

Although P\textsubscript{dnaK} was the most effective native promoter for expressing gadB2 and producing GABA, it was not as effective as P\textsubscript{tacM}. There are two transcription initiation sites in P\textsubscript{dnaK}, P\textsubscript{1}\textsubscript{dnaK} promoter is recognized by σ\textsuperscript{A}, P\textsubscript{2}\textsubscript{dnaK} promoter is recognized by σ\textsuperscript{E} and σ\textsuperscript{H}; meanwhile, P\textsubscript{dnaK} is directly repressed by HspR (Ehira et al. 2009; Šilar et al. 2016). Therefore, cis-regulatory elements may be present in the flanking region of P\textsubscript{dnaK}. Then various length of P\textsubscript{dnaK} with extended flanking sequence was tested for its strength. In addition, the coden sequence downstream of initiation coden, especially the following two codens was shown to be crucial for translational efficiency (Stenstrom et al. 2001). Based on this knowledge, bicistronic expression cassette, which includes a leader peptide and a second RBS between 5'-UTR and target gene, was explored and shown to be effective for increasing the expression activity of some native promoters (Mutalik et al. 2013; Zhao et al. 2016b). Then P\textsubscript{dnaK} prolonged to the downstream sequence of initiation coden and carried additional optimal RBS and AS of R4a (P\textsubscript{dnaK-2SD}) was tested for its strength.

The three gadB2-expressing strains under the prolonged P\textsubscript{dnaK} promoters, i.e. P\textsubscript{dnaK(+1)} prolonged to downstream 60 bp, P\textsubscript{dnaK(-1)} prolonged to upstream 60 bp and P\textsubscript{dnaK(-2)} prolonged to upstream 180 bp, as well as the strain under the bicistronic P\textsubscript{dnaK} promoter (P\textsubscript{dnaK2SD-B2}) grew and consumed glucose in a similar manner with the strain P\textsubscript{dnaK}-B2 (Fig. 6a, b); meanwhile, the
pH variation of these four strains was also similar to PdnaK-B2. However, the gadB2-expressing strain under the P_{dnaK(−3)} promoter that prolongs to upstream 240 bp grew and consumed glucose much slowly than PdnaK-B2, perhaps due to the reason that tedious fragment might increase the growth burden of bacteria. Meanwhile, its pH value during GABA conversion stage was significantly lower than other strains. The GABA production of PdnaK(+1)-B2 and PdnaK(−1)-B2 (about 16 g/L) was comparable to that of PdnaK-B2 (Fig. 6e) and similar amount of Glu (7–10 g/L) was remained (Fig. 6d), although their GAD activity and gadB2 transcription level were somewhat lower than those of PdnaK-B2 (Fig. 6c, f), indicating the similar activity of P_{dnaK(±1)}, P_{dnaK(−1)} and P_{dnaK} promoters for producing GABA. But the GABA production of PdnaK(−2)-B2 decreased by 40% and that of PdnaK(−3)-B2 decreased greatly to only 1.3 g/L; meanwhile, more Glu was remained and the total amount of Glu and GABA also decreased, likely due to the further decrease of GAD activity in these two strains, especially in PdnaK(−3)-B2, indicating the repression of P_{dnaK} as it extended to upstream 180 and 240 bp. Therefore, the activity of P_{dnaK} did not increase as P_{dnaK} region extended. Furthermore, although the GAD activity of PdnaK2SD-B2 was always higher than that of PdnaK-B2 (Fig. 6c), its gadB2 transcription level (Fig. 6f) and GABA production (Fig. 6e) was lower than PdnaK-B2. Therefore, bicistronic architecture of P_{dnaK} was not beneficial for gadB2 expression and GABA production in C. glutamicum.

Coexpression of gadB1mut and gadB2 by optimal promoter and RBS for production of GABA

Even controlled by the robust promoter (P_{tacM}) and RBS sequence (R4a), expression of gadB2 only was not sufficient for converting all the synthesized Glu into GABA and approximately 5 g/L Glu was remained at the end of fermentation. Therefore, another GAD gene, L. brevis gadB1, was co-expressed with gadB2, both under the robust promoter of P_{tacM} and RBS sequence of R4a. Considering that as the active pH range of GadB1 was broadened to near-neutral pHs after mutagenesis, GABA production

![Fig. 6](image-url)
increased, especially in gadB<sup>T17I/D294G/E312S/Q346H</sup>-expressing strain (Shi et al. 2014), this gadB1 mutant (gadB1<sup>mut</sup>) was expressed here instead of wild-type gadB1.

The gadB1<sup>mut</sup>-expressing strain R4a-B1<sup>mut</sup> grew and consumed glucose a little slowly, while the gadB2–gadB1<sup>mut</sup>-coexpressing strain R4a-B2B1<sup>mut</sup> grew and consumed glucose a little faster than the gadB2-expressing strain R4a-B2 (Fig. 7a, b). The pH value of R4a-B1<sup>mut</sup> varied similarly with that of R4a-B2, whereas the pH of R4a-B2B1<sup>mut</sup> varied a little differently and increased to a lower level at the later stage of fermentation (Fig. 7c). The GAD activity of R4a-B1<sup>mut</sup> (49.7±1.8 U/g DCW) and R4a-B2B1<sup>mut</sup> (127±18 U/g DCW) was 55% and 3.0-fold higher than that of R4a-B2 (32.0±2.5 U/g DCW), respectively. Compared with R4a-B2, more GABA (25.2±3.0 g/L) was produced and nearly no Glu (0.6±0.2 g/L) was remained in R4a-B2B1<sup>mut</sup>, whereas in R4a-B1<sup>mut</sup>, less amount of GABA and similar amount of Glu were produced (Fig. 7d, e). Meanwhile, the total amount of Glu and GABA of R4a-B2B1<sup>mut</sup> was a little higher, whereas that of R4a-B1<sup>mut</sup> was a little lower than that of R4a-B2 (Fig. 7f). Furthermore, in R4a-B2B1<sup>mut</sup>, the highest amount of GABA (26.5±1.0 g/L) and the highest amount of both Glu and GABA (290±35 mM) were obtained at 60 h and decreased thereafter, indicating the decomposition and consumption of GABA and Glu after 60 h, likely due to the exhaust of glucose. Therefore, coexpression of gadB1<sup>mut</sup> and gadB2 under the optimal P<sub> tacM </sub>Promoter and R4a RBS sequence was effective for production of GABA in C. glutamicum.

**Discussion**

This study aims to ascertain an optimal combination of promoter and RBS sequence applying for the expression of GAD gene(s) and production of GABA in C. glutamicum. The expression level of heterologous genes has been illustrated to be influenced by multiple factors, including gene dosage, promoter strength, secondary structure of mRNA and RBS sequence (Stenstrom et al. 2001; Salis et al. 2009). To obtain the optimal expression element, successive investigation of RBS sequences and promoters was conducted.

For all the RBS sequences tested in this study, their strength for translating GadB2 and producing GABA was...
dramatically different (Fig. 1c, b). These RBS sequence can be used to translate protein in *C. glutamicum* at different levels. Two RBS sequences stronger than the frequently used RBS sequence of R, i.e. R4 and R6, were obtained here. Meanwhile, R4 showed the highest strength, whereas R9 and R3 showed the lowest strength. Similarly, the RBS sequence of R4 (GAAAGGAGA) was reported to exhibit higher expression level of NhhBA and specific activity of NHase in *C. glutamicum* (Kang et al. 2014). However, the RBS sequence of R3 (GAAAGGCGA) generated a slightly lower NhhBA level and NHase activity than R4, whereas here it resulted in the greatly decreased GAD activity and GABA production. In addition, the RBS sequence of R9 with AS of 8 nt (GAAAGGAGGttgggaca) was reported to be a strong RBS for expressing *via* genes and producing violacein in *C. glutamicum* (Sun et al. 2016), meanwhile, the anti-SD sequence at the 3′-end of the 16S rRNA of *C. glutamicum* (Sun et al. 2016) was described as 5′-CCUCCUUUC-3′ (Martín et al. 2003), whereas in our study R9 was too weak to express GadB2 and produce GABA. Therefore, the translation efficiency of different proteins through the identical RBS sequence of R3 or R9 may be dramatically different. However, the strong RBS sequences of R (AGAAGGAGG) and R7 (GAAAGGAGA) used previously (Shi et al. 2013; Zhang et al. 2015b) were also effective for translating GadB2 and producing GABA. Besides RBS sequence, the AS between RBS and translational start codon also influenced GadB2 activity and GABA production significantly, with the AS of 6 nt the most efficient one (Fig. 2e). This is in consistent with previous study on the spacer length of *C. glutamicum* by transcriptome analysis, which showed 6 nt the maximum number (Pfeifer-Sancar et al. 2013). The RBS sequence and AS determine the affinity and accessibility of ribosome to RBS. At the beginning of translation, ribosome directly binds to RBS. A weaker secondary structure, i.e. fewer base pairs at RBS, can strengthen the accessibility and affinity of ribosome to RBS and thus improve the translation efficiency (Isaacs et al. 2004). Here, through optimizing RBS sequence and AS for translating GadB2, GAD activity increased by 156% and GABA production increased by 82% compared to the classic strong RBS of R and AS of 7 nt.

The strength of promoters tested in this study was also dramatically different as expressing *gadB2* and producing GABA (Figs. 3, 4, 5). These promoters with different strength can be applied to provide different level of gene expression in *C. glutamicum*. *P* _tacM_ showed the highest strength here, followed by *P* _dnaK_ and *P* _lilvE_ whereas *P* _lilvE_ and *P* _dnaK_ are σB-dependent (Larisch et al. 2007; Pátek and Nešvera 2011). *P* _dnaK_ is recognized by σH, σ7 and σ11, while *P* _clgR_ is recognized by σH and σ11 (Šilar et al. 2016). *P* _clgR_ is dependent on σM and σ11 (Ehira et al. 2009), while *P* _trxB1_ and *P* _sufR_ are σH-specific (Dostálková et al. 2017). Even with prolonged length and bicistronic architecture, the strength of *P* _dnaK_ did not enhance (Figs. 5e, 6), although bicistronic expression architecture of 12 genes of *C. glutamicum* had been proven to be more efficient than monocistronic expression part (Zhao et al. 2016b). Despite this, several novel promoters that were as strong as *P* _tacM_ and *P* _clgR_, i.e. *P* _dnaK_ and *P* _clgR_, were found in this study. *P* _tacM_ and *P* _clgR_ have been generally used as strong promoters for enhancing gene expression in *C. glutamicum* (Ravasi et al. 2012; Pátek et al. 2013; Vogt et al. 2015; Man et al. 2016). However, the widely applied strong promoter *P* _kad_ (Becker et al. 2011; Lee et al. 2014; Kim et al. 2015; Man et al. 2016) showed very weak ability for expressing *gadB2* and producing GABA. *P* _cg3141 (P_hmp) which exhibited the highest inducibility for expression of a reporter, sfGFP during the transition phase between exponential and stationary phases in *C. glutamicum* (Kim et al. 2016) also showed much weak ability for GAD activity and GABA production. Therefore, all the native promoters tested here did not work as well as the strong synthetic promoter *P* _tacM_ partially due to the complex regulation of these native promoters by transcriptional regulators, such as regulation of *P* _gapA_ by GlxR and SugR, regulation of *P* _dtsR1_ by GlxR, repression of *P* _dnaK_, *P* _clgR_ and *P* _clgR_ by HspR, repression of *P* _gbh_ by GlxR, AmrR, FarR and ArgR, repression of *P* _sufR_ by SufR (Schroder and Tauch 2010). Recently, the strong synthetic promoter *P* _E3h_ was employed for producing single-chain variable fragment of antibody (Yim et al. 2014), GABA (Choi et al. 2015) and 5-aminovaleric acid (Shin et al. 2016), while *P* _I30_ was proven to be more suitable than *P* _I36_ for the production of cadaverine in *C. glutamicum* (Oh et al. 2015). Therefore, synthetic promoters will be tested for *gadB2* expression and GABA production in the future.

Finally, two GAD genes, i.e. *gadB2* and *gadB1mut*, were co-expressed in *C. glutamicum*, both under the optimal *P* _tacM_ promoter and R4a RBS sequence. GAD activity increased greatly by 3.0-fold; consequently, GABA
production increased to more than 25 g/L and all Glu was converted to GABA (Fig. 7d, e). However, the GABA production was not high enough. The GABA titer here was somewhat lower than that of GADΔpknG (Okai et al. 2014) and recombinant C. glutamicum strain harbouring pHGmut (Choi et al. 2015) which expressed E. coli GAD and much lower than that of GABA6C and GABA6F (Jorge et al. 2017) whose putrescine pathway was engineered (Table 3). The GABA volumetric productivity here was comparable to that of strains reported previously with GAD activity and significant lower than that of strains with engineered putrescine pathway. In addition, the GABA yield on glucose here was lower than that of GADΔpknG but comparable to that of other recombinant C. glutamicum strains. Considering that more GABA will be produced if the Glu production is high enough, fed-batch fermentation of R4a-B2B1mut will be carried out in the future.

Table 3  GABA production in several recombinant C. glutamicum strains

| Strains                  | GAD   | GAD ΔpknG | Harbouring pHGmut | R4a-B2 | R4a-B2B1mut | GABA6C | GABA6F |
|--------------------------|-------|-----------|--------------------|--------|-------------|--------|--------|
| Cultivation in           | Shake flask | Fed-batch | Shake flask         | Fed-batch | Putrescine |
| Pathway                  | GAD   | GAD       | GAD                | GAD    | Putrescine  |
| Cultivation time (h)     | 120   | 120       | 72                 | 72     | 60          | 64     | 69     |
| GABA titer (g/L)         | 3.1 ± 0.5 | 3.1 ± 0.4 | 3.86 ± 0.9         | 20.2 ± 0.3 | 26.5 ± 1.0 | 59.7   | 63.2   |
| GABA volumetric productivity (g/L/h) | 0.108 | 0.259 | 0.536 | 0.281 | 0.442 | 1.34 | 1.13 |
| GABA yield on glucose (g/g) | 0.156 | 0.511 | 0.320 | 0.212 | 0.269 | 0.24 | 0.24 |

References

Okai et al. (2014) Choi et al. (2015) This work Jorge et al. (2017)

Acknowledgements

The authors are thankful to the technical staff of the food safety lab of State Key Laboratory of Food Science and Technology, China.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

Not applicable.

Consent for publication

Not applicable.

Ethics approval and consent to participate

This article does not contain studies with human participant or animals performed by any of the authors.

Funding

This work was supported by national first-class discipline program of Light Industry Technology and Engineering (LITE2018-10).

Publisher’s Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Received: 7 April 2018 Accepted: 13 April 2018 Published online: 18 April 2018

Abbreviations

GAD: glutamate decarboxylase; Glu: L-glutamate; GABA: γ-aminobutyric acid; RBS: ribosomal binding site; AS: aligned spacing; LAB: lactic acid bacteria; Lys: L-lysine; SD: Shine–Dalgarno; dnaK: rRNA gene.

Authors’ contributions

FS conceived and designed the experiments, coordinated the study, analyzed the data and contributed to the writing of the manuscript. ML performed the measurements and experiments and analyzed the data. YL participated in the writing of the manuscript. All authors read and approved the final manuscript.

Author details

1 State Key Laboratory of Food Science and Technology, Jiangnan University, 1800 Lihu Avenue, Wuxi 214122, China. 2 Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, China. 3 International Joint Laboratory on Food Safety, Jiangnan University, Wuxi 214122, China. 4 National Engineering Laboratory for Cereal Fermentation Technology, Jiangnan University, Wuxi 214122, China.

References

Amador E, Castro JM, Correa A, Martin JF (1999) Structure and organization of the rmd operon of ‘Brevibacterium lactofermentum’: analysis of the 16S rRNA gene. Microbiology 145:915–924

Becker J, Zelder O, Hafner S, Schroder H, Wittmann C (2011) From zero to hero-design-based systems metabolic engineering of Corynebacterium glutamicum for γ-glutamate. Biotechnol Bioeng 110:2154–2165

Choi JW, Yim SS, Lee SH, Kang TJ, Park SJ, Jeong KJ (2015) Enhanced production of γ-aminobutyrate (GABA) in recombinant Corynebacterium glutamicum by expressing glutamate decarboxylase active in expanded pH range. Microb Cell Fact 14:21

Dostalova H, Holatko J, Busche T, Rucka L, Rapoport A, Halada P, Nelvra J, Kalinowski J, Patek M (2017) Assignment of sigma factors of RNA polymerase to promoters in Corynebacterium glutamicum. Mol Microbiol 113:671–686

Ehira S, Teramoto H, Inui M, Yukawa H (2009) Regulation of GABA biosynthesis by expressing glutamate decarboxylase active in expanded pH range. Microb Cell Fact 8:20

Huang Y, Su L, Wu J (2016) Pyridoxine supplementation improves the activity of recombinant glutamate decarboxylase and the enzymatic production of γ-aminobutyric acid. PLoS ONE 11:e0157466
Isaacs FJ, Dwyer DJ, Ding C, Pervouchine DD, Cantor CR, Collins JJ (2004) Engineered riboregulators enable post-transcriptional control of gene expression. Nat Biotechnol 22:841–847

Jorge JM, Leggewie C, Wendisch VF (2016) A new metabolic route for the production of gamma-aminobutyric acid by Corynebacterium glutamicum from glucose. Amino Acids 48:2519–2531

Jorge JM, Nguyen AQ, Perez-Garcia F, Kind S, Wendisch VF (2017) Improved fermentative production of gamma-aminobutyric acid via the putrescine route: systems metabolic engineering for production from glucose, amino sugars, and xylose. Biotechnol Bioeng 114:862–873

Kang MS, Han SS, Kim MY, Kim BY, Huh JP, Kim HS, Lee JH (2014) High-level expression in Corynebacterium glutamicum of nitrite hydratase from Rhodococcus rhodochrous for acrylamide production. Appl Microbiol Biotechnol 98:4379–4387

Kim SY, Lee J, Lee SY (2015) Metabolic engineering of Corynebacterium glutamicum for the production of γ-ornithine. Biotechnol Bioeng 112:416–421

Kim MJ, Yim SS, Choi JW, Jeong KJ (2016) Development of a potential stationary-phase specific gene expression system by engineering of SigB-dependent cg3141 promoter in Corynebacterium glutamicum. Appl Microbiol Biotechnol 100:4473–4483

Larsch C, Nakunst D, Huser AT, Tauch A, Kalinowski J (2007) The alternative sigma factor SigB of Corynebacterium glutamicum modulates global gene expression during transition from exponential growth to stationary phase. BMC Genomics 8

Lee JH (2014) Development and characterization of expression vectors for Corynebacterium glutamicum. J Microbiol Biotechnol 24:70–79

Leuchtenberger W, Huthmacher K, Drauz K (2005) Biotechnological production of amino acids and derivatives: current status and prospects. Appl Microbiol Biotechnol 69:1–8

Li H, Cao Y (2010) Lactic acid bacterial cell factories for gamma-aminobutyric acid. Amino Acids 39:1107–1116

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔct method. Methods 25:402–408

Man Z, Xu M, Rao Z, Guo J, Yang T, Zhang X, Xu Z (2016) Improved fermentation of gamma-aminobutyric acid by Corynebacterium glutamicum. J Biotechnol 214:191–198

Ravasi P, Peiru S, Gramajo H, Menzeliza HG (2012) Design and testing of a synthetic biology framework for genetic engineering of Corynebacterium glutamicum. Microbiol Cell Fact 11:147

Ryter JW, Helmark S, Chen J, Lezey MJ, Solem C, Jensen PR (2014) Synthetic promoter libraries for Corynebacterium glutamicum. Appl Microbiol Biotechnol 98:2617–2623

Salis HM, Mirsky EA, Voigt CA (2009) Automated design of synthetic ribosome binding sites to control protein expression. Nat Biotechnol 27:946–950

Schneider J, Niermann K, Wendisch VF (2011) Production of the amino acids l-glutamate, l-lysine, l-ornithine and l-arginine from albacins by recombinant Corynebacterium glutamicum. J Biotechnol 154:191–196

Shen J, Chen J, Jensen PR, Solem C (2017) A novel genetic tool for metabolic optimization of Corynebacterium glutamicum: efficient and repetitive chromosomal integration of synthetic promoter-driven expression libraries. Appl Microbiol Biotechnol 101:4737–4746

Shi F, Li Y (2011) Synthesis of γ-aminobutyric acid by expressing Lactobacillus brevis-derived glutamate decarboxylase in the Corynebacterium glutamicum strain ATCC 13032. Biotechnol Lett 33:2469–2474

Shi F, Xie B, Jiang J, Li Y, Li, Xie Y (2013) Enhancement of γ-aminobutyric acid production in recombinant Corynebacterium glutamicum by co-expressing two glutamate decarboxylase genes from Lactobacillus brevis. J Ind Microbiol Biotechnol 40:1285–1296

Shi F, Xie B, Jiang J, Wang N, Li Y, Wang X (2014) Directed evolution and mutagenesis of glutamate decarboxylase from Lactobacillus brevis Lb85 to broaden the range of its activity toward a near-neutral pH. Enzyme Microb Technol 61:62–65

Shi F, Ni Y, Wang N (2016) Metabolism and biotechnological production of gamma-aminobutyric acid (GABA). In: Vandamme EJ, Revueltla JL (eds) Industrial biotechnology of vitamins, biopigments, and antioxidants. Wiley, Weinheim, pp 445–468

Shi F, Zhang M, Li Y (2017a) Overexpression of psc or deletion of mfdh for improving production of γ-aminobutyric acid in recombinant Corynebacterium glutamicum. World J Microbiol Biotechnol 33:122

Shi X, Chang C, Ma S, Cheng Y, Zhang J, Gao Q (2017b) Efficient biocconversion of L-glutamate to γ-aminobutyric acid by Lactobacillus brevis resting cells. J Ind Microbiol Biotechnol 44:697–704

Shin JH, Park SH, Oh YH, Choi JW, Lee MW, Cho JS, Jeong KJ, Joo JC, Yu JP, Park SJ, Lee SY (2016) Metabolic engineering of Corynebacterium glutamicum for enhanced production of 5-aminovaleric acid. Microb Cell Fact 15:174

Silar R, Holátko J, Rucka L, Rapoport A, Dostálová H, Kadeřábková P, Nešvera J, Pátek M (2016) Use of in vitro transcription system for analysis of Corynebacterium glutamicum promoters recognized by two sigma factors. Curr Microbiol 73:401–408

Stenstrom CM, Holmgren E, Isaksson LA (2001) Cooperative effects by the initiation codon and its flanking regions on translation initiation. Gene 273:259–265

Sun H, Zhao D, Xiong B, Zhang C, Bi C (2016) Engineering Corynebacterium glutamicum for violacein hyper production. Microbiol Cell Fact 15:148

Teramoto H, Watanabe K, Suzuki N, Inui M, Yukawa H (2011) High yield secretion of heterologous proteins in Corynebacterium glutamicum using its own Tat-type signal sequence. Appl Microbiol Biotechnol 91:677–687

Voigt ML, Krumback K, Wang WC, van Ooyen J, Noack S, Klein B, Bott M, Egelgell L (2015) The contest for precursors: channeling isoleucine synthesis in Corynebacterium glutamicum without byproduct formation. Appl Microbiol Biotechnol 99:791–800

Wang N, Yi Y, Shi F (2015) Deletion of gadA or pyc improves production of γ-aminobutyric acid and its precursor γ-glutamate in recombinant Corynebacterium glutamicum. Biotechnol Lett 37:1473–1481

Wong CG, Bottiglieri T, Snead OC (2003) GABA, gamma-hydroxybutyric acid, and neurological disease. Ann Neurol 54:532–532

Wu Q, Qin HM, Law YS, Khafipour E, Shah NP (2017) Common distribution of gad operon in Lactobacillus brevis and its gadA contributes to efficient GABA synthesis toward cytosolic near-neutral pH. Front Microbiol 8:206
Xu D, Tan Y, Shi F, Wang X (2010) An improved shuttle vector constructed for metabolic engineering research in Corynebacterium glutamicum. Plasmid 64:85–91
Yim SS, An SJ, Kang M, Lee JH, Jeong KJ (2013) Isolation of fully synthetic promoters for high-level gene expression in Corynebacterium glutamicum. Biotechnol Bioeng 110:11
Yim SS, An SJ, Choi JW, Ryu AJ, Jeong KJ (2014) High-level secretory production of recombinant single-chain variable fragment (scFv) in Corynebacterium glutamicum. Appl Microbiol Biotechnol 98:273–284
Zhang B, Zhou N, Liu YM, Liu C, Lou CB, Jiang CY, Liu SJ (2015a) Ribosome binding site libraries and pathway modules for shikimic acid synthesis with Corynebacterium glutamicum. Microb Cell Fact. 14:71
Zhang L, Jia H, Xu D (2015b) Construction of a novel twin-arginine translocation (Tat)-dependent type expression vector for secretory production of heterologous proteins in Corynebacterium glutamicum. Plasmid 82:50–55
Zhang B, Ren LQ, Yu M, Zhou Y, Ye BC (2017a) Enhanced l-ornithine production by systematic manipulation of l-ornithine metabolism in engineered Corynebacterium glutamicum S9114. Bioresour Technol 250:60–68
Zhang W, Zhao Z, Yang Y, Liu X, Bai Z (2017b) Construction of an expression vector that uses the aph promoter for protein expression in Corynebacterium glutamicum. Plasmid 94:1–6
Zhao A, Hu X, Li Y, Chen C, Wang X (2016a) Extracellular expression of glutamate decarboxylase B in Escherichia coli to improve gamma-aminobutyric acid production. AMB Expr 6:55
Zhao Z, Liu X, Zhang W, Yang Y, Dai X, Bai Z (2016b) Construction of genetic parts from the Corynebacterium glutamicum genome with high expression activities. Biotechnol Lett 38:2119–2126

Submit your manuscript to a SpringerOpen journal and benefit from:

- Convenient online submission
- Rigorous peer review
- Open access: articles freely available online
- High visibility within the field
- Retaining the copyright to your article

Submit your next manuscript at ➤ springeropen.com