Review

Integrative expression vectors with Pg grac promoters for inducer-free overproduction of recombinant proteins in *Bacillus subtilis*

Dinh Thi Minh Tran¹,c,f,g,¹, Trang Thi Phuong Phan¹,b,g,*, Thanh Thi Ngoc Doan¹,d, Thuoc Linh Tran¹,g, Wolfgang Schumann¹,e, Hoang Duc Nguyen¹,f,g,*

¹ Center for Bioscience and Biotechnology, University of Science, 227 Nguyen Van Cu Dist. 5, Ho Chi Minh City, Viet Nam
² Laboratory of Molecular Biotechnology, University of Science, 227 Nguyen Van Cu Dist. 5, Ho Chi Minh City, Viet Nam
³ Department of Biology, Ho Chi Minh City University of Education, 280 An Duong Vuong, Dist. 5, Ho Chi Minh City, Viet Nam
⁴ Agriculture and Food Technology Faculty, Tien Giang University, 119, Ap Bac, My Tho City, Tien Giang, Viet Nam
⁵ Institute of Genetics, University of Bayreuth, D-95446, Bayreuth, Germany
⁶ Department of Microbiology, University of Science, 227 Nguyen Van Cu Dist. 5, Ho Chi Minh City, Viet Nam
⁷ Vietnam National University, Ho Chi Minh City, Viet Nam

A R T I C L E   I N F O

Article history:
Received 3 May 2020
Received in revised form 28 September 2020
Accepted 8 October 2020

Keywords:
Inducer-free
Integrative expression vector
Pg grac01 promoter
Pg grac100 promoter
Pg grac212 promoter
pH T vector

A B S T R A C T

Inducer-free integrative vectors are often used to create *B. subtilis* strains for industrial purposes, but employing strong promoters to produce high levels of recombinant proteins in *B. subtilis* results in high leaky expression that can hamper cloning in *Escherichia coli*. To overcome the problem, we used strong IPTG-inducible Pg grac promoters harboring lac operators to construct inducer-free integrative vectors able to integrate into the *B. subtilis* genome at either the lac operon or the amyE locus, or both and examined their ability to repress the β-galactosidase (BgaB) gene in *E. coli* and to overexpress BgaB in *B. subtilis*. The Pg grac01 vectors could repress bgaB expression about 24-fold in *E. coli* to low background levels. The integrated Pg grac01-bgaB constructs exhibited inducer-free expression and produced 8% of total cellular proteins, only 1.25 or 1.75 times less compared with their cognates as plasmids. The stronger promoters, Pg grac100-bgaB and Pg grac212-bgaB yielded 20.9% and 42% of total intracellular proteins after 12 h of incubation, respectively. Incorporation of the Pg grac212-bgaB into both amyE and lac operons resulted in BgaB expression up to 53.4%. In conclusion, integrative vectors containing the Pg grac promoter family have great potential for inducer-free overproduction of recombinant proteins in *B. subtilis*.

© 2020 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

1. Introduction

*B. subtilis* is presently the best-characterized Gram-positive bacterium [1]. Its biochemistry, physiology and genetics have been studied intensively for more than fifty years [2]. As a result, a great deal of vital information concerning its transcription and translation mechanisms, genetic manipulation, and large-scale fermentation has been acquired [3]. In addition, it has a long history of industrial use because of its excellent growth on cheap carbon sources, and robustness under industrial conditions [4]. Another advantage is that *B. subtilis* is regarded as a Generally Recognized as Safe (GRAS) organism that lacks endotoxins and is non-pathogenic [5,6]. Furthermore, it has no significant bias in codon usage [3]. Therefore, it has become an ideal bacterial ‘factory’ for recombinant protein production and a large variety of expression vectors have been created. The vectors may be inducible or inducer-free (constitutive or autoinducible) [7–9]. Inducer-free expression vectors have recently been gaining increased popularity because of the high costs [10] of many inducer compounds and problems with their toxicity [11].

Traditional expression systems utilized high copy number plasmids introduced into *B. subtilis* to create recombinant strains for heterologous protein expression [3]. However, plasmid-less engineered *B. subtilis* strains are preferred in industrial applications due to their stability and lower ecological risk [12]. Formerly, plasmid-free recombinant *B. subtilis* strains were constructed by homologous recombination between the target sequence in the chromosome and the homologous flanking sequences sandwiching the fragment of interest mediating ectopic insertion of the

Abbreviations: BgaB, β-galactosidase; LB, Luria broth; IPTG, isopropylthiogalactoside; MUG, methylumbelliferyl β-D-galactopyranoside; MCS, multiple cloning site.

* Corresponding authors at: Center for Bioscience and Biotechnology, University of Science, 227 Nguyen Van Cu Dist. 5, Ho Chi Minh City, Viet Nam.

E-mail addresses: pptrang@hcmus.edu.vn (T.T.P. Phan), ndhoang@hcmus.edu.vn (H.D. Nguyen).

¹ These authors contributed equally to this work.

https://doi.org/10.1016/j.btre.2020.e00540
2215-017X/© 2020 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).
desired genes into the bacterial chromosome [3]. Recently, several new vectors have been developed resulting in more efficient integration. Some of them allow production of recombinant proteins by incorporating many copies of the recombinant gene at different sites in the bacterial genome [5,13]. For example, an integrated vector engineered with the strong inducer-free promoter NBP3510 exhibited β-galactosidase (BgaB) and GFP (green fluorescent protein) production of 43% and 30% of the total cellular proteins, respectively [14].

The unsolved problem in the generation of useful vectors allowing robust B. subtilis expression systems is their leaky expression in E. coli. Most of the expression vectors for B. subtilis are shuttle-vectors because the cloning steps must be carried out in E. coli [3], which can sometimes result in unexpectedly high protein expression levels [15,16], and, in the worst case, kill the E. coli cells. Previous publications did not mention whether strong promoters for B. subtilis also cause high background expression in E. coli. Here, we constructed inducer-free expression vectors allowing the integration of the recombinant gene into the B. subtilis genome. These vectors carried strong promoters and their expression level in B. subtilis was comparable to that from high copy number replicative plasmids while expression in E. coli remained relatively low. In addition, double-copy insertion strains were generated by the integration of the recombinant gene into two different neutral loci. The best resulting strains had an expression level of over 50% of the total intracellular proteins.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

Plasmids, oligonucleotides, and strains used in this study are shown in Table 1. The E. coli strain OmniMAX (Invitrogen) was used as the recipient in all cloning experiments and to determine the background expression levels. All recombinant B. subtilis strains used to analyze the expression of the bgaB gene were derived from B. subtilis 1012. Cultures were initiated from single-colony inocula grown on LB agar plates. Cells were routinely grown in Luria broth (LB) at 37 °C with shaking at 200 rpm. Where necessary, the antibiotics ampicillin at 100 μg/mL for E. coli and chloramphenicol at 10 μg/mL for B. subtilis were added to recombinant strains harboring replicative plasmids.

2.2. Construction of integrative vectors

2.2.1. Expression vectors integrating into the lacA locus

We first created the three basic inducer-free integrative expression vectors, pHT2171, pHT2184 and pHT2188, and then, the bgaB gene was introduced into these vectors to obtain pHT2172, pHT2185 and pHT2189. pHT2171 was constructed by inserting the cassette containing the lacO3 operator and the Pgrac01 promoter (amplified from pHT2134 as the template, a derivative of pHT2071 [19] carrying a new MCS, with ON2195/ON2194) into the pHT1305 empty vector which contains sequences to allow integration at the lacA locus on the B. subtilis genome without any promoter. pHT2184 was constructed by insertion of the cassette harboring lacO3 and the Pgrac100 promoter into the pHT1326 backbone. This backbone has no promoter but contains the homologous sequences allowing integration into the lacA locus on the B. subtilis genome. pHT2184 was first cleaved with SalI and BamHI to remove Pgrac100. Then, it was ligated to Pgrac212 amplified using the pHT2080 template to create pHT2188.

2.2.2. Inducer-free integrative vectors containing Pgrac01-bgaB, Pgrac100-bgaB, and Pgrac212-bgaB

To construct pHT2172, pHT2185, and pHT2189, we amplified the bgaB gene using the primer pairs ON2134 and ON941 with pNDH33-bgaB as a template. The BamHI/AatII-treated PCR product was introduced into pHT2171, pHT2184, and pHT2188 at the BamHI and AatII sites, respectively.

2.2.3. Expression vectors able to integrate into the amyE locus

To construct plasmids pHT2170, pHT2176, and pHT2177, we removed lacI together with the lacO3 sequence from plasmids pHT2115, pHT2118, and pHT2119, respectively, and inserted the lacO3 sequence between the SnuBI and SacI restriction sites by using the two complementary oligonucleotides ON1975 and ON1976.

2.3. Generation of E. coli and B. subtilis recombinant strains

Expression vectors were confirmed by DNA sequencing. The correct vectors were transformed into E. coli OmniMAX as described in [22] and into B. subtilis 1012 as described elsewhere [23]. Recombinant B. subtilis strains generated by double crossover events were screened by PCR using specific oligonucleotide pairs. Fig. 2A and D shows specific primers with the length of the PCR products for the strains carrying Pgrac01 that integrate into amyE and lacA loci. The same PCR approach has been used to confirm the integration of the expression cassettes containing Pgrac100 and Pgrac212.

2.4. Measurement of BgaB expression levels in E. coli and B. subtilis

Recombinant strains were streaked on LB agar plates with the appropriate antibiotic: ampicillin at 100 μg/mL for E. coli and chloramphenicol at 10 μg/mL for B. subtilis harboring replicative plasmids, and no antibiotic for B. subtilis strains carrying integrated sequences. A single colony was inoculated into a culture tube containing 5 mL LB medium and antibiotic and shaken overnight at 200 rpm at 37°C. Cultures of each strain were replicated using three separate colonies. The OD600 of the pre-culture was measured and an appropriate volume of pre-culture of each clone was transferred to 30 mL LB medium containing the appropriate antibiotic in 100 mL shake flasks to give an OD600 of 0.1 and incubated with shaking at 37°C. When the OD600 of the culture reached 0.8–1, the cells were divided into two sub-cultures and one of them was induced by the addition of IPTG to a final concentration of 1 mM. Cells were collected by centrifugation at 0 h just before induction and at 2, 4, 6, 8, 10, 12 h after induction. The OD600 of all sub-cultures was monitored and a volume equivalent to an OD600 of 2.4 was pipetted into 1.5 mL Eppendorf tubes, centrifuged, and the supernatants removed. Samples were prepared for activity measurements and for SDS-PAGE analysis. BgaB activity was measured as described in [19].

For SDS-PAGE analyses, cell pellets were lysed by the addition of 100 μL lysis buffer (25 mM SDS, 250 mM sucrose) with an addition of 2.5 μL lysozyme at 50 mg/mL. The mixtures were vortexed thoroughly and incubated at 37°C for 5 min. After that, 25 μL of 5X sample buffer (10 mL Tris – HCl pH 6.8, 1.54 g dithiothreitol, 0.4 g SDS, 4 mL glycerol, bromophenol blue, and dH2O up to 20 mL) was added. The samples were mixed well and heated at 95°C for 5 min followed by centrifugation at 15871 rcf for 5 min. Aliquots of 8 μL of each sample were applied to each well on SDS-PAGE gels [24].
Table 1
Bacterial strains, plasmids and oligonucleotides used in this study.

| Strain or plasmid | Genotype | Source/reference |
|-------------------|----------|------------------|
| E. coli OmniMAX   | F'proAB lacI lacZ ΔM15 Tn10(Tet^r) Δ(ccdAB) mcrA Δ(mrr-hsdRMS-merC) φ80(lacZΔM15 Δlac2Y都不会 argF) U169 endA1 recA1 supE44 thi-1 gyrA96 relA1 tonA panD | Invitrogen |
| Bacillus subtilis 1012 | leuA8 metB5 tprC2 hsdRMI | This study |
| B. subtilis HT2170 | Recombinant B. subtilis 1012 with the expression cassette of pHT2170 integrated at the amyE locus | This study |
| B. subtilis HT2176 | Recombinant B. subtilis 1012 with the expression cassette of pHT2176 integrated at the amyE locus | This study |
| B. subtilis HT2177 | Recombinant B. subtilis 1012 with the expression cassette of pHT2177 integrated at the amyE locus | This study |
| Bs/pHCMS05- bgaB | Recombinant B. subtilis 1012 carrying pHCMC05-bgaB | This study |
| Bs/pHT01-bgaB | Recombinant B. subtilis 1012 carrying pHT01-bgaB | This study |
| Bs/pHT2071 | Recombinant B. subtilis 1012 carrying pHT2071 | This study |
| E. coli pHT01-bgaB | Recombinant E. coli OmniMAX carrying pHT01-bgaB | This study |
| E. coli/pHT1379 | Recombinant E. coli OmniMAX carrying pHT1379 | This study |
| E. coli/pHT2071 | Recombinant E. coli OmniMAX carrying pHT2071 | This study |
| E. coli/pHT2170 | Recombinant E. coli OmniMAX carrying pHT2170 | This study |
| pHT1305 Pgr0101-bgaB, inducible, replicative | Lab stock and obtained from Ms. Hanh |
| pH11326 | An empty vector that lacks promoters and reporter genes, carries a neomycin resistance gene and can integrate into the B. subtilis chromosome at the lacA locus | Lab stock and obtained from Ms. Hanh |
| pH11379 Pgr01- multiple cloning site (MCS), ΔlacI, allows integration at the amyE locus, served as a negative control | Lab stock and obtained from Ms. Hanh |
| pH12071 Pgr01-bgaB, ΔlacI, replicative | Colony PCR pH12185, pH12189 |
| pH12115 Pgr01-bgaB, intact lacI, allows integration at the amyE locus | Colony PCR pH12170, pH12176, pH12177 |
| pH12118 Pgr100-bgaB, intact lacI, allows integration at the amyE locus | For sequencing pH12171 |
| pH12119 Pgr212-bgaB, intact lacI, allows integration at the amyE locus | For sequencing pH12172, pH12176, pH12189 |
| pH12170 Pgr01-bgaB, ΔlacI, allows integration at the amyE locus | For sequencing pH12172, pH12176, pH12189 |
| pH12171 Pgr01-MCS, ΔlacI, allows integration at the lacA locus, served as a negative control | For sequencing pH12172, pH12176, pH12189 |
| pH12172 Pgr01-bgaB, ΔlacI, allows integration at the lacA locus | For sequencing pH12172, pH12176, pH12189 |
| pH12176 Pgr100-bgaB, ΔlacI, allows integration at the amyE locus | For sequencing pH12172, pH12176, pH12189 |
| pH12177 Pgr212-bgaB, ΔlacI, allows integration at the amyE locus | For sequencing pH12172, pH12176, pH12189 |
| pH12184 Pgr100-MCS, ΔlacI, allows integration at the lacA locus | For sequencing pH12172, pH12176, pH12189 |
| pH12185 Pgr100-bgaB, ΔlacI, allows integration at the lacA locus | For sequencing pH12172, pH12176, pH12189 |
| pH12188 Pgr212-MCS, ΔlacI, allows integration at the lacA locus | For sequencing pH12172, pH12176, pH12189 |
| pH12189 Pgr212-bgaB, ΔlacI, allows integration at the lacA locus | For sequencing pH12172, pH12176, pH12189 |

**Oligonucleotides**

| Sequence | Used for |
|----------|----------|
| ON469 GCGGTCTCTGGTTGCTCG | To confirm the insertion of the expression cassette at 5’ amyE |
| ON470 AAGCGGTCCGGATTAAGCTAC | To confirm the insertion of the expression cassette at 3’ amyE |
| ON745 CCACGTCAGAGTCAGCTCAAGATTCCCTATCATTTATCAGCGG | To confirm the insertion of bgf in B. subtilis genome |
| ON881 TCATAGCCTGATATTTGATAC | Colony PCR pH12185, pH12189 |
| ON900 GGCGGATCTCTCTCAATGTTTAAGGAAAGGAC | Colony PCR pH12170, pH12176, pH12177 |
| ON934 AGAACGCGAGAACGATAAGTTCATGCTAC | For sequencing pH12171 |
| ON972 TGCGGATCCGATCTGAGATGAAATG | For sequencing pH12172, pH12176, pH12189 |
| ON977 GAGCGATGCTCTGAGATGAAATG | For sequencing pH12172, pH12176, pH12189 |
| ON1207 GAGCTTTCCTCCCTATAGAATG | For sequencing pH12172, pH12176, pH12189 |
| ON1289 CCTTCCGACCCGCAATTCGCT | Colony PCR pH12171 |
| ON1354 AACGCAGATCCGATCTGAGATGAAATG | Colony PCR pH12171 |
| ON1441 CTGATTCGATCTGAGATGAAATG | Colony PCR pH12171 |
| ON1442 GATCTGCCGATTCTGGCTAC | Colony PCR pH12171 |
| ON1479 GATGATCAGATCTGAGATGAAATG | Colony PCR pH12171 |
| ON1896 CGGTTGATCCGACTTTCAGCT | Colony PCR pH12171 |
| ON1975 CATTATCAGGATCGGCGCCGCT | Colony PCR pH12171 |
| ON1976 AGCGTTGATCCGACTTTCAGCT | Colony PCR pH12171 |
| ON2134 GGGCGGGGCGGATCCGCTTAC | Colony PCR pH12171 |
| ON2194 GGGCGGGGCGGATCCGCTTAC | Colony PCR pH12171 |
| ON2195 GGGCGGGGCGGATCCGCTTAC | Colony PCR pH12171 |

ΔlacI, deletion of partial or full lacI gene; Pgr01- (another name is Pgruc) [20]; Pgruc100 [21] and Pgruc212 [18] are the names of different promoters. Fig. 4D shows the alignment of the promoter sequences.
3. Results

3.1. Background expression levels of Pgrac01-bgaB constructs in E. coli

Expression vectors for *B. subtilis* are shuttle-vectors, and the cloning steps are carried out in *E. coli*. However, a vector that could drive a high level of expression in *B. subtilis* often results in a high level of expression in *E. coli* even in the absence of inducers. Previously, we reported a new strategy to construct inducer-free plasmids based on IPTG-inducible promoters. By deleting part of the *lac* gene in IPTG-inducible pH vectors, the resulting plasmids could express the target proteins in the absence of IPTG in *B. subtilis* while still repressing the background expression in *E. coli* through the chromosomal *lac* gene [19]. To generate expression vectors for *B. subtilis* which express the recombinant protein at high levels in *B. subtilis* while performing very low expression in *E. coli*, we used this strategy to develop inducer-free integrative expression vectors based on Pgrac promoters as reported earlier [19]. The promoters are flanked by two *lac* operators, *lacO1* at the downstream and *lacO3* at upstream of the promoters, which promote the repression of leaky expression by DNA-loop formation in the presence of the LacI repressor [25]. Besides, to increase the stability of the vectors as well as to lower the background expression in *E. coli*, we introduced the rop gene into the vectors. The Rop protein decreases the copy number of ColEI-like plasmids by stabilizing the RNAI-RNAII duplex [26].

An integrative expression vector harboring the Pgrac01 promoter and the bgaB reporter gene was constructed, and the leaky expression level was compared with that of some other replicative plasmids.

While pH701-bgaB (Pgrac01-bgaB, containing *lac*) could repress leaky expression in *E. coli* OmniMAX about 16-fold, pH72071 (Pgrac01-bgaB, Δ*lacI*) could repress it only about 3-fold, and pH72170 (Pgrac01-bgaB, Δ*lacI, rop*) could repress it about 24-fold (Fig. 1A). These results demonstrate that the combination of two *lac* operators together with the rop gene in the pH72170 plasmid created optimal repression of background expression in *E. coli*. The leaky expression of the bgaB gene from the inducer-free integrative expression vectors was the lowest among these vectors analyzed (Fig. 1B). Moreover, the leaky expression from pH72170 was approximately similar to pH71379 which served as the negative control which did not express bgaB. These results demonstrated that pH72170 could repress the leaky expression in *E. coli* thereby allowing the cloning steps. The conceptual figure (Fig. 1C) shows the repression of the Pgrac promoter by plasmid and chromosomal *lacI* and control of plasmid copy via rop gene in *E. coli* cloning strain.

3.2. Inducer-free expression of Pgrac01–bgaB integrated into the *B. subtilis* genome

The expression cassette of the vector pH72170 consisted of two homologous sequences of the *amy*E gene from *B. subtilis* genome, the spcC gene for selection, the Pgrac01 promoter and the bgaB reporter gene. This vector does not include an origin of replication for *B. subtilis*; therefore, when it is transformed into *B. subtilis* cells in the presence of spectinomycin, the expression cassette is integrated into the genome by either a single or a double crossover event. The latter was confirmed by PCR (Fig. 2A, B) and the resulting strain was named *B. subtilis* HT2170. The expression cassette was stably maintained in the genome of this strain even in the absence of spectinomycin. The bgaB expression level in *B. subtilis* HT2170 in the absence of IPTG and antibiotics was tested and compared with those of some other replicative plasmids carrying the same or a different promoter. HT2170 synthesized the BgaB protein in a manner different from strains carrying pH701-bgaB or pHCMC05-bgaB, but in the same way as with pH72071. While pH701-bgaB and pHCMC05-bgaB expressed bgaB only in the presence of IPTG, pH72170 synthetized BgaB similarly in the absence or presence of IPTG (Fig. 3A). SDS-PAGE analyses (Fig. 3B) confirmed this result. These results indicate that pH72170 can efficiently express bgaB in an inducer-free manner. The best performance of pH72170 was 6.66 × 10⁴ methylumbelliferyl β-D-galactopyranoside (MUG) units while that of pHCMC05-bgaB with the Pspac promoter reached only 0.47 × 10⁴ MUG units. The BgaB activity of pH72170 was about 14-fold higher than that obtained with the multi-copy replicative Pspac plasmid. The SDS-PAGE results (Fig. 3B) also confirmed that the expression level of the inducer-free integrative vector pH72170 was much higher than that of pHCMC05-bgaB. However, the BgaB activity of pH72170 was lower than that obtained with replicative plasmids carrying the same promoter. The BgaB activity of pH72170 was about 80 % of pH701-bgaB after induction with 1 mM IPTG and about 50 % of that obtained with pH72071 (Fig. 3A). In addition, SDS-PAGE analysis revealed that BgaB expressed by pH72170 accounted for only 8% of the total protein while pH72071 expressed 14 %, and pH701-bgaB about 10 % (Fig. 3B).

The expression of *B. subtilis* HT2170 was 1.25 or 1.75 times less than plasmid expression carrying the same promoter, most probably because of the lower copy number. Therefore, to generate integrative vectors whose expression level was comparable with that of multi-copy plasmids, we next constructed integrative vectors with stronger promoters.

3.3. High expression levels of the bgaB reporter gene using stronger Pgrac promoters

Next, we aimed to construct inducer-free integrative vectors with high-level expression by using the strongest promoters from the Pgrac library. On example is the engineered Pgrac100 promoter whose UP element, the -35 and -15 sequences have been mutated allowing intracellular accumulation of BgaB up to 30 % [27]. Pgrac212 is structurally similar to Pgrac01 containing modifications at the controllable stabilizing element (CoSE; the region from +1 to the RBS) [15] resulting in BgaB levels within the same range as compared to Pgrac100 [21]. Therefore, Pgrac100 and Pgrac212 were chosen. The highest BgaB activities were 6.9 × 10⁴ MUG units for the vector carrying the Pgrac01 promoter, 9.1 × 10⁴ MUG units for those with Pgrac100, and 14.4 × 10⁴ MUG units for those with Pgrac212 (Fig. 4A). Using the stronger promoters, bgaB expression increased 1.3 up to 2.1-fold. Analysis of Fig. 4B and 4C by Alpha Ease 4.0 showed that BgaB expressed by Pgrac100 vectors accounted for 20.9 % and Pgrac212 vectors accounted for 42 % of the total intracellular proteins after 12 h. BgaB expressed by HT2176-Pgrac100 accounted for 9% of the total cellular protein at 2 h, 12 % at 4 h and 20.9 % at 12 h (Fig. 4B). Similarly, the amount of BgaB produced by HT2177-Pgrac212 was low at the early stage of the culture, but rose rapidly with time-11 % of the total protein at 2 h, 32.7 % after 4 h, finally reaching 42 % after 12 h, or about 4-fold over time in culture (Fig. 4C).

3.4. Increasing expression levels by integrating Pgrac212-bgaB into both *amy*E and *lacA* loci

Another way to increase the expression level in *B. subtilis* strains carrying the expression unit in the genome is to increase the copy number of the expression unit. Several methods are available for multi-copy insertions of the target gene. Among them, integration at two and more different chromosomal sites may be the most stable [28], so the expression cassette was inserted into both the *amy*E and the *lacA* loci to increase the magnitude of expression of the target gene. The vector was first transformed into competent *B.
subtilis cells and the integration occurred at the amyE locus in the presence of spectinomycin. Next, a second vector with homologous sequences to the lacA locus was transformed into the strain already carrying one copy of the expression unit at the amyE locus in the presence of neomycin. The recombinant strains possessed two expression cassettes in the genome were confirmed by PCR (Fig. 2C, D), and bgaB expression was evaluated by the MUG assay and by SDS-PAGE.

Integration of the bgaB gene into both the amyE and the lacA loci doubled the copy number of the gene and the synthesis of BgaB from both loci was much higher than the expression from either the amyE or the lacA locus under control of the same promoter. Strains with expression cassettes containing Pgrac01, Pgrac100, or Pgrac212 integrated at the two loci expressed BgaB at levels of 23.4 %, 24 %, and 53.4 %, of total proteins, respectively. The expression of BgaB was increased from 1.1 to 1.3-fold (Fig. 5A). The strain with Pgrac212 expression cassettes inserted into both loci synthesized...
Fig. 2. **Confirmation of integration by PCR.** (A) Schematic diagram shows the sites of oligonucleotides used for checking the double crossover at both *amyE* in *B. subtilis* recombinant strains. (B) Electrophoresis of PCR products using *B. subtilis* HT2170 as template on 2% agarose gel, 1: PCR products by ON1976/ON470, 2: PCR products by ON954/ON745, 3: PCR products by ON469/ON979. (C) Electrophoresis of PCR products of *B. subtilis* strain with integration at both *amyE* and *lacA* loci on 2% agarose gel, 1: PCR products by ON1441/ON1479, 2: PCR products by ON954/ON745, 3: PCR products by ON1896/ON1442, 4: PCR products by ON469/ON979, 5: PCR products by ON1976/ON470. (D) Schematic diagram shows the sites of oligonucleotides used for PCR to check the double crossover at both *amyE* and *lacA* loci in *B. subtilis* recombinant strains. The length of the PCR products are indicated in the Figure.

Fig. 5. **Increasing expression levels of BgaB by integrating constructs at both *amyE* and *lacA* loci in the *B. subtilis* 1012 genome.** (A) SDS-PAGE with different promoters: P01, P_grac_01, P100, P_grac_100, P212, P_grac_212; (B) SDS-PAGE with P_grac_212-bgaB integrated at both *amyE* and *lacA* loci. Recombinant strains were cultured in LB medium without antibiotics until the mid-log phase. Samples were harvested at 0 h (when OD600 reached 0.8-1) and 2 h - 12 h later.
BgaB at 11% after 2 h of induction, 34.7% after 4 h and continued increasing to 53.4% at 12 h (Fig. 5B).

4. Discussion

Due to its many favorable characteristics, _B. subtilis_ serves as an excellent cell factory for the production of heterologous proteins. However, competent _B. subtilis_ cells for cloning experiments are present only in low numbers resulting in poor transformation efficiency. The competence problem has been overcome by performing the cloning steps in _E. coli_ using _B. subtilis_–_E. coli_ shuttle vectors [3], but leaky protein expression in _E. coli_ can hamper the cloning efficiency. The strong promoters that allow a high level of expression in _B. subtilis_ also drive leaky expression to high levels in _E. coli_. Many regulatable vectors for strong protein synthesis in _B. subtilis_ result from the incorporation of a promoter with the lac operator [29–32]. This type of hybrid promoter is used in IPTG-inducible vectors to prevent unwanted expression in _E. coli_. However, the difference between the presence of the lacI gene in _B. subtilis_ and in the _E. coli_ genome allows lacO-hybrid promoters to be used to create vectors that are inducer-free in _B. subtilis_ but remain controllable in _E. coli_. In a previous study, we reported a new strategy for generating inducer-free replicative plasmids [19] to achieve high-level expression in _B. subtilis_ while suppressing leakiness in _E. coli_; but the level of background expression in _E. coli_ still remained high. These vectors were based on CoE1-like plasmids and the rop gene could be used to reduce the expression of leaky promoters in _E. coli_ by lowering the plasmid copy number.

The gene encoding the Rop protein is removed in most vectors to increase their copy number in _E. coli_ [33], but we chose to introduce it into our vectors because they were constructed for recombinant protein synthesis in _B. subtilis_ where low plasmid copy number in _E. coli_ would not be a problem. On the contrary, it helps to reduce background expression in this host. In this study, the combination of the rop gene and the two lac operators in our vectors led to 24-fold repression in recombinant _E. coli_ carrying pHT2170 with the Pgrac01 promoter compared to only 3-fold with the previously reported strategy, significantly decreasing the background expression. After 4 h, the leaky BgaB was only around 230 MUG units, 19-fold lower than with pHT2071 (Pgrac01, replicative, inducer-free, high copy number) and 4-fold lower than pHT01-BgaB (Pgrac01, replicative, IPTG-inducible, high copy number). The conceptual figure (Fig. 1C) shows the background in _E. coli_ and the inducible or inducer-free expression in _B. subtilis_ in the host.

Repli-cative plasmids can have problems with stability and safety however, so bioengineers are turning more to vectors integrated into the host’s genome. A single integrated expression cassette may not produce the desired expression level, so efforts are being made to increase the copy number in the genome in order to boost expression. Ten copies of an mpr^R^ cassette in which the CSP gene was placed between the promoter of the _B. amyloliquefaciens_ rplU-rpmA gene and the Rho-independent transcription terminator were ectopically inserted into designated (3 copies) and random (7 copies) sequences into the recipient’s DNA. The resulting bacterial strain produced approximately 0.5 g/L of secreted CSP after cultivation in flasks with starch-containing media, and its performance was comparable to an analogous strain in which the mpr^R^ cassette was carried on a multi-copy plasmid [12]. In another study, nine copies of an arg^C^ cassette containing the _Krebsiella bacillus_ pycnus arginate gene regulated by the strong promoter P43 were inserted into the recipient’s genome. Tests showed that the highest arginase activity (145 U/mL) was obtained from flask cultures, and this segregation-stable strain could efficiently hydrolyze L-arginine with a 97.2% molar yield, suggesting a potential application for the food industry [28]. A strong promoter was engineered that allowed synthesis of BgaB and sfGFP to levels of 43% and 30% of intracellular proteins, respectively. It was also used to allow the secretion of methyl parathion hydrolase (MPH) and chlorothalonil hydrolytic dehalogenase (Chd) to a level of 0.3 g/L (144 U/mL) and 0.27 g/l (4.4 U/mL) using shake-flask culture conditions [14]. In our study, the strong Pgrac promoters were used to generate effective _B. subtilis_ – _E. coli_ inducer-free integrative vectors. The best performance of plasmid-
less strain with a single genomic copy of the BgaB expression cassette produced target protein to 42 % of total protein. With ectopic insertion into both amyE and lacA loci, the BgaB yield reached 53.4 % of total protein. A series of different integrative vectors with a variety of expression levels were created to meet different protein expression needs.

Inducible-free expression vectors (constitutive and auto-inducible) avoid the need to add an inducer to the culture medium thereby lowering the production cost. Constitutive promoters are not suitable for the production of toxic proteins, but auto-inducible promoters are ideal for large-scale commercial protein production. Such promoters induce expression of the target gene from the late log phase to the stationary phase with no requirement for an inducer, which facilitates high-yield production of heterologous proteins at low cost [34]. The silencing of the lacI gene in designated vectors allowed constitutive expression in B. subtilis. The best performance was obtained with vectors expressing the target protein at low levels during early culture stages but switching to high production when host cells reach the late log phase. As demonstrated here, B. subtilis carrying the Pprac212 cassette expressed BgaB protein only up to 11 % during the first two hours after induction, then the yield increased to 30 % over the next two hours when the cells begin to enter stationary phase. These positive results show the potential commercial value of our newly constructed vectors. These efforts also constitute an extension of our previous investigation of B. subtilis as a vaccine delivery vector. We showed that the expression of small amounts of LTB in the cytoplasm or anchored on the cell surface by a sorbte [35,36] could induce a humoral immune response in mice [37]. The use of a vector carrying Pprac212 linked to a gene encoding the human rhinovirus 3C protease resulted in the production of recombinant protein up to 16 % in B. subtilis after IPTG induction [38]. Recent reports using different expression systems showed the expression of various recombinant proteins in B. subtilis such as α-amylase, PhoA, single-chain variable antibody fragment, RNase barnase, trehalase synthase, human FGF21 [39–41]. Therefore, our inducible-free expression system could be a cost-effective solution for synthesizing recombinant proteins or vaccines for animals.

Funding sources and acknowledgements

This work was supported by the Vietnam National Foundation for Science and Technology Development (NAFOSTED) under grant number 106-NN.02-2015.24. The equipment was provided by TWAS under research grant 14–201 RG/BIOS/G, TWAS. W. Schumann would like to thank the SES for financial support to travel to Vietnam. The authors would like to thank Mr. Hanh for kindly providing plasmid pHIT1305, pHIT1326, and pHIT1379 for this study.

CRediT authorship contribution statement

Dinh Thi Minh Tran: Investigation, Writing - original draft. Trang Thi Phuong Phan: Investigation, Resources, Project administration. Thanh Thi Ngoc Doan: Validation, Resources. Thuc Linh Tran: Supervision. Wolfgang Schumann: Writing - review & editing. Hoang Duc Nguyen: Conceptualization, Supervision, Methodology.

Declaration of Competing Interest

The authors report no declarations of interest.

References

[1] J. Van Djil, M. Hecker, Bacillus subtilis: from soil bacterium to super-secretion cell factory. Microb. Cell Fact. 12 (2013) 1, doi:10.1186/1475-2859-12-3.
[2] X. Yan, H.-J. Yu, Q. Hong, S.-P. Li, Cre/lox system and PCR-based gene engineering in Bacillus subtilis, Appl. Environ. Microbiol. 74 (2008) 5556–5562, doi:10.1128/AEM.01556-08.
[3] W. Schumann, Production of recombinant proteins in Bacillus subtilis, Adv. Appl. Microbiol. 62 (2007) 137–189, doi:10.1016/S0065-2164 (07)62006-1.
[4] Y. Liu, L. Liu, J. Li, G. Du, J. Chen, Synthetic biology toolbox and chassis development in Bacillus subtilis, Trends Biotechnol. 37 (2019) 548–562, doi:10.1016/j.tibtech.2018.10.005.
[5] K. Huang, T. Zhang, B. Jiang, X. Yan, W. Mu, M. Miao, Overproduction of Hummelichipaciitrus pycnus arginase with multi-copy insertion of the argRΨc cassette into the Bacillus subtilis chromosome, Appl. Microbiol. Biotechnol. 101 (2017) 6039–6048, doi:10.1007/s00253-017-8355-9.
[6] Y. Gu, X. Xu, Y. Wu, T. Niou, Y. Liu, J. Li, G. Du, L. Liu, Advances and prospects of Bacillus subtilis cellular factories: from rational design to industrial applications, Metab. Eng. 50 (2018) 109–121, doi:10.1016/j.ymben.2018.05.006.
[7] C. Guan, W. Cui, J. Cheng, L. Zhou, J. Guo, X. Hu, G. Xiao, Z. Zhou, Construction and development of an auto-regulatory gene expression system in Bacillus subtilis, Microb. Cell Fact. 14 (2015) 150, doi:10.1186/s12934-015-0341-2.
[8] X. Yu, J. Xu, X. Liu, X. Chu, P. Wang, J. Tian, N. Wu, Y. Yan, Identification of a highly efficient stationary phase promoter in Bacillus subtilis, Sci. Rep. 5 (2015) 1–9, doi:10.1038/srep18405.
[9] C. Zhou, B. Ye, S. Cheng, L. Zhao, Y. Liu, J. Jiang, X. Yan, Promoter engineering enables overproduction of foreign proteins from a single copy expression cassette in Bacillus subtilis, Microb. Cell Fact. 18 (2019) 111, doi:10.1186/s12934-019-1159-0.
[10] H. Liu, X. Wang, S. Yang, R. Wang, T. Wang, Saturation mutagenesis and self-inducible expression of trehalose synthase in Bacillus subtilis, Biotechnol. Prog. 2019, e102526, doi:10.1002/bptp.2019.e102526.
[11] R. Panahi, E. Vashheghi-Farahani, S.A. Shojaosadati, B. Bambai, Auto-inducible expression system based on the SigB-dependent ohrB promoter in Bacillus subtilis, Mol. Biol. 48 (2014) 852–857, doi:10.1016/j.molbi.2013.11.032.
[12] Y.A. Yomantas, E.G. Abalakina, L.I. Golubeva, L.Y. Gorbacheva, S.V. Mashko, Overproduction of Bacillus amyloglucosanis extracellular glatamylandopeptidase as a result of ectopic multi-copy insertion of an efficiently-expressed mpr gene into the Bacillus subtilis chromosome, Microb. Cell Fact. 10 (2011) 64, doi:10.1186/1475-2859-10-64.
[13] Y.A. Yomantas, E.G. Abalakina, L.I. Golubeva, L.Y. Gorbacheva, S.V. Mashko, Overproduction of Bacillus amyloglucosanis extracellular glatamylandopeptidase as a result of ectopic multi-copy insertion of an efficiently-expressed mpr gene into the Bacillus subtilis chromosome, Microb. Cell Fact. 10 (2011) 64, doi:10.1186/1475-2859-10-64.
[14] C.L. Zhou, B. Ye, S. Cheng, L. Zhao, Y. Liu, J. Jiang, X. Yan, Promoter engineering enables overproduction of foreign proteins from a single copy expression cassette in Bacillus subtilis, Microb. Cell Fact. 18 (2019) 111, doi:10.1186/s12934-019-1159-0.
[15] T.T. Phan, H.D. Nguyen, W. Schumann, Construction of a 5’-controllable stabilizing element (CoSE) for over-production of heterologous proteins at high levels in Bacillus subtilis, J. Biotechnol. 168 (2013) 32–39, doi:10.1016/j.jbti.2013.07.031.
[16] T.T. Phan, T.T. Phan, T.K. Huyhn, N.T.K. Dang, P.T.K. Huyhn, T.M. Nguyen, T.T. T. Truong, T.L. Tran, W. Schumann, H.D. Nguyen, Development of inducer-free expression plasmids based on IPTG-inducible promoters for Bacillus subtilis, Microb. Cell Fact. 16 (2017) 130, doi:10.1186/s12934-017-0747-0.
[17] H. Saito, T. Shibata, T. Ando, Mapping of genes determining nonpermissiveness and host-specific restriction to bacteriophages in Bacillus subtilis Marburg, Mol. Gen. Genet. 170 (1979) 117–122.
[18] T.T. Phan, H.D. Nguyen, W. Schumann, Construction of a 5’-controllable stabilizing element (CoSE) for over-production of heterologous proteins at high levels in Bacillus subtilis, J. Biotechnol. 168 (2013), doi:10.1016/j.jbti.2013.07.031.
[19] T.T. Phan, T.T. Phan, T.K. Huyhn, N.T.K. Dang, P.T.K. Huyhn, T.M. Nguyen, T.T. T. Truong, T.L. Tran, W. Schumann, H.D. Nguyen, Development of inducer-free expression plasmids based on IPTG-inducible promoters for Bacillus subtilis, Microb. Cell Fact. 16 (2017) 130, doi:10.1186/s12934-017-0747-0.
[20] H.D. Nguyen, Q.A. Nguyen, R.C. Ferreira, L.C.S. Ferreira, L.T. Tran, W. Schumann, Construction of plasmid-based expression vectors for Bacillus subtilis exhibiting full structural stability, Plasmid. 54 (2005) 241–248, doi:10.1016/j.plasmid.2005.05.001.
[21] T.T. Phan, L.T. Tran, W. Schumann, H.D. Nguyen, Development of Pprac100-based expression vectors allowing high protein production levels in Bacillus subtilis and relatively low basal expression in Escherichia coli, Microb. Cell Fact. 14 (2015) 72, doi:10.1186/s12934-015-0255-5.
[22] H. Inoue, H. Nojima, H. Okayama, High efficiency transformation of Escherichia coli with plasmids, Gene 96 (1990) 23–28, doi:http://dx.doi.org/10.1016/0378-1119(90)80336-p.

[23] T. Phan, P. Huynh, T. Truong, H. Nguyen, A generic protocol for intracellular expression of recombinant proteins in Bacillus subtilis, in: N.A. Burgess-Brown (Ed.), Heterologous Gene Expression in E.Coli: Methods and Protocols, Springer, New York, NY, 2017, pp. 325–334, doi:http://dx.doi.org/10.1007/978-1-4939-6887-9_21.

[24] T. Phan, P. Huynh, T. Truong, H. Nguyen, A generic protocol for intracellular expression of recombinant proteins in Bacillus subtilis, Methods Mol. Biol. 1586 (2017) 325–334, doi:http://dx.doi.org/10.1007/978-1-4939-6887-9_21.

[25] M.C. Mossing, M.T. Record, Upstream operators enhance repression of the lac promoter, Science 233 (1986) 889–892, doi:http://dx.doi.org/10.1126/science.3090685.

[26] M. Camps, Modulation of ColE1-like plasmid replication for recombinant gene expression, Recent Pat. DNA Gene Seq. 4 (2010) 58–73.

[27] T.T.P. Phan, H.D. Nguyen, W. Schumann, Development of a strong intracellular expression system for Bacillus subtilis by optimizing promoter elements, J. Biotechnol. 157 (2012) 167–172, doi:http://dx.doi.org/10.1016/j.jbiotec.2011.10.006.

[28] K. Huang, T. Zhang, B. Jiang, X. Yan, W. Mu, M. Miao, Overproduction of Rummeliibacillus pycus arginine with multi-copy insertion of the arg^{+ve} cassette into the Bacillus subtilis chromosome, Appl. Microbiol. Biotechnol. 101 (2017) 6039–6048, doi:http://dx.doi.org/10.1007/s00253-017-8355-9.

[29] D.G. Yansura, D.J. Henner, Use of the Escherichia coli Lac repressor and operator to control gene expression in Bacillus subtilis, Proc. Natl. Acad. Sci. U.S.A. 81 (1984), doi:http://dx.doi.org/10.1073/pnas.81.2.439.

[30] T.P. Phan, H.D. Nguyen, W. Schumann, Novel plasmid-based expression vectors for intra- and extracellular production of recombinant proteins in Bacillus subtilis, Protein Expr. Purif. 46 (2006), doi:http://dx.doi.org/10.1016/j.pep.2005.07.005.

[31] P.T. Chen, J.-F. Shaw, Y.-P. Chao, T.-H. David Ho, S.-M. Yu, Construction of chromosomally located T7 expression system for production of heterologous secreted proteins in Bacillus subtilis, J. Agric. Food Chem. 58 (2010) 5392–5399, doi:http://dx.doi.org/10.1021/jf100445a.

[32] S.M. Castillo-Hair, M. Fujita, O.A. Igoshin, J.J. Tabor, An engineered B. Subtilis inducible promoter system with over 10,000-fold dynamic range, ACS Synth. Biol. 8 (2019) 1673–1678, doi:http://dx.doi.org/10.1021/acssynbio.8b00469.

[33] G. Cesareni, M.A. Muesing, B. Polisky, Control of ColE1 DNA replication: the rop gene product negatively affects transcription from the replication primer promoter, Proc Natl Acad Sci U.S.A. 79 (1982) 6313–6317.

[34] X. Yu, J. Xu, X. Liu, X. Chu, P. Wang, J. Tian, N. Wu, Y. Fan, Identification of a highly efficient stationary phase promoter in Bacillus subtilis, Sci. Rep. 5 (2015) 18405, doi:http://dx.doi.org/10.1038/srep18405.

[35] H.D. Nguyen, W. Schumann, Establishment of an experimental system allowing immobilization of proteins on the surface of Bacillus subtilis cells, J. Biotechnol. 122 (2006) 473–482, doi:http://dx.doi.org/10.1016/j.jbiotec.2005.09.012.

[36] H.D. Nguyen, T.T.P. Phan, W. Schumann, Analysis and application of Bacillus subtilis sortases to anchor recombinant proteins on the cell wall, AMB Express 1 (2011) 22, doi:http://dx.doi.org/10.1186/2191-0855-1-22.

[37] J.D. Pacez, H.D. Nguyen, W.B. Luiz, R.C.C. Ferreira, M.E. Shrogo-Almeida, W. Schuman, L.C.S. Ferreira, Evaluation of different promoter sequences and antigen sorting signals on the immunogenicity of Bacillus subtilis vaccine vehicles, Vaccine 25 (2007) 4671–4680, doi:http://dx.doi.org/10.1016/j.vaccine.2007.04.021.

[38] V.D. Le, T.T.P. Phan, T.M. Nguyen, L. Brunsfeld, W. Schumann, H.D. Nguyen, Using the IPTG-inducible PyrC212 promoter for overexpression of human Rhinovirus 3C protease fusions in the cytoplasm of Bacillus subtilis cells, Curr. Microbiol. (2019), doi:http://dx.doi.org/10.1007/s00284-019-01783-9.

[39] J. Heinrich, C. Drewniak, E. Neugebauer, H. Kellner, T. Wiegert, The YosW signal peptide directs efficient secretion of different heterologous proteins fused to a StreptI-SUMO tag in Bacillus subtilis, Microb. Cell Fact. 18 (2019) 31, doi:http://dx.doi.org/10.1186/s12934-019-1078-0.

[40] H. Liu, H. Liu, S. Yang, R. Wang, T. Wang, Improved expression and optimization of trehalose synthase by regulation of PPyh in Bacillus subtilis, Sci. Rep. 9 (2019) 1–10, doi:http://dx.doi.org/10.1038/s41598-019-43172-z.

[41] D.L. Li, G. Fu, R. Tu, Z. Jin, D. Zhang, High-efficiency expression and secretion of human FG21 in Bacillus subtilis by intercalation of a mini-cistron cassette and combinatorial optimization of cell regulatory components, Microb. Cell Fact. 18 (2019) 17, doi:http://dx.doi.org/10.1186/s12934-019-1066-4.