Development of inducer-free expression plasmids based on IPTG-inducible promoters for *Bacillus subtilis*

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**Abstract**

**Background:** Besides *Escherichia coli*, *Bacillus subtilis* is an important bacterial species for the production of recombinant proteins. Recombinant genes are inserted into shuttle expression vectors which replicate in both *E. coli* and in *B. subtilis*. The ligation products are first transformed into *E. coli* cells, analyzed for correct insertions, and the correct recombinant plasmids are then transformed into *B. subtilis*. A major problem using *E. coli* cells can be the strong basal level of expression of the recombinant protein which may interfere with the stability of the cells. To minimize this problem, we developed strong expression vectors being repressed in *E. coli* and inducer-free in *B. subtilis*.

**Results:** In general, induction of IPTG-inducible expression vectors is determined by the regulatory lacI gene encoding the LacI repressor in combination with the lacO operator on the promoter. To investigate the inducer-free properties of the vectors, we constructed inducer-free expression plasmids by removing the lacI gene and characterized their properties. First, we examined the ability to repress a reporter gene in *E. coli*, which is a prominent property facilitating the construction of the expression vectors carrying a target gene. The β-galactosidase (*bgaB* gene) basal levels expressed from Pgrac01-*bgaB* could be repressed at least twice in the *E. coli* cloning strain. Second, the inducer-free production of BgaB from four different plasmids with the Pgrac01 promoter in *B. subtilis* was investigated. As expected, BgaB expression levels of inducer-free constructs are at least 37 times higher than that of the inducible constructs in the absence of IPTG, and comparable to those in the presence of the inducer. Third, using efficient IPTG-inducible expression vectors containing the strong promoter Pgrac100, we could convert them into inducer-free expression plasmids. The BgaB production levels from the inducer-free plasmid in the absence of the inducer were at least 4.5 times higher than that of the inducible vector using the same promoter. Finally, we used gfp as a reporter gene in combination with the two promoters Pgrac01 and Pgrac100 to test the new vector types. The GFP expression levels could be repressed at least 1.5 times for the Pgrac01-gfp+ inducer-free construct in *E. coli*. The inducer-free constructs Pgrac01-gfp+ and Pgrac100-gfp+ allowed GFP expression at high levels from 23 × 10^4 to 32 × 10^4 RFU units and 9–13% of total intracellular proteins. We could reconfirm the two major advantages of the new inducer-free expression plasmids: (1) Strong repression of the target gene expression in the *E. coli* cloning strain, and (2) production of the target protein at high levels in *B. subtilis* in the absence of the inducer.

**Conclusions:** We propose a general strategy to generate inducer-free expression vector by using IPTG-inducible vectors, and more specifically we developed inducer-free expression plasmids using IPTG-inducible promoters in *E. coli* and inducer-free in *B. subtilis*.
Background

The rod-shaped Gram-positive soil bacterium *Bacillus subtilis* is an attractive host for the production of recombinant proteins of biotechnological interests. Because of its easy handling, including the development of novel expression systems, high-cell-density growth and its classification as a generally recognized as safe (GRAS) organism based on the lack of pathogenicity and the complete absence of endotoxins.

The most important element of expression vectors is the promoter. Three types of promoters have been developed for the expression of recombinant genes: (i) constitutive, (ii) autoinducible, and (iii) inducible ones [1]. To generate expression vectors either new promoters can be isolated from bacterial genomes and tested or existing promoters can be engineered to enhance their strength. One example is the widely used promoter Pspac [2], the first IPTG-inducible promoter for *B. subtilis* that consist of *E. coli* lacO operator and an early promoter of the *B. subtilis* phage SPO-1 in combination with the regulatory element, *E. coli* LacI repressor. To improve the IPTG-inducible expression vector, the Pgrac promoter [3], a derivative of the groESL promoter of *B. subtilis* was generated. By optimizing its UP element and the −35, −16, −10 and +1 regions, recombinant proteins accumulated up to about 30% of the total cellular proteins [4]. Similar modifications have been carried out with the core region of the aprE promoter of *B. subtilis* [5] and the cry3Aa [6] promoter, resulting in improved transcription activities in both cases.

Auto-inducible and constitutive expression vectors belong to the category of inducer-free expression vectors that harbor auto-inducible or constitutive promoters. One auto-inducible expression vector is based on the srfA promoter which displayed a cell-density dependent expression pattern. While the recombinant protein was present at a low level at the early exponential growth phase, it was highly expressed at the late exponential as well in the stationary phase [7]. The productivity of the PspFα promoter could be further enhanced by promoter engineering and by using the spore mutant strain BSG1682. This strain carries a deletion of the gene coding for sigma F [8].

IPTG-inducible promoters are widely used and well characterized to study gene expression in *B. subtilis*. However, using the same promoters for the construction of different types of vectors for inducible and inducer-free expression has not been reported so far. In this work, we converted IPTG-inducible into inducer-free expression vectors and experimentally proved their two important properties: (1) low basal level of expression in *E. coli* due to efficient repression, and (2) production of recombinant proteins at high levels in the absence of the inducer.

Results and discussion

Construction of the inducer-free expression vectors and control of basal levels in *E. coli*

The pHTO1 expression vector and its derivatives contain the IPTG-inducible Pgrac (now Pgrac01) promoter [9]. Here, the promoter is flanked by two lacO operators, where one operator (lacO1) is located immediately downstream of the promoter and the second (lacO3) near the end of the lacI gene (Fig. 1a). Binding of the LacI repressor to both operators increases repression as shown elsewhere [10]. To convert this IPTG-inducible into an inducer-free expression vector for *B. subtilis*, we partially or fully deleted the lacI gene and preserving lacO3 as shown diagrammatically (Fig. 1b). As an example, the inducer-free expression plasmid pH1655 was constructed (Fig. 1c).

Most of the expression vectors for *B. subtilis* are shuttle vectors which replicate both in *E. coli* and in *B. subtilis*. The cloning steps are carried out in *E. coli*, and the final expression vector containing the recombinant gene is then transformed into *B. subtilis* followed by its expression of the recombinant gene. In general, efficient expression vectors with strong promoters for *B. subtilis* also allow high protein production in *E. coli*. If constitutive or auto-inducible promoters are used, the basal level of expression in *E. coli* can create a problem depending on the expression level and the type of recombinant protein. Therefore, we constructed new expression vectors
allowing repression in *E. coli* cloning strains. For the cloning purpose, the *E. coli* strain OmniMAX carries the *lacI* allele on an F' factor and should be able to repress expression from the pHt vectors carrying the *Pgrac01* promoter flanked by the two *lac* operators, *lacO1* and *lacO3* (see Fig. 1b). To test this assumption, plasmids pHt01-*bgaB* (control), and four inducer-free expression vectors containing *Pgrac01* promoter, pHt1655, pHt2071, pHt1660 and pHt1663 were transformed into *E. coli* OmniMAX. Cells of the five strains were grown in LB medium till the mid-log phase. Then, either no or 1 mM IPTG was added, and samples were withdrawn at t = 0 (immediately before addition of IPTG) and 2 and 4 h later. We used the *bgaB* gene coding for a heat-stable β-galactosidase (BgaB) as reporter gene [11]. The BgaB activities were determined in all samples, and the results are presented in Fig. 2. While the basal level of expression in the absence of the inducer IPTG remained low and comparable with all five plasmids analyzed, it could be induced after addition of IPTG from $1.3 \times 10^4$ to $2.4 \times 10^4$ 4 h after induction depending on the plasmid type (Fig. 2). The BgaB expression levels could be repressed at least 6.0-, 4.5-, 2.0-, 5.6- and 5.5-folds for pHt10- *bgaB*, pHt1655, pHt2071, pHt1660 and pHt1653, respectively. Among them, the basal level remained low in the absence of IPTG for plasmid pHt1660. Based on these data, plasmid pHt1660 should be used as a vector plasmid for cloning of genes whose products could harm the *E. coli* cells. In summary, the new expression vectors were able to repress target gene expression at least twice in *E. coli*, which might facilitate the cloning steps to generate stable recombinant vectors.

**Fig. 1** Conversion of an IPTG-inducible into an inducer-free expression vector for *B. subtilis*. **a** Schematic representation of the location of two *lacO* operators, e.g., in pHt01-*bgaB* and repression of the target gene by the presence of LacI (circle) encoded by *lacI* gene. **b** Removal of the *lacI* gene resulted in the inducer-free expression plasmid, such as pHt1655. **c** Map of the pHt1655 vector.

**Fig. 2** BgaB activities of five plasmids with the *Pgrac01* promoter in *E. coli*. The bacterial cells were grown in LB medium at 37 °C to the mid-logarithmic growth phase. Then, the culture was split into two subcultures and was further incubated in the absence of IPTG (0 mM) and the others induced with 1 mM IPTG. Samples were taken immediately after addition of IPTG (0 h) and 2 and 4 h later (2, 4 h). The β-galactosidase activity was measured in all samples and expressed as MUG units × 10^4.
Next, the new appropriate recombinant vectors were transformed into *B. subtilis*.

**Inducer-free production of BgaB from plasmids with the Pgrac01 promoter in *B. subtilis***

The four plasmids pHT01 (Pgrac01, no *bgaB* gene), pHT01-*bgaB* (Pgrac01-*bgaB*, inducible), pHC02-*bgaB* (PlepA-*bgaB*, inducer-free) and pHT1655 (inducer-free) were transformed into the *B. subtilis* strain 1012. The strains were grown in LB medium in the presence of chloramphenicol to the mid-log phase. Then, IPTG was added at 0 (control), 0.01, 0.1 and 1.0 mM to each culture. Aliquots were collected 2 h later, and the BgaB activity or SDS-PAGE analysis were carried out as described in Materials and methods. First, using SDS-PAGE, we analyzed with the amount of BgaB produced by *B. subtilis* harboring plasmids carrying the Pgrac01 promoter: the empty vector pHT01, the IPTG-inducible vector pHT01-*bgaB*, and the inducer-free expression vector pHT1655. While no BgaB protein was synthesized with pHT01 as to be expected (Fig. 3a), it was IPTG-inducible in the presence of pHT01-*bgaB*, and synthesized in the presence and absence of IPTG with plasmid pHT1655 (Fig. 3a). Quantification of the BgaB protein bands using the ImageJ program revealed that BgaB accumulated up to 14% of cellular proteins for pHT1655 and equal to that of pHT01-*bgaB* in the presence of IPTG. In the case of the activities, the *B. subtilis* strain carrying pHT01 produced a very low level of β-galactosidase activities. While the amount of enzyme was also negligible for pHT01-*bgaB* in the absence of IPTG, it increased to 8 to 10 × 10^4 units in the presence of 0.1 and 1.0 mM of IPTG (Fig. 3b). In contrast, cells harboring the pHT1655 plasmid produced up to 9 × 10^4 units both in the absence and presence of IPTG. These results demonstrate that the *B. subtilis* strain carrying the plasmid pHT1655 produced BgaB in the absence of IPTG were 37 times higher as compared to plasmid pHT01-*bgaB* and at levels comparable to that after 1 mM IPTG-induction. We also analyzed expression of the *bgaB* gene from the plasmid pHC02-*bgaB* [12] where the gene is under control of the constitutive promoter PlepA from *B. subtilis*. The BgaB production level of the PlepA-*bgaB* construct was only at 0.14 × 10^4 units (Fig. 3b). In comparison with the inducer-free pHT1655 plasmid, the BgaB production levels was 50 times higher than that of pHC02-*bgaB*. We conclude that the newly constructed vector pHT1655 allows BgaB production at high levels in an inducer-free manner.

Next, we investigated whether the spacer length between the two lacO operators, lacO1 and lacO3, will influence the expression level. The operator lacO1 is located downstream of the promoter PgroES and lacO3 upstream of the promoter (Fig. 1a, b). Naturally, there is a lacO3 the end of the lacI gene. By deletion of part of

![Fig. 3](image-url)
the *lacI* gene on the plasmids, we increased the spacer length from 143 bp (pHT1655) to 280 (pHT2071), 548 (pHT1660) and 787 bp (pHT1653), and measured the β-galactosidase activities as described. For all the inducer-free plasmids, BgaB activities in the absence of IPTG are in the same range with the inducible plasmid pHT01-*bgaB* in the presence of 1 mM IPTG (Figs. 3b, 4a). The 280 bp spacer (pHT2071) resulted in an increase from about $8 \times 10^4$ in pHT1655 to $14 \times 10^4$ units (Fig. 4a). This result would agree with the report that the different spacer lengths between *lacO*1 and *lacO*3 would enhance or reduce the target gene expression depending on the helix formation [13]. However, SDS-PAGE analysis of BgaB expression did not show significant differences between samples from four plasmids, and equal to that from pHT1655 (Fig. 3a). In summary, the results provided strong evidence that plasmids with the entire or partially deleted *lacI* gene could allow BgaB production at high levels in the absence of IPTG.

**Development of inducer-free expression vector using another IPTG-inducible plasmid with the strong promoter Pgrac100 for B. subtilis**

So far, inducer-free plasmids have been successfully created based on the IPTG-inducible Pgrac01 promoter. However, many other IPTG-inducible promoters were created based on a genetic modification of the Pgrac01 promoter, and many resulting promoters conferred high recombinant protein production levels in *B. subtilis* [4, 14]. Among them, the Pgrac100 promoter was used to develop IPTG-inducible expression vectors allowing remarkably high protein production levels in *B. subtilis* and a relatively low basal expression level in *E. coli* [15]. Therefore, we next analyzed whether the vector with the

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**Fig. 4** Influence of the spacer lengths between *lacO*1 and *lacO*3 on the β-galactosidase activity. The plasmids with different spacer lengths pHT1655 (143 bp), pHT2071 (280 bp), pHT1660 (548 bp) and pHT1653 (787 bp) carrying Pgrac01 (a) and pHT100 (inducible), pHT1656 (88 bp), pHT2079 (225 bp), pHT1674 (493 bp), pHT1654 (732 bp) harbouring Pgrac100 (b) were constructed. The deleted DNA region of *lacI* gene in the plasmids with Pgrac01 are identical to those with Pgrac100. The plasmids were transformed separately into *B. subtilis* 1012, and all strains were cultivated to an OD of 0.8. Then, the cultures were subdivided into two subcultures where IPTG was added at 1 mM to one of them. The BgaB activities were measured at time points 0 (immediately before addition of IPTG) and 2 and 4 h later.
strong synthetic Pgrac100 promoter could be converted into efficient inducer-free expression plasmids.

To answer this question, we constructed four additional Pgrac100-bgaB inducer-free vectors, named pHT1654, pHT1656, pHT1674 and pHT2079 and fused the bgaB reporter gene immediately downstream of the promoters. The spacer lengths between lacO1 and lacO3 are varied from 88 bp (pHT1656) to 225 bp (pHT2079), 493 bp (pHT1674) and 732 bp (pHT1654), in which the deleted DNA regions of lacI gene were identical to those in pHT1655 (Pgrac01-bgaB, 143 bp spacer), pHT2071 (Pgrac01-bgaB, 280 bp spacer), pHT1660 (Pgrac01-bgaB, 548 bp spacer), pHT1653 (Pgrac01-bgaB, 787 bp spacer), respectively. B. subtilis cells carrying these plasmids were then grown in LB medium till the mid-log phase, split into two subcultures where one remained untreated and the second was induced by addition of 1 mM IPTG. Samples were withdrawn immediately before adding the inducer IPTG and 2 and 4 h later. The β-galactosidase activities were measured in all samples and are presented in Fig. 4b. As can be seen from this figure, the BgaB activities expressed from the inducer-free plasmids with Pgrac100 in the absence of IPTG are comparable to those from Pgrac01 and reached between 9 × 10^4 and 12 × 10^4 units after 4 h. These values are comparable to those obtained with plasmid pHT100 after 4 h after induction (Fig. 4b) and at least 4.5 times higher than that without induction. The expression levels of the Pgrac01-bgaB construct were comparable to Pgrac100-bgaB plasmids because these constructs contain strong promoters, and the activities of BgaB in the samples with high expression levels are not linear with the strengths of the promoters [16]. BgaB protein band analysis from SDS-PAGE using the imageJ programme showed that BgaB expression levels could reach around 25% of the total cellular proteins which correspond with the inducible vector pHT100 as reported [4]. We conclude that another IPTG-inducible expression vector with the strong promoter could be converted into an inducer-free expression plasmid.

**Application of inducer-free expression vectors to produce GFP in the cytoplasm of B. subtilis**

In the last step, to check for the two major advantages of the inducer-free expression plasmids, we used another intracellular reporter protein, GFP, in combination with both promoters, Pgrac01 and Pgrac100. The two inducible expression vectors pHT100-gfp+ (Pgrac01-gfp+) and pHT1168 (Pgrac100-gfp+) were used as controls and four inducer-free expression plasmids, pHT1650 (Pgrac01-gfp+, 548 bp spacer), pHT1651 (Pgrac01-gfp+, 787 bp spacer), pHT1655 (Pgrac100-gfp+, 493 bp spacer) and pHT1696 (Pgrac100-gfp+, 732 bp spacer) were generated.

Next, we analyzed for the first advantage of the inducer-free vector that is the ability to repress the reporter gene expression in the E. coli cloning strain OmniMAX. Cells of the six strains were grown in LB medium till the mid-log phase. Then, either no or 1 mM IPTG was added, and samples were withdrawn at t = 0 (immediately before addition of IPTG) and 2 and 4 h later. The GFP fluorescence were measured in all samples, and the results are presented in Fig. 5a. The basal expression levels of the inducible vectors pHT100-gfp+ (Pgrac01-gfp+) and pHT1168 (Pgrac100-gfp+) ranking from 0.5 × 10^4 to 1.2 × 10^4 RFU were comparable to the Pgrac100-gfp+ inducer-free constructs, reaching 1.3 × 10^5 RFU (Fig. 5a) after 2 h of incubation. The GFP expression levels could be repressed at least 1.5 times for the Pgrac01-gfp+ inducer-free construct. Also, when using the E. coli OmniMAX strain carrying plasmid pLacI (Novagen) that produces more LacI repressor, the reporter gene expression could be repressed at least 10 times (data not shown). Similar approaches using E. coli cloning strains with high levels of LacI expression could be used to reduce the basal levels, for example applying a lacR21 strain [17]. These results confirmed that new inducer-free expression plasmids were able to retain at low basal levels or repress target gene expression in E. coli.

Then, we checked for the second advantage of the inducer-free vector, the ability allowing reporter gene expression in the absence of the inducer. We measured the GFP activities expressed in B. subtilis with the IPTG-inducible plasmids pHT100-gfp+ and pHT1168 and the four inducer-free plasmids pHT1650, pHT1651, pHT1695 and pHT1696. The GFP fluorescence could be induced about 100-fold with pHT100-gfp+ and about 15-fold with pHT1168 in B. subtilis. With the remaining four inducer-free plasmids, GFP activities accumulated from 23 × 10^4 to 32 × 10^4 RFU units in the 2 h samples and were comparable between the samples in the presence and absence of the inducer (Fig. 5b). The SDS-PAGE analysis also agreed with the previous data (Fig. 5c) and GFP production of about 9–13% of the total cellular proteins. The results reconfirmed that the newly developed inducer-free expression plasmid could allow GFP expression in the absence of inducer. They further reconfirmed two major benefits of the newly developed vectors: (i) to obtain a low basal level of recombinant proteins in E. coli, and (ii) to allow high protein production levels in B. subtilis in the absence of the inducer.

**Conclusions**

This study proposed a general strategy to convert IPTG-inducible into inducer-free expression vectors. Specifically, we developed novel inducer-free expression...
plasmids containing IPTG-inducible promoters, Pgrac01 and Pgrac100, and checked for the production of the two reporter proteins BgaB and GFP. The novel inducer-free expression plasmids allowed high production levels of recombinant proteins in B. subtilis without the addition of inducer and at the same time maintained a low level
of background expression or even repressed the recombinant gene expression in *E. coli*. The novel inducer-free expression plasmids are extended versions of the currently available IPTG-inducible expression vectors for *B. subtilis*, in which all these vectors use the same cognate promoters. This achievement will be a major milestone pushing forward the use of *B. subtilis* expression systems for over-production of recombinant proteins.

**Methods**

**Bacterial strains, plasmids and growth conditions**

*Escherichia coli* strain OmniMAX (Invitrogen) was used as a recipient in all cloning experiments and to determine expression levels. *B. subtilis* strain 1012 [18] was used to analyze expression of the two reporter genes *bgaB* and *gfp*. A list of the plasmids and oligonucleotides used in this study is shown in Table 1. Cells were routinely grown

### Table 1 Bacterial strains, plasmids and oligonucleotides used in this study

| Bacterial strains | Genotype | Source/reference |
|-------------------|----------|------------------|
| *E. coli* OmniMAX | \(F^+\) [proAB lacIΔ(lacZΔM15 Tn10(TetR) Δ(80lacZ)ΔM15 Δ(lacZYA-argF)U16 9 endA1 recA1 supE44 thi-1 gyrA96 relA1 tonA panD] used for cloning | Invitrogen |
| *B. subtilis* 1012 | leuA8 metB5 trpC2 hsrM1 | [18] |

| Plasmids | Description | Source/references |
|----------|-------------|------------------|
| pHCMC02-\(bgaB\) | P\(lepA\)-\(bgaB\), inducer-free expression plasmid | [12] |
| pNDH33-\(bgaB\) | P\(grac01\)-\(bgaB\), inducible | [3] |
| p\(lacI\) | Expression of LacI, used with *E. coli* cloning strain | Novagen |
| pH01 | P\(grac01\), inducible | [9] |
| pH01-\(bgaB\) | P\(grac01\)-\(bgaB\), inducible | [9] |
| pH74 | P\(grac01\), Strep-tag II, inducible | [9] |
| pH10-\(gfp\) | P\(grac01\)-\(gfp\)+, inducible | [9] |
| pH100 | P\(grac100\)-\(bgaB\), inducible | [4] |
| pH255 | P\(grac100\), inducible | [4] |
| pH1168 | P\(grac100\)-\(gfp\)+, inducible | [4] |
| pH1650 | P\(grac01\)-\(gfp\)+, Δ\(lac\), lacO1-lacO3 548 bp | This study |
| pH1651 | P\(grac01\)-\(gfp\)+, Δ\(lac\), lacO1-lacO3 787 bp | This study |
| pH1653 | P\(grac01\)-\(bgaB\), Δ\(lac\), lacO1-lacO3 787 bp | This study |
| pH1654 | P\(grac100\)-\(bgaB\), Δ\(lac\), lacO1-lacO3 732 bp | This study |
| pH1655 | P\(grac01\)-\(bgaB\), Δ\(lac\), lacO1-lacO3 143 bp | This study |
| pH1656 | P\(grac100\)-\(bgaB\), Δ\(lac\), lacO1-lacO3 88 bp | This study |
| pH1657 | Basic plasmid, used to construct pH1655 | This study |
| pH1658 | Basic plasmid, used to construct pH1656, pH1681, pH1682 | This study |
| pH1660 | P\(grac01\)-\(bgaB\), Δ\(lac\), lacO1-lacO3 548 bp | This study |
| pH1661 | Basic plasmid, used to construct pH1660 | This study |
| pH1674 | P\(grac01\)-\(bgaB\), Δ\(lac\), lacO1-lacO3 493 bp | This study |
| pH1695 | P\(grac01\)-\(gfp\)+, Δ\(lac\), lacO1-lacO3 493 bp | This study |
| pH1696 | P\(grac100\)-\(gfp\)+, Δ\(lac\), lacO1-lacO3 732 bp | This study |
| pH2071 | P\(grac01\)-\(bgaB\), Δ\(lac\), lacO1-lacO3 280 bp | This study |
| pH2079 | P\(grac01\)-\(bgaB\), Δ\(lac\), lacO1-lacO3 255 bp | This study |
| ON224 | CCGGATGACGTCGAATTCTAAACCTTCCCGGTTCATCATG | pH1655, pH1660, pH1656 |
| ON925 | GAATTAGCTTGGTACCAAAGGAGGTAAGGATCACTAG | To amplify promoter P\(grac100\) to construct pH1674 |
| ON926B | GACGTCGACTCTAGACATGGATCCTTCCTTTAATTGG | To amplify promoter P\(grac100\) to construct pH1674 |
| ON941 | AAAGGAGGAAGGATCCATGAATGTGTTATC | This study |
| ON985 | CAATTGCGTTGCGCTCACTGCCAGCGCT | This study |
| ON986 | AGCGCTGGCTGGCCATCTGGCCAGCGCT | This study |
| ON1975 | CAATTGCGTTGCGCTCACTGCAGCCAGCT | This study |
| ON1976 | AGCGCTGGCTGGCCATCTGGCCAGCGCT | This study |

P\(grac01\) (another name is P\(grac\)) and P\(grac100\) are the name of two different promoters.
in Luria broth (LB) at 37 °C under aeration and shaking at 200 rpm. Antibiotics were added where appropriate (ampicillin at 100 µg/mL for *E. coli* and chloramphenicol at 10 µg/mL for *B. subtilis").

**Construction of expression vectors**

**Basic inducer-free expression vectors**

To generate the first two basic inducer-free expression vectors, we removed the *lacI* and the *lacO3* sequences from plasmids pHT24 and pHT255, respectively, and inserted *lacO3* between the *KpnI* and *SacI* restriction sites by using the complementary oligonucleotides (ON) ON985 and ON986, resulting in the new plasmids pHT1657 and pHT1658. The third basic expression vector, pHT1661, was constructed by cutting pHT24 with *SnaBI* and *EcoRV* followed by Klenow enzyme treatment and religation with T4 DNA ligase to remove part of the *lacI* gene.

**Inducer-free plasmids containing Pgrac01-bgaB and Pgrac01-gfp+**

To construct pHT1655 and pHT1660, we amplified the *bgaB* gene using the primer pairs, ON224 and ON941 with pNDH33-*bgaB* as a template [3]. The *BamHI/AatII*-treated PCR product was introduced into pHT1657 and pHT1661 at the *BamHI* and *AatII* sites, respectively. To construct pHT1651 and pHT1653, we cut pHT01-*bgaB* [9] and pHT10-*gfp+* with *SnaBI/Apal*, treated the DNAs with Klenow enzyme and religated to remove part of the *lacI* gene. To construct plasmid pHT2071, we removed *lacI* together with the *lacO3* sequence from plasmid pHT01-*bgaB* and inserted the *lacO3* sequence between the *SnaBI* and *SacI* restriction sites by using the two complementary oligonucleotides ON1975 and ON1976. To obtain pHT1650, we treated pHT10-*gfp+* with *SnaBI/EcoRV* and followed by religation to remove part of the *lacI* gene.

**Inducer-free Pgrac100-bgaB plasmids**

To generate pH1654 and pH2079, we digested pH100 with *SnaBI/Apal* or *SnaBI/Sacl*, followed by treatment with Klenow enzyme and religation with T4 DNA ligase to remove major parts of the *lacI* gene. To construct pH1656, we amplified the *bgaB* gene using the primer pairs ON224 and ON941 with pNDH33-*bgaB* as a template. The *BamHI/AatII*-treated PCR product was introduced into pH1658 cut with *BamHI* and *AatII*. To construct pH1764, we amplified the Pgrac100 promoter using the primer pair ON925 and ON926B and pH100 as a template. The *KpnI/BamHI*-treated PCR product was ligated into pH1660 cut with *KpnI* and *BamHI*. To construct pH1695, we cut pH1168 with *SnaBI/EcoRV* and religated to remove a major part of *lacI* gene. To construct pH1696, we cut pH1168 with *SnaBI/Apal*, treated with Klenow and followed by religation to remove a major part of the *lacI* gene.

**Measurement of the BgaB and GFP production levels in *E. coli* and *B. subtilis***

Three single colonies of each strain were cultured in 5 mL LB liquid medium containing the appropriate antibiotic and shaken overnight at 200 rpm at room temperature (27 °C). A 1 mL pre-culture of each clone was transferred to 30 mL LB medium containing the appropriate antibiotic in 100 mL shake flasks and incubated at 37 °C at 200 rpm. When the OD600 of the culture reached 0.6–1, the cells were induced by addition of IPTG at the indicated concentrations. Aliquots of the cells were harvested before (0 h) and 2 or 4 h after addition of IPTG. The cells were collected in Eppendorf tubes at an OD600 of 2.4 after centrifugation. Samples were prepared for activity measurements and SDS-PAGE analyses. The cells were lysed by addition of lysozyme, and sample buffer was added to 150 µL and 8 µL each were applied to SDS-PAGE.

To prepare the samples for measurements of GFP and BgaB activities, the collected *E. coli* cells were resuspended in 300 µL PBS (140 mM NaCl, 2.7 mM KCl, 10 mM NaHPO4·2H2O, 1.8 mM KH2PO4). Then, 12 µL of chloroform and 6 µL of 0.1% SDS were added followed by shaking for 1 h. *B. subtilis* cells were lysed in 300 µL PBS containing 1 mg/mL of lysozyme and incubated at 37 °C for 2 h. All samples were centrifuged at 10,000 rpm for 5 min and used for determination of the activities.

GFP fluorescence were measured by using a microplate fluorometer (Clariostar, BMG LabTech) and a 384-well plate (Black) with an excitation wavelength at 470 ± 8 nm and an emission wavelength at 515 ± 8 nm. Determination of the GFP expression was calculated as relative fluorescence unit (RFU) divided by the OD600 of dGFP/OD600. All data were averaged from three independent samples of each time point [7].

To quantify BgaB activities, 20 µL of the supernatant were added into each well of a 384-well plate (Black) containing 80 µL of Z-buffer (60 mM Na2HPO4·2H2O, 40 mM NaH2PO4·2H2O, 10 mM KCl, 1 mM MgSO4), 25 µL of 1 mg/mL 4-Methylumbelliferyl β-p-galactopyranoside (MUG) in dimethyl sulfoxide (DMSO) were added to each well, and the samples were incubated at 55 °C for 15 min. The reaction was stopped with 30 µL of 1 M Na2CO3. The amount of fluorescence generated by β-gal-dependent MUG hydrolysis was quantified in a microplate fluorometer (Clariostar, BMG Labtech), using as a blank reference the assay with a cell-free culture medium sample. Arbitrary units of β-gal activity (MUG units) were calculated as follows: (VI/Vs) × F360/460/ (t × OD600); VI, the volume of the lysis from the cell
samples; Vs the volume of the samples used for the assay; F_{360/460}, fluorescence signals measure with an excitation wavelength at 360 ± 8 nm and an emission wavelength at 460 ± 8 nm; t, incubation time; OD_{600}, OD of the collected samples [19].

Authors’ contributions
HDN, TTPP, and WS designed the experiments; DTMT, TTPP, TRH, NTKD, PTKH, TMN and TTTT performed the experiments, DTMT, TTPP, HND and TLT, analyzed the data; WS and HDN prepared the manuscript. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request. The materials, the pHT vectors and bacterial strains will be available via Center for Bioscience and Biotechnology or associated companies such as HTBioTec, Vietnam or TWAS.

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