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Development of inducer-free expression plasmids using IPTG-inducible P*spac* promoter for *Bacillus subtilis*

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Abstract:

An inducer-free expression vector for the low expression levels in *Bacillus subtilis (B. subtilis)* is necessary for fundamental research. In this study, we constructed inducer-free expression plasmids carrying P*spac*, a well-known IPTG-inducible promoter, by removing a part of the *lacI* gene. Then, we analysed the expression of the target genes *bgaB* and *gfp*$^+$ in *B. subtilis.* Western blot experiments demonstrated that the reporters from the inducer-free plasmids with P*spac* could be produced at low levels in *B. subtilis* strains and were equivalent to their corresponding inducible constructs with 1 mM IPTG. The reporter activities showed that inducer-free expression from the P*spac* promoter was dramatically less than that of the inducer-free plasmids with the strong promoters P*grac*01 and P*grac*100 reported previously, about 16.2 to 20.3 times for BgaB and 24.7 to 34.3 times for GFP$^+$, respectively. In conclusion, the inducer-free expression vectors carrying P*spac* promoters allow the constitutive expression of heterologous recombinant proteins at low levels in *B. subtilis*.

Keywords: ***Bacillus subtilis,*** **inducer-free, pHT vector, P*spac*, weak promoter.**

Classification number: **3.5**

Introduction

B. subtilis is an important host for the production of heterologous proteins because of its advantages such as easy handling, safe, non-pathogenic, endotoxin-free, effective protein secretion mechanisms, and industrial fermentation. Besides, *B. subtilis* is a model organism for studying Gram-positive bacteria and the biological systems of cellular differentiation, stress responses, and multicellular organization [1, 2]. Thus, scientists have paid more attention to its expression systems for industrial applications and basic research [3].

Fundamental research has shown it is essential to have a weak promoter that can be controlled to express low levels of recombinant proteins within the cells. IPTG-inducible P*spac*, a well-characterized hybrid promoter, is composed of the *B. subtilis* phage SPO-1 promoter and the *E. coli lac* operator, which leads to transcription activation for low levels of gene expression. The P*spac* promoter allows low-expression levels of reporter expression [4, 5], which is approximately 50 times weaker than the P*grac* promoter. Many plasmids containing the IPTG-inducible P*spac* promoter, such as pHCMC05, pAL01, and the improved plasmid pHT2002 [6], are suitable to express a modest amount of the heterologous protein in the induction of IPTG in *B. subtilis.* One example of requiring low protein expression level is sortase, which is a membrane-associated protein needed for anchoring recombinant proteins to the cell wall. Low levels of sortase are necessary to avoid membrane clogging [7]. The weak promoter P*spac* is also an appropriate choice when the recombinant protein, or a protein of interest in any pathway, is harmless to the host cells after overproduction.

Induction of protein expression is stimulated by the addition of the inducer IPTG, a non-metabolizable analogue

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of allolactose. After induction, the RNA polymerase enzyme specially transcribes the coding sequence of the protein of interest present in the expression plasmid under the control of the promoter. IPTG is very useful to control gene expression in many microorganisms. However, IPTG has several limitations: (i) it requires cell culture monitoring to ensure that IPTG is added at the appropriate time. Because the induction point varies significantly from one recombinant protein to another, the process becomes challenging to manipulate, particularly when several proteins are expressed in parallel (e.g., for a screening study) and (ii) it presents toxicity limitations that affect cell viability [8]. Therefore, inducers are sometimes not necessary for low and continuous protein expression in the cell.

Some auto-inducible and constitutive expression vectors were constructed such that heterologous proteins can be expressed in *B. subtilis* during continuous culture without adding the inducer. In the last few years, inducer-free expression systems were developed by deleting a part of or the entire *lacI* gene in the vector carrying the IPTG-inducible promoters, P*grac*01, P*grac*57, and P*grac*100 [9, 10]. These strong inducer-free expression vectors have shown a prospective yield of recombinant proteins for industrial and medical applications. However, an inducer-free expression vector system based on a weak promoter controlling the expression of heterologous proteins at low levels has not yet been studied. In this work, inducer-free expression plasmids were developed by deleting the *lacI* gene on plasmids carrying the P*spac* promoter, which allows the system to express proteins of interest without using any inducers. Two widespread reporter genes, *bgaB* and *gfp*$^+$, were used to investigate the expression levels of these vectors.

Materials and methods

Strains, plasmids and growth conditions

Escherichia coli OmniMAX™ was used as the host for gene cloning and *B. subtilis* 1012 was used for gene expression and integration. The final concentrations of antibiotics were as follows, in mg/l: ampicillin (Amp), 100 for *E. coli*; and chloramphenicol (Cm), 10 for *B. subtilis*. The strains were cultivated in Luria-Bertani (LB) medium consisting of 1% tryptone, 0.5% yeast extract, and 0.5% NaCl. Strains were cultivated at 37°C in shaking flasks at 200 rpm. The cell density was determined by measuring the OD_{600} with an S-20 spectrophotometer (Boeco, Germany). Table 1 shows a list of the plasmids and oligonucleotides used in this study.

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

Bacterial strains	**Genotype**	**Source/references**
E. coli OmniMAX	F′ {*pro*AB *lac*I^q *lac*ZΔM15 *Tn*10(Tet^R) Δ(*ccd*AB)} *mcr*A Δ(*mrr hsd*RMS-*mcr*BC) Φ 80(*lac*Z)ΔM15 Δ(*lac*ZYA-*arg*F)U16 9 *end*A1 *rec*A1 *sup*E44 *thi*-1 *gyr*A96 *rel*A1 *ton*A *pan*D; used for cloning	Invitrogen
B. subtilis 1012	*leuA8 metB5 trpC2 hsrM1*	Mobitec
Plasmids	**Description**	**Source/references**
pHCMC05	P*spac* promoter, no reporter gene, negative control	[4]
pHCMC05-bgaB	P*spac-bgaB*, inducible, used to construct pHT1672	[4]
pHT1675	P*grac*100-*gfp,* Δ*lacI, lacO1-lacO3* 406 bp; used to construct pHT1692	This study
pHT2002	P*spac-bgaB*, inducible	[6]
pHT1535	P*spac-gfp*$^+$, inducible plasmid	This study
pHT1692	P*spac-gfp*$^+$, Δ*lacI* 406 bp	This study
pHT1672	P*spac-bgaB*, Δ*lacI* 787 bp	This study
Oligonucleotide	**Sequence 5' → 3'**	**Used for**
ON1896	CGGTTCGATCTTGCTCCAACTG	pHT 1672, plasmid sequencing
ON925	GAATTAGCTTGGTACCAAAGGAGGTAAGGATCACTAG	pHT1672, screening *E. coli* colonies
ON1278	GGCCATGACGTCTTTGTAAAGCTCATCCATGCCATGTGT	
ON780	CCCGCGGTCAGCTAGCCTAAACCTTCCCGGCTTCATCATGCTC	pHT1672, screening *B. subtilis* colonies
ON779	AAAGGAGGAAAGATCTATGAATGTGTTATCCTCAATTTGTTAC	
ON1512	ATCTCCATGGACGCGTGACG	pHCMC05, receiving P*spac* promoter
ON1249	CGTTTCCACCGGAATTAGCTTG	
ON926B ON1273	GACGTCGACTCTAGACATGGATCCTTCCTCCTTTATATGG CCCGGTACCCACTTCCTAGAATATATATTATGTAAACAAAGGAGGTA AGGATCACTAG	pHT1692, screening *E. coli* colonies
ON1280	GGCCATGACGTCTTATTTGTAAAGCTCATCCATGCCATGTGT	pHT1692, screening *B. subtilis* colonies
ON867B	GTGAAGGTGATGCTACAAACGGAAAGCTTACCCTTAAA	

Materials

Taq DNA polymerase and enzymes, including *Sna*BI, *Apa*I, T4 DNA ligase, *Kpn*I, *Bam*HI, Alkaline phosphatase, and Klenow, were supplied by Thermo Scientific. PCR kit, cloning kit, and basic materials for molecular biology were supplied by Qiagen, Thermo Scientific, Sigma-Aldrich, Merck-Millipore, and BioBasic. The plasmid pHCMC05-*bgaB*, and the plasmid pHT1675 were used as the origin plasmids to create the inducer-free plasmids. The plasmid pHCMC05 without the *bgaB* or *gfp*$^+$ gene was used as a negative control. All primers for PCR were described in Table 1.

Construction of the inducer-free expression plasmid based on the Pspac promoter

The plasmid pHCMC05-*bgaB* [4], a popular IPTG-inducible plasmid based on the P*spac* promoter, was engineered by removing 787 bp from the *lacI* gene to generate the auto-inducible plasmid pHT1672 (Fig. 1A). First, the plasmid pHCMC05-*bgaB* was treated by the restriction enzymes *Sna*BI and *Apa*I, in which *Apa*I created sticky end products while *Sna*BI created blunt end products. Second, the sticky ends were deleted by the Klenow Fragment to optimize the ligation reaction. Then, the ligation reaction of these products was carried out by T4 ligase and subsequently transformed into *E. coli*. The transformants were analysed by colony PCR using restriction analysis. Finally, the gene sequence of pHT1672 was confirmed by an improved Sanger method using an ON1896 primer on a Big Dye™ terminator (Macrogen - Korea). The structure of the pHT1672 plasmid is shown in Fig. 1B.

Transformation of recombinant plasmids into B. subtilis 1012 competent cells

The procedure for the transformation of recombinant plasmids into *B. subtilis* 1012 competent cells was carried out as described elsewhere [11]. First, the *B. subtilis* 1012 competent cells were shaken in 50-ml flasks containing 10 ml of LS medium at 50 rpm in a 30°C-shaking incubator for 2 h. Then, 100 µl of 0.1 M ethylene glycol tetraacetic acid (EGTA) was added into the prepared 50-ml flask with *B. subtilis* competent cells and this flask was kept at room temperature (25°C) for 10 min. After that, 1 ml of *B. subtilis* competent cells was removed and transferred into a 1.5-ml tube containing the recombinant plasmid. Next, this tube was shaken at 200 rpm in 37°C for 2 h before being centrifuged at 7000 rpm for 5 min to receive the recombinant cells. The cells were resuspended in the left supernatant and spread on LB agar plates containing 10 µg/ml Cm and 40 µg/ml 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). The plates were incubated at 37°C overnight. Then, *B. subtilis* colonies were screened using ON780/ON779 primers by colony PCR. Finally, the LB liquid media was used for breeding *B. subtilis* cells from selected colonies.

Evaluation of expression of the reporter protein

The evaluation protocol of BgaB expression has been described in previous articles [6, 9, 12]. First, three single colonies of each recombinant *B. subtilis* 1012 strain were cultured in LB liquid medium. The shaking culture flask of each clone was incubated at 37°C at 200 rpm to the mid-log phase when the OD_{600} of the culture reached 0.8-1. Then, IPTG was added at 0 (control), 0.1, and 1.0 mM to each culture. The cells were collected at 0 h (before induction), 2 h, and 4 h after induction for measurement of the BgaB activity and Western Blot analysis. In terms of this purpose, the volume of cell suspension was received at OD of 1.2 and 2.4, respectively.

For the investigation of BgaB activity, *B. subtilis* cells were lysed in 500 µl of a LacZ buffer containing 200 µg/ml of lysozyme and incubated at 37°C for 2 h. All samples were centrifuged at 10000 rpm for 5 min before the determination of the activities. The BgaB activity was represented by MUG units, which were calculated by measuring the fluorescence intensity at E_x/E_m=360/460 nm. We used 4-methylumbelliferyl-β-D-Galactopyranoside (MUG) as a substrate to realize the presence of the target protein β-galactosidase through the fluorescence. A microplate fluorometer (Clariostar, BMG Labtech) was used to measure the amount of fluorescence created by β-gal-dependent MUG hydrolysis, in which a culture medium sample without cell was used as a blank reference. The β-galactosidase activity (MUG units) were calculated by the following equation: $(V_l/V_s)\times F_{360/460}/(t\times OD_{600})$; where V_l is the volume of cell lysate; V_s is the sample volume used for the assay; $F_{360/460}$ is the fluorescence signals measured with the excitation - emission wavelengths of 360±8 nm and 460±8 nm, respectively; t is the reaction time (30 min); and OD_{600} is the OD of the cell samples at 600 nm (OD_{600}=1.2) [9, 12].

In Western Blot analyses, separation of the cell's proteins was conducted by SDS-PAGE and the transferation of these proteins to the nitrocellulose membrane was performed using

a transfer apparatus (Bio-Rad). Western Blot was carried out with primary antibodies that were complementary to the BgaB that were created in the mice in our lab, while the secondary antibody Anti-Mouse IgG - Peroxidase antibody was supplied by Sigma. Five-percent skimmed milk was used for blocking and antibody incubation, in which the concentration of primary antibodies was 1:20000 while the concentration of secondary antibodies was 1:10000. The proteins were stained by using a Pierce™ ECL Western Blotting Substrate (Thermo Scientific) and the chemiluminescent signal was detected by the iBright™ CL1500 Imaging System (Invitrogen).

Inducer-free expression of GFP at a low level in B. subtilis

In this procedure, the plasmid pHT1675, an inducer-free plasmid based on the P*grac*100 promoter, was engineered to form pHT1692. Firstly, the P*spac* gene was received from the basic plasmid pHCMC05 by using PCR with the primer pair ON1512/ON1249. Next, the PCR products were treated by *Kpn*I/*Bam*HI enzymes while the origin plasmid pHT1675 was treated with *Kpn*I/*Bam*HI and alkaline phosphatase to remove the P*grac*100 gene. Then, the enzymatic products were ligated by T4 DNA ligase, resulting in the pHT1692 vector containing the P*spac* promoter. Finally, we checked the GFP expression levels of this self-inducible *B. subtilis* expression system to demonstrate its potential application in basic research.

For the investigation of GFP activity, *B. subtilis* cells were lysed in a 500 µl PBS buffer that contained 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4, and 400 µg/ml lysozyme. Then, these mixtures were incubated at 37°C for 2 h. Immediately after centrifugation at 10000 rpm for 5 min, all samples were used for determination of the activities. GFP fluorescence was measured by using a microplate fluorometer (Clariostar, BMG LabTech) and a 384-well plate (Black) with an excitation wavelength of 470±8 nm and an emission wavelength of 515±8 nm. The determination of the GFP expression was calculated as the relative fluorescence unit (RFU) divided by the OD_{600}. All data were averaged from three independent samples of each time point [9]. The Western Blot was conducted with the primary antibodies against the GFP created in the mice in our lab and the secondary antibody Anti-Mouse IgG (whole molecule)-Peroxidase antibody was supplied by Sigma. The Western Blot procedure used is described above.

Results and discussion

Construction of the inducer-free expression plasmid pHT1672 based on Pspac promoter

The inducer-free plasmid pHT1672 was constructed successfully by deleting 787 bp between *Apa*I and *Sna*BI from the *lacI* gene of the plasmid pHCMC05-*bgaB* (Fig. 1). The results of DNA sequencing analysed by the Clone Manager showed 100% sequence homology between the analysed and designed DNA sequences of pHT1672. Because the plasmid lacks the regulatory gene, it will express the target protein in *B. subtilis* cells at the maximum level of the promoter without an inducer.

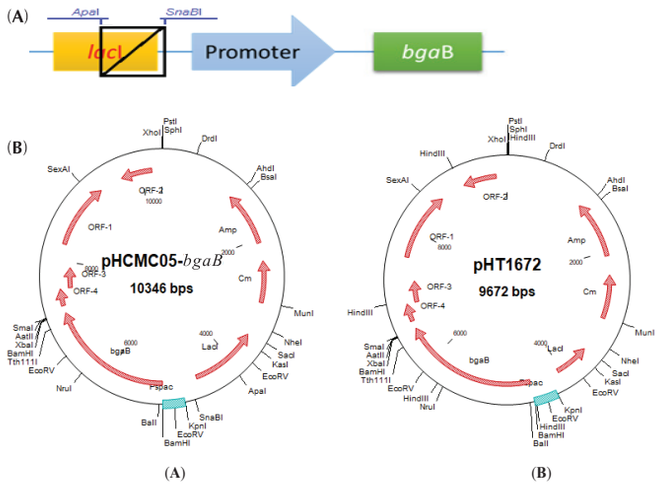

Fig. 1. The development of an inducer-free expression vector from an IPTG-inducible expression vector for *B. subtilis*. (**A**) A schematic depicting the deletion of a part of the *lacI* gene from the IPTG-inducible vector resulting in an inducer-free expression vector and (**B**) a map of the pHCMC05-*bgaB* (inducible vector) and pHT1672 (inducer-free vector).

Non-inducible expression of BgaB from plasmid pHT1672 with the Pspac promoter in B. subtilis

In this experiment, we investigated the expression levels of the target protein in *B. subtilis,* which contains pHT1672 under the control of the P*spac* promoter by using the inducer IPTG. *B. subtilis* strains containing the origin plasmid pHCMC05-*bgaB* and the inducible expression plasmid based on the P*spac* promoter pHT2002 [9] were also tested for comparison. *B. subtilis* with pHCMC05, a similar expression system without the *bgaB* gene, was used as a negative control. The *BgaB* expression of these four *B. subtilis* strains are shown in Fig. 2.

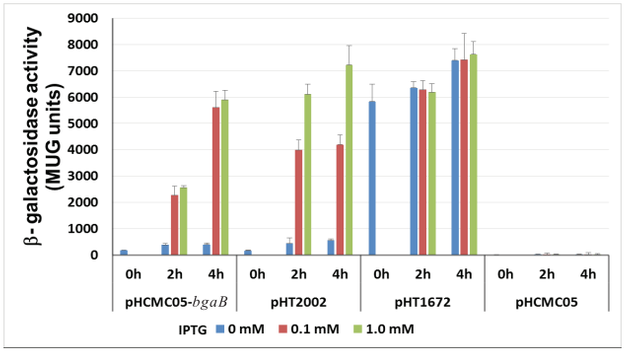

Fig. 2. The *BgaB* expression of the inducible and inducer-free plasmids in *B. subtilis* 1012. The production of the reporter protein *BgaB* over the four different *B. subtilis* strains haboring surveyed vectors pHCMC05-*bgaB* (origin plasmid, P*spac*, inducible), pHT2002 (P*spac*, inducible), pHT1672 (P*spac*, inducible-free), and pHCMC05 (P*spac*, negative control without *bgaB* gene), were evaluated in the presence of different IPTG concentrations (0, 0.1, 1.0 mM IPTG). The activities of β-galactosidase (MUG units) was measured in all samples. All cultures were grown three times and each experiment was repeated at least twice under similar conditions. Error bars denote standard deviations.

At the same time we collected the aliquots, the MUG units of the *B. subtilis* strain containing the inducer-free plasmid pHT1672 were equivalent in spite of different IPTG concentrations. After 2 and 4 h, the expression level of *BgaB* from the inducer-free plasmid pHT1672 in the absence of IPTG were compared to those of the origin inducible plasmids, pHCMC05-*bgaB* and pHT2002, with 1 mM IPTG. As shown in Fig. 2, the MUG units of pHT1672 after 2 h without induction were about 16.5 times higher than those of pHCMC05-*bgaB*. These ratios reached about 18.6 for samples received after 4 h of culture. In addition, the expression levels of *B. subtilis* containing the inducible-free construct pHT1672 were at least 13.2 times higher than that of pHT2002 without induction after 2 and 4 h. After 4 h induction with 1.0 mM IPTG, the value of β-galactosidase activity was equivalent when the comparison of different strains pHT1672 and 2002 was performed. It could be deduced that the deletion of the *lacI* gene has no effect on the strength of the P*spac* promoter and the conversion from inducible to inducer-free plasmids in *B. subtilis*.

The *BgaB* expression levels of the inducer-free plasmid based on the P*spac* promoter pHT1672 were about 16.2 to 20.3 times lower than that of the inducer-free plasmids based on the P*grac*01 and P*grac*100 promoter [9]. A previous study [6] showed that the heterologous *bgaB* could be induced for the expression at modest amounts in the *B. subtilis* containing pHT2002 by using IPTG as inducer. As a result of this study, we concluded that the inducible-free plasmid pHT1672 could express the recombinant protein *BgaB* at a low level without the addition of IPTG. This auto-inducible system can control the expression of target proteins continuously in the *B. subtilis* host strain without an inducer.

The expression of heterologous proteins controlled by the P*spac* promoter in *B. subtilis* was so low that they could not be detected by SDS-PAGE (Fig. 3A). This result was comparable with a previous study [6]. To confirm whether or not *BgaB* was expressed from these four *B. subtilis* strains, the Western Blot was conducted with the aliquots of these cells and the volume of the cell suspension was received at an OD of 2.4 after 4 h of induction. The presence of *BgaB* is shown in Fig. 3.

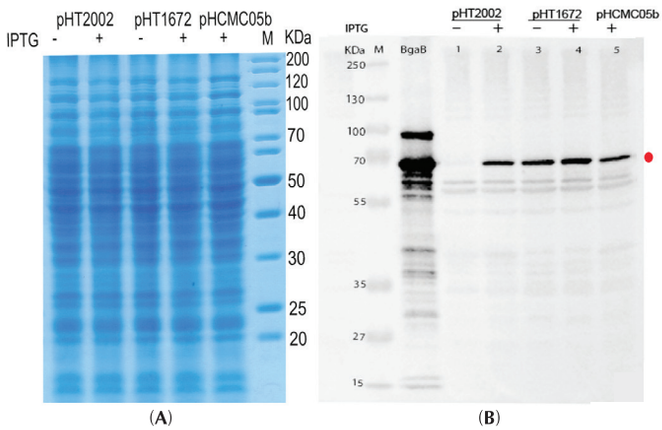

Fig. 3. The Western Blot results showing the *BgaB* expression of inducer-free plasmids based on the P*spac* promoter in *B. subtilis* 1012. The aliquots of surveyed *B. subtilis* strains (pHT1672, pHT2002) in the induced conditions (+, 1 mM IPTG) and the non-induced conditions (-, 0 mM IPTG). The origin plasmid pHCMC05- *bgaB* (pHCMC05b) was used as a positive control. The three different bacterial strains containing the surveyed vectors were cultured in an LB liquid medium at 37°C to the mid-logarithmic growth phase. Then, each culture was divided into two subcultures, where one was continuously incubated without an IPTG induce (0 mM) and the other was in inducible conditions with 1 mM IPTG (the positive controls were induced at 1 mM IPTG). The samples were collected 4 h after induction. The size of the *BgaB* lane was about 78 kDa. (**A**) The SDS-PAGE result shows fuzzy *BgaB* lines that are difficult to identify and (**B**) The Western Blot result clearly reflects the expression of *BgaB* in the *B. subtilis* strains.

As shown in Fig. 3, we confirmed that using polyclonal antibodies developed in our laboratory could detect a 78 kDa protein corresponding to the molecular size of *BgaB*. The thickest band of each line was *BgaB*, while the other fuzzy bands were non-specific binding proteins of the cells. The *BgaB* expression levels of the two surveyed *B. subtilis* strains that contained the inducer-free plasmids (pHT1672, pHT2002) were similar. Besides, there was no significant difference between the *BgaB* bands of these strains in the presence or absence of the inducer IPTG. The expression level of heterologous protein *BgaB* in the *B. subtilis* strain pHT1672 was equivalent to the level of that in the *B. subtilis* strain pHT2002, which is an inducer-free plasmid based on P*spac* promoter recently published in Ref. [6]. These levels were higher than that of the positive control pHCMC05-*bgaB*. Thus, we conclude that the inducer-free vectors based on the P*spac* promoter could express modest amounts of the heterologous protein. Therefore, these vectors are suitable for basic research to express proteins for metabolic engineering or membrane proteins.

Inducer-free production of GFP from plasmid pHT1692 with the Pspac promoter in B. subtilis

The target protein using an inducer-free expression system is continuously expressed in the *B. subtilis* host strain without inducer. Therefore, protein overexpression under strong promoter systems might cause a change of the protein's structure, resulting in inactivation of the protein's function [12]. Basic research has shown that it is important to use an inducer-free vector to allow the target heterologous protein to be produced continuously at low levels. To reconfirm the potential application of inducer-free plasmids based on the P*spac* promoter, the expression of another target protein was also investigated. For this study, the auto-inducible plasmid pHT1692 was created by engineering the origin of the inducer-free plasmid pHT1675 (P*grac*100-*gfp, ΔlacI, lacO1-lacO3* 406 bp) [9]. First, the P*spac* gene was received from the basic plasmid pHCMC05 by using PCR with the primer pair ON1512/ON1249. Next, the PCR products were treated with *Kpn*I/*Bam*HI enzymes while the origin plasmid pHT1675 was treated with *Kpn*I/*Bam*HI and alkaline phosphatase to remove the P*grac*100 promoter. Then, the enzymatic products were ligated by T4 DNA ligase, resulting in the pHT1692 vector consisting of a P*spac* promoter. The activity of GFP produced by the *B. subtilis* strain with an inducer-free vector based on P*spac* promoter pHT1692 was evaluated by fluorescent spectrometry (plate reader). *B. subtilis* with pHCMC05, a similar expression system without *gfp*$^+$ gene, was used as a negative control. The *B. subtilis* strain containing the inducible expression plasmid based on the P*spac* promoter pHT1535 was also tested for comparison. The GFP expression of these *B. subtilis* strains is shown in Fig. 4.

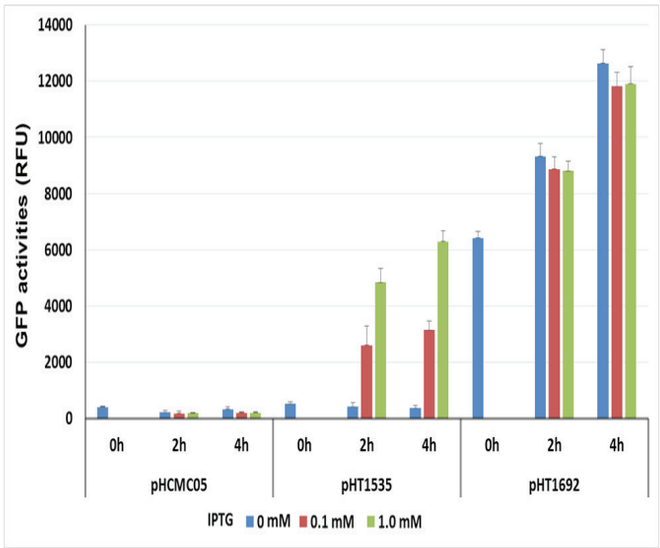

Fig. 4. The GFP expression of the inducible and inducer-free plasmids in *B. subtilis* 1012. The production of the reporter protein GFP in the different *B. subtilis* strains contained surveyed vectors pHT1535 (P*spac,* inducible), pHT1692 (P*spac,* inducible-free), and pHCMC05 (P*spac,* negative control without *gfp+* gene) was evaluated in the presence of different IPTG concentrations (0, 0.1, 1.0 mM IPTG). The activities of GFP (RFU units) was measured in all samples. All cultures were grown three times and each experiment was repeated at least twice with similar conditions. Error bars denote standard deviations.

GFP activities of the *B. subtilis* strain pHT1692 changed from 6416 to 12640 RFU and were significantly different between the surveyed samples in non-induced conditions by time. The activity of GFP measured from the strain pHT1692 achieved the highest activity of 12640 RFU, which was 2-fold higher than that of the inducible plasmid pHT1535. The expression levels of the strain pHT1692 were about 24.7 to 34.3 less than that of some inducer-free plasmids based on *Pgrac*01 and *Pgrac*100 previously reported in Ref. [9].

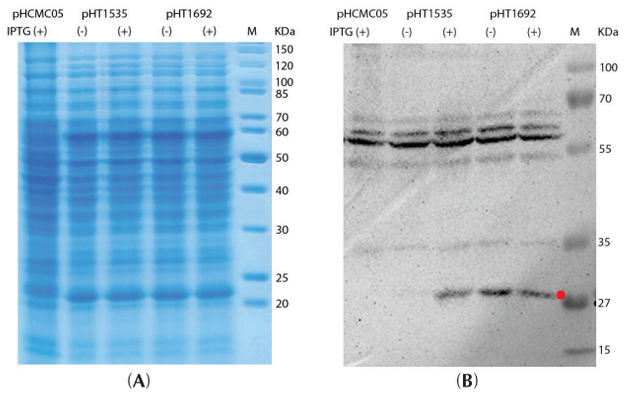

Fig. 5. The Western Blot results showing the GFP expression of inducer-free plasmids based on the P*spac* promoter in *B. subtilis* 1012. The suspension of the surveyed *B. subtilis* strains (pHT1692, pHT1535) under induced conditions (+, 1 mM IPTG) and non-induced conditions (-, 0 mM IPTG). The plasmid pHCMC05 without the *gfp*$^+$ gene was used as a negative control. The three different bacterial strains containing the surveyed vectors were cultured in an LB liquid medium at 37°C to the mid-logarithmic growth phase. Then, each culture was divided into two subcultures where one was continuously incubated without IPTG inducer (0 mM) and the other was under inducible conditions with 1 mM IPTG (the positive controls were induced at 1 mM IPTG). The samples were collected 4 h after induction. The size of the GFP lane was about 27 kDa. (**A**) The SDS-PAGE result shows fuzzy GFP lines that are difficult to identify and (**B**) The Western Blot result clearly reflects the expression of GFP in the *B. subtilis* strains.

The Western Blot results (Fig. 5B) also corresponded with previous publications and the sample of surveyed strains (pHT1672, pHT1535) indicated that the GFP protein was present in small quantities. These results certainly demonstrate that the newly constructed inducer-free expression plasmid based on the P*spac* promoter could allow low-level GFP expression without controlling.

Conclusions

P*spac*, a well-known IPTG-inducible promoter for *B. subtilis*, is suitable for studying the role of proteins that are produced at modest concentrations in the cell. We successfully engineered a P*spac* cassette by deleting a part of the *lacI* gene to create the inducer-free expression plasmids, pHT1692 and pHT1672, that express recombinant reporter proteins at low levels in *B. subtilis* without the addition of IPTG. The inducer-free expression plasmids for low protein expression in *B. subtilis* could be useful for investigating heterologous proteins at low levels.

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COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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