

Construction of expression plasmid for *Bacillus subtilis* using *Pspac* promoter and *BgaB* as a reporter

Phan Thi Thu Hanh¹, Nguyen Ngoc Yen Nhi¹, Le Thuy Tien¹, Chu Thi Bich Phuong^{1,2}, Le Thi Phuong Ngan¹, Phan Thi Phuong Trang¹, Hoang Duc Nguyen^{1,*} 

ABSTRACT

Introduction: In basic research, it is essential to use an inducible promoter which can be controlled to express a small amount of protein for studying their roles in the cell. *Pspac*, a well-known weak promoter for *Bacillus subtilis*, uses isopropyl β -D-1-thiogalactopyranoside (IPTG) as an inducer. However, plasmids carrying this promoter such as pHCMC05 still have a disadvantage which harbors a repetitive DNA fragment of about 200 bp, resulting in structural instability in *Escherichia coli*, causing difficulty during cloning. **Methods:** In this study, we constructed a plasmid that does not carry the repetitive sequences and investigated plasmid structural stability in *E. coli*, then measured the β -galactosidase reporter gene (*bgaB*) expression in *B. subtilis*. **Results:** The constructed plasmid pHT2002 was stable over 56 generations while pHCMC05-*bgaB* was structurally unstable and ultimately lost after 42 generations. *BgaB* activities and Western-blot indicated that *BgaB*-coding gene under control of IPTG-inducible promoter *Pspac* could be expressed at low levels. **Conclusion:** The study demonstrated that the new expression plasmid without the repeated sequences retained its structural stability in *E. coli* facilitating the cloning step. The expression plasmid with *Pspac* promoter for *B. subtilis* could be used to express a modest amount of the heterologous protein in the presence of IPTG.

Key words: *Bacillus subtilis*, *Pspac*, weak promoter, low expression, *BgaB*, pHCMC05

¹Center for Bioscience and Biotechnology, University of Science-VNUHCM

²Faculty of Pharmacy - Ho Chi Minh City University of Technology (HUTECH)

Correspondence

Hoang Duc Nguyen, Center for Bioscience and Biotechnology, University of Science-VNUHCM

Email: ndhoang@hcmus.edu.vn

History

- Received: 2018-12-21
- Accepted: 2019-04-24
- Published: 2019-06-19

DOI :

<https://doi.org/10.32508/stdj.v22i2.1284>



Copyright

© VNU-HCM Press. This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International license.



INTRODUCTION

Bacillus subtilis is a Gram-positive bacterium considered as a microbial cell factory. *B. subtilis* (i) is recognized as GRAS microorganism, which is non-pathogenic and does not produce endotoxin; (ii) capable of secreting directly into culture medium; (iii) has a large amount of well understood genetic information; (iv) its vector expression system has been established¹. For overexpression of recombinant proteins, the most easy-to-use expression system in *B. subtilis* is the pHT system, for examples pHT plasmids carrying *Pgrac* (also called *Pgrac01*)² with the *Pgrac100* promoter³, delivered by MoBiTec and the *Pgrac212* increased the mRNA half-life by three times that of *Pgrac*⁴. However, in practice, scientists sometimes need vectors for low inducible expression, for example for expression of some proteins for metabolic engineering or some functional membrane proteins. Therefore, it is essential to use an inducible promoter which can be controlled to express a modest amount of proteins for studying their roles in the cell. *Pspac* promoter is an appropriate option for this purpose, which is a weak promoter, approximately 50 times weaker than the *Pgrac* promoter^{2,5}.

Current available plasmid carrying *Pspac* promoter, pHCMC05 consists of a repeated sequence DNA repeat of about 117 bp (TAACCTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCTGTGCCAGCTGCATTAATGAATCGGCCAACCGGC-C-GGGGAGAGCGGTTTTCGCTATTGGGCGC)⁶. This repeated sequence was detected to be related to the structural instability when amplifying in *E. coli*, which could promote the homologous recombination causing the alteration of plasmid structure and the deletion of target genes⁷. According to those reasons, we aim to construct *E. coli* structurally stable plasmid carrying *Pspac* promoter that could be applied to express a small amount of protein by addition of the inducer in *B. subtilis*. In this study, we constructed plasmid pHT2002, carrying *Pspac* promoter and not containing repeated sequence. The structural stability of pHT2002 was investigated along with plasmid pHCMC05-*bgaB* based on the expression of the β -galactosidase (*bgaB*) reporter gene in *E. coli* and restriction map analysis. Also, we checked the presence of *BgaB* protein by Western-blot and measured the *BgaB* activities expressed under

Cite this article : Thu Hanh P T, Yen Nhi N N, Tien L T, Phuong C T B, Ngan L T P, Trang P T P, Nguyen H D. **Construction of expression plasmid for *Bacillus subtilis* using *Pspac* promoter and *BgaB* as a reporter.** *Sci. Tech. Dev. J.*; 22(2):239-246.

the control of the IPTG-inducible *Pspac* promoter in *B. subtilis*.

MATERIALS - METHODS

Bacterial strains and growth conditions

E. coli OmniMAXTM (F' {*proAB lacI^q lacZ*ΔM15 *Tn10*(Tet^R) Δ(*ccdAB*)} *mcrA* Δ(*mrr* *hsdRMS-mcrBC*) Φ 80(*lacZ*)ΔM15 Δ(*lacZYA-argF*)U169 *endA1* *recA1* *supE44* *thi-1* *gyrA96* *relA1* *tonA* *panD*) (Invitrogen) was used for gene cloning.

B. subtilis 1012 (*leuA8 metB5 trpC2 hsdRM1*) (Morbitec) was used for expression of recombinant plasmids.

The bacterial cells were cultured in Luria-Bertani (LB) broth at 37°C. Antibiotics were added at the appropriate concentration: ampicillin (Fisher BioReagents) at 100 μg/ml, chloramphenicol (Sigma) at 10 μg/ml.

Construction of the expression plasmid

The plasmid pHT2002 was different from pHCMC05-*bgaB*^{8,9} (Figure 1A) at 117 bp repeated sequence, which was removed in pHT2002. To generate the pHT2002 plasmid, we inserted the coding sequence for the *Pspac* promoter into the backbone plasmid pHT212⁸. Plasmid pHT212 derives from pHCMC05 and contains promoter *Pgrac212* and the *bgaB* gene, which does not have the repeated sequence. The development of plasmid pHT212 from pHCMC05 was described elsewhere⁸. First, the coding sequence of the *Pspac* gene was amplified by PCR using ON632 (TAGGCGGGCTGCCCGGGGACG) and ON1249 (CGTTTCCACCGGAATTAGCTTG) (Macrogen) with pHCMC05 as a template. The 370 bp amplified sequence was cleaved with *Bam*HI (Thermo Scientific) and *Kpn*I (Thermo Scientific) and ligated into pHT212 treated with the same enzymes resulting in pHT2002. The structure of the plasmid pHT2002 is shown in Figure 1B.

Investigation of the structural stability of the vector in *E. coli*

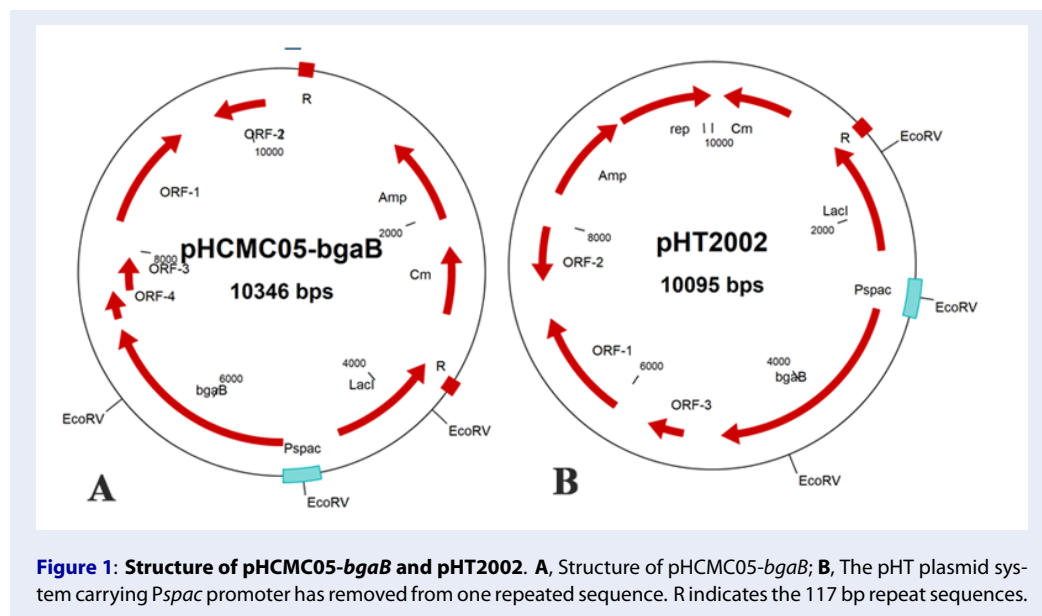
The structural stability of the two plasmids pHCMC05-*bgaB* and pHT2002 was investigated in *E. coli* OmniMAX via expression of BgaB on Xgal (5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside) (Thermo Scientific) plates in the presence of ampicillin (100 μg/ml). A single blue colony of the chosen strain of *E. coli*, carrying the respective plasmid, was taken from an agar plate

inoculated into 10 ml LB-broth with ampicillin (LB-Amp) and incubated 12 hours on an orbital shaker at 37 °C, 250 rpm. The cells were subcultured by transferring 5 μl culture into 10 ml fresh LB-Amp every 12 hours for eight times. At the same time, the cultures were diluted to 10⁶ times and spread on LB-Amp-Xgal plates. The appearing of the white colonies on the LB-Amp-Xgal plate indicated that the original plasmid was not in *E. coli* cells. In parallel, the plasmids from these subcultured were extracted for restriction analysis, allowing for comparisons of DNA fragment sizes at different subcultures.

Expression of the reporter β-galactosidase

Expression of the reporter protein BgaB using pHT2002 under the regulation of *Pspac* with IPTG inducer was measured in *B. subtilis* 1012. *B. subtilis* 1012 strains carrying the recombinant plasmids pHT2002 and pHT1512 (negative) was cultured in an LB medium containing the appropriate antibiotic. We followed the protocol as described elsewhere¹⁰. Briefly, the bacterial cells were grown to the mid-logarithmic growth phase and then induced with 0.1 mM, 1 mM IPTG. The cells were collected before induction (0 hour), after 2 hours and 4 hours of induction in Eppendorf tubes at an OD₆₀₀ of 2.4 after centrifugation. Samples were prepared for activity measurements and SDS-PAGE analysis. The BgaB activities were represented by MUG unit because of using MUG substrate and measuring the fluorescence intensity at Ex/Em = 360/460 nm. We used the multi-mode microplate reader Clario Star machine (BMG LABTECH), 384-well black plate with clear flat bottoms to measure the MUG fluorescence signals. All data were averaged from three independent samples of each time point. Mean values are given together with the standard deviations using Excel with the function STDEV.

For Western blot, 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. The protein from SDS-PAGE was transferred to nitrocellulose membrane (Bio-Rad) using a Bio-Rad apparatus. Western-Blot was performed with primary antibodies that were resistant to BgaB developed in mice (raised by the lab), used at a dilution of 1: 2000; and used at a dilution of 1: 10000 for the secondary antibody Anti-Mouse IgG (whole molecule)-Peroxidase antibody (Sigma). The signals from the blot were developed using TMB substrate (Sigma).



RESULTS

The removal of the 117 bp repeated sequence results in the stable structure of the plasmid carrying *Pspac-bgaB* promoter in *E. coli*.

We investigated the structural stability of two plasmids pHCMC05-*bgaB* and pHT2002 in *E. coli* for 48 generations after eight subcultures. The cells were cultured/subcultured in the LB-Amp medium and then spread on LB-Amp-Xgal plate. The blue colonies indicated that the plasmid remained stable in the *E. coli* cells and the white colonies indicated the structural instability.

The result showed that all colonies containing pHT2002 after 7 (the 1st culture), 28 (the 4th subculture) or 56 (the 8th subculture) generations exhibited a blue phenotype (Figure 2), demonstrating its structural stability. According to the results in Figure 2, white colonies appeared on the plate of pHCMC05-*bgaB* in the 4th subculture and completely white in the 8th subculture. Thus, these plates consisted of *E. coli* containing plasmid pHCMC05-*bgaB*, which was altered in structure leading to the loss of *bgaB* gene sequence due to the homologous recombination between the repeats. At this point, the predicted altered structure of pHCMC05-*bgaB* was named pHCMC05-*bgaB* Delta. The homologous recombination at the repeated regions of pHCMC05-*bgaB* resulting in two plasmids, but only plasmid pHCMC05-*bgaB* Delta could be selected because of the presence of Ampicillin resistant gene.

To clarify the results of observation of white-blue colonies associated with the structural alteration leading to the loss of the target gene *bgaB*, we extracted plasmids in the cells from the subcultures. Then we performed the restriction analysis with *EcoRV* for the plasmids from the 1st culture, 4th, 6th, 8th subculture. By calculation, *EcoRV* cut the plasmids pHCMC05-*bgaB* and pHT2002 into 3 fragments (Figures 3 and 4), while the pHCMC05-*bgaB* Delta does not have *EcoRV* site. Figure 3 showed the size of each of the fragments when plasmids were cleaved with this enzyme. All restriction digestions were carried out in two hours at 37 °C. The samples were analyzed by agarose gel electrophoresis of which results were shown in Figure 4.

Figure 4 showed the electrophoresis gel that after being cleaved with *EcoRV* enzyme, the digest bands of the plasmid from the cells carrying pHCMC05-*bgaB* started to change from the 6th subculture (after 42 generations). A bright band appeared as about over 3000 bp corresponded to the size of the modified plasmid of pHCMC05-*bgaB* with no *EcoRV* restriction site in the plasmid. In the 8th subculture (after 56 generations), only the band with size corresponded to the homologous recombinant structure was found. The results indicated that over 56 generations, pHCMC05-*bgaB* completely lost the target gene *bgaB*. On the contrary, pHT2002 cut with

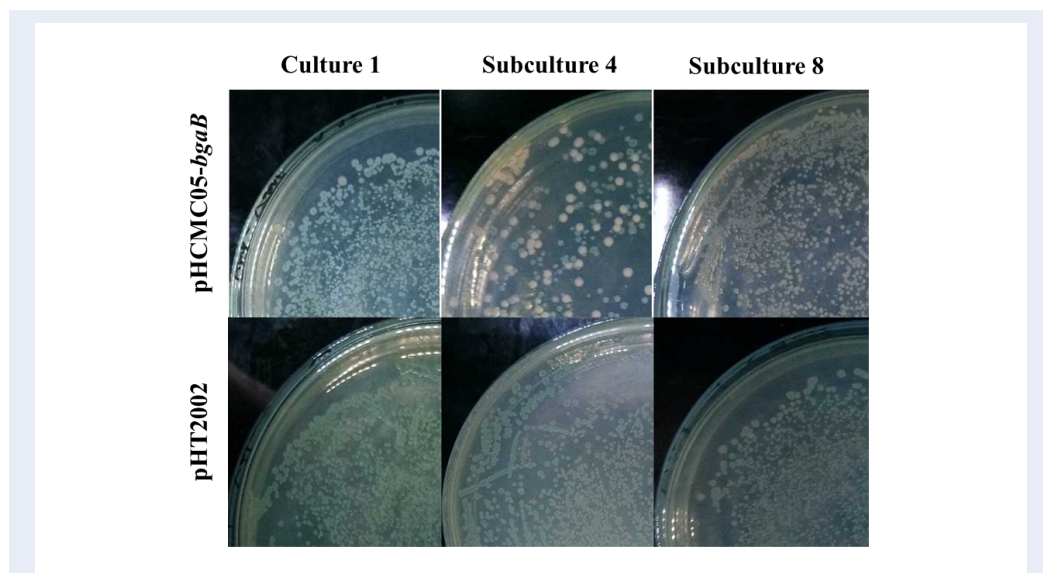


Figure 2: Blue-white colonies of *E. coli* carrying pHCMC05-*bgaB* and pHT2002 over generations. *E. coli* harbor two plasmids containing *Pspac-bgaB* were grown in LB medium for over generations involving subcultures after 12 hours and then plated on LB-Amp plates containing Xgal. Culture 1, the 1st culture; subculture 4 (28 generations), the cells were subcultured 4 times; subculture 8, the cells were subcultured 8 times (56 generations).

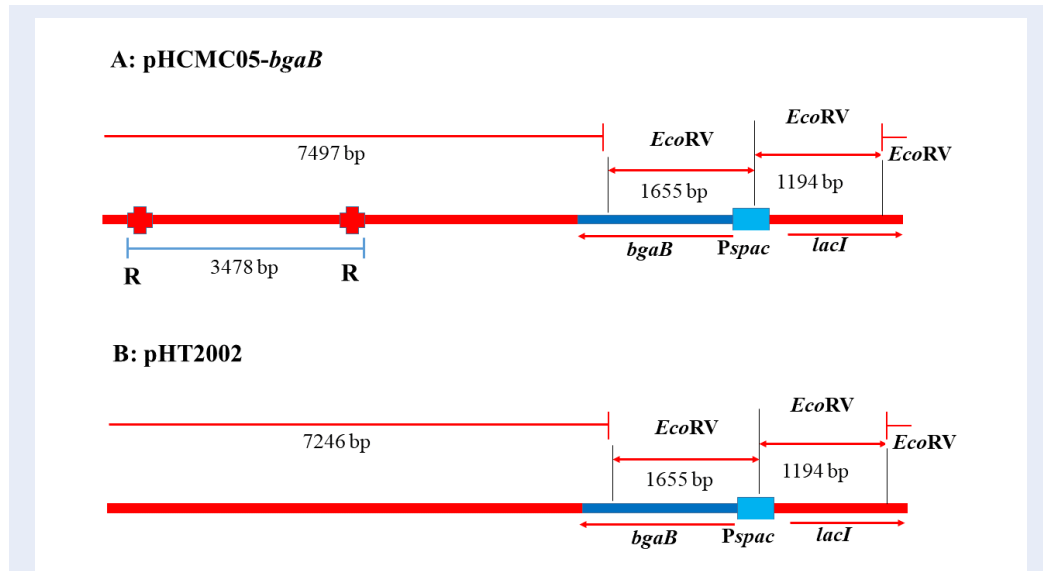


Figure 3: The resulting fragments of pHT2002 and pHCMC05-*bgaB* when cut with *EcoRV*. A, digesting pHCMC05-*bgaB* with *EcoRV* would result in 3 bands: 7497 bp, 1655 bp, 1194 bp. B, digesting pHT2002 with *EcoRV* would result in 3 bands: 7246 bp, 1655 bp, 1194 bp. The red cross symbols (R) indicated the repeated regions, which will be circled resulting in the altered plasmid pHCMC05-*bgaB* Delta with the size of 3269 bp.

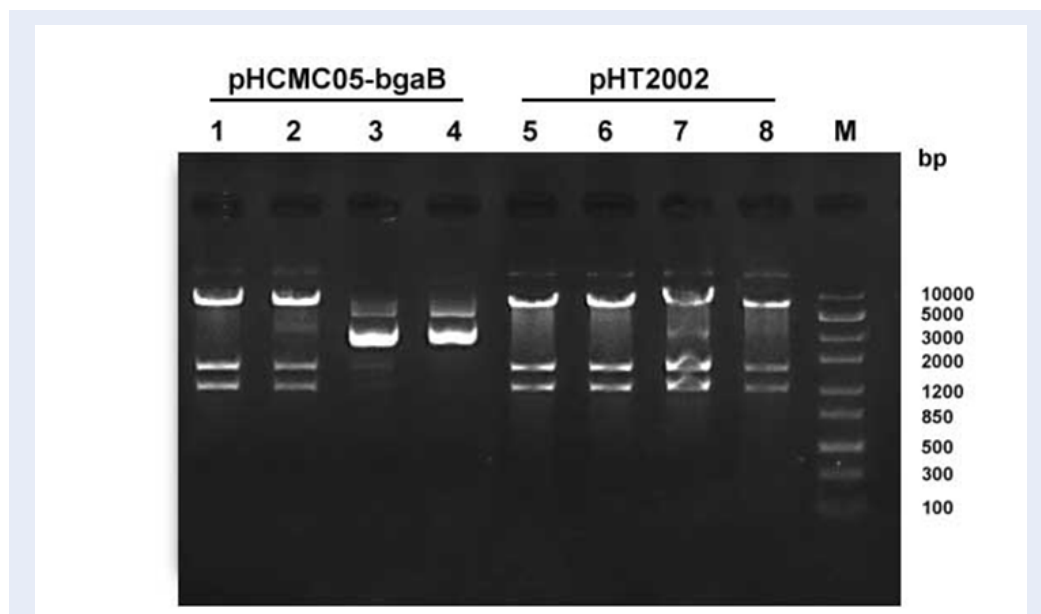


Figure 4: Analysis of the structural stability of plasmids pHT2002 and pHCMC05-*bgaB*. Single colonies of *E. coli* OmniMAX carrying pHT2002 or pHCMC05-*bgaB* were grown in 12 hours in LB-Amp at 37°C. 5 µl aliquot of the culture were transferred to new 10 ml medium for 12 hours incubation, which was repeated 8 times. The cells from the culture or subculture were collected for plasmid preparation. Plasmids were prepared, cut with *EcoRV*, and analyzed by agarose gel electrophoresis. Lanes 1, 2, 3, 4: pHT2002 cut by *EcoRV*. Lanes 5, 6, 7, 8: plasmid from cell carrying pHCMC05-*bgaB* cut by *EcoRV*. Lanes 1, 5, plasmid from the 1st culture; lane 2, 6, the 4th subculture; lane 3, 7, the 6th subculture; lane 4, 8, the 8th subculture.

EcoRV enzyme showed bands as predicted after the 8th subculture, which proved that this plasmid was structurally stable for at least 56 generations. These results led to the conclusion that plasmid pHT2002 had a stable structure over generations in *E. coli*.

The induction of plasmid pHT2002 to express BgaB in *B. subtilis*

For this experiment, we aim to examine the expression levels of target protein BgaB in *B. subtilis*/pHT2002 under the control of the *Pspac* promoter by using inducer IPTG. *B. subtilis*/pHT1512 containing the corresponding expression system but lacking the *bgaB* gene used as a negative-control strain. The *B. subtilis* strains were grown in liquid media and the cells were collected for β -galactosidase activity. Figure 5 showed the BgaB activities of these two *B. subtilis* strains. Compared with *B. subtilis*/HT1512 strain, *B. subtilis*/pHT2002 showed the expression of BgaB, which increased in the presence of 0.1 mM and 1 mM IPTG and over incubation time 2 hours, 4 hours. The result indicated that the expression level of *B. subtilis*/pHT2002 regulated by the *Pspac* promoter via an IPTG inducer. In comparison

with *Pgrac* promoters using the same assay, the expression levels of BgaB under control of *Pspac* promoter from pHT2002 was less than 3000 times that of from *Pgrac01*¹⁰. The results showed that the heterologous *bgaB* gene could be induced for the expression at modest levels by the addition of IPTG.

Result of Western blot

Since we could not detect the protein expression via SDS-PAGE because of a low level of protein expression, we used the Western Blot to detect the target BgaB protein. Figure 6, lane 5 showed a thick band corresponding to BgaB size (78 kDa) for the *B. subtilis*/pHT2002 sample induced with 1mM IPTG, while there are light bands for the other samples. This result re-confirmed that *Pspac* is a weak promoter to regulate low levels of protein expression. This result is consistent with the objective of this study.

DISCUSSION

We could show here that the newly constructed plasmid which contained *Pspac* promoter reached three objectives which are (i) stable in structure, (ii) low

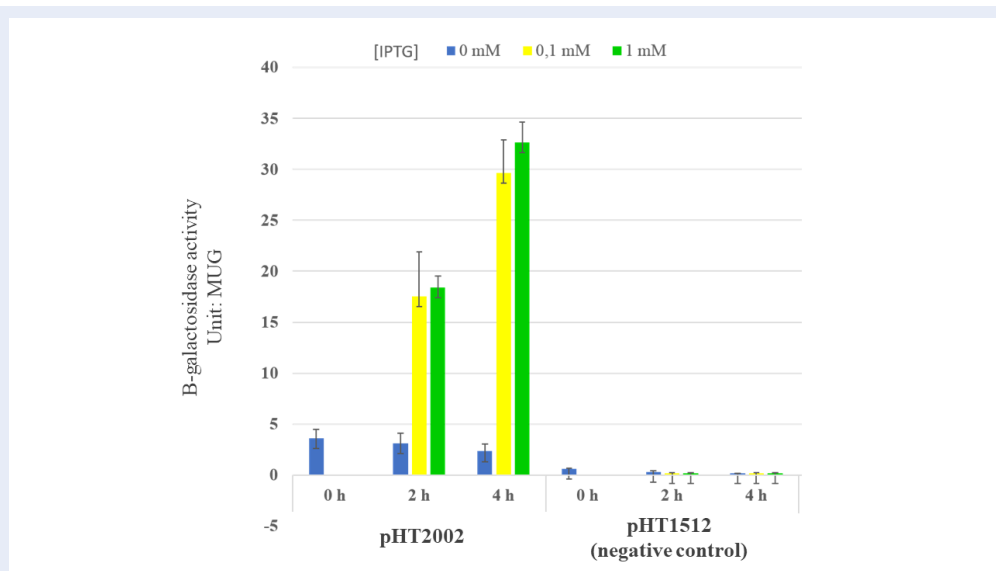


Figure 5: Reporter assay of BgaB activity in strain *B. subtilis* /pHT2002. The empty vector pHT1512 served as a negative control. The bacterial cells carrying these vectors were grown in LB medium at 37°C to the mid-logarithmic growth phase. Then, the culture was split into three subcultures, where one was further incubated in the absence of IPTG (0 mM) and other two induced with 0.1 and 1 mM IPTG. Samples were collected after 0 hour (before induction), 2 hours, 4 hours (after induction). BgaB activities from the collected samples were measured on 384-well plate. All data were averaged from three independent samples of each time point. Mean values are given together with the standard deviations.

protein expression and (iii) controllable by using inducer. It is the first successful step to carry out further researches to test the expression with other reporter proteins.

In terms of application, this plasmid is available for protein expression on the surface of the cell such as sortases, which have been used to anchor heterologous proteins on the cell wall of different Gram-positive bacterial species. Sortase A, in particular, is a membrane-anchored transpeptidase¹¹. The excess of this protein expression will cause membrane congestion¹². Therefore, it is required to have a low expression system.

Another example is PrkC protein kinase, which is responsible for triggering spore germination in response to muropeptides. PrkC exerts its effects through signal transduction pathways involving phosphorylation of its substrates. They need only a small amount, and depending on the stage of the cell they need to be expressed¹³.

We are interested in using engineered bacterial expression systems for fundamental researches concerned about studying protein structures and functions in cells, and the finding suggests that this plasmid development is suitable for that purpose.

CONCLUSION

The study demonstrated that the new expression plasmid without the repeated sequences conferred its structural stability in *E. coli*, which facilitate the cloning step. On the other hand, the expression plasmid with *Pspac* promoter could be used to express a modest amount of the heterologous protein in the presence of IPTG in *B. subtilis*.

ABBREVIATIONS

Amp: Ampicillin

B. subtilis: *Bacillus subtilis*

bp: base pair

bgaB: β -galactosidase

Cm: Chloramphenicol

DNA: Deoxyribose Nucleic Acid

E. coli: *Escherichia coli*

IPTG: Isopropyl β -D-Thiogalactopyranoside

kDa: kilo Dalton

LB: Luria-Bertani medium

M: Marker

mRNA: messenger Ribonucleic Acid

MUG: 4-Methylumbelliferyl β -D-Galactopyranoside

OD: Optical Density

PCR: Polymerase Chain Reaction

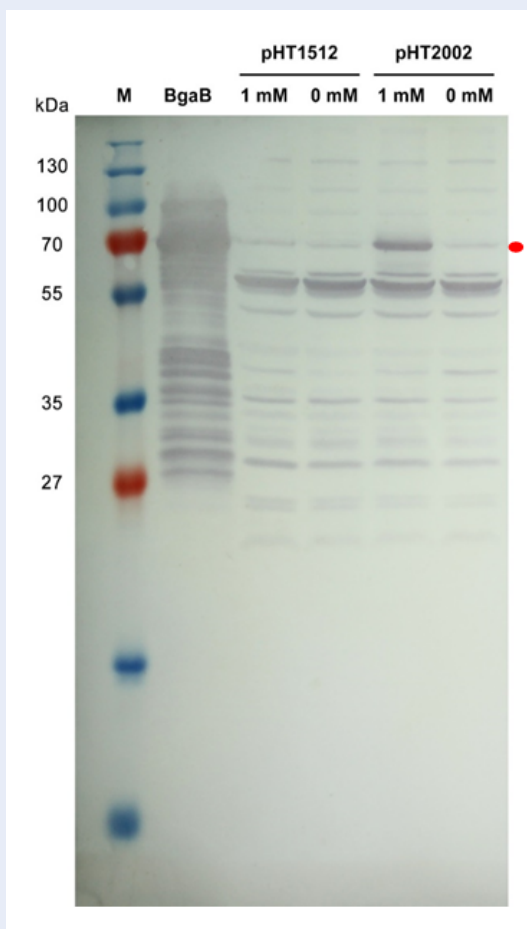


Figure 6: Detection of the BgaB protein by immunoblot analysis. *B. subtilis*/pHT2002 and *B. subtilis*/pHT1512 (negative control) were cultured without (0 mM) or with 1 mM IPTG for 4 hours. The cells were lysed and analyzed by SDS-PAGE. Protein samples were transferred to nitrocellulose membrane. BgaB was detected by using primary antibody raised in mice and Secondary antibody Anti-mouse IgG peroxidase antibody. Detection of protein BgaB was shown in lane 2. Lane 1: Prestained molecular weight standards. Lanes 3, 4: *B. subtilis*/pHT2002 induced with 0 and 1 mM IPTG. Lane 5, 6. *B. subtilis*/pHT1512 induced with 0 and 1 mM IPTG. Red dot, the position of the BgaB proteins.

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

TMB: 3,3',5,5'-Tetramethylbenzidine

Xgal: 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

Ex/Em: excitation/emission

COMPETING INTERESTS

There is no conflict of interest.

AUTHORS' CONTRIBUTIONS

Phan Thi Thu Hanh performed experiments under the supervision of Hoang Duc Nguyen. All the authors designed experiments, analyzed the data and completed the paper.

ACKNOWLEDGMENTS

This research was partially funded by University of Science, Vietnam National University Ho Chi Minh City for the labor cost, grant number (T2018-41). The materials were supported by the National Foundation for Science and Technology Development (NAFOS-TED) under Grant Number 106-NN.02-2015.24.

REFERENCES

1. Harwood CR. *Bacillus subtilis* and its relatives: molecular biological and industrial workhorses. *Trends in biotechnology*. 1992;10:247–256. PMID: 1368322.
2. Phan TTP, Nguyen HD, Schumann W. Novel plasmid-based expression vectors for intra- and extracellular production of

- recombinant proteins in *Bacillus subtilis*, Protein Expression and Purification. 2006;46:189–195. PMID: 16125412. Available from: [10.1016/j.pep.2005.07.005](https://doi.org/10.1016/j.pep.2005.07.005).
3. Phan TTP, Tran LT, Schumann W, Nguyen HD. Development of Pgrac100-based expression vectors allowing high protein production levels in *Bacillus subtilis* and relatively low basal expression in *Escherichia coli*. Microbial Cell Factories. 2015;14. PMID: PMC4446860. Available from: [10.1186/s12934-015-0255-z](https://doi.org/10.1186/s12934-015-0255-z).
 4. Phan TTP, Nguyen HD, Schumann W. Construction of a 5'-controllable stabilizing element (CoSE) for over-production of heterologous proteins at high levels in *Bacillus subtilis*. Journal of Biotechnology. 2013;168:32–39.
 5. Yansura DG, Henner DJ. Use of the *Escherichia coli* lac repressor and operator to control gene expression in *Bacillus subtilis*. Proc Natl Acad Sci U S A. 1984;81(2):439–443. PMID: 6420789.
 6. Nguyen HD, Phan TTP, Schumann W. Expression Vectors for the Rapid Purification of Recombinant Proteins in *Bacillus subtilis*. Current Microbiology. 2007;55:89–93. PMID: 17624574. Available from: [10.1007/s00284-006-0419-5](https://doi.org/10.1007/s00284-006-0419-5).
 7. Bzymek M, Lovett ST. Instability of repetitive DNA sequences: the role of replication in multiple mechanisms. Proc Natl Acad Sci U S A. 2001;98(15):8319–8325. PMID: PMC37438. Available from: [10.1073/pnas.111008398](https://doi.org/10.1073/pnas.111008398).
 8. Trang PTP. Construction and analysis of novel controllable expression vectors for *Bacillus subtilis*; 2007.
 9. Nguyen DH. Construction of plasmid-based expression and secretion vectors and study of the immobilization of proteins on the surface of *Bacillus subtilis* cells; 2006.
 10. Tran DTM, Phan TTP, Huynh TK, et al. Development of inducer-free expression plasmids based on IPTG-inducible promoters for *Bacillus subtilis*. Microbial Cell Factories. 2017;16(1):130. PMID: PMC5526301. Available from: [10.1186/s12934-017-0747-0](https://doi.org/10.1186/s12934-017-0747-0).
 11. Wu Z, Hong H, Zhao X, Wang X. Efficient expression of sortase A from *Staphylococcus aureus* in *Escherichia coli* and its enzymatic characterizations. Bioresources and Bioprocessing. 2017;4.
 12. Wardenburg JB, Patel RJ, Schneewind O. Surface Proteins and Exotoxins Are Required for the Pathogenesis of *Staphylococcus aureus* Pneumonia. Infection and Immunity. 2007;75:1040–1044.
 13. Pompeo F, Byrne D, Mengin-Lecreux D, Galinier A. Dual regulation of activity and intracellular localization of the PASTA kinase PrkC during *Bacillus subtilis* growth. Scientific Reports. 2018;8.

