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ORIGINAL ARTICLE

# Validation of reference genes for the normalization of RT-qPCR gene expression in *Bacillus siamensis* 1021 grown in different culture media

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## ABSTRACT

**Background and Objectives:** House-keeping genes are generally selected as reference genes in gene expression analysis. However, some genes may not be stably expressed across all experimental conditions. Thus, this study aimed to validate seven house-keeping genes for gene expression analysis in *Bacillus siamensis* 1021.

**Materials and Methods:** Strain 1021 was grown in potato dextrose broth, nutrient broth and mineral salt medium. Reverse-transcription quantitative PCR was used to determine Cq values of seven reference genes including *gyrA*, *gyrB*, *ssb* and *dnaB*, *rpsU*, gat_Yqey and *udp* in these media. Expression stability of these genes was analyzed, using geNorm and Normfinder applications. The target gene *ftsZ* was used for assessment of the best candidate genes.

**Results:** Based on geNorm and Normfinder, *ssb* was the most-stably expressed gene, while *udp* was the least-stably expressed gene. Pairwise variation indicated the combination of *ssb*, *gyrA*, *gyrB* and gatB_Yqey was suitable for the normalization of *ftsZ* expression. *ftsZ* expression in potato dextrose broth and mineral salt medium was higher than that in nutrient broth. In contrast, the normalization against *udp* resulted in an under- and overestimation of *ftsZ* expression in potato dextrose broth and mineral salt medium, respectively.

**Conclusion:** The combination of *ssb*, *gyrA*, *gyrB* and gatB_Yqey was the best candidate for normalization of target gene expression in *B. siamensis* 1021 in these media. This study emphasized the significance of reference gene validation for gene expression analysis and provided a guideline for future gene expression studies in *B. siamensis*.

**Keywords:** *Bacillus*; Reverse-transcription quantitative polymerase chain reaction; Gene expression; Culture media

## INTRODUCTION

Reverse-transcription quantitative PCR (RT-qPCR) is a relatively simple and inexpensive technique that is generally used to determine the expression level of target genes. It has been applied for research in various field, e.g., agricultural science, biomedical science, molecular genetics, etc. It is also used for assessing differential gene expression under different conditions in various tissues and organisms. The expression level of a target gene is normalized against reference genes, whose expression is relatively stable across tested conditions (1). In previous studies, house-keeping genes were selected as reference genes because of their constitutive expression. They were also required for basic growth and cellular functions under various conditions (2). However, they may be differentially expressed under different growth conditions or in distinct organisms. For example, in *Pseudomonas brassicacearum* GS20, *gmk* expression stability was less affected by changes in temperature, while *gyrA* was the most suitable refer-

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ence gene under different iron concentrations in the medium (3). In contrast, the more stably expressed genes in *Pseudomonas aeruginosa* were *proC* and *rpoD* (4). Thus, the minimum information for publication of quantitative real-time PCR experiments (MIQE) indicated that all reference genes must be tested for their expression stabilities across cell and tissue types as well as experimental conditions (5). The use of a single reference gene for normalization must be avoided (5). This is excepted only when a gene was provided with evidence of its invariable gene expression under the experimental design (5).

Various algorithms and applications were available for testing gene expression stability of candidate genes. These included geNorm, Normfinder, Bestkeeper and RefFinder (6). Among these, geNorm and Normfinder were widely used in previous studies. It analyzed the expression stability of a given set of reference genes. The minimum number of reference genes was also determined, based on the calculated geometric mean (7). Normfinder used the model-based approach to analyze the expression variation of candidate genes (8). It calculated the stability value of each candidate gene and determined the best combination of two candidate genes (8). A previous study in *Acanthamoeba* spp. showed that geNorm and Normfinder consistently indicated that the 18S rRNA and HPRT genes were the most stably expressed reference genes (6). However, geNorm suggested different numbers of reference genes to be used in different *Acanthamoeba* spp. strains (6).

*Bacillus siamensis* was first described as a Gram-positive, rod-shaped bacterium isolated from salted crabs in Thailand (9). Based on a previous genomic study, it was found closely related to *Bacillus velezensis* and *Bacillus amyloliquefaciens* (10). Several strains of *B. velezensis* and *B. amyloliquefaciens* were demonstrated for their antagonistic and biocontrol activities against various phytopathogens and plant diseases (11-13). Similar to its relatives, strains of *B. siamensis* were recently proposed as potential biocontrol agents against many pathogenic fungi, e.g. *Alternaria alternata, Rhizoctonia solani, Verticillium lateritium, Macrophomina phaseolina* (14-16). The antagonistic activities of several *B. siamensis* strains were derived from the production of nonribosomal peptides (NRPs) (14, 17). This class of lipopeptides was synthesized by modular nonribosomal peptide synthetases (NRPSs) (18). Previous studies in other *Bacillus* species suggested that NRP production correlated with expression of NRPS genes (19-20). Several regulatory genes of NRP biosynthesis were also determined (21-22). Similar to NRPS genes, expression levels of these regulatory genes affected NRP biosynthesis in *B. subtilis* ZK0 (23). Thus, gene expression analysis could provide a better understanding on the regulation of NRP production. *B. siamensis* 1021 was previously isolated and identified, based on the phylogenetic analysis of the 16S rRNA gene (24). The production of antibiotic compounds of this strain against *Pyricularia oryzae*, the causative agent of rice blast, depended on culture media (24). However, little was known about the regulation and expression of NRPS genes in *B. siamensis.* For accurate quantification of NRPS genes in strain 1021, reference genes must be validated. Thus, the aim of the present study was to identify the most stably expressed reference genes among seven reference genes, including *gyrA, gyrB, ssb, dnaB, rpsU,* gat_Yqey and *udp.* The reference genes could be used for the future analysis of NRPS gene expression in this strain. This study also provided a guideline for gene expression analysis in other *B. siamensis* strains.

## MATERIALS AND METHODS

**Bacterial strain and culture.** *B. siamensis* strain 1021 was grown in nutrient agar (NA; Sisco Research Laboratories, India) at 30°C for 24 hours to obtain single colonies. A single colony was used for inoculation in 10 ml of nutrient broth (NB; Sisco Research Laboratories, India) and grown at 30°C for 24 hours, on a rotary shaker at 160 rpm. The light absorbance of the cell suspension was determined at the 600 nm wavelength ($OD_{600}$). Cell concentration was adjusted to 0.1 $OD_{600}$, and 100 µL of this suspension was used for inoculation in 50 ml of NB, potato dextrose broth (PDB; Himedia, India) and mineral salt medium (MSM) (17). The bacterial cultures were grown at 30°C for 60 hours on a rotary shaker at 160 rpm. The cultures were sampled at 8, 12, 24, 36, 48 and 60 hours post inoculation for growth determination. Cell suspension was serially diluted and grown on NA at 30°C for 18 hours for colony enumeration.

**RNA extraction and cDNA synthesis.** For RNA extraction, 2 mL of cell suspension in PDB, NB and MSM were collected at 48 hours post inoculation. The

cells were harvested by centrifugation at 13,500 rpm for 10 minutes. Favorprep Tissue Total RNA mini kit (Favorgen, Taiwan) was used for RNA extraction, according to the manufacturer's protocol. RNA solution was treated with RNase-free DNase I (Geneaid, Taiwan) and purified with RNA Cleanup kit (Geneaid, Taiwan), according to the manufacturer's protocols. RNA quality and concentration was determined using NanoDrop spectrophotometer (ThermoFisher Scientific, USA). Purified RNA solution was used for agarose gel electrophoresis to determine the absence of contaminating genomic DNA. cDNA was synthesized from one µg of RNA and random hexamer primers, using iScript Select cDNA Synthesis kit (Bio-Rad, USA). The temperature profile followed the manufacturer's protocol.

**Reference gene selection and primer design.** Whole-genome sequence of strain 1021 (assembly accession number: GCF_014534735.1) was obtained from the GenBank database. The annotated nucleotide sequences of *gyrA*,*grB*, *dnaB*, *rpsU*, *ssb*, gatB_Yqey, *udp* and *ftsZ* genes were obtained. These genes functioned in DNA replication (*gyrA*, *gyrB*, *ssb* and *dnaB*), translation (*rpsU* and gat_Yqey) and the epimerization of UDP-N-acetylglucosamine (*udp*). Primers were manually designed and used for qRT-PCR (Table 1).

**Reverse-transcription quantitative PCR (RT-qPCR).** Equal volumes of all cDNA samples were pooled to obtain the 1× cDNA solution. This solution was used for preparation of five-fold serial dilutions (1/5×, 1/25× and 1/125×). All RT-qPCR reactions were performed, using Luna Universal qPCR Master Mix (New England Biolabs, USA), according to the manufacturer's protocol. Detection of amplification products was based on SYBR Green fluorescence. Two µl of cDNA solutions of each concentration were used as the template. The temperature profile included one cycle of 95°C for 60 seconds and 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds. Melt-curve analysis was done from 60°C to 95°C with a 0.5°C increment. The reactions were done on CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA). Cq (quantitation cycles) values were determined from three technical replicates and used for generating the standard curves with the logarithmic scale of the cDNA concentrations. *r2* and PCR efficiency (E) (25) of the primers for each gene were determined. E was determined based on the slopes of the standard curves: $E\ (\%) = (10^{-(1/\text{slope})} - 1) \times 100\%$ (25).

**Expression data and reference gene stability.** cDNA samples obtained from PDB, NB and MSM were diluted five folds. Cq values of the seven can-

**Table 1.** Primers used in this study.

| Gene | Accession number | Primer sequence (5'-3') | Tm (°C) | Product size (bp) | *r2* 0.998 | PCR efficiency |
|---|---|---|---|---|---|---|
| *gyrA* | MBD0406835 | F: CGATTAACCGGTCTGGAGCG | 62.5 | 71 | | 99.3% |
| | | R: AGCTCGGCGATAAGCGCAAC | 62.5 | | 0.998 | |
| *gyrB* | MBD0406836 | F: GCCGGCGGTAAATTTGACGG | 62.5 | 72 | | 99.4% |
| | | R: TACGACAGACGCCCCTACAC | 62.5 | | 0.995 | |
| *dnaB* | MBD0406901 | F: TTCAAGCGACAACCGCCAGC | 62.5 | 64 | | 104.5% |
| | | R: CCGTGCCAGCGATTTTAGCG | 62.5 | | 0.998 | |
| *rpsU* | MBD0407962 | F: GCTCTTCGTCGCTTCAAACGC | 63.2 | 66 | | 98.6% |
| | | R: TTCGCGCTTTCTTGCTTCTTGC | 62.1 | | 0.998 | |
| *ssb* | MBD0407252 | F: GGATACAACGAAGGAAACAGCG | 62.1 | 61 | | 99.3% |
| | | R: GATTATCATTTTGGCCTCCGCC | 62.1 | | 0.993 | |
| gatB_Yqey | MBD0407961 | F: CGAGCTCGAAAGCGGACATG | 62.5 | 83 | | 101.8% |
| | | R: GTTAATCACGCTGCCGTCAGC | 63.2 | | 0.999 | |
| *udp* | MBD0407318 | F: AATCCCGCAGTACGTGAGGC | 62.5 | 55 | | 98.6% |
| | | R: GCACTCTGTCCGAATCAGCG | 62.5 | | 0.996 | |
| *ftsZ* | MBD0406176 | F: GCCGAAGCCGCTAAAAAGGC | 62.5 | 70 | | 108.5% |
| | | R: AAACACCTTGCGCTCCGTCG | 62.5 | | | |

didate genes were determined, using RT-qPCR. A single threshold baseline was set, using the CFS Manager software (Bio-Rad, USA). The Cq values in each sample was converted to relative quantities (RQ), using the formula $RQ = E^{-\Delta Cq}$ (6). ΔCq was determined as ΔCq = Cqmin – Cqsample, where Cqmin was the lowest Cq value for each gene and Cqsample was the Cq value of each sample. The RQ values were subsequently used for calculation of gene expression stability, using the geNorm (7) and Normfinder (8). Pairwise variation $V_{n/n+1}$ was performed, as described previously (7).

**Validation of reference genes.** Relative *ftsZ* gene expression of strain 1021 in each medium was normalized against the selected reference genes, using the ΔΔCq method (26). Bacteria grown in NB medium was used as the reference group, because NB is the general medium for growing bacteria.

**Statistical analysis.** All experiments were performed in triplicates. Statistical analysis was carried out, using IBM SPSS Statistics Version 28.0. Analysis of variance (ANOVA), followed by multiple comparisons with Tukey's test, was used to determine statistically significant differences ($P < 0.05$).

## RESULTS

**Differential growth of strain 1021 in PDB, NB and MSM.** Strain 1021 grew differently in PDB, NB and MSM media (Fig. 1). Growth of strain 1021 in these media was comparable up to 24 hours post inoculation. At 36 hours, bacterial growth in PDB (2.3 + 0.14 $\times 10^8$ CFU/mL) became considerably higher than that observed in the other two media. Additionally, growth in MSM medium (1.1 + 0.14 × $10^8$ CFU/ml) became significantly higher than that in NB (5.8 × $10^7$ CFU/mL). At 48 hours, growth in PDB slightly increased to 2.5 + 0.17 × $10^8$ CFU/mL and was approximately four and two folds of that in NB (5.8 + 0.06 $\times 10^7$ CFU/mL) and MSM (1.08 + 0.11 × $10^8$ CFU/mL), respectively.

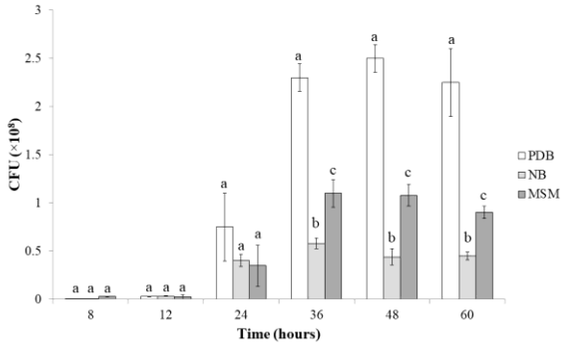


**Fig. 1.** Growth of strain 1021 in PDB, NB and MSM media at 30°C on a rotary shaker at 160 rpm. The cultures were grown for 60 hours. Error bars indicate standard deviations. Different letters indicate statistically significant differences between samples at the same growth period.

**Reference gene analysis for RT-qPCR.** Seven reference genes, *gyrA, gyrB, dnaB, rpsU, ssb*, gatB_Yqey and *udp*, were selected for the analysis of their gene expression stability in the three media. Based on the standard curves, the *r2* and PCR efficiency values of the primers ranged from 0.993 to 0.999 and from 98.6% to 104.5%, respectively (Table 1). From the melt-curve analysis, a single peak and a single $T_m$ value was obtained from each primer pair (Fig. 2). The $T_m$ values ranged from 80.0°C to 82.5°C. This indicated there was only one type of PCR product amplified in each PCR reaction.

Cq values of the house-keeping genes of strain 1021 grown in PDB, NB and MSM were determined for the calculation of gene expression stability, using geNorm and Normfinder programs (Table 2). Based on geNorm, the expression stability values (*M*) of each reference gene ranged from 0.672 (*ssb*) to 1.314 (*udp*) (Table 2). Low M values indicated high levels of gene expression stability, while high *M* values signified low levels of gene expression stability (15). Thus, *ssb* was the most stably expressed gene, while *udp* was the least stably expressed gene. Stepwise exclusion of the least stably expressed reference genes was performed by the program geNorm. The combination of *gyrA* and *gyrB* displayed the lowest *M* value (Fig. 3). To determine the number of required reference genes, pairwise variation (Vn/n+1) was calculated. The variation level at 0.15 was previously proposed as the cut-off level where lower variation levels indicated the inclusion of an additional reference gene was not required (7). We found that V4/5 (0.13) and V5/6 (0.14) were below the cut-off level (Fig. 4). This suggested that the combination of the four most-stably expressed genes, *ssb*, *gyrA*, *gyrB* and gatB_Yqey, was the best candidates for normalization of target gene expression. Consistently, Normfinder (8) suggested that *ssb* (stability value = 0.086) and *udp* (0.740) were the most- and the least-stably expressed genes (Table 2). Additional-

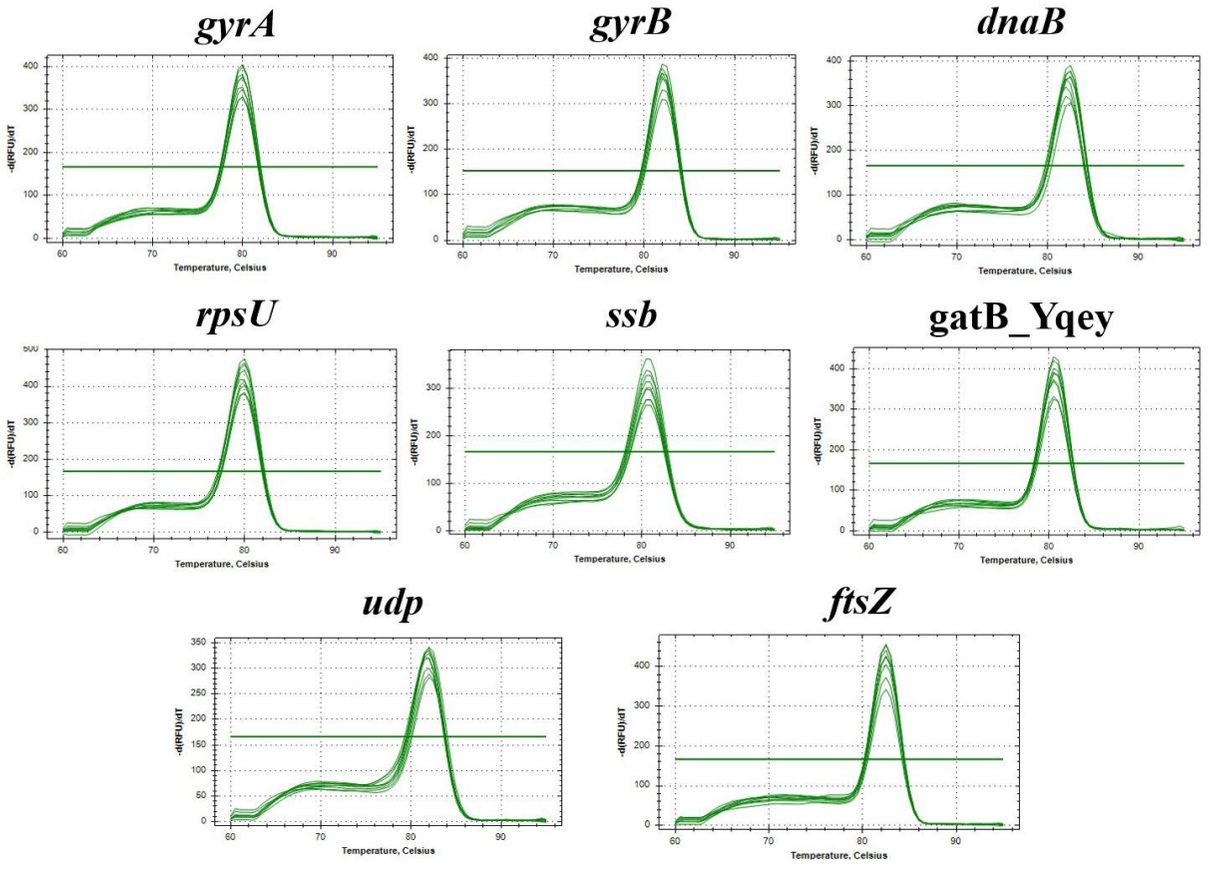


**Fig. 2.** Melt-curves of the amplification product from the candidate genes *gyrA, gyrB, dnaB, rpsU, ssb*, gatB_Yqey and *udp* and the target gene *ftsZ*. A single peak indicated the presence of one type of amplification product in each reaction.

**Table 2.** Expression stability of seven reference genes obtained from geNorm and Normfinder.

| Gene | Expression stability | |
|---|---|---|
| | geNorm | Normfinder |
| *ssb* | 0.672 | 0.086 |
| *gyrA* | 0.796 | 0.373 |
| gatB_Yqey | 0.828 | 0.381 |
| *rpsU* | 0.908 | 0.476 |
| *gyrB* | 0.709 | 0.258 |
| *dnaB* | 1.053 | 0.545 |
| *udp* | 1.314 | 0.740 |

ly, the combination of *ssb* and *gyrB* was suggested by Normfinder with the stability value at 0.128.

***ftsZ* expression analysis in strain 1021.** The *r2* and PCR efficiency of the *ftsZ* primers were 0.996 and 108.5%, respectively (Table 1). The melt-curve analysis revealed a single peak and a single Cq value which suggested there was only one PCR product

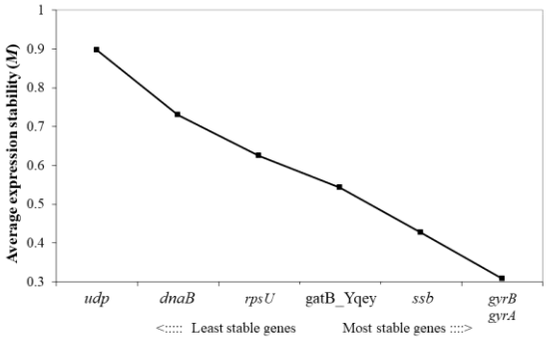


**Fig. 3.** Ranking of the reference genes based on the average expression stability (*M*) after stepwise exclusion of the least-stably expressed gene by geNorm.

from the *ftsZ* primers (Fig. 2). Relative gene expression of *ftsZ* in PDB, NB and MSM was normalized against the combination of four genes (*ssb-gyrA-gyrB*-gatB_Yqey) (Fig. 5). *ftsZ* expression of strain 1021 in PDB (2.40 + 0.37) was comparable to that in MSM (2.37 + 0.65), whereas the expression level in

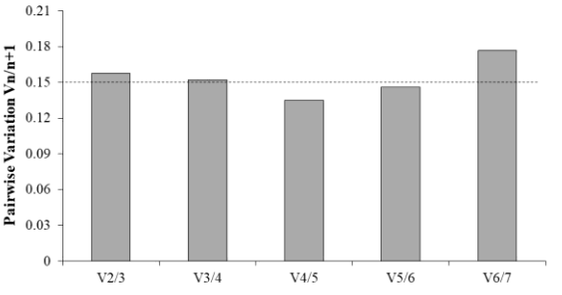


**Fig. 4.** Pairwise variation (Vn/n+1) was calculated with geNorm. The analysis determined the optimum number of reference genes required for accurate normalization of target gene expression. Vn/n+1 values lower than the cut-off value (0.15) indicated the addition of additional reference genes would not yield a significant improvement of normalization.

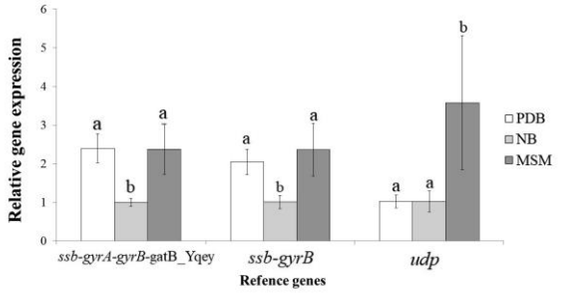


**Fig. 5.** Relative expression levels of the *ftsZ* gene of strain 1021 grown in PDB, NB and MSM, when normalized against different reference genes. Different letters indicate statistically significant differences ($P < 005$) between media with the same reference genes.

NB (1.00 + 0.10) was significantly lower. A similar result was observed with the normalization against the combination of two genes (*ssb-gyrB*) that was suggested by Normfinder. In contrast, normalization against *udp*, the least-stably expressed gene, resulted in a different expression pattern. *ftsZ* expression in MSM (3.58 + 1.73) was overestimated, while an underestimation of *ftsZ* expression was observed in PDB (1.02 + 0.16). *ftsZ* expression in PDB was insignificantly different from that in NB.

## DISCUSSION

RT-qPCR is an important tool for studying gene expression and regulation. Reliable and stably expressed reference genes are crucial for an accurate estimation of target gene expression levels. The MIQE guideline suggested all reference genes used in an expression analysis must be validated for their expression stability in the experimental design (5). Previously, the expression stability of eleven genes were determined in *Bacillus cereus* ATCC 14579 and *Bacillus thuringiensis* subsp. *konkukian* 97-27. Based on geNorm and Normfinder programs, the study reported that the gatB_Yqey gene could be used as a single reference gene for normalization of gene expression throughout the bacterial life cycle in brain heart infusion medium (27). In contrast, another study of *B. subtilis* used the 16S rRNA gene as the reference gene for quantifying expression of genes involved in bacterial adaptation to phosphate-limiting conditions (28). However, a survey on the use of 16S rRNA as the reference gene indicated its poor expression stability in many validation studies of various bacterial species (29). The survey also found that genes belonging to DNA replication were experimentally validated in a higher proportion of previous studies (29). Additionally, up to the time of the preparation of this manuscript, no reference genes were not yet experimentally validated in other *B. siamensis* strains. Thus, for gene expression analysis of *B. siamensis* 1021, we aimed to examine the expression stability of various house-keeping genes that were involved in DNA replication (*ssb, gyrA, gyrB, dnaB*) and other cellular processes (*rpsU*, gatB_Yqey, *udp*).

Based on the expression stability levels obtained from geNorm and Normfinder, we found that the *ssb, gyrA, gyrB* and gatB_Yqey genes were the four most stably expressed genes. The V4/5 variation level (0.13) was below the cut-off level (7). It also suggested four genes were required for the normalization of target gene expression in strain 1021. The *udp* gene was the least stably expressed in this study, based on geNorm and Normfinder. In contrast, *udp* was the second stably expressed gene in *B. cereus* ATCC 14579 and *B. thuringiensis* subsp. konkukian 97-27 at various time point of their growth in brain heart infusion medium (27). This distinction emphasized the significance of reference gene analysis prior to a gene expression study.

Strain 1021 grew differently in PDB, NB and MSM. A higher growth was observed in PDB and MSM, compared to that in NB medium, after 24 hours post inoculation. This was rather counter-intuitive, since NB is often used for growing bacteria. However, several previous studies used PDB for growing strains

of *B. pumilus*, *Bacillus natto* and *B. subtilis* for bioactive compound production (30-32). MSM was also included in this study because it was used for the optimization of secondary metabolite production by *B. siamensis* SCSIO 05746 (17). FtsZ is an initiating protein for the formation of the Z ring at the site of cell division in bacteria and directly involved in bacterial growth (33). In *Escherichia coli*, its expression oscillated during the cell cycle and reached the maximum during DNA replication (33). Thus, this gene was selected as the target gene for validation of the selected reference genes.

Based on the pairwise variation analysis by geNorm, the combination of *ssb*, *gyrA*, *gyrB* and gatB_Yqey were tested for the normalization of *ftsZ* expression. Based on this strategy, *ftsZ* expression was considerably higher in PDB and MSM, compared to NB. The normalization against the combination of two reference genes suggested by Normfinder (*ssb*-*gyrB*) also yielded a similar result. This *ftsZ* expression pattern positively correlated with strain 1021 growth in PDB, NB and MSM. It also suggested that the combination of the four reference genes was a suitable normalization strategy in this experimental setting. This was supported by previous studies which showed that the level of *ftsZ* expression changed during cell cycle. In *Prochlorococcus*, *ftsZ* mRNA level reached the maximum during DNA replication. Its protein level also peaked at the time of cell division (34). Another study demonstrated the growth inhibitory effect of bacilysin, obtained from *Bacillus amyloliquefaciens* FZB42, on *Microcystis aeruginosa* (35). It showed that *ftsZ* transcription in *M. aeruginosa* was reduced during the prolonged treatment of bacilysin. Our expression analysis suggested PDB and MSM had a stimulating effect on both strain 1021 growth and its *ftsZ* expression. It also supported our proposal of the combinations of the reference genes *ssb*, *gyrA*, *gyrB* and gatB_Yqey for normalization of target gene expression in this strain. These reference genes were potential candidates for future gene expression analysis in strain 1021 and other *B. siamensis* strains.

## CONCLUSION

Seven reference genes were tested for their expression stability in *Bacillus siamensis* 1021, grown in PDB, NB and MSM. The combination of *ssb*, *gyrA*, *gyrB* and gatB_Yqey was found as the best candidate for the normalization strategy in this experimental design. This was supported by the correlation between strain 1021 growth and *ftsZ* expression in these media. In contrast, we found that the use of the least stably expressed gene *udp* resulted in a different pattern of *ftsZ* expression level. Our study indicated the significance of reference gene analysis and provided the candidates for further gene expression studies in *B. siamensis* 1021.

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