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Assay and characterization of a strong promoter element from *B. subtilis*

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Abstract

A new strong promoter fragment isolated from *Bacillus subtilis* was identified and characterized. Using the heat stable β-galactosidase as reporter, the promoter fragment exhibited high expression strength both in *Escherichia coli* and *B. subtilis*. The typical prokaryotic promoter conservation regions were found in the promoter fragment and the putative promoter was identified as the control element of *yxiE* gene via sequencing assay and predication of promoter. To further verify and characterize the cloned strong promoter, the putative promoter was sub-cloned and the β-Gal directed by the promoters was high-level expressed both in *E. coli* and *B. subtilis*. By means of the isolated promoter, an efficient expression system was developed in *B. subtilis* and the benefit and usefulness was demonstrated through expression of three heterologous and homogenous proteins. Thus, we identified a newly strong promoter of *B. subtilis* and provided a robust expression system for genetic engineering of *B. subtilis*.

Keywords: *Bacillus subtilis*; Expression vector; Heat stable β-galactosidase; Strong promoter; Sub-clone

Bacillus subtilis is a non-pathogenic Gram-positive bacterium and is generally regarded as safe organism (GRAS). *B. subtilis* has long been exploited for industrial and biotechnological application [1,2]. With the completion of sequencing of the *B. subtilis* genome, post-genomic studies were stimulated. Many coding gene were gradually identified and recognition of regulation machinery and element was enhanced in *B. subtilis* [3]. Undoubtedly, all these knowledge accelerated the biotechnological application of *B. subtilis* in industry field, of which the regulation element, promoter, played an important role in genetic engineering of *B. subtilis*, i.e. control element of expression vector [4].

In genetic engineering of *B. subtilis*, plasmid backbone and promoter are two basic elements. Plasmid instability was once one barrier in genetic manipulation [5], but recently many a convenient vector systems of *B. subtilis* have been developed for genetic manipulation [4,6,7]. This further makes the *B. subtilis* a potential bacillus in genetic engineering and industrial application.

A lot of information of *B. subtilis* promoter has been acquired and several of them has been exploited to be used as control element in the construction of expression vector in *B. subtilis* [8–10], of which some constitutive promoters, such as P43 promoter and phage promoter, were widely investigated and characterized. Amongst the P43 promoter was used as a common control element in the construction of expression vector in *B. subtilis* and was considered as a strong promoter [10]. It is in the interest of the biotechnological application and industry to seek new strong promoters. Here, a promoter isolated from *B. subtilis*

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exhibited high expression for reporter gene both in *Escherichia coli* and *B. subtilis*, and demonstrated higher expression strength than P43 promoter.

By using of promoter trapping vector to clone promoter fragment from the bacteria genome DNA is a frequently approach [10,11], of which the β-galactosidase (β-Gal) was one of usually used reporters [12]. In this study, a promoter fragment isolated from *B. subtilis* by using β-Gal as reporter demonstrated strong promoter activity both in *E. coli* and *B. subtilis*. The promoter fragment was sequenced and the putative promoter was predicted. The typical prokaryotic promoter conservation region was found. Sub-cloned promoter from the fragment further verified the putative promoter and used it to construct expression vector in *B. subtilis*.

Materials and methods

Strains, plasmids, and growth conditions. *Bacillus subtilis* 1A747 was kindly provided by the Bacillus Genetic Stock Center. *E. coli* DH5α was purchased from Novagen (Darmstadt, Germany). The plasmids used in this study were listed in Table 1. All strains were incubated at 37 °C in Luria–Bertani (LB) medium. When required, medium for *E. coli* and *B. subtilis* were supplemented with 50 μg/mL spectinomycin, 100 μg/mL ampicillin or 5 μg/mL chloromycetin, respectively.

General DNA manipulation. The isolation and manipulation of recombinant DNA was performed using standard techniques [13]. All enzymes were commercial preparations, and used as recommended by the manufacturers (TOYOBO, Japan).

Transformation of E. coli and *B. subtilis*. Transformation of *E. coli* cells was performed as previously described [13]. *B. subtilis* cells were transformed by electroporation [4,14].

Construction of plasmids. Using pSI3423 as template, the 320-bp and 313-bp promoter fragments were amplified through polymerase chain reaction (PCR) with the two primer pairs, P3-up/P3-down (5′-TTGGGCCCATTTAATTGAAG-3′; 5′-GCGAATTCGCTCTTCCCG-3′) and P3-up (N)/P3-down (N) (5′-TTGGGCCCATCATTTAATTGAAGC-3′; 5′-TTGAATTCCCGCCTTT CGGACTG-3′), respectively. The restriction sites *Apa*I and *Eco*RI were introduced into the amplified fragments by the primers (underline). And then the two fragments were cloned into pGEM-T vector, yielding pGJT-78 and pGJT-436. Excised from the pGJT-78 and pGJT-436 with *Apa*I and *Eco*RI, the two promoter fragments were cloned into the corresponding sites of pLJ-2, respectively, resulting in pYG78 and pGJ436. The promoter excised from pGJT-78 with *Apa*I and *Eco*RI, cloned into the corresponding sites of pGJ103, resulting in expression vector pYG123. To compare the efficiency of the expression system to that of P43 system, the *bgaB* gene was excised from pLJ-2 with *Eco*RI and *Sac*I, and then cloned into the downstream of P43 in pGJ288, respectively, resulting in pGJ-*bgaB*. Using BI1 (5′-TTGAATTCGTGACAATTGCATCGTC-3′) and BI2 (5′-TTGGATCCCTTATTCAAAAGTCACCG-3′) as primers, the *bioI* was PCR amplified from *B. subtilis* 1A747 chromosome DNA. Digested by the *Eco*RI and *Bam*HI, the amplified fragment was cloned into the pYG123, resulting in pYGI. The *vgb* gene was excised from pGJ203 with *Eco*RI and *Sac*I and cloned into the corresponding sites of pYG123, resulting in pYG-*vgb*. To demonstrate the expression difference between the system constructed in this study and P43 promoter system, the *Eco*RI- *Sac*I-treated *bioI* gene and *vgb* gene were cloned into the corresponding sites of pGJ288, respectively, yielding pGJ-*bioI* and pGJ-*vgb*.

β-Gal activity assay. The β-Gal activity assay was carried out as previously described [15,16]. Samples were prepared at different cultured time and β-Gal activity was measured. The activity was given as Miller units per mL sample (Miller U/mL).

SDS–PAGE assay. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of the β-Gal was performed as described previously [13]. The protein fractions were analyzed on SDS/12% polyacrylamide gels that were stained with Coomassie brilliant blue R-250.

Promoter sequence predication and analysis. The promoter fragments were sequenced by Aoke Corp. (Beijing, China). The sequence analysis was performed online with NCBI blast (http://www.ncbi.nih.gov), and promoters were predicted online by using of softberry (http://www.softberry.com).

Results and discussion

Characterization of strong promoter isolated from B. subtilis

In our previous work, the *B. subtilis* promoter library was created using the promoter trapping vector with *bgaB* [16] as reporter gene, of which the DNA fragments from *B. subtilis* genome DNA digested by *sau*3AI were inserted into the *Bam*HI site upstream of β-Gal coding gene. The library was screened and obtained when the appropriate promoters had been inserted upstream of the promoterless β-Gal gene,

Table 1
Plasmids used in this study

Plasmid	Relevant characteristics	Reference or source
pLJ-2	Cm^R, promoter-less *bgaB*	Lab stock
pShuttleI	Cm^R, promoter-less *bgaB* (with its native RBS)	Lab stock
pSI3423	The cloned promoter upstream the *bgaB*	Lab stock
pGJT-78	pGEMT-vector with the sub-cloned promoter	This work
pGJT-436	pGEMT-vector with the sub-cloned promoter	This work
pYJ78	pLJ-2 with the sub-cloned promoter	This work
pGJ436	pLJ-2 with the sub-cloned promoter	This work
pGJ103	*E. coli–B. subtilis* shuttle vector	[4]
pYG123	*B. subtilis* expression vector directed by PyxiE	This work
pGJ288	pGJ103 with P43 promoter between *Apa*I and *Eco*RI	Lab stock
pGJ-*bgaB*	pGJ288 with *bgaB* downstream P43	This work
pYGI	*bioI* under control of PyxiE in pYG123	This work
pGJ203	*vgb* gene donor plasmid	[4]
pYG-*vgb*	Expression vector of *vgb* gene directed by PyxiE	This work
pGJ-*bioI*	Expression vector of *bioI* gene directed by P43	This work
pGJ-*vgb*	Expression vector of *vgb* gene directed by P43	This work

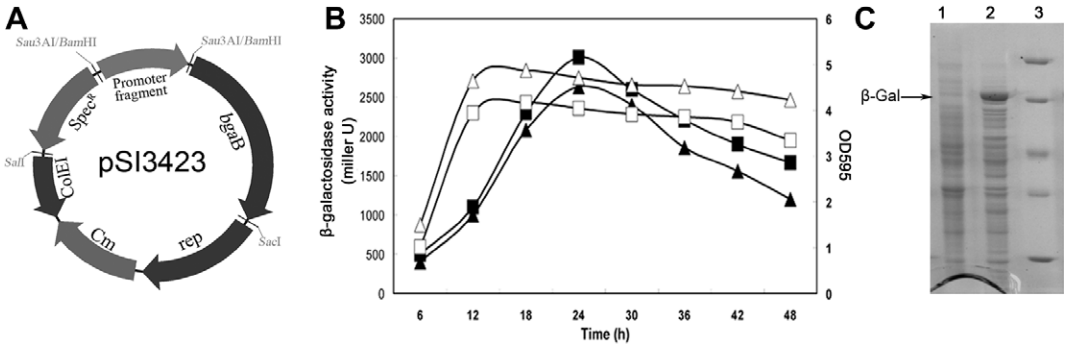

Fig. 1. (A) Relevant features of pSI3423. The *bgaB* represents β-Gal encoding region; ColE1, *E. coli* ColE1 replicon; Cm, chloramphenicol-resistance marker; Spec^R, spectinomycin-resistance marker; Rep, replicon in *B. subtilis*; promoter fragment, inserted promoter-activity fragment P3423 from *B. subtilis* chromosome DNA through partial digestion with *Sau*3AI; The unique restriction enzyme sites are marketed on the outside of the map. (B) β-Gal production from pSI3423 both in *E. coli* and *B. subtilis*. Solid square (■) and solid triangle (▲) represent β-Gal production from pSI3423 in *E. coli* and *B. subtilis*, respectively; hollow square (□) and hollow triangle (△) represent the growth curves of pSI3423 in *E. coli* and *B. subtilis*. (C) SDS–PAGE analysis of β-Gal from pSI3423. Lane 1, crude extract of *B. subtilis* 1A747 harboring pShuttleI harvested at 24 h (this served as a negative control); lane 2, crude extract of *B. subtilis* 1A747 harboring pSI3423 harvested at 24 h; lane 3, molecular mass markers (top to bottom: 116, 66, 45, 35, and 25 kDa). β-Gal is indicated by the arrow.

from which one recombinant, pSI3423 (Fig. 1A), demonstrated high expression strength for β-Gal. For further characterizing the cloned promoter, the β-Gal production driven by the cloned promoter fragment, P3423, was quantitative determination both in *E. coli* and *B. subtilis* (Fig. 1B) in this study. The *B. subtilis* 1A747 harbouring pShuttleI was used as the negative control, and the β-Gal activity from *B. subtilis* 1A747 harbouring pShuttleI was not detected both in *E. coli* and *B. subtilis*. The β-Gal driven by the P3423 reached maximum at 24 h in *E. coli* and *B. subtilis* (3417 and 2877 Miller U/mL, respectively). To compare the strength between the cloned promoter fragment in this study and commonly used strong promoter in *B. subtilis*, the β-Gal production driven by P43 promoter [4] was investigated. The production of β-Gal driven by the isolated promoter fragment was 2 times of that by P43 promoter at 24 h (1457 Miller U/mL). Thus, this suggested, we clone a strong promoter fragment from *B. subtilis*. The SDS–PAGE analysis of the β-Gal from the pSI3423 in *B. subtilis* revealed a distinct band with a molecular mass of approximately 70 kDa (Fig. 1C, lane 2) that was accordance with calculated molecular weight of β-Gal, further confirming that the β-Gal were successfully expressed under the control of P3423.

Sequencing and analysis of the cloned promoter fragments

To insight into the cloned promoter fragment, P3423 was analyzed by means of restriction enzyme digestion first. The result revealed that the inserted fragment upstream of the reporter gene, *bgaB*, was about 600 bp in the recombinant pSI3423. Subsequently, sequencing of cloned promoter fragment confirmed that the inserted fragment is 672 bp long in pSI3423, and *bgaB* gene was located at downstream of this fragment.

Sequence analysis showed that the inserted P3423 in pSI3423 contained three fragments (Fig. 2A) corresponding to the genome DNA of *B. subtilis* 168 (NC_ 000964.2), i.e. 3′ end partial sequence of *bglH* coding gene, 5′ end partial of *yxiE* coding gene with its non-coding region (1–368 bp) and partial internal region of *gltB* coding gene (365–473 bp) as well as *pgk* gene (468–668 bp), in which the probably control element of *yxiE* was included in P3423.

Online prediction of promoter indicated that the P3423 contained prokaryotic promoter conservation regions with high possibility score, in which the putative promoter located at upstream of *yxiE* gene. The space between the classic −35 box (TTGACA) and −10 box (AATAA) is 20 bp and a typical Shine–Dalgarno (SD) sequence (GGCGG) was found 7 bp upstream the start codon (ATG) of *yxiE* gene (Fig. 2B). Thus, this suggested the promoter cloned in this study is the control element of *yxiE* gene and *bgaB* was driven by the promoter in the pSI3423. There has been no report about the control element of *yxiE* gene so far.

To experimentally confirm the cloned promoter, the promoter of *yxiE* (P*yxiE*) was sub-cloned and cloned into the promoter probe vector to examine its function and efficiency. The *bgaB* gene was under the control of P*yxiE* in resultant recombinant pGJ436. Surprisingly, the β-Gal production from pGJ436 both in *E. coli* and *B. subtilis* were rather low (310 Miller U/mL in *E. coli* and 220 Miller U/mL in *B. subtilis*, respectively). Obviously, this result exists dramatic difference with that as expected. To finding out the reason, the sequence of express cassette in pGJ436 was checked. To maintain the space of 7 bp between SD of P*yxiE* and start condon of *bgaB* in pGJ436, the downstream of SD was designed to be replaced by the sequence of *Eco*RI site. This probably destroyed the function of ribosome binding site (RBS). Thus, we constructed the pYG78, in which the SD sequence and seven nucleotides downstream the SD of P*yxiE* was maintained and located upstream of *bgaB*. Though there are 13 bp space between the SD and the start codon of *bgaB* (7 bp of downstream the SD plus 6 bp of the *Eco*RI site), the β-Gal production

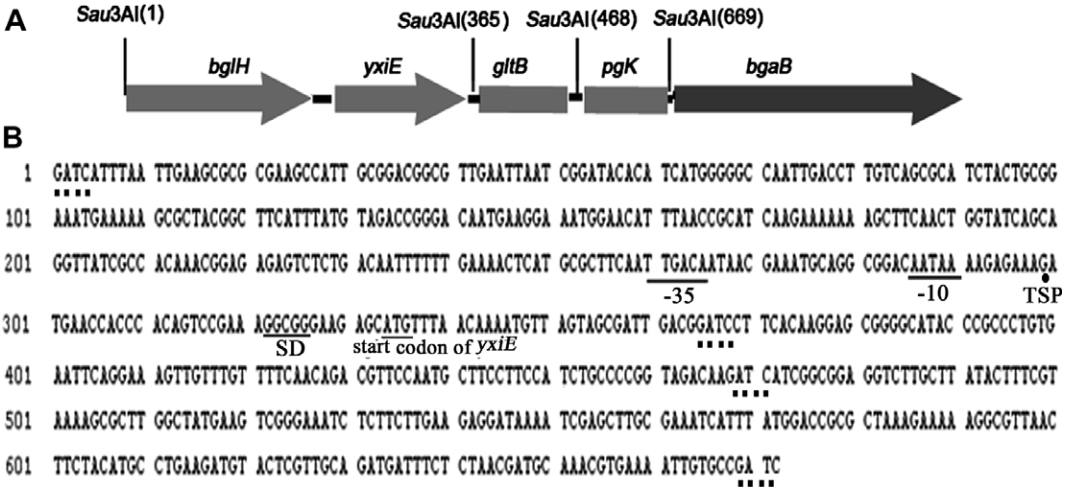

Fig. 2. (A) Map of inserted promoter activity fragment upstream the *bgaB* of pSI3423. *bglH*, 3′ partial fragment of *bglH* coding gene; *yxiE*, 5′ partial fragment of *yxiE* coding gene; *gltB* and *pgK*, internal partial fragment of *gltB* and *pgK* coding gene, respectively; *bgaB*, coding gene for β-Gal. The restriction site *Sau*3AI and the location in the inserted fragment are indicated. (B) Sequence of the inserted fragment P3423. The conservation region of promoter is indicated by underlined (−10, GACAAT; −35, TTGACA). TSP represents the transcription start point; SD, Shine–Dalgarno box; the start codon of *yxiE* gene was indicated and the dots indicate the sequence of *Sau*3AI.

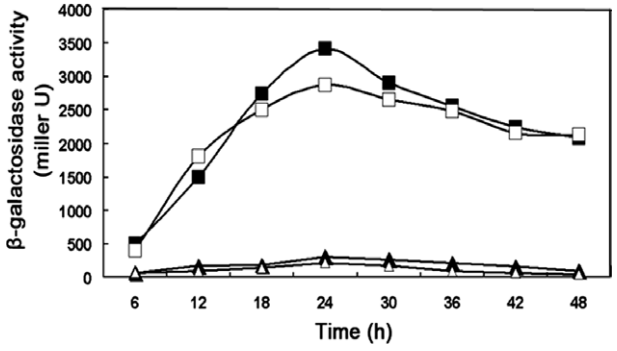

Fig. 3. Production of β-Gal from pYG78 and pGJ436 both in *E. coli* and *B. subtilis*. Solid square (■) and hollow square (□) represent β-Gal production from pYG78 in *E. coli* and *B. subtilis*, respectively; solid triangle (▲) and hollow triangle (Δ) represent β-Gal production from pGJ436 in *E. coli* and *B. subtilis*, respectively.

from pGJ78 (Fig. 3) demonstrated similar trend with the original plasmid pSI3423 both in *E. coli* and *B. subtilis* (the β-Gal production from pSI3423 seen 1st section of results and discussion), in which the β-Gal production from pYG78 reached 3009 Miller U/mL and 2630 Miller U/mL in *E. coli* and *B. subtilis* at 24 h, respectively.

Thus, this validated the cloned promoter P*yxiE* and, meanwhile confirmed our speculation that the disorder of RBS in pGJ436 caused the low-level expression of *bgaB*. Since this newly isolated and characterized promoter demonstrated high expression efficiency, the P*yxiE* provided a potential control element in expression system of *B. subtilis*.

Construction of expression vector directed by the cloned promoter

Using the characterized promoter P*yxiE* in this study and the *E. coli–B. subtilis* shuttle vector pGJ103 [4], the expression vector of *B. subtilis*, pYG123, was constructed (Fig. 4A). In pYG123, the P*yxiE* was used as control element and several common unique restriction sites allowed target gene to be cloned conveniently into the downstream of the promoter in *E. coli* and expressed in *B. subtilis*.

To facilitate a rigorous comparison between the expression system directed by P*yxiE* and commonly used strong promoter system, the expression of *bgaB* gene driven by P43 and P*yxiE*, respectively, was characterized in *B. subtilis*. Table 2 demonstrated that the production of β-Gal from *B. subtilis* harbouring pYG78 and *B. subtilis* harbouring pGJ-*bgaB* driven by P*yxiE* and P43 promoter, respectively, both reached maximum at 24 h in *B. subtilis*. While, the β-Gal production directed by P*yxiE* was far higher than that by P43 (2729 Miller U/mL vs 1426 Miller U/mL). SDS–PAGE assay showed that there are the distinct target protein (β-Gal) bands at about 70 kDa for crude protein from the P*yxiE* and P43 system (Fig. 4B). The difference of amount of β-Gal bands from SDS–PAGE was visual (Fig. 4B, lanes 2 and 3 vs lanes 4 and 5) and accordance with the above-mentioned result.

To further validate the expression system developed in this study, the *bioI* gene involved in biotin biosynthesis of *B. subtilis* [17,18] and *vgb* gene coding for coding for *Vitreoscilla* haemoglobin [19] were expressed by this strong promoter system in *B. subtilis*. Fig. 4C and 4D demonstrated both genes were soluble expressed driven by P*yxiE* and P43 system as expected. Furthermore, the expression levels of two genes (*bioI* and *vgb*) directed by P*yxiE* system were higher visually than that by P43 system (Fig. 4C, lane 1 vs lane 2; Fig. 4D, lane 1 vs lane 2).

Promoters are important regulatory elements in the genome. They control the spatial and temporal expression of genes and their expression strength [6,7]. Meanwhile, strong promoter played an important role in biotechnological application and industry. We characterized a new

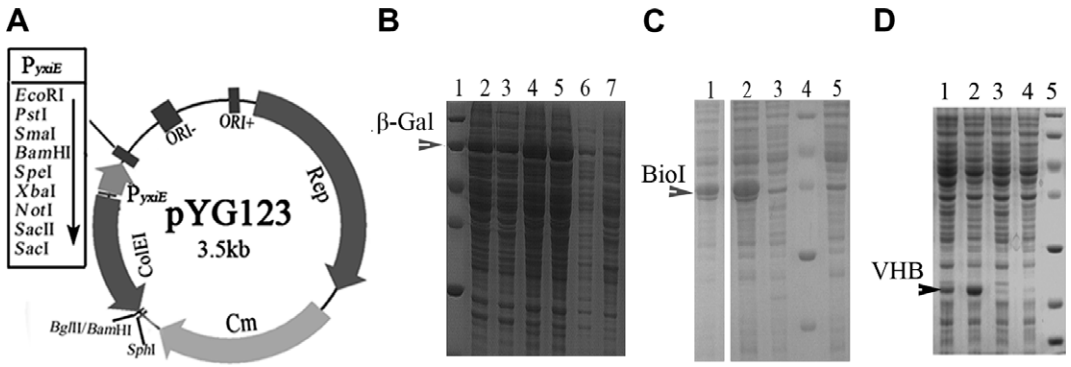

Fig. 4. (A) Map of expression vector pYG123. ColE1, *E. coli* ColE1 replicon; Cm, chloramphenicol-resistance marker; Rep, replicon in *B. subtilis*; P*yxiE*, promoter of *yxiE* gene. The unique restriction enzyme sites are marked on the outside of the map. (B) SDS–PAGE analysis of β-Gal production driven by the P*yxiE* system and P43 system in *B. subtilis* harvested after 24 h cultured. Lane 1, molecular mass markers (top to bottom: 116, 66, 45, 35, and 25 kDa); lanes 2 and 3, β-Gal production from P43 system; lanes 4 and 5, β-Gal production from P*yxiE* system; lane 6, crude extract of *B. subtilis* 1A747 harboring pGJ288 (this served as a negative control); lane 7, crude extract of *B. subtilis* 1A747 harboring pYG123 (this served as a negative control). (C) SDS–PAGE analysis of expression *bioI* gene directed by the P*yxiE* system and P43 system in *B. subtilis* at 24 h. Lanes 1 and 2, the expression of *bioI* gene driven by the P43 system (pGJ-*bioI*) and P*yxiE* system (pYGI), respectively; lanes 3 and 5, the crude extract of *B. subtilis* 1A747 harboring pGJ288 and pYG123, respectively (this served as a negative control); lane 4, molecular mass markers (top to bottom: 116, 66, 45, 35, and 25 kDa). (D) SDS–PAGE analysis of expression *vgb* gene directed by the P*yxiE* system and P43 system in *B. subtilis* at 24 h. Lanes 1 and 2, the expression of *vgb* directed by P43 system (pGJ-*vgb*) and P*yxiE* system (pYG-*vgb*), respectively; lanes 3 and 4, the crude extract of *B. subtilis* 1A747 harboring pGJ288 and pYG123, respectively (this served as a negative control); lane 5, molecular mass markers (top to bottom: 116, 66, 45, 35, 25, 18, and 14 kDa).

Table 2
β-Gal production driven by P*yxiE* system and P43 system

Strains	β-Gal activity (Miller U/mL)							
	6 h	12 h	18 h	24 h	30 h	36 h	42 h	48 h
1A747 (pYG78)	400	1800	431	729	621	362	160	130
1A747 (pGJ-*bgaB*)	259	853	1181	1426	1267	1164	1026	957
1A747 (pYG123)	ND	ND	3	11	7	ND	ND	ND
1A747 (pGJ288)	ND	ND	ND	5	3	ND	ND	ND

ND, no detected.

constitutive promoter isolated from *B. subtilis* and this promoter demonstrated high strength compared to the common strong promoter P43. Through sequencing and analysis of promoter, the isolated promoter was identified as the control element of *yxiE* gene of *B. subtilis*. The isolated promoter is a potential control element for expression system in *B. subtilis*. The expression system directed by the P*yxiE* was constructed and characterized. Using *bgaB* gene as reporter, the benefits of the system was demonstrated through strict comparison to the P43 promoter system in *B. subtilis*. The efficiency and usefulness of the system developed in this study was further validated through high-level expression of other two heterologous and homologous proteins in *B. subtilis*. In conclusion, we characterized a strong promoter-activity fragment newly isolated from *B. subtilis*, and the putative promoter was identified as the control element of *yxiE* gene. Since the P*yxiE* demonstrated high expression activity, the expression system directed by P*yxiE* was constructed in *B. subtilis*. Furthermore, three proteins were successfully expressed directed by this system in *B. subtilis* to demonstrate the effectiveness of the P*yxiE* promoter system. Thus, we characterized and identified a new strong promoter of *B. subtilis*, and provided a efficient expression system in *B. subtilis*.

Acknowledgments

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