Generation of an Artificial Double Promoter for Protein Expression in *Bacillus subtilis* through a Promoter Trap System

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### Abstract

*Bacillus subtilis* is an attractive host for production of recombinant proteins. Promoters and expression plasmid backbones have direct impacts on the efficiency of gene expression. To screen and isolate strong promoters, a promoter trap vector pShuttleF was developed in this study. Using the vector, approximately 1000 colonies containing likely promoters from *Bacillus licheniformis* genomic DNA were obtained. Amongst them, pShuttle-09 exhibited the highest β-Gal activities in both *Escherichia coli* and *B. subtilis*. The activity of pShuttle-09 in *B. subtilis* was eight times that of the P43 promoter, a commonly used strong promoter for *B. subtilis*. A sequence analysis showed that pShuttle-09 contained P<sub>luxS</sub> and truncated luxS in-frame fused with the reporter gene as well as another fragment upstream of P<sub>luxS</sub> containing a putative promoter. This putative promoter was a hybrid promoter and its β-Gal activity was higher than P<sub>luxS</sub>. Reconstructing the hybrid promoter from pShuttle-09 to P<sub>luxS</sub> further improved the β-Gal production by 60%. The usefulness of our promoter trap system is likely due to random shuffling and recombination of DNA fragments and adoption of a rapid and high-throughput screening. Thus, our data provide additional evidence to support the concept of using a promoter trap system to create new promoters.

### Citation

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### Introduction

*Bacillus subtilis* is a non-pathogenic gram-positive bacterium and has been an attractive host for production of recombinant proteins from both prokaryotic and eukaryotic origins [1], [2], [3]. The suitability and popularity of *B. subtilis* as one of main hosts for recombinant protein production is due to several reasons: generally recognized as safe as a result of its lack of pathogenicity and the absence of endotoxins; capable of secreting functional proteins directly into culture media, easy for genetic manipulation and handling, and capable of large-scale fermentation [2], [4], [5], [6].

*B. subtilis* own proteins can be over expressed in *B. subtilis* in the order of several grams per liter [3]. However, secretion of heterologous proteins in *B. subtilis* is usually low [7]. Thus, efficient production of high-value recombinant proteins in *B. subtilis* remains a major challenge. To circumvent the problem, strong promoters, a variety of translation/secretion signals, and transcription terminators are continuously being explored. In particular, several strong promoters have been reported [8], [9], [10], [11], [12], [13], [14], [15]. In spite of the progress, it is still beneficial to find even more potent promoters.

Promoters are important regulatory elements in a genome and control spatial and temporal expression of genes and their expression strength. In bacteria, a promoter is recognized by RNA polymerases and associated sigma factors, which may be recruited to the promoter by regulatory proteins binding to specific sites in the promoter. There are several ways to find new promoters. The advent of DNA sequencing has significantly accelerated sequencing of many bacterial genomes and prediction of promoters. However, it is not yet possible to scan a bacterial genome sequence and readily predict the expression behavior of genes belonging to a regulon, largely because an accurate prediction of promoters is difficult [16]. Alternatively, a promoter trap vector, which is a plasmid containing a multiple cloning site at the 5’ end of a promoter-less marker gene, can be used to identify promoters [17], [18], [19]. An unidentified DNA fragment is cloned into the multiple cloning site and expression of the marker gene is monitored to identify active promoter elements in the unidentified DNA fragment. Promoter trap systems have been successfully used to identify promoters for *B. subtilis* either from *B. subtilis* itself or from other bacillus species [20], [21], [22], [23].

A promoter trap system could perform shuffling and recombination of DNA fragments and screen of strong promoters simultaneously. However, this has never being reported. Fortunately, when a promoter trap system was used in this study to screen Sau3A I cut fragments from *B. licheniformis* genomic DNA for potential promoters, a strong double promoter was identified, containing a normal promoter and a hybrid promoter due to shuffling and recombination. The putative double promoter was further improved and used for protein expression both in *E. coli*
Our results show that promoter trap systems could be suitable for finding novel strong promoters.

**Materials and Methods**

**Bacterial strains, plasmids and growth conditions**

*B. subtilis* 1A747 (*B. subtilis* PY79) was a gift from Bacillus Genetic Stock Centre of the Ohio State University. *B. pumilus* BYG, *B. licheniformis* A061, *B. amyloliquefaciens* and *B. megaterium* W-5 were isolated by our laboratory. The bacterial strains were isolated from effluent of a paper mill in Hubei province of China and partial 16S rDNA sequencing was used for identification of species in the genus Bacillus. The plasmids used in this study are listed in Table 1. *E. coli* DH5α was purchased from Novagen (Darmstadt, Germany). The bacterial strains were cultured in the Luria-Bertani (LB) medium at 37°C. The following concentrations of antibiotics were used for selection: 100 μg/mL ampicillin (Amp), 5 μg/mL chloramphenicol (Cm) and 50 μg/mL spectinomycin (Spec).

**PCR and DNA manipulation**

Polymerase chain reaction (PCR) primers and oligonucleotides used in this study are listed in Table 2. Isolation and manipulation of recombinant DNA was performed using standard techniques [24]. All enzymes were from Promega China (Beijing, China) and used as recommended by the manufacturer. The transformation of *E. coli* and *B. subtilis* was performed by electroporation [24], [25].

**Construction of a promoter trap vector**

Using the primer pair bgaB-1 and bgaB-2, the *bgaB* coding region was PCR amplified from the plasmid pDL [6]. The amplified fragment was flanked by BamH I and Sac I at 5' and 3' ends, respectively. The promoter trap vector was assembled as follows. Firstly, the obtained *bgaB* digested by BamH I and Sac I was cloned into corresponding sites of pGJ103, yielding pGJ-Bga. Then, the *Apa I*-BamH I-treated spectinomycin resistance gene, which was PCR amplified from the pDG1728 [26] using the primer pair spec-1 and spec-2, was inserted into corresponding sites of pGJ-Bga, resulting in a promoter trap vector pShuttleF.

**Cloning of promoter fragments**

The genomic DNA of *B. licheniformis* was partially digested with Sau3A I, and then ligated with the promoter trap vector pShuttleF treated with the BamH I and alkaline phosphatase. The ligation mixture was transformed into *E. coli* DH5α and recombinants harbouring promoter fragments were screened on the LB solid medium supplemented with X-Gal (20 μg/mL).

**Sequencing and analysis**

Promoters were sequenced by AuGCT Biotechnology (Beijing, China). The sequence analysis was performed online with NCBI blast 2.0 (www.ebi.ac.uk); promoter region was predicted by the BPROM program (Softberry Inc., Mount Kisco, NY, USA; http://linux1.softberry.com).

### Table 1. Plasmids used in this study.

| Plasmids | Relevant characteristics | Sources |
|----------|--------------------------|---------|
| pGJ103   | *E. coli*- *B. subtilis* shuttle vector | [6] |
| pDL      | *bgaB* gene donor        | [6] |
| pDG1728  | specR gene donor         | [27] |
| pShuttleF| Promoter-trapping vector | This study |
| pB43     | P43 promoter donor       | This study |
| pShuttle-09| Recombinant cloned promoter fragment | This study |
| pShuttle-P43| pShuttleF inserted P43 Promoter | This study |
| pShuttle-luxi | *bgaB* directed by PluxS of *B. licheniformis* in shuttle vector | This study |
| pShuttle-luxS | *bgaB* directed by PluxS of *B. subtilis* in shuttle vector | This study |
| pShuttle-luxP | *bgaB* directed by PluxS of *B. pumilus* in shuttle vector | This study |
| pShuttle-luxM | *bgaB* directed by PluxS of *B. megaterium* in shuttle vector | This study |
| pShuttle-Hyb | *bgaB* directed by hybrid promoter from pShuttle-09 | This study |
| pShuttle-Fus1 | pShuttleF harbouring cloned promoter without 5’end of luxS CDS | This study |
| pShuttle-PLapS | pShuttle-F harbouring combined promoter P<sub>LapS</sub> | This study |
| pShuttle-PLapP | pShuttle-F harbouring combined promoter P<sub>LapP</sub> | This study |
| pShuttle-PLapL | pShuttle-F harbouring combined promoter P<sub>LapL</sub> | This study |
| pLus-Hyb | Expression vector based on P<sub>LapS</sub> | This study |
| pLus-His | Expression vector for expression purification | This study |
| pLu-bga | pLux-Hyb harbouring *bgaB* | This study |
| pLuHis-bga | pLux-His harbouring *bgaB* | This study |
| pLu-biol | pLux-Hyb harbouring *biol* | This study |
| pLuHis-biol | pLux-His harbouring *biol* | This study |

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Sub-cloning of promoter regions

Using the primer pair P43-1/P43-2, the P43 promoter was PCR amplified from plasmid pB43. The resultant promoter fragment was digested with Apa I and BamH I, and cloned into corresponding sites of pShuttleF, resulting in pShuttle-P43. By means of primer pairs luxS-up/luxS-down, luxP-up/luxP-down and luxM-up/luxM-down, three luxS promoter fragments were cloned from genomic DNA of B. subtilis 1A747, B. pumilus BYG and B. megaterium W-5, respectively. The corresponding amplicons were then cloned into pShuttleF digested with Apa I and BamH I, generating pShuttle-luxS, pShuttle-luxP and pShuttle-luxM. Using insert-1/luxL-down as primers, the P\textsubscript{luxS-i} was amplified from pShuttle-09 and cloned into pShuttleF, resulting in pShuttle-luxi. The bgaB contained Shine–Dalgarno box (SD) was PCR amplified
from pDL by using bgaB-fus and bgaB-2 and cloned into pshuttleF, resulting in pDET-1. Then, the hybrid promoter amplified from pShuttle-09 (using the primer pair insert-3/insert-2) was cloned into pDET-1 treated with Apa I and BamH I, resulting in pShuttle-Hyb. By using the primer pair lux-2/insert-2, an isolated promoter fragment (deletion of 5’ coding region of luxS) was amplified from pShuttle-09 and cloned into pshuttleF, yielding pShuttle-Fus1.

Reconstruction of cloned promoter

The reconstruction of promoter was carried out by the splicing by overlapping extension PCR. Four pairs of primers, apr-1/apr-2, apr-3/apr-4, apr-5/apr-6 and apr-7/apr-8, were employed to PCR amplified −35 region of promoter Papr (promoter for the alkaline protease gene) from genomic DNA of B. subtilis, B. pumilus, B. licheniformis and B. amyloliquefaciens, respectively, yielding Papr-1, Papr-2, Papr-3 and Papr-4. Four pairs of primers (insert1-up/lux-2, insert2-up/lux-2, insert3-up/lux-2 and insert4-up/lux-2) were used to amplify the Plac and −10 region of the hybrid promoter from pShuttle-09. The upstream primers were designed about 15 bp sequence homogenous with 3’ end of Papr-1, Papr-2, Papr-3 and Papr-4, respectively, in fragments luxF-1 to luxF-4. Other four pairs of primers (apr1-down/apr-2-down, apr-3/apr-4-down, apr-5/apr-6-down and apr-7/apr-8-down) were employed to amplify promoters from PaprS-1, PaprP-1, PaprL-1 and PaprA-1 respectively. The downstream primers were designed about 15 bp sequence homogenous with 5’ end of −10 region of the hybrid promoter, resulting fragment PaprS-2, PaprP-2, PaprL-2 and PaprA-2. With the mixture pairs of Papr-1/luxF-2, Papr-2/luxF-3, Papr-3/luxF-4 and Papr-4/luxF-5 as templates, there were which there were about 30 bp overlap in each mixture pair, four overlapping PCR amplifications were facilitated by means of apr1/lux-2, apr-3/lux-2, apr-5/lux-2 and apr-7/lux-2, respectively. Recombined promoters PaprS, PaprP, PaprL and PaprA were digested with Apa I and BamH I and cloned into pShuttleF, respectively, resulting in pShuttle-PaprS, pShuttle-PaprP, pShuttle-PaprL and pShuttle-PaprA.

Construction of expression vectors

Using primers apr-1 and laps-down, the promoter PaprS flanked by Apa I and EcoR I was PCR amplified from pShuttle-PaprS. The resultant promoter was digested with Apa I and EcoR I and cloned into corresponding sites of pGJ103, yielding the expression vector pLas-Hyb. To introduce 6-His tag, synthetic oligonucleotides Tag-1 and Tag-2 were annealed. The annealed fragment with adhesive ends of Xba I and Sac I at 5’ and 3’ ends respectively was inserted into the Xba I-Sac I treated pLas-Hyb, resulting in pLas-His.

Using primer pairs bgaB-3/bgaB-2 and bgaB-3/bgaB-T, two bgaB fragments were PCR amplified from pShuttleF, resulting in bgaF-1 flanked with EcoR I and Sac I and bgaF-2 with EcoR I and BamH I. bgaF-2 did not contain the termination codon. The bgaF-1 and bgaF-2 were cloned into pLas-Hyb and pLas-His, respectively, yielding pLa-bga and pLasHis-bga. Similarly, the bioI gene were PCR amplified from B. subtilis 1A747 genomic DNA by using primer pairs bioI-1/bioI-2 and bioI-1/bioI-3, resulting in bioIF-1 with EcoR I and BamH I and bioIF-2 with EcoR I and Xba I. The resultant fragments were inserted into pLas-Hyb and pLas-His, yielding pLa-bioI and pLasHis-bioI.

β-Gal activity assay

The β-Gal activity assay was carried out as previously described [27]. Samples were taken at different time points of culture and the β-Gal activity was measured. The activity was expressed as Miller units per mL sample (Miller U/mL). For each assay, three independent experiments were performed with two replicates. Statistical tests were carried out by the SPSS (Statistical Product and Service Solutions) software. Data are presented as means±S.D.

Results

Screening and isolation of the strong promoter fragments from B. licheniformis chromosomal DNA

To screen and isolate strong promoter elements, a promoter trap vector pShuttleF (Figure 1A) was constructed on an E. coli-B. subtilis shuttle vector pGJ103. The pShuttleF contained bgaB coding for a heat stable β-galactosidase (β-Gal) as a reporter gene. In addition, an alternative resistance selection marker, spectinomycin, was introduced into the pShuttleF beside the chloramphenicol resistance marker. Both spectinomycin and chloramphenolic resistance genes could confer the resistance selection either in E. coli or B. subtilis. Finally, an engineered BamH I restriction site was designed next to the start codon of bgaB in order to clone DNA fragments digested by the Sau3A I. Validation of the pShuttleF was achieved by inserting a commonly used strong promoter P43 (2, 6) in B. subtilis upstream of bgaB. The resultant pShuttle-P43 was transformed into E. coli DH5α and B. subtilis 1A747 and blue colonies on solid LB plate supplemented with X-gal were isolated. The bgaB directed by the P43 in pShuttleF was duly expressed in E. coli and B. subtilis (Figures 1B and 1C).

In order to identify new promoters, Sau3A I-digested B. licheniformis DNA fragments were inserted into the BamH I site of the promoter trap vector pShuttleF and resultant plasmids were electro-transformed into E. coli DH5α. Two hundred colonies were picked from about 1000 colonies according to visible coloration on the X-gal plate screen in the presence of both antibiotics. The β-Gal activities by these recombinants after 24 h of culture ranged from 300 Miller U/mL to 14016 Miller U/mL. Amongst them, the recombinant pShuttle-09 demonstrated the highest β-Gal production. The selected 200 colonies were also electo-transformed into the B. subtilis 1A747. Ten blue colonies were selected to measure the β-Gal activities. As shown in Figure 1C, pShuttle-09 again showed the highest β-Gal activity with the activity of 16417±300 Miller U/mL at 24 h, which was eight times of that from the P43 promoter system in B. subtilis. To further verify the reporter gene product driven by pShuttle-09, SDS-PAGE analysis of the β-Gal from the pShuttle-09 both in E. coli and B. subtilis was carried out. Coomassie blue staining revealed a distinct band with a molecular mass of approximately 70 kDa, which corresponded to the molecular weight of β-Gal (data not shown). Taken together, these results suggested that pShuttle-09 contained a putative strong promoter from B. licheniformis chromosomal DNA and drove expression of the reporter gene both in E. coli and B. subtilis.

Sequence analysis, predication and characterization of the cloned promoter fragment

To characterize the putative promoter DNA element in pShuttle-09, the inserted fragment was enzyme cut and sequenced. The sequencing result showed that the inserted fragment was 505 bp long. Sequence alignment showed that the inserted sequence in pShuttle-09 belonged to B. licheniformis genome sequence (NC_CP000002.3) with 96% of homology. According to the annotation of B. licheniformis genomic DNA sequences, the inserted sequence contained two fragments (Figure 2A) corresponding to a partial sequence of the coding region of ybjB gene.
(about 109 bp) and a 5' end partial sequences of luxS (about 200 bp).

Sequence analyses (Figure 2B) also showed truncated luxS gene was fused with bgaB and a typical SD was present 7 bp upstream of the start codon of the luxS gene. It contained typical −35 and −10 elements recognized by a Sigma Factor σA. Therefore, the promoter P_{luxS} might be the control element of bgaB in pShuttle-09. However, further in silico prediction of promoters (Figure 2B) indicated two conserved prokaryotic promoter regions with high probability scores in the cloned fragment. The first putative promoter (P_{luxS}) was located in the 5' non-translational region of luxS gene. Interestingly, the second putative promoter was located on two sides of the second Sau3AI cutting site in pShuttle-09. The −35 element was located within the coding region of ylyB, while

Figure 2. A map (A) and the sequence (B) of an inserted fragment in pShuttle-09. (A) ylyb CDS is the coding sequence of ylyb; luxS CDS represents the coding sequence of luxS; Partial luxS includes 5' non-translated region and an 87 bp coding sequence of luxS. (B) The conservative regions of two putative promoter (−10 and −35) are underlined. The Sau3AI restriction sites are indicated. The beginnings of open reading frames of luxS and bgaB under the putative promoters are indicated by arrows. doi:10.1371/journal.pone.0056321.g002
the −10 element was located upstream of the core promoter region of luxS.

In order to determine the effect of the first putative promoter (P_{luxS}) on the reporter gene expression, a P_{luxS} vector was constructed by sub-cloning a 200 bp P_{luxS} fragment into pShuttle-F. For facilitating a rigorous comparison, promoters of luxS were also cloned from genomic DNA of B. subtilis, B. pumilus and B. megaterium. The β-Gal production driven by P_{luxS} from B. subtilis, B. pumilus and B. megaterium were about 78%, 63% and 70% of that from B. licheniformis (Figure 3A), respectively. However, the β-Gal production driven by P_{luxS} from B. licheniformis was only about 27% of that from pShuttle-09 in B. subtilis (Figure 3A). These data strongly suggested that P_{luxS} is not a major promoter for β-Gal expression in pShuttle-09.

Analysis of the second putative promoter showed the −10 region was located at 6 bp in the second Sau3A I-cutting region and −35 region was located at 86 bp in the first Sau3A I-cutting region in pShuttle-09. A partial sequence of the coding sequence of the yfbB gene by itself could not have the promoter activity, since it did not contain complete promoter elements. It appears that the hybrid promoter was unintentionally created in pShuttle-09. Subsequently, this hybrid promoter was sub-cloned to determine whether it can direct the expression of β-Gal or not. As shown in Figure 3B, the β-Gal production driven by the hybrid promoter was about 67% of that from pShuttle-09 in B. subtilis. It seems that both the hybrid promoter and P_{luxS} contributed additively to expression of β-Gal in pShuttle-09.

Improvement of the cloned double promoter through reconstruction of the hybrid promoter element

In order to further improve efficacy of the double promoter, the second Sau3A I-cutting region containing P_{luxS} and another −10 region was assembled with −35 regions of P_Apr from B. subtilis, B. pumilus, B. licheniformis and B. amyloliquefaciens, respectively. The corresponding promoters, P_{LapS}, P_{LapP}, P_{LapL} and P_{LapT}, were used to drive the expression of β-Gal. The results (Figure 3C) indicated that β-Gal activity driven by P_{LapT} was 1.6 fold higher than that from pShuttle-09 after 24 h culture. On the other hand, the production of β-Gal from P_{LapL}, P_{LapP} and P_{LapL} was only about 81%, 32% and 33% of that from pShuttle-09, respectively. Subsequently, P_{LapT} was used for construction of expression vectors.

Construction of expression vector using the improved promoter P_{LapT}

To further exploit its application in B. subtilis, the strong promoter P_{LapT} was sub-cloned as a truncated fragment flanked with engineered Apa I and EcoR I restriction sites, with the EcoR I site close to the SD. The resultant promoter was assembled to E. coli-B. subtilis shuttle vector pGJ103, generating an expression vector pLu-hyb (Figure 4A). In order to easily detect and purify expressed protein, another vector pLu-His (Figure 4B) was constructed through introducing 6-his tag at the end of MCS in pLu-Hyb.

To determine efficiencies of pLu-hyb and pLu-His, bgaF-1 and bgaF-2 were cloned into pLu-hyb and pLu-His, respectively, to facilitate β-Gal expression. The production of β-Gal from the pLu-bga reached 26037±1037 Miller U/mL (Figure 4C), indicating that the β-Gal was highly expressed. SDS-PAGE analyses of β-Gal from crude extract of B. subtilis harbouring pLu-bga (Figure 4D) or purified β-Gal from B. subtilis harbouring pLuHis-bga (Figure 4E) further verified that β-Gal was successfully expressed. Similarly, baiL gene involved in biotin biosynthesis pathway of B. subtilis was successfully expressed using two similar expression vectors (pLu-biol and pLuHis-biol) in B. subtilis (Figure 5). Therefore, these results further demonstrated the effectiveness of the promoter systems for protein expression.

Discussion

The major finding from this study is that an artificial double promoter was obtained from B. licheniformis through a promoter trap system. The double promoter contained P_{luxS} and a hybrid promoter and can be used for protein expression in B. subtilis. These results support the notion that it is possible to shuffle, generate and select a strong prokaryotic promoter simultaneously, by applying a promoter trap system to randomly cut DNA fragments from a genome.

The interest in promoters stems from myriad opportunities for controlling gene expression. Our promoter trap system was designed to increase the stringency of screening by employing the coding region of bgaB rather than the bgaB gene with a SD as a reporter. So far, the promoter-less bgaB gene has been used as a reporter in several promoter trap systems [11], [22]. However, others usually used bgaB gene with a SD. In our system, promoter fragments inserted into the upstream of bgaB could not drive

Figure 3. β-Gal activities from P_{luxS} (A), the hybrid promoter (B) and the reconstructed promoters (C). (A) Production of β-Gal from pShuttle-09, pShuttle-lux, pShuttle-luxS, pShuttle-luxP and pShuttle-luxM in B. subtilis 1A747 after 24 h culture. Different letters on columns indicate significant differences (p<0.05). (B) Production of β-Gal from pShuttle-Hyb (gray columns) and pShuttle-Fus-1 (black columns) in B. subtilis 1A747 after different hours of culture. Different letters on columns in the same time point indicate significant differences (p<0.05). (C) Production of β-Gal from pShuttle-Fus1, pDE-P_{LapS}, pDE-P_{LapP} and pDE-P_{LapP} in B. subtilis 1A747 after 24 h culture. Different letters on columns indicate significant differences (p<0.05).

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expression of β-Gal unless the cloned promoter fragments contain SD. This design might limit the number of recombinant clones which can be screened. Nevertheless, approximately 1000 blue recombinant colonies were observed in this study. The strength of cloned promoters was conveniently determined via coloration caused by production of β-Gal when a recombinant harbouring a promoter fragment was cultured on solid LB supplemented with X-gal. Further quantitative determination of putative promoter strength was easily achieved by measuring the activity of β-Gal.

*B. licheniformis* genomic DNA was used as a potential promoter source in this study. Recombinant protein expression in *B. subtilis* can be driven by promoters from *B. subtilis* or from other bacillus species. To date, several strong exogenous promoters for the *B. subtilis* system have been isolated and used to construct expression vectors [3], [12], [28]. For example, exogenous promoters Pxyl and Pspac have been widely used as expression control elements in *B. subtilis* [12], [28]. Our results support the notion that other bacillus species could be good sources of strong promoters for *B. subtilis*.

Our results exemplified the potential of a promoter trap system to create a strong hybrid promoter. A hybrid promoter is defined in this study as a promoter consisting of different core elements from two different genes. One promoter identified in this study is a hybrid promoter assembled artificially from a −35 region from the coding sequence of *ylyB* gene and a −10 element from non-coding sequence of *luxS* gene. Our hybrid promoter is artifically created and quite different from reported hybrid promoters, which contain elements from two different promoters. It is well known that a hybrid promoter could be far more efficient than either one of the parental promoters [29]. Recently, Kim [30] constructed a hybrid promoter, BJ27UP, from a strong promoter BJ27D88 and a fragment of the tac promoter in order to express *B. licheniformis* aminopeptidase in *B. subtilis* and found that the activity of the hybrid promoter was increased approximately threefold in comparison with the tac promoter. Nevertheless, the study on hybrid promoters is limited, presumably due to lack of suitable ways to create hybrid promoters.

The results from this study also demonstrated the capability of a promoter trap system to create a double promoter. Interestingly, both the hybrid promoter and P*luxS* promoter in our study contributed to transcription of the reporter gene in an additive way. A double promoter in this study is defined as the combination of two promoters which control transcription of same gene. This definition is different from most overlapping promoters often seen in bacteria. Analyzing the database of the *E. coli* genome, Bendtsen [31] found 14% of the identified ‘forward’ promoters overlap with a promoter oriented in the opposite direction. These overlapping
promoters are arranged in three different ways: (i) each promoter transcribes a gene and one or more regulatory proteins are identified which affect transcription of at least one of the promoters directly; (ii) each promoter transcribes a gene but no regulatory proteins are known to bind the promoter region; and (iii) only one of the promoters transcribes an annotated gene, while the other promoter could transcribe a regulatory antisense RNA or act as a regulatory RNAP binding site which interferes with the promoter transcribing the annotated gene [31]. However, some overlapping promoters do control same gene. For example, Wang and Doi [32] detected two overlapping promoters for the chloramphenicol acetyltransferase gene and these two promoters are arranged in three different ways: (i) each promoter transcribes a gene and one or more regulatory proteins are identified which affect transcription of at least one of the promoters directly; (ii) each promoter transcribes a gene but no regulatory proteins are known to bind the promoter region; and (iii) only one of the promoters transcribes an annotated gene, while the other promoter could transcribe a regulatory antisense RNA or act as a regulatory RNAP binding site which interferes with the promoter transcribing the annotated gene [31]. However, some overlapping promoters do control same gene. For example, Wang and Doi [32] detected two overlapping promoters for the chloramphenicol acetyltransferase gene and these two promoters are recognized by two different RNA polymerase holoenzymes. The authors further proposed that genes expressed during both growth and sporulation may be regulated by tandem overlapping promoters whose transcription initiation points are either identical or very close. The possible benefit of double promoters to increase transcription has been explored in biotechnological application. Most studies have shown that two or more tandem promoters could significantly improve the expression level of heterogeneous genes. Widner [33] found that gene expression was distinctly increased under the control of two or three tandem promoters in contrast to one alone in B. subtilis. Kang [34] also reported that two expression systems constructed by sequential alignment of a constitutive promoter for either $\alpha$-amylase from B. subtilis NA64 or maltogenic amylase from B. licheniformis downstream of the Hpa II promoter elevated the TSAGT productivity by 11- and 12-fold, respectively, in comparison with the single Hpa II promoter system. Li [35] tried to construct multiple core-tac-promoters (MCPtacs) in tandem and found that integration of the phaCAB genes with the 5 copies of core-tac-promoters resulted in an engineered E. coli that can accumulate 23.7% polyhydroxybutyrate of the cell dry weight in batch cultivation. On the other hand, Wu [36] did not observe improvement of expression, when two tandem-linked promoters were used. Our results support the idea to use double promoters to increase production efficiency of recombinant proteins. Whether the double promoter in this study is targeted by same RNA polymerase or different RNA polymerases is not known at this time and worthy of further investigation.

In conclusion, our data support the concept of using a promoter trap system to discover new promoters. The usefulness of a promoter trap system is likely due to random shuffling and recombination of DNA fragments and adoption of a rapid and high-throughput screening. It is tempting to speculate that more novel promoters could be found if reaction conditions for cutting and ligation could be adjusted to increase chances for shuffling DNA fragments.

**Author Contributions**

Conceived and designed the experiments: XZ YC MY. Performed the experiments: MY WZ SJ PC. Analyzed the data: MY WZ YC. Contributed reagents/materials/analysis tools: MY WZ SJ. Wrote the paper: XZ YC MY.

**References**

1. Schallmey M, Singh A, Ward OP. (2004) Developments in the use of Bacillus species for industrial production. Curr Microbiol 50:1–17.
2. Zhang XZ, Cui ZL, Hong Q, Li SP. (2005) High-level expression and secretion of methyl parathion hydrolase in Bacillus subtilis WB8100. Appl Environ Microbiol 71: 4101–4103.
3. Schumann W. (2007) Production of recombinant proteins in Bacillus subtilis. Adv Appl Microbiol 62:137–189.
4. Nguyen HD, Schumann W. (2006) Novel plasmid-based expression vectors for intra- and extracellular production of recombinant proteins in Bacillus subtilis. Prot Expr Purif 46:189–195.
5. Li W, Pan JG, Park SH, Choi SK. (2010) Development of a stationary phase-specific autoinducible expression system in Bacillus subtilis. J Biotechnol 149:16-20.
6. Yang MM, Zhang WW, Zhang XF, Cen PL. (2006) Construction and characterization of a novel maltose inducible expression vector in Bacillus subtilis. Biotechnol Lett 28:1713–1718.
7. Zhu FM, Ji SY, Zhang WW, Li W, Cao BY, et al. (2008) Development and Application of a Novel Signal Peptide Probe Vector with PGA as Reporter in Bacillus subtilis WB700: Twenty-Four Tat Pathway Signal Peptides from Bacillus subtilis were monitored. Mol Biotechnol 39: 225–230.
8. Meijer WJJ, Salas M. (2004) Relevance of UP elements for three strong Bacillus subtilis phase phi29 promoters. Nucleic Acids Res 32:1166–1176.
9. Zhong AL, Liu H, Yang MM, Gong YS, Chen H. (2007) Assay and characterization of a strong promoter element from B. subtilis. Biochem Biophys Res Commun 354: 90–95.
Development of Recombinant Protective Antigen-Based Vaccines. Infect Immun 71: 801–813.

19. Cui ZL, Zhang XZ, Zhang ZH, Li SP. (2004) Construction and application of a promoter-trapping vector with methyl parathion hydrolase gene mpd as the reporter. Biotechnol Lett 26: 1113–1118.

20. Aoki S, Kondo T, Ishiura M. (2002) A promoter-trap vector for clock-controlled genes in the cyanobacterium Synechocystis sp. PCC 6803. J Microbiol Methods 49: 265–274.

21. James AC, Philip ES, Patricia R, Abdallah FE, Claude FG. (2003) An enhanced GFP reporter system to monitor gene expression in Borrelia burgdorferi. Microbiol 149: 1819–1828.

22. Phan TT, Nguyen HD, Schumann W. (2010) Establishment of a simple and rapid method to screen for strong promoters in Bacillus subtilis. Prot Expr Purif 71: 174–178.

23. Wang YG, Xia QY, Gu WL, Sun JB, Zhang H, et al. (2011) Isolation of a strong promoter fragment from endophytic Enterobacter cloacae and verification of its promoter activity when its host strain colonizes banana plants. Appl Microbiol Biotechnol 93: 1585–1599.

24. Maniatis T, Frisch EF, Sambrook J. (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY

25. Guerov-Feury AM, Frauchner N, Stragier P. (1996) Plasmids for ectopic integration in Bacillus subtilis. Gene 180: 57–61.

26. Hirata H, Fukazawa T, Negoro S, Okada H. (1986) Structure of a β-galactosidase gene of Bacillus stearothermophilus. J Bacteriol 166: 722–727.

27. Hartl B, Wehel W, Wiegel T, Homuth G, Schumann W. (2001) Development of a new integration site within the Bacillus subtilis chromosome and construction of compatible expression cassettes. J Bacteriol 183: 2696–2699.

28. de Boer HA, Comstock IJ, Vasser M. (1983) The tac promoter: a functional hybrid derived from the trp and lac promoters. Proc Nat Acad Sci 80: 21–25.

29. Kim YH, Lee BR, Lee YP. (2011) Secretory overproduction of the aminopeptidase from Bacillus licheniformis by a novel hybrid promoter in Bacillus subtilis. World J Microbiol Biotechnol 27: 2747–2751.

30. Bendtzen RM, Erdosz J, Csanaveszki Z, Svemingeren NL, Snarpen K, et al. (2011) Direct and indirect effects in the regulation of overlapping promoters. Nucleic Acids Res 39: 6879–6885.

31. Hartl B, Wehel W, Wiegel T, Homuth G, Schumann W. (2001) Development of a new integration site within the Bacillus subtilis chromosome and construction of compatible expression cassettes. J Bacteriol 183: 2696–2699.

32. Wang PZ, Doi RH. (1984) Overlapping promoters transcribed by Bacillus subtilis sigma 55 and sigma 37 RNA polymerase holoenzymes during growth and stationary phases. J Biol Chem 259: 8619–8625.

33. Widner B, Thomas M, Sternberg D, Lammon D, Behr R, et al. (2000) Development of marker-free strains of Bacillus subtilis capable of secreting high levels of industrial enzymes. J Ind Microbiol Biotechnol 25: 204–212.

34. Kang HK, Jiang JH, Shim JH, Park JT, Kim YW, et al. (2010) Efficient constitutive expression of thermostable 4-α-glucanotransferase in Bacillus subtilis using dual promoters. World J Microbiol Biotechnol 26: 1915–1918.

35. Li MJ, Wang JS, Geng YP, Li YK, Wang Q, et al. (2012) A strategy of gene overexpression based on tandem repetitive promoters in Escherichia coli. Microb Cell Fact 11:19.

36. Wu SM, Feng C, Zhong J, Huan LD. (2011) Enhanced production of recombinant nattokinase in Bacillus subtilis by promoter optimization. World J Microbiol Biotechnol 27: 99–106.