# Development of an inducible T7 expression system in Bacillus subtilis ATCC 6051a for production of α-L-arabinofuranosidase

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# Abstract

Background: Xylan is the second most abundant polysaccharide biomass on the earth, the polymers have a backbone of β-1,4-linked xylose residues with various side-chain substitutions, such as arabinose, acetic acid, glucuronic acid, and other esterified groups, thus, the removal of arabinose side groups by α-L-arabinofuranosidases is helpful in various industrial processes involving xylan treatment. Bacillus subtilis ATCC 6051a is known for its excellent capacity of secretory production of recombinant peptides, however, poor experience in genetic manipulation and lack of universal expression elements impede this strain for wider application.

Results: Xylose inducible comK was integrated into the genome of B. subtilis ATCC 6051a, and the transformation efficiency of the engineered strain B. subtilis 164S was increased by more than 1000 folds. B. subtilis 164S was further modified to generate B. subtilis 164T7P which incorporates a D-xylose inducible T7 RNA polymerase. The recombinant GFP expressed by 164T7P is more than thirteen times that achieved by P43 promoter, representing the most efficient expression system that had been ever reported in B. subtilis . Subsequently, abfA1 , encoding a glycoside hydrolase (GH) family 51 enzyme was cloned and overexpressed in 164T7P. The activity of recombinant α-L-arabinofuranosidase (AbfA1) reached 90.6±2.0 U mL -1 in the fermentation broth. Using p NPA as a substrate, kinetic parameters of the crude enzyme were Km of 1.4±0.1 mM and kcat of 139.4 s −1 . The optimum temperature and pH of the recombinant AbfA1 towards p NPA were observed to be 45 °C and pH 6.5, respectively.

Conclusion: With enhanced cellular competence and introduction of T7 RNA polymerase, B. subtilis ATCC 6051a was engineered as a versatile cell tool for recombinant production of heterologous peptides employing T7 promoter. The novel expression kit demonstrated a very low level of leaky expression of target genes. The efficiency and applicability of the system were demonstrated by high-level production of a bacterial type α-L-arabinofuranosidase.


# Background

Given that their biological nature of nonpathogenic GRAS (generally recognized as safe), Bacillus subtilis species serve as one type of the most widely used cell factories, especially for the production of recombinant enzymes applicable in food-processing industry. B. subtilis strains are thus the most characterized gram-positive bacteria, which is intensively studied in the fields of genetics, biochemistry, physiology and fermentation technologies [1–3]. Due to the native transformability, B. subtilis strain 168 and its protease deficient subspecies are commonly used for lab-scale production of proteins. Nevertheless, the less domesticated strain B. subtilis ATCC 6051a exhibited much better secretory capability and superior growth properties in complex mediums than B. subtilis 168 [4–6], however, engineering in B. subtilis ATCC 6051a was greatly limited or retarded by its poor transformation performance.

The genome sequence of B. subtilis ATCC 6051a was published and compared side by side with B. subtilis 168 [6], which revealed that the severely reduced competence of B. subtilis ATCC 6051a was likely caused by a frameshift mutation in comP that encodes a two-component sensor kinase, which activates an operon related to competence forming in B. subtilis by turning on expression of its downstream ComK, a decisive regulator for natural competence development [7, 8]. It has been reported that the overexpression of ComK was capable of upturning the transformation efficiency for nearly 1000 times [9–11]. However, the attempt of raising the ComK level in B.subtilis ATCC 6051a had never been reported previously. On the other side, the repertoire of efficient promoters in B. subtilis is really limited. The most known promoter applied in B. subtilis is P43 [12], which is an artificial element widely utilized. Some other novel promoters include Pgrac [13], PyxiE [14], Pglv [15], dual promoter PgsiB–PHpaII [16] and pShuttle-09 [17], but none of them is comparable to phage derived T7 promoter in transcriptional strength. The T7 expression system employing the T7 promoter was first introduced in Escherichia coli and displayed high efficiency and specificity in heterologous expression of target peptides [18, 19]. Studies have shown that the engineered T7 expression system was able to improve the expression level of heterologous peptides in Bacillus [20–22], although it has not tested in undomesticated B. subtilis strain, such as in B.subtilis ATCC 6051a. Another important feature of a promoter is its controllability. Since it has been confirmed in E. coli, IPTG inducible element was also introduced in Gram-positive strains, in promoter such as Pspac. However, it is known that the control on the leaky expression run by Pspac or other lacO containing promoters was not desirable as expected to be [23]. Thus, the efforts to seek a stable, tightly controllable and highly-active promoter are vital and urgent for the further expanding of the applicability of B. subtilis in the production of recombinant proteins.

In this study, comk cassette was integrated into the chromosome of B. subtilis ATCC 6051a, creating an easy-to-manipulate strain B. subtilis 164S, thereafter, a revised cloning strategy based on ‘Simple Cloning’ [24] was invented, enabling convenient seamless cloning of shuttle vectors. To create a host cell utilizing T7 promoter, DNA cassette encoding T7 RNA polymerase under $P_{xylA}$, a xylose inducible promoter, was integrated into the genome B. subtilis 164S, generating B. subtilis 164T7P. The efficiency of the T7 promoter in B. subtilis was demonstrated by the expression of GFP and an α-L-arabinofuranosidase encoded by abfA1 from B. licheniformis.

# Results And Discussions

### Engineering *B. subtilis* ATCC 6051a to raise its natural competence by expressing recombinant *comK*

Compared with traditional genetic manipulation through circular plasmid mediated homologous recombination, double crossover by homologous fragments exchange through linear DNA eases the handling of genome engineering in prokaryotic cells in terms of efficiency and labor intensity. However, comparing with circular plasmid transformation, homologous integration through linear DNA requires much higher transformation efficiency. Therefore, it was an intimidating task to perform one step gene replacement in *B. subtilis* ATCC 6051a, a genetically indocile, however industrial favored strains. The alignment analysis using the genomic sequences of *B. subtilis* ATCC 6051a and *B. subtilis* 168 revealed a

frameshift mutation from AA to A that leads to functional disability of ComP in *B. subtilis* ATCC 6051a, subsequently reducing the cellular level of ComK [6], one of the key activators modulating natural competence in *Bacillus*. Overexpression of ComK was proposed and confirmed to improve the transformation efficiency even earlier [9-11, 24, 25]. Thus, we proposed to perform homologous expression of ComK in 6051a by cloning a copy of the native *comk* gene under a xylose inducible promoter, $P_{xylA}$ from *B. megaterium* [9], then inserting the cassette into 6051a's chromosome via double-crossover recombination. However, that could not be directly accomplished duo to host cells' low transformation efficiency. Therefore, we constructed pMK4-comk, a self-replicating plasmid containing the expression cassette, and transformed the shuttle vector into *B.* 6051a (as shown in Fig. 1a, more details are shown in Additional file 1: Fig S1). With the lifted transformation efficiency gained through the plasmid mediated expression of ComK, linear *comk* cassette together with kanamycin resistance marker (*kan*) was integrated into the genome and replaced native *nprE* in 6051a (Fig. 1b). Finally, a plasmid expressing Cre integrase was transformed into the *bacillus* mutant (Fig. S1), creating a markerless strain, which was named as *B. subtilis* 164S.

## Enhanced transformation efficiency upon induction of ComK

The transformation efficiency of *B. subtilis* 164S was determined by transforming 1.0 μg of pMK4 DNA (circular DNA of 5585 bp in length) to properly prepared competence cells. As the result of induced expression of ComK by culturing cells in medium containing D-xylose, the transformation efficiency reached $3\times10^4$ transformants per μg DNA, which increased more than one thousand times over 6051a (data not shown).

To determine the recombination efficiency through double crossover, linear DNAs with varying sizes of homologous fragments flanking four different open reading frames (encoding *spoII AC*, *aprE*, *amyE* and *srfAC*, respectively, Table 1) were prepared by fusion PCR [26, 27]. The numbers of transformants obtained after transformation into 164S or 6051a using each linear DNA were counted. As seen in Table 1, 164S transformants' colony numbers in all experiments fell into a range of 70~160, in contrast, no transformant was obtained from 6051a using the same linear DNA (data not shown).

Previous studies postulated that the minimal length of DNA fragments required for homologous recombination was around 400~500 bp [27, 28], it has never been confirmed in industrial *bacillus* strain such as 6051a. To determine the relationship between the length of the homologous flanking region and the double crossover efficiency, we prepared linear DNAs for in-frame deletion of *spoII AC* with different sizes of homologous arms (200 to 1000 bp, respectively, Fig. 2a), used them for gene replacement in 164S. As presented in Fig. 2b, linear DNA mediated transformations in 164S were DNA size dependent, longer DNA arms evoked much higher recombination efficiency in 164S, and the minimal length to initiate an efficient DNA exchange event was about 400 bp, that is in agreement with the previous report [27].

Table 1 The efficiency of linear DNA mediated transformation in *B. subtilis* 164S

| Integration loci | Homology length (bp) | | Transformation efficiency[a] |
|---|---|---|---|
| | Upstream | Downstream | |
| *spoII AC* | 1010 | 1012 | $1.6\times10^2$ |
| *aprE* | 619 | 600 | $0.9\times10^2$ |
| *amyE* | 815 | 807 | $1.3\times10^2$ |
| *srfAC* | 928 | 1008 | $0.7\times10^2$ |

[a] Transformation experiments were repeated three times, the transformation efficiency was calculated as the average number of $Erm^R$colonies formed on plates per μg of linear DNAs.

## Seamless cloning method for one step construction of Bacillus expression vector

Seamless cloning is advantageous in preparing faithful DNA constructs. The method takes advantage of restriction-independent cloning strategies, such as overlap PCR, DNA recombination or DNA ligation using type IIs restriction enzymes. Available commercial kits for seamless cloning include Golden Gate cloning technology, Gibson assembly, or In-fusion Cloning. Additionally, an enzyme-free cloning method known as “Simple Cloning” was developed and proved to have high transformation efficiency in *B. subtilis* SCK6 [24]. According to the report, ~$10^7$ transformants per μg of DNA multimer can be achieved. Here we slightly modified the method and developed it as one step seamless method for the construction of desired plasmids. As demonstrated in Fig. 3a, the backbone plasmid was linearized by restriction enzyme, it joined with the insert through overlap PCR using primers without restriction site sequences that originally existed on the plasmid DNA; during PCR program, the unmatched sequences would be automatically trimmed by 3'- 5' exonuclease of high-fidelity DNA polymerase; after transformation into *B. subtilis* cell, linear DNA multimers generated by PCR trigger efficient *in vivo* splicing that leads to formation of circular DNA [24].

Construction of pMK4-T7-abfA1 demonstrated the feasibility of the novel cloning strategy. First, pMK4-T7 was digested with BamHI to give linear double strand DNAs that were used in PCR reaction for preparation DNA multimers, the PCR system also included PCR prepared *abfA1* fragment containing homologous ends to the vector DNA, however without BamHI residue site that originally existed immediate downstream of T7 promoter on pMK4-T7; PCR generated multimers were subsequently transformed into 164S, that promptly produced transformants harboring circularized pMK4-T7-abfA1. As shown in Fig. 3b, the DNA sequencing confirmed BamHI site was eliminated on generated pMK4-T7-abfA1. The transformation efficiency can be over $1.0\times10^6$ transformants per μg of DNA multimer, which was lower than the report [24] but sufficient for most strain engineering jobs.

## Construction of inducible T7 expression system in *B. subtilis*

T7 RNA polymerase over-expressing strains, such as BL21 (DE3) or JM109 (DE3), were required for T7 promoter guided robust expression in *E. coli*, for the same reason, T7 RNA polymerase has to be

expressed in *B. subtilis* if T7 promoter is recruited for transcription of target genes [20-22]. However, most bacterial systems utilize IPTG as the expression switch, however, IPTG is costly and unhealthy if not removed from the products, thereby limiting the system's application in the food or pharmaceutical field [29]. In this study, a D-xylose inducible T7 expression system was constructed. As demonstrated in Fig. 4a, we first assembled an artificial cassette encoding T7 RNA polymerase under the inducible promoter $P_{xylA}$, delivery of the linear DNA into 164S knocked out *aprE* in 164S and replaced it with the DNA cassette expressing T7 RNA polymerase. A copy of *xylR* was included in the cassette to stop the "leaky expression" of the polymerase when D-xylose is not available. 164T7P was tested in the expression of GFP (cloned in plasmid pMK4-T7-gfp, as seen in Additional file 2: Fig S2). Since P43 was recognized as a strong and constitutive promoter in *B. subtilis* [12, 30], a 164T7P control strain harboring the plasmid pMK4-P43-gfp (Additional file 2: Fig S2) was made for comparison between $P_{xylA}$ and P43. To examine GFP expression levels, the average fluorescence intensity in cells grown upon the presence of different concentrations of D-xylose was determined in a microplate reader. As shown in Fig. 4b, the level of heterologous expressed GFP in 164T7P correlated with the concentration of D-xylose supplemented in mediums (Fig. 4b and Fig. 4c). It showed that in medium with 1.0% D-xylose, the fluorescence intensity of GFP expressed by T7 promoter reached $2.83\times10^7$ au, which is more than 13 times of that achieved by P43 promoter ($2.08\times10^6$ au) (Fig. 4c, Additional file 3: Fig. S3), besides, in the absence of xylose (Fig. 4b, Fig. 4c), recombinant *B. subtilis* cells exhibited very low level of background expression of GFP, emitting fluorescence equivalent of only 1% that induced level, whereas, lacO/IPTG inducible control system in *B. subtilis* displayed a rather high level of leaky expression [23].

## Heterologous expression and characterization of AbfA1

L-arabinosyl residues are widely distributed in hemicelluloses as one of the main side chains. Therefore, α-L-arabinofuranosidase (EC 3.2.1.55) is indispensable for the full degradation of polysaccharide xylan into xylose [31-33]. Through preliminary search and screening (data not shown), an ORF (*abfA1*) from the *B. licheniformis* ATCC 14580 encoding putative *abfA1* was found and cloned into the plasmid pMK4-T7 using Simple and Seamless method, *in vivo* generated plasmid pMK4-T7-abfA1 rendered *B. subtilis* 164T7P ability of producing arabinofuranosidase (AbfA1) using mRNA transcribed by inducible T7 expression system. For full induction, 10 g $L^{-1}$ of D-xylose was supplemented into the culture growing at 37ºC. After the cells were spun down, 10 μL of supernatant was analyzed by SDS-PAGE. As seen in Fig. 5a, a high level of α-L-arabinofuranosidase was secreted out of host cells, with an estimated mass of 56.9 kDa. The total protein concentration in the supernatant was measured to be 0.8 g $L^{-1}$ in Bradford assay, and the recombinant AbfA1 represented more than 90% of the total protein in the supernatant(Fig. 5a). The activity and the kinetic parameters of AbfA1 were determined using *p*NPA (4-nitrophenyl α-L-arabinofuranoside) as the substrate. The maximum activity during fermentation was measured to be 90.6±2.0 U $mL^{-1}$ at 52 h (Fig. 5b), with a Km value of 1.4±0.1 mM and the kcat value of 139.4 $s^{-1}$. The enzyme remained to be active in the pH range of 3.5-9.0 with an optimal pH at 6.5 (Fig. 5c). Its optimal temperature was determined to be 45 ºC while working between 25-55 ºC (Fig. 5d). This is by far the first report on the heterologous secretion expression of α-L-arabinofuranosidase in *B. subtilis*. It is worth

mentioning that the protein sequence of AbfA1 showed neither Sec- nor Tat- signal peptide when analyzed by SignalP-5.0 Server [34]. And, even the score of the prediction of non-classical protein secretion on AbfA1 is rather low (<0.5) [35], we still speculate that it is secreted through a non-classical pathway when expressed in *B. subtilis* [36].

# Conclusion

We developed a xylose-inducible expression system in B. subtilis ATCC 6051a. First, we improved industrial B. subtilis' natural competence through the inducible expression of Comk that was placed under the control of a $P_{xylA}$ promoter; then, we integrated T7 RNA polymerase behind another $P_{xylA}$ promoter on 6051a's genome. The constructed T7 RNAP expression system exhibited high efficiency of T7 promoter-specific protein's expression in engineered 6051a strain, that included GFP and a bacterial arabinofuranosidase, the latter was secreted out of host cells unexpectedly, apparently through a non-classical secretion pathway since it lacks any known secretive signal peptide. Also, we modified a seamless cloning strategy, enabling one-step, rapid and precise construction of expression vectors in B. subtilis. These efforts not only established an attractive expression platform with high efficiency but also provided benefits for DNA or genome manipulation in previously imbecile B. subtilis ATCC 6051a.

# Materials And Methods

### Strains, plasmids, reagents, and cultivation conditions

Bacteria strains and plasmids used in this study are listed in Table 1. *B. subtilis* ATCC 6051a was purchased from ATCC; *E.coli* DH5α was used for plasmid cloning and propagation. All strains were cultivated in LB medium at 37 ºC. For induced expression of recombinant peptides in *B. subtilis*, 0.3 mL of seed culture was transferred into 30 ml of fresh LB loaded in a 250-mL shaking flask. When necessary, 1.0-10 g $L^{-1}$ of D-xylose, 100 μg $mL^{-1}$ ampicillin, 10 μg $mL^{-1}$ erythromycin, 20 μg $mL^{-1}$ kanamycin or 10 μg $mL^{-1}$ chloramphenicol were supplemented into mediums.

Table 1 Strains and plasmids used in this work.

| Strain or plasmid | Description | Source/reference |
|---|---|---|
| *B. subtilis* | | |
| 1A976 | $Erm^R$, *B. subtilis* 1A751 derivate, *lacA::PxylA-comK* | [ 9] |
| 6051a | Wild type *B. subtilis* strain | ATCC |
| 164K | $Km^R$, *B. subtilis* 6051a derivate, *nprE*::*PxylA- comk-lox71-kan-lox66* | This work |
| 164S | 6051a derivate, *nprE*::PxylA-*comk* | This work |
| 164T7P | 6051a derivate, *nprE*::*PxylA-comk*, *aprE*::*PxylA-T7P*, expressing T7 RNA polyerase and ComK under PxylA | This work |
| *E.coli* | | |
| DH5α | Cloning strain | Lab stock |
| BL21(DE3) | Expression strain | Lab stock |
| Plasmid | | |
| pMK4 | *E. coli–B. subtilis* shuttle vector, $Amp^R$ for *E. coli*; $Cm^R$ for *B. subtilis* | BGSC |
| pMK4-comk | *comk* cloned behind $P_{xylA}$ promoter in pMK4 | This work |
| pGSNE | $Amp^R$, integration plasmid carrying *comk* cassette and fragments for *nprE*deletion | This work |
| pGSNE-comk | pGSNE carrying *comk* cassette containing *PxylA-comk* and erythromycin resistance gene *ermC* fused between *lox71* and *lox66* | This work |
| pGSNE-comk | $Amp^R$, Integration plasmid carrying *comk* cassette containing *nprE*homologous arm | This work |
| pDGC | $Amp^R$, $Km^R$, integration plasmid pDG148 containing *cre* behind the promoter Pspac | [ 27] |
| pMK4-cre | Shuttle vector pMK4 with *cre* under control of Pspac | This work |
| pUCK-syn-sigF | $Amp^R$ , Plasmid for *spoIIAC*-knock-out | This work |
| pMD19T (Simple) | $Amp^R$, Cloning vector | Takara |
| pMD19T-aea | pMD19T carrying fragments for *aprE* deletion | This work |
| pMD19T-T7P | pMD19T-aea carrying T7 RNA polymerase expression cassette | This work |
| pMK4-T7 | pMK4 carrying T7 promoter | This work |
| pMK4-T7-gfp | pMK4 carrying *gfp* under control of T7 promoter | Lab stock |
| pMK4-T7-abfA1 | pMK4 carrying *abfA1* under control of the T7 promoter | This work |
| pMK4-P43-gfp | pMK4 carrying *gfp* under control of the P43 promoter | Lab stock |

$Amp^R$, ampicillin resistance; $Cm^R$, chloramphenicol resistance; $Erm^R$, erythromycin resistance; $Km^R$, kanamycin resistance.

### DNA manipulation techniques

The isolation and manipulation of recombinant DNA were performed using standard techniques. DNA synthesis (Additional file 4: Table S1) and sequencing were performed by Sangon Biotech Co., Ltd. (Shanghai, China). Unless otherwise indicated, the plasmids were constructed with ClonExpress® II One Step Cloning Kit (Vazyme Biotech Co.Ltd. Nanjing, China). Restriction enzymes and other molecular biology reagents were purchased from Thermo Fisher Scientific. High-fidelity DNA polymerase 2× Phanta® Master Mix was purchased from Vazyme Biotech. TA cloning kit was purchased from TaKaRa Biotechnology (Dalian) Co., Ltd. The SDS-PAGE Preparation kit was purchased from Sangon Biotech Co., Ltd.

### Construction of *B. subtilis* 164S

The $P_{xylA}$ comk cassette was amplified by PCR using the genomic DNA of *B. subtilis* 1A976 as the template with the primer pair Comk-F/Comk-R. The PCR product was digested with PstI and ligated into the corresponding site of pMK4 to generate pMK4-comk, which was transformed into *B. subtilis* ATCC 6051a using the method previously described [37]. pMK4-comK was then transformed into 6051a to raise its integration efficiency, generated transformant was named as 6051a/pMK4-comK. A DNA fragment *PxylA-comk-lox71-kan-lox66* was chemically synthesized, then digested with KpnI/NheI and ligated into the same enzymes treated integration vector, pGSNE, to generate pGSNE-comk. pGSNE contains DNA fragments flanking the ORF of *nprE* derived from *6051a*, that enable homologous recombinant and leads to null deletion of *nprE* and insertion of target DNA carried on pGSNE. Therefore, after transforming NcoI-linearized pGSNE-comK into 6051a/pMK4-comK, obtained colonies were resistant to kanamycin and chloramphenicol, and they were verified to bear *comk* cassette on the locus of *nprE*. The mutant 6051a was cultivated and passaged two generations in LB without antibiotic, then diluted and spread onto plain LB plates. Formed colonies were screened for their sensitivity to chloramphenicol to check whether the plasmid pMK4-comk was cured or not. The plasmid-free mutant was designated as *B. subtilis* 164K. To further eliminate the *kan* cassette from 164K, a Cre/Lox recombination system was employed [27]. First, a DNA cassette containing *cre* behind Pspac was prepared using pDGC as the template with the primer pair Cre-F/Cre-R, the PCR product was digested with EcoRI and ligated into pMK4 to generate pMK4-cre; then the vector was transformed into 164K, that elicited the homologous recombination of *loxP* sites in 164K upon the expression of Cre recombinase and resulted in elimination of *kan* from the chromosome. Next, the vector cure procedure was performed to remove pMK4-cre from the transformant, which finally generated *B. subtilis* 164S.

### Generation of linear DNAs for gene replacement in *B. subtilis*

Linear DNAs used for gene replacement contain an erythromycin resistance gene as the selective marker which is surrounded by homologous DNAs [26, 27]. For example, *apr-erm-apr* was used for *apr* (*apr* encodes alkaline protease in the strain) deletion in 6051a. To prepare *apr-erm-apr*, *ermC* cassette DNA was first amplified using vector pUCK-syn-sigf as the template with the primer pairs Erm-F/Erm-R, then the primer pairs ApU-F/ApU-R and ApD-F/ApD-R were used to prepare flanking regions of *apr*. Above mentioned three fragments contains ending sequences homologous to each other and were put into PCR tube for overlap PCR that was done using 2 × Phanta® Master Mix. The obtained PCR products were treated with DpnI, and followed by purification with the Axygen DNA purification kit. With the same strategy, linear DNAs for gene replacement of *spoII AC*, *amyE* or *srfAC* were also prepared; and the primers used were listed in Additional file 4: Table S1.

### Quantitative transformation efficiency assay for *B. subtilis*

The transformation of *B. subtlilis* 164S or *B. subtlilis* 164T7P was performed using the method described in the previous study with minor modification [9]. Briefly, 0.5 mL of a fresh culture grown in LB was inoculated into 4.5 ml of pre-warmed LB containing 1% (m/v) D-xylose, followed by incubation at 37 $^{o}$C on a rotary shaker for 1.5 h, or until $OD_{600}$ reached ~1.0. 100 μL of fresh culture was immediately used for transformation by mixing with ~100 ng of linear or circular DNA. The DNA-cells mixture was incubated at 37 $^{o}$C with rotary shaking for 2 h before spreading onto LB agar plates with an appropriate antibiotic. The linear DNAs prepared for gene replacement (*apr*, *amyE*, *spoIIAC* or *srfAC*) were comprised of a DNA cassette encoding drug resistance and two flanking fragments for homologous recombination.

### Construction of *B. subtlilis* 164T7P

A DNA cassette encoding T7 RNA polymerase was prepared by PCR using the genome DNA of *E. coli* BL21 (DE3) as the template with the primer pair F77-F/F77-R. A DNA fragment containing $P_{xylA}$ promoter and *xylR* was amplified from vector pMK4-comk using the primer pair Fxy-F/Fxy-R. Overlap PCR was performed to obtain a fused DNA fragment, which was then digested with XmaI/KpnI before ligating with the same enzymes treated pMD19T-aea. The generated plasmid (pMD19T-aea-T7P) was linearized by ApaLI before transforming into *B. subtilis* 164S. Colonies formed on the LB agar plate containing erythromycin were verified for DNA insertion of a cassette encoding T7 RNA polymerase on the locus of *aprE*. The loss of the drug resistance (*ermC*) was achieved by Cre recombinase using the procedure described early. Finally, the markerless strain derived from 164S was named as 164T7P since it harbors a DNA insert on its chromosome for expression of T7 RNA polymerase.

### Construction of *B. subtilis* strains expressing GFP or α-L-arabinofuranosidase

pMK4-T7-gfp and pMK4-T7-abfA1 were constructed for heterologous expression of GFP and the arabinofuranosidase respectively. First, we cloned a DNA fragment that contains a T7 promoter, T7 terminator and some restriction cloning sites, into pMK4 to produce pMK4-T7. Next, pMK4-T7 was linearized with BamHI and EcoRI, which generated linear DNAs for the preparation of the multimer DNAs,

using the overlap PCR based on the 'Simple Cloning' method described earlier, and, the insert DNA encoding *gfp* or *abfA1* was required for the overlap PCR, they were also prepared by PCR. The previously constructed pMK4-P43-gfp was used as the template for PCR preparation of the *gfp* insert, the template DNA to prepare *abfA1* insert was chemically synthesized using the sequence derived from *B. licheniformis* ATCC 14580 (accession number NZ_CP034569) with the primer pair ArbF-F/Arbf-R. Finally, the generated multimer DNAs were transformed into *B. subtilis* 164T7P, creating the strain for expression of GFP or AbfA1.

### Fluorescence measurements

Culture samples were diluted properly with normal saline and in sterile saline solution and aliquoted in a volume of 200 μL into each well of 96-well plates. The GFP fluorescence of the samples were measured using the Synergy™ H1 Reader (Biotek) with an excitation source of 485 nm and an emission window of 520 nm.

### Enzymatic assay of recombinant α-L-arabinofuranosidase

The activity of α-L-arabinofuranosidase was detected in 50 μL of the reaction solution (buffered with 50 mM phosphate/citrate, pH 7.0), which used 1.0 mg/mL of *p*NPA (4-nitrophenyl α-L-arabinofuranoside) as the substrate. The solution was prewarmed at 40 ºC for 5 min, followed by mixing with 100 μL of properly diluted enzyme and incubating at 45 ºC for 5 min, then the reaction was stopped by adding 150 μL of 1M $Na_2CO_3$. The reaction will release *p*-nitrophenol that can be detected using a spectrophotometer at a wavelength of 410 nm, and one unit of the enzyme was defined as the amount of enzyme (mL) required to releases 1 μM *p*-nitrophenol per minute [31-33]. The effect of pH and temperature on α-L-arabinofuranosidase's activity was analyzed on a temperature range of 25–65 $^{o}$C and a pH range of 3.5-9.0, respectively. To prepare buffers for the reactions, sodium citrate buffer was used to maintain a pH from 3.5-5.5, potassium phosphate buffer for pH 6.0-8.0, and Tris-HCl buffer for pH 8.5-9.0, respectively. The kinetic parameters of the enzyme (Km, Vmax, and kcat) were determined using different *p*NPA concentrations (ranging from 0.05 to 7.0 mM). The Km and Vmax were calculated by non-linear regression analysis with Origin 8.0 Pro software. The total protein content in the supernatants of the fermentation broth was determined by Bradford assay [38].

### SDS-PAGE

The liquid samples of whole-cell extract or cell-free supernatants were mixed with 5× Protein Loading Buffer（313 mmol/L Tris-HCl; pH6.8; 0.05% Bromophenol blue; 50% glycerol; 10% SDS）with a ratio of 4:1 (V/V). The samples were then treated in boiling water bath for 10 min. After cooling down, samples with a volume of 10 μl each were loaded on the wells in SDS-polyacrylamide gels prepared by SDS-PAGE Preparation Kit. Staining and destaining of the gels were performed as previously described [39].

# Additional Files

Additional file 1: Fig. S1 Detailed diagram of engineering *B. subtilis* ATCC 6051a to *B. subtilis* 164S.

Additional file 2: Fig. S2. Key features of GFP expression plasmids pMK4-T7-gfp and pMK4-P43-gfp, respectively.

Additional file 3: Fig. S3 SDS-PAGE analysis of whole cell extracts.

Additional file 4: Table S1. Primers used in this work.

# Abbreviations

**LB:** Luria–Bertani; **BGSC**: Bacillus Genetic Stock Center; **GFP**: green fluorescent protein; **IPTG**: isopropyl-β-D-thiogalactopyranoside; **SDS-PAGE**: dodecyl sulfate, sodium salt (SDS)-polyacrylamide gel electrophoresis

# Declarations

### Authors' contributions

MHJ and JSS designed the research. MHJ performed most of the experiments. MHJ, SJL, YHL, HYW, QC, JPS, and JB helped with the experiment and discussion. MHJ, SJL and JSS analyzed the data and prepared the manuscript. All authors read and approved the final manuscript.

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### Competing interests

The authors declare that they have no competing interests.

### Availability of data and materials

All data generated and analyzed during this study are included in this published article and its additional files.

### Consent for publication

Not applicable.

### Ethics approval and consent to participate

Not applicable.

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# Figures

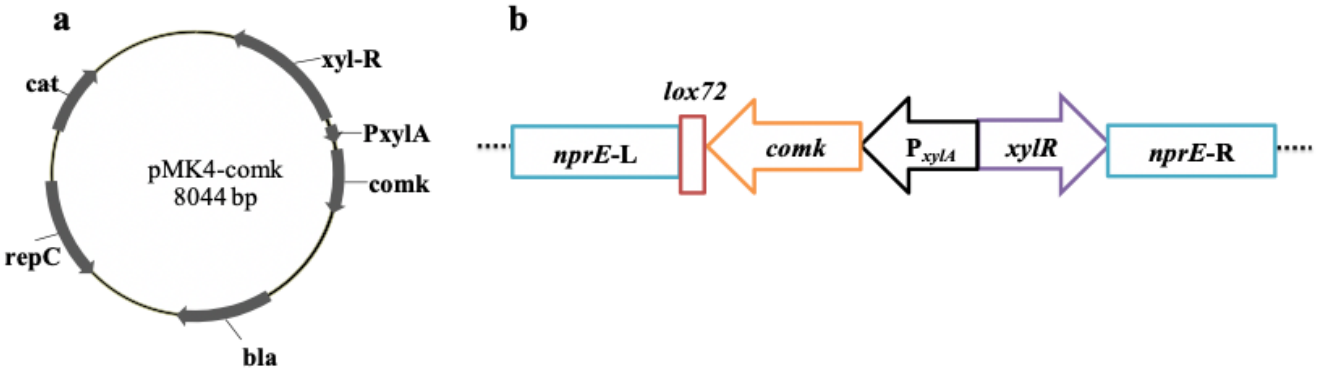
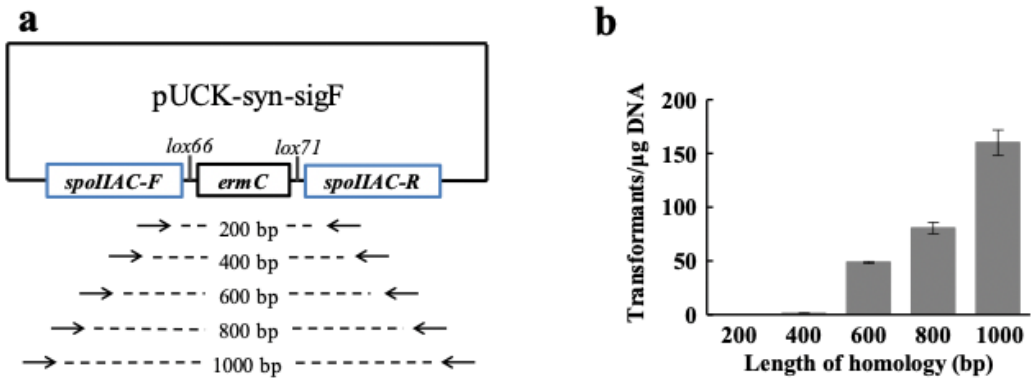


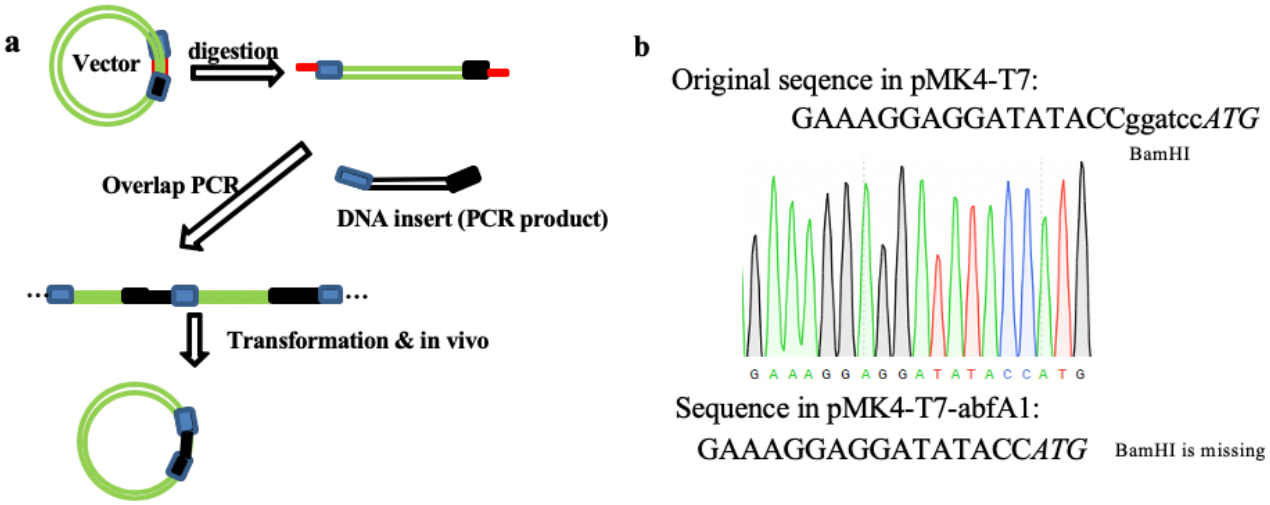

**Figure 1**

Genetic elements for converting B.subtilis ATCC 6051a to 164S. a. The plasmid map of pMK4-comk. b. Illustration of integrated comK cassette in the chromosome of 164S.



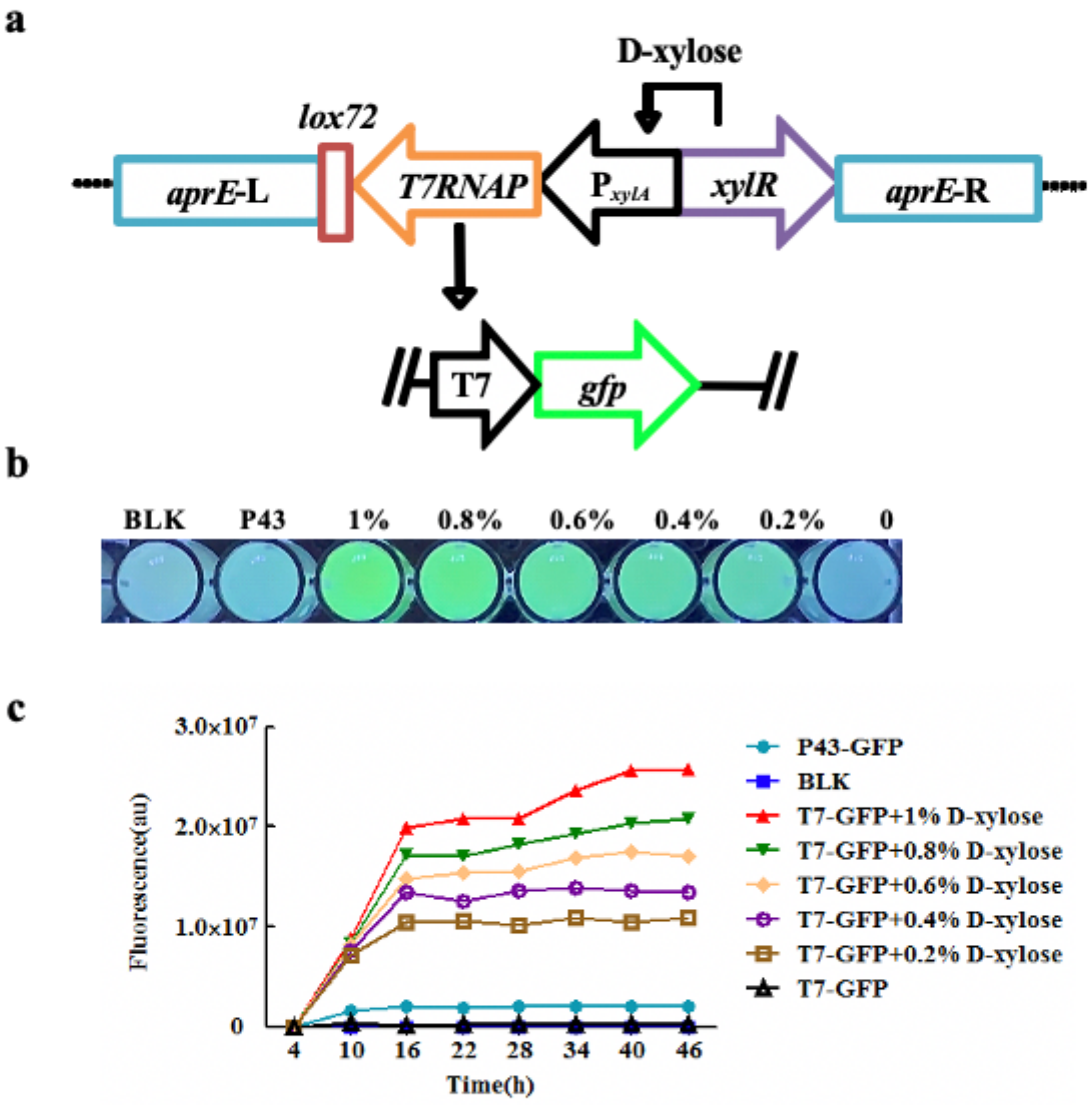

## Figure 2

In-frame deletion of spoII AC in B. subtilis 164S. a. Schematic demonstration of PCR preparation of linear DNAs for knockout of spoII AC. pUCK-syn-sigF, containing ermC and flanking regions of spoII AC, was used as the PCR template for PCR preparation of linear DNAs. b. Transformation efficiency calculated in experiments of spoII AC knockout in B. subtilis 164S using linear DNAs bearing homologous arms of different sizes.




## Figure 3

Simple and seamless cloning method applied in B.subtilis. a. Scheme of multimer preparation by overlap PCR that removes the residual sequences of restriction site used for linearization of the backbone vector. Red bar represents the restriction site; Blue and black bar represent the homologous sequences. b. The removal of BamHI was confirmed by DNA sequencing on constructed pMK4-T7-abfA1.




## Figure 4

Construction of D-xylose inducible T7 expression system in B. subtilis. a. The circuit design of D-xylose induced T7 expression system. b. Color recorded for the recombinant B. subtilis cultures expressing GFP under different concentration of D-xylose, in comparison with control group strain (164ST7P bearing

pMK4-P43-GFP) and BLK (164ST7P bearing pMK4-T7). c. GFP fluorescence measured using cultures grown in mediums containing different concentrations of D-xylose (164T7P bearing pMK4-T7-GFP), together with control group strain (164ST7P bearing pMK4-P43-GFP) and BLK (164ST7P bearing pMK4-T7).

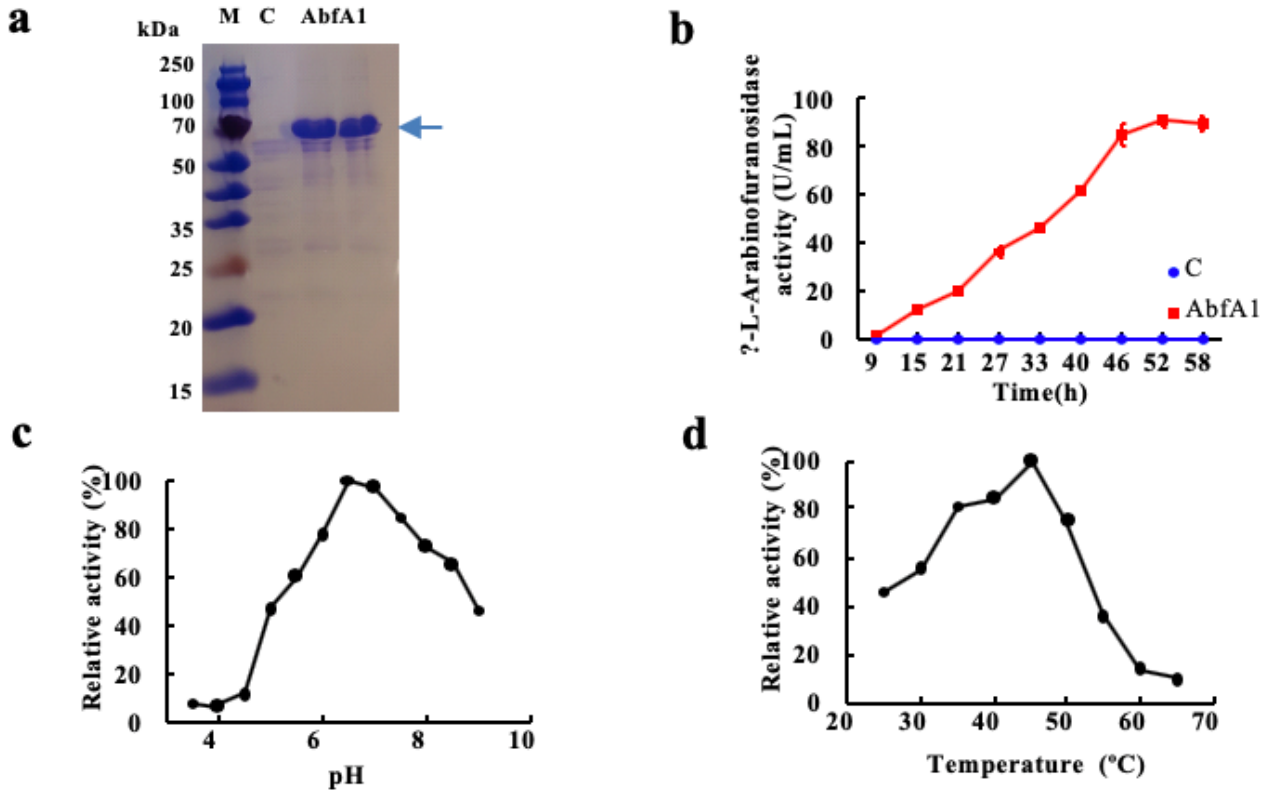



**Figure 5**

Heterologous production of α-L-arabinofuranosidase by the improved B. subtilis ATCC 6051a employing the T7 transcription system. a. The SDS-PAGE analysis of cell-free supernatant derived from broth cultures of 164T7P transformants. M: standard protein marker; C: control sample (164T7P bearing pMK4-T7); AbfA1: A164T7P bearing pMK4-T7-abfA1; b. Detected enzymatic activity in the broth culture of 164T7P transformants. C: control sample (164T7P bearing pMK4-T7); AbfA1: A164T7P bearing pMK4-T7-abfA1; Extracellular AbfA1 activity of B. subtilis clones; c. The effect of pH on the activity of purified AbfA1; d. The effect of the temperature on the activity of purified AbfA1.

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