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Engineering *Bacillus subtilis* ATCC 6051a for the production of recombinant catalases

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Abstract: Catalases are a large group of enzymes that decompose hydrogen peroxide to oxygen and hydrogen, and have been applied widely in numerous areas. *Bacillus subtilis* ATCC 6051a is a well-known host strain for high level secretion of heterologous peptides. However, the application of 6051a was seriously hampered by insufficient transformation efficiency. In this study, D-xylose inducible *comK* was integrated into the genome of *B. subtilis* ATCC 6051a, generating 164S, a mutant owns a transformation efficiency of 1 000-fold higher than its parent strain, thus allowing gene replacement by double crossover recombination using linear dsDNAs. The efficiency of the flanking arms for homologous recombination was then analyzed. We found that 400 bp was the minimal length of homologous fragments required to initiate efficient recombination in the 164S strain. In addition, DNA cassettes encoding two mesophilic catalases (Orf 2-62 and Orf 2-63) from *B. licheniformis* were integrated onto 164S. The catalytic properties of recombinant Orf 2-62 and Orf 2-63 were analyzed, and were found to be predominantly secreted into the fermentation broth, although they obviously lack any known secretory signal peptide. This work demonstrated that *B. subtilis* 164S is an excellent cell tool, not only for its superior secretion capacity, but also for its convenience in genetic modification.

Keywords: *Bacillus subtilis* ATCC 6051a, Transformation efficiency, Comk, Catalase

Introduction

Bacillus subtilis is a microorganism that has been classified as Generally Recognized As Safe and serves as a common microbial host for the production of enzymes that are utilized in the food-processing industry (Schallmey et al., 2004; van Dijl & Hecker, 2013). Due to its superior capability for transformation, *B. subtilis* strain 168, and its protease deficient mutants, are often selected for the laboratory production of proteins (Zeigler et al., 2008). However, these laboratory strains are inferior to less domesticated strains of *B. subtilis*, such as *B. subtilis* ATCC 6051a, both in terms of secretory capability and growth properties when cultivated in complex media (Jeong et al., 2015; Kabisch et al., 2013; Zhang et al., 2016). Nevertheless, the production of exogenous proteins in *B. subtilis* ATCC 6051a has rarely been reported; this is most probably due to our limited knowledge of gene manipulation in this strain.

Generally, the transformation of nondomesticated *Bacillus* strains can be partly solved by tedious laboratory work and by attempting a range of transformation protocols, such as weakening the cell wall or disturbing the fluidity of the cell membrane using glycine, DL-threonine (DL-Thr), or Tween 80 (Ito & Nagane, 2001; Peng et al., 2009; Zhang et al., 2011), or by modifying the methylation pattern of foreign DNAs in order to avoid the restrictions imposed by the native restriction–modification (R–M) system (Bai et al., 2018; Yasui et al., 2009; Zhang et al., 2011). Due to low transformation efficiency, traditional DNA replacement within a microbial genome needs to be performed using circular DNA in two rounds of selection. However, this practice is time consuming and very inefficient, especially when the target gene plays a role in cell growth or proliferation. In such cases, it is possible that a deletion strain may never be obtained following the second round of recombination events. In contrast, the linear DNA mediated-double crossover technique provides a rapid and efficient method for replacing DNA. However, for nondomesticated strains, it is likely that the transformation efficiency will never reach a level that is suitable for linear DNA-mediated recombination. Previous research has shown that the length of the homologous arms of linear DNA exerts significant effects on transformation efficiency (Melnikov & Youngman, 1999). However, it is difficult to perform studies with recalcitrant strains unless a fundamental change is made to the transformability of these strains.

Previous research published the sequence of the *B. subtilis* ATCC 6051a genome and compared this with the *B. subtilis* 168 genome (Jeong et al., 2015). This previous study revealed that the severely reduced competence of *B. subtilis* ATCC 6051a was likely to be caused by a frameshift mutation in *comP* that encodes a two-component sensor kinase that activates an operon that is related to the formation of competence in *B. subtilis* by turning on the downstream expression of ComK, a decisive regulator for the development of natural competence (Berka et al., 2002; Van Sinderen & Venema, 1994). Previous work has reported that the upregulation of the *comK* gene led to an increase in the transformation efficiency by almost 1000-fold (Rahmer et al., 2015; Shi et al., 2013; Zhang & Zhang, 2011); however, similar investigations have yet to be carried out for the 6051a strain.

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Table 1. The Bacterial Strains and Plasmids Used in This Study

Strain or plasmid	Description	Source/reference
B. subtilis		
1A976	Erm^R, *B. subtilis* 1A751 derivate, *lacA*::P_{xylA}-*comk*	Zhang and Zhang (2011)
6051a	Wild type *B. subtilis* strain	ATCC
164K	Km^R, *B. subtilis* 6051a derivate, *nprE*::P_{xylA}-*comk-lox71-kan-lox66*	This study
164S	6051a derivate, *nprE*::P_{xylA}-*comk*	This study
62CAT	164S derivate, *aprE*::P_{holin}-*orf 2–62*	This study
63CAT	164S derivate, *aprE*::P_{holin}-*orf 2–63*	This study
E. coli		
DH5*α*	Cloning strain	Commercially available
BL21(DE3)	Expression strain	Commercially available
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 study
pGSNE	Amp^R, integration plasmid carrying *comk* cassette and fragments for *nprE* deletion	This study
pGSNE-comk	pGSNE carrying *comk* cassette containing P_{xylA}-*comk* and erythromycin resistance gene *ermC* fused between *lox71* and *lox66*	This study
pGSNE-comk	Amp^R, integration plasmid carrying *comk* cassette containing *nprE* homologous arm	This study
pDGC	Amp^R, Km^R, integration plasmid pDG148 containing *cre* behind the promoter Pspac	Yan et al. (2008)
pMK4-cre	Shuttle vector pMK4 with *cre* under control of Pspac	This study
pUCK-syn-sigF	Amp^R, plasmid for *spoIIAC*-knock-out	This study
pMD19T (Smiple)	Amp^R, plasmid for TA cloning	TaKaRa
pTA-aea (pMD19T-aea)	Amp^R, plasmid for *aprE*-knock-out	This study
pMK4-Pholin-gfp	Shuttle vector pMK4 with *gfp* under control of P_{holin}	Lab stock
pTA-aeaP	Amp^R, plasmid for integrative expression carrying promoter P_{holin}	This study
pTA-aeaP-62cat	Amp^R, plasmid pTA-aeaP carrying *orf cat62*	This study
pTA-aea-63cat	Amp^R, plasmid pTA-aeaP carrying *orf cat63*	This study

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

Catalase (EC 1.11.1.6, CAT) is an enzyme that is widely distributed in a range of different organisms. This enzyme protects living cells from the damage and destruction caused by the production of reactive oxygen species; catalase achieves this by catalyzing the dismutation of hydrogen peroxide (H_2O_2) into water and oxygen (Aebi, 1974). Thus, CATs have the ability to remove excess H_2O_2, a widely used oxidizer in numerous industrial processes. Therefore, CATs are used to dislodge the remaining H_2O_2 after bleaching in the textile and paper industries (Shi et al., 2008). In the food industry, CATs are applied in food processing to prevent oxidation. CATs have been used as active ingredients for medical disinfection, therapeutic applications, they can also be used as biosensors (Philibert et al., 2016). CATs could be applied in a wide range of different circumstances and environments. Consequently, there is an urgent need to be able to synthesize a wider range of CATs to meet market demand. Previous research has demonstrated that *B. subtilis* catalase 1 (KatA) can be secreted into a culture medium without a signal peptide and was subsequently characterized as a nonclassically secreted protein (Naclerio et al., 1995; Wang et al., 2016). Previous research, using shuttle vectors, led to the successful overexpression of *B. subtilis* CAT KatA and KatX2 in *B. subtilis* WB600 and *B. subtilis* 168 strains (Philibert et al., 2016). However, the heterologous expression of foreign peptides based on extra-genomic DNA molecules is genetically unstable; the loss of plasmids from host cells tends to occur frequently (Bron et al., 1991).

In the present study, we constructed a high-efficiency ATCC 6051a mutant (164S) by integrating a copy of *comk* cassette into the genome. Then, we investigated the length requirement for effective double crossover recombination using *B. subtilis* 164S. Finally, two catalases were cloned from *Bacillus licheniformis*, a thermostable strain of bacteria, and expressed in *B. subtilis* 164S using a constitutive promoter acquired from a Bacillus phage.

Materials and Methods

Bacterial Strains, Plasmids, Primers, and Growth Conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *B. subtilis* 6051a was acquired from ATCC. Our plasmid cloning work involved *Escherichia coli* DH5*α*. The primers used in this study are listed in Supplementary Table S1. All of the strains used in this study were cultivated in Luria–Bertani (LB) medium at 37°C and 200 rpm. When required, 100 μg ml^{-1} of ampicillin, 10 μg ml^{-1} of erythromycin, 20 μg ml^{-1} of kanamycin, 10 μg ml^{-1} of chloramphenicol or 10 g l^{-1} of D-xylose, were supplemented into the culture medium; 2% agar was also added to the liquid medium in order to prepare solid medium.

DNA Manipulation and Reagents

DNA synthesis and sequencing were performed by Sangon Biotech Co., Ltd. (Shanghai, China). Unless otherwise indicated, the plasmids were constructed with a ClonExpress® II One Step Cloning Kit (Vazyme Biotech Co., Ltd. Nanjing, China). A TA cloning kit was purchased from TaKaRa Biotechnology (Dalian) Co., Ltd. Restriction enzymes and DNA ligase were purchased from Thermo Fisher Scientific Inc., USA. High-fidelity DNA polymerase 2 × Phanta® Master Mix was purchased from Vazyme Biotech. The PAGE Gel Fast Preparation Kit was purchased from EpiZyme Biotech (Shanghai).

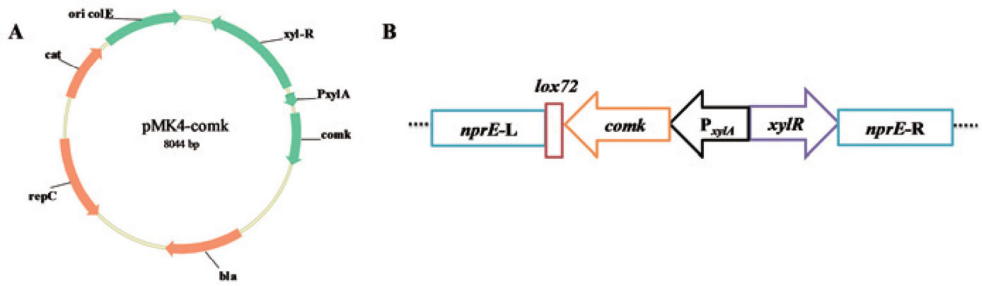

Fig. 1. The elements used to improve the transformation efficiency of *B. subtilis* ATCC 6051a. (A) Key features of plasmid pMK4-comk. (B) The genotype of *B. subtilis* 164S. The native *nprE* (originally between *nprE*-L and *nprE*-R) was replaced by an introduced P_{xylA}-*comk* cassette.

Construction of *B. subtilis* 164S

The P_{xylA}-comk cassette was amplified by polymerase chain reaction (PCR) using the genomic DNA of *B. subtilis* 1A976 (Zhang & Zhang, 2011) as a template and a specific primer pair (Comk-F/Comk-R). The PCR product was digested with *PstI* and ligated into the corresponding site of the pMK4 plasmid to generate a pMK4-comk construct, which was then transformed into *B. subtilis* ATCC 6051a using a method described previously (Xue et al., 1999). The pMK4-comK construct was then transformed into the 6051a strain in an attempt to increase the efficiency of its integration; the generated transformant was referred to as 6051a/pMK4-comK. We then chemically synthesized a DNA fragment, P_{xylA}-*comk-lox71-kan-lox66*, which was subsequently digested with *KpnI/NheI* and ligated into the integration vector, pGSNE (previously digested with the same enzymes) to generate a pGSNE-comk construct. The pGSNE construct contains DNA fragments flanking the operon of *nprE* (encoding neutral protease) derived from *6051a*, that enables homologous recombination and leads to the null deletion of *nprE* and the insertion of the target DNA carried by pGSNE. Following the transformation of *NcoI*-linearized pGSNE-comK into 6051a/pMK4-comK, we found that the colonies obtained were resistant to kanamycin and chloramphenicol. These colonies were validated by colony PCR, demonstrating the presence of the *comk* cassette at the *nprE* locus. The mutant 6051a was then cultivated, passaged through two generations in LB without antibiotic, diluted, and then spread onto plain LB plates. Colonies were subsequently screened for their sensitivity to chloramphenicol to check whether the plasmid pMK4-comk was functional or not. The plasmid-free mutant was designated as *B. subtilis* 164 K. To further eliminate the *kan* cassette from 164 K, a Cre/Lox recombination system was employed (Yan et al., 2008). First, a DNA cassette containing a *cre* fragment under the control of the Pspac promoter, was prepared using pDGC (Yan et al., 2008) as a template a specific primer pair (Cre-F/Cre-R). The PCR product was then digested with *EcoRI* and ligated into the pMK4 plasmid to generate a pMK4-cre construct. Then, the vector was transformed into a previously constructed 164 K (Supplementary Fig. S1); this elicited the homologous recombination of *loxP* sites in 164 K upon the expression of Cre recombinase and resulted in the elimination of *kan* from the chromosome. Next, a vector cure procedure was performed to remove pMK4-cre from the transformants. In brief, a single colony of the recombinant strain was cultivated at 37°C and 200 rpm; the growing culture was passaged every 4 hr by diluting a 30 μl aliquot into 3 ml of fresh LB without antibiotics. After three passages, 200 μl of the final culture was diluted and plated onto LB plates without antibiotics, and incubated at 37°C overnight. The colonies formed were then screened for a loss in resistance against chloramphenicol and kanamycin. The resultant recombinant strain that was sensitive to both chloramphenicol and kanamycin was further confirmed using PCR, and designated as *B. subtilis* 164S.

Table 2. The Efficiency of Linear DNA-Mediated Transformation in *B. subtilis* 164S

Integration loci	Length of homology (bp)		Transformation efficiency[a]
	Upstream	Downstream	
spoII AC	1010	1012	1.6×10^2
aprE	619	600	0.9×10^2
amyE	815	807	1.3×10^2
srfAC	928	1008	0.7×10^2

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

Preparation of Linear DNAs for Gene Replacement in *B. subtilis*

Numerous linear DNAs were prepared by PCR for transformation. These contained a resistance gene as a selective marker and surrounded by DNA fragments for homologous recombination (Shevchuk et al., 2004; Yan et al., 2008). For example, *aprU-erm-aprD* was used for *apr* (encoding alkaline protease in the strain) deletion in 6051a. First, *ermC* and flanking homologous fragments (*aprU* and *aprD*) needed to be prepared by PCR. *ermC* was amplified using the vector pUCK-syn-sigf as a template and specific primer pairs (Erm-F/Erm-R). In a similar manner, specific primer pairs (ApU-F/ApU-R and ApD-F/ApD-R) were used to prepare *aprU* and *aprD*. Then, the PCR products were pooled together so that we could perform an overlapping PCR. This is because the products contained ending sequences that were homologous to each other. This PCR was carried out using 2 × Phanta® Master Mix and a specific primer pair (ApU-F/ApD-R). The obtained PCR products were digested with *DpnI* and then purified by the Axygen DNA purification kit. Similarly, we also prepared linear DNAs for the gene replacement of *spoII AC*, *amyE* or *srfAC*. The primers used for these reactions are given in Supplementary Table S1.

Quantitative Transformation Efficiency Assay for *B. subtilis*

The transformation of *B. subtlilis* 164S was performed using established methods but applied with minor modifications (Zhang & Zhang, 2011). In brief, 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-

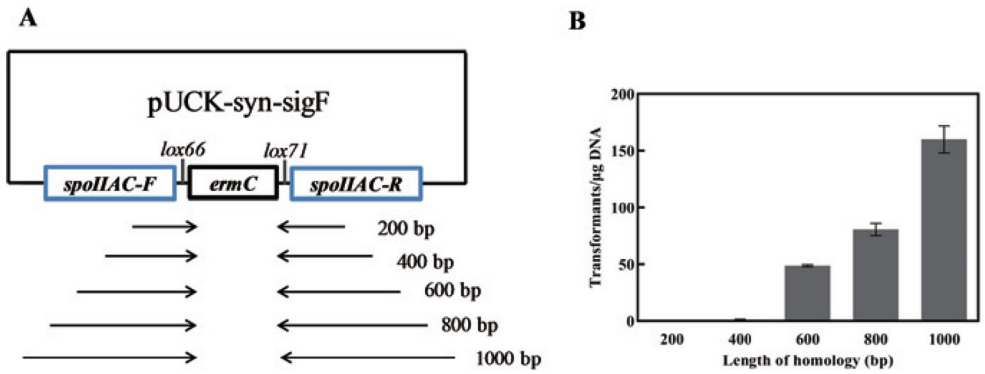

Fig. 2. The in-frame deletion of *spoII AC* in *B. subtilis* 164S. (A) Schematic demonstration of the PCR preparation of linear DNAs for the knockout of *spoII AC*. The pUCK-syn-sigF plasmid, containing an *ermC* and flanking regions for *spoII AC*, were used as the PCR template for the preparation of linear DNAs; the sizes of the flanking fragments varied from 200 to 1 000 bp. (B) Transformation efficiency in experiments involving *spoII AC* knockout in *B. subtilis* 164S using linear DNAs bearing homologous arms of different sizes.

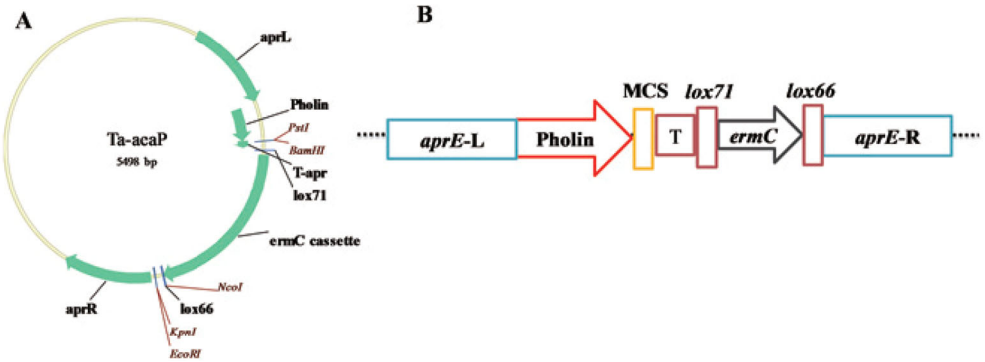

Fig. 3. The integrative Ta-aeaP plasmid. (A) The key features of plasmid Ta-aeaP. (B) A schematic showing the integrative elements of plasmid Ta-aeaP; *aprE*-L and *aprE*-R represent homologous fragments upstream and downstream of *aprE*, respectively; Pholin represents the promoter that was used to drive the expression of holing; T represents the terminator of *aprE*; *ermC* represents the erythromycin-resistant gene expression cassette.

xylose. This was then incubated at 37°C on a rotary shaker for 1.5 hr, or until the OD_{600} reached ~1.0. Then, 100 μl of fresh culture was aliquoted for transformation by mixing with ~100 ng of linear or circular DNA. The mixture of DNA and cells were then incubated at 37°C with rotary shaking for 2 hr before spreading onto LB agar plates with an appropriate antibiotic.

Construction of Catalase-Producing Strains

A three-fragment dsDNA, including the upstream and downstream regions of *aprE* and a copy of the *ermC* cassette, was prepared by overlapping PCR; the product obtained was then inserted into pMD19T via TA cloning, thus generating a pTA-aea construct (pMD19T-aea). This construct was then used as a backbone plasmid for the cloning of catalase genes, as well as P_{holin}, which was then amplified by PCR using the Pho-F/Pho-R primer pair and pMK4-Pholin-gfp as the template. The P_{holin} fragment was cloned into pTA-aea at the *PstI* restriction site using ClonExpress® II One Step Cloning Kit; this generated a pTA-aeaP construct. Next, we amplified two catalase encoding fragments, *orf 2-62* and *orf 2-63*; these were amplified using genomic DNA from *B. licheniformis* J-bac as the template and the 62-F/62-R and 63-F/63-R primer pair. The PCR products were then inserted into the pTA-aeaP construct between the *PstI* and *BamHI* restriction sites using the ClonExpress® II One Step Cloning Kit. The plasmids generated were named as pTA-aeaP-62cat and pTA-aeaP-63cat, respectively. pTA-aeaP-62cat and pTA-aeaP-63cat were then either digested by *PciI* or *BsaI*, to produce linearized dsDNAs for transformation into 164S. The transformants were selected by erythromycin-containing LB plates. A specific primer pair (62-F/62-R or 63-F/63-R) was used for PCR in order to confirm that the heterologous genes had been inserted appropriately. Finally, the transformants were transformed with pMK4-cre; this induced the elimination of the erythromycin resistance gene, thus generating *B. subtilis* 62CAT and *B. subtilis* 63CAT.

Production and Characterization of Recombinant Catalases

Single colonies of *B. subtilis* 62CAT and 63CAT were inoculated into 50 ml of LB broth in 250 ml flasks and cultivated for 60 hr at 37°C with gentle shaking at 200 rpm. The cultures were sampled at different incubation times and used for enzymatic assays. In order to prepare cell free supernatants, the collected samples were centrifuged at 4 000 × *g* for 10 min at 4°C. Collected cells were washed twice with 50 mM phosphate buffer saline (PBS, pH 8.0) and lysed by ultrasound sonication on ice for 10 min with a 6 s interval and 3 s of ultrasonication. The lysates were then centrifuged at 6 000 × *g* for 10 min at 4°C, thus creating cytoplasmic samples.

Next, we assayed the catalase activity of recombinant Orf 2-62 or Orf 2-63 in both the cytoplasm and supernatant, as described previously (Philibert et al., 2016; Shi et al., 2008). In brief, we used a spectrophotometer to monitor the amount of H_2O_2 that could be decomposed by the catalase added in the

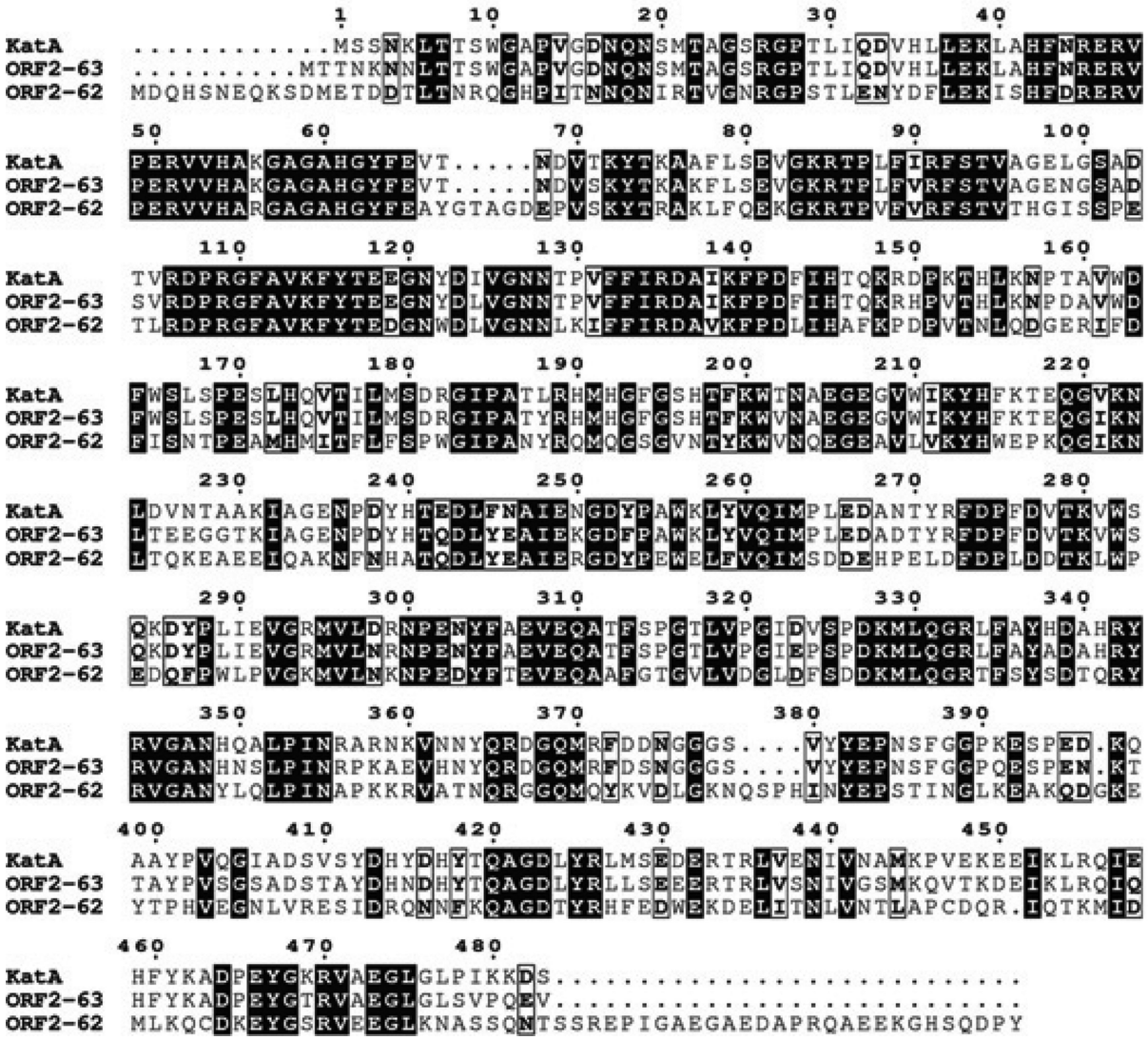

Fig. 4. Multiple sequence alignment of catalases from *B. licheniformis* J-bac with KatA. Sequences were aligned using Multalin (http://multalin.toulouse.inra.fr/multalin/multalin.html), and the image was created using ESPript (http://espript.ibcp.fr/ESPript/ESPript/).

reaction mixture; this was detected by measuring the absorbance at 240 nm. One unit of catalase activity was defined as the amount that degrades 1 μmol of H_2O_2 per min at a pH of 8.0 and 37°C. The reaction mixture contained 50 mM of PBS buffer (pH 8.0), 10 mM H_2O_2, and 0.1 ml of the enzyme solution, in a total volume of 3.0 ml. The effect of pH and temperature on the activity of recombinant catalase was analyzed across a wide range of temperature (25–65°C) and pH range (4.5–9.0). Sodium citrate, PBS, and Tris–HCl buffer, were used to create pH ranges of 4.5–5.5, 6.0–8.0, and 8.5–9.0, respectively. Protein samples were tested by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Results and Discussion

Improving the Transformation Efficiency of *B. subtilis* ATCC 6051a

Traditionally, genetic manipulation has been carried out by homologous recombination mediated by circular plasmids. However, double crossover techniques, using double-strand linear DNAs (dsDNAs), provide us with a much simpler option for genomic engineering in prokaryotic cells that is much more efficient and less labor intensive than more traditional methodology. However, dsDNA-mediated recombination events require much higher levels of transformation efficiency. For this reason, it was a significant challenge to perform one-step gene replacement in *B. subtilis* ATCC 6051a. Alignment analysis using the genomic sequences of *B. subtilis* ATCC 6051a and *B. subtilis* 168 revealed a frameshift mutation (from AA to A) that led to the aberrant functional ability of ComP in *B. subtilis* ATCC 6051a, thus s reducing the subsequent cellular expression of level of ComK (Jeong et al., 2015); this is one of the key activators modulating the natural competence of *Bacillus*. Previous work has confirmed that the overexpression of ComK can improve transformation efficiency (Rahmer et al., 2015; Shi et al., 2013; You et al., 2012; Zhang & Zhang, 2011). Thus, we intended to introduce the homologous expression of ComK in the 6051a strain by placing a copy of the native *comk* gene into the genome of 6051a under the control of P_{xylA}, a D-xylose inducible promoter from *B. megaterium* (Zhang & Zhang, 2011). The mediation of recombination by linear DNA would be preferable for the genomic integration of the *comk* cassette; however, this could not be accomplished directly due to the low transformation efficiency

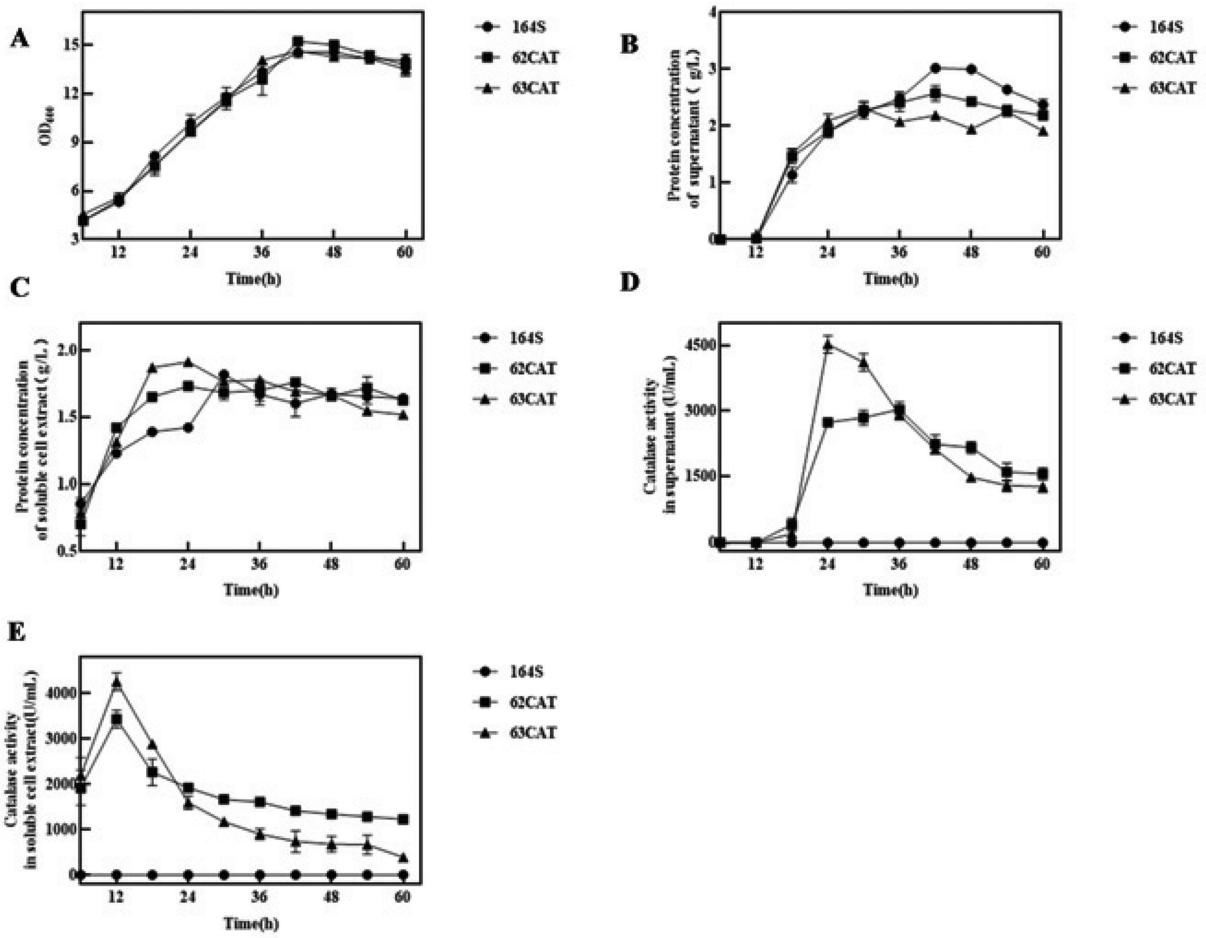

Fig. 5. The heterologous expression of *B. licheniformis* catalases in *B. subtilis*. (A) Cell growth curve of *B. subtilis* 164S, 62CAT, and 63CAT. (B) Measurement of the protein concentrations of cell-free supernatant samples from164S, 62CAT, and 63CAT. (C) Measurement of soluble cell extract samples. The protein concentrations were detected by Modified BCA Protein Assay Kit purchased from Sangon Biotech (Shanghai) Co., Ltd. (D) Measurement of the enzymatic activities in supernatant samples from 164S, 62CAT, and 63CAT. (E) Measurement of enzymatic activity in soluble cell extract samples. Error bars represent standard deviation from the mean value of triple experiments.

of these cells. In order to solve this problem, pMK4-comK, a self-replicating plasmid expressing ComK, was constructed and transformed into the 6051a (as shown in Fig. 1A; further details are shown in Supplementary Fig. S1). With improved transformation efficiency, a linear *comk* cassette, along with a kanamycin resistance gene (*kan*), was successfully integrated into the genome at the locus of the native *nprE* in the 6051a strain (Fig. 1B). To remove the selective marker (*kan*), a plasmid expressing Cre integrase was transformed into the *Bacillus* mutant (Supplementary Fig. S1), leading to a marker-free strain; this was referred to as *B. subtilis* 164S.

Double Crossover Efficiency, as Triggered by Linear dsDNAs in *B. subtilis* 164S

First, we checked the transformation efficiency of the circular plasmid by transforming 1.0 μg of pMK4 (circular DNA; 5585 bp in length) into competent *B. subtilis* 164S cells. We found that the presence of D-xylose in the medium during the preparation of 164S competent cells led to an increase in the transformation efficiency (up to 3 × 10^4 transformants per μg of DNA). This represented more than a 1 000-fold increase in efficiency compared with the parent strain (Supplementary Fig. S2). Next, to determine the recombination efficiency by double crossover, we used fusion PCR to prepare a range of linear DNAs with varying sizes of homologous fragments that flanked four different open reading frames (encoding *spoII AC*, *aprE*, *amyE*, and *srfAC*, respectively, Table 2) (Shevchuk et al., 2004; Yan et al., 2008). We then counted the numbers of transformants obtained after transformation into 164S or 6051a using each linear DNA. As shown in Table 2, the number of 164S transformants, across all experiments, fell into the 70–160 range. In contrast, the same linear DNA produced no transformants in the 6051a strain. Previous studies postulated that the minimal length of DNA fragments required for homologous recombination was approximately 400–500 bp (Dubnau, 1993; Yan et al., 2008). However, this has never been confirmed with industrial strains of *Bacillus*, such as 6051a. To investigate the effect of the size of the homologous flanking region on recombination efficiency, we prepared a range of linear DNAs for the in-frame deletion of *spoII AC* with different sizes of homologous arms (200–1 000 bp, respectively, Fig. 2A); our aim was to use these DNAs for gene replacement in 164S. As presented in Fig. 2B, linear DNA-mediated transformations in 164S were dependent on DNA size; longer DNA arms evoked much higher levels of recombination efficiency in 164S. The minimal length of DNA to initiate an efficient DNA exchange event was approximately 400 bp; this concurs with previous findings (Yan et al., 2008). Nevertheless, we found that the transformation efficiency was approximately 100-fold higher

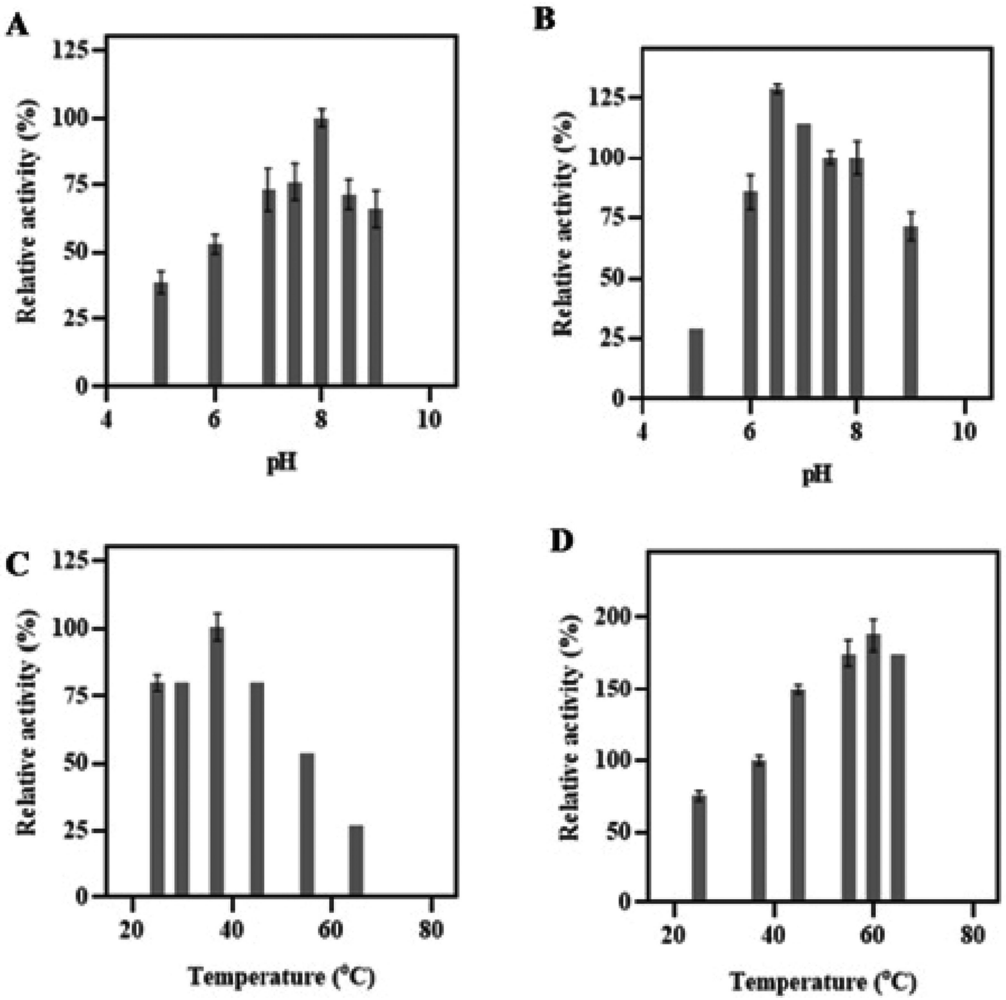

Fig. 6. Temperature and pH curves for Orf 2-62 and Orf 2-63 recombinant catalases. The pH properties of recombinant Orf 2-62 (A) and Orf 2-63 (B); 100% enzyme activity was set at pH 8.0. The temperature properties of recombinant Orf 2-62 (C) and Orf 2-63 (D); 100% enzyme activity was set at 37°C. Error bars represent standard deviation from the mean value of triple replicates.

when using DNAs with 1 000 bp of homologous arms when compared with arms that were 400 bp in length (Fig. 2B).

Construction of Catalase-Expressing Strains

We constructed Ta-aeaP (Fig. 3A) and used this as a template for the preparation of linear dsDNAs that were subsequently used for the integration of DNA inserts at the locus of *aprE* (encoding the ORF of *B. subtilis* alkaline protease). In this strategy, *aprL* and *aprR* were used for homologous integration, Ta-aeaP contained an artificial promoter, P_{holin}, which was a modification of a promoter that drives the expression of viral holin within the *B. subtilis* prophage region of Φ105 (Armentrout & Rutberg, 1971; Leung & Errington, 1995). Fig. 3B demonstrates the linear structure of Ta-aeaP; this also contained an MCS (multiple cloning site) that was designed for the cloning of target genes.

In thermophilic strain *Bacillus licheniformis* J-bac, five catalase homologs were found (data not shown). Orf 2-62 and Orf 2-63 are two of them that are closer to KatA (a vegetative catalase in *B. subtilis* 168). The sequence homology between Orf 2-63 and KatA is 86.5%, while the similarity between Orf 2-62 and KatA is 46.8% (Fig. 4). In consideration expression and folding efficiency, *orf 2-62* and *orf 2-63* were constructed for heterologous expression. Previous research has shown that *B. subtilis* KatA is a typical nonclassical secretory protein (Wang et al., 2016). Therefore, both catalase genes were directly cloned downstream of P_{holin}, without a secretory peptide encoding sequence upstream of these genes.

Expression of Heterologous Catalases in *B. subtilis*

The Ta-aeaP-62cat and Ta-aeaP-63cat (Supplementary Fig. 3A, B) constructs were engineered and used to generate recombinant strains expressing the two forms of catalase. The *B. subtilis* 164S, 62CAT and 63CAT were cultivated in shaking flasks, and their growth behavior and enzymatic profiles were monitored. The samples of whole-cell extracts or cell-free supernatants were prepared for SDS-PAGE analysis (Supplementary Fig. S4). It has to be noted that the total intrinsic activity of native catalases from *B. subtilis* 164S was found to be very low in our assays (<10 U/ml, data not shown). Both forms of catalase (Orf 2-62 and Orf 2-63) were detected in both whole cell extracts and supernatants (Supplementary Fig. S4), thus indicating that both enzymes were secreted from cells, at least to some extent. To gain further insight into the relative amounts of enzymes secreted from the cells, we examined the cell growth and analyzed the enzymatic activities of the enzymes both in and outside of cells (Fig. 5). As can be seen, the growth profiles of *B. subtilis* 164S, 62CAT, and 63CAT

were similar to each other (Fig. 5A). Protein levels were mostly similar, while at some time points, protein concentrations were found to be different, not only in supernatant (Fig. 5B) but also in soluble cell extracts (Fig. 5C). Interestingly, the catalase activity profile in both supernatants and soluble cell extracts did not match with their corresponding profiles related to protein concentrations (Fig. 5D, E). As seen in Fig. 5D, during the initial 12 hr period of fermentation, there was almost no secretion of catalase. However, the maximal activity of the recombinant catalases, both from *B. subtilis* 62CAT and 63CAT, appeared at earlier growth stage, 24 hr in supernatant samples and 12 hr in soluble cell extracts, indicating that the recombinant catalases were quickly accumulated inside cells and later secreted out of the cells in a nonclassical pathway. The maximal activity of recombinant Orf 2-62 in the supernatant reached a maximum of 3033.5 ± 120.6 U/ml at 36 hr. In contrast, the activity of Orf 2-63 in the culture supernatant peaked at 24 hr, and reached a maximum of 4523.4 ± 143.6 U/ml (Fig. 5D). Finally, the maximum activity of total Orf 2-62 reached 4648.6 ± 99.8 U/ml at 36 hr, while the maximum activity of Orf 2-63 was 6122.0 ± 246.8 U/ml at 24 hr.

Next, we carried out further analysis on the Orf 2-62 and Orf 2-63 recombinant enzymes (Fig. 6). The optimum pH of Orf 2-62 was pH 8.0, with an optimal temperature of 37°C. This enzyme was active between pH 6.0 and pH 9.0 with a temperature range of 25–50°C (>50% of maximum activity retained). Orf 2-63 exhibited maximal activity at pH 6.5 at 60°C and was active between pH 6.0 and pH 9.0, with a temperature range of 37–65°C (>50% of maximum activity retained). Thus, Orf 2-62 and Orf 2-63 covered a wide range of temperatures, thus highlighting the fact that a thermophile may adapt to temperature changes in a highly flexible manner by combining peptide pools and mesophilic/thermophilic isozymes. In this way, these bacteria are able to deal with changing temperatures in certain niches. From a practical point of view, the combination of Orf 2-62 and Orf 2-63 provides us with a much broader capability of deploying these enzymes in a range of applications, including industries related to environmental treatment, food or chemicals.

Conclusion

In the present study, we successfully converted an industrial strain of *B. subtilis* by integrating a copy of the comK cassette into the bacterial genome. We found that the transformation efficiency of the generated strain (164S) was 1 000-fold higher than the parent strain (*B. subtilis* ATCC 6051a). Systematic analysis then revealed that the minimal size of the related homologous fragments needed to be at least 400 bp in length in order to initiate a linear dsDNA-mediated gene replacement event in *B. subtilis*. The engineered *B. subtilis* strain was then used for the heterologous expression of two catalases from a thermophilic *B. licheniformis*. Both catalases were efficiently secreted into the medium during fermentation even though they lacked any known secretion signal. Orf 2-62 was identified as the most active enzyme at 37°C, even though it was derived from a thermophilic strain of *Bacillus*, thus reflecting the fact that microorganisms may have evolved and taken advantage of isozymes that can adapt to different temperatures and pH ranges, and therefore have better chances of survival in the natural environment.

Supplementary Material

Supplementary material is available online at *JIMB* (*www.academic.oup.com/jimb*).

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Conflict of Interest

The authors declare no conflict of interest.

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