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 *comK* 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 1,000-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.
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\textsubscript{2}O\textsubscript{2}) into water and oxygen (Aebi, 1974). Thus, CATs have the ability to remove excess H\textsubscript{2}O\textsubscript{2}, a widely used oxidizer in numerous industrial processes. Therefore, CATs are used to dislodge the remaining H\textsubscript{2}O\textsubscript{2} after bleaching in the textile and paper industries (Shi et al., 2008). However, the heterologous expression of foreign peptides led to the successful overexpression of KatA, 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 DH5α. 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\textsuperscript{-1} of ampicillin, 10 μg ml\textsuperscript{-1} of erythromycin, 20 μg ml\textsuperscript{-1} of kanamycin, 10 μg ml\textsuperscript{-1} of chloramphenicol or 10 g l\textsuperscript{-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).

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

| Strain or plasmid | Description | Source/reference |
|-------------------|-------------|------------------|
| B. subtilis 1A976 | Erm\textsuperscript{R}, B. subtilis 1A751 derivate, lacA: P\textsubscript{spac}-comk | Zhang and Zhang (2011) |
| 6051a | Wild type B. subtilis strain | ATCC |
| 164K | Km\textsuperscript{R}, B. subtilis 6051a derivate, nprE: P\textsubscript{spac}-comk-lox71-kan-lox66 | This study |
| 164S | 6051a derivate, nprE: P\textsubscript{spac}-comk | This study |
| 62CAT | 164S derivate, aprE-P\textsubscript{halin}-orf 2–62 | This study |
| 63CAT | 164S derivate, aprE-P\textsubscript{halin}-orf 2–63 | This study |
| E. coli DH5α | Cloning strain | commercially available |
| BL21(DE3) | Expression strain | commercially available |
| Plasmid pMK4 | E. coli–B. subtilis shuttle vector, Amp\textsuperscript{R} for E. coli; Cm\textsuperscript{R} for B. subtilis | BGSC |
| pMK4-comk | comk cloned behind P\textsubscript{spac} promoter in pMK4 | This study |
| pGSNE | Amp\textsuperscript{R}, integration plasmid carrying comk cassette and fragments for aprE deletion | This study |
| pGSNE-comk | pGSNE carrying comk cassette containing P\textsubscript{spac}comk and erythromycin resistance gene ermC fused between lox71 and lox66 | This study |
| pGSNE-comk | Amp\textsuperscript{R}, integration plasmid carrying comk cassette containing nprE homologous arm | This study |
| pDGC | Amp\textsuperscript{R}, Km\textsuperscript{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\textsuperscript{R}, plasmid for spoilAC-knock-out | This study |
| pMD19T (Smiple) | Amp\textsuperscript{R}, plasmid for TA cloning | TaKaRa |
| pTA-aea (pMD19T-aea) | Amp\textsuperscript{R}, plasmid for aprE-knock-out | This study |
| pMK4-Pholin-gfp | Shuttle vector pMK4 with gfp under control of P\textsubscript{halin} | Lab stock |
| pTA-aeaP | Amp\textsuperscript{R}, plasmid for integrative expression carrying promoter P\textsubscript{halin} | This study |
| pTA-aeaP-62cat | Amp\textsuperscript{R}, plasmid pTA-aeaP carrying orf cat62 | This study |
| pTA-aeaP-63cat | Amp\textsuperscript{R}, plasmid pTA-aeaP carrying orf cat63 | This study |

Erm\textsuperscript{R}, erythromycin resistance; Km\textsuperscript{R}, kanamycin resistance; Amp\textsuperscript{R}, ampicillin resistance; Cm\textsuperscript{R}, chloramphenicol resistance.
Construction of B. subtilis 164S

The Pspac-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, PGSNE-comK-lox71-knu-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 Ncol-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, passed 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 knu 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 Pspsac 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 knu 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.

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 (AprU-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. subtilis 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-
xylose. This was then incubated at 37°C on a rotary shaker for 1.5 hr, or until the OD<sub>600</sub> 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 <i>aprE</i> and a copy of the <i>ermC</i> 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 <i>P</i><sub>holin</sub>, which was then amplified by PCR using the Pho-F/Pho-R primer pair and pMK4-Pholin-gfp as the template. The <i>P</i><sub>holin</sub> 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, <i>orf 2-62</i> and <i>orf 2-63</i>; these were amplified using genomic DNA from <i>B. licheniformis</i> 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 <i>B. subtilis</i> 62CAT and <i>B. subtilis</i> 63CAT.

### Production and Characterization of Recombinant Catalases

Single colonies of <i>B. subtilis</i> 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<sub>2</sub>O<sub>2</sub> 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://esprit.ibcp.fr/ESPrpt/ESPrpt/).

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$_2$O$_2$ 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$_2$O$_2$, 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* ATCC6051a 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 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 from 164S, 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 $3 \times 10^6$ transformants per μg of DNA). This represented more than a 1000-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 spoI 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 spoI AC with different sizes of homologous arms (200–1000 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...
when using DNAs with 1 000 bp of homologous arms when com-
pared 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 nonclassi-
cal 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 recombi-
nant 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 in-
sight into the relative amounts of enzymes secreted from the cells, we examined the cell growth and analyzed the enzymatic activi-
ties 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 proteins related to protein concentrations (Fig. SD, E). As seen in Fig SD, 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. SD). 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 1000-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.

**References**

Aebi, H. (1974). Catalase. In Methods of enzymatic analysis (pp. 673–684). Elsevier. https://doi.org/10.1016/B978-0-12-091302-2.00032-3

Armentrout, R. W. & Rutberg, L. (1971). Heat induction of prophage ϕ105 in Bacillus subtilis: Replication of the bacterial and bacteriophage genomes. *Journal of Virology*, 8(4), 455–468. https://doi.org/10.1128/JVI.8.4.455-468.1971

Bai, H., Deng, A., Liu, S., Cui, D., Qui, Q., Wang, L., Yang, Z., Wu, J., Shang, X., Zhang, Y., & Wen, T. (2018). A novel tool for microbial genome editing using the restriction-modification system. *ACS Synthetic Biology*, 7(1), 98–106. https://doi.org/10.1021/acssynbio.7b00254

Berka, R. M., Hahn, J., Albano, M., Draskovic, I., Persuh, M., Cui, X. J., Sloma, A., Widner, W., & Dubnau, D. (2002). Microarray analysis of the Bacillus subtilis K-state: Genome-wide expression changes dependent on ComK. *Molecular Microbiology*, 43(5), 1331–1345. https://doi.org/10.1046/j.1365-2958.2002.02833.x

Bron, S., Meijer, W., Holsappel, S., & Haima, P. (1991). Plasmid instability and molecular-cloning in Bacillus subtilis. *Research in Microbiology*, 142(7–8), 875–883. https://doi.org/10.1016/0923-2508(91)90068-I

Dubnau, D. (1993). Genetic exchange and homologous recombination in Bacillus subtilis and other gram-positive bacteria: Biochemistry, physiology, and molecular genetics (pp. 553–584). Wiley. https://doi.org/10.1128/9781555818388.ch39

Ito, M. & Nagane, M. (2001). Improvement of the electrotransformation efficiency of facultatively alkaliphilic Bacillus pseudofirmus OF4 by high osmolarity and glycine treatment. *Bioscience, Biotechnology, and Biochemistry*, 65(12), 2773–2775. https://doi.org/10.1271/bbb.65.2773

Jeong, H., Sim, Y. M., Park, S.-H., & Choi, S.-K. (2015). Complete genome sequence of Bacillus subtilis strain ATCC 6051a, a potential host for high-level secretion of industrial enzymes. *Genome Announcements*, 3(3), e00532–15. https://doi.org/10.1128/genomeA.00532-15

Kabisch, J., Thuemer, A., Huebel, T., Popper, L., Daniel, R., & Schweder, T. (2013). Characterization and optimization of Bacillus subtilis ATCC 6051 as an expression host. *Journal of Biotechnology*, 163(2), 97–104. https://doi.org/10.1016/j.jbiotec.2012.06.034

Leung, Y.-C. & Errington, J. (1995). Characterization of an insertion in the phage ϕ105 genome that blocks host Bacillus subtilis lysis and provides strong expression of heterologous genes. *Gene*, 154(1), 1–6. https://doi.org/10.1016/0378-1119(94)00874-R

Melnikov, A. & Youngman, P. J. (1999). Random mutagenesis by recombinational capture of PCR products in Bacillus subtilis and Acinetobacter calcoaceticus. *Nucleic Acids Research*, 27(4), 1056–1062. https://doi.org/10.1093/nar/27.4.1056
Van Sinderen, D. & Venema, G. (1994). *Bacillus subtilis* vegetative catalase is an extracellular enzyme. *Applied and Environmental Microbiology*, 61(12), 4471–4473. https://doi.org/10.1128/AEM.61.12.4471-4473.1995

Peng, D., Luo, Y., Guo, S., Zeng, H., Ju, S., Yu, Z., & Sun, M. (2009). Elaboration of an electroporation protocol for large plasmids and wild-type strains of *Bacillus thuringiensis*. *Journal of Applied Microbiology*, 106(6), 1849–1858. https://doi.org/10.1111/j.1365-2672.2009.04151.x

Philibert, T., Rao, Z., Yang, T., Zhou, J., Huang, G., Irene, K., & Samuel, N. (2016). Heterologous expression and characterization of a new heme-catalase in *Bacillus subtilis* 168. *Journal of Industrial Microbiology & Biotechnology*, 43(6), 729–740. https://doi.org/10.1007/s10529-007-9510-7

Rahmer, R., Heravi, K. M., & Altenbuchner, J. (2015). Construction of a super-competent *Bacillus subtilis* 168 using the Pmt/A-comKS inducible cassette. *Frontiers in Microbiology*, 6, 1431. https://doi.org/10.3389/fmicb.2015.01431

Schallmey, M., Singh, A., & Ward, O. P. (2004). Developments in the use of *Bacillus* species for industrial production. *Canadian Journal of Microbiology*, 50(1), 1–17. https://doi.org/10.1111/j.1751-7915.2010.00230.x

Shevchuk, N. A., Bryksin, A. V., Nusinovich, Y. A., Cabello, F. C., Sutherland, M., & Ladisch, S. (2004). Construction of long DNA molecules using long PCR-based fusion of several fragments simultaneously. *Nucleic Acids Research*, 32(2), 19e, 1–19. https://doi.org/10.1093/nar/gnh014

Shi, T., Wang, G., Wang, Z., Fu, J., Chen, T., & Zhao, X. (2013). Establishment of a markerless mutation delivery system in *Bacillus subtilis* stimulated by a double-strand break in the chromosome. *PLoS One*, 8(11), e81370. https://doi.org/10.1371/journal.pone.0081370

Shi, X., Feng, M., Zhao, Y., Guo, X., & Zhou, P. (2007). Overexpression, purification and characterization of a recombinant secretory catalase from *Bacillus subtilis*. *Biotechnology Letters*, 30(1), 181–186. https://doi.org/10.1007/s10529-007-9510-7

van Dijl, J. M. & Hecker, M. (2013). *Bacillus subtilis*: From soil bacterium to super-secreting cell factory. *Microbial Cell Factories*, 12(1), 3. https://doi.org/10.1186/1475-2859-12-3

Van Sinderen, D. & Venema, G. (1994). comK acts as an autoregulatory control switch in the signal transduction route to competence in *Bacillus subtilis*. *Journal of Bacteriology*, 176(18), 5762–5770. https://doi.org/10.1128/jb.176.18.5762-5770.1994

Wang, G., Xia, Y., Song, X., & Ai, L. (2016). Common non-classically secreted bacterial proteins with experimental evidence. *Current Microbiology*, 72(1), 102–111. https://doi.org/10.1007/s00284-015-0915-6

Xue, G.-P., Johnson, J. S., & Dalrymple, B. P. (1999). High osmolarity improves the electro-transformation efficiency of the gram-positive bacteria *Bacillus subtilis* and *Bacillus licheniformis*. *Journal of Microbiological Methods*, 34(3), 183–191. https://doi.org/10.1002/cjce.5450780403

Yan, X., Yu, H.-J., Hong, Q., & Li, S.-P. (2008). Cre/lox system and PCR-based genome engineering in *Bacillus subtilis*. *Applied and Environmental Microbiology*, 74(17), 5556–5562. https://doi.org/10.1128/AEM.01156-08

Yasui, K., Kano, Y., Tanaka, K., Watanabe, K., Shimizu-Kadota, M., Yoshikawa, H., & Suzuki, T. (2009). Improvement of bacterial transformation efficiency using plasmid artificial modification. *Nucleic Acids Research*, 37(1), e3. https://doi.org/10.1093/nar/gkn884

You, C., Zhang, X.-Z., & Zhang, Y.-H. P. (2012). Simple cloning via direct transformation of PCR product (DNA multimer) to *Escherichia coli* and *Bacillus subtilis*. *Applied and Environmental Microbiology*, 78(5), 1593–1595. https://doi.org/10.1128/AEM.07105-11

Zeigler, D. R., Pragai, Z., Rodriguez, S., Chevreux, B., Muffler, A., Albert, T., Bai, R., Wyss, M., & Perkins, J. B. (2008). The origins of 168, W23, and other *Bacillus subtilis* legacy strains. *Journal of Bacteriology*, 190(21), 6983–6995. https://doi.org/10.1128/JB.00722-08

Zhang, G. Q., Bao, P., Zhang, Y., Deng, A. H., Chen, N., & Wen, T. Y. (2011). Enhancing electro-transformation competency of recalcitrant *Bacillus amyloliquefaciens* by combining cell-wall weakening and cell-membrane fluidity disturbing. *Analytical Biochemistry*, 409(1), 130–137. https://doi.org/10.1016/j.ab.2010.10.013

Zhang, K., Duan, X., & Wu, J. (2016). Multigene disruption in undomesticated *Bacillus subtilis* ATCC 6051a using the CRISPR/Cas9 system. *Scientific Reports*, 6(1), 27943. https://doi.org/10.1038/srep27943

Zhang, X.-Z. & Zhang, Y. H. P. (2011). Simple, fast and high-efficiency transformation system for directed evolution of cellulase in *Bacillus subtilis*. *Microbial Biotechnology*, 4(1), 98–105. https://doi.org/10.1111/j.1751-7915.2010.00230.x