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Original Research Article

A recombineering system for *Bacillus subtilis* based on the native phage recombinase pair YqaJ/YqaK

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ABSTRACT

Bacillus subtilis plays an important role in fundamental and applied research, and it has been widely used as a cell factory for the production of enzymes, antimicrobial materials, and chemicals for agriculture, medicine, and industry. However, genetic manipulation tools for *B. subtilis* have low efficiency. In this work, our goal was to develop a simple recombineering system for *B. subtilis*. We showed that genome editing can be achieved in *B. subtiliis* 1A751 through co-expression of YqaJ/YqaK, a native phage recombinase pair found in *B. subtilis* 168, and the competence master regulator ComK using a double-stranded DNA substrate with short homology arms (100 bp) and a phosphorothioate modification at the 5′-end. Efficient gene knockouts and large DNA insertions were achieved using this new recombineering system in *B. subtilis* 1A751. As far as we know, this is the first recombineering system using the native phage recombinase pair YqaJ/YqaK in *B. subtilis*. In conclusion, this new recombineering system provides a simple and fast tool for genetic manipulation of *B. subtilis*, and it will promote studies of genome function, construction of production strains, and genome mining in *B. subtilis*.

1. Introduction

Bacillus subtilis is a Gram-positive, non-pathogenic, endospore-forming, and rod-shaped bacterium, and studies and application of this microorganism can be traced back more than one hundred years [1]. Its strong capacity for protein expression and secretion, production of antibacterial non-ribosomal peptides, and lack of endotoxins, make *B. subtilis* suitable for wide-ranging applications in many fields [2]. In industry, *B. subtilis* is the most widely used strain in the production of industrial enzyme preparations [3]. It is estimated that *Bacillus* enzymes, such as proteases, amylases, lipases, and cellulases, account for more than 50% of the total industrial enzyme market [4]. In agriculture, *B. subtilis* is an important component of many biopesticides, biofertilizers, and feed additives, and it plays an important role in preventing and controlling plant diseases, promoting crop growth, and regulating animal intestinal microecology [2]. In medicine, various antimicrobial lipopeptides, such as, polymyxins, surfactins, and fengycins, have been identified from *Bacillus* or *Paenibacillus* spp. [5]. *B. subtilis* is also the most important heterologous expression host after *Escherichia coli* and *Saccharomyces cerevisiae* [6].

B. subtilis was one of the first microorganisms to have a complete whole-genome sequence [7]. As more and more genome sequences for *B. subtilis* strains become available, researchers hope to gain more insight into the molecular mechanism of specific biochemical pathways at the genome level and to genetically modify industrial production strains to improve product yield and activity. In synthetic biology research, genome reduction and minimum genome engineering efforts have greatly promoted the optimization of *B. subtilis* as a “cell factory” [6]. However, achieving the above goals requires efficient genome engineering tools. The traditional genome editing technique used for *B. subtilis* is RecA-dependent single and double homologous recombination (HR), which requires long flanking homology arms (HAs) (> 1 kb) and is time-consuming, lowly efficient and laborious [8]. Furthermore, the recently developed CRISPR-Cas9-based system has also been used in *B. subtilis*, but it has low efficiency due to off-target effects [9–14]. Thus, efficient high-fidelity genome editing tools are needed for *B. subtilis*.

Recombineering is mediated by phage-encoded homologous recombinases, such as Redα/Redβ/Redγ from the lambda phage Red operon and RecE/RecT from Rac prophage [15,16]. The most important advantage of recombineering over RecA-dependent HR is that short HAs

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Table 1
Bacterial strains and plasmids used in this study.

Strains	Genome type	Ref. or source
E. coli GB2005	(HS996, Δ*recET*, Δ*ybcC*). The endogenous recET locus and the DLP12 prophage ybcC, which encodes a putative exonuclease similar to the Red*α*, were deleted	(Fu et al. 2012)
E. coli GB05-dir	GB2005, P_{BAD}-ETgA at the *ybcC* locus	(Fu et al. 2012)
B. subtilis 168	Wild type	DSMZ
B. subtilis 1A751	*B. subtilis* 168 derivative, *his nprR2 nprE18* △*aprA3* △*eglS102* △*bglT bglSRV*	BGSC
Plasmids	**Characteristics**	**Ref. or source**
pAD123	pBR322 ori, repB ori, CmR, AmpR, gfp	BGSC
pSC101-P_{BAD}-gbaA	pSC101 replicon, used for amplification of P_{BAD} promoter	(Fu et al. 2012)
pWYE598	gp34.1-gp35-gp36	(Sun et al. 2015)
pAD123-P_{BAD}-gam-cm	pBR322 ori, repB ori, P_{BAD} promoter, redgam, cmR	This study
pAD123-P_{BAD}-gam-yqaJ-K-cm	Recombinase YqaJ-K tested vector in *E. coli* GB2005	This study
pAD123-P_{BAD}-gam-gp34.1–35–36-cm	Recombinase Gp34.1–35–36 tested vector in *E. coli* GB2005	This study
pAD123-P_{BAD}-gba-cm	Recombinase gba tested vector in *E. coli* GB2005	This study
pAD123-P_{xylA}-gfp-cm	Xylose inducible promoter test vector in *B. subitlis*	This study
pAD123-P_{xylA}-comK-cm	ComK induced vector in *B. subitlis*	This study
pAD123-P_{xylA}-comK-yqaJ-K-cm	ComK, recombinase YqaJ-K co-expression vector	This study
pAD123-P_{xylA}-comK-gp34.1–35–36-cm	ComK, recombinase Gp34.1–35–36 co-expression vector	This study
pAD123-P_{xylA}-comK-gba-cm	ComK, recombinase Gam-Beta-Alfa co-expression vector	This study
pAD123-P_{xylA}-comK-Plu2934–2935–2936-cm	ComK, recombinase Plu2934–2935–2936 co-expression vector	This study
pAD123-P_{xylA}-comK-yqaI-J-K-cm	ComK, recombinase YqaI-J-K co-expression vector	This study
pAD123-P_{xylA}-comK-yqaJ-K-L-cm	ComK, recombinase YqaJ-K-L co-expression vector	This study
pAD123-P_{xylA}-comK-gam-yqaJ-K-cm	ComK, recombinase gam-YqaJ-K co-expression vector	This study
pAD123-P_{xylA}-comK-Plu2934-yqaJ-K-cm	ComK, recombinase Plu2934-YqaJ-K co-expression vector	This study
pAD123-P_{xylA}-comK-yqaK-cm	ComK, recombinase YqaK co-expression vector	This study
pAD123-P_{xylA}-comK-gp35-cm	ComK, recombinase Gp35 co-expression vector	This study
pAD123-Spect	pBR322ori, repB ori, Spect	This study

(only 50 bp length), which can be constructed simply using PCR methods, are required to achieve high HR efficiency. The Red system can be applied efficiently in *E. coli* and some closely related Gram-negative species, such as *Yersinia, Shigella*, and *Salmonella*. However, its application has been limited in more distantly related bacteria, such as *Burkholderia* and *Pseudomonas* species [17,18] and especially Gram-positive bacteria [19]. Thus, host-specific phage-derived recombination systems need to be developed. In recent years, recombineering systems based on phage or prophage recombinases for many species, such as *Photorhabdus, Clostridium*, and *Burkholderia*, have been established [18,20,21].

In 2012, application of the Red system in *B. subtilis* ATCC6633 was reported [22]. Using this system, knockout of the 37 kb *myc* biosynthetic gene cluster (BGC) was achieved; the single-stranded DNA (ssDNA) substrate had short HAs (70 bp) with a 5′-terminal phosphorothioate modification, which protects the ssDNA from exonuclease degradation. However, the recombination efficiency was still low. Thus, there was still a need to search for native phage or prophage recombinases and developing a *B. subtilis* host-specific recombineering system. In 2015, Wen's team reported a *B. subtilis* recombineering system based on the recombinase GP35, which is a RecT homologous protein from the *B. subtilis* phage SPP1 [23]. Using the GP35-based recombineering system, a ssDNA with HAs of 500 bp was used as the substrate, and the recombination efficiency was nearly 100-fold higher than the Red system. However, this recombineering system, consisting of only ssDNA recombinase and lacking a double-stranded DNA (dsDNA) exonuclease and RecBCD inhibitor, requires ssDNA as a substrate and long HAs (500 bp). Producing such ssDNA substrates is very labor intensive. Hence, it is not easy to for labs to use this recombineering system.

In this study, we developed a new recombineering system for *Bacillus* based on a pair of RecET-like recombinases, YqaJ/YqaK (hereafter YqaJK), obtained from a *B. subtilis* 168 prophage. Optimized transformation efficiency of *B. subtilis* 168 was achieved by overexpression of the competence master regulator ComK. This new recombineering system, which consists of co-expression of YqaJK and ComK and using a dsDNA substrate with a phosphorothioate modification at the 5′-end, was developed in *B. subtilis* 1A751. Using this system, efficient gene knockouts and large DNA insertions were achieved.

2. Materials and methods

2.1. Bacterial strains, plasmids, and culture conditions

Bacterial strains and plasmids used in this study are listed in Table 1 [16,23-25]. The primers used for construction of plasmids or colony PCR verification were synthesized by Sangon Biotech (Shanghai, China) (Table S1). DNA markers, DNA polymerases, and restriction enzymes were bought from New England Biolabs (Ipswich, MA, USA). Inducers and antibiotics were supplied by Sangon Biotech (Shanghai, China).

E. coli and *B. subtilis* strains were incubated in liquid Luria–Bertani (LB) medium or on LB agar plates containing 1.2% (w/v) agar. *E. coli* strains were cultivated at 37 ˚C, and *B. subtilis* strains were cultivated at 30 ˚C. The working concentrations of antibiotics are listed in Table S2.

2.2. Bioinformatic analysis

The protein sequences of RecT (WP_000166319.1), Red*β* (WP_001350280.1), and Plu*β* (WP_011147155.1) homologs were examined using PSI-BLAST in the NCBI non-redundant protein sequence database [26]. The selected recombination proteins encoded by genes adjacent to the exonuclease protein gene of the operon in the *Bacillus* or *Bacillus* phage genomes are shown in Table S3.

2.3. Construction of plasmids

All recombinase expression plasmids contained two replication origins, pBR322 and repB, allowing for replication in both *E. coli* and *B. subtilis,* and all plasmids were constructed by linear-linear HR in *E. coli* GB05-dir (Figs. S1–S3) [16,27]. Recombinase expression plasmids used in *E. coli* GB2005 contain the pBR322 and repB origins and the arabinose inducible promoter P_{BAD}. The original plasmid pAD123 was digested with *Kpn*I and *Pst*I to give a 4.2 kb linear fragment. The *yqaJ-yqaK* fragment was amplified by PCR using the genomic DNA of *B. subtilis* 168 as template and yqaK-F/yqaK-R as primers. The araC-P_{BAD}-gam fragment was amplified from the plasmid pSC101-P_{BAD}-gbaA by PCR using oligonucleotides pBAD-g-F and pBAD-g-R. Then, the three

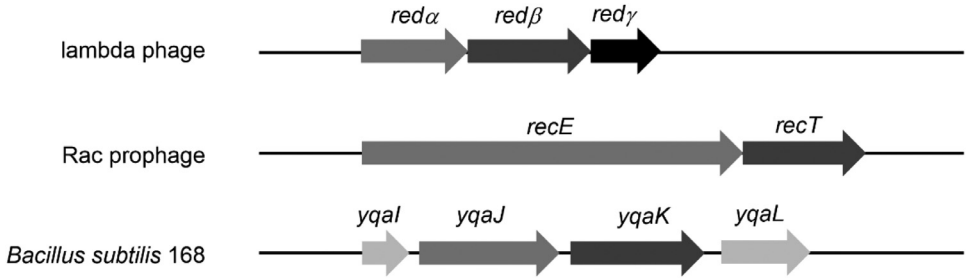

Fig. 1. Red/ET recombinase pairs from lambda phage and Rac prophage and their homologs yqaJ and yqaK from *B. subtilis* 168. Arrows with the same shade represent genes with similar functions or classification. All genes are drawn to scale.

linear DNA fragments were co-transformed into *E. coli* GB05-dir to construct pAD123-P_{BAD}-gam-yqaJ-K-cm. pAD123-P_{BAD}-gam-gp34.1–35-cm and pAD123-P_{BAD}-red$\gamma\beta\alpha$-cm were constructed similarly to pAD123-P_{BAD}-gam-yqaJ-K-cm.

The competence inducible vector and the recombinase expression plasmids used in *B. subtilis* 1A751 contained the pBR322 and repB origins and the xylose-inducible promoter P_{xylA} (Figs. S2 and S3) [28]. The plasmid pAD123-P_{xylA}-gfp-cm was digested by *BamH*I and *Pst*I to yield a linear fragment. The *comK* fragment was amplified by PCR using the genomic DNA of *B. subtilis* 168 as template and comK-F1/comK-R1 as primers. Then, the two linear fragments were co-transformed into *E. coli* GB05-dir to construct pAD123-P_{xylA}-comK-cm, which was further digested by *Pst*I to give a 5.5 kb linear fragment. The *yqaJ-yqaK* fragment was amplified by PCR using the genomic DNA of *B. subtilis* 168 as template and yqaJ-yqaK-F2/yqaJ-yqaK-R2 as primers. Then, the two linear fragments were co-transformed into *E. coli* GB05-dir to construct pAD123-P_{xlyA}-comK-yqaJ-K-cm. Other recombinase expression plasmids were constructed in a way similar to that of pAD123-P_{xlyA}-comK-yqaJ-K-cm.

2.4. Competent cell preparation and recombineering

Recombinase expression plasmids were electroporated into *E. coli* GB2005 and *B. subtilis* 1A751. Electrocompetent cell preparation and recombineering in *E. coli* GB2005 were performed according to our previously established protocol [16]. For *B. subtilis* 1A751 containing a recombinase expression plasmid, 50 μl of overnight culture was diluted into 1 ml fresh LB medium with chloramphenicol (5 μg/ml) at 30 ˚C. When the OD_{600} value of the culture was 1.0, 50 μl of 20% D-xylose was added into the culture to a final concentration of 1%. Two hours later, the super-competent cells were centrifuged at 9000 rpm for 1 min. Then, the supernatants were discarded, the cell pellets were resuspended in 1 ml LB, and 1 μg of *SpecR* fragment with HAs of different lengths was added. The cells were incubated at 30 ˚C for 1.5 h without shaking and then 100 μl of cells was spread on an LB plate containing the appropriate antibiotic. The plates were incubated at 30 ˚C for 12–15 h until the colonies were visible, and the clones were checked by colony PCR.

3. Results and discussion

3.1. Bioinformatic analysis of the endogenous phage recombinase pairs in Bacillus

The recombinase GP35, a RecT homologous protein from the *B. subtilis* phage SPP1, was used previously to perform recombination in *B. subtilis* [23]. However, this recombineering system consisting only of a recombinase, needs ssDNA with HAs of 500 bp as the substrate, which is difficult to prepare. Thus, in this study, we aimed to identify endogenous phage recombinase pairs consisting of a recombinase and an exonuclease. The protein sequences of RecT, Redβ, and Pluβ were used as queries in PSI-BLAST searches against the non-redundant protein sequence database to scan the genomes and phage genomes of *Bacillus* for candidate recombinase pairs [26]. Many candidate recombinase pairs were identified (Table S3). The most common system is the YqaJK system, which is homologous to the RecE/RecT system and exists in a variety of *Bacillus* species. Therefore, we selected the YqaJK system for subsequent research.

The YqaJK operon in the genome of the model strain *B. subtilis* 168 (GenBank: AL009126.3) encodes a putative nuclease (YqaJ), a putative DNA recombination protein (YqaK), a DNA binding protein (YqaL), and a hypothetical protein (YqaI) (Fig. 1). YqaK (protein ID: CAB14569.1; locus tag: BSU_26280) was 26% identical to RecT, 20% identical to Pluβ, and 15% identical to Redβ. YqaJ (protein ID: CAB14570.1; locus tag: BSU_26290) was 17% identical to Redα and 16% identical to the truncated RecE. Thus, we selected the YqaJK operon from the genome of the

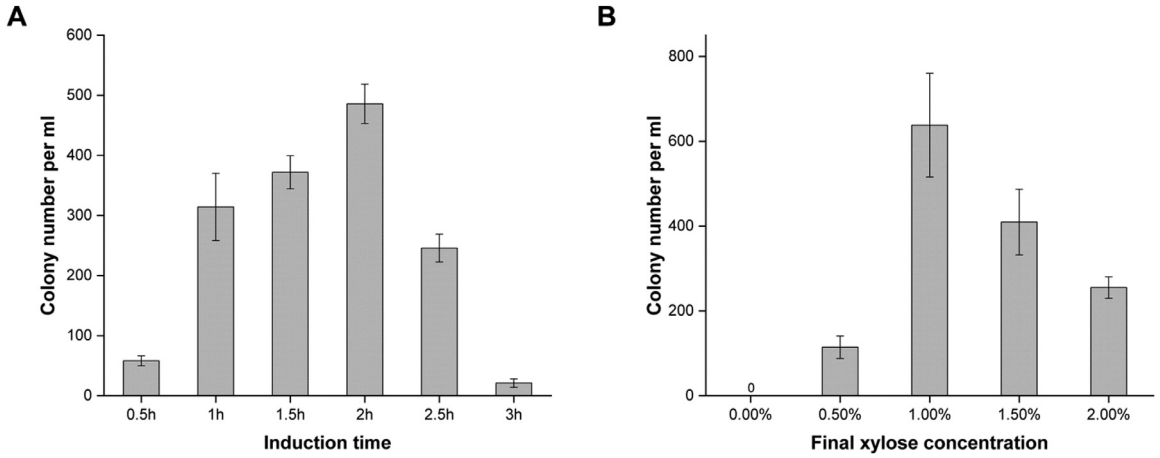

Fig. 2. Transformation efficiency of super-competent *B. subtilis* 1A751 cells expressing ComK. (A) Effect of the induction time on transformation efficiency. (B) Final concentration of D-xylose on transformation efficiency. Error bars, SD; $n = 3$.

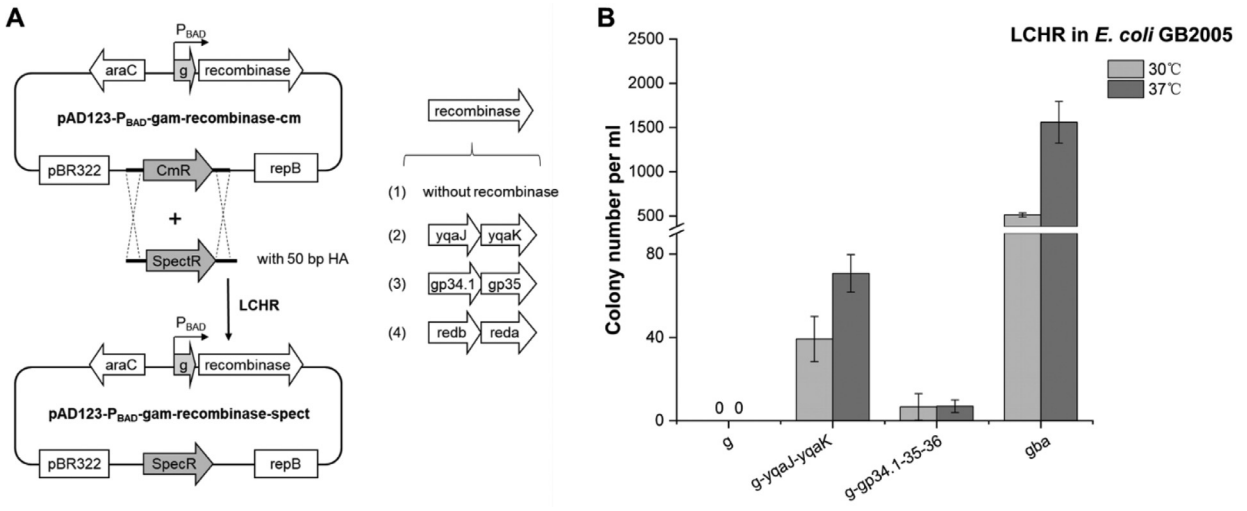

Fig. 3. Recombineering efficiencies of different recombinases combined with Redγ in *E. coli* GB2005. (A) Diagram showing the plasmid modification assay (linear plus circular homologous recombination [LCHR]) used to test the efficiencies of the recombination systems in *E. coli*. A PCR product carrying the spectinomycin resistance gene (*spectR*) flanked by 50 bp HAs (represented by bold lines) was integrated into the expression plasmid, replacing the chloramphenicol resistance gene (*cmR*). (B) Functional characterization of recombinases in *E. coli* using the LCHR assay at 30 ˚C or 37 ˚C. g, Redγ; gba, Red$\gamma\beta\alpha$; g-yqaJ-yqaK, Redγ-YqaJ-YqaK; g-gp34.1–35–36, Redγ-Gp34.1-Gp35-Gp36. Error bars, SD; $n = 3$.

model strain *B. subtilis* 168 to develop a recombineering system for *B. subtilis*.

3.2. Optimization of transformation efficiency in B. subtilis 1A751

The prerequisite for efficient DNA HR in bacteria is a high transformation efficiency [29]. However, transformation of *B. subtilis* is not as easy as that of *E. coli*. To achieve a high transformation efficiency and decrease the amount of labor required, super-competent *B. subtilis* 1A751 cells were prepared by overexpressing the competence master regulator ComK under the control of the xylose-inducible promoter P_{xylA} (Fig. S2) [30]. The transformation efficiency of *B. subtilis* 1A751 cells was then optimized. Firstly, different induction times were tested. The results showed that the transformation efficiency of *B. subtilis* 1A751 reached its maximal level after 2 h of induction by xylose (Fig. 2A). Next, different concentrations of the inducer xylose were tested, and the optimum working concentration was found to be 1% (Fig. 2B). This simple and fast natural transformation method established for *B. subtilis* 1A751 avoids the need for the complex and low-efficiency electrotransformation method.

3.3. Efficiency of recombineering systems in E. coli

Redγ has been proved to be able to significantly enhance the recombineering efficiency by inhibiting the exonuclease and helicase activities of the RecBCD complex, which rapidly degrades linear dsDNA

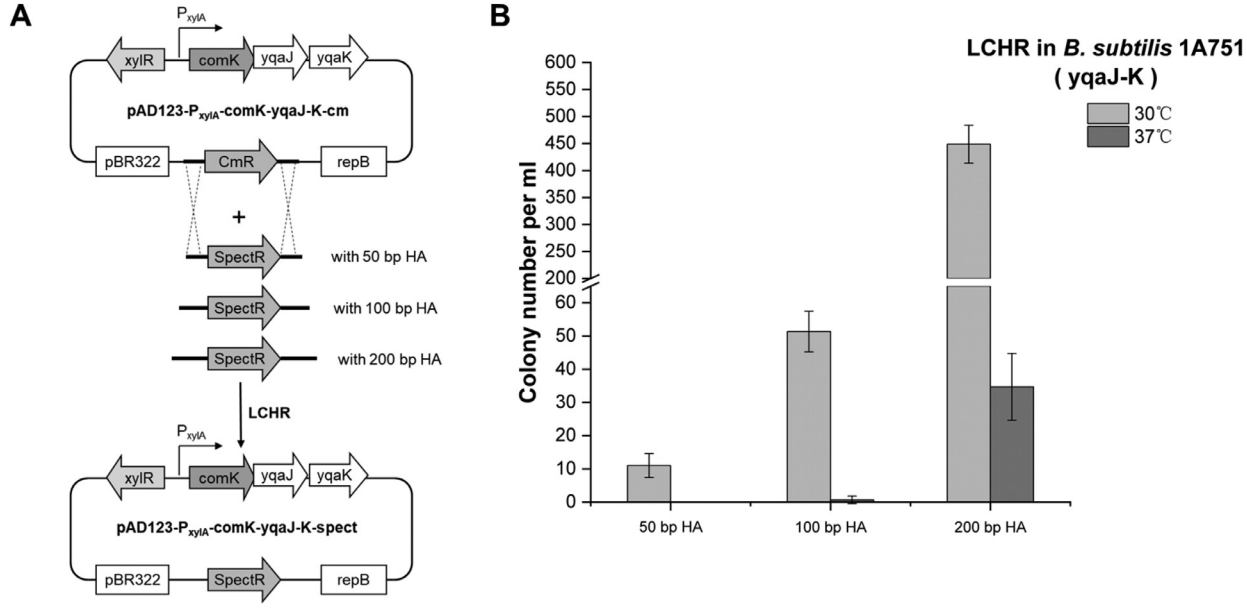

Fig. 4. Recombination efficiency of the YqaJK system when using dsDNA substrates with HAs of different length (50 bp, 100 bp, and 200 bp) in *B. subtilis* 1A751. (A) Diagram of the plasmid modification assay (linear plus circular homologous recombination [LCHR]) used to test recombination efficiency in *B. subtilis* 1A751. A PCR product carrying the spectinomycin resistance gene (*spectR*) flanked by 50, 100, or 200 bp HAs (represented by bold lines) was integrated into the expression plasmid, replacing the chloramphenicol resistance gene (*cmR*). (B) Functional characterization of the YqaJK system in *B. subtilis* 1A751 using the LCHR assay at 30 ˚C or 37 ˚C. Error bars, SD; $n = 3$.

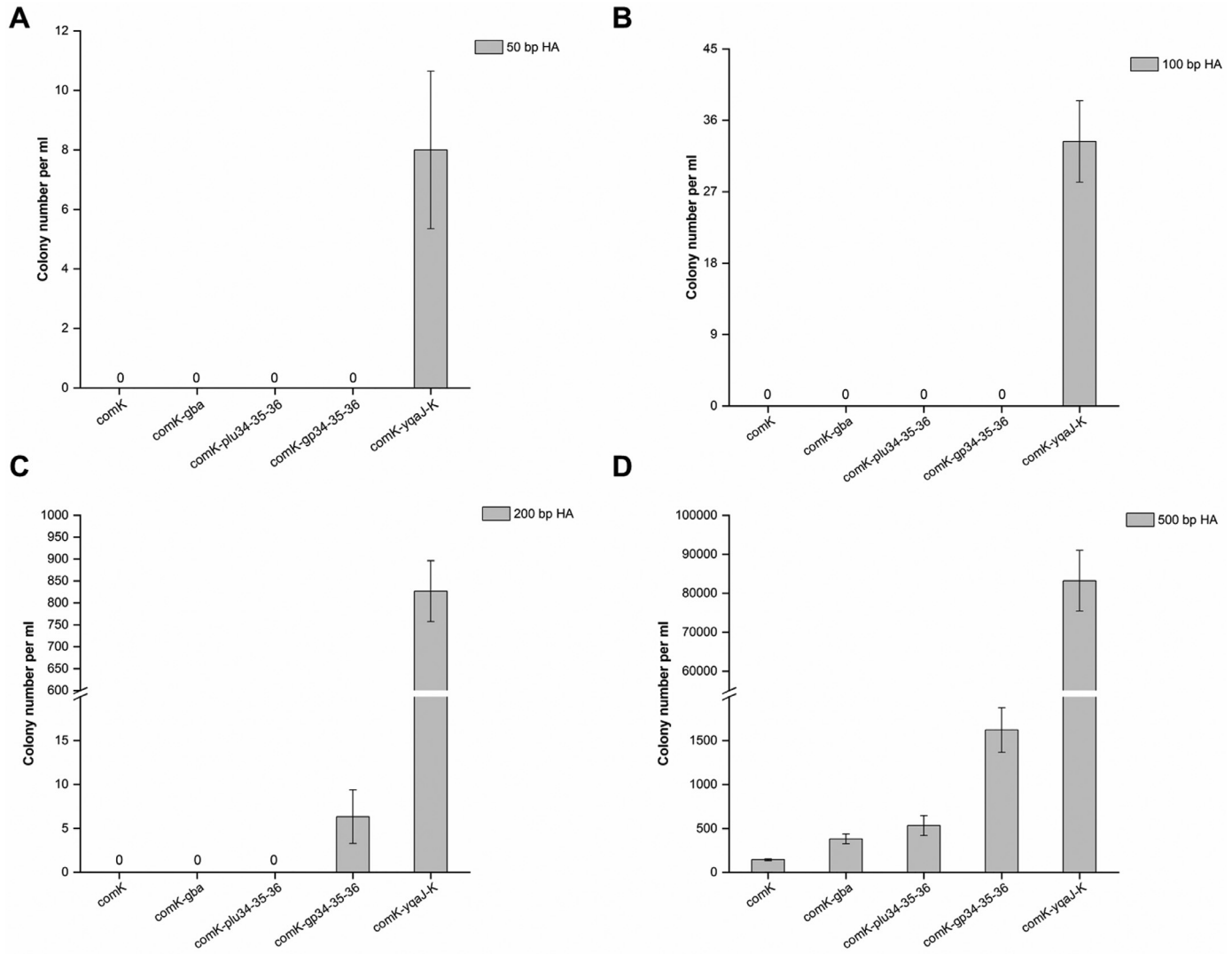

Fig. 5. Comparison of the recombination efficiencies of different recombinase systems using dsDNA substrates with HAs with different lengths, 50 bp (A), 100 bp (B), 200 bp (C), and 500 bp (D), using a plasmid modification assay in *B. subtilis* 1A751 at 30 ˚C. Error bars, SD; $n = 3$.

[31]. Thus, in this study, the recombinase pairs YqaJ-K, GP34.1–35, and Red$\beta\alpha$ combined with Redγ were cloned into recombinase expression plasmids (Fig. S1). The recombination efficiencies of the three recombineering systems, Red$\gamma\beta\alpha$ (as a positive control), Redγ-YqaJ-K, and Redγ-GP34.1–35–36, were compared using a plasmid modification assay, linear plus circular HR (LCHR), in *E. coli* GB2005 (Fig. 3A). Redγ without recombinase was included as a negative control. In the plasmid modification assay, a PCR product carrying the spectinomycin resistance gene (*spectR*) flanked by 50 bp HAs was integrated into a recombinase expression plasmid, replacing the chloramphenicol resistance gene (*cmR*) (Fig. 3A). Recombineering efficiency was determined by counting the number spectinomycin-resistant colonies, and generation of the recombinant plasmid pAD123-P_{BAD}-gam-recombinase-spect was verified by restriction analysis. When optimizing the recombination efficiency of recombinases, the culture temperatures of bacteria are always considered. *E. coli* is grown at 37 ˚C, while *B. subtilis* normally grows at 30 ˚C. Thus, the recombination efficiency of recombinases under both 30 ˚C and 37 ˚C were tested. The results showed that both the YqaJK and GP34.1–35 systems could mediate recombination assisted by Redγ and that the recombination efficiency was higher under 37 ˚C than 30 ˚C in *E. coli* GB2005 (Fig. 3B). In addition, the recombination efficiency of Redγ-YqaJ-K was 6 – 10 fold higher than that of the Redγ-GP34.1–35 system but lower than that of Red$\gamma\beta\alpha$ system. This result further confirmed the host specificities of recombineering systems. To sum up, the YqaJK system had the ability to mediate HR using short HAs, and it can be used to develop a simple and efficient recombineering system in *B. subtilis*.

3.4. *Efficiency of plasmid modification using the recombineering systems in* B. subtilis

Using the optimized transformation method, YqaJK and ComK were co-expressed under the inducible promoter P_{xylA} (Fig. S3) in *B. subtilis* 1A751, which is derived from *B. subtilis* 168. Recombination efficiencies of HAs with different lengths (50 bp, 100 bp, and 200 bp) were compared using a plasmid modification assay in *B. subtilis* 1A751 (Fig. 4A). The results showed that co-expression of YqaJK and ComK could mediate LCHR using a substrate with short HAs (50 bp) and that recombination efficiency increased linearly as the HA length increased under 30 ˚C in *B. subtilis* 1A751 (Fig. 4B). Furthermore, recombination efficiency of this system was higher at 30 ˚C than 37 ˚C. This indicates that it is feasible to construct a fast and simple recombineering system without preparation of competent cells via co-expression of YqaJK and ComK in *B. subtilis*.

To compare the efficiencies of recombinase systems from various phages in *B. subtilis*, Red$\gamma\beta\alpha$ from *E. coli* λ phage, Plu34–35–36 from *Pho-*

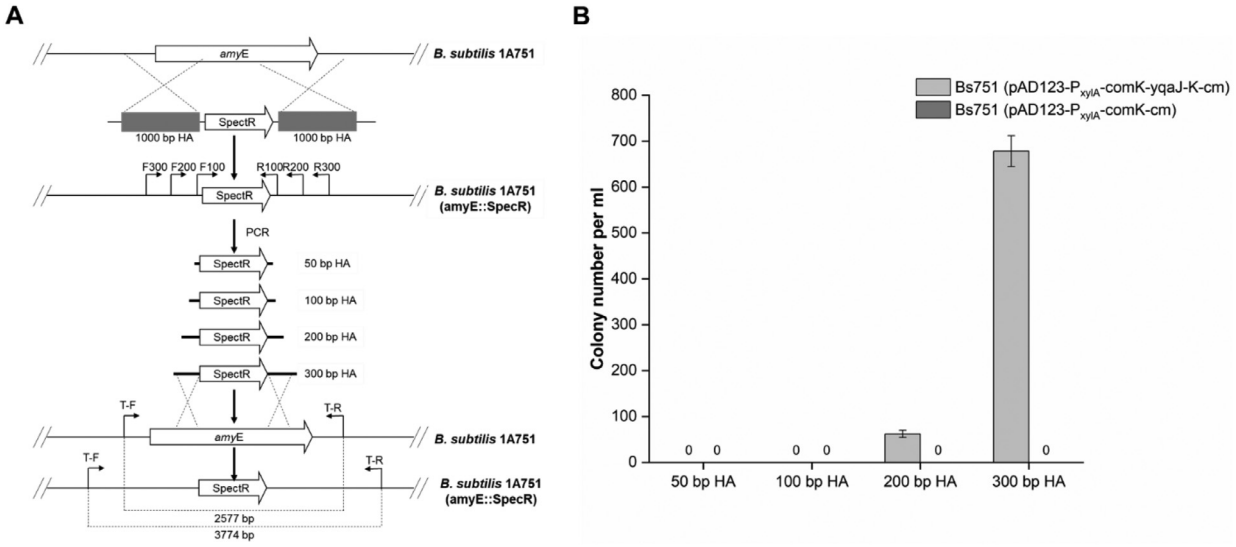

Fig. 6. Recombineering efficiencies of the YqaJK system using dsDNA substrates with HAs of different lengths (50 bp, 100 bp, 200 bp, and 300 bp) in *B. subtilis* 1A751. (A) Diagram of the genome editing assay used to test recombineering efficiency in *B. subtilis* 1A751. A PCR product carrying the spectinomycin resistance gene (*spectR*) flanked by 50, 100, 200, or 300 bp HAs (represented by bold lines) was integrated into the genome of *B. subtilis* 1A751, replacing the *amyE* gene. (B) Recombination efficiency of the YqaJK system in a genome editing assay in *B. subtilis* 1A751 at 30 °C. Error bars, SD; $n = 3$.

torhabdus luminescens prophage [20], and Gp34.1–35–36 from *B. subtilis* phage SPP1 were co-expressed with ComK under the inducible promoter P_{xylA} in *B. subtilis* 1A751. The recombination efficiencies of the recombinase systems using dsDNA substrates with different HA lengths (50 bp, 100 bp, 200 bp, and 500 bp) were compared using a plasmid modification assay (Fig. 5). The YqaJK system co-expressed with ComK showed the highest recombination efficiency when compared with the other recombinase systems, and only the YqaJK system could mediate LCHR using short HAs (50 bp and 100 bp) (Fig. 5A and B). Although Gp34.1–35–36 from the *B. subtilis* phage SPP1 could mediate LCHR using 200 bp HAs, the recombination efficiency was very low compared with that of the YqaJK system (Fig. 5C). The recombination efficiencies of the YqaJK system using dsDNA substrates with 500 bp HAs were 50-fold, 154-fold, 215-fold, and 567-fold higher compared with the Gp34.1–35–36 system, Plu$\alpha\beta\gamma$ system, Red$\alpha\beta\gamma$ system, and RecA-dependent recombination system, respectively (Fig. 5D). These results indicated that the recombination efficiency of the YqaJK system is better than that of other recombinases so far used in *B. subtilis* and suggest it could be used to engineer the genome of *B. subtilis*.

3.5. *Optimization of recombineering for genome editing in* B. subtilis

The recombination efficiencies of the YqaJK system using dsDNA substrates with different HA lengths (50 bp, 100 bp, 200 bp, and 500 bp) were compared using a genome editing assay in *B. subtilis* 1A751 (Fig. 6). In this assay, a PCR product carrying *spectR* flanked by HAs was integrated into the genome of *B. subtilis* 1A751, replacing the amylase gene *amyE* (Fig. 6A). The recombination efficiency of the YqaJK system in the genome editing assay was drastically lower compared with that in the plasmid modification assay (Fig. 6B). This indicated that the YqaJK system cannot mediate genome editing using dsDNA substrates with short HAs (< 100 bp) using natural transformation conditions (10^5–10^6 CFU/μg DNA) and that further optimization is needed.

Although phosphorothioate modification at the 5′-end of the lagging strand was reported to be able to improve recombination efficiency, preparation of ssDNA with phosphorothioate modification is difficult and tedious for long ssDNA substrates [23]. dsDNA substrates with phosphorothioate modification at the 5′-end can be easily generated by PCR amplification using primers with the first four internucleotide linkages at the 5′-end being phosphorothioated. Thus, here, to investigate the effects of phosphorothioation on recombineering efficiency, four different modified dsDNA substrates were obtained by PCR using different combinations of unmodified primers and primers with the first four internucleotide linkages at the 5′-end being phosphorothioated: (i) a *spectR* PCR substrate with phosphorothioate modification at the 5′ ends of both the leading strand and lagging strand (referred to as SS); (ii) a *spectR* PCR substrate with phosphorothioate modification at the 5′ end of only the lagging strand (SO); (iii) a *spectR* PCR substrate with phosphorothioate modification at the 5′ end of only the leading strand (OS); (iv) a *spectR* PCR substrate without phosphorothioate modification (OO). The recombination efficiencies of the YqaJK system using these four modified dsDNA substrates were compared by performing plasmid modification assays with 200 bp HAs (Fig. 7A) and genome editing assays with 300 bp HAs (Fig. 7B) in *B. subtilis* 1A751. The results showed that the recombination efficiency of the YqaJK system using SO was highest; the efficiency was almost 10-fold higher than that of OO in the plasmid modification assay and 2.5-fold higher in the genome editing assay. The recombination efficiencies of the YqaJK system using SO and SS were comparable in both the plasmid modification assay and genome editing assay.

Next, a *spectR* PCR substrate with short HAs (100 bp) with different phosphorothioate modifications was used to modify the genome of *B. subtilis* 1A751 using the YqaJK system (Fig. 7C). Knockout of the amylase gene *amyE* was achieved using both SS and SO (Fig. 7D). In conclusion, under natural competent conditions, the YqaJK system can mediate gene knockout in *B. subtilis* 1A751 using dsDNA substrates with short HAs (100 bp) with a phosphorothioate modification at the 5′-end.

In the search for recombination proteins in *B. subtilis* 168, two hypothetical proteins, YqaI and YqaL, were considered to be functionally associated with their adjacent recombinases. Therefore, the YqaI-YqaJ-YqaK system and YqaJ-YqaK-YqaL system were constructed to investigate the function of YqaI and YqaL. The recombination efficiencies of these systems for plasmid modification using dsDNA substrates with 200 bp HAs were compared in *B. subtilis* 1A751 (Fig. S4). Neither YqaI nor YqaL were necessary for recombineering in *B. subtilis*. The above results indicate that YqaJK system is an ideal recombineering system with high efficiency.

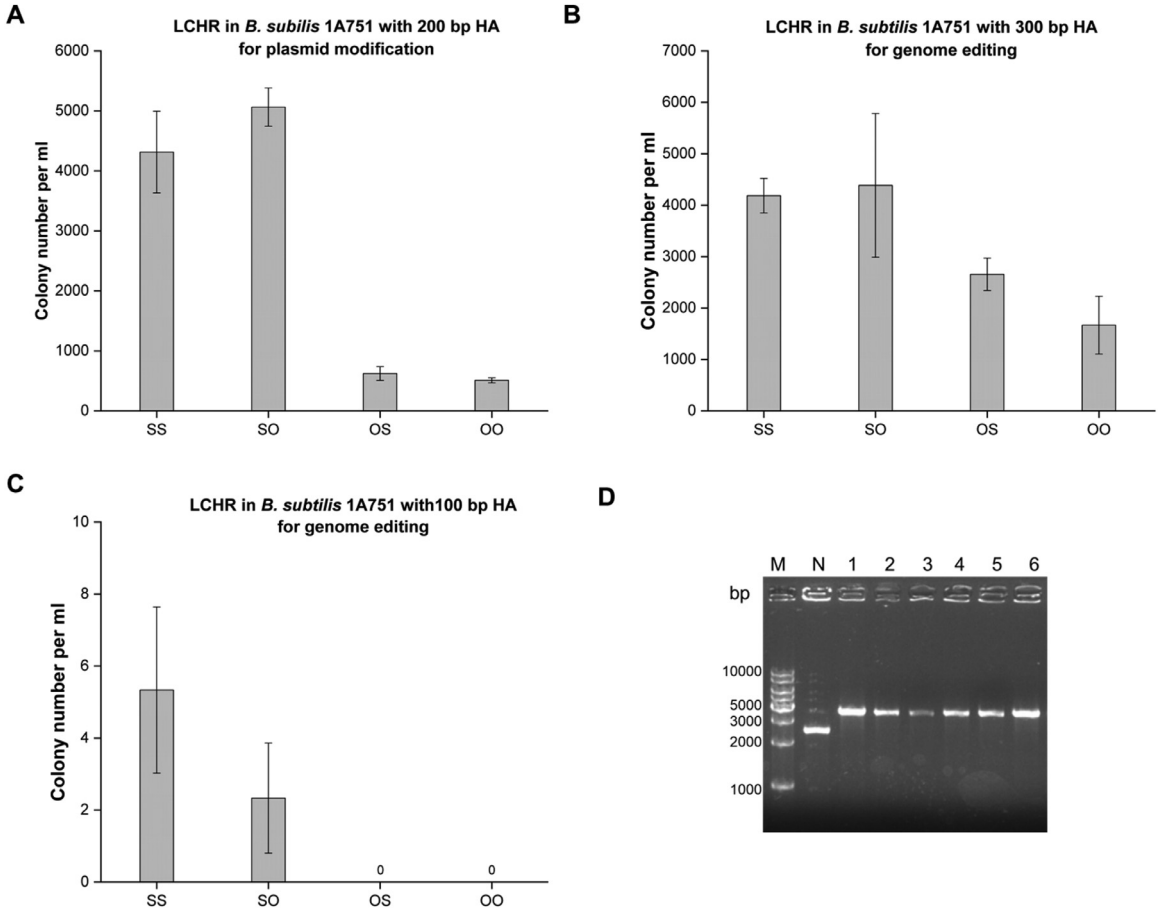

Fig. 7. Recombination efficiencies of the YqaJK system using dsDNA substrates with different phosphorothioate modifications in *B. subtilis* 1A751. (A-C) Recombination efficiencies of the YqaJK system in a plasmid modification assay using dsDNA substrates with 200 bp HAs (A), 300 bp HAs (B), and 100 bp HAs (C) in *B. subtilis* 1A751. SS, a *spectR* PCR substrate with phosphorothioate modification at the 5′ ends of both the leading strand and lagging strand; SO, a *spectR* PCR substrate with phosphorothioate modification at the 5′ end of only the lagging strand; OS, a *spectR* PCR substrate with phosphorothioate modification at the 5′ end of only the leading strand; OO, a *spectR* PCR substrate with no phosphorothioate modification of either strand. Error bars, SD; $n = 3$.

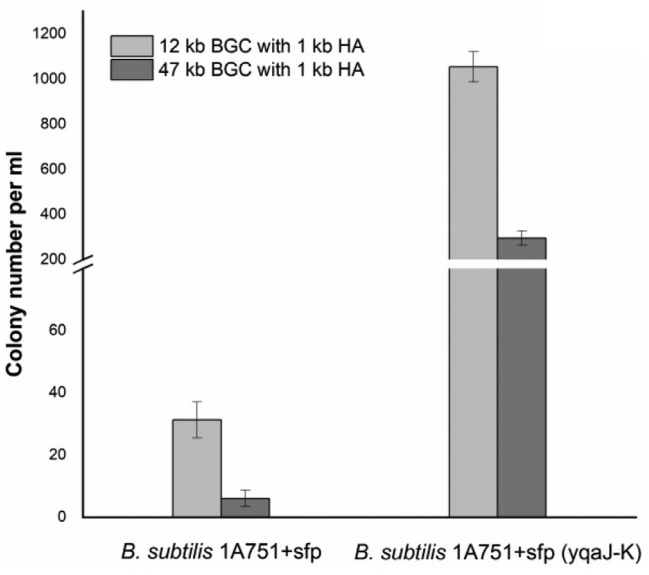

Fig. 8. Efficiency of homologous recombination of large-sized DNA fragments induced by the YqaJK system. Error bars, SD; $n = 3$.

3.6. *Efficient gene knockout and large DNA insertion in* B. subtilis *1A751*

The *srfAA* gene, the first gene of the surfactin BGC, was successfully knocked out using the YqaJK system co-expressed with ComK (Fig. S5). This result further confirmed that the YqaJK system could mediate efficient gene knockout or deletion in *B. subtilis* 1A751 using dsDNA substrates with short HAs (100 bp) and a phosphorothioate modification at the 5′-end.

B. subtilis has been reported to be a suitable heterologous host for expression of BGCs of compounds from *Bacillus* and related genera [5]. We previously cloned several BGCs (> 40 kb) from *Bacillus* species. However, efficiency of the integration of the BGCs into the genome of *B. subtilis* 1A751-sfp via traditional recombination is very low [32]. Here we found that the efficiency of integration of large DNA fragments (12 kb and 47 kb) into the genome of *B. subtilis* 1A751 was 50-fold higher using YqaJK system than when using traditional recombination (Fig. 8). The YqaJK system will facilitate genome mining and the discovery of new natural products from *Bacillus* species.

4. Conclusions

Bacillus species have been developed into an important platform for the production of a variety of biochemicals and enzymes. Although *B.*

subtilis plays an important role in basic and applied research, the availability of efficient genetic tools lags far behind that for *S. cerevisiae* and *E. coli*, which are by far the most widely used cell factories. Thus, *B. subtilis*, an important and fascinating manufacturing platform, requires more efficient high-fidelity genetic engineering tools.

In this study, we have reported a new recombineering system for *B. subtilis* based on the native phage recombinase pair YqaJK from *B. subtilis* 168. Our optimization of the recombination efficiency of this recombineering system led to the following conclusions. First, the YqaJK system is a better recombineering system than other recombinase systems so far used in *B. subtilis*, demonstrating that the YqaJK system could serve as an efficient genome manipulation tool for *B. subtilis*. Second, phosphorothioate modification at the 5′-end of the lagging targeting strand has been shown to improve the recombination efficiency [22,33], and here we found that this was also the case for the YqaJK system: SO and SS dsDNA substrates had higher recombination efficiencies and could mediate gene knockout in *B. subtilis* 168 using 100 bp HAs. Although Shen and co-workers reported successful genome editing by beta recombination using ssDNA with short HAs and phosphorothioate modification, preparation of ssDNA is tedious and difficult if long DNA substrates are needed [22]. Thus, this work provides a simple and fast method using phosphorothioate-modified dsDNA.

Since the CRISPR-Cas9 system was first reported as a eukaryotic genome editing tool in 2013, thousands of studies have successfully applied this system to edit the genomes of many different organisms [34]. However, this strategy shows low efficiency mostly due to the off-target effects of sgRNAs or the weak capacity of the native homology-directed repair system in some organisms [35]. Here, we expected that combining the YqaJK system and CRISPR-Cas9 system would improve the efficiency of genome editing. However, no improvement of the genome editing efficiency in *B. subtilis* was observed (data not shown).

To sum up, we developed a recombineering system for *B. subtilis* based on the phage recombinase pair YqaJK. A fast and efficient transformation method was developed by over-expressing the competence master regulator ComK. Recombination efficiency was greatly enhanced by using dsDNA with a 5′ phosphorothioate modification. Using this new recombineering system, gene knockout can be achieved without construction of a gene knockout vector or the need for overlapping PCR to obtain a gene knockout vector with HAs, and recombineering in *B. subtilis* 1A751 can be accomplished in only 2 days. Furthermore, the efficiency of large DNA insertion can be highly improved by using the YqaJK system in *B. subtilis* 1A751. This will promote research into genome function, construction of production strains, genome mining, and heterologous expression of BGCs in *B. subtilis*.

Data Availability Statement

Data will be made available on request.

Author Contribution Statement

Q.L., R.L., Y.Z., and J.F. designed the experiments. Q.L., R.L., H.S., R.Y., Q.S., Q.C., and X.W. performed the experiments. Q.L., R.L., A.L., Y.Z., and J.F. wrote the manuscript with help from all authors.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Given their roles as Editor-in-Chief and Guest Editor, respectively, Dr. Youming Zhang and Dr. Jun Fu had no involvement in the peer review of this article, and had no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to Dr. Shengbiao Hu.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.engmic.2023.100099.

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