Bacillus subtilis genome editing using ssDNA with short homology regions

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ABSTRACT

In this study, we developed a simple and efficient Bacillus subtilis genome editing method in which targeted gene(s) could be inactivated by single-stranded PCR product(s) flanked by short homology regions and in-frame deletion could be achieved by incubating the transformants at 42°C. In this process, homologous recombination (HR) was promoted by the lambda beta protein synthesized under the control of promoter P_{RM} in the lambda cl857 P_{RM}–P_{R} promoter system on a temperature sensitive plasmid pWY121. Promoter P_{R} drove the expression of the recombinase gene cre at 42°C for excising the floxed (lox sites flanked) disruption cassette that contained a bleomycin resistance marker and a heat inducible counter-selectable marker (hewl, encoding hen egg white lysozyme). Then, we amplified the single-stranded disruption cassette using the primers that carried 70 nt homology extensions corresponding to the regions flanking the target gene. By transforming the respective PCR products into the B. subtilis that harbored pWY121 and incubating the resultant mutants at 42°C, we knocked out multiple genes in the same genetic background with no marker left. This process is simple and efficient and can be widely applied to large-scale genome analysis of recalcitrant Bacillus species.

INTRODUCTION

Bacillus subtilis and its closely related species are important cell factories for the production of industrial enzymes, antibiotics, insecticides and so on (1,2). Bacillus species take in and integrate exogenous linear DNA using the natural competence when they enter the stationary growth phase (3,4). However, efficient transformation of competent cells requires at least 400–500 bp of homologous arms (3,5) and preparation of efficient competent cells is difficult for some Bacillus strains (6), which has led to the development of other transformation strategies including phage transduction (7), protoplast fusion (8) and the simple and efficient electroporation methods (9). The rapid development in genome-sequencing technologies accentuates the need for efficient gene function analysis and genome engineering, and from the practical point of view, it would be highly desirable to develop a method allowing multiple markerless modification of the genome with short homologous DNA stretches.

Recombination efficiency and homology requirement are limited by substrate DNA availability and recombination activity. Linear double-stranded DNA (dsDNA) molecules introduced into B. subtilis are prone to degradation by the rapid and processive AddAB helicase–nuclease (10,11), unless the Chi site (5'-AGCGG-3') is reached (12,13). Chi site recognition by RecBCD, the counterpart of AddAB in Escherichia coli, coordinates the preferential loading of the recombinase protein RecA onto the resulting chi-containing single-stranded DNA (ssDNA) (14). RecA facilitates homologous recombination (HR) in E. coli between DNA molecules with 20–40 bp of homology (15,16). However, in B. subtilis, both the RecA-dependent HR (17,18) and the RecA-independent HR involving the single-strand annealing protein (SSAP) are of low efficiency. Datta et al. (19) reported that, when expressed in E. coli, the activity of SSAP from B. subtilis is 1/100 of that from E. coli, and 1/1000 of phage lambda beta protein (19). Beta protein plays a central role in the lambda Red system in which protein gamma inhibits the E. coli RecBCD exonuclease V, Exo creates ssDNA by degrading linear dsDNA in the 5′ to 3′ direction and the beta protein protects the ssDNA from exonuclease attack and promotes annealing of the ssDNA to the complementary regions of the replication fork in order to generate the recombinant (20,21). The lambda Red system facilitates site-directed chromosome

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modification using PCR products that carry short homology extensions in E. coli (20, 22), Salmonella enteric (23), Pseudomonas aeruginosa (24) and many other bacteria.

Recently, lambda beta protein has been found to mediate recombination through a fully single-stranded intermediate which preferentially binds to the lagging strand during DNA replication (25, 26). Theoretically, beta recombination requires only ssDNA and beta protein. Compared with E. coli, B. subtilis contains less ssDNA exonuclease (27). The ssDNA-specific 5’ exonuclease RecJ (also named YrvE) and NrnA degrade ssDNA from the 5’-end (28,29), but their activity can be diminished by phosphorothioate modification (26,30). The exononuclease YhaM degrades ssDNA from the 3’-end (31), but it is repressed by LexA during exponential growth (32). The above discoveries provide a new option for HR enhancement in B. subtilis by electroporating ssDNA into the exponentially growing B. subtilis cells that express beta protein.

The disruption cassettes inserted into the chromosome can be deleted by site-specific recombination systems such as Cre/loxP (33) and Flp/FRT (34). The recombination of the Cre/loxP system is more efficient than the Flp/FRT system (35). The Cre recombinase inverts a region of DNA between two divergently oriented loxP sites or excises the DNA when the two surrounding loxP sites are convergently oriented. This deletion leaves an intact loxP site leading to genetic instability in further modification. This problem can be avoided by using a pair of mutant lox sites (lox71/lox66) which can be recombined into a double-mutant lox72 site that shows strongly reduced binding affinity for Cre, allowing for repeated gene deletion in a single genetic background (36–38).

Though efficient, Cre recombination does not guarantee DNA sequence excision in 100% of chromosomes (39); therefore, counter-selectable markers are needed to eliminate the cells that carry the intact disruption cassettes. Four kinds of counter-selectable markers have been reported for use in B. subtilis, based on the following genes: upp (40), blai (41), mazF (42) and ysbC (43). However, application of upp or blai requires a strain with a specific mutation in the chromosome, whereas mazF and ysbC are difficult to be maintained in E. coli, which limits the use of these counter-selectable markers.

The novel counter-selectable marker described here was based on the hewl gene which is small, active against Bacillus species and distantly related to Bacillus genes (44). The well-known observation that eukaryotic proteins containing multiple disulfide bonds were difficult to fold in B. subtilis may have discouraged efforts to counter-select B. subtilis cells by intracellular expressing hewl (45). It was later discovered that the denatured lysozyme has unique and potent microbicidal properties (46–48), and the functions were mainly conferred by the internal helix–loop–helix domain (87–114 sequence of hen lysozyme) (49). The bactericidal potency of lysozyme is not only due to muramidase activity but also to its cationic and hydrophobic properties (46). We placed gene hewl after promoter PR as a temperature-inducible counter-selectable marker. The PR promoter has been shown to be functional in B. subtilis and is efficiently repressed by CI857 repressor protein at 30°C and derepressed at 42°C (50). In this case, hewl can be switched off or on by temperature shift.

Here, we described a procedure based on the mechanisms of lambda beta annealing and Cre recombination that allowed us to inactivate a specific gene with a single-stranded PCR product containing an antibiotic resistance marker and a counter-selectable marker. The single-stranded PCR product was generated using primers with 70 nt homology extensions. After transformation of PCR product and primary selection of the mutant, the floxed markers could be excised by Cre recombination which was expressed at 42°C, and the cells with intact disruption cassette could be completely killed by hen egg white lysozyme. Curing of the temperature-sensitive replicon pWY121 could be easily achieved by incubating the transformants at 50°C.

MATERIALS AND METHODS

Bacterial strains, plasmids and primers

The bacterial strains and plasmids used in this study are listed in Table 1. The services of primer synthesis (Table 2) and DNA sequencing were provided by Invitrogen (Shanghai, China) and Generay (Shanghai, China).

Culture and growth conditions

Escherichia coli DH5α and B. subtilis strains were cultured at 37°C in Luria–Bertani (LB) or LBG medium (LB medium containing 2% glucose). When required, antibiotics were added to the growth media at the following concentrations: ampicillin, 100 μg/ml; bleomycin, 50 μg/ml for E. coli and 20 μg/ml for B. subtilis; erythromycin 300 μg/ml for E. coli and 5 μg/ml for B. subtilis.

DNA manipulation techniques

DNA manipulation and E. coli transformation were performed using standard techniques (53). Restriction enzymes, T4-ligases and DNA markers were purchased from New England Biolabs (NEB).

Electroporation of B. subtilis

Electroporation of B. subtilis was carried out according to the method described by Zhang et al. (54), with minor modifications. An overnight LB culture of the B. subtilis cells was diluted 100-fold to fresh LBG medium. When it reached an OD600 (optical density at 600 nm) of 0.2, the culture was supplemented with DL-Threonine, Glycine and Tween 80 at final concentrations of 1.0, 2.0 and 0.03%, respectively, and continued to be shaken for 1 h. The culture was then cooled on ice for 20 min and centrifuged at 5000 g for 10 min at 4°C. Cells were washed twice with ice-cold electroporation buffer (0.5 M trehalose, 0.5 M sorbitol, 0.5 M mannitol, 0.5 mM MgCl2, 0.5 mM K2HPO4 and 0.5 mM KH2PO4, pH 7.2) and resuspended in electroporation medium at 1/100 of the original culture volume. For electroporation, an ice-cold 2 mm cuvette containing 100 μl competent cells and 2 μl
Table 1. Strains and plasmids

<table>
<thead>
<tr>
<th>Strains or plasmid</th>
<th>Characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Escherichia coli DH5α</td>
<td><em>Φ80lacZ ΔM15 recA1 endA1 gyr96 thi-1 hsdR17 (rK−, mK+) supE44 relA1 deoR</em></td>
<td>Promega</td>
</tr>
<tr>
<td>Bacillus subtilis ATCC633</td>
<td>Wild-type, produces mycosubtilin</td>
<td></td>
</tr>
<tr>
<td>Bacillus subtilis AL135</td>
<td>Bacillus subtilis ATCC633/AbnR</td>
<td>This work</td>
</tr>
<tr>
<td>Bacillus subtilis AB211</td>
<td>Bacillus subtilis ATCC633/AbnB</td>
<td>This work</td>
</tr>
<tr>
<td>Bacillus subtilis AM336</td>
<td>Bacillus subtilis ATCC633/AbnB</td>
<td>This work</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGEM-T easy</td>
<td>Amp&lt;sup&gt;a&lt;/sup&gt;, cloning vector</td>
<td>Promega</td>
</tr>
<tr>
<td>pE194</td>
<td>Erm&lt;sup&gt;a&lt;/sup&gt;, B. subtilis/E. coli shuttle vector</td>
<td>This work</td>
</tr>
<tr>
<td>pGE194</td>
<td>Amp&lt;sup&gt;a&lt;/sup&gt;, Erm&lt;sup&gt;a&lt;/sup&gt;, <em>B. subtilis-E. coli</em> shuttle vector containing the P&lt;sub&gt;RM&lt;/sub&gt;-P&lt;sub&gt;R&lt;/sub&gt; promoter system for expression in <em>B. subtilis</em></td>
<td>This work</td>
</tr>
<tr>
<td>pGEP</td>
<td>Amp&lt;sup&gt;a&lt;/sup&gt;, Erm&lt;sup&gt;a&lt;/sup&gt;, pGEP containing λ cI857</td>
<td>This work</td>
</tr>
<tr>
<td>pGECC</td>
<td>Amp&lt;sup&gt;a&lt;/sup&gt;, Erm&lt;sup&gt;a&lt;/sup&gt;, pGEC containing <em>cre</em></td>
<td>This work</td>
</tr>
<tr>
<td>pKD46</td>
<td>Amp&lt;sup&gt;a&lt;/sup&gt;, Red expression plasmid, PBAD gam bet exor psIC101</td>
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<tr>
<td>pCP20</td>
<td>Amp&lt;sup&gt;a&lt;/sup&gt;, cat cI857 k P&lt;sub&gt;R&lt;/sub&gt; flp psIC101 oriTS</td>
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</tr>
<tr>
<td>pWY121</td>
<td>Amp&lt;sup&gt;a&lt;/sup&gt;, Erm&lt;sup&gt;a&lt;/sup&gt;, B. subtilis recombination vector encoding λ cI857, λ β, λ exo and cre recombinase genes.</td>
<td>This work</td>
</tr>
<tr>
<td>pMD19</td>
<td>Amp&lt;sup&gt;a&lt;/sup&gt;, cloning vector</td>
<td>Takara</td>
</tr>
<tr>
<td>pDGHCZ</td>
<td><em>B. subtilis</em> integration vector, ble, ere</td>
<td>39</td>
</tr>
<tr>
<td>pMDB19</td>
<td>Amp&lt;sup&gt;a&lt;/sup&gt;, Ble&lt;sup&gt;a&lt;/sup&gt;, pMD19 containing lox&lt;sub&gt;71&lt;/sub&gt;-ble-lox&lt;sub&gt;66&lt;/sub&gt; cassette</td>
<td>This work</td>
</tr>
<tr>
<td>pMDB-857</td>
<td>Amp&lt;sup&gt;a&lt;/sup&gt;, pMDB19 containing λ cI857 gene in the NdeI site</td>
<td>This work</td>
</tr>
<tr>
<td>pQRBL</td>
<td>Amp&lt;sup&gt;a&lt;/sup&gt;, Ble&lt;sup&gt;a&lt;/sup&gt;, pMDB-857 with λ cI857-blew-hw-P&lt;sub&gt;R&lt;/sub&gt;-lox&lt;sub&gt;66&lt;/sub&gt; cassette</td>
<td>This work</td>
</tr>
</tbody>
</table>

<sup>a</sup>Amp<sup>a</sup>: Ampicillin-resistance, Erm<sup>a</sup>: Erythromycin resistance, Ble<sup>a</sup>: Bleomycin resistance, hew<sub>e</sub>: hen egg white lysozyme gene.

Table 2. Primer sequences

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>5′ caacttggaacgctgctac3′</td>
</tr>
<tr>
<td>P2</td>
<td>5′ gtcagcaagctgtagaag3′</td>
</tr>
<tr>
<td>P3</td>
<td>5′ tagtcgacacagtggagcaaaaag3′</td>
</tr>
<tr>
<td>P4</td>
<td>5′ ggatccgaagctgtagaag3′</td>
</tr>
<tr>
<td>P5</td>
<td>5′ cattcgagatggtgaaattg3′</td>
</tr>
<tr>
<td>P6</td>
<td>5′ gaaatccatagtaagat3′</td>
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<tr>
<td>P7</td>
<td>5′ cggggacctcgtacgagc3′</td>
</tr>
<tr>
<td>P8</td>
<td>5′ aactcgagcactcttactcctg3′</td>
</tr>
<tr>
<td>P9</td>
<td>5′ ggagactatacaatcagagc3′</td>
</tr>
<tr>
<td>P10</td>
<td>5′ gcggggactatgacgattgacg3′</td>
</tr>
<tr>
<td>P11</td>
<td>5′ ggtgagctgtagaag3′</td>
</tr>
<tr>
<td>P12</td>
<td>5′ aggattgaggttggatg3′</td>
</tr>
<tr>
<td>P13</td>
<td>5′ a-g-t-t-aagatgactactactctc3′</td>
</tr>
<tr>
<td>P14</td>
<td>5′ ggtgagctgtagaag3′</td>
</tr>
<tr>
<td>P15</td>
<td>5′ a-g-t-t-aagatgactactactctc3′</td>
</tr>
<tr>
<td>P16</td>
<td>5′ caggtgagaatgcactcttactcctg3′</td>
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<tr>
<td>P17</td>
<td>5′ c-c-c-c-tgcgagctgctacgctttactg3′</td>
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<tr>
<td>P18</td>
<td>5′ acaggggcctacagca3′</td>
</tr>
<tr>
<td>P19</td>
<td>5′ cacactttacg3′</td>
</tr>
<tr>
<td>P20</td>
<td>5′ ggctgactactagactttactg3′</td>
</tr>
<tr>
<td>P21</td>
<td>5′ ccttttaacctggactactctc3′</td>
</tr>
</tbody>
</table>

<sup>a</sup>Phosphorothioate modification was indicated by ‘-’

DNA (25 ng/µl) was shocked by a single 12.5 kV/cm pulse generated by Gene Pulser (Bio-Rad Laboratories, Richmond, CA, USA), with the resistance and capacitance set at 200 Ω and 25 µF, respectively. One milliliter of LB broth containing 0.5 M sorbitol and 0.38 M mannitol was immediately added to the cuvette. The culture was incubated at 37°C for 3 h to allow expression of the antibiotic resistant genes and was then spread onto LB agar plates supplemented with appropriate antibiotics.

Construction of pWY121

To construct a temperature-sensitive vector, a corresponding replication origin and an erythromycin resistance cassette were amplified from plasmid pE194 using primer pair P1/P2, and the blunt-ended PCR product was cloned into the Nael site of pGEM-T easy, forming pGE194. The λ cI857 P<sub>RM</sub>-P<sub>R</sub> promoter system was synthesized in the form of Sall/NheI-rbs-P<sub>RM</sub>-P<sub>R</sub>-rbs-XbaI-NdeI, with ribosome binding sites (rbs, ’taaggag’) and restriction
enzyme cutting sites properly integrated at both extremities. The Sall-NdeI digested product was inserted into the corresponding sites of pGE194, forming pGEP. Gene cl857 was amplified from plasmid pCP20 using primer pair P3/P4, with the NheI site introduced by primer P3 and the SalI site introduced by primer P4. The NheI-Sall digested PCR product was then cloned into the corresponding sites of pGEP to yield pGEPC which was digested with XbaI–NdeI and ligated with the PCR product of cre. The cre gene was amplified from pDGICZ using primer pair P5/P6 and digested with XbaI–NdeI. The resulting plasmid was named pGECC. Finally, lambda beta gene was cut from pKD46 using Sall–Neol and inserted into the corresponding sites of pGECC, overlapping the start codon of beta with the stop codon of cl857 in the order of ATGA, thus forming pWY121 (Figure 1A).

Construction of pQRBL

The bleomycin resistance cassette was PCR amplified from pDGICZ using primers P7 and P8, with EcoRI-lox71 incorporated into P7 and Sall-lox66 incorporated into P8. After EcoRI–Sall digestion, cassette lox71-ble-lox66 was ligated into the corresponding region of pMD19, yielding pMDB19. Lambda cl857 gene was PCR amplified from pCP20 using primer P9 and P10, both primers containing the NdeI site. After NdeI digestion, cl857 was inserted into the NdeI site of pMDB19, forming pMDB-857. A counter-selectable marker cassette BglII-P4-hewl-BglII was synthesized without signal peptide sequence for intracellular expression of hen egg white lysozyme. After digestion with BglII, cassette P4-hewl was inserted into the BamHI site that was presented immediately downstream of the stop codon of the bld gene in pMDB-857. The resulting plasmid was named pQRBL (Figure 1B).

ssDNA generation

Since the complete genome sequence of B. subtilis ATCC6633 was not available, the determination of replication orientation and design of PCR primers were based on the published genome sequence of B. subtilis subsp. spizizenii str. W23, which is 99.995% identical with the draft sequence of strain 6633 (55). The ssDNA of the PCR products were generated according to the method established by Tang et al. (56), with the following modifications: 100 pg PCR product amplified in 50 µl PCR solution [consisting of 25 µl 2× Long Taq Mix (Dongshe Biotec, Co. Ltd., China), 0.5 µg pQRBL, 2 pmol each forward and reverse primers] was used as the template for the generation of ssDNA in the same PCR system containing one primer. The cycling program was 94°C for 3 min for DNA denaturation, followed by 30 cycles of 94°C for 30s, 55°C for 30s and 72°C for 1 min. The ssDNA was purified using QIAEX II Kit (Qiagen, Germany).

Gene disruption

The basic principles of gene disruption are illustrated in Figure 2. Bacillus subtilis ATCC6633 transformants carrying plasmid pWY121 were made electro-competent for the transformation of the single-stranded PCR products that target gene aabr. Cassette lox71-ble-lox66 was PCR amplified from pMDB19 with primer pair P11/P12; P11 and P12 each contains 70 nt extension homologous to regions adjacent to aabr. The PCR product was used as template for the generation of single-stranded PCR products using primer P11, P12 or P13. The P11 amplified strand was complementary to the leading strand of aabr during replication, the P12 amplified strand was complementary to the lagging strand of aabr and the P13 was the modified version of P12, with the first four internucleotide linkages at the 5'-end being phosphorothioated. After electroporation with these PCR products, the transformants were selected against bleomycin at 30°C and were cultured for 24 h in LB broth at 42°C and 170 rpm. Aliquots of 100 µl culture were spread on LB plates with or without bleomycin to determine the in-frame deletion frequency.

To test the efficiency of hewl as a counter-selectable marker, the PCR product of cassette ble-hewl (lox site free) was amplified from pQRBL with primers P14 and
P15 (each containing 70 nt extension homologous to regions adjacent to abrB), and was used as template for ssDNA generation with P15. The resulting ssDNA was transformed into ATCC6633/pWY121 and selected against bleomycin. The desired mutant ATCC6633::ble-hewl was named B. subtilis AL135.

The in-frame abrB deletion mutant was regenerated using cassette lox71::ble-hewl-lox66. The cassette was first amplified from pQRBL with primer pair P11/P12 and the PCR product was used as template for ssDNA generation with primer P13. The abrB deletion mutant was named B. subtilis AB211. Strain AB211 containing pWY121 was made electro-competent for the disruption of the ~37 kb mycosubtilin synthetase gene cluster myc. The lox71::ble-hewl-lox66 cassette amplified with primer pair P16/P17 (each containing 70 nt extension homologous to regions adjacent to myc) and was used as template for generation of ssDNA using P17. After transforming the P17 amplified ssDNA into AB211/pWY121, the bleomycin-resistant colonies were grown in LB broth at 42°C for 24h and then spread on LB agar plates.

Cell viability determination

The Bacillus subtilis ATCC6633 wild-type carrying pWY121 and the mutant AL135 containing pWY121 were cultured in 200 ml LB broth by shaking at 30°C and 170 rpm. The OD600 was measured using a Nanodrop 2000C spectrophotometer (Thermo Scientific, Wilmington, DE, USA). When the OD600 reading reached 0.6, cultures were immediately transferred to a 42°C incubator for continued shaking and OD600 detection. Cultures of AL135 were periodically sampled and plated on LB agar for colony forming units (CFU) enumeration.

Mutation verification

Mutations of abrB were checked by southern hybridization. Genomic DNA of B. subtilis wild-type and mutant strains were digested with EcoRV endonuclease, electrophoresed in 1.0% agarose gel and transferred onto nylon membranes (Roche, Germany). A DNA probe was prepared using Digoxigenin-labeled dUTP and specific PCR products amplified from the genomic DNA of the wild-type strain with primer pair P18/P19. Hybridization and detection were performed according to the manufacturer’s protocol (Roche, Germany). Deletion of myc cluster was checked by PCR reaction with primer pair P20/P21.

RESULTS

Improvement of electroporation condition

High transformation efficiency is the prerequisite for efficient DNA recombination, but traditional electroporation protocols for B. subtilis usually produce an efficiency of $10^4 - 10^6$ (9, 57–59) which was improved in the present study. Cell walls of B. subtilis were weakened by adding glycine and DL-threonine, cell-membrane fluidity was elevated by supplementing Tween 80 according to Zhang et al. (54), and $10^5$ CFU/µg pE194 DNA were obtained. The transformation efficiency was increased to $10^7$ CFU/µg pE194 DNA with the addition of 0.5 M trehalose to the electroporation medium (Table 3). Trehalose, in combination with an equal amount of sorbitol and mannositol has recently been reported to significantly improve the transformation efficiency by protecting B. subtilis cells from electric shock damage (59).

Proper timing of competent cell preparation is important for ssDNA-directed beta recombination because of the requirement of DNA replication; hence, cells were collected from early log phase of growth, during which DNA replicated actively and the 3′-5′ internucleotide linkages phosphorothioated, became resistant to 3′-5′ exonucleases (25) and increased its potential for crossing the lipid bilayer (60).

Description of the ssDNA-directed genome editing system in B. subtilis

In this system, we constructed the plasmid pMDB19 as a PCR template for the generation of single-stranded PCR products. Beta protein was encoded by the low-copy
plasmid pWY121 that contained the temperature inducible promoter system $\lambda e^{-}857$-PR$_{RM}$-P$_R$ (50) and the protein was expressed from the strong promoter PR$_{RM}$ by fusing behind $\lambda e^{-}857$. It is worth noting that, while the drug-resistant marker is sometimes useful to maintain stably the transformant, it is necessary for it to be deleted for the multiple manipulation of the genome (42). The product of $\lambda e^{-}857$ represses promoter P$_R$ at 30°C and the repression can be relieved at 42°C. Therefore, we placed the cre recombinase gene after P$_R$ for conditional in-frame deletion. Cre excises the marker gene flanked by the convergently oriented lox sites. Hence, after beta recombination at 30°C, the in-frame deletion can be easily achieved by switching the temperature to 42°C.

Transformants carrying plasmid pWY121 were made electro-competent for the transformation of PCR products. After primary selection against bleomycin, mutants were cultured in LB broth at 42°C for 24 h (Erythromycin should be added to the LB broth to maintain pWY121 if further modifications were required). A portion of the mutant culture was then spread on LB plates and incubated at 30°C for bleomycin sensitivity test to calculate the marker deletion frequency. Samples were finally colony-purified non-selectively at 30°C and then tested for loss of plasmid pWY121 based on erythromycin sensitivity. The problem of the genome editing system was, after Cre recombination, a part of the cell population still contained the intact resistance marker. Selection of the strain that had lost resistance was time-consuming due to the absence of positive selection, making counter-selectable marker instrumental for improving this system.

Construction of the marker-eviction hewl cassette

To test the feasibility of using hewl as a counter-selectable marker in B. subtilis, we inserted the $e^{-}857$ gene into plasmid pMDB19 to yield plasmid pMDB-857 and the PR-hewl fragment was synthesized and inserted into pMDB-857 between ble and lox66 to form the new cassette lox71-ble-hewl-lox66. The effect of hewl as counter-selectable marker was tested by plotting the cell viability profile of B. subtilis AL135 which contained a $\Delta$abrB:ble-hewl mutation and a free plasmid pWY121. Figure 3 shows that the growth rate of AL135 slowed down after temperature shift from 30°C to 42°C, and that the OD600 of the culture decreased 4 h later. The living cells of AL135 were periodically enumerated. We observed that AL135 could not form colonies on LB plate after 12 h of induction.

Disruption of abrB gene and myc gene cluster

Disruption of gene abrB using the traditional method of cloning the whole DNA in E. coli and inserting the resistance marker into the middle of the gene failed, because the promoter of abrB was too strong and abrB with its native promoter was toxic to E. coli. Therefore, we used pMDB19 as PCR template to delete the 285 bp abrB gene, leaving intact the terminator and promoter for yabC and metS (Figure 4). The lox71-ble-lox66 cassette on pMDB19 was amplified using primers that carried 70 nt homology extensions matching the flanking regions of abrB. The ssDNAs were generated and separately introduced into strain ATCC6633 that expressed beta protein. During beta-mediated integration of exogenous DNA, the lagging targeting strand was more efficient than the leading targeting strand (2.8 $\times$ 10$^3$ versus 0.3 $\times$ 10$^3$), implicating the preferential annealing of ssDNA to the lagging strand. Phosphorothioate modification at the 5’-end of the lagging targeting strand improved the recombination efficiency to 1.4 $\times$ 10$^4$ CFU/µg DNA (Figure 5).

After beta recombination, the bleomycin-resistant mutants were cultured in antibiotic free LB broth at 42°C to allow expression of Cre in order to delete the disruption cassette. Cre recombination occurred at a frequency of about 85.3% (Table 4), with the desired in-frame deletion mutants mixed with the insertional mutants and a counter-selectable marker was required to eliminate the cells carrying intact disruption cassette.
Plasmid pQRBL was thus constructed to provide cassette lox71-ble-hewl-lox66 that contained the Pr-driven gene hewl encoding hen egg white lysozyme. Intracellular expression of the lysozyme during incubation at 42°C killed the cells with intact disruption cassette.

Bacillus subtilis ATCC6633 without gene abrB was reconstituted using cassette lox71-ble-hewl-lox66. The in-frame deletion efficiency turned out to be 100%, albeit the insertional inactivation efficiency was not as high as lox71-ble-lox66 (Table 4). Plasmid pWY121 was maintained in abrB mutant strain AB211, and the ~37 kb mycosubtilin synthetase gene cluster myc was deleted in the same way.

**DISCUSSION**

Our method for editing B. subtilis genome is based on the lambda Red system (20). In the preliminary study, we expressed the Red genes (γ, β and exo) under IPTG inducible promoter Pspac on a pE194 derived plasmid, but recombination with dsDNA that carried short homology (less than 100 bp) was inefficient and non-specific, probably due to the failure of Gam to inhibit AddAB (counterpart of E. coli RecBCD). It was reported that AddAB in Coxiella burnetii did not interact with the lambda gamma protein (61).

It was recently discovered that lambda beta-mediated recombination occurred through a fully single-stranded intermediate which preferentially targeted the lagging strand during DNA replication (25,26). This new mechanism of the Red system inspired us to try ssDNAs. Basically, ssDNA-directed recombination required only the beta protein, and this simplified processing reduced degradation of DNA. During beta recombination with single-stranded disruption cassette carrying various homology lengths, bleomycin resistance gene with 70 nt homology extensions was observed to be sufficient for B. subtilis genome editing (data not shown).

Here, we constructed the recombination plasmid pWY121 in which beta and cre were cloned under control of Prm and Pr, separately, on a pE194-derived low-copy number and temperature-sensitive plasmid. This construction allowed expression of beta at 30°C and cre at 42°C, so that, after beta-mediated integration of the disruption cassette, the marker gene could be Cre-deleted by temperature shift and no additional transformation was required. Meanwhile, template plasmid pQRBL was constructed to equip ssDNA generation with a disruption cassette that contained the antibiotic resistance marker ble and the counter-selectable marker hewl, and was flanked by lox sites. The length of the cassette was 1081 bp, about the average of Okazaki fragments.

Beta recombination with lagging targeting strand was more efficient than that with leading targeting strand (Figure 5). This strand preference implied that ssDNA preferentially annealed at the replication fork for lagging strand synthesis. The 5' homology arm of the lagging targeting strand should anneal after the 3' homology arm of the ssDNA, because its complementary region was exposed later at the replication fork. Therefore, the 5' homology arm is more important than the 3' homology arm (25). Phosphorothioate modification at the 5'-end of ssDNA improved the recombination efficiency significantly (Figure 5), by conferring exonuclease resistance to ssDNA (26, 30).

**Table 4. The efficiencies of abrB mutation using different cassettes**

<table>
<thead>
<tr>
<th>Cassette</th>
<th>Insertional inactivationa</th>
<th>In-frame deletionb</th>
</tr>
</thead>
<tbody>
<tr>
<td>lox71-ble-lox66</td>
<td>(1.4 ± 0.8) × 10⁴/µg DNA</td>
<td>85.3%</td>
</tr>
<tr>
<td>lox71-ble-hewl-lox66</td>
<td>(3.7 ± 1.5) × 10⁴/µg DNA</td>
<td>100.00%</td>
</tr>
</tbody>
</table>

aThe Beta-mediated insertional inactivation efficiency was calculated as the number of Ble colonies/µg of PCR products.
bThe Cre-mediated in-frame deletion efficiency was calculated as (1-Nr/Nt) × 100%. Nr, number of Ble colonies in 100 µl 42°C treated culture; Nt, number of total colonies in 100 µl 42°C treated culture.
Cre recombinase excised the disruption cassette at a high frequency and the cells with intact disruption cassette could be extirpated by the hewl-encoded thermostable lysozyme (Table 4). Notably, during 42°C incubation, hewl could be expressed from the Cre-recombined residual plasmid, and this accumulation of the lysozyme to the lethal concentration took about 4 h during which sufficient parent cells were divided. Although direct incubation of mutants carrying the lox71-ble-hewl-lox66 cassette at 42°C yielded positive in-frame deletion mutants, short time (2–3 h) incubation of the mutants at 30°C before induction at 42°C was more efficient, probably due to the activated cell division that allowed more hewl-free mutant strains to be produced before the parent cells were destroyed by the lysozyme. Cre recombination between lox71 and lox66 sites left a 69 nt scar (Figure 2). As drawn, this scar contained an idealized ribosome binding site for downstream gene expression. Stop codons existed on both directions in the scar but all of them were in one reading frame on each direction. Therefore, pQRBL could be used to generate non-polar deletions. The scar also possessed a residual lox72 site which was resistant to Cre recombinase and did not affect further manipulations in the same genome (37,38).

In summary, the present results demonstrate that lambda beta protein could be successfully applied to short homology-directed HR in B. subtilis and by analogy, to genome editing in more organisms, given that the recombinase is well expressed and the donor DNA is finely protected. Furthermore, the exploitation of the cI857-P<sub>Rm</sub>-P<sub>R</sub>-hewl system as a counter-selectable marker makes genome editing in B. subtilis more straightforward.

ACCESSION NUMBERS

The sequences reported in this article were deposited in the GenBank database [accession nos. JN798465 (pWY121), JN798466 (pQRBL)].

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REFERENCES


