Bacillus subtilis genome editing using ssDNA with short homology regions

Yang Wang¹, Jun Weng¹, Raza Waseem¹, Xihou Yin², Ruifu Zhang¹ and Qirong Shen¹,*

¹Department of Plant Nutrition, College of Resource and Environmental Sciences, Nanjing Agricultural University, No.1 Weigang Road, Nanjing 210095, Jiangsu Province, P.R. China and ²Department of Pharmaceutical Sciences, College of Pharmacy, Oregon State University, Pharmacy Building Room 203, Corvallis, OR 97331–3507, USA

Received October 4, 2011; Revised March 4, 2012; Accepted March 5, 2012

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 PRM in the lambda cI857 PRM–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 exonucleolytic 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
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 *S*. 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 exoribonuclease YhaM degrades ssDNA from the 3'-ends by phosphorothioate modification (26,30). The 0-fold markers could be excised by Cre recombinase 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

| Strains or plasmid | Characteristics<sup>a</sup> | Reference |
|--------------------|-------------------------------|-----------|
| **Bacterial strains**<br>Escherichia coli DH5α | Φ80lacZ ΔM15 recA1 endA1 gyr96 thi-1 hsdR17 (rK<sup>-</sup>, mK<sup>+</sup>) supE44 relA1 deoR | Promega |
| Bacillus subtilis ATCC633 | Wild-type, produces mycosubtilin | 51 |
| Bacillus subtilis AL135 | Bacillus subtilis ATCC633::ble-hwel | This work |
| Bacillus subtilis AB211 | Bacillus subtilis ATCC633ΔabrB | This work |
| Bacillus subtilis AM336 | Bacillus subtilis ATCC633ΔabrB Δmyc | This work |
| **Plasmids** | | |
| pGEM-T easy | Amp<sup>+</sup>, cloning vector | Promega |
| pEG194 | Erm<sup>+</sup>, Bacillus/Staphylococcus plasmid vector | 52 |
| pGE194 | Amp<sup>+</sup>, Erm<sup>+</sup>, B.subtilis–E.coli shuttle vector | This work |
| pGEP | Amp<sup>+</sup>, Erm<sup>+</sup>, B.subtilis–E.coli shuttle vector containing the P<sub>RM</sub>–P<sub>R</sub> promoter system for expression in B.subtilis | This work |
| pGEPC | Amp<sup>+</sup>, Erm<sup>+</sup>, pGEP containing λ cI857<sup>+</sup> | This work |
| pGECC | Amp<sup>+</sup>, Erm<sup>+</sup>, pGECC containing cI857<sup>+</sup> | This work |
| pKD46 | Amp<sup>+</sup>, Red expression plasmid, PBAD gam bet exo ori pSC101 | 20 |
| pCP20 | Amp<sup>+</sup>, cat cI857<sup>+</sup> K<sub>R</sub> φ80 D lacZ<sub>a</sub>ΔlacYA-argF)U169 phiA | 20 |
| pWY121 | Amp<sup>+</sup>, Erm<sup>+</sup>, B. subtilis recombination vector encoding λ cI857<sup>+</sup>, λ β, κ exo and cre recombinase genes. | This work |
| pMD19 | Amp<sup>+</sup>, cloning vector | Takara |
| pDGHCZ | B. subtilis integration vector, ble, cre | 39 |
| pMDB19 | Amp<sup>+</sup>, Ble<sup>+</sup>, pMD19 containing lox71-ble-lox66 cassette | This work |
| pMDB-857 | Amp<sup>+</sup>, pMDB19 containing λ cI857<sup>+</sup> gene in the NdeI site | This work |
| pQRL | Amp<sup>+</sup>, Ble<sup>+</sup>, pMDB-857 with lox71-ble-hwel-P<sub>R</sub>-lox66 cassette | This work |

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

### Table 2. Primer sequences

| Primer | Sequence<sup>b</sup> |
|--------|----------------------|
| P1     | 5’ cacattgaacacgctgca3’ |
| P2     | 5’ gccgaaagggtcgaaga3’ |
| P3     | 5’ tagtactgctagacacaa3’ |
| P4     | 5’ tggctagacgaatctgca3’ |
| P5     | 5’ ctagtctgatgccaaaggct3’ |
| P6     | 5’ gaattccatatgtaaatct3’ |
| P7     | 5’ cgggtctagatctgactatct3’ |
| P8     | 5’ gacctgacctcctacgtaa3’ |
| P9     | 5’ ggggatctgatcgaaca3’ |
| P10    | 5’ ggggatctgatcgaaca3’ |
| P11    | 5’ ggtctgatcgaacacgctgca3’ |
| P12    | 5’ ggtctgatcgaacacgctgca3’ |
| P13    | 5’ ggtctgatcgaacacgctgca3’ |
| P14    | 5’ ggtctgatcgaacacgctgca3’ |
| P15    | 5’ ggtctgatcgaacacgctgca3’ |
| P16    | 5’ ggtctgatcgaacacgctgca3’ |
| P17    | 5’ cceccctggctccataccgct3’ |
| P18    | 5’ acaggaaatacttacca3’ |
| P19    | 5’ ccaacattttggcttta3’ |
| P20    | 5’ gctgtacactgttagta3’ |
| P21    | 5’ cctgtatagctgctgta3’ |

<sup>b</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 pEG194 using primer pair P1/P2, and the blunt-ended PCR product was cloned into the NaeI site of pGEM-T easy, forming pGE194. The λ cI857<sup>+</sup> P<sub>RM</sub>–P<sub>R</sub> promoter system was synthesized in the form of Sall-NheI-ble-P<sub>R</sub>-P<sub>RM</sub>-ble-NheI-XbaI-NdeI, with ribosome binding sites (rbs, ‘taaggagg’) and restriction...
enzyme cutting sites properly integrated at both extremities. The Sall-Ndel digested product was inserted into the corresponding sites of pGEP194, 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–SalI digested PCR product was then cloned into the corresponding sites of pGEP to yield pGEPB, which was digested with XbaI–Ndel and ligated with the PCR product of cre. The cre gene was amplified from pDGICZ using primer pair P5/P6 and digested with XbaI–Ndel. The resulting plasmid was named pGECB. Finally, lambda beta gene was cut from pKD46 using Sall–NcoI and inserted into the corresponding sites of pGECB, 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 pMD19B. Lambda cl857 gene was PCR amplified from pCP20 using primer P9 and P10, both primers containing the Ndel site. After Ndel digestion, cl857 was inserted into the Ndel site of pMDB19, forming pMDB857. A counter-selectable marker cassette BglII-Pe-hewl-BglII was synthesized without signal peptide sequence for intracellular expression of hen egg white lysozyme. After digestion with BglII, cassette Pe-hewl was inserted into the BamHI site that was presented immediately downstream of the stop codon of the ble gene in pMDB857. 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 (Dongsheng Biotech 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 30 s, 55°C for 30 s 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 *abrB*. 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 *abrB*. 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 *abrB* during replication, the P12 amplified strand was complementary to the lagging strand of *abrB* 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 ΔabrB::ble-hewl was named *B. subtilis* AL135.

The in-frame *abrB* deletion mutant was regenerated using cassette *lox*71-*ble*-hewl-*lox*66. 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 *lox*71-*ble*-hewl-*lox*66 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 24 h 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% 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 mannitol 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' exonuclease YhaM was repressed by LexA (32). Use of ssDNA is a necessary requirement for successful beta recombination in *B. subtilis* since AddAB enzymes degrade dsDNA and hinder the recombination. The ssDNA, with the 5'-end 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 λ cI857-PRM-P_R (50) and the protein was expressed from the strong promoter P_RM by fusing behind λ cI857. 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 λ cI857 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 hewl into plasmid pMDB19 to yield plasmid pMDB-857 and the P_R-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 ΔabrB::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 merS (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 × 10^3 versus 0.3 × 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 × 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.
the bracket indicates the length of the predicted PCR test product.

cassette
lox71-ble-lox66

cassette
lox71-ble-hewl-lox66

the two EcoRV restriction sites flanking mutation, the sizes in the brackets indicate the DNA lengths between the lagging strand; 4. ssDNA complementary to the lagging strand; 3. ssDNA complementary to the leading strand; 2. ssDNA complementary to the lagging strand with four consecutive phosphorothioated bonds at the 5'-end.

Plasmid pQRBL was thus constructed to provide cassette lox71-ble-hewl-lox66 that contained the P<sub>R</sub>-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 constructed using cassette lox71-ble-hewl-lox66. (Table 4). Plasmid pWY121 was thus 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.

**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 P<sub>RM</sub> and P<sub>R</sub>, 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 |
|-------------------------------|-------------------------------|-------------------------------|
| Insertional inactivation<sup>a</sup> | In-frame deletion<sup>b</sup> |
| lox71-ble-lox66 | (1.4 ± 0.8) × 10<sup>3</sup>/µg DNA | 85.3% |
| lox71-ble-hewl-lox66 | (3.7 ± 1.5) × 10<sup>2</sup>/µg DNA | 100.00% |

<sup>a</sup>The Beta-mediated insertional inactivation efficiency was calculated as the number of Ble<sup>c</sup> colonies/µg of PCR products.

<sup>b</sup>The Cre-mediated in-frame deletion efficiency was calculated as (1-Nr/Nt) × 100%. Nr, number of Ble<sup>c</sup> 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 cultivated by the hewl-encoded thermo-stable 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-PRM-P_R-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)].

ACKNOWLEDGEMENTS

Prof. Feirong Gu and David D. Myrold are thanked for their critical reading of the article and Dr Xin Yan for providing plasmids pE194 and pDGICZ.

FUNDING

Funding for open access charge: Priority Academic Program Development of Jiangsu Higher Education Institutions; Chinese Ministry of Science and Technology (2011BAD11B03-02) and Chinese Ministry of Agriculture (201103004).

Conflict of interest statement. None declared.

REFERENCES

1. Bron,S., Meima,R., van Dijl,J.M., Wipat,A. and Harwood,C. (1999) Molecular biology and genetics of Bacillus spp. In: Demain,A.L. and Davies,J.E. (eds), Manual of Industrial Microbiology and Biotechnology, 2nd edn. ASM Press, Washington, DC, pp. 392–416.
2. Schallmeier,M., Singh,A. and Ward,O.P. (2004) Developments in the use of Bacillus species for industrial production. Can. J. Microbiol, 50, 1–17.
3. Dubnau,D. (1993) Genetic exchange and homologous recombination. In: Sonenshein,A.L., Hoch,J.A. and Losick,R. (eds), Bacillus subtilis and Other Gram-positive Bacteria: Biochemistry, Physiology, and Molecular Genetics. ASM, Washington, DC, pp. 555–584.
4. Dubnau,D. and Lovett,C.M. Jr (2002) Transformation and recombination. In: Sonenshein,A.L., Hoch,J.A. and Losick,R. (eds), Bacillus subtilis and Its Closest Relatives: from Genes to Cells. ASM, Washington, DC, pp. 453–471.
5. Melnikov,A. and Youngman,P.J. (1999) Random mutagenesis by recombinational capture of PCR products in Bacillus subtilis and Acinetobacter calcoaceticus. Nucleic Acids Res., 27, 1056–1062.
6. Nijland,R., Burgess,J.G., Errington,J. and Veening,J.W. (2010) Transformation of environmental Bacillus subtilis isolates by transiently inducing genetic competence. PloS ONE, 5, e9724.
7. Yashin,R.E. and Young,F.E. (1974) Transduction in Bacillus subtilis by bacteriophage SPPI. J. Virol., 14, 1343–1348.
8. Chang,S. and Cohen,S.N. (1979) High frequency transformation of Bacillus subtilis protoplasts by plasmid DNA. Mol. Gen. Genet., 168, 111–115.
9. Brigidi,P., De Rossi,E., Bertarini,M.L., Riccardi,G. and Matteuzzi,D. (1990) Genetic transformation of intact cells of Bacillus subtilis by electroporation. FEBS Microbiol. Lett., 67, 135–138.
10. Kooistra,J., Huijema,B.J. and Venema,G. (1993) The Bacillus subtilis AddAB genes are fully functional in Escherichia coli. Mol. Microbiol., 7, 915–923.
11. Hajime,B.J., Venema,G. and Kooistra,J. (1996) The C terminus of the AddA subunit of the Bacillus subtilis ATP-dependent DNase is required for the ATP-dependent exonuclease activity but not for the helicase activity. J. Bacteriol., 178, 5086–5091.
12. Chedin,F., Noirot,P., Biaudet,V. and Ehrlich,S.D. (1988) A five-nucleotide sequence protects DNA from exonucleaseolytic degradation by AddAB, the RecBCD analogue of Bacillus subtilis. Mol. Microbiol., 29, 1369–1377.
13. Chedin,F., Ehrlich,S.D. and Kowalezykowski,S.C. (2000) The Bacillus subtilis AddAB helicase/nuclease is regulated by its cognate Chi sequence in vitro. J. Mol. Biol., 298, 7–20.
14. Anderson,D.G. and Kowalezykowski,S.C. (1997) The translocating RecBCD enzyme stimulates recombination by directing RecA protein onto ssDNA in a γ-regulated manner. Cell, 90, 77–86.
15. Watt,V.M., Ingles,C.J., Urdea,M.S. and Rutter,W.J. (1985) Homology requirements for recombination in Escherichia coli. Proc. Natl Acad. Sci. USA, 82, 4766–4772.
16. King,S.R. and Richardson,J.P. (1986) Role of homology and pathway specificity for recombination between plasmids and bacteriophage. Mol. Gen. Genet., 204, 141–147.
17. Hofmeester,J., Israeli-Reches,M. and Dubnau,D. (1983) Integration of plasmid pE194 at multiple sites on the Bacillus subtilis chromosome. Mol. Gen. Genet., 189, 58–68.
18. Bashkirov,V.I., Khasanov,F.K. and Prozorov,A.A. (1987) Illegitimate recombination in Bacillus subtilis: nucleotide sequences at recombinant DNA junctions. Mol. Gen. Genet., 210, 578–580.
19. Dutta,S., Costantino,N., Zhou,X. and Court,D.L. (2008) Identification and analysis of recombineering functions from Gram-negative and Gram-positive bacteria and their phages. Proc. Natl Acad. Sci. USA, 105, 1626–1631.
20. Datserik,A.A. and Wanmer,B.L. (2000) One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc. Natl Acad. Sci. USA, 97, 6640–6645.
21. Murphy,K.C. and Marinus,M.G. (2010) RecA-independent single-stranded DNA oligonucleotide-mediated mutagenesis. F1000 Biol. Rep., 2, 56.
22. Murphy,K.C., Campellone,K.G. and Poteete,A.R. (2000) PCR-mediated gene replacement in Escherichia coli. Gene, 246, 321–330.
43. Defoor,E., Kryger,M.B. and Martinussen,J. (2007) The orotate transporter encoded by oroP from Lactococcus lactis is required for orotate utilization and has utility as a food-grade selectable marker. Microbiology, 153, 3645–3659.

44. Abdou,A.M., Higashigushi,S., Aboueleinin,A.M., Kim,M. and Ibrahim,H.R. (2007) Antimicrobial peptides derived from hen egg lysozyme with inhibitory effect against Bacillus species. Food Control, 18, 173–178.

45. Yoshimura,K., Toibana,A., Kikuchi,K., Kobayashi,M., Hayakawa,T., Nakahama,K., Kikuchi,M. and Ikehara,M. (1987) Differences between Saccharomyces cerevisiae and Bacillus subtilis in secretion of human lysozyme. Biochem. Biophys. Res.Commun., 145, 712–718.

46. Pellegrini,A., Thomas,U., von Fellenberg,R. and Wild.P. (1992) Bactericidal activity of lysozyme and aprotinin against Gram-negative and Gram-positive bacteria related to their basic character. J. Appl. Bacteriol., 72, 180–187.

47. Ibrahim,H.R., Higashigushi,S., Junjea,L.R., Kim,M. and Yamamoto,T. (1996) A structural phase of heat-denatured lysozyme with novel antimicrobial action. J. Agric. Food Chem., 44, 1416–1423.

48. Ibrahim,H.R., Matsuzaki,T. and Aoki,T. (2001) Genetic evidence that antibacterial activity of lysozyme is independent of its catalytic function. FEBS Lett., 506, 27–32.

49. Ibrahim,H.R., Thomas,U. and Pellegrini,A. (2001) A helix-loop-helix peptide at the upper lip of the active site cleft of lysozyme confers potent antimicrobial activity with membrane permeabilization action. J. Biol. Chem., 276, 43767–43774.

50. Breitling,R., Sorokin,A.V. and Behnke,D. (1990) Temperature-inducible gene expression in Bacillus subtilis mediated by the c857-encoded repressor of bacteriophage lambda. Gene, 93, 30–34.

51. Duitman,E.H., Hamoen,L.W., Rembold,M., Venema,G., Seitz,H., Saenger,W., Bernhardt,F., Reinhardt,R., Schmidt,M., Ullrich,C. et al. (1999) The mycosubtilin synthetase of Bacillus subtilis ATCC 6633: a multifunctional hybrid between a peptide synthetase, an amino transferase, and a fatty acid synthase. Proc. Natl Acad. Sci. USA, 96, 13294–13299.

52. Horinouchi,S. and Weisblum,B. (1982) Nucleotide sequence and functional map of pE184, a plasmid that specifies inducible resistance to macrolide, lincosamide and streptogramin type B antibiotics. J. Bacteriol., 150, 804–814.

53. Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

54. Zhang,G., Bao,P., Zhang,Y., Deng,A., Chen,N. and Wen,T. (2011) Enhancing electro-transfer and transfection efficiency of recalcitrant Bacillus anoxylipasefaciens by combining cell-wall weakening and cell-membrane fluidity disturbing. Anal. Biochem., 409, 130–137.

55. Zeigler,D.R. (2011) The genome sequence of Bacillus subtilis subsp. spizizenii W23: insights into speciation within the B. subtilis complex and into the history of B. subtilis genetics. Microbiology, 157, 2013–2041.

56. Tang,X., Morris,S.L., Langone,J.J. and Bockstahler,L.E. (2006) Simple and effective method for generating single-stranded DNA targets and probes. BioTechniques, 40, 759–763.

57. Stephenson,M. and Jarrett,P. (1991) Transformation of Bacillus subtilis by electroporation. Biotechnol. Tech., 5, 9–12.

58. Xue,G.P., Johnson,J.S. and Dalrymple,B.P. (1999) High osmolality improves the electro-transformation efficiency of the Gram-positive bacteria Bacillus subtilis and Bacillus licheniformis. J. Microbiol. Methods, 34, 183–191.

59. Cao,G.Q., Zhang,X.H., Zhong,L. and Lu,Z.X. (2011) A modified electro-transformation method for Bacillus subtilis and its application in the production of antimicrobial lipopeptides. Biotechnol. Lett., 33, 1047–1051.

60. Hughes,J.A., Bennett,C.F., Cook,P.D., Guinouso,C.J., Mirabelli,C.K. and Juliano,R.L. (1994) Lipid membrane permeability of 2-modified derivatives of phosphorothioate oligonucleotides. J. Pharmacol. Sci., 83, 597–600.

61. Mertens,K., Lantsheer,L., Ennis,D.G. and Samuel,J.E. (2008) Constitutive SOS expression and damage-inducible AddAB-mediated recombinational repair systems for Coxiella burnetii as potential adaptations for survival within macrophages. Mol. Microbiol., 69, 1411–1426.