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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 *cI857* 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 recombinase 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 (χ_{Bs}, 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

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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 exoribonuclease 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 P_R as a temperature-inducible counter-selectable marker. The P_R 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 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 OD_{600} (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 $MgCl_2$, 0.5 mM K_2HPO_4 and 0.5 mM KH_2PO_4, 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[a]	Reference
Bacterial strains		
Escherichia coli DH5α	Φ80d*lacZ ΔM15 recA1 endA1 gyrA96 thi-1 hsdR17 (rK⁻, mK⁺) supE44 relA1 deoR Δ(lacZYA-argF)U169 phoA*	Promega
Bacillus subtilis ATCC6633	Wild-type, produces mycosubtilin	51
Bacillus subtilis AL135	*Bacillus subtilis* ATCC6633*ΔabrB*::ble-*hewl*	This work
Bacillus subtilis AB211	*Bacillus subtilis* ATCC6633 *ΔabrB*	This work
Bacillus subtilis AM336	*Bacillus subtilis* ATCC6633 *ΔabrB Δmyc*	This work
Plasmids		
pGEM-T easy	Amp^r, cloning vector	Promega
pE194	Erm^r, Bacillus/Staphylococcus plasmid vector	52
pGE194	Amp^r, Erm^r , *B.subtilis–E.coli* shuttle vector	This work
pGEP	Amp^r, Erm^r , *B.subtilis–E.coli* shuttle vector containing the P_{RM}-P_R promoter system for expression in *B.subtilis*	This work
pGEPC	Amp^r, Erm^r , pGEP containing *λ cI857*	This work
pGECC	Amp^r, Erm^r , pGEPC containing *cre*	This work
pKD46	Amp^r, Red expression plasmid, PBAD gam bet exo ori pSC101	20
pCP20	Amp^r, cat *cI857* λ P_R flp pSC101 oriTS	20
pWY121	Amp^r, Erm^r, *B. subtilis* recombination vector encoding *λ cI857*, *λ β*, *λ exo* and *cre* recombinase genes.	This work
pMD19	Amp^r, cloning vector	Takara
pDGICZ	*B. subtilis* integration vector, *ble*, *cre*	39
pMDB19	Amp^r, Ble^r, pMD19 containing *lox71-ble-lox66* cassette	This work
pMDB-857	Amp^r, pMDB19 containing *λ cI857* gene in the NdeI site	This work
pQRBL	Amp^r, Ble^r, pMDB-857 with *lox71-ble-hewl*-P_R-*lox66* cassette	This work

[a]Amp^r: Ampicillin-resistance, Erm^r: Erythromycin resistance, Ble^r: Bleomycin resistance, *hewl*: hen egg white lysozyme gene.

Table 2. Primer sequences

Primer	Sequence[a]
P1	5′ cacttgtaaccagtccgtccac 3′
P2	5′ gtccagaaggtcgatagaaag 3′
P3	5′ tagctagccgtatgagcacaaaaaagaaaccattaac 3′
P4	5′ gcgtcgacagaatccatgccgacacgttcagccagcttcccagccagcgttgcgagtgcagtactcatccaaacgtctcttcag 3′
P5	5′ ctagtctagagatgtccaatttactgaccgt 3′
P6	5′ ggaattccatatgtaagatttaaatgcaaccgt 3′
P7	5′ cggaattctaccgttcgtatagcatacattatacgaagttatcttgatatggcttttatatgtg 3′
P8	5′ acgcgtcgacctccttaccgttcgtataatgtatgctatacgaagttatggatcctcagtcctgctcctcgg 3′
P9	5′ ggggaattcatatggcaaccattatcaccgccagagg 3′
P10	5′ ggggaattcatatgtcatcagccaaacgtctcttcaggc 3′
P11	5′ gtcgatcgcaatgttccaaagcgtgaagtgtatgatgcttaccatattcagcaataaaaaaaacgttcttgaacgacggccagtgaattc 3′
P12	5′ aggtaaggattttgtcgaataatgacgaagaaaaatataatttaaacaaataatcatctcttgggaggagtgcaggtcgacctccttac 3′
P13	5′ a-g-g-t-aaggattttgtcgaataatgacgaagaaaaatataatttaaacaaataatcatctcttgggaggagtgcaggtcgacctccttac 3′
P14	5′ gtcgatcgcaatgttccaaagcgtgaagtgtatgatgcttaccatattcagcaataaaaaaaacgttcttgatatggctttttatatgtg 3′
P15	5′ a-g-g-t-aaggattttgtcgaataatgacgaagaaaaatataatttaaacaaataatcatctcttgggaggagggatctcaccgcaagggat 3′
P16	5′ cgagttgcaacgtcatctgtgtccgtccctggcagttttatctgccgcgggcttctcacccttgctcttgtgcaggtcgacctccttac 3′
P17	5′ c-c-c-c-tgtccgctaatccgctgcttcatcattcagcaggtaaactttatgggtgaagttaaagcgttcttcaacgacggccagtgaattc 3′
P18	5′ acaggaaaacattacacctcaggagt 3′
P19	5′ cacaactttatgccgtttttctgtcg 3′
P20	5′ gcctgtaactggctagaatc 3′
P21	5′ cctgtatcgtcttagccagc 3′

[a]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 NaeI site of pGEM-T easy, forming pGE194. The *λ cI857* P_{RM}-P_R promoter system was synthesized in the form of SalI-NheI-rbs-P_{RM}-P_R-rbs-XbaI-NdeI, with ribosome binding sites (rbs, 'taaggagg') and restriction

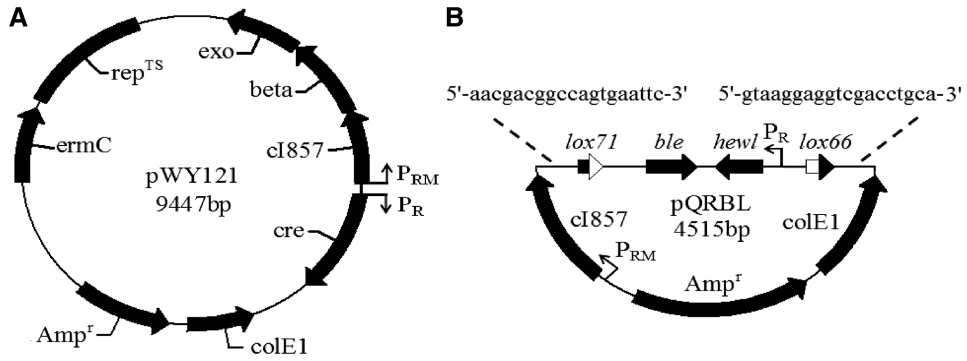

Figure 1. Plasmids used for gene disruptions in *B. subtilis*. (**A**) Recombinase genes (*beta* and *cre*) were cloned into pGE194, with a temperature-sensitive replicon (rep^{TS}) and erythromycin resistance gene (*ermC*). Lambda *beta* and *exo* were fused downstream of *cI857*, under control of P_{RM}, and *cre* was driven by P_R. (**B**) A disruption cassette was cloned into pMD19 carrying *cI857* to generate pQRBL, the disruption cassette contains bleomycin resistance gene (*ble*), hen egg white lysozyme (*hewl*) encoding gene under control of promoter P_R and *lox71*/*lox66* site at each extremity. Priming sequences for PCR amplification were indicated.

enzyme cutting sites properly integrated at both extremities. The SalI-NdeI digested product was inserted into the corresponding sites of pGE194, forming pGEP. Gene *cI857* 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 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 SalI–NcoI and inserted into the corresponding sites of pGECC, overlapping the start codon of *beta* with the stop codon of *cI857* 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 SalI-*lox66* incorporated into P8. After EcoRI–SalI digestion, cassette *lox71-ble-lox66* was ligated into the corresponding region of pMD19, yielding pMDB19. Lambda *cI857* gene was PCR amplified from pCP20 using primer P9 and P10, both primers containing the NdeI site. After NdeI digestion, *cI857* was inserted into the NdeI site of pMDB19, forming pMDB-857. A counter-selectable marker cassette BglII-P_R-*hewl*-BglII was synthesized without signal peptide sequence for intracellular expression of hen egg white lysozyme. After digestion with BglII, cassette P_R-*hewl* was inserted into the BamHI site that was presented immediately downstream of the stop codon of the *ble* 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 (Dongsheng 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 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

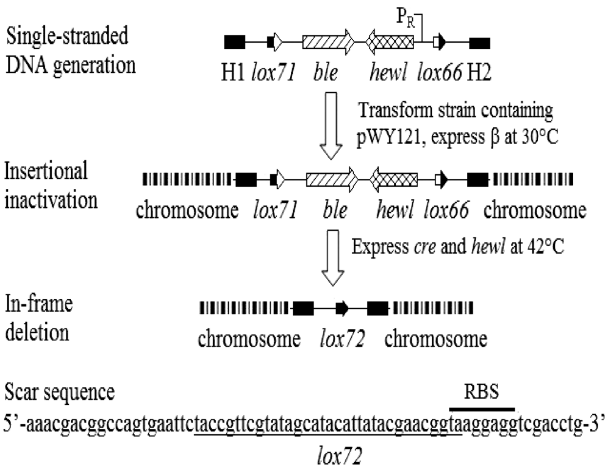

Figure 2. Gene disruption strategy. H1 and H2 refer to the homology extensions. Scar sequence indicates the 69 bp DNA region remained after Cre-mediated excision of the disruption cassette, *lox* sites and RBS (ribosome binding site) are marked.

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 *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 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 OD_{600} reading reached 0.6, cultures were immediately transferred to a 42°C incubator for continued shaking and OD_{600} 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.

Table 3. Effects of growth media and electroporation medium on the transformation efficiency of *B. subtilis*

Growth medium	Electroporation medium	Transformation efficiency (CFU/μgDNA)
LB	ETM	$(1.28 \pm 0.14) \times 10^4$
LBG	ETM	$(1.75 \pm 0.26) \times 10^4$
LBG + (Gly, Thr, Tween80)	ETM	$(7.31 \pm 0.94) \times 10^5$
LBG + (Gly, Thr, Tween80)	ETM + 0.5 M trehalose	$(1.04 \pm 0.19) \times 10^7$

ETM: 0.5 M sorbitol, 0.5 M mannitol, 0.25mM KH_2PO_4, 0.25mM K_2HPO_4 and 0.5mM $MgCl_2$.

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 5′–3′ 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

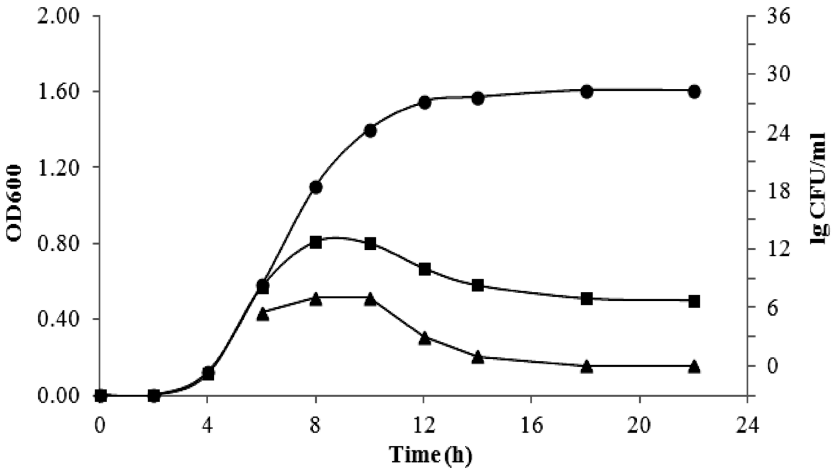

Figure 3. Viability profile of *B. subtilis* ATCC6633 wild-type and *abrB*::*ble-hewl* mutant AL135, both contain pWY121. Incubation temperature was shifted from 30°C to 42°C when the OD600 reached 0.6. Growth curves of wild-type (filled circle) and mutant AL135 (filled square) and logs of AL135 colony forming units (lg CFU/ml) were plotted against time (filled triangle).

plasmid pWY121 that contained the temperature inducible promoter system *λ cI857*-P_{RM}-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 the *cI857* gene 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 *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 (2.8×10^3 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.

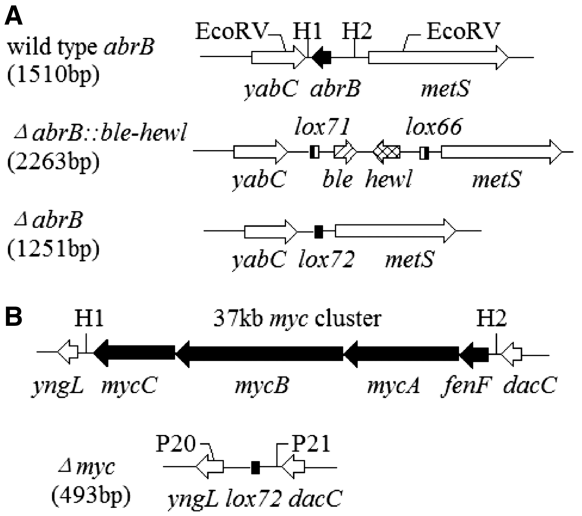

Figure 4. Structure and verification of gene mutation. (**A**) *abrB* mutation, the sizes in the brackets indicate the DNA lengths between the two EcoRV restriction sites flanking *abrB*. (No EcoRV site exists in cassette *lox71-ble-hewl-lox66*.) (**B**) Deletion of *myc* cluster, the size in the bracket indicates the length of the predicted PCR test product.

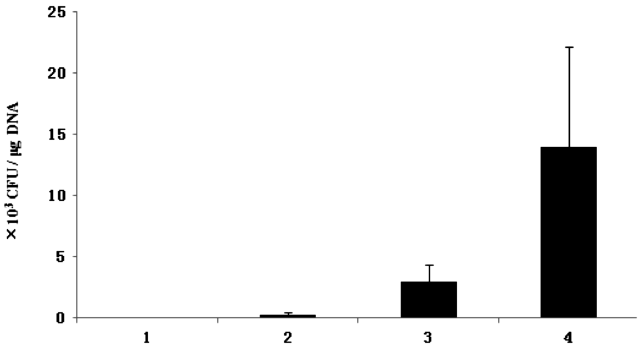

Figure 5. Bleomycin-resistant colonies after inactivation of *abrB* using cassette *lox71-ble-lox66* based PCR products: 1. dsDNA; 2. ssDNA complementary to the leading strand; 3. ssDNA complementary to the lagging strand; 4. ssDNA complementary to the lagging strand with four consecutive phosphorothioated bonds at the 5′-end.

Plasmid pQRBL was thus constructed to provide cassette *lox71-ble-hewl-lox66* that contained the P_R-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 reconstructed 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).

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

	Insertional inactivation[a]	In-frame deletion[b]
lox71-ble-lox66	$(1.4 \pm 0.8) \times 10^4$/μg DNA	85.3%
lox71-ble-hewl-lox66	$(3.7 \pm 1.5) \times 10^3$/μg DNA	100.00%

[a]The Beta-mediated insertional inactivation efficiency was calculated as the number of Ble^r colonies/μg of PCR products.
[b]The Cre-mediated in-frame deletion efficiency was calculated as (1-Nr/Nt) × 100%. Nr, number of Ble^r colonies in 100 μl 42°C treated culture; Nt, number of total colonies in 100 μl 42°C treated culture.

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_{RM} and P_R, 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).

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_{RM}-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)].

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