

## Cre/lox System and PCR-Based Genome Engineering in *Bacillus subtilis*<sup>▽†</sup>

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**We have developed a fast and accurate method to engineer the *Bacillus subtilis* genome that involves fusing by PCR two flanking homology regions with an antibiotic resistance gene cassette bordered by two mutant *lox* sites (*lox71* and *lox66*). The resulting PCR products were used directly to transform *B. subtilis*, and then transient Cre recombinase expression in the transformants was used to recombine *lox71* and *lox66* into a double-mutant *lox72* site, thereby excising the marker gene. The mutation process could also be accomplished in 2 days by using a strain containing a *cre* isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG)-inducible expression cassette in the chromosome as the recipient or using the *lox* site-flanked cassette containing both the *cre* IPTG-inducible expression cassette and resistance marker. The in vivo recombination efficiencies of different *lox* pairs were compared; the *lox72* site that remains in the chromosome after Cre recombination had a low affinity for Cre and did not interfere with subsequent rounds of Cre/*lox* mutagenesis. We used this method to inactivate a specific gene, to delete a long fragment, to realize the in-frame deletion of a target gene, to introduce a gene of interest, and to carry out multiple manipulations in the same background. Furthermore, it should also be applicable to large genome rearrangement.**

*Bacillus subtilis* is the best-characterized gram-positive bacterial organism: its biochemistry, physiology and genetics have been studied intensely for more than 50 years. *B. subtilis* and the closely related *Bacillus* species are nonpathogenic and free of endotoxins, and their fermentation technology has been well characterized, making them important cell factories for industrial enzymes, fine biochemicals, antibiotics, and insecticides (8, 31). The completion of the sequencing and annotation of the *B. subtilis* 168 genome supplies a complete view of the *B. subtilis* protein machinery, inspiring new approaches for analyzing biochemical pathways (23, 25). Postgenomic studies require simple and highly efficient tools to enable genetic manipulation. Classically, these chromosomal modifications have been achieved by a method that uses a positive selection marker, usually an antibiotic resistance marker, which is generated by insertion of the marker gene into the chromosome. When introducing multiple modifications into the same background, it is better to evict the selection marker gene, usually through a single-crossover event. Selection of the strain that has lost marker gene is tedious without the aid of counterselectable markers that, under appropriate growth conditions, can promote the death of the microorganisms that harbor them. Until now, four counterselectable markers have been described (6, 12, 17, 38) that improve the genetic manipulations of *B. subtilis* by allowing the subsequent excision of the selection marker, coupled with positive selection. The genetic manipulations described above, however, are mainly based on

restriction enzyme and DNA ligase-dependent vector construction, which require about 1 to 2 weeks before a marker-free modification can be achieved.

A simple PCR-based method for gene replacement was used on a genome-wide scale in *Saccharomyces cerevisiae* and *Escherichia coli*, depending on a mitotic recombination system and bacteriophage-encoded recombination systems, respectively (4, 5, 11, 29). In contrast to *E. coli*, *B. subtilis* naturally develops a physiological state of competence, in which it has the ability to bind, take up, and integrate exogenous linear DNA efficiently (15). Integration of linear DNA, however, requires a minimal length of homology of about 400 to 500 bp (15), compared to just 30 to 50 bp in *S. cerevisiae* and *E. coli* (5, 11). In addition, transformation frequency in *B. subtilis* falls off sharply as the size of transforming DNA decreases (14). These behaviors preclude the use of DNA molecules with short homology extensions in a gene replacement method in *B. subtilis*. Thus, genetic manipulations were mainly based on the cloning of segments from every target site into an integrative vector. Recently, some researchers have started using joining PCR (17) or long-flanking homology PCR (LFH PCR) techniques (35) to fuse long-flanking homologous regions with a selection marker to delete a given target gene in *B. subtilis* (16, 27). These PCR-based methods greatly improve mutation efficiency, but selection markers are left behind. Furthermore, the fidelity of PCR amplification should also be considered, as any undesired PCR amplification errors that arise in the flanking homology can be introduced into the chromosome of *B. subtilis* through a double-crossover event. In 2004, Shevchuk et al. reported a procedure for fast and precise fusion of several fragments into a linear DNA construct, and for shorter projects (<4 kb), the accuracy is expected to be better since high-fidelity PCR can be used (32).

Site-specific recombination systems such as Flp/*FRT* (7) and Cre/*lox* (1) have much higher recombination efficiency than the endogenous recombination systems, making them ideal for many genetic manipulation strategies. The Cre/*loxP* recombi-

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FIG. 1. Schematic representation of mutant *lox66* and *lox71* sites, which, after Cre-mediated recombination, result in a double-mutant *lox72* site. Boldfaced sequences are mutated in comparison to the native *loxP* site. Solid arrows between pairs of *lox* sites indicate the relative Cre-mediated recombination efficiencies in the forward and reverse directions. “X” indicates recombination between two *lox* sites. Arrows with dotted lines depict the orientation of the 8-bp spacer.

nation system is a simple two-component system currently recognized as a powerful DNA recombination tool (24). Cre recombinase catalyzes reciprocal site-specific recombination between two *loxP* sites without requiring any host cofactors or accessory proteins. A DNA sequence that is flanked by *loxP* sites is excised when the *loxP* sites are convergently oriented or inverted when the *loxP* sites are divergently oriented. Notably, the use of native *loxP* sites for consecutive rounds of manipulations in the same background would lead to the integration into the genome of multiple *loxP* sites that could still be recognized by Cre. To minimize genetic instability, a pair of mutant *lox* sites (Fig. 1), a right element mutant *lox* site (*lox66*) and a left element mutant *lox* site (*lox71*) are usually used (2). Recombination of *lox71* and *lox66* results in a double-mutant *lox72* site that has a strongly reduced binding affinity for Cre, allowing for repeated manipulations in a single genetic background (26, 34).

Here, combining the mutant Cre/*lox* system and Shevchuk’s (32) fusion PCR method, we developed a fast and accurate *B. subtilis* genome engineering procedure that enabled us to inactivate a specific gene, to delete a long fragment, to realize the in-frame deletion of a target gene, to introduce a gene of interest, and to do multiple manipulations in the same background. The flanking homology could be shortened to approximately 450 bp, and together with the 500-bp zeocin resistance (*Zeo*<sup>r</sup>) *lox71-zeo-lox66* cassette, the resulting PCR-fused fragment was about 1,500 bp. This length remarkably reduced the number of the errors generated by PCR and ensured the accuracy of the method since the undesired PCR amplification errors arising in the flanking homology may be introduced into the chromosome of *Bacillus subtilis*. Using the strain containing a *cre* isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible expression cassette in the chromosome as the recipient or using the *lox* site-flanked cassette containing the *cre* IPTG-inducible expression cassette and resistance marker, the mutation procedure could be finished in 2 days, allowing it to be used for genome-scale mutagenesis.

#### MATERIALS AND METHODS

**Bacterial strains, plasmids, oligonucleotides, and DNA sequencing.** The bacterial strains and plasmids used in this study are listed in Table 1. All *B. subtilis* recombinant strains were derived from the Marburg 168 *trpC2* strain. The synthesis of oligonucleotides (see Table S1 in the supplemental material) and DNA sequencing were performed by Invitrogen Biotechnology Co., Ltd. *Taq* DNA polymerase, PrimeSTAR HS DNA polymerase, and TA cloning vectors were purchased from TaKaRa Biotechnology (Dalian) Co., Ltd.

**Culture, growth conditions, and phenotypic characterization.** All microorganisms were grown at 37°C in Luria-Bertani (LB) broth or on LB agar. Minimal medium (MM) was used for auxotrophy determination (20). To check histidine auxotrophy, MM was supplemented with histidine or histidinol at a concentration of 50 μg/ml. Amylase expression by *B. subtilis* colonies was detected by growing colonies overnight on LB agar containing 1% starch and then staining the plate with iodine, as described elsewhere (10). Methyl parathion hydrolase (MPH) hydrolyzes methyl parathion to bright yellow *p*-nitrophenol, which was

detected by incubating the strains on LB agar containing methyl parathion (250 μg/ml) (9). *E. coli* transformation was performed as described by Sambrook et al. (30). *B. subtilis* transformation was performed by the competent cell method (3). The following antibiotics were used for selections: ampicillin, 100 μg/ml; spectinomycin, 100 μg/ml; erythromycin, 0.3 μg/ml; and zeocin, 20 μg/ml.

**DNA manipulation techniques.** The isolation and manipulation of recombinant DNA were performed using standard techniques. All enzymes were commercial preparations and used as specified by the supplier (TaKaRa Biotechnology Co., Ltd.).

**Plasmids.** The *lox71-spc-lox66* cassette was amplified by PCR using pDG1730 (18) as a template and oligonucleotide pair P1/P2 as primers; *lox71* and *lox66* sites were introduced by the forward and reverse primers, respectively. The PCR-generated fragment was TA cloned into pMD18-T, yielding plasmid p7S6 (Fig. 2A). With p7S6 as template, the three primer pairs (forward/reverse) P3/P2,

TABLE 1. Strains and plasmids used in this study

<i>B. subtilis</i> strain or plasmid	Characteristic(s)	Source or reference
<b>Strains</b>		
168	<i>trpC2</i>	BGSC
BSPC1	Δ <i>amyE::lox72</i>	This work
BSPC2	Δ <i>prophage 3::lox72</i>	This work
BSPC3	Δ <i>hisZ::lox72</i>	This work
BSPC4	Δ <i>amyE::(lox72-spc-lox72)</i> cassette)	This work
BSPC5	Δ <i>amyE::(lox72-spc-lox71)</i> cassette)	This work
BSPC6	Δ <i>amyE::(lox72-spc-lox66)</i> cassette)	This work
BSPC7	Δ <i>amyE::(lox71-spc-lox66)</i> cassette)	This work
BSPC8	Δ <i>amyE::lox72</i> Δ <i>prophage 3::lox72</i> Δ <i>hisZ::lox72</i>	This work
BSPC9	Δ <i>amyE::(mpd</i> expression cassette, <i>lox72</i> )	This work
BSPC10	Δ <i>amyE::(Pspac-cre spc lacI)</i>	This work
BSPC11	Δ <i>amyE::(Pspac-cre spc lacI)</i>	This work
	Δ <i>hisZ::(lox71-zeo-lox66)</i> cassette)	
BSPC12	Δ <i>hisZ::(lox71-zeo-lox66)</i> cassette)	This work
BSPC13	Δ <i>lacA::(Pspac-cre spc lacI)</i>	This work
<b>Plasmids</b>		
pMD18-T	Amp <sup>r</sup> ; MCS	Takara
p7S6	pMD18-T ligated with <i>lox71-spc-lox66</i> cassette	This work
p2S6	pMD18-T ligated with <i>lox72-spc-lox66</i> cassette	This work
p2S1	pMD18-T ligated with <i>lox72-spc-lox71</i> cassette	This work
p2S2	pMD18-T ligated with <i>lox72-spc-lox72</i> cassette	This work
pPICZαA	<i>Zeo</i> <sup>r</sup> ; <i>P. pastoris</i> expression vector	Invitrogen
p7Z6	pMD18-T containing <i>lox71-zeo-lox66</i> cassette	This work
pTS	Em <sup>r</sup> Amp <sup>r</sup> ; temperature sensitive in <i>B. subtilis</i>	This work
pTSC	pTS containing <i>Pspac-cre</i> expression cassette	This work
pDG148	Amp <sup>r</sup> Km <sup>r</sup> ; <i>B. subtilis-E. coli</i> shuttle expression vector	33
pDGC	pDG148 containing <i>cre</i> fused to <i>Pspac</i> promoter	This work
pDG1730	Amp <sup>r</sup> Spc <sup>r</sup> ; <i>B. subtilis amyE</i> locus integration vector	18
pDGI	pDG1730 containing IPTG-inducible expression elements	This work
pDGC	pDGI containing <i>cre</i> under control of <i>Pspac</i>	This work
pDGCZ	pDGC, Sh <i>ble</i>	This work
pAX01	Em <sup>r</sup> Amp <sup>r</sup> ; <i>B. subtilis lacA</i> locus integration vector	19
pAXC	Amp <sup>r</sup> Spc <sup>r</sup> ; pAX01 containing <i>cre</i> IPTG-inducible expression cassette	This work
pM7Z6M	pMD18T-simple ligated with PCR-fused fragment containing <i>lox71-spc-lox66</i> cassette and homology-flanking <i>amyE</i> site	This work
pUBC19	Amp <sup>r</sup> Km <sup>r</sup> ; <i>B. subtilis-E. coli</i> shuttle vector	39
pPhisZ	pUBC19 containing <i>hisZ</i> gene and its promoter region	This work
pP43NMK	Amp <sup>r</sup> Km <sup>r</sup> ; <i>B. subtilis</i> expression vector, <i>mpd</i> expression cassette	39

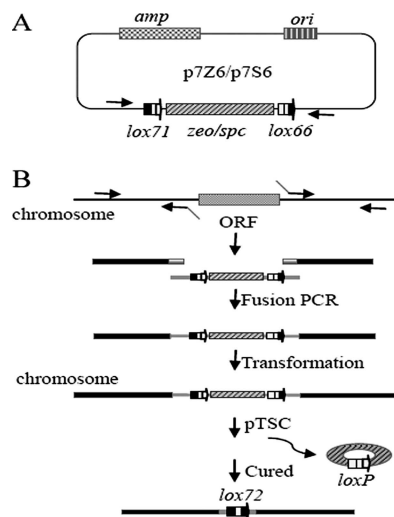


FIG. 2. Vectors containing antibiotic resistance marker cassettes (A) and scheme for Cre/lox and PCR-based mutation delivery in *B. subtilis* (B). (A) *ori*, ColE1 replication origin; *amp*, Amp<sup>r</sup> marker; *zeo/spc*, Zeo<sup>r</sup> or Spc<sup>r</sup> marker; horizontal solid arrows, primer binding site. (B) (i) *lox71-spc/zeo-lox66*, *lox71-spc-lox66/lox71-zeo-lox66* cassette. The front and back regions flanking the target to be deleted are PCR amplified, gel purified and fused by PCR. (ii) PCR-fused products are directly used to transform *B. subtilis*, and Spc<sup>r</sup> or Zeo<sup>r</sup> transformants are selected. (iii) pTSC is introduced into a Spc<sup>r</sup> or Zeo<sup>r</sup> clone, and the constitutively expressed Cre recombinase mediates the recombination between *lox71* and *lox66*. (iv) pTSC is eliminated to get the target strain by patching a transformant on antibiotic-free LB agar and incubating it at 51°C.

P1/P4, and P3/P4 were used to amplify fragments the *lox72-spc-lox66*, *lox71-spc-lox72*, and *lox72-spc-lox72* fragments, respectively. These fragments were each cloned into pMD18-T to generate p2S6, p1S2, and p2S2. The promoter of the *B. subtilis* DNA-binding protein Hbsu gene *hbs* (28) and two mutant *lox* sites (*lox71* and *lox66*) were added to the Zeo<sup>r</sup> gene *Sh ble* by PCR using pPICZαA (<http://www.invitrogen.com>) as a template and oligonucleotides P5, P6, and P7 as primers. The PCR-produced *lox71-zeo-lox66* cassette was cloned into pMD18-T to create p7Z6 (Fig. 2A).

To construct a temperature-sensitive vector for this study, the plasmids pUC19 carrying an ampicillin resistance (Amp<sup>r</sup>) marker (37) and pE194 (21) carrying an erythromycin resistance (Em<sup>r</sup>) marker were each digested with NdeI, ligated together, and transformed into *E. coli* DH5α. Transformants were selected on LB agar containing both ampicillin (100 μg/ml) and erythromycin (300 μg/ml). Transformants were examined by restriction digestion to identify an appropriate shuttle plasmid containing both pUC19 and pE194 fragments. One recombinant, pTS, was confirmed by further restriction enzyme digestion. The Cre recombinase gene *cre* was amplified using the primer pair P8/P9, with the HindIII site and the Shine-Dalgarno sequence (UAGGAGG) introduced by the forward primer and the SphI site introduced by the reverse primer. The HindIII/SphI-digested product was then cloned into the corresponding site of pDG148 (33) to yield pDGC, putting *cre* under the control of the *Pspac* promoter. *Pspac-cre* expression cassette (without *lacI*) was excised from pDGC with EcoRI/SphI and ligated into pTS, also cut with EcoRI/SphI, yielding plasmid pTSC. For the elimination of pTSC from *B. subtilis*, a transformant was patched on antibiotic-free LB agar and incubated at 51°C (13): the resulting colonies all lost pTSC.

For complementation analysis of the *hisZ* gene in-frame deletion mutation, the *hisZ* gene and its promoter region was amplified with the primer pair P32/P33. The PCR product was digested by BamHI/PstI and ligated into the corresponding sites of the *B. subtilis-E. coli* shuttle vector pUBC19 (39), yielding pHisZ.

For integration of the *cre* IPTG-inducible expression cassette into the *B. subtilis* chromosome at the *amyE* site, plasmid pDGIC was constructed as follows. The EcoRI- and BamHI-flanked fragment containing an IPTG-inducible *Pspac* promoter and the *lac* repressor-encoding gene *lacI* were transferred from pDG148 to the corresponding sites of pDG1730, giving pDGI; then, the *cre* gene was amplified using the primer pair P8/P9 above, and the HindIII/SphI-digested

PCR product was cloned into the corresponding sites of pDGI to put *cre* under the control of the IPTG-inducible *Pspac* promoter and to yield pDGIC. For insertion of *cre* IPTG-inducible expression cassette at the *lacA* locus of *B. subtilis*, the *cre* IPTG-inducible expression cassette and Spc<sup>r</sup> marker were PCR amplified using pDGIC as template and P34 and P35 as primers. The SmaI-digested PCR product was ligated with pAX01 (19), which was also digested by SmaI, to generate pAXC. To generate a *lox*-free big cassette containing the *cre* IPTG-inducible expression cassette and the Zeo<sup>r</sup> marker, the Zeo<sup>r</sup> marker was amplified using primer pair P36/P37, and the BamHI-digested PCR product was inserted downstream of the *lacI* gene in pDGIC, yielding pDGICZ.

#### PCR-based fusion of antibiotic resistance marker cassette with LFH regions.

Fusion of the antibiotic resistance marker cassette with LFH regions by PCR was done as described by Shevchuk et al. (32). In brief, it was carried out as follows. Marker cassettes were amplified from vector p7Z6 or p7S6 with primer pair P10/P11 or P12/P13. Two primer pairs were used to amplify ~450-bp or ~900-bp DNA fragments flanking the region to be mutated at its front and back ends. Extensions of 25 to 40 nucleotides (nt) that were complementary to the 5' and 3' ends of the amplified marker cassette were added to the 5' end of the reverse and forward primers of the front and back flanking regions, respectively. Three fragments were amplified using PrimeSTAR HS DNA polymerase, and all PCR products were gel purified with extraction from agarose using the AxyPrep DNA gel purification and extraction kit (Axygen). The purified marker cassette fragment could be used repeatedly. The steps were as follows. Step A included 12 μl water, 5 μl PrimeSTAR buffer (5×), 2 μl deoxynucleoside triphosphates (dNTP) mix (2.5 mM each), 2.5 μl (25 ng) front flanking fragment, 2.5 μl (25 ng) back flanking fragment, 0.5 μl (5 ng) marker cassette fragment, and 0.5 μl PrimeSTAR HS DNA polymerase. Step B included 32 μl water, 10 μl PrimeSTAR buffer, 4 μl dNTP mix, 1 μl forward primer of front flanking fragment, 1 μl reverse primer of back flanking fragment, 1 μl of unpurified PCR product from step A, and 1 μl PrimeSTAR HS DNA polymerase. The resulting PCR product was analyzed by electrophoresis in 1% agarose.

**Nucleotide sequence accession numbers.** The sequences of plasmids p7S6, p7Z6, pTSC, and pDGICZ have been submitted to NCBI under accession no. EU541493, EU541492, EU864234, and EU864235, respectively.

## RESULTS

**Scheme for Cre/lox system and PCR-based genetic modification system in *B. subtilis*.** The *lox71-spc-lox66* and *lox71-zeo-lox66* resistance marker cassettes were first cloned into the pMD18-T vector to yield the marker cassette templates p7S6 and p7Z6 (Fig. 2A). Zeocin is rarely used in *B. subtilis* for selection, but we found it worked very well at 20 μg/ml. The rationale of this system for genetic modification is shown in Fig. 2B. The front and back regions flanking the target to be deleted were amplified by PCR with the appropriate primers, the *lox71-spc-lox-66* and *lox71-zeo-lox66* cassettes, and these two region fragments were fused by PCR, the PCR products were directly used to transform *B. subtilis* competent cells, and Spc<sup>r</sup> or Zeo<sup>r</sup> transformants were selected. pTSC was introduced into an Spc<sup>r</sup> or Zeo<sup>r</sup> clone, and most (>90%) of the Em<sup>r</sup> transformants were spectinomycin and zeocin sensitive (Spc<sup>s</sup> Zeo<sup>s</sup>), as the constitutively expressed Cre recombinase efficiently promoted the recombination between *lox71* and *lox66*, thereby deleting the resistance marker cassette and leaving a 34-bp double-mutant *lox72* site in the chromosome of *B. subtilis*; then pTSC was eliminated to get the target strain. Note that the *lox71* should be designed upstream of *lox66*, so that, after recombination between them, the double-mutant *lox72* site is left in the chromosome and the wild-type *loxP* site is eliminated with the resistance marker cassette. The whole process can be accomplished in about 4 days.

**Knockout of the *amyE* gene and deletion of the prophage 3 region.** To test the feasibility of this strategy, we first used it to knock out the well-known *amyE* gene, which is usually used as an integration site (18). The upstream and downstream frag-



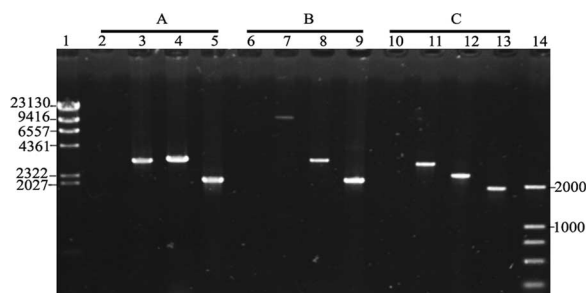


FIG. 3. Confirmation of the *amyE* knockout (A), prophage 3 deletion (B) and *hisZ* in-frame deletion (C) by PCR. The PCR products were analyzed by agarose gel electrophoresis. Lanes 1 and 14, DNA markers. (A) P14 and P17 were used as primers, and water (lane 2), 168 (lane 3), 168 [ $\Delta amyE::(lox71-spc-lox66)$ ] (lane 4), and BSPC1 ( $\Delta amyE::lox72$ ) (lane 5) were used as templates. (B) P18 and P21 were used as primers, and water (lane 6), 168 (lane 7), BSPC7 [ $\Delta amyE::(lox71-spc-lox66)$ ] (lane 8), and BSPC2 ( $\Delta$ prophage 3:*lox72*) (lane 9) were used as templates. (C) P21 and P25 were used as primers, and water (lane 10), 168 (lane 11), 168 [ $\Delta hisZ::(lox71-zeo-lox66)$ ] (lane 12), and BSPC3 ( $\Delta hisZ::lox72$ ) (lane 13) were used as templates.

ments flanking *amyE* were PCR amplified using *B. subtilis* 168 chromosomal DNA as the template and oligonucleotide pairs P14/P15 and P16/P17 as primers, respectively. The *lox71-spc-lox66* cassette was PCR amplified using p7S6 as the template and oligonucleotide pair P10/P11 as primers. According to the procedure described above, the 1,166-bp fragment of the *amyE* gene was deleted, and a double-mutant *lox72* site remained. The resulting mutant, BSPC1, was confirmed by PCR amplification with primers P14 and P17 (Fig. 3A), sequencing of homologous regions (data not shown), and detection of  $\alpha$ -amylase activity (halo assay). These results suggest that the strategy described above worked efficiently.

DNA sequencing of the *B. subtilis* chromosomal *rmE* region revealed a 13-kb region with a lower G+C content and lower gene density between the *groESL* operon and the *gut* operon (22); this region was called prophage 3, which contains the genes for both BsuM modification (*ydiO* and *ydiP*) and BsuM restriction (*ydiR*, *ydiS*, and *ydiA*) (36). Here, we used the method described above to delete the prophage 3 region (SubtiList coordinates bp 652840 to 664137). Two primer pairs, P18/P19 and P20/P21, were used to amplify the upstream and downstream fragments flanking prophage 3 from *B. subtilis* 168 chromosomal DNA. The *lox71-spc-lox66* fragment was already

gel purified above. An ~11-kb fragment was deleted, and *lox72* remained, yielding strain BSPC2, which was confirmed by PCR amplification with primers P18 and P21 (Fig. 3B) and sequencing of homologous regions (data not shown).

**In-frame deletion of the *hisZ* gene in the histidine operon.** An in-frame deletion can inactivate a protein by removing its central part while preserving the signals for translation regulation, which minimizes the chance of exerting a polar effect on the expression of the downstream genes. *hisZ*, the first gene of the *B. subtilis* histidine operon (Fig. 4), was used to generate an in-frame deletion. Primer pairs P22/P23 and P24/P25 were designed to amplify upstream and downstream regions from *B. subtilis* chromosomal DNA. The *lox71-zeo-lox66* cassette was amplified using p7Z6 as template and P12 and P13 as primers. The primers were designed so that, after Cre-mediated excision of the *lox*-flanked Zeo<sup>r</sup> marker, the remnant open reading frame (ORF) contained a translatable scar sequence in-frame with the *hisZ* gene initiation codon, its N-terminal 6-nt coding region, and its C-terminal 30-nt coding region (Fig. 4). Translation from the  $\Delta hisZ$  Shine-Dalgarno sequence and start codon was expected to produce a 43-residue peptide with the HisZ N-terminal MetPheMet, 31 scar-specific residues, and 9 residues of the HisZ C terminus (Fig. 4). The mutation process and the remnant ORF of *hisZ* were confirmed by PCR amplification (Fig. 3C) and DNA sequencing. The resulting  $\Delta hisZ$  strain, BSPC3, required histidine for growth in MM. For complementation analysis, pUBC19 (control) and pPhisZ were separately introduced into BSPC3 to generate BSPC3(pUBC19) and BSPC3(pPhisZ). BSPC3(pPhisZ) grew well in MM, but BSPC3(pUBC19) required histidine for growth. The *hisZ* and *hisG* genes overlap by 8 nt, and *hisG* and *hisD* overlap by 4 nt. The addition of histidinol, which is converted in histidine by histidinol dehydrogenase (HisD), restored the growth of BSPC3 in MM. These results prove that few secondary mutations were caused by *hisZ* gene in-frame deletion.

**Shortening the length of the PCR-fused fragment.** The disadvantage of the PCR-based allele replacement strategy is as obvious as its advantage. It is more time saving than a cloning-based procedure, but as the amplified fragment lengthens, the number of undesired errors increases. Here, PCR-generated errors in the flanking homology regions were not expected to be introduced into the *B. subtilis* chromosome through a double-crossover event. On the premise of getting recombination-mediated transformants, we managed to reduce the length of the resulting PCR-fused fragment. Integration of linear DNA

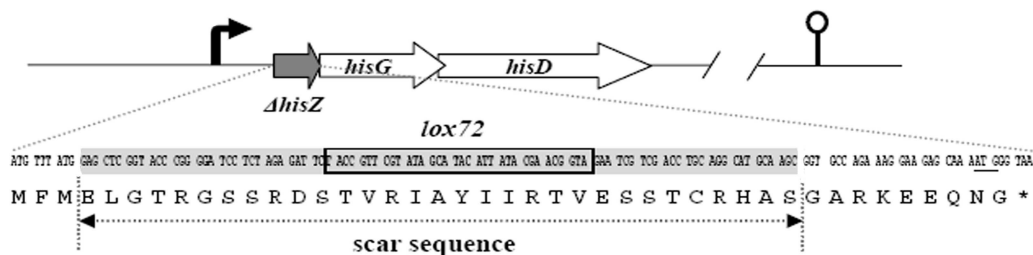


FIG. 4. In-frame deletion of *hisZ* gene in the histidine operon. The designed structure of the *hisZ* in-frame deletion. The ORFs are represented by open arrows, the promoter region is represented by a bent arrow, and the transcription terminator is represented by a lollipop. Cre-mediated excision of the *lox*-flanked zeocin-resistant marker is predicted to create a translatable scar sequence that is in frame with the *hisZ* gene (N-terminal 9-nt coding region and C-terminal 30-nt coding region). Translation from the  $\Delta hisZ$  Shine-Dalgarno sequence and start codon is expected to produce a 43-residue peptide with the HisZ N-terminal MetPheMet, 31 scar-specific residues, and 9 residues of the HisZ C terminus. The scar sequence is shaded, and the *lox72* site is boxed.

TABLE 2. Recombination-mediated transformation efficiencies of the PCR-fused fragments in *B. subtilis*

Integration site	Homology length (bp)		Transformation efficiency <sup>a</sup>
	Upstream	Downstream	
<i>amyE</i>	401	401	$0.9 \times 10^2$
	986	931	$1.7 \times 10^3$
<i>hisZ</i>	514	512	$0.5 \times 10^2$
	900	916	$1.5 \times 10^3$

<sup>a</sup> The transformation efficiency was calculated as the number of Zeo<sup>r</sup> colonies/μg of PCR-fused products.

into *B. subtilis* requires a minimal length of homology of about 400 to 500 bp (15), so the recombination-mediated integration efficiencies of the fusion fragments containing ~450-bp flanking homology regions were investigated here. Four primer pairs were used to generate different lengths of the homology regions (upstream/downstream regions of *amyE* and *hisZ* sites) flanking the *lox71-zeo-lox66* cassette (500 bp): P14/P15 (986/931 bp), P26/P27 (401/401 bp), P22/P25 (900/916 bp), and P28/P29 (514/512 bp). As shown in Table 2, under our conditions, PCR-generated products with homology of about 900 bp on either side were sufficient to promote effective recombination-mediated transformation ( $\sim 10^3/\mu\text{g}$ ), and products with homology of ~450 bp gave approximately tens of transformants ( $<10^2/\mu\text{g}$ ). This result implies that, with a larger amount of PCR products added, products with homology of ~500 bp also work well. A short Zeo<sup>r</sup> marker *lox71-zeo-lox66* cassette (500 bp) was constructed; this cassette was found to work well in *B. subtilis*. So the length of the resulting PCR-fused fragment was shortened to about 1,500 bp, thereby decreasing the number of the errors generated by PCR amplification and the possibility of introducing undesired mutations into the regions flanking the target site in the chromosome of *B. subtilis*. The 1,500-bp PCR-fused products amplified with PrimeSTAR HS DNA polymerase were TA cloned and sequenced. No error was found in two of the three sequenced clones, and one error arose in the primer region of the last clone (data not shown), which may have been a result of the synthesis of the long oligonucleotide.

**Comparing the in vivo Cre-mediated recombination efficiencies of different *lox* pairs.** Cre-mediated recombination between *lox71* and *lox66* generates a wild-type *loxP* site and a double-mutant *lox72* site that has low affinity for Cre (2). To investigate the potential effect of the *lox72* site remaining in the chromosome on the repeated manipulations in the same background, we compared the in vivo Cre-mediated recombination efficiencies of different *lox* pairs (*lox72/lox72*, *lox71/lox72*, *lox72/lox66*, and *lox71/lox66*). The *lox72-spc-lox72*, *lox71-spc-lox72*, *lox72-spc-lox66*, and *lox71-spc-lox66* cassettes were each integrated into the *B. subtilis* chromosome at the *amyE* site by the method described above, yielding strains BSPC4, BSPC5, BSPC6, and BSPC7, respectively. These four strains were each transformed with plasmids pTSC and pTS (control) and incubated at 37°C for 24 h. Then, 100 transformants of each strain were transferred into LB broth containing 100 μg/ml spectinomycin in 96-well plates and incubated at 37°C with shaking; Spc<sup>s</sup> transformants (resulting from the loss of the

TABLE 3. In vivo recombination efficiencies of different *lox* pairs

<i>lox</i> pair	Spc <sup>s</sup> transformants (pTSC/pTS) <sup>a</sup>	Efficiency (%) <sup>b</sup>	Relative efficiency vs. <i>lox71/lox66</i>
<i>lox71/lox66</i>	92/0	92	1
<i>lox72/lox71</i>	36/0	36	0.41
<i>lox72/lox66</i>	38/0	38	0.39
<i>lox72/lox72</i>	10/0	10	0.11

<sup>a</sup> The strains containing different *lox* pairs were each transformed with plasmids pTSC and pTS (control) and incubated at 37°C for 24 h on LB agar containing erythromycin.

<sup>b</sup> The efficiency of Cre-mediated recombination was calculated as the no. of Spc<sup>s</sup> transformants/no. of all tested transformants (100).

spectinomycin cassette during Cre-mediated recombination between two *lox* sites) were counted after 24 h. As shown in Table 3, none of the checked transformants harboring pTS was Spc<sup>s</sup>, indicating that the recombination of different *lox* pairs was Cre mediated. Among them, *lox71/lox66* had the highest Cre-mediated recombination efficiency, and about 92% of the transformants had undergone a Cre-mediated recombination event between *lox71* and *lox66*, losing the Spc<sup>r</sup> marker; on the contrary, 90% of the BSPC4 [ $\Delta amyE::(lox72-spc-lox72)$ ] transformants were still Spc<sup>r</sup>, which validated the low affinity of *lox72* to Cre recombinase and also suggested that *lox72/lox72* has a low Cre-mediated recombination efficiency in vivo. Compared with the recombination efficiency of *lox71/lox66*, the relative Cre-mediated recombination efficiencies were approximately 0.41 for *lox71/lox72* and about 0.39 for *lox72/lox66*. So, the main effect of the *lox72* site remaining in the chromosome on multiple mutations in the same background is its recombination with the *lox71* or *lox66* sites subsequently introduced. The *lox71/lox66* pair has higher recombination efficiency, however, than *lox71/lox72* or *lox72/lox66*. Furthermore, if the *lox72* sites are physically far from *lox71* or *lox66*, *lox72* sites left from previous manipulations should have little effect on the next manipulation. To prove this point, we successfully introduced a prophage 3 deletion and a *hisZ* in-frame deletion mutation into BSPC1 ( $\Delta amyE::lox72$ ), yielding BSPC8 ( $\Delta amyE::lox72 \Delta hisZ::lox72 \Delta$ prophage 3::*lox72*).

#### Introduction of the *mpd* gene into *B. subtilis* chromosome.

This system can also be used to conveniently introduce a gene of interest into a target site without selection markers. There were two multiple cloning sites (MCS) flanking the *lox71-zeo-lox66* cassette in the plasmid p7Z6. The resulting PCR fusion fragment (*amyE* upstream region, *lox71-zeo-lox66* cassette, and *amyE* downstream region) containing the same MCS between the regions of homology and the *lox71-zeo-lox66* cassette was TA cloned into pMD18T-simple, which lacks an MCS, giving pM7Z6M. The *mpd* (MPH gene from *Plesiomonas* sp. strain M6) (40) expression cassette was PCR amplified using plasmid pP43NMK (39) as the template and oligonucleotide pair P30/P31 as primers and inserted into the MCS of pM7Z6M at EcoRI and BamHI sites. The resulting plasmid, pAZP43M (see Fig. S1 in the supplemental material), was linearized and introduced into *B. subtilis* competent cells. Then, the *lox71-zeo-lox66* cassette was evicted through transient Cre recombinase expression, yielding strain BSPC9. A yellow halo (*p*-nitrophenol) around the BSPC9 colony indicated the functional expres-

sion of the *mpd* gene (see Fig. S2 in the supplemental material).

**Integration of the *cre* IPTG-inducible expression cassette into the *B. subtilis* chromosome.** To further simplify the mutation procedure, the plasmid pDGIC was linearized and introduced into *B. subtilis* 168 to generate BSPC10, in which the IPTG-inducible *cre* expression cassette was integrated at the *amyE* site. In so doing, we bypassed the introduction and elimination of plasmid pTSC. The *lox71-zeo-lox66* cassette was inserted at the *hisZ* site of BSPC10 and *B. subtilis* 168, respectively, yielding BSPC11 [ $\Delta amyE::(\text{P}_{spac}\text{-cre } spc \text{ } lacI) \Delta hisZ::(\text{lox71-zeo-lox66 } \text{cassette})$ ] and BSPC12 [ $\Delta hisZ::(\text{lox71-zeo-lox66 } \text{cassette})$ ]. BSPC11 and BSPC12 (control) were each transferred into LB broth or LB broth containing 1 mM IPTG and incubated for 8 h, and then cells were diluted and spread on LB agar. One hundred colonies of each treatment were transferred into LB broth containing zeocin. All IPTG-induced BSPC11 cells were Zeo<sup>s</sup>. Without the addition of IPTG, approximately 10% of BSPC11 cells were Zeo<sup>s</sup>. With or without the addition of IPTG, all BSPC12 (control) cells were Zeo<sup>r</sup>. This result suggests that IPTG-induced *cre* expression could efficiently promote the recombination of *lox71/lox66* and that any leaky expression of *cre* from the IPTG-inducible *Pspac* promoter could also work in some cells. Inserting the *cre* gene at the frequently used *amyE* site may compromise the use of this site for other insertions, so we transferred the *cre* IPTG-inducible expression cassette to the *lacA* locus to produce BSPC13.

As an alternative, we tried to put the *cre* IPTG-inducible expression cassette inside the *lox71-zeo-lox66* cassette so that, after the induction, the Cre recombinase would excise both the marker gene and the *cre* gene. We could not accomplish this construction, however, even in *E. coli* strain JM109 (*lacI<sup>q</sup>*), probably due to leaky *cre* expression from the IPTG-inducible *Pspac* promoter. To solve this problem, we constructed the vector pDGICZ to generate a big *lox*-free cassette containing the *cre* IPTG-inducible expression cassette and Zeo<sup>r</sup> marker, and then, in the PCR fusion process, *lox71* and *lox66* were added to the both sides of the big cassette by the primer pair P38/P39. The *amyE* gene was successfully deleted using this strategy. (Primer pairs P14/P40 and P41/P17 were used to amplify upstream and downstream regions of *amyE*, respectively.) In this manner, after the induction, both the marker gene and the *cre* gene were excised, obviating the need for the introduction and elimination of plasmid pTSC. The length of the resulting PCR fragment increased (4 to 5 kb), however, so the accuracy of this approach was possibly affected.

## DISCUSSION

In this study, we developed a Cre/lox system and PCR-based strategy for genetic manipulation of the *B. subtilis* chromosome that could rapidly introduce mutations and eliminate the selection marker. This method bypasses the traditional time-consuming vector construction procedure and overcomes the drawback of methods that leave selection markers behind. Furthermore, the length of the resulting PCR-fused fragment could be shortened to approximately 1,500 bp, which remarkably reduces the errors generated by PCR amplification and the possibility of introducing undesired mutations into the regions flanking the targeted site in the chromosome of *B. subtilis*. However, it should be noted that sequencing of the

flanking regions is necessary and our approach only reduces the number of clones that have to be sequenced to identify a clone without errors. Introduction of a *cre* expression temperature-sensitive vector promoted the efficient recombination between two single-mutant *lox* sites and created a double-mutant *lox72* site that did not affect further manipulations; three mutations were successfully introduced into the same background by this method.

To further simplify our scheme, an IPTG-inducible *cre* expression cassette was integrated at the *amyE* site of the *B. subtilis* chromosome. IPTG-induced *cre* expression could efficiently promote the recombination between *lox71* and *lox66*. Therefore, the whole mutation procedure could be accomplished in 2 days when using BSPC11 as the recipient. As a result, genome-scale mutations could be carried out efficiently in *B. subtilis*, as has been done for the construction of *Escherichia coli* K-12 in-frame, single-gene-knockout mutants (4). The IPTG-inducible *cre* gene was ligated with the Zeo<sup>r</sup> marker to produce a big *lox* site-free cassette, and *lox71* and *lox66* were added to both sides of the big cassette by the primers. After induction, the Cre recombinase would excise both the marker gene and the *cre* gene, eliminating the need for the introduction and elimination of pTSC.

An about-11-kb prophage 3 region was successfully replaced using the *lox71-spc-lox66* cassette flanked by upstream and downstream homologies (~900 bp each). When a much longer region needs to be deleted, however, this procedure may not work. Another strategy should work in this case: first, *lox71* and *lox66* would be placed at the two sides of the target region, and then transiently expressed Cre recombinase would delete the target region together with the selection markers. Alternatively, when the *lox71* and *lox66* are divergently oriented, the target region will be inverted, a property that could be exploited to create large genome rearrangements.

In conclusion, this method is simpler and more applicable than existing methods for genome engineering in *B. subtilis*. The strains BSPC10 and BSPC13, as well as the plasmids p7S6, p7Z6, pTSC, and pDGICZ, will be accessible from the *Bacillus* Genetic Stock Center (<http://www.bgsc.org>) (accession no. 1A872, 1A873, ECE202, ECE203, ECE204, and ECE205).

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