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Efficient and Precise Construction of Markerless Manipulations in the *Bacillus subtilis* Genome

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We have developed an efficient and precise method for genome manipulations in *Bacillus subtilis* that allows rapid alteration of a gene sequence or multiple gene sequences without altering the chromosome in any other way. In our approach, the *Escherichia coli* toxin gene *mazF*, which was used as a counter-selectable marker, was placed under the control of a xylose-inducible expression system and associated with an antibiotic resistance gene to create a "*mazF*-cassette". A polymerase chain reaction (PCR)-generated fragment, consisting of two homology regions joined to the *mazF*-cassette, was integrated into the chromosome at the target locus by homologous recombination, using positive selection for antibiotic resistance. Then, the excision of the *mazF*-cassette from the chromosome by a single-crossover event between two short directly repeated (DR) sequences, included in the design of the PCR products, was achieved by counter-selection of *mazF*. We used this method efficiently and precisely to deliver a point mutation, to inactivate a specific gene, to delete a large genomic region, and to generate the in-frame deletion with minimal polar effects in the same background.

Keywords: *Bacillus subtilis*, *mazF*, high-fidelity fusion PCR, markless genome manipulation

Bacillus subtilis, one of the most extensively studied model microorganisms, displays superior ability to produce various secretary enzymes and has long been exploited for industrial and biotechnological applications [26, 31]. The completion of the sequencing and annotation of the *B. subtilis* 168 genome starts a new era of researching in functions of new genes and the complex biochemical pathways [19, 20]. Postgenomic studies, especially the emerging field of genome reduction by rationally designed stepwise deletions of chromosomal regions, require simple and highly efficient tools that allow repeated gene manipulations in the same strain.

Classically, these chromosomal modifications could be achieved by a method using a positive selection marker, usually an antibiotic resistance gene generated by the insertion of a selection marker gene into the chromosome. When introducing multiple modifications into the same background, the number of chromosomal modifications is limited by the number of available resistance genes; alternatively, if the same resistance gene is used, the eviction of the gene by a single-crossover event prior to further genetic manipulation is required. Selection of the strain that has lost a marker gene is tedious without the aid of counter-selectable markers that, under appropriate growth conditions, can promote the death of the microorganisms that harbor them. Counter-selectable markers are often instrumental in the construction of clean and unmarked mutations in bacteria [24]. Until now, four similar methods [8, 10, 13, 34] have been described that allow the subsequent excision of the selection marker coupled with positive selection in *B. subtilis*. The approaches using the *upp* gene [13] and the *blaI* gene [8], respectively, as a counter-selectable marker need to construct a strain with a mutation for a specific gene in the chromosome. Because new mutants must also be prepared when these methods are applied to different strains, these two methods can therefore only be used in strains that have a clear genetic background, which limits their application. The toxin gene *mazF*, which encodes an ACA-specific mRNA interferase in *Escherichia coli*, was initially developed as a counter-selectable marker in *B. subtilis* by Zhang *et al.* [34]. This method can satisfy the strong demand for a universal delivery system that can be applied in any *Bacillus* species without requiring any

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prior modification to the host. However, it is not compatible with large-scale approaches since it is based on restriction enzyme and DNA ligase-dependent vector construction, which requires about 2 weeks before a marker-free modification can be achieved. In addition, the *mazF* gene was placed under the control of the *spac* expression system [33], which has been widely recognized for its low induction rate and capacity to allow significant expression in the absence of an inducer in *B. subtilis* [7, 17, 29]. Moreover the significant leakiness of expression of *mazF* may result in the accumulation of spontaneous *mazF*-resistant mutants, which decreases the possibility of isolating the right colonies with designed mutation.

Simple and highly efficient polymerase chain reaction (PCR)-based methods for one-step disruption or modification of genes were used in *Saccharomyces cerevisiae* and *E. coli* at a genome-wide scale, depending on a mitotic recombination system and bacteriophage-encoded recombination systems, respectively [4, 6, 11, 22, 23]. Recently, a procedure combining the site-specific recombination system (Cre/*lox* system) [1] and high-fidelity fusion PCR method [27] was developed to efficiently introduce unmarked mutations into the *B. subtilis* chromosome [32]. After eliminating the selection marker from the mutated locus, however, it would leave remnant sequences (scars) at the targeted locus that can be problematic under certain conditions. Considering all the existing methods, an ideal strategy for large-scale manipulations in the *B. subtilis* genome should be simple, precise, and not altering the genome in any other way.

Here, we describe a simple and no-scar-leaving *B. subtilis* genome engineering procedure based on the use of *mazF* as a counter-selectable marker and high-fidelity fusion PCR method that has enabled us to do successive manipulations precisely into the chromosome: deliver an unmarked point mutation, inactivate a specific gene, delete a large genomic region, and generate an in-frame deletion with minimal polar effects. In our strategy, the toxin gene *mazF* was placed under the control of a *Bacillus megaterium*-derived *xyl* expression system, which was apparently superior to the *spac* expression system for its particularly tight transcriptional regulation and higher induction rate [17, 18]. The transforming linear DNA molecule is PCR-generated and no cloning step is required, which greatly saves the proceeding time. In our hands, the mutation procedure could be finished in about 4 days, allowing it to be used for genome-scale mutagenesis.

Materials and Methods

Bacterial Strains and Growth Conditions

The bacterial strains used in this study are listed in Table 1. All *B. subtilis* recombinant strains were derived from the Marburg 168 *trpC2*. Strains were grown at 37°C in Luria–Bertani (LB) broth or on LB agar. Minimal medium (MM) (1.0 g/l of NaCl, 1.0 g/l of NH_4NO_3, 0.5 g/l of KH_2PO_4, 1.5 g/l of K_2HPO_4, and 0.1 g/l of $MgSO_4 \cdot 7H_2O$) supplemented with 0.4% glucose and 0.01% of the required amino acids was used for auxotrophy determination [16]. To check histidine auxotrophy, MM was supplemented with histidine or histidinol at a concentration of 50 μg/ml. Amylase expression by

Table 1. Strains and plasmids used in this study.

Strain or plasmids	Characteristics[a]	Reference
B. subtilis		
168	trpC2	BGSC
BSMC1	Δ*amyE*::*mazF*-cassette	This work
BSMC1E	*amyE* (Gln190 to His)	This work
BSMC2	Δ*amyE*(Gln190 to His)::*mazF*-cassette	This work
BSMC2E	Δ*amyE*	This work
BSMC3	Δ*amyE*; Δprophage 3:: *mazF*-cassette	This work
BSMC3E	Δ*amyE*; Δprophage 3	This work
BSMC4	Δ*amyE*; Δprophage 3; Ä*hisZ*::*mazF*-cassette	This work
BSMC4E	Δ*amyE*; Δprophage 3; Ä*hisZ*	This work
Plasmids		
pMD18-T	Amp^r; MCS	Takara
PDGIEF	Amp^r; Spc^r; *mazF* under the control of *spac* expression system	[34]
pAX01	Em^r; Ampr; *B. subtilis lacA* locus integration vector	[17]
PDGRE	Amp^r; Spc^r; *xyl* expression system	This work
PDGREF	Amp^r; Spc^r; *mazF* under the control of *xyl* expression system	This work
pPhisZ	pUBC19 containing *hisZ* gene and its promoter region	[32]
pUBC19	Amp^r; Km^r; *B. subtilis–E. coli* shuttle vector	[32]

[a]Amp^r, ampicillin resistance; Spc^r, spectinomycin resistance; MCS, multiple clone site.

Table 2. Primers used in this study.

Primer	Sequence
P1	5-aaggatccctaacttatagggtaacac-3
P2	5-aagaattcgcggccgcattagcatgccattgagatctcatttccccctttgatttttag-3
P3	5-aaagatctatggtaagccgatacgtacccgatat-3
P4	5-aagcatgcctacccaatcagtacgttaat-3
P5	5-atgtttgcaaaacgattcaaaacctctttactgc-3
P6	5-tctttttttaagtgttacccctataagttagacttgtgtattgtgtgtattccagtca-3
P7	5-ctaacttataggggtaacacttaaaaaagaa-3
P8	5-ttataatttttttaatctgttatttaaatagtttatag-3
P9	5-aactatttaaataacagattaaaaaaattataagtatgactggaatacacacaataca-3
P10	5-cgcctccctcaggtctggaaaagaaaaga-3
P11	5-atgtttgcaaaacgattcaaaacctctttactg-3
P12	5-acggatatcatcatcgctcatccatgtcgacgatcagaccagtttttaatttgtg-3
P13	5-tcgacatggatgagcgatgatgatatccgtctaacttataggggtaacacttaaaaaag-3
P14	5-aactatttaaataacagattaaaaaaattataatcgacatggatgagcgatgatgata-3
P15	5-ccttaaacgcctgtcgtctggtccattat-3
P16	5-ttttaaagcaagaagttcaccaattcttaggtcg-3
P17	5-ctttcaagtcaatatattgatcatgtttagaattttcacctcacatcgcttatcg-3
P18	5-ctaaacatgatcaatatattgacttgaaagctaacttataggggtaacacttaaaaaag-3
P19	5-aactatttaaataacagattaaaaaaattataactaaacatgatcaatatattgacttg-3
P20	5-acatcttaaaggtcatccaatgggcaagtg-3
P21	5-atcatatcccgtaacggcgacgctg-3
P22	5-ttacccattttgctcttcctttctggcacccatgccgtgcggtttttcaaacata-3
P23	5-ggtgccagaaaggaagagcaaaatgggtaactaacttatagggtaacacttaaa-3
P24	5-tatttaaataacagattaaaaaaattataacaaagtggttcttcaagatttatcg-3
P25	5-gcgggctgtctatttcgatgcagtcaaat-3

B. subtilis colonies was detected by growing colonies overnight on an LB plate containing 1% starch and then staining the plate with iodine, as described elsewhere [9].

Oligonucleotides, PCR Amplification, and DNA Sequencing

The synthesis of oligonucleotides (Table 2) 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.

DNA Manipulation Techniques

The isolation and manipulation of recombinant DNA was performed using standard techniques. All enzymes were commercial preparations and used as specified by the suppliers [TaKaRa Biotechnology (Dalian) Co., Ltd.]. *E. coli* transformation was performed as described by Sambrook *et al.* [25]. *B. subtilis* transformation was performed by the natural competence method [2]. Selections were as follows: ampicillin (Amp), 100 μg/ml; spectinomycin (Spc), 100 μg/ml.

Construction of the *mazF*-Cassette

The *mazF*-cassette was assembled from different plasmids in the following way. The 1,471-bp xylose expression cassette containing the xylose-inducible promoter P*xylA* and the repressor encoding gene *xylR* was PCR-amplified using vector pAX01 [17] DNA as the template and oligonucleotide pair P1/P2 as primers, flanked with Bgl, SphI, and EcoRI restriction sites distal to the P*xylA* promoter and with BamHI site close to the *xylR* repressor gene. The BamHI/EcoRI fragment of the xylose expression cassette was cloned into the corresponding sites of the integration vector PDGIEF [34], generating PDGRE. In parallel, the *mazF* gene was amplified using oligonucleotide pair P3/P4 as primers and *E. coli* JM109 chromosomal DNA as the template, with Bgl introduced by the forward primer, and the SphI site introduced by the reverse primer; the Bgl/SphI-digested PCR product was then cloned into the corresponding sites of PDGRE to yield PDGREF, in which the *mazF* gene was placed next to the spectinomycin resistance gene (*Spc*) to form the *mazF*-cassette (Fig. 1). The 2.8-kb *mazF*-cassette was PCR-amplified from PDGREF using appropriate primers in Table 2.

PCR-Based Triple Fusion of *mazF*-Cassette with Homology Regions

Fusion of the *mazF*-cassette with two homology regions by PCR followed the method described by Shevchuk *et al.* [27]. The general procedure of PCR fusion is described in Fig. 2. The *mazF*-cassette was amplified from vector PDGREF with the appropriate primer pair.

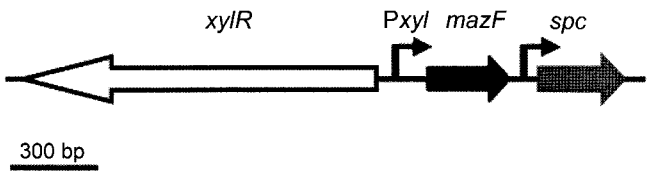

Fig. 1. Schematic representation of the *mazF*-cassette.
It is composed of the *mazF* gene under the control of the *xyl* expression system, associated with a spectinomycin resistance marker.

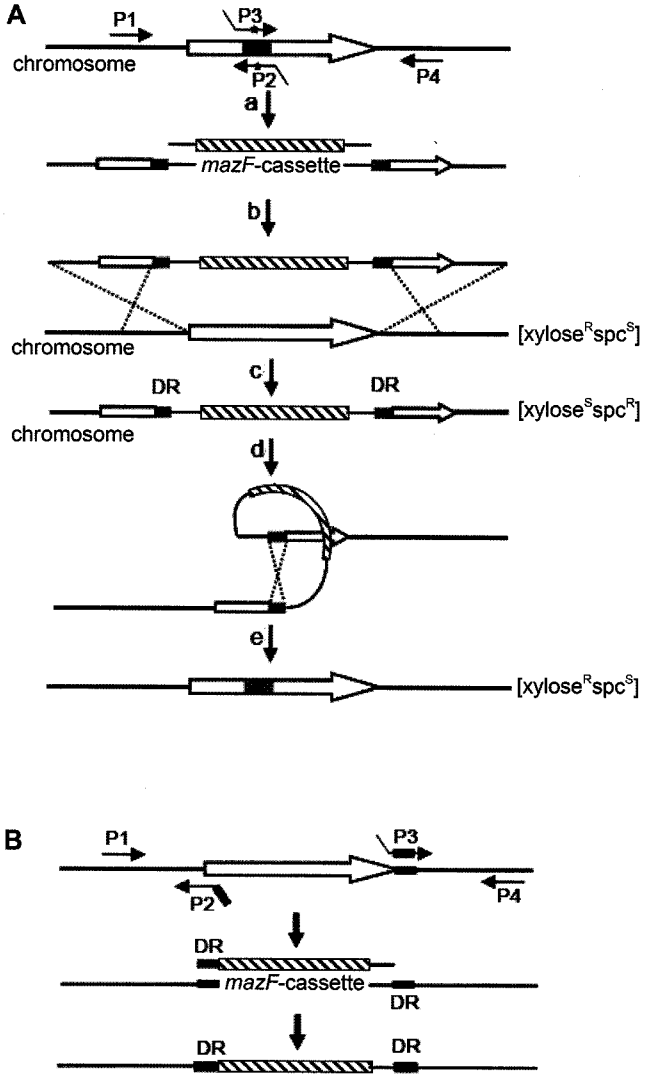

Fig. 2. Scheme for point-mutation delivery (**A**) and deletion of chromosomal region (**B**) in *B. subtilis* using the *mazF*-cassette.
A. (a) The front and back homology fragments carrying the mutation site (*) are amplified by PCR with primer pairs P1/P2 and P3/P4, respectively. (b) Then, a triple fusion PCR reaction joins them with the *mazF*-cassette. (c) The PCR-fused products are used directly to transform *B. subtilis*, and integration of the *mazF*-cassette by a double-crossover event yields xylose^S spc^R transformants. (d, e) The excision of the *mazF*-cassette through a single-crossover event between the 30-bp direct repeats (DR) generates a xylose^R spc^S strain that carries only the desired mutation with no other modification in the chromosome. **B.** The primer positions and DR region to generate a deletion are indicated. The 30-bp region of P2, which creates the DR, is homologous to the remote P3 priming site. The remainder of the procedure is essentially the same as above.

The ~500-bp or ~1,000-bp upstream and downstream DNA fragments flanking the region to be mutated were amplified using two primer pairs and the *B. subtilis* 168 chromosome as template. Overlapping ends of 30 nucleotides (nt) that were identical to the 5' and 3' ends of the *mazF*-cassette were designed in the 5' end of the reverse and forward primers of the upstream and downstream flanking regions, respectively. Three fragments were amplified using PrimeSTAR HS DNA Polymerase, and all PCR products were gel-purified with

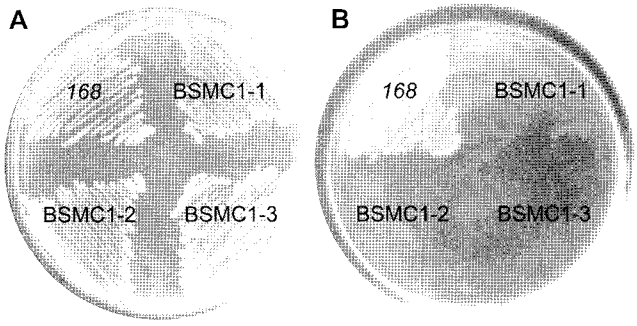

Fig. 3. A test of the feasibility of the *mazF* gene as a counter-selectable marker under the control of the *xyl* expression system.
Strains *B. subtilis* 168 and three BSMC1 (xylose^S spc^R) colonies were streaked on an LB plate in the absence (**A**) and presence (**B**) of 2% xylose.

extraction from agarose using the Gel purification AxyPrep DNA gel extraction kit (Axygen). The concrete triple fusion PCR steps were as follows. Step A: 12 μl water, 5 μl PrimeSTAR buffer (5×), 2 μl 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. Cycling parameters: initial denaturation, 98°C 2 min; subsequent steps, 98°C 15 s, annealing at 56°C 15 s, extension at 72°C 3 min, 15 cycles total; hold at 4°C. Step B: 15.5 μl water, 5 μl PrimeSTAR buffer, 2 μl dNTP mix, 0.5 μl forward primer of front flanking fragment, 0.5 μl reverse primer of back flanking fragment, 1 μl of unpurified PCR product from step A, and 0.5 μl PrimeSTAR HS DNA Polymerase. Cycling parameters: initial denaturation 98°C 2 min; subsequent steps, 98°C 15 s, annealing at 68°C 15 s, extension at 72°C 4 min, 29 cycles total; final additional extension at 72°C 5 min; hold at 4°C. The resulting PCR product was analyzed by electrophoresis in 1% agarose. Note that the purified *mazF*-cassette fragment could be used repeatedly for delivering different point mutations into the chromosome but the forward primer of the *mazF*-cassette should be redesigned in stepwise deletion of chromosomal regions without any scar-leaving.

Construction of Mutant Strains

All the mutated strains were constructed with the procedure described in Fig. 2. Fusion of the *mazF*-cassette with two homology regions was achieved by the triple fusion PCR strategy described in detail above. The PCR product was used to transform *B. subtilis* 168 competent cells, and xylose^S spc^R colonies were isolated on LB medium supplemented with 100 μg/ml spectinomycin. The integration of the *mazF*-cassette at the correct locus of the chromosome was confirmed by PCR for 2–4 individual colonies. These xylose^S spc^R strains could not form colonies on the LB medium supplemented with 2% xylose, indicating that *mazF* can be used as a counter-selectable marker under the control of the *xyl* expression system (Fig. 3). Then, the strains with the expected structure were used for eviction of the *mazF*-cassette. The optimum procedure to obtain cells that have lost the *mazF*-cassette (xylose^R spc^S) is as follows. Aliquots (50–100 μl) of a 10-fold diluted culture grown in LB at 37°C for 12–24 h were spread on LB plates containing 2% xylose and incubated 8–16 h at 37°C. Higher concentrations of xylose did not improve the proportion of *mazF*-cassette pop-out. It is worth noting that the xylose^R colonies can result from several types of

events: (i) the loss of the *mazF*-cassette by a single-crossover event between two short directly repeated (DR) sequences; (ii) the inactivation of the *mazF* gene in the *mazF*-cassette; and (iii) spontaneous mutations that confer resistance to xylose. However, only the loss of the *mazF*-cassette by pop-out gives rise to xylose^R spc^S cells. To obtain the pop-out clones, 50 xylose^R colonies were picked on LB plates containing 100 μg/ml spectinomycin only, and incubated 24 h at 37°C. Only the colonies that did not grow were picked from the corresponding xylose plate. The isolated xylose^R spc^S colonies were identified by PCR method with the appropriate primer pair, and the presence of the desired mutation in the chromosome of these cells was confirmed by DNA sequencing. In our hands, the mutation procedure could be finished in about 4 days, allowing it to be used for genome-scale mutagenesis.

Nucleotide Sequence Accession Number

The sequences of plasmids PDGREF have been submitted to NCBI under Accession No. FJ946915.

RESULTS

Rationale for the Mutation Delivery System in *B. subtilis*

To obtain an efficient and precise genome manipulation system compatible with high throughput procedures, we took advantage of the efficient homologous integration of linear DNA fragments in the *B. subtilis* chromosome [12]. The rationale for this mutation delivery system is shown in Fig. 2. To introduce point mutations into the chromosome (Fig. 2A), the front and back homology fragments carrying the mutation site are amplified by PCR with appropriate primers, so that a 30-bp region of the front fragment is homologous to the back fragment. Then, a triple fusion PCR reaction joins the *mazF*-cassette with these two fragments, generating the molecule carrying the *mazF*-cassette flanked by 30-bp direct repeats (DR) comprising the mutation site. The PCR products are used directly to transform *B. subtilis* 168 competent cells, and spectinomycineresistant transformants are selected. The wild-type target gene is replaced by the *mazF*-tagged mutated copy by a double-crossover event. Excision of the *mazF*-cassette by a single-crossover event between the two 30-bp DR (pop-out) generates xylose^R cells that can be selected from the population by plating on xylose-containing medium. The pop-out events can be distinguished from other mutations conferring xylose resistance, because they lead to the loss of antibiotic resistance. Thereby, the xylose^R spc^S colonies that carry the desired mutation can be identified by screening the xylose^R colonies (see Materials and Methods).

For knock out of genes of interest, in-frame deletion, and genome reduction (Fig. 2B), the 30-bp DR sequence, which is comprised in the back homology fragment, is designed as an overlapping region for fusion PCR both in the backward primer of the front homology fragment and forward primer of the *mazF*-cassette, respectively. The remainder of the procedure is essentially the same as above.

The most important feature of this system is that series of well-designed mutations can be introduced into the same background without altering the genome in any other way.

Evaluation of the *xyl* Expression System in *B. subtilis* and Point-Mutagenesis of the *amyE* Gene

In order to test the feasibility of this strategy, we first used it to construct a point mutation in the well-known *amyE* gene [15] that changed Gln190 to His (CAA to CAC). The *mazF*-cassette was PCR-amplified using plasmid PDGREF as the template and oligonucleotide pair P7/P8 as primers. The 584-bp upstream and 500-bp downstream fragments of the *amyE* gene were amplified by PCR using *B. subtilis* 168 chromosomal DNA as template and two oligonucleotide pairs P5/P6 and P9/P10 as primers, respectively. The primers P6 and P9 carried the CAA-CAC mutation (Table 2). According to the procedure described above, the spc^R strain BSMC1 (Table 1) was selected after integration of the *mazF*-cassette into the chromosome. The double-crossover event was confirmed by PCR amplification with primers P5 and P10 (Fig. 4A). The fact that BSMC1 was unable to grow on the medium containing 2% xylose suggests that *mazF* can be used as a counter-selectable marker under the control of the *xyl* expression system (Fig. 3). Cultures from two independently isolated xylose^S spc^R colonies were subjected to xylose selection (see Materials and Methods), and the loss of the *mazF*-cassette by pop-out was detected in 50% and 28% of the xylose^R colonies.

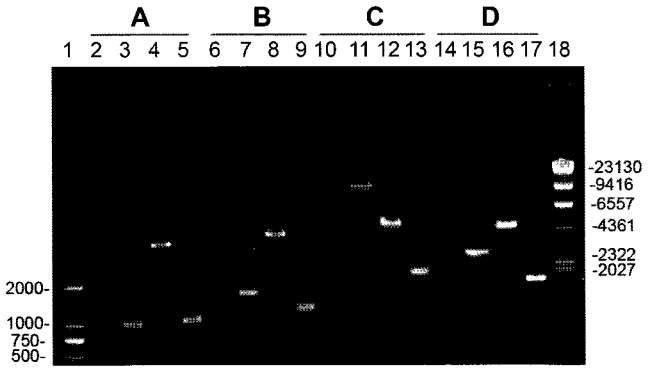

Fig. 4. Confirmation of the *amyE* point mutation (**A**), *amyE* knockout (**B**), prophage 3 deletion (**C**), and *hisZ* in-frame deletion (**D**) by PCR.

The PCR products were analyzed by agarose gel electrophoresis. Lanes 1 and 18, DNA markers. **A**. P5/P10 as primers; water (lane 2), 168 (lane 3), BSMC1 (Δ*amyE*::*mazF*-cassette) (lane 4), and BSMC1E (*amyE*190) (lane 5) as templates. **B**. P11/P15 as primers; water (lane 6), BSMC1E (lane 7), BSMC2 (Δ*amyE*::*mazF*-cassette) (lane 8), and BSMC2E (Δ*amyE*) (lane 9) as templates. **C**. P16/P20 as primers; water (lane 10), BSMC2E (Δ*amyE*) (lane 11), BSMC3 (Δ*amyE*, Δ*prophage 3*::*mazF*-cassette)(lane 12), and BSMC3E (Δ*amyE*, Δprophage 3) (lane 13) as templates. **D**. P21/P25 as primers; water (lane 14), BSMC3E (Δ*amyE*, Δprophage 3) (lane 15), BSMC4 (Δ*amyE*, Δprophage 3, Δ*hisZ*:: *mazF*-cassette) (lane 16), and BSMC4E (Δ*amyE*, Δprophage 3, Δ*hisZ*) (lane 17) as templates.

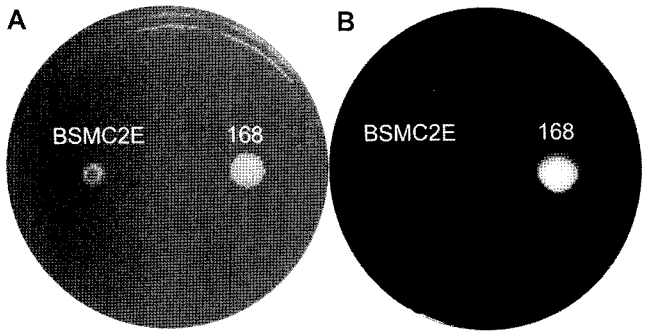

Fig. 5. Confirmation of *amyE* knockout by halo assay.
B. subtilis 168 and BSMC2E were grown on LB plate containing 1% starch (**A**) and was stained with iodine to detect the α-amylase activity (**B**).

Three resulting mutants, BSMC1E (xylose^R spc^S), were confirmed by PCR amplification with primers P5/P10 (Fig. 4A), *via* sequencing of homologous regions (data not shown). In two cases, the *amyE* gene carried solely the expected mutation, yielding the Gln190 to His (CAA to CAC), whereas the remaining clone carried one additional mutation, probably generated during the PCR amplification. The results above suggest that this strategy can introduce a point mutation efficiently into the chromosome.

Knockout of the *amyE* Gene and Deletion of the Prophage 3 Region

The 521-bp upstream and 600-bp downstream fragments of the *amyE* gene were PCR-amplified using *B. subtilis* 168 chromosomal DNA as template and two oligonucleotide pairs P11/P112 and P14/P15 as primers, respectively. The *mazF*-cassette was PCR-amplified using plasmid PDGREF as template and oligonucleotide pair P13/P8 as primers. The triple fusion PCR products were used to transform BSMC1E competent cells. The integration of the *mazF*-cassette into the chromosome inactivated *amyE*, yielding xylose^S spc^R colonies (BSMC2). Cultures from two colonies were subjected to xylose selection. For both cultures, 65% of the colonies had lost the *mazF*-cassette. Two xylose^R spc^S clones (BSMC2E) were confirmed by PCR amplification with primers P11/P15 (Fig. 4B), sequencing of deletion junctions (data not shown), and α-amylase activity (Fig. 5).

To test the feasibility of our strategy for deletion of a large chromosomal region, we deleted the prophage 3 (652840–664137-bp SubtiList coordinates) region [21, 30] in the *B. subtilis* genome. Two primer pairs, P16/P17 and P19/P20, were used to amplify the 1,000-bp upstream and 1,084-bp downstream fragments flanking prophage 3 from *B. subtilis* 168 chromosomal DNA. The *mazF*-cassette was PCR-amplified using plasmid PDGREF as the template and oligonucleotide pair P18/P8 as primers. The strain BSMC2E (Δ*amyE*) was used to delete the prophage 3 region. The xylose^S spc^R strain was subjected to xylose selection, yielding 38% of *mazF*-cassette pop-out. An ~11-kb fragment was deleted in the resulting strain BSMC3E, which was confirmed by PCR-amplification with primers P16/P20 (Fig. 4C) and sequencing of deletion junctions (data not shown).

In-Frame Deletion of *hisZ* Gene in the Histidine Operon

An in-frame deletion inactivates a protein by removing its central part while preserving the signals for translation regulation. This strategy minimizes the chance of exerting a polar effect on the expression of the downstream genes. Here, we used the *hisZ* gene, the first gene of the *B. subtilis* histidine operon (Fig. 6), to generate an in-frame deletion with our system. A deletion disrupting the *hisZ* frame had a strong polar effect on the expression of the downstream *hisD* gene, whereas an in-frame *hisZ* deletion had no polar effect [28]. The primer pairs, P21/P22 and P24/P25, were used to amplify the 916-bp upstream and 920-bp downstream regions from *B. subtilis* chromosomal DNA. The *mazF*-cassette was amplified using plasmid PDGREF as the template and P23/P8 as primers. The primers were designed so that, after pop-out of the *mazF*-cassette, the remnant open reading frame (ORF) contained 10 N-terminal and 10 C-terminal amino acids of the HisZ (Fig. 6). The integration of the *mazF*-cassette into the chromosome inactivated

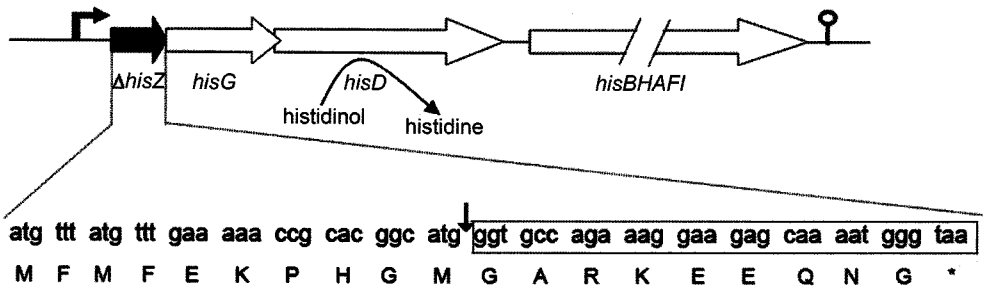

Fig. 6. In-frame deletion of the *hisZ* gene in the histidine operon: schematic representation of the *B. subtilis* histidine operon.
The open reading frames (ORFs) are represented by open arrows, the promoter region is represented by a bent arrow, and the transcription terminator is represented by a lollipop. A blow-up view of the remnant ORF (10 N-terminal amino acids and 10 C-terminal amino acids) encoded by Δ*hisZ* (dashed arrow) is represented (bottom line). DNA and amino acid sequences are indicated. The deletion junction is marked by a vertical arrow, and the direct repeats (DR) correspond to the boxed sequence.

hisZ, yielding xylose^S spc^R His$^-$ colonies. Cultures from two colonies were subjected to xylose selection, and the loss of the *mazF*-cassette by pop-out was detected in 25% and 38% of xylose^R colonies. The mutation process and the deletion junctions were confirmed by PCR-amplification (Fig. 4D) with primer pair P21/P25 and DNA sequencing. The remnant ORF in Δ*hisZ* encoded the expected 20 amino-acid peptide (Fig. 6). The resulting Δ*hisZ* strain, BSMC4E, required histidine for growth in minimal media. In the histidine operon, 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), also restored the growth of BSMC4E in minimal media, indicating that expression of the *hisD* gene downstream of Δ*hisZ* was not affected. For complementation analysis, pUBC19 (control) and pPhisZ were separately introduced into BSMC4E to generate BSMC4E (pUBC19) and BSMC4E (pPhisZ). BSMC4E (pPhisZ) grew well in minimal media, but BSMC4E (pUBC19) required histidine for growth. The results above realized that our system allowed efficient introduction of in-frame deletions with minimal polar effect in the *B. subtilis* chromosome.

DISCUSSION

In this study, we have developed a simple and highly efficient strategy for precise genetic manipulation of the *B. subtilis* chromosome that could rapidly deliver mutations without altering the genome in any other way. This strategy is based on the high-fidelity fusion PCR method and the use of the *mazF* gene, encoding an endoribonuclease, as a counter-selectable marker. This method bypasses the traditional time-consuming restriction/ligation-dependent vector construction procedure and overcomes the drawback of methods that leave selection markers behind. Furthermore, the toxin gene *mazF* was placed under the control of a *Bacillus megaterium*-derived *xyl* expression system, which was apparently superior to the *spac* expression system for its particularly tight transcriptional regulation and higher induction rate. We used this method step by step to deliver a point mutation, to inactivate a specific gene, to delete a large genomic region, and to realize the in-frame deletion in the same strain. Thus, our method provides an efficient way to combine multiple mutations.

Notably, the PCR-based methods greatly improve mutation efficiency; however, 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. With the high-fidelity fusion-PCR strategy developed by Shevchuk *et al.* [27], the error rate of PCR fusion is very low in our hands. Furthermore, the length of the resulting PCR-fused fragment could be shortened to approximately 3,900 bp, which can reduce the errors generated by PCR amplification and the possibility of introducing undesired mutations into the regions flanking the targeted site. However, it should be noted that sequencing of the flanking regions is necessary to identify a clone without errors. For best specificity of the triple fusion product, the length of homologous sequences introduced into the adjacent ends of DNA fragments to be fused should be designed at least 30 bp with similar annealing temperature as in step A, and the two primers should be designed with a high annealing temperature (68°C with a melting temperature of 70–72°C) as in step B.

Compared with existing delivery systems, our method is well adapted to genome-scale approaches in *B. subtilis* for the following three advantages. First, using the toxin gene *mazF* as a counter-selectable marker makes it unnecessary for a specific prerequisite strain before delivering mutations. Second, the mutation-delivery/cassette-excision cycle can introduce clean and unmarked mutations without altering the genome in any other way. Third, the use of high-fidelity fusion-PCR makes the time-consuming cloning procedure unnecessary, and allows the construction of a large number of mutations in parallel.

The emerging field of synthetic genomics is expected to facilitate the generation of microorganisms with the potential to achieve a sustainable society [5, 14]. One approach towards this goal is the reduction of microbial genomes by rationally designed deletions to create simplified cells with predictable behavior that act as a platform to build in various genetic systems for specific purposes. Challenges to reduce the genome size by the stepwise introduction of large-scale deletions have been undertaken for *B. subtilis* [3, 30]. Takuya Morimoto *et al.* [21] reported that depletion of 874 kb (20%) of the *B. subtilis* genomic sequence remarkably enhanced the productivity of recombinant proteins. Their results demonstrate the effectiveness of synthetic genomics in creating novel and useful bacteria for industrial use [21]. In our hands, an about 11-kb prophage 3 region was successfully deleted that allows our method to stepwise delete large genomic regions for genome reconstruction.

Even though *mazF* was placed under the control of the *xyl* expression system, there were still some xylose^R clones without the loss of the *mazF*-cassette on xylose selection, indicating *mazF*-resistant mutations in these clones. To enhance the counter-selectable efficiency, the procedure for xylose selection was optimized. Despite that *mazF*-resistant mutation happened in a fraction of xylose^R cells, the pop-out clones with the loss of *mazF*-cassette are still very sensitive to the expression of *mazF*, such that the selection efficiency is not influenced for the next circle of manipulation.

In conclusion, this method is simpler and more applicable than existing methods for no-scar-leaving genome engineering in *B. subtilis*. The plasmid PDGREF will be accessible from the *Bacillus* Genetic Stock Center (http://www.bgsc.org).

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