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A versatile mini-*mazF*-cassette for marker-free targeted genetic modification in *Bacillus subtilis*

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ABSTRACT

There are some drawbacks for *MazF*-cassette constructed in previous reports for marker-free genetic manipulation in *Bacillus subtilis*, including cloning-dependent methodology and non-strictly controlled expression system. In our study, the modifications on *mazF*-cassette are carried out, such as using mini Zeocin resistance gene as positive-selectable marker and strictly controlled xyl promoter from the *B. subtilis* to replace non-strictly controlled IPTG-inducible Pspac or xyl promoter from *Bacillus megaterium*. Then the mini-*mazF*-cassette was successfully applied to knock-out the *amyE* gene, to delete a 90-kb gene cluster, and to knock-in a green fluorescent protein expression cassette employing a cloning-independent methodology, without introducing undesirable redundant sequences at the modified locus in the *B. subtilis* 1A751. Besides, the mini-*mazF*-cassette could be used repeatedly to delete multiple genes or gene clusters with only a 2- to 2.5-kb PCR-fused fragment, which largely reduced the frequency of nucleic acid mutations generated by PCR compared to previous reports. We further demonstrated that the frequency of spontaneous *mazF*-resistant mutants was lower, and the frequency of generating desired clones was nearly 100%. The entire procedure for marker-free genetic manipulation using the mini-*mazF*-cassette can be finished in about 3 days. This modified cassette has remarkable improvement compared to existing approaches and is applicable for available manipulating *Bacillus* species chromosomes.

1. Introduction

Bacillus subtilis, a generally considered safe (GRAS) bacterium, is widely used for production of industrial enzymes because of its superior ability to secrete large amounts of proteins into the supernatant (Schallmey et al., 2004). Since the completion of the sequencing and annotation of the *B. subtilis* 168 strain genome, more than 44 *Bacillus* species have been sequenced and deposited in National Center for Biotechnology Information (NCBI) databases (Earl et al., 2007). Post-genomic studies require simple and highly efficient tools to allow genetic manipulation. Routinely, antibiotic resistance or other selectable marker genes are used as positive selection marker in chromosomal modifications. However, the insertion of a selectable gene often modifies the physiology of the manipulated strain. Moreover, the less available resistance genes make it impossible for multiple modifications in the same strain. Thus, the deletion of the selectable marker genes is required. Currently, two main methods are used for chromosomal modification without introducing selection marker. One is based on site-specific recombination, and the other is the counter-selection system. The drawback of the first method is that it leaves a specific redundant sequence (Suzuki and Nakayama, 2011; Yan et al., 2008). A specific instrument is usually required for constructing the unmarked mutations without introducing redundant sequence in bacteria using counter-selectable markers (Reyrat et al., 1998).

Currently, several counter-selectable markers, such as the *upp* (Fabret et al., 2002), *blaI* (Brans et al., 2004), *AraR* (Liu et al., 2008), or *hewl* (Wang et al., 2012) genes, could be used to delete antibiotic markers in specific *B. subtilis* strain, these markers were limited to be used in strains with specific genetic backgrounds. Zhang et al. (2006) constructed a universal, unmarked delivery system using the *Escherichia coli mazF* gene as a counter-selection marker, which could be applied in any *Bacillus* species without any prior modification. MazF is an mRNA interferase that specifically cleaves single-stranded mRNAs at ACA sequences, resulting in inhibited protein synthesis and cell growth arrest (Zhang et al., 2006). Once MazF is expressed in the host, programmed cell death occurs in both prokaryotes (including *E. coli*) and eukaryotes (Kolodkin-Gal et al., 2007; Yang et al., 2009). The drawbacks of this method are the requirement for a cloning-dependent methodology, and a long experimental time of about 2 weeks. Although the IPTG-inducible Pspac system combined with a high-fidelity fusion PCR method was used by Morimoto et al. (2009) to delete sequences from 8.5 kb to 128 kb, limitations remained in this method, such as a lower amount of leakiness and 4.0-kb PCR-fused fragment, which might increase the frequency of

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nucleic acid mutations and the difficulty in assembly of different genetic fragments.

Thus, a strictly controlled promoter would be preferable to replacing the IPTG-inducible Pspac promoter. PxylA is a promoter which is repressed by XylR (xylose-responsive repressor proteins). In presence of Xylose, XylR leaves the operator sequence and the Pxyl is active. A particularly strict transcription regulation system of xyl from *B. megaterium* and a high-fidelity fusion PCR method was used to efficiently introduce unmarked mutations into the *B. subtilis* chromosome (Yu et al., 2010). However, there were some limitations, such as the long fused fragment of approximately 3900 bp, and spontaneous mazF-resistant mutants generated (Morimoto et al., 2009; Yu et al., 2010; Zhang et al., 2006).

Previous reports showed that the inducible Pxyl promoter from the *B. subtilis* had tight transcriptional regulation (Daniel et al., 1998; Lewis and Marston, 1999). The xyl promoter from *B. subtilis* W23 showed the higher induction/repression ratio than that from *B. megaterium* (246–279-fold versus 150–200-fold) (Bhavsar et al., 2001; Hartl et al., 2001). Zeocin resistance, encoded by a mini gene (Yan et al., 2008), could be used to shorten the length of the counter-selection cassette. In our study, a "mini-*mazF*-cassette," which contain *mazF*, zeocin resistance gene and Pxyl promoter from *B. subtilis*, was constructed using the method of high-fidelity fusion PCR, we further verified its efficiency by knocking-out the *amyE* gene, deleting a 90-kb gene cluster, and knocking-in a green fluorescent protein expression cassette.

2. Materials and methods

2.1. Bacterial strains, plasmids, and oligonucleotides

The bacterial strains and plasmids used in the study are listed in Table 1. The specific primers (Table 2) used for PCR amplification were synthesized by Invitrogen Biotechnology Co., Ltd.

Table 1
Strains and plasmids used in this study.

Strain or plasmid	Relevant properties	Reference
Escherichia coli JM109	recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi (lac-proAB)F′[traD36 proAB + lacI^q lacZ△M15]	(Sambrook et al., 1989)
Bacillus subtilis		
1A751	*his nprR2 nprE18 DaprA3 DeglS102 DbglT bglSRV*	BGSC
ZPM6	1A751 derivate, zeo^R, amyE::(zeo,Pxyl,mazF)	This work
ZPMPS	1A751 derivate, zeo^R, amyE::(zeo,Pspac,mazF)	This work
ZPMBm	1A751 derivate, zeo^R, amyE::(zeo,Pxyl-Bm,mazF)	This work
ZPM61	1A751 derivate, zeo^R, amyE::(DR,zeo,Pxyl,mazF,DR)	This work
ZPM61S	1A751 derivate, amyE::DR	This work
ZPMG	1A751 derivate, zeo^R, amyE::(P43-GFP,zeo,DR, Pxyl,mazF,DR)	This work
ZPMGS	1A751 derivate, amyE::(P43-GFP,DR)	This work
ZPM62	1A751 derivate, zeo^R, Skin,pro7::(spoIVCB, yraKF, zeo, Pxyl,mazF, yqcl)	This work
ZPM62	1A751 derivate, zeo^R, Skin, pro7::(spoIVCB, yraKF)	This work
Plasmids		
pDGCIZ	Amp^r,Spc^r; pDG1730 derivate, containing zeocin resistance	(Yan et al., 2008)
pSG1729	Amp^r,Spc^r; containing Pxyl promoter	(Lewis and Marston, 1999)
pHCMC05	Amp^r,Cm^r; containing Pspac promoter	(Nguyen et al., 2005)
pBE2P43GFP	Amp^r,Kan^r,pBE2 containing P43 promoter and GFP gene	Lab stock

Amp^r, ampicillin resistance; Spc^r, spectinomycin resistance; zeo^R, zeocin resistance, Kan^r, kanamycin resistance.
Cm^r, chloromycetin resistance.
BGSC, Bacillus Genetic Stock Center (www.BGSC.org).

Table 2
Primers used in this study.

Primer	Sequence(5′–3′)
P1	ggcGTCGACGGATCCGAATTCAAGCTTCAGTCCTGCTCCTCGGCCAC
P2	TTCATGAAAGACTTGATATGGCTTTTTATATGTG
P3	CATATCAAGTCTTTCATGAAAAACTAAAAAAAATATT
P4	ACCAGATCCTCCTTTAGATGCATTTTATGTCATATTGTA
P5	CATCTAAAGGAGGATCTGGTAATGGTAAGCCGATA
P6	ggcTCTAGACTACCCAATCAGTACGTTAAT
P7	AATCAATAATGGACCAGACGACAGTCCTGCTCCTCGGCCAC
P8	AGTCTTCAAAAAATCAAATAAGGAGT
P9	**TCGTCTGGTCCATTATTGATTTGATAAACG**CTTAACCTCATTGGAAATCGCG
P10	TGATTGGGTAGTCTAGAGCCCAGATGCGAATACAACAAAAGC
P11	GTAAGTCCCGTCTAGCCTTGCCCTC
P12	TACCACCTATCTTAACCTCATTGGAAATCGCG
P13	ATGAGGTTAAGATAGGTGGTATGTTTTCGCTTG
P14	**TCGTCTGGTCCATTATTGATTTGATAAACG**TTATTTGTATAGTTCATCCATGCC
P15	TGATTGGGTAGTCTAGAGCCCAGATGCGAATACAACAAAAGC
P16	GTAAGTCCCGTCTAGCCTTGCCCTC
P17	ATCAGCCCCTGCACCGCCTTCCTCG
P18	GAATTGCTCGACACCTGTCACCATCGTCACC
P19	TGACAGGTGTCGAGCAATTCATGGAAGACCTTA
P20	TCGTCTGGTCCATTATTGATTCATCTCACATTGCGTTCTCGT
P21	TGATTGGGTAGTCTAGAGCCGAGCAATTCATGGAAGACCTTA
P22	TTGCGTTCTCGTTAGTGAAAAGT
P23	AGCATGTCGAACTGGTACTGGC
P24	GTATCCGTGTCCGCAGGCAGC
P25	ATGAGTAAAGGAGAAG
P26	TTATTTGTATAGTTCATC
P27	CATATCAAGTCTTTCATGAACTAACTTATAGGGGTAACACTTAA
P28	ACCAGATCCTCCTTTAGATGTTAGATATCACTAGTTTGGACCATT
P29	CATATCAAGTCTTTCATGAACCAGTGAATTCGAGCTCAGGCC
P30	ACCAGATCCTCCTTTAGATGTAATTGTTATCCGCTCACAATTCC

2.2. Culture and growth conditions

All *B. subtilis* strains were grown in Luria–Bertani (LB) or low-salt Luria–Bertani (LLB) medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl, pH 7.5). To test the efficiency of promoter transcription regulation system, the strains were grown overnight in LLB containing zeocin and 1% glucose, followed by dilution 1:10 in fresh LB containing 1% glucose in the next morning, then grown for 2–3 h until an OD_{600} of 0.4, approximately 1.2×10^9 viable cells/ml were plating on LB agar containing 1% xylose or 1 mM IPTG. When necessary, the final concentrations of antibiotics were 100 μg/ml ampicillin and 20 μg/ml zeocin.

2.3. DNA manipulation techniques

The isolation and manipulation of recombinant DNA was performed using standard techniques. All enzymes were commercially prepared, and PrimeSTAR HS DNA polymerase (Takara) was used. The *B. subtilis* competent cells were obtained by the two-step method (Cutting and Vander-Horn, 1990).

2.4. Construction of recombinant B. subtilis containing the mini-mazF-cassette

The zeocin resistance gene, the 150-bp PxylA from *B. subtili*, and the *mazF* gene were assembled to construct mini-mazF-cassette (Fig. 1A). The three fragments were PCR-amplified using vector pDGICZ, pSG1729 and *E. coli* JM109 chromosomal DNA as template, P1/P2, P3/P4, and P5/P6 as primers, respectively. At the terminal of P2 and P4, there are 20 nucleotides (nt) being identical to the ends of P3 and P5, respectively. P4 was introduced the Shine-Dalgarno sequence (AAAGGAGGAUCUGGUA), P1 was flanked with *Sal*I, *Bam*HI, *Eco*RI, and *Hin*dIII restriction sites and P6 with *Xba*I restriction sites at the 3′-end of the *mazF* gene. Products were gel-purified using AxyPrep DNA gel

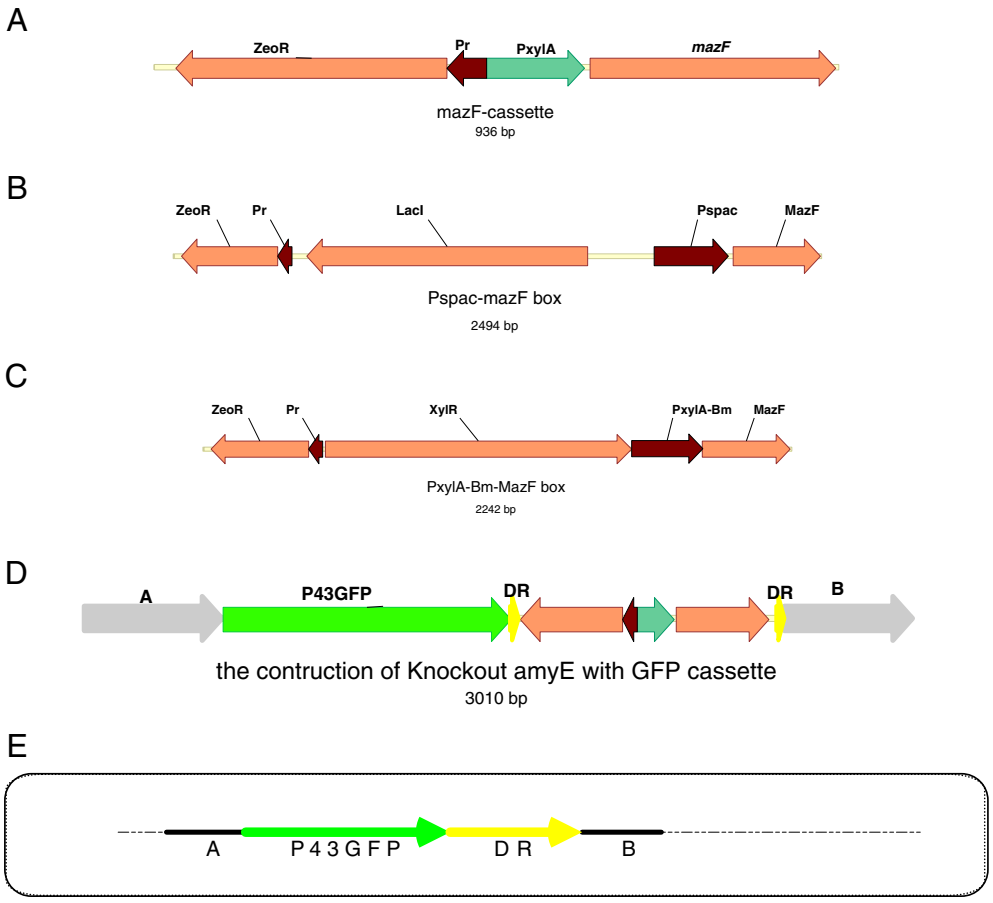

Fig. 1. Construction of the *mazF*-cassette, *Bacillus subtilis* ZPMG zeo^R, *amyE*::(P43-GFP, zeo, DR, Pxyl, mazF, DR), *B. subtilis* ZPMGS *amyE*::(P43-GFP, DR). (A) Schematic representation of the mini-*mazF*-cassette. (B) Schematic representation of the Pspac-*mazF*-cassette. (C) Schematic representation of the PxylA-Bm-*mazF*-cassette. Zeo^R, zeocin resistance; Pr, promoter of the *B. subtilis* DNA-binding protein HBsu gene hbs; PxylA, promoter of the *B. subtilis xylose-induced* expression system; mazF, *Escherichia coli* toxin gene. Pspac,a promoter of the *lacI*-*Pspac* system from pHCMC05. PxylA-Bm,a promoter of the XylR-PxylA' system from *Bacillus megaterium*. (D) Schematic representation of tetraploid fusion PCR product. (E) Schematic representation of the ZPMGS chromosome. A: upstream sequence; B: downstream sequence; DR: direct repeat sequence; P43GFP: green fluorescent protein expression cassette.

extraction kit (Axygen). These three PCR fragments were fused in the order zeo-Pxyl-mazF with the method of splicing by overlapped extension PCR (SOE-PCR) (Shevchuk et al., 2004). Various transformants were obtained using 10 μg recombinant PCR product in the three independent duplicate tests.

The fused PCR product was digested with *Sal*I and *Xba*I and inserted into the corresponding sites of pSG1729. The ligation mixture was transformed into competent *B. subtilis* 1A751 cells, and the recombinant *B. subtilis* containing mini-*mazF*-cassette were selected with zeocin and named *B. subtilis* ZPM6.

In order to evaluate the strict regulation of different promoter, PxylA from the *B. subtilis* was replaced with IPTG-inducible Pspac and PxylA from *B. megaterium* (PxylA-Bm), respectively. DNA for the lacI gene and Pspac promoter was PCR-amplified from the pHCMC05 with primers P29/P30. The 1,471-bp xylose expression cassette containing the xylose-inducible promoter PxlyA and the repressor encoding gene xylR was PCR-amplified using vector pDGICZ DNA as the template and P27/P28 as primers. The three amplified DNA fragments (PCR1-zeoR sequence with primer pair P11/P2, PCR2-Pspac sequence from pHCM05 or PCR2-PxlyA sequence from pDGICZ, and PCR3-mazF sequence with primer pair P5/P8) were ligated in the order zeo-Pspac-mazF (Fig. 1B) or zeo-PxylA-Bm-mazF (Fig. 1C) with the method described above, respectively. The two fusion PCR products were transformed into competent *B. subtilis* 1A751 cells and yielded the recombinant strain *B. subtilis* ZPMPS and ZPMBm, respectively.

2.5. Construction of the amyE deletion strains

In order to construct the amyE deletion strain (rationale as Fig. 2A), we first fused two homologous regions of the amyE gene to the mini-*mazF*-cassette. The mini-*mazF*-cassette was amplified from the genome of *B. subtilis* ZPM6 with P7/P6 primers. The 500-bp upstream (A) and downstream (B) DNA fragments of the *amyE* gene flanking region were amplified using P8/P9 and P10/P11 as primers and *B. subtilis* 1A751 chromosome as template. Overlapping fragments of 20 nt were added to the 5′-end of the reverse and the forward primers to match the 5′-and 3′-ends of the mini-*mazF*-cassette. Fusion of the mini-*mazF*-cassette with fragment A and B was achieved by the triple fusion PCR strategy described above.

The fusion PCR product (A-DR-mazF-DR-B) was transformed into *B. subtilis* 1A751 competent cells, and xylose^S Zeo^R colonies were selected on LB medium supplemented with 20 μg/ml zeocin. The integration of the mini-*mazF*-cassette at the correct locus of the chromosome was confirmed by PCR for 2–4 individual colonies, as well as by detecting amylase activity with the method of iodine staining according to Zhang et al. (2006). The *amyE* gene deletion strain was named *B. subtilis* ZPM61. Cells with the mini-*mazF*-cassette (xylose^R Zeo^S) deleted were obtained by spreading aliquots (50–100 μl) of a 10-fold diluted colonies on LB plates containing 1% xylose and incubating 8–16 h at 37 °C, the mini-*mazF*-cassette was excised by intra-molecular homologous

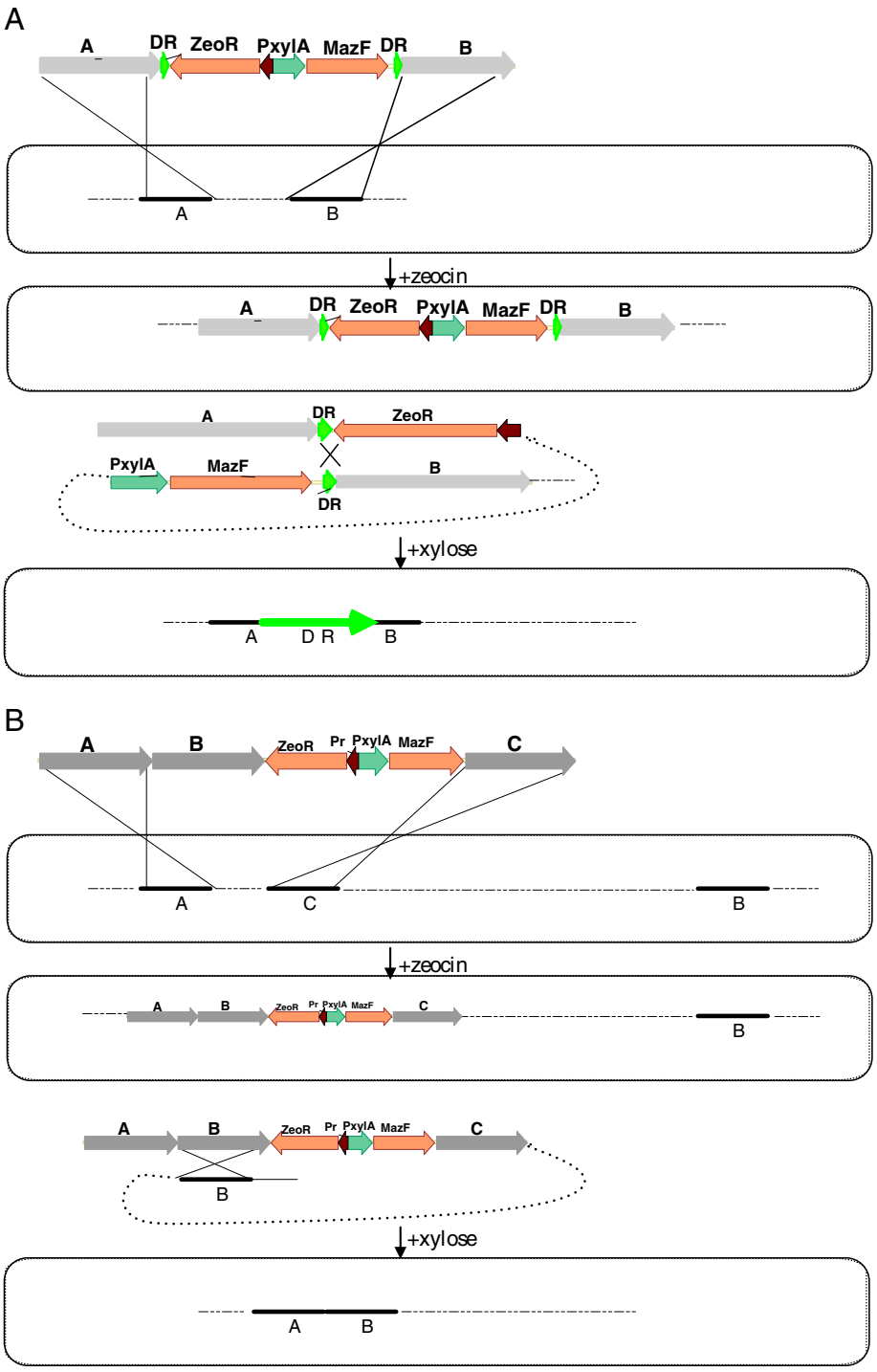

Fig. 2. Scheme for deletion of gene (A) and gene cluster (B) in *Bacillus subtilis* using the *mazF*-cassette. A: upstream sequence; B: downstream sequence; C: internal homology fragment of the gene cluster; DR: direct repeat sequence; Zeo^R, zeocin resistance; Pr, promoter of the *B. subtilis* DNA-binding protein HBsu gene hbs; PxylA, promoter of the *B. subtilis* xylose-induced expression system; mazF, *Escherichia coli* toxin gene.

recombination (DR). PCR examination indicated that xylose-resistant cells were marker-free, yielding the recombinant strain *B. subtilis* ZPM61S.

In order to evaluate the strict regulation of PxylA from the *B. subtilis*, construction of the amyE deletion strain with zeo-Pspac-mazF or zeo-PxylA-Bm-mazF as *mazF*-cassette was carried out. The transformants with $xylose^S$ Zeo^R and deleted *mazF*-cassette ($xylose^R$ Zeo^S) were selected as described above.

2.6. Construction of the strains with deletion of the skin and prophage 7 region

For deletion of large fragments such as a gene cluster, a similar procedure was performed (Fig. 2B). In our study, the skin and prophage 7 region, a non-essential *B. subtilis* gene cluster (Kobayashi et al., 2003), was targeted for deletion. The 500-bp upstream and downstream DNA fragments flanking gene cluster were amplified with gene *spoIVCB* and *yraKF* as template, P17/P18 and P19/P20 as primers, respectively. Then

they fused to a new generated homologous fragment. An internal sequence (fragment C) of about 500 bp, which is located 4–5 kb from the end of the upstream or downstream fragments, was chosen as a new homology fragment; it was amplified using *yqcI* gene as template, P21/P22 as primers. The two homologous fragments were fused with the mini-*mazF*-cassette for efficient deletion of the large fragment. The fusion PCR product was used to directly transform *B. subtilis* 1A751 competent cells, and the recombinant cells were confirmed as above and designated *B. subtilis* ZPM62. Mini-*mazF*-cassette was evicted as described above and designated *B. subtilis* ZPM62S.

2.7. Construction of the strains with unmarked knock-in of green fluorescent protein (GFP) expression cassettes

The green fluorescent protein (GFP) expression cassette was amplified from vector pBE2P43GFP using the primers P13/P14. The *gfp* gene was under the control of the P43 promoter from *B. subtilis* 1A751. The upstream (A) was amplified as above. A fusion PCR fragment (DR-mazF-DR-B), containing the mini-*mazF*-cassette, DR sequence, and downstream homologous recombination regions of the *amyE* gene, was amplified from *B. subtilis* ZPM61Δ*amyE* with the primer P7/P11. Three fragments were fused by overlapped extension PCR (SOE-PCR). The PCR product (A-P43GFP-DR-mini-*mazF*-cassette-DR-B) (Fig. 1D) as used to transform *B. subtilis* 1A751 competent cells, and the recombinant strain with zeo^R was designated *B. subtilis* ZPMG and the strain without zeo^R was designated ZPMGS. Mini-*mazF*-cassette was evicted as above.

2.8. Nucleotide sequence accession number

The sequences of mini-*mazF*-cassette have been submitted to NCBI (JN388939). *B. subtilis* ZPM6 can be ordered from the Bacillus Genetic Stock Center (http://www.bgsc.org) with the accession no. 1A1057.

3. Results

3.1. Construction of recombinant B. subtilis containing the mini-mazF-cassette

The constructed 936-bp mini-*mazF*-cassette was digested with *Sal*I and *Xba*I and inserted into pSG1729. The ligation mixture was directly transformed into *B. subtilis* 1A751. The selected zeocin-resistant *B. subtilis* strain named ZPM6 could not form colonies on LB medium supplemented with 1% xylose (Fig. 3A and B). The mini-*mazF*-cassette was further confirmed by PCR amplification with primer P7/P6 (Fig. 4A, lanes 2 and 3). These results showed that the mini-*mazF*-cassette is successfully constructed and inserted into the *B. subtilis* 1A751. Moreover, when the expression of MazF in mini-*mazF*-cassette is induced, it can inhibit *B. subtilis* proliferation, suggesting that the mini-*mazF*-cassette can be used as a counter-selectable marker in *B. subtilis*.

3.2. Construction of the amyE deletion strains

To explore the feasibility of our strategy, the deletion of *amyE* gene in *B. subtilis* was carried out. The two homologous fragments were fused to the mini-*mazF*-cassette to generate a 1970-bp triple-fusion PCR product (A-DR-mazF-DR-B) containing two 30-bp DR sequences. The fusion PCR products were transformed into *B. subtilis* 1A751 competent cells, the integration of the mini-*mazF*-cassette into the chromosome happened, inactivating *amyE* and obtaining 31–68 zeocin-resistant transformants (named *B. subtilis* ZPM61).

Five selected colonies from the zeocin-resistant transformants were subjected to xylose for further selection. After the seconded cycle of homologous recombination, xylose^R Zeo^S colones were obtained; 96% of the total clones ($n = 23$) had lost the mini-*mazF*-cassette, and five selected colonies were further confirmed by PCR (Fig. 4A, lanes 4 and 5) and showed no α-amylase activity in a starch-plate assay (Fig. 3C and D). These results showed that mini-*mazF*-cassette could be used to effectively disrupt a gene of interest and subsequently evict the selectable markers.

3.3. Strict regulation of xyl promoter from B. subtilis W23 among mini-mazF-cassette

To evaluate the strict regulation of the mini-*mazF*-cassette, after 4 h incubation of ZPM6 in LLB + zeocin, dilutions were spread on LB medium with or without 1% xylose. No xylose^R clones were obtained, indicating there was no or very low expression of MazF in the host in the absence of xylose inducer (Fig. 3E and F).

To compare the stringency of regulation of different promoters, the PxylA from the *B. subtilis* in the mini-*mazF*-cassette was replaced with IPTG-inducible Pspac and PxylA from *B. megaterium*, respectively. Transformation efficiency of three different assembled mazF-cassette with different promoter was shown in Table 3. The transformants were checked for correct integration by testing for amylase activity and (or) PCR amplification. The mini-*mazF*-cassette with PxylA from the *B. subtilis* was showed a 3-fold increase in transformation frequency compared to other two *mazF*-cassettes. Besides, most transformants of other two *mazF*-cassettes were killed when LB medium was supplied with xylose, as no fragments were excised by intra-molecular homologous recombination. The spontaneous *mazF*-resistant mutants of the mini-*mazF*-cassette with PxylA from the *B. subtilis* were no more than 5 (per 10^5 cells) among selected xylose^S Zeo^R colonies compared to other cassettes (Table 3, Fig. 3E and F). These results suggested the xyl promoter from *B. subtilis* W23 showed the higher induction/repression ratio and the lower frequency of spontaneous *mazF*-resistant mutants than the promoters with IPTG-inducible Pspac system or *xyl* expression system from *B. megaterium*.

3.4. Construction of the strain with deletion of the skin and prophage 7 region

For deletion of large fragments such as a gene cluster, a similar procedure was performed. The skin and prophage 7 region, a non-essential *B. subtilis* gene cluster (Kobayashi et al., 2003), was targeted for deletion. The 116 genes in the skin and prophage 7 region contained about 90 kb. The 500-bp upstream DNA fragments of the *spoIVCB* gene and downstream DNA fragments of the *yraKF* were amplified. In addition, an internal 500-bp DNA region of the *yqcI* gene was used as the new back homology fragment. The three fragments were fused with the mini-*mazF*-cassette to generate an approximately 2500-bp fusion PCR product, which was used to directly transform *B. subtilis* 1A751 competent cells, then 28–65 zeocin-resistant transformants were selected. Cultures from zeocin-resistant colonies were subjected to xylose for further selection. Intra-molecular homologous recombination occurred after plating on LB medium supplemented with 1% xylose. After PCR detection, 98% of the total clones ($n = 44$) were marker-free, and the colonies were further confirmed by PCR (Fig. 4D). Using the primers for the mini-*mazF*-cassette (about 930 bp), a band of about 1.0 kb indicated that the mini-*mazF*-cassette inserted into the ZPM62 genome (Fig. 4D, lane 11) but disappeared in the ZPM62S (Fig. 4D, lane 12). Using the primers for the *yqcI* gene, similar results were obtained (Fig. 4D, lanes 13 and 14). These results showed that mini-*mazF*-cassette could be used to effectively disrupt a gene cluster and subsequently evict the selectable markers.

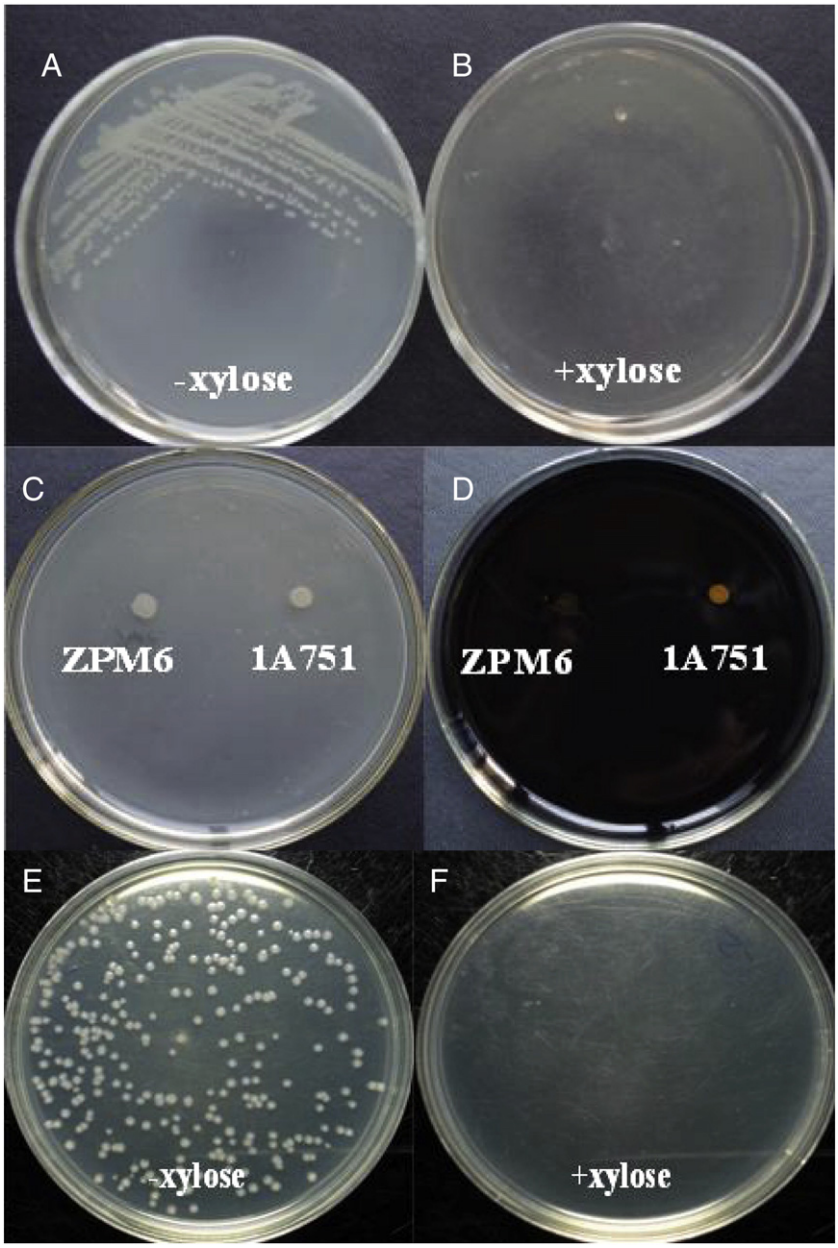

Fig. 3. Feasibility test of the *mazF*-cassette. *Bacillus subtilis* ZPM6 was streaked on an LB plate in the absence (A) and presence (B) of 1% xylose. *B. subtilis* 1A751 and *B. subtilis* ZPM6 were grown on LB plates containing 1% starch (C and D); one plate (D) was stained with iodine to detect α-amylase activity, indicated by a transparent zone. Preculture of *B. subtilis* ZPM6 was coated on an LB plate in the absence (E) and presence (F) of 1% xylose.

3.5. Construction of the strains with unmarked knock-in of green fluorescent protein (GFP) expression cassettes

The GFP gene was placed under the control of P43 promoter of *B. subtilis*. The chromosomal integration of the GFP expression cassette and the *mazF*-cassette (about 3.1 kb) into the *B. subtilis* 1A751 *amyE* gene locus, and the eviction of the *mazF*-cassette from the genome, were performed as described above. This yielded the 20–60 zeocin-resistant ZPMG strain (Fig. 1D), 96% of the xylose^S Zeo^R transformants showed fluorescence emission, after the seconded cycle of homologous recombination, yielding the zeocin-sensitive ZPMGS strain (Fig. 1E). After the elimination of the mini-*mazF*-cassette, one copy of the DR sequence, the P43 promoter, and GFP gene remained in the ZPMGS strain genome, occupied the locus of *amyE* gene (Fig. 1E). Fluorescence microscopy demonstrated the expression of GFP in ZPMGS (Fig. 5). PCR with primers for the GFP expression cassette (about 1.0 kb) and the partial *amyE* gene are in Fig. 4B and C. The GFP gene in ZPMGS (about 0.8 kb) (Fig. 4B, lane 8) was the same length as the fragment obtained by using pBE2P43GFP as DNA template (Fig. 4B, lane 6). The amplified fragments using primers P23/P24 for the partial *amyE* gene in ZPM61S (about 0.5 kb) and ZPMGS are in Fig. 4C. In contrast to lane 9, a band of about 1.6 kb in lane 10 indicated that the GFP expression cassette and one copy of the DR sequence were inserted into the *amyE* gene locus of the ZPMGS genome. The presence of the smaller product was appeared to the reasons that the genomic template is too much. These results showed that *mini-mazF*-cassette could be used to effectively construct the strains with unmarked knock-in of green fluorescent protein (GFP) expression cassettes.

4. Discussion

In this study, a versatile mini-*mazF*-cassette was constructed using a PCR-fusion method, for marker-free genetic manipulation of the *B. subtilis*. This cassette contains the *E. coli mazF* gene encoding an endoribonuclease

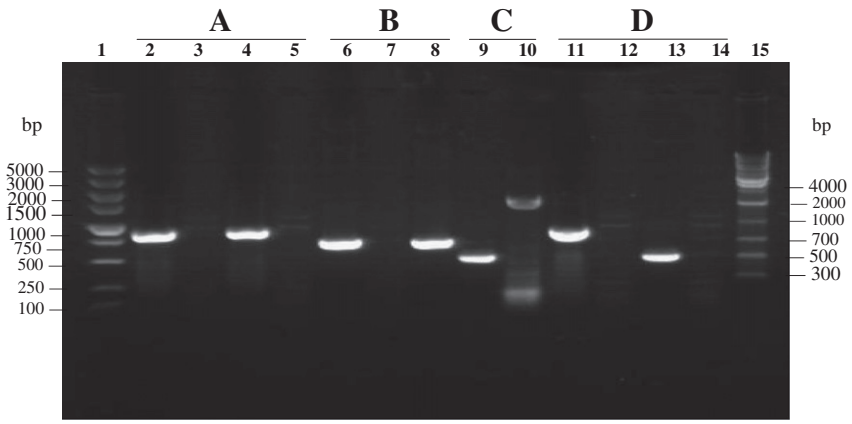

Fig. 4. Confirmation of the *mazF*-cassette. (A) Introduction of a green fluorescent protein expression cassette (B); *amyE* deletion (C); Skin and Pro7 deletion (D); Lane 1: DL5000. Lane 15: 1 kb DNA ladder. (A) Primer pair P7/P6 used to amplify the *mazF*-cassette. Lane 2: ZPM6 (positive control, about 1.0 kb). Lane 3: 1A751 (negative control). Lane 4: ZPM61. Lane 5: ZPM61S. (B) Primer pair P25/P26 used to amplify the *gfp* gene. Lane 6: P43GFP (positive control, about 0.8 kb). Lane 7: ZPM61S. Lane 8: ZPMGS; (C) Primer pair P23/P24 used to amplify the *amyE* gene. Lane 9: ZPM61S (about 0.5 kb). Lane 10: ZPMGS (about 1.6 kb); (D) Primers used to amplify the *mazF*-cassette (P7/P6) and yqcI gene (P21/P22). Lane 11: ZPM62 (about 1.0 kb). Lane 12: ZPM62S, Lane 13: ZPM62 (about 0.5 kb), Lane 14: ZPM62S.

as a counter-selection marker. Using this cassette could replace the traditional, time-consuming vector construction procedure. As a proof of the feasibility of mini-*mazF*-cassette, we successfully knocked out the *amyE* gene and a 90-kb gene cluster, knocked-in a GFP expression cassette, without introducing unwanted redundant sequences at the modification locus.

Previous reports have indicated that *E. coli mazF* as a counter-selection marker may produce spontaneous *mazF*-resistant mutants (Yu et al., 2006) because of accumulation of MazF in the host in the absence of inducer. In this study, we selected the inducible Pxyl promoter from the *B. subtilis* as a candidate for the tight transcriptional regulation of the toxic *mazF* gene, the frequency of spontaneous *mazF*-resistant strains was less than 5% (Table 3, Fig. 3E and F). The presumable reasons are as follows: first, the xyl promoter from *B. subtilis* W23 has higher induction/repression ratio (Bhavsar et al., 2001; Hartl et al., 2001). Second, the introduction of a DNA sequence derived from *B. subtilis* into the *B. subtilis* genome should have no side effect on cell growth and chromosome replication. Third, the xylose utilization pathway is not essential for energy production and a deficiency in this pathway did not affect the growth of *B. subtilis* cells in the absence of xylose (Bhavsar et al., 2001).

Compared with the mazF-cassette described by Yu et al. (2010) and Morimoto et al. (2009), the mini-*mazF*-cassette constructed in this study has three advantages. Firstly, mini-*mazF*-cassette could be used repeatedly to delete multiple genes or gene clusters with only 2- to 2.5-kb PCR-fused fragments, which can reduce the frequency of nucleic acid mutations generated by PCR. In addition, the mini-*mazF*-cassette showed a 3-fold higher transformation frequency compared to other two mazF-cassettes. Secondly, the *mazF* gene was placed under the control of the well-characterized *B. subtilis* *xyl* regulatory system, which is apparently superior to the *spac* expression system and *B. megaterium xyl* regulatory system (Bhavsar et al., 2001). The spontaneous frequency of the *mazF*-resistant strains was low, and more than 95% of the total clones (the number is about 20) had lost the *mazF*-cassette, whereas only 50% in the previously published system (Yu et al., 2010). Third, The *xyl* operons of several Gram-positive bacteria are transcriptionally regulated by xylose-responsive repressor proteins (XylR) (Dahl et al., 1995). The XylR proteins show considerable sequence similarities in *B. megaterium*, *B. amyloliquefaciens*, *B. pumilus*, *B. cereus*, *B. licheniformis*, *B. halodurans*, *B. coagulans*, *B. anthracis*, *Staphylococcus xylosus*, *Tetragenococcus halophila*, and *Lactobacillus pentosus* (Dahl et al., 1995; Lokman et al., 1997; Rodionov et al., 2001; Rygus et al., 1991; Takeda et al., 1998), indicating that the transcriptional regulation of this operon could be exploited in those species, especially *Bacillus*.

In conclusion, the mini-*mazF*-cassette could significantly improve the manipulation of multiple and marker-free genetic modification in *B. subtilis*, and this method might be applicable in other *Bacillus* species.

Table 3
Comparative analysis the strict regulation of the *mazF* gene.

mazF-cassette[a]	strains	Primary transformation[b] (Zeocin^R/μg DNA)	Transformants/10^5 cell[c] at [Xylose] or IPTG
PxylA-Bs	ZPM6	16.4	<5
Pspac	ZPMSP	5.8	24
PxynA-Bm	ZPMBm	6.2	11

[a] The mazF gene was under control the different promoters.
[b] The number of transformants per 1 μg of recombinant PCR product used to transform *Bacillus subtilis* 1A751 cells.
[c] At the time of plating, the ZPM6, ZPMSP, and ZPMBm culture contained 1.2×10^9 viable cells/ml in LB plus 1% glucose, three independent duplicate tests were performed.

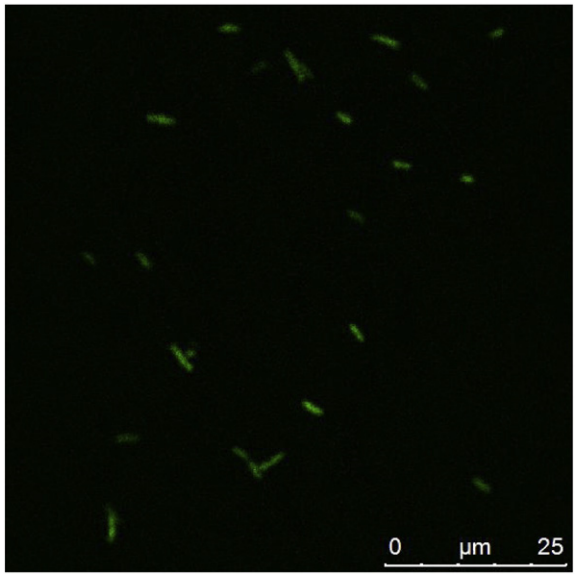

Fig. 5. Microscopy for GFP in ZPMGS.

Conflict of interest

None declared.

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