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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.

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#### 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). Postgenomic 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 sitespecific recombination, and the other is the counter-selection system.

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

Amp<sup>r</sup>, ampicillin resistance; Spc<sup>r</sup>, spectinomycin resistance; zeo<sup>R</sup>, zeocin resistance, Kan<sup>r</sup>, kanamycin resistance.

Cm<sup>r</sup>, 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	CATATCAAGTCTTTCATGAAAAACTAAAAAAAAATATT			
P4	ACCAGATCCTCCTTTAGATGCATTTTATGTCATATTGTA			
P5	CATCTAAAGGAGGATCTGGTAATGGTAAGCCGATA			
P6	ggcTCTAGACTACCCAATCAGTACGTTAAT			
P7	AATCAATAATGGACCAGACGACAGTCCTGCTCCTCGGCCAC			
P8	AGTCTTCAAAAAATCAAATAAGGAGT			
P9	TCGTCTGGTCCATTATTGATTTGATAAACGCTTAACCTCATTGGAAATCGCG			
P10	TGATTGGGTAGTCTAGAGCCCAGATGCGAATACAACAAAAGC			
P11	GTAAGTCCCGTCTAGCCTTGCCCTC			
P12	TACCACCTATCTTAACCTCATTGGAAATCGCG			
P13	ATGAGGTTAAGATAGGTGGTATGTTTTCGCTTG			
P14	TCGTCTGGTCCATTATTGATTTGATAAACGTTATTTGTATAGTTCATCCATGCC			
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  $1.2 \times 10^9$  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-mazFcassette

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 *SalI*, *Bam*HI, *Eco*RI, and *Hind*III restriction sites and P6 with *XbaI* restriction sites at the 3'-end of the *mazF* gene. Products were gel-purified using AxyPrep DNA gel

extraction kit (Axygen). These three PCR fragments were fused in the



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<sup>S</sup> Zeo<sup>R</sup> and deleted *mazF*-cassette (xylose<sup>R</sup> Zeo<sup>S</sup>) 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 *yqcl* 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* ZPM61 $\Delta$ *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<sup>R</sup> was designated *B. subtilis* ZPMG and the strain without zeo<sup>R</sup> 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 zeocinresistant *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 counterselectable 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<sup>R</sup> Zeo<sup>S</sup> 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  $\alpha$ -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 minimazF-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<sup>R</sup> 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 mazFcassette 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 mazFresistant mutants of the mini-mazF-cassette with PxylA from the *B. subtilis* were no more than 5 (per 10<sup>5</sup> cells) among selected xylose<sup>8</sup> Zeo<sup>R</sup> 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 nonessential 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 yqcl 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 yqcl 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 streake931.7(d)-242.8(o)19.6(n)-231.8(a)0(n)-219.8(L)0(B)-225.1(p) (C) bilis bit ii(a) A 75(b) and 20.5(a) iii(a) A 75(b) and 20.5(a) ii

# 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 zeocinresistant ZPMG strain (Fig. 1D), 96% of the xylose<sup>S</sup> Zeo<sup>R</sup> 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 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 muc**Ts**hese results showed that *mini-mazF*-cassette cfuld be used to effectively construct the strains with unmarke9614.ad knock-in off**greers**cent 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 *E. coli mazF* gene encoding an endorib**onutàiast**he 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 counterselection 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 clwm13.50TD0Tco.7(ao1[(l)0(i)4-2.4(t)-13(n)]TJ0-1.3159T[.7(on)-21.3(ly4-519.2(b)-11.-ly4-2.4(b)-11.-ly4

#### Conflict of interest

None declared.

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#### References

- Bhavsar, A.P., Zhao, X., Brown, E.D., 2001. Development and characterization of a xylosedependent system for expression of cloned genes in *Bacillus subtilis*: conditional complementation of a teichoic acid mutant. Appl. Environ. Microbiol. 67, 403–410.
- Brans, A., Filee, P., Chevigne, A., Claessens, A., Joris, B., 2004. New integrative method to generate *Bacillus subtilis* recombinant strains free of selection markers. Appl. Environ. Microbiol. 70, 7241–7250.
- Cutting, S.M., Vander-Horn, P.B., 1990. Genetic analysis. In: Harwood, C.R., C. S. M. (Eds.), Molecular Biological Methods for Bacillus. Wiley Press, Chichester, pp. 27–75.
- Dahl, M.K., Schmiedel, D., Hillen, W., 1995. Glucose and glucose-6-phosphate interaction with Xyl repressor proteins from *Bacillus spp.* may contribute to regulation of xylose utilization. J. Bacteriol. 177, 5467–5472.
- Daniel, R.A., Harry, E.J., Katis, V.L., Wake, R.G., Errington, J., 1998. Characterization of the essential cell division gene ftsL(yIID) of *Bacillus subtilis* and its role in the assembly of the division apparatus. Mol. Microbiol. 29, 593–604.
- Earl, A.M., Losick, R., Kolter, R., 2007. Bacillus subtilis genome diversity. J. Bacteriol. 189, 1163–1170.
- Fabret, C., Ehrlich, S.D., Noirot, P., 2002. A new mutation delivery system for genome-scale approaches in *Bacillus subtilis*. Mol. Microbiol. 46, 25–36.
- Hartl, B., Wehrl, W., Wiegert, T., Homuth, G., Schumann, W., 2001. Development of a new integration site within the Bacillus subtilis chromosome and construction of compatible expression cassettes. J. Bacteriol. 183, 2696–2699.
- Kobayashi, K., Ehrlich, S.D., Albertini, A., Amati, G., Andersen, K.K., Arnaud, M., Asai, K., Ashikaga, S., Aymerich, S., Bessieres, P., Boland, F., Brignell, S.C., Bron, S., Bunai, K., Chapuis, J., Christiansen, L.C., Danchin, A., Debarbouille, M., Dervyn, E., Deuerling, E., Devine, K., Devine, S.K., Dreesen, O., Errington, J., Fillinger, S., Foster, SJ., Fujita, Y., Galizzi, A., Gardan, R., Eschevins, C., Fukushima, T., Haga, K., Harwood, C.R., Hecker, M., Hosoya, D., Hullo, M.F., Kakeshita, H., Karamata, D., Kasahara, Y., Kawamura, F., Koga, K., Koski, P., Kuwana, R., Imamura, D., Ishimaru, M., Ishikawa, S., Ishio, I., Le Coq, D., Masson, A., Mauel, C., Meima, R., Mellado, R.P., Moir, A., Moriya, S., Nagakawa, E., Nanamiya, H., Nakai, S., Nygaard, P., Ogura, M., Ohanan, T., O'Reilly, M., O'Rourke, M., Pragai, Z., Pooley, H.M., Rapoport, G., Rawlins, J.P., Rivas, L.A., Rivolta, C., Sadaie, A., Sadaie, Y., Sarvas, M., Sato, T., Saxild, H.H., Scanlan, E., Schumann, W., Seegers, J.F., Sekiguchi, J., Sekowska, A., Seror, SJ., Simon, M.,

Stragier, P., Studer, R., Takamatsu, H., Tanaka, T., Takeuchi, M., Thomaides, H.B., Vagner, V., van Dijl, J.M., Watabe, K., Wipat, A., Yamamoto, H., Yamamoto, M., Yamamoto, Y., Yamane, K., Yata, K., Yoshida, K., Yoshikawa, H., Zuber, U., Ogasawara, N., 2003. Essential *Bacillus subtilis* genes. Proc. Natl. Acad. Sci. U. S. A. 100, 4678–4683.

- Kolodkin-Gal, I., Hazan, R., Gaathon, A., Carmeli, S., Engelberg-Kulka, H., 2007. A linear pentapeptide is a quorum-sensing factor required for mazEF-mediated cell death in *Escherichia coli*. Science 318, 652–655.
- Lewis, P.J., Marston, A.L., 1999. GFP vectors for controlled expression and dual labelling of protein fusions in *Bacillus subtilis*. Gene 227, 101–110.
- Liu, S., Endo, K., Ara, K., Ozaki, K., Ogasawara, N., 2008. Introduction of marker-free deletions in *Bacillus subtilis* using the AraR repressor and the ara promoter. Microbiology 154, 2562–2570.
- Lokman, B.C., Heerikhuisen, M., Leer, R.J., van den Broek, A., Borsboom, Y., Chaillou, S., Postma, P.W., Pouwels, P.H., 1997. Regulation of expression of the *Lactobacillus pentosus* xylAB operon. J. Bacteriol. 179, 5391–5397.
- Morimoto, T., Ara, K., Ozaki, K., Ogasawara, N., 2009. A new simple method to introduce marker-free deletions in the *Bacillus subtilis* genome. Genes Genet. Syst. 84, 315–318.
- Nguyen, H.D., Nguyen, Q.A., Ferreira, R.C., Ferreira, L.C., Tran, L.T., Schumann, W., 2005. Construction of plasmid-based expression vectors for *Bacillus subtilis* exhibiting full structural stability. Plasmid 54, 241–248.
- Reyrat, J.M., Pelicic, V., Gicquel, B., Rappuoli, R., 1998. Counterselectable markers: untapped tools for bacterial genetics and pathogenesis. Infect. Immun. 66, 4011–4017.
- Rodionov, D.A., Mironov, A.A., Gelfand, M.S., 2001. Transcriptional regulation of pentose utilisation systems in the *Bacillus/Clostridium* group of bacteria. FEMS Microbiol. Lett. 205, 305–314.
- Rygus, T., Scheler, A., Allmansberger, R., Hillen, W., 1991. Molecular cloning, structure, promoters and regulatory elements for transcription of the *Bacillus megaterium* encoded regulon for xylose utilization. Arch. Microbiol. 155, 535–542.
- Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Schallmey, M., Singh, A., Ward, O.P., 2004. Developments in the use of *Bacillus* species for industrial production. Can. J. Microbiol. 50, 1–17.
- Suzuki, E., Nakayama, M., 2011. VCre/VloxP and SCre/SloxP: new site-specific recombination systems for genome engineering. Nucleic Acids Res. 39, e49.
- Takeda, Y., Takase, K., Yamato, I., Abe, K., 1998. Sequencing and characterization of the xyl operon of a Gram-positive bacterium, *Tetragenococcus halophila*. Appl. Environ. Microbiol. 64, 2513–2519.
- Wang, Y., Weng, J., Waseem, R., Yin, X., Zhang, R., Shen, Q., 2012. Bacillus subtilis genome editing using ssDNA with short homology regions. Nucleic Acids Res.
- Yan, X., Yu, H.J., Hong, Q., Li, S.P., 2008. Cre/lox system and PCR-based genome engineering in *Bacillus subtilis*. Appl. Environ. Microbiol. 74, 5556–5562.
- Yang, J., Jiang, W., Yang, S., 2009. mazF as a counter-selectable marker for unmarked genetic modification of *Pichia pastoris*. FEMS Yeast Res. 9, 600–609.
- Yu, H., Yan, X., Shen, W., Shen, Y., Zhang, J., Li, S., 2010. Efficient and precise construction of markerless manipulations in the *Bacillus subtilis* genome. J. Microbiol. Biotechnol. 20, 45–53.
- Zhang, X.Z., Yan, X., Cui, Z.L., Hong, Q., Li, S.P., 2006. mazF, a novel counter-selectable marker for unmarked chromosomal manipulation in *Bacillus subtilis*. Nucleic Acids Res. 34, e71.