Introduction of marker-free deletions in *Bacillus subtilis* using the AraR repressor and the *ara* promoter

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We have developed a system for the induction of marker-free mutation of *Bacillus subtilis*. The system features both the advantages of the use of antibiotic-resistance markers for mutant selection, and the ability to efficiently remove the markers, leaving unmarked mutations in the genome. It utilizes both a selective marker cassette and a counter-selective marker cassette. The selective marker cassette contains a chloramphenicol-resistance gene and the *araR* gene, which encodes the repressor for the arabinose operon (*ara*) of *B. subtilis*. The counter-selective marker cassette consists of a promoterless neomycin (Nm)-resistance gene (*neo*) fused to the *ara* promoter. First, the chromosomal *araR* locus is replaced with the counter-selective marker cassette by double-crossover homologous recombination and positive selection for Nm resistance. The selective marker cassette is connected with upstream and downstream sequences from the target locus, and is integrated into the upstream region of the target locus by a double-crossover event. This integration is also positively selected for, using chloramphenicol resistance. In the resultant strain, AraR, encoded by *araR* on the selective marker cassette, represses the expression of *neo* in the absence of L-arabinose. Finally, the eviction of the selective marker cassette together with the target locus is achieved by an intra-genomic single-crossover event between the two downstream regions of the target locus, and can be selected for based on Nm resistance, because of the excision of *araR*. The counter-selective marker cassette remaining in the genome, whose expression is switched on or off based on the excision or introduction of the selective marker cassette, is used again for the next round of deletion. Using this system, the 3.8 kb *iolS*–*csbC* region and the 41.8 kb *hutM*–*csbC* region have been efficiently and successfully deleted, without leaving markers in the target loci. The positive selection and simple procedure will make it a useful tool for the construction of multiple mutations.

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INTRODUCTION

Bacillus subtilis is one of the most important bacterial strains for industrial protein production, because various proteins are secreted directly into the culture medium rather than being trapped in a periplasmic space, as is frequently the case for *Escherichia coli* and other Gram-negative bacteria (Harwood *et al.*, 1990). The complete sequencing and annotation of the *B. subtilis* genome (Kobayashi *et al.*, 2003; Kunst *et al.*, 1997) has facilitated the construction of artificial mutant strains for industrial and scientific uses (Harwood & Wipat, 1996). Multiple mutations are required in many cases. Mutant selection

relies mostly on antibiotic-resistance markers; however, the number of markers available for use in *B. subtilis* is limited. To circumvent this problem, several methods have been developed to produce unmarked mutations in *B. subtilis* (Bloor & Cranenburgh, 2006; Brans *et al.*, 2004; Fabret *et al.*, 2002; Leenhouts *et al.*, 1996; Zhang *et al.*, 2006) and other micro-organisms (Bloor & Cranenburgh, 2006; Leenhouts *et al.*, 1996; Malaga *et al.*, 2003; White *et al.*, 2007). A method of selectable marker gene excision by Xer recombination has been reported recently, in *E. coli* and *B. subtilis*. The native Xer site-specific recombinase, RipX/CodV in *B. subtilis*, recognizes the *dif* sites and acts to resolve the two directly repeated *dif* sites to a single site (Bloor & Cranenburgh, 2006). Leenhouts *et al.* (1996) have described a method to generate unlabelled gene

Abbreviations: Cm, chloramphenicol; Nm, neomycin; SOE-PCR, splicing by overlapped extension PCR; Sp, spectinomycin.

replacements in *Lactococcus lactis* and *B. subtilis*, using a conditional replication plasmid as the integration vector and *E. coli lacZ* as the reporter. However, the selections of the unmarked mutants were conducted with negative selection. The method involving the *upp* cassette, described by Fabret *et al.* (2002), tends to result in many false-positive colonies in our experience, so that it is not adequate for the efficient construction of multiple mutants. Two other methods use, respectively, the heterologous genes *blaI* from *Bacillus licheniformis* and *mazF* from *E. coli* for counter selection (Brans *et al.*, 2004; Zhang *et al.*, 2006), and specific direct repeat DNA sequences, used for homologous recombination, are introduced into the genome during every round of manipulation. For multiple modifications of the *B. subtilis* genome, dozens of mutations might be performed. If many direct repeat sequences are left in the genome, intra-genomic homologous recombinations are more likely to occur, which might result in the deletion or inversion of genomic regions. The purpose of this study is to construct a marker-free multiple mutation system by positive selection, without heterologous DNA fragments remaining in the target sequence.

We describe here a method that involves a selective marker cassette carrying an antibiotic-resistance gene and a gene for a repressor active in *B. subtilis* cells. Upstream and downstream sequences from the target locus are placed in front of the cassette, which is then inserted into the upstream region of the target locus by homologous recombination (Fig. 1). This cassette is designed to be removed later, and can be used subsequently for another round of mutation. Another antibiotic-resistance gene, whose expression is controlled by the repressor, is used as a counter-selective marker for excision of the selective marker cassette along with the target DNA sequence. The sequence downstream of the target locus, similar to the downstream sequence introduced with the selective marker cassette, acts as a specific site for homologous recombination, which makes it possible for no heterologous DNA sequences to remain at the mutation locus. When the selective marker cassette is removed, the counter-selective marker gene is released from repression, so that antibiotic resistance is expressed. The counter-selective marker remains in the *B. subtilis* genome after the deletion of the target locus and is used again for the next round of deletion manipulation.

For this method we selected the gene that encodes the repressor of the arabinose metabolism genes, namely *araR* (Mota *et al.*, 1999, 2001; Sa-Nogueira & Mota, 1997), for use in the selective marker cassette, and the promoter of the *araBDLMNPQ-abfA* operon (Sa-Nogueira *et al.*, 1997) for use in the counter-selective marker cassette. This new mutation system will be convenient for the introduction of multiple mutations, and we discuss its use in large-scale deletion mutations of the *B. subtilis* genome.

METHODS

Bacterial strains and oligonucleotides. The bacterial strains used in this study are listed in Table 1. All *B. subtilis* recombinant strains were *B. subtilis* 168 derivatives. The primers used for PCR amplification were synthesized by Sigma Genosys and are listed in Table 2.

Culture and growth conditions. All organisms were cultured in Luria–Bertani (LB) broth or on 1.5 % (w/v) agar plates supplemented with the appropriate antibiotics when required. Chloramphenicol (Cm), 10 µg ml⁻¹, neomycin (Nm), 20 µg ml⁻¹, and spectinomycin (Sp), 100 µg ml⁻¹, were used to select resistant *B. subtilis* cells. All bacterial cultures were incubated at 37 °C, with shaking at 180 r.p.m. for liquid cultures. Spizizen minimal medium was used as the basal medium for the preparation of competent cells, as described elsewhere (Anagnostopoulos & Spizizen, 1961).

DNA manipulation techniques. The isolation and manipulation of recombinant DNA was performed using standard techniques. Enzymes were commercial preparations and were used as specified by the supplier (Takara). Chromosomal DNA was prepared using the Microbial Genomic DNA Isolation kit (MO Bio). PCRs were performed in final volumes of 50 µl containing 1.25 U Pyrobest DNA polymerase (Takara), 1 mM MgSO₄, 0.2 mM dNTP and 0.4 µM of each primer. The amplification program consisted of 30 cycles of 10 s at 98 °C, 30 s at 55 °C, 1 min for every 1 kb of amplified product at 72 °C, followed by a final 5 min at 72 °C. All PCRs were performed in a Perkin-Elmer 9700 thermal cycler. The PCR products were analysed by electrophoresis in 1 % (w/v) agarose gels. PCR products were purified with a PCR Purification kit (MO Bio).

Construction of strains AR1 and AR2. Strain AR1, in which the coding region of *araA* was substituted with a promoterless *neo* gene (Fig. 2), was constructed from strain 168 as follows. The 0.8 kb promoterless *neo* gene fragment was amplified from pUB110 (McKenzie *et al.*, 1986) using the primers Nmf and Nmr. Approximately 1.0 kb of each of the flanking sequences upstream (UParaA) and downstream (DNaraA) of the coding region of *araA* was amplified by PCR from the *B. subtilis* genome, using the primers araA/N5f and araArv, and araAfw and araA/N3r, respectively. The UParaA and DNaraA fragments overlapped with *neo* by 20 bp at the upstream and downstream ends, respectively. These three PCR fragments were then ligated in the order UParaA-*neo*-DNaraA by splicing by overlapped extension PCR (SOE-PCR) (Ho *et al.*, 1989; Liu *et al.*, 2007) using the primer set araAfw2 and araArv2. In order to minimize the minor products of PCR ligation, these primers were designed using sequences 12 bp from the ends of a similar product that would be amplified using the araAfw and araArv primers. The 2.8 kb PCR product was then introduced into *B. subtilis* cells by competent cell transformation, and an Nm-resistant transformant was selected from an LB agar plate containing 0.4 % L-arabinose (Sa-Nogueira *et al.*, 1997) and designated AR1 (Fig. 2).

Strain AR2, in which the *araR* gene was substituted with the Sp-resistance gene (*spm*), was constructed from strain AR1. The *spm* gene was amplified from pDG1727 (Guerout-Fleury *et al.*, 1995) using the primers spf and spr. Approximately 1.0 kb of each of the flanking sequences upstream (UParaR) and downstream (DNaraR) of *araR* was amplified by PCR from the *B. subtilis* genome using the primers araRfw and araR/Spr, and araR/Spf and araRrv, respectively. The UParaR fragment and DNaraR fragments overlapped with *spm* by 20 bp at the upstream and the downstream ends, respectively. The three PCR fragments were then ligated in the order UParaR-*spm*-DNaraR by SOE-PCR (Ho *et al.*, 1989; Liu *et al.*, 2007) using the primer set araRfw2 and araRrv2. The 3.2 kb PCR product was then

Fig. 1. Strategy for the construction of unmarked deletion mutants using selective and counter-selective marker cassettes. UP and DN represent the 1 kb DNA sequences flanking the upstream and downstream regions of the deletion target, respectively. P_{ara}, promoter of the *ara* operon; *neo*, Nm-resistance gene; *cat*, Cm-resistance gene; *araR*, the gene encoding the repressor protein of the *ara* operon; *araA*, the first gene in the *ara* operon. Crossed lines indicate a recombination event. The white pentagons marked G represent the 1 kb downstream region of UP, which is used as one of the homologous recombination sites for selection marker cassette introduction. The broken lines sandwiched by the white pentagon marked G and a grey pentagon indicate the deletion target. The DN sequence is introduced together with the selective marker cassette, and becomes the site of recombination for the excision of the selective marker cassette and the target sequence.

used for transformation of strain AR1, and an Sp-resistant transformant was selected and designated AR2 (Fig. 2).

Construction of the selective marker cassette. The selective marker cassette containing the Cm-resistance gene (*cat*) and *araR* was constructed by PCR amplification. The 0.9 kb *cat* fragment was amplified from pC194 (Horinouchi & Weisblum, 1982) using the primers *cat*/csbCDf and *cat*r (Table 2). The 1.3 kb *araR* fragment, including the promoter and coding regions, was amplified from the *B. subtilis* genome using the primers *araR*f/Cm and *araR*r. The resulting fragment overlapped by 20 bp with the downstream end of the *cat* fragment. The *cat* and *araR* fragments were then ligated

by SOE-PCR (Ho *et al.*, 1989; Liu *et al.*, 2007), using the primers *cat*/csbCDf and *araR*r, to obtain the 2.1 kb selective marker cassette.

Construction of the counter-selective marker cassette. The 1.1 kb DNA fragment P_{ara}-*neo* (Fig. 2) was amplified from the AR1 genome using the primers Paraf and Nmr, for use as the counter-selective marker cassette.

Transformation with PCR fragments. Transformation of *B. subtilis* 168 with PCR fragments was performed using competent cells, as described by Anagnostopoulos & Spizizen (1961).

Table 1. *B. subtilis* strains used in this study

All strains were derived in the course of this study.

Strain	Description*
168	<i>trpC2</i>
AR1	<i>trpC2</i> Δ <i>araA::neo</i> (promoterless)
AR2	<i>trpC2</i> Δ <i>araA::neo</i> (promoterless) Δ <i>araR::spm</i>
AR3	<i>trpC2</i> Δ <i>araR::P_{ara-neo}</i>
AR31	<i>trpC2</i> Δ <i>araR::P_{ara-neo}</i> , DN- <i>cat-araR</i> was inserted between <i>iolR</i> and <i>iolS</i>
AR32	<i>trpC2</i> Δ <i>araR::P_{ara-neo}</i> , Δ <i>hutM::DN-cat-araR</i> *
AR31d	<i>trpC2</i> Δ <i>araR::P_{ara-neo}</i> Δ <i>iolS-csbC</i>
AR32d	<i>trpC2</i> Δ <i>araR::P_{ara-neo}</i> Δ <i>hutM-csbC</i>

*DN, expresses the 1 kb fragment downstream of *csbC*.

DNA sequence determination. The DNA sequences of cassettes, and the junctions of upstream and downstream regions of deletion targets in the deletion mutants were determined. The dideoxy chain-termination method was used, with a BigDye Terminator v3.1 Cycle Sequencing kit and a 3730XL DNA sequencer (Applied Biosystems).

The deletion junctions were amplified from mutants and the PCR products were used as templates.

RESULTS AND DISCUSSION

Construction of an antibiotic marker controlled by AraR

In *B. subtilis*, the genes for L-arabinose utilization, including those in the *araABDLMNPQ-abfA* operon (Sa-Nogueira *et al.*, 1997) and the divergently arranged *araE/araR* genes (Sa-Nogueira & Ramos, 1997; Sa-Nogueira & Mota, 1997), are induced by L-arabinose and negatively controlled by AraR (Mota *et al.*, 1999, 2001). We selected the *B. subtilis* *araR* gene and one of its target promoters as candidates for utilization in the selective and counter-selective marker cassettes in this study for the following reasons. First, the arabinose utilization pathway is not essential for energy production, and a deficiency in the pathway does not affect the growth of *B. subtilis* cells in the absence of arabinose. Second, the introduction of a DNA sequence derived from *B. subtilis* into the *B. subtilis* genome should have no effect on cell growth and

Table 2. Primers used in this study

Primer	Sequence (5'→3')
araAfw	AAGTAGAACATGGCACGTCG
araAfw2	GGTACCGAAACATGAGCGTC
araArv	GGGGAGCTCATGGTATGCGC
araArv2	GACGCTAAAAATTGGACCGT
araA/N3r	TGACCTCTAATAATTGTAAAGTGTTCTCATTTCCCGTGA
araA/N5f	ATTATTATTGGTCCATTTCATGTAAACTGCCCTTTCTCAT
araRfw	TAGTAAGTAATCGCGTTTCAT
araRfw2	TTCTGAGACCCGGCTTAAAC
araRr	TTATTCATTTCAGTTTTCGTG
araRrv	CGGGCAGAGGAATGGGGCTA
araRrv2	CTGCCACCGCAGTTTTCGATT
araRf/Cm	CTGCCCCGTTAGTTGAAGGCATTTTCTGTCAATGTTTTC
araR/Spf	ACAATAAACCCCTTGATATGATTTGAGCCTGAGTTGATCA
araR/Spr	ATTCACGAACGAAAAATCGATTGACAGAAAAATGCAACAAG
catr	GCTGTAATATAAAAAACCTTC
cat/csbCDf	GATGTTTTTCGCGATCAGCTTCTTCAACTAAAGCACCCAT
csbCrv	GGCATGGTCGATTCTGAGGA
csbCrv2	AGCTGATCGCGAAAAACATC
csbC-Df	TCTTCAGGAGAACAGCAGCCAACGGTTTAAAAAGAAGAAA
hutMfw	AAAATGAGCGACCTGATCGC
iolfw	TTACATACAAGCAACCATTT
iolfw2	TTCTAATCGCATAATACCCA
iolrv	AACTACCTTTATTTTATCGG
iolrv2	TATTTAGGAATTTATCGCAC
iol/CRf	CACGAAAACTGAATGAATAAATCCATTTTATTCTAGGAGG
iol/csbCUr	TTTCTTCTTTTTAAACCGTTGATAGACTCTCAACACTATT
Nmf	GTGAATGGACCAATAATAAT
Nmr	TTAACAATTATTAGAGGTCA
Paraf	ATTGGAGCTCGGGCTGGC
spf	ATCGATTTTCGTTTCGTG
spr	CATATGCAAGGGTTTATTG

Fig. 2. (a) Diagrams of the selective and counter-selective marker cassettes used in this study. (b) Mutant strains derived from *B. subtilis* 168 (wild-type). P_{ara} , promoter of the *ara* operon; *neo*, Nm-resistance gene; *cat*, Cm-resistance gene; *araR*, the gene encoding the repressor protein of the *ara* operon; *araA*, the first gene in the *ara* operon; *spm*, Sp-resistance gene.

chromosome replication. Third, the mechanism of AraR regulation has been extensively studied in recent years.

To evaluate the stringency of regulation by AraR of an antibiotic-resistance gene under the control of the *ara* operon promoter, we replaced the *araA* gene with the promoterless Nm-resistance gene (*neo*) to produce the strain AR1 (Fig. 2). AR1 was selected for Nm resistance in the presence of 0.4 % (w/v) L-arabinose, and confirmed to be sensitive to Nm in the absence of L-arabinose (Table 3). Next, the *araR* gene in AR1 was deleted and replaced with the Sp-resistance gene *spm*, to produce the Sp-resistant transformant AR2 (Fig. 2). AR2 was confirmed to be resistant to Nm whether L-arabinose was present in the medium or not (Table 3). It was encouraging to find that the *neo* gene fused to the *ara* promoter (P_{ara} -*neo*) was constitutively expressed in the absence of the *araR* gene. These results indicated that the expression of P_{ara} -*neo* could be stringently controlled by *araR*. In other words, the expression of P_{ara} -*neo* could be switched on or off, depending on the presence or absence of *araR* in the genome, under growth conditions without L-arabinose.

Construction of a mutation system using AraR and P_{ara} -*neo*

Our goal was to construct an efficient system to make multiple mutations using the P_{ara} -*neo* gene as the counter-selective marker. This marker would be used to select for

deletion of the selective marker cassette, consisting of an antibiotic-resistance gene and *araR*.

The 1.1 kb DNA fragment of P_{ara} -*neo* (Fig. 2) was amplified from the AR1 genome using the primers ParaF and Nmr, to be used as the counter-selective marker cassette. It was ligated between upstream and downstream regions (~1 kb each) from the *araR* gene by SOE-PCR. The 3.1 kb fragment thus obtained was introduced into *B. subtilis* 168 cells by competent cell transformation, and mutants were selected on an LB agar plate containing Nm.

Table 3. Resistance of mutant strains to Nm

+, *araR* gene present; –, *araR* gene absent; S, sensitive to Nm; R, resistant to Nm.

Strain	<i>AraR</i>	Nm resistance	
		–Arabinose	+Arabinose
AR1	+	S	R
AR2	–	R	R
AR3	–	R	R
AR31	+	S	R
AR32	+	S	R
AR31d	–	R	R
AR32d	–	R	R

An Nm-resistant transformant was confirmed by PCR and designated AR3 (Fig. 2). Deletion of *araR* made it possible for the $P_{ara-neo}$ gene in the counter-selective marker cassette to be expressed, because of the absence of the AraR repressor. However, if the *araR* gene were reintroduced into the AR3 strain, we would expect the expression of $P_{ara-neo}$ to be repressed, as shown in Table 3. In other words, a selective marker cassette containing *araR* and an antibiotic-resistance gene would transform strain AR3 to be sensitive to Nm. Then, deletion of the selective marker cassette from the transformant could be positively selected for on the basis of resistance to Nm. Therefore it would be possible to introduce multiple mutations into strain AR3 by the repeated process of introduction (along with target gene sequences) and rescue of the selective marker cassette containing *araR*.

Markerless deletions of genomic regions from AR3

Strain AR3 was used as a starting strain for markerless deletions. First, we tried to delete the 3.8 kb region of *iolS-csbC*, as shown in Fig. 1. Approximately 1.0 kb of each of the flanking sequences upstream of *iolS* (UP) and downstream of *csbC* (DN) was amplified by PCR from the *B. subtilis* genome using the primers *iolfw* and *iol/csbCUr*, and *csbC-Df* and *csbCrv*, respectively. These primers were designed so that the downstream end of the UP fragment overlapped with the upstream end of the DN fragment by 20 bp. The UP and DN fragments were ligated by SOE-PCR (Ho *et al.*, 1989), using the primers *iolfw* and *csbCrv2*, to obtain a 2.0 kb fragment. The 2.1 kb selective marker cassette consisting of the *cat* and *araR* genes was constructed by PCR as described in Methods. A 1.0 kb fragment (labelled G in Fig. 1) from the upstream region of the *iolS* gene (the upstream end in the deletion target region) was amplified by PCR from the *B. subtilis* genome using the primers *iol/CRf* and *iolrv*. These three fragments of 2 kb, 2.1 kb (the selective marker cassette) and 1 kb, which overlapped one another by 20 bp, were then ligated by SOE-PCR (Ho *et al.*, 1989) using the primer set *iolfw2* and *iolrv2*. The resulting 5.1 kb PCR fragment was used to transform strain AR3 by the competent cell method (Anagnostopoulos & Spizizen, 1961). A transformed strain was obtained by selection for Cm resistance, and designated AR31 (Fig. 1). Introduction of the selective marker cassette together with the DN fragment into the genome immediately upstream of the deletion target was verified by PCR analysis (Fig. 3). Thus, in the genome of strain AR31, a DN fragment was located at each end of the region consisting of the selective marker cassette and the deletion target (Fig. 1). We verified that AR31 was sensitive to Nm as a consequence of the integration into the genome of the selective marker cassette containing *araR* (Table 3).

Strain AR31 was incubated in LB broth for 4 h at 37 °C to induce intra-genomic recombination at the two homolog-

Fig. 3. PCR confirmation of deletions in the mutant genomes. M, molecular mass markers, with fragment sizes of 12, 10, 8, 6, 4, 3, 2, 1.5, 1 and 0.5 kb from top to bottom. (a) Confirmation of AR31. Lanes: 1, amplification of UP-DN-*cat* (2.9 kb); 2, amplification of DN-*cat-araR* (3.0 kb); 3, amplification of *cat-araR-G* (3.0 kb); 4, amplification of DN-*cat-araR-G* (4.0 kb) (Fig. 1). (b) Confirmation of AR31d. Lanes 1–6 represent the amplification of the UP-DN (2.0 kb; Fig. 1) region in six colonies. (c) Confirmation of AR32. Lanes 1–4 show a similar analysis to that described for (a). (d) Confirmation of AR32d. Lanes 1–6 show the analysis of six colonies as described for (b).

ous DN fragments, and then plated onto LB agar plates containing Nm, after making appropriate dilutions. After incubation at 37 °C for 1 day, Nm-resistant colonies appeared with a frequency of 1.8×10^{-4} colonies per cell plated. This result coincided well with our other results for intra-genomic recombination efficiency (S. Liu and others, unpublished data). One hundred of the Nm-resistant colonies were replicated onto LB agar containing Cm, and 96 of these colonies were found to be Cm sensitive. It is likely that the selective marker cassette (*cat* and *araR*) was deleted from the genome in these cells. Furthermore, six of the Nm-resistant but Cm-sensitive recombinants were randomly selected for confirmation of the deletion by PCR, using the primers *iolfw* and *csbCrv*. As expected, a 2 kb PCR fragment was amplified in all six recombinants (Fig. 3b). This was the expected size of the fragment after the deletion of the *iolS-csbC* region. The *csbCrv* primer is

The 41.8 kb region between the *hutM* and *csbC* genes was also tested for deletion by the same method as that described above for the 3.8 kb *iolS*–*csbC* region. For the deletion of *iolS*–*csbC*, the selective marker cassette was inserted with the DN sequence upstream of *iolS*, which was at the 5' end of the deletion target. However, for the *hutM*–*csbC* deletion, the DN fragment and selective marker cassette were used to replace the *hutM* gene locus. This was the only difference between the two deletion approaches.

The 2060- and 2000-base DNA sequences at the junctions between the upstream and downstream regions of the

Fig. 4. Determination of the DNA sequences around the deletion targets of AR31d and AR32d. The partial sequences of UP-DN from AR31d and AR32d together with their upstream and downstream sequences of 60–70 bases are shown. (a) AR31d, the UP-DN and its upstream and downstream sequences of 60 and 70 bases, respectively, were determined. The location of the 3.8 kb deletion target is indicated. (b) AR32d, the UP-DN and its upstream and downstream sequences of 70 bases, respectively, were determined. The location of the 41.8 kb deletion target is indicated. The UP-DN sequences are shown in the boxes. The upstream sequences of 60–70 bases and the downstream sequences of 70 bases are shown outside the boxes. The primers used for the amplification of the PCR fragments for sequencing are underlined, and these were also used for the PCR confirmation in Fig. 3(b, d). The numbers above the sequences indicate the position in the whole *B. subtilis* genome (see <http://bacillus.genome.jp/>).

deletion targets in the AR31d and AR32d mutants were amplified by PCR using the primer sets iolfw and csbCrv, and hutMfw and csbCrv, respectively, and determined by DNA sequencing. The partial sequences were shown in Fig. 4. The 3.8 and 41.8 kb fragments between UP and DN were deleted as expected in AR31d and AR32d, respectively. The UP-DN fragments in AR31 and AR32 (Fig. 1) could not be amplified using the same primer sets as above under the PCR conditions for 2 kb fragment amplification, because the reverse primer of csbCrv was 70 bases downstream of the DN sequence. The results indicated that the target regions were deleted accurately at the expected sites.

The method described above has the following advantages: (1) both steps in the mutation strategy, including the introduction of the selective marker cassette and the excision of the target region along with the selective marker cassette, are positively selected for by antibiotic resistance (Cm and Nm resistance, respectively); (2) there is no heterologous DNA fragment left in the genome at the site of excision of the selective marker cassette; (3) it is possible to modify the genome repeatedly for an unlimited number of times. In this report, we tested the procedure using deletions of genetic loci. The same procedure can also be used for point mutations, substitutions, additions and other modifications of the *B. subtilis* genome. The deletion strategy described in this method has the important advantage that, due to the introduction of the downstream sequence (DN) from the deletion target (Fig. 1), it is possible for intra-genomic homologous recombination to occur, enabling the excision of the selective marker cassette. Our unpublished data indicate that regions of up to 50 kb can be excised by intra-genomic homologous recombination in the presence of homologous DNA sequences of at least 0.5 kb, with efficiencies of around 10^{-4} recombinants per cell plated. This recombination rate is much higher (about three to four orders of magnitude) than that of transformation with PCR fragments. If the DN fragment is not introduced together with the selective marker cassette, one more step of transformation with a PCR fragment must be conducted, in order to excise the selective marker cassette and the deletion target. In this case, even if the selective marker cassette is present, the Nm-resistant colonies occur with frequencies of 10^{-7} to 10^{-8} , similar to the transformation efficiencies of PCR fragments. It is apparent that the selection for excision of the selective marker cassette will be difficult after transformation using a PCR fragment containing only the UP and DN fragments. Moreover, the selection of unmarked deletion mutants is from LB cultures, in which the viable cell count will be over 10^8 cells ml⁻¹ after an incubation of 4 h. This implies that there will be 10^4 unmarked deletion mutants per millilitre in this LB culture. Thus, our results indicate that the method described above can be used simply, easily and precisely for multiple rounds of deletion mutagenesis of the *B. subtilis* genome, without any heterologous sequences remaining at the sites of mutagenesis.

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