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LETTER TO THE EDITOR

Targeted gene disruption by use of a group II intron (targetron) vector in *Clostridium acetobutylicum*

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Dear Editor:

Clostridium acetobutylicum, a gram-positive, anaerobic, spore-forming bacterium, is capable of using a wide variety of carbon sources to produce acetone, butanol and ethanol. To improve solvent productivity of *C. acetobutylicum*, metabolic engineering is considered as a useful tool in developing strains with industrially desirable characteristics. However, to date, there are few useful methods for genetic manipulation of *C. acetobutylicum*, especially for gene disruption. To our knowledge, two types of vectors, including non-replicative and replicative integrative plasmids, have been developed for gene-inactivation in *C. acetobutylicum*. By using non-replicative integrative plasmids, *buk* and *solR* genes of *C. acetobutylicum* were inactivated [1, 2]. However, due to their low frequencies of transformation and recombination, the non-replicative integrative plasmids are usually transformed at less than 1 integrative transformant per mg plasmid DNA. To obtain the integrative mutant, it may require higher transformation frequencies up to 10^5, but the typical transformation frequencies were reported at 10^3 [3]. Harris *et al.* described the construction of a replicative integrative plasmid pETSPO and its application in the disruption of gene *spo0A* which could not be inactivated by using the non-replicative integrative plasmid [4]. With the functional replication origin in *C. acetobutylicum*, pETSPO increases opportunity for homologous recombination, but it is still time-consuming to screen for double crossover integration. Therefore, a more efficient tool for targeted gene inactivation in the *C. acetobutylicum* is much needed.

Recently, a new strategy was developed to construct gene inactivation mutants by using group II intron-based Targetron technology. The mobile group II intron, originating from the *Lactococcus lactis* L1.LtrB intron, has been successfully used in a wide range of bacteria including *Clostridium perfringens* [5]. Without a proper replicon and/or promoter, the targetron plasmid pJIR750ai for *C. perfringens* from Sigma Aldrich was not applicable for gene disruption in the *C. acetobutylicum* directly (data not shown). Therefore, a modified targetron plasmid pSY6 was created by cloning the *L1.LtrB* group II intron fragment into the pIMP1-ptb, which was an *E. coli-C. acetobutylicum* shuttle vector containing a *ptb* (phosphotransbutyrylase) promoter [6].

The gene *buk*, encoding the butyrate kinase, catalyzes the production of butyrate, and the gene *solR* located on the megaplasmid of the strain, encodes a putative repressor of solvent formation genes [7, 8]. pSY6-buk and pSY6-solR vectors, constructed based on pSY6 (Figure 1A and Supplementary information, Figure S1), were electroporated into *C. acetobutylicum* ATCC 824, respectively. Then, the cells were incubated overnight to induce the intron invasion (See Supplementary information, Materials and Methods). The overnight cultures were spread onto CGM medium (25 μg/ml erythromycin) and the transformants were analyzed

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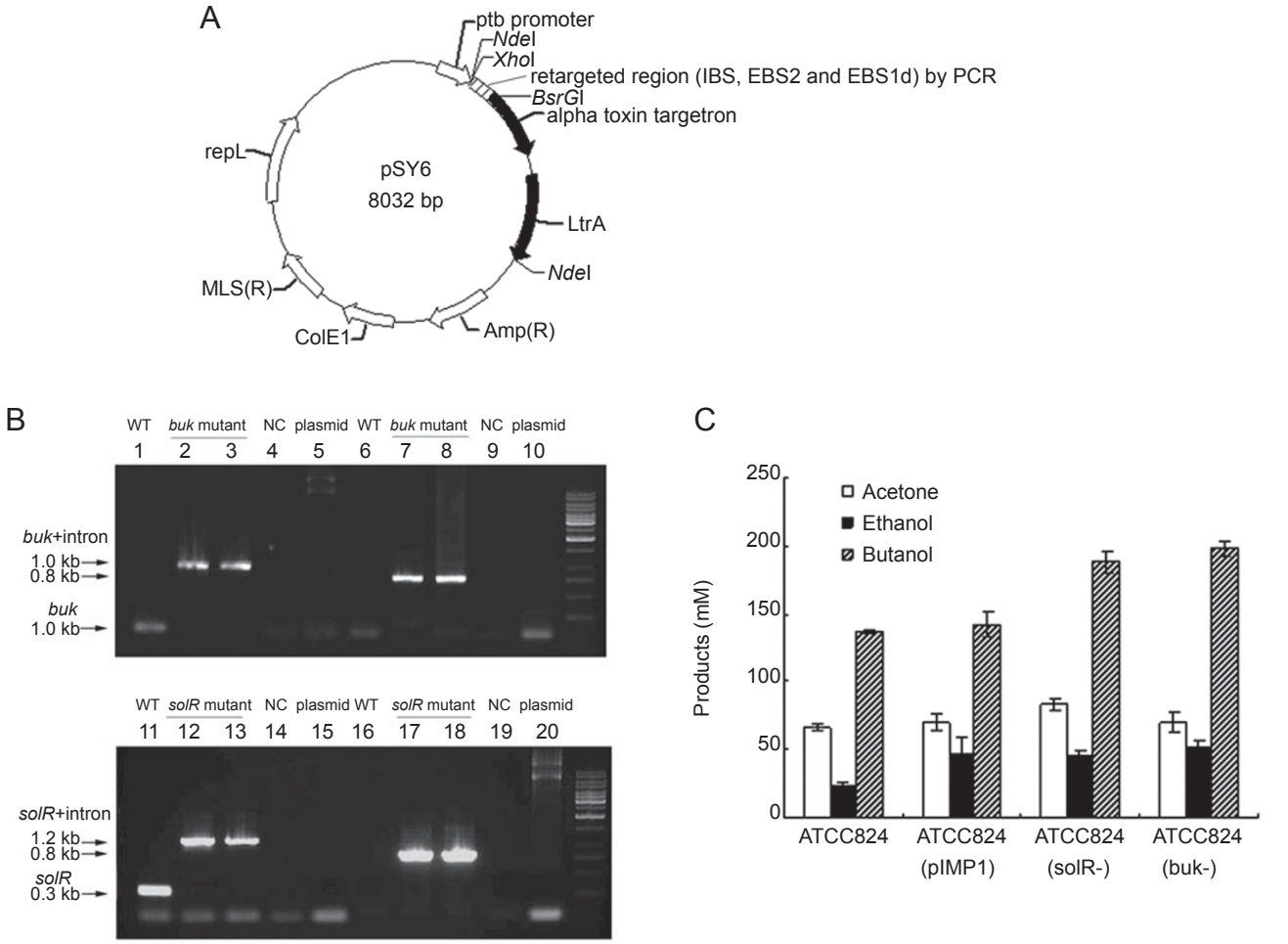

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Plasmid	DNA (μg)	Number of transformants	Insertion frequency (%)	Number of insertions/μg DNA	Reference
pJC4BK	15	13	-	<1	[1]
pDHSR	4	0	0	0	This study
pSY6-buk	1	770	62%	474	This study
pSY6-solR	1	450	25%	113	This study

Figure 1 (A) The targetron plasmid pSY6 for gene disruption in *C. acetobutylicum*. pSY6 is a derivative tagetron plasmid which was constructed by inserting the alpha toxin targetron and *ltrA* gene from pJIR750ai into the *E. coli-C. acetobutylicum* shuttle plasmid pIMP1-ptb and it can be generated into pSY6-buk and pSY6-solR by modifying the retargeted region(IBS, EBS2, and EBS1d). IBS: intron binding site; EBS2: exon binding site 2; EBS1d: exon binding site 1/δ; ColE1: ColE1 origin of replication; MLS(R): macrolide-lincosamide-streptogramin B resistance gene; Amp(R): ampicillin resistant; repL: the origin of replication from pIMP1. **(B)** Confirmation of *buk* and *solR* gene disruption in the genome of *C. acetobutyliucm* by PCR. WT: the genome of wild type *C. acetobuylicum* 824; NC: negative control; plasmid: the targetron plasmid pSY6-buk and pSY6-solR for *buk* and *solR* disruption, respectively; lanes 1-5, using primers (buk-for-s49/50 and buk-rev-s49/50) flanking the 49/50 site of gene *buk*; 6-10, using primers (buk-rev-s49/50 and buk49/50s-EBS2) to perform the junction PCR; lanes 11-15, using primers (solR-for-a468/469 and solR-rev-a468/469) flanking the 468/469 site of gene *solR*; 16-20, using primers (solR-for-a468/469 and solR-a468/469-EBS2) to perform the junction PCR. Arrows indicate the intron insertion in the mutant. The bands at the bottom of the gel appear as primer dimmers. **(C)** Comparison of the solvent production between the mutants and wild type strain. **(D)** Gene insertion efficiencies of non-replicative integrative plasmids DNA and targetron plasmids DNA.

using colony PCR (See Supplementary information, Figure S2). The PCR results showed that the intron insertion frequency was 62% for *buk* mutant and 25% for *solR* mutant, respectively. However, no transformant was obtained with the non-replicative integrative plasmid (Figure 1D). To further verify the targeted gene inactivation, DNA sequencing of the PCR products amplified from genomic DNA of mutants (Figure 1B) confirmed the presence of L1.LtrB intron integrated in the sense strand at the site between nt 49 and 50 of *buk* from its translation start codon and in the antisense strand at the site between nt 468 and 469 of *solR* from its translation start codon, respectively (See Supplementary information, Figures S3 and S4). Information on all bacterial strains, plasmids and PCR primers could be found in Supplementary information, Table S1.

Solvent production of the mutants was determined by fermentation analysis to identify the phenotype changes caused by the gene inactivation (See Supplementary information, Figure S5). As indicated in Figure 1C, *buk* mutant and *solR* mutant produced 44% and 37% more butanol than wild type, respectively. These results were consistent with the previously published data [7, 8].

Here, we reported the construction of targetron plasmid pSY6 and its successful application in gene-inactivation in *C. acetobutyliucm*, which provides a new genetic tool for gene disruption in this anaerobic organism. The group II intron can insert into defined sites at high efficiencies through a mechanism termed retrohoming independent of homologous recombination [5], which circumvented the low frequency of homologous recombination in *C. acetobutylicum*. The plasmid pSY6, containing the replicon from pIMP1, could be cured by repeated transferring in the fresh CGM medium without antibiotics [9]. Therefore, it is possible to construct multiple knock-outs consecutively in *C. acetobutylicum* because no selection marker will remain in the plasmid-cured mutant. As a conclusion, this new technology could improve the efficiency of genetic manipulation in *C. acetobutylicum* and has the potential to be widely applied in the metabolic engineering of *C. acetobutylicum*. During the revision of this manuscript, Heap *et al*. also adapted a mutagenesis system based on the mobile group II intron from the *ltrB* gene of *Lactococcus lactis* (Ll.ltrB) to function in clostridial hosts [10]

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(**Supplementary Information** is linked to the online version of the paper on the Cell Research website.)