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Short communication

# Markerless chromosomal gene deletion in *Clostridium beijerinckii* using CRISPR/Cas9 system

Yi Wang[a,b,1], Zhong-Tian Zhang[c], Seung-Oh Seo[a,b], Kijoong Choi[a], Ting Lu[b,d], Yong-Su Jin[a,b], Hans P. Blaschek[a,b,e,*]

[a] Department of Food Science and Human Nutrition, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA
[b] Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA
[c] Department of Chemistry, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA
[d] Department of Bioengineering, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA
[e] The Integrated Bioprocessing Research Laboratory (IBRL), University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

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ABSTRACT

The anaerobic spore-forming, gram-positive, solventogenic clostridia are notorious for being difficult to genetically engineer. Based on CRISPR/Cas9 assisted homologous recombination, we demonstrated that clean markerless gene deletion from the chromosome can be easily achieved with a high efficiency through a single-step transformation in *Clostridium beijerinckii* NCIMB 8052, one of the most prominent strains for acetone, butanol and ethanol (ABE) production. This highly efficient genome engineering system can be further explored for multiplex genome engineering purposes. The protocols and principles developed in this study provided valuable references for genome engineering in other microorganisms lacking developed genetic engineering tools.



Solventogenic clostridia are industrially significant microorganisms with great potentials for producing biofuels and biochemicals (Jones and Woods, 1986). Metabolic engineering based on designed genetic perturbations is a great strategy for studying the complex metabolism of solventogenic clostridia as well as constructing strains with desirable phenotypes. Various genetic engineering tools for the manipulation of solventogenic clostridia have recently been developed, however, many limitations still exist for efficient use of these tools. For example, the genetic tools based on mobile group II introns (ClosTron or TargeTron) have been employed by various research groups in many different strains (Heap et al., 2010; Jang et al., 2012; Wang et al., 2013). These technologies achieve disruption of genes by inserting an intron fragment at the chromosomal targeting site. One limitation of these methods, however, is that, their efficiency heavily relies on the precise prediction of insertion sites using a specific algorithm, and it can be very challenging to find an optimal target site when the interesting insertion sequence is short (for example <400 bp). In addition, the gene knockout achieved with this method is virtually not a true deletion of gene, but rather an interruption of the gene function, whose residue activity may be variable based on the insertion site and the feature of the gene of interest (Al-Hinai et al., 2012; Wang et al., 2013). Furthermore, it has been reported that intron insertions achieved through these methods, especially for the ones at the sense orientation, may be spliced back out by the intron-encoding protein (Frazier et al., 2003); thus a complete curing of the plasmid must be achieved after the desirable mutant is obtained. Recently, a counter selection marker-assisted genetic engineering system has been developed by Al-Hinai et al. (2012). Allelic-exchange-based chromosomal gene deletion and integration can be obtained with the assistance of the inducible counter selection marker *mazF*, a toxin gene from *Escherichia coli*. However, with this system, an antibiotic marker is still required to be integrated into chromosome for selection purposes. Although the marker may be 'flipped out' by the FLP recombinase expressed on an additional plasmid (which makes the whole transformation process laborious and complicated), a remarkable scar is still left behind. Moreover, the

* Corresponding author at: The Integrated Bioprocessing Research Laboratory (IBRL) University of Illinois at Urbana-Champaign, RM 34, 1207 W Gregory Drive, Urbana, IL 61801, USA. Tel.: +1 217 333 8224; fax: +1 217 244 2517.
*E-mail address:* blaschek@illinois.edu (H.P. Blaschek).
[1] Current address: 215 Tom E. Corley Building, Biosystems Engineering Department, Auburn University, Auburn, AL 36849, USA.

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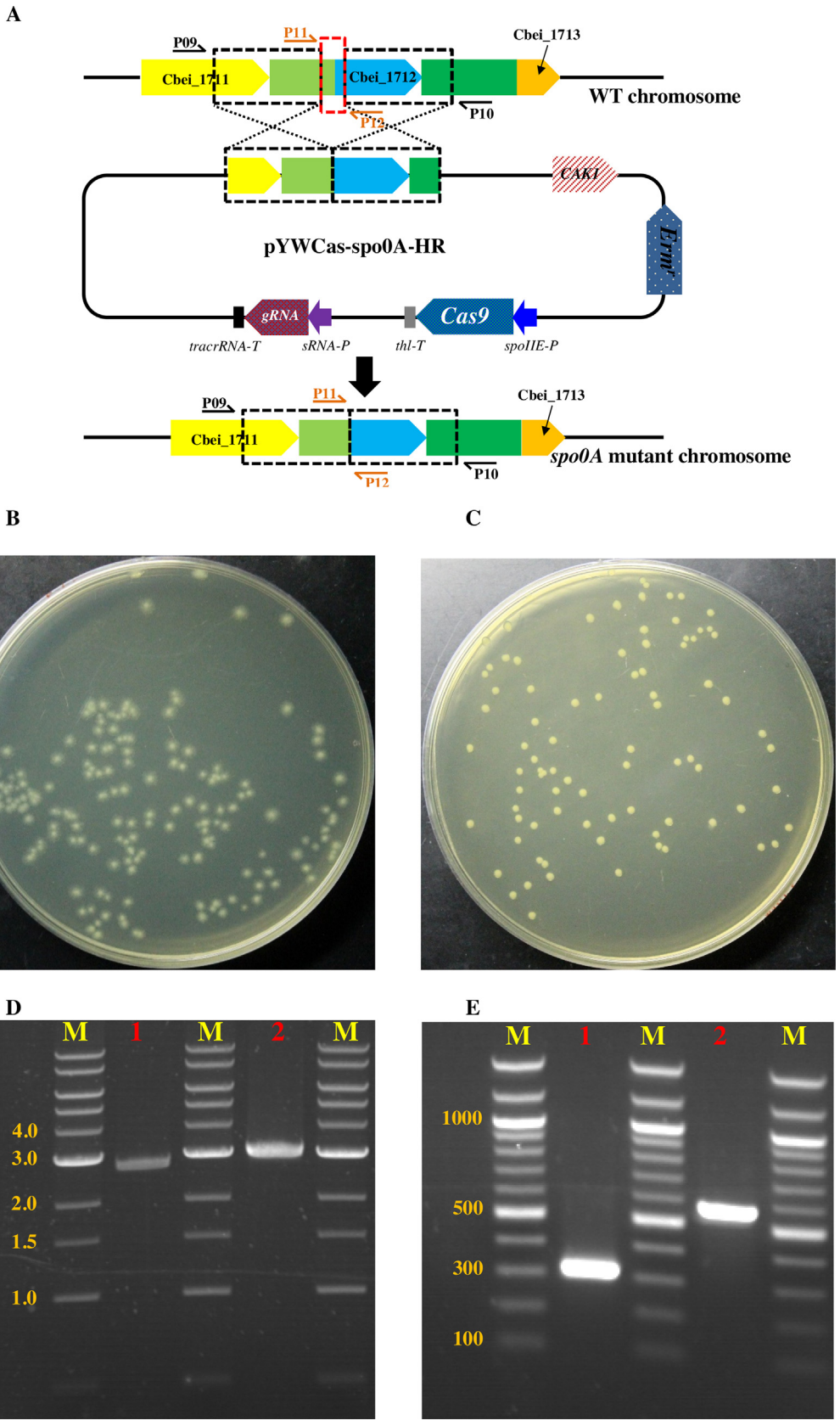


**Fig. 1.** Deletion of gene fragment from the chromosome of *Clostridium beijerinckii* using CRISPR/Cas9 system. (A) The customized CRISPR/Cas9 vector targeting on *spo0A* gene (Cbei_1712) in *C. beijerinckii* achieved a markerless gene fragment deletion via a homologous recombination event. The boxed regions (with black dash lines) show the approximate regions of homology (~1 kb each) incorporated in the CRISPR/Cas9 vector. The region covered by the red dash line box indicated the gene sequence that has been deleted. Putative deletion mutants were screened by two rounds of PCRs with the indicated primers. *spoIIE*-P: the promoter of *spoIIE* gene (Cbei_0097); Cas9: the *Streptococcus pyogenes* Cas9 ORF amplified from the plasmid pMJ806 (purchased from Addgene) (Jinek et al., 2012); *thl*-T: terminator of thiolase gene (Cbei_0411); *sRNA*-P: the promoter of the small RNA gene (sCbei_5830) (Chen et al., 2011); gRNA: the chimeric gRNA (Mali et al., 2013), with a 20-nt guiding sequence (5′-ATAATAAAAGAATGGACAAA-3′)