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Deletion of pyruvate decarboxylase gene in *Zymomonas mobilis* by recombineering through bacteriophage lambda red genes

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ABSTRACT

Zymomonas mobilis ZM4 is a gram negative ethanologenic bacterium used in several biotechnological applications. Metabolic engineering in this bacterium is limited because of the available genome engineering tools. In the present study, we report genome engineering in this bacterium using bacteriophage lambda Red genes. Stability of plasmid replicons RK2 (pSIM9) and pBBR1 (pSIM7) containing the lambda Red genes was found to be 78% and 74%, respectively. We demonstrate successful deletion of pyruvate decarboxylase gene by recombineering in *Z. mobilis*. The deletion was confirmed by PCR and by estimating the metabolites formed. Ethanol, which was the main product in wild type cells, was formed in almost negligible amount in the *pdc*-deleted mutant. The developed *Δpdc Z. mobilis* cells can be exploited for production of desired bioproducts by expression of suitable enzymes that can regenerate NAD^+.

1. Introduction

Zymomonas mobilis is a Gram negative, rod-shaped, ethanologenic bacterium in which glucose metabolism occurs by the Entner-Doudoroff pathway. It contains a number of desirable characteristics like high sugar uptake and ethanol production rates, relatively low biomass production and high ethanol tolerance, which make it an ideal platform for both metabolic engineering and commercial-scale production of desirable bio-products.

A number of genome engineering approaches in this bacterium have been used for metabolic engineering leading to the production of various compounds (He et al., 2014). For example, a *SacC* mutant was constructed *via* the insertional mutagenesis method to obtain higher yield of levan (Senthilkumar et al., 2004). In this study, the *sacC* gene was deleted by electroporation of the suicide plasmid and then plating after overnight growth allowing single and double cross-over events to happen. Recently, an engineered *Z. mobilis* strain was also constructed for isobutanol production *via* metabolic pathway engineering: 2-ketoisovalerate decarboxylase (*kivd*) gene and alcohol dehydrogenase (*adhA*) gene from *Lactococcus lactis* were introduced into *Z. mobilis* ZM4, which led to isobutanol accumulation (He et al., 2014).

Since *Z. mobilis* cannot utilize xylose as its substrate, it was incorporated together with two operons encoding xylose assimilation and pentose phosphate pathway enzymes such as xylose isomerase, xylulokinase, transketolase, and transaldolase (Zhang et al., 1995).

Thus, the methods that have been used so far are homologous recombination, insertional mutation, Flp recombinase based methods, fusion PCR based methods, and transposon mutagenesis (He et al., 2014). Cao et al. (2016) demonstrated curing of plasmids using CRISPR/Cas9 system. The *cas9* gene from *Streptococcus pyogenes* was cloned under the control of *pdc* promoter and expressed in *Z. mobilis*. The replicase gene of the plasmids native to *Z. mobilis* was disrupted by electroporating the *cas9* expressing plasmid and the sgRNA (Cao et al., 2016). The results suggest the possibility of genome engineering in *Z. mobilis* using CRISPR/Cas9 based system.

Recently, recombineering in *Z. mobilis* has been demonstrated using Enterobacteriophage RecET system (Wu et al., 2017). However, there are no reports on genome engineering in this bacterium using bacteriophage lambda Red genes. Recombineering is a rapid method for genome engineering in bacteria, in which bacteriophage recombination proteins assist direct targeting of genes in bacteria. Based on the homology of short stretch of nucleotides, the target sequence in bacterial genome is replaced by the desired sequence. The applicability of this method has been demonstrated in a number of gram negative bacteria such as *Vibrio cholerae* (Yamamoto et al., 2009) and *Pseudomonas* (Swingle et al., 2010). In *V. cholerae*, recombineering has been demonstrated using bacteriophage lambda Red enzymes, which is a preferred method due to several advantages. Spontaneous mutations can be avoided using this strategy by transient expression of red genes to circumvent undesired genetic alterations. Unlike Flp/*FRT* and Cre/*lox* recombination systems, recombineering does not require specific flanking sites for genetic modifications to occur. Further, it excludes the

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Table 1
Bacterial strains and Plasmids used in the present study.

Bacterial strain/Plasmid	Size	Genotype	Source/Reference
Bacterial strain			
Zymomonas mobilis ZM4 (MTCC accession no. 2427)			Microbial Type Culture Collection
Escherichia coli DH5α			Invitrogen
Plasmid			
pSIM5	6171 bp	pSC101 repAts, CmR	Datta et al., 2006
pSIM7	5676 bp	pBBR1, CmR	Datta et al., 2006
pSIM9	6435 bp	pRK2trfAts, CmR	Datta et al., 2006
pRFB110	6624 bp	KnR	Fekete and Chattoraj, 2005

chances of random integration as in case of transposon mutagenesis, since it is a highly targeted approach based on sequence homology. Moreover, a homologous region of as small as 40 bp is enough to undergo recombination events which makes it easier and less time-consuming method. However, there are no reports describing recombineering in *Zymomonas mobilis* using bacteriophage lambda Red system. A rapid method for genome engineering in this bacterium will be useful for various scientific and industrial applications such as increase in production of metabolites and microbial process development as mentioned by Doelle et al. (1993). Considering this, we have optimized the protocol and demonstrate recombineering in *Z. mobilis* using lambda Red genes in the present study.

2. Materials and methods

Bacterial strains and plasmids used in the study are listed in Table 1. *Zymomonas mobilis* was maintained in Rich Medium (RM) (glucose 20 g/l, yeast extract 10 g/l and KH_2PO_4 2 g/l). Primers used in the study are listed in Table 2.

2.1. Preparation of electrocompetent cells

For preparing electrocompetent cells, single colonies of *Z. mobilis* were inoculated in RM medium. Overnight grown culture (0.5 ml) was inoculated into 50 ml RM and incubated at 200 rpm, 30 °C until OD_{600} reached 0.4–0.5. The 50 ml culture was partitioned equally in Oak ridge tubes. The tubes were immediately transferred to ice and swirled to cool down followed by centrifugation at 6700 rpm, 4 °C for 7 min. The obtained pellets were re-suspended in 1 ml chilled autoclaved double distilled water. Further, 30 ml chilled autoclaved double distilled water was added and mixed gently. They were centrifuged again and finally re-suspended in 1 ml chilled double distilled water. To concentrate the cells, centrifugation was performed at 12,000 rpm, 4 °C for 30 s. About 25 µl glycerol (10%) was added and mixed. These 1000× concentrated competent cells were used for recombineering.

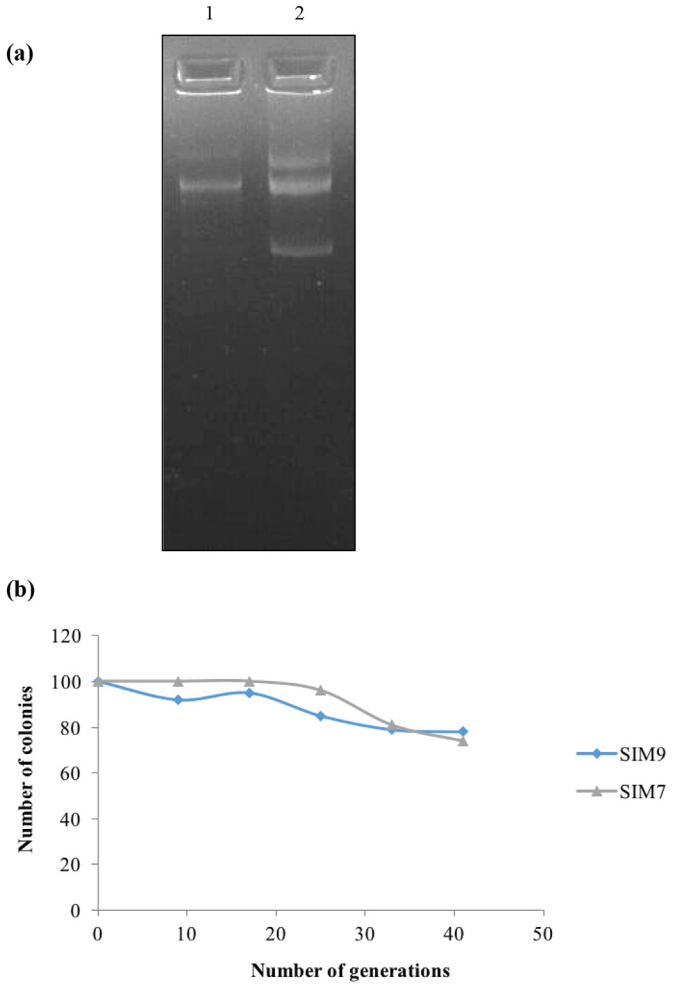

Fig. 2. (a) Plasmids pSIM7 and pSIM9 were checked on 1% agarose gel after transformation in *Z. mobilis* ZM4. Lane 1, pSIM7; lane 2, pSIM9. (b) Stability of pSIM7 and pSIM9 in *Z. mobilis*.

Table 2
Primers used in the present study.

Primer Name	Sequence (5′- 3′)
pdcRecF	ATGAGTTATACTGTCGGTACCTATTTAGCGGAGCGGCTTGTCCAGATTGGTCAAGCTTTTAAGGTTTAACGGTTGTGG (81 MER)
pdcRecR	CTAGAGGAGCTTGTTAACAGGCTTACGGCTGTTGGCGGCAGCAACGCGCTTACCAAGCTTGCATGCAGATTGCAGCATTACAC (86 MER)
pdcScreenR	TGCTTGTCCTCGATGTAATCAG
kanInternalF	ACCATGATATTCGGCAAGCAGGCATCGCCATG
pdcF	GACTCATATGATGAGTTATACTGTCGGTACCTATTTAGCGGAGCG
pdcR	GTCACTCGAGGAGGAGCTTGTTAACAGGCTTACGGCTGTTGGCGG

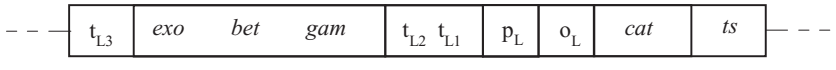

Fig. 1. Segment of lambda bacteriophage genome present in pSIM plasmids for recombineering. The Red genes exo, *bet* and *gam* are expressed under the control of lambda thermosensitive repressor (ts). t_L, transcription terminators; p_L, promoter; o_L, operator; *cat*, chloramphenicol resistance gene. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)