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Development of Cre-*lox* based multiple knockout system in *Deinococcus radiodurans* R1

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Abstract−The extremophilic bacterium *Deinococcus radiodurans* R1 has been considered as an attractive microorganism due to its remarkable tolerance to various external stresses. Considering the nature of *D. radiodurans* R1, it has potential as a platform microorganism for industrial applications, including biorefinery and bioremediation process. However, *D. radiodurans* R1 is well known for its hard genetic manipulation. Thus, much effort has been made to develop efficient genetic engineering tools for making *D. radiodurans* R1 suitable for industrial platform microorganism. Although a plasmid-based single gene knockout method has been reported, development of multiple gene knockout system has not yet been reported. Here we report, for the first time, Cre-*lox* based rapid and efficient multiple knockout method for metabolic engineering of *D. radiodurans* R1. Also, deletion of *dr0053* gene was successfully achieved within seven days to make biofilm overproducing strain.

Keywords: *Deinococcus radiodurans* R1, Cre-*lox*, Multiple Knockout Method, Genetic Engineering, Biofilm Production

INTRODUCTION

Metabolic engineering and synthetic biology have been receiving much attention as a platform technology to make microorganism means of solution for global issues such as energy and environmental problems [1-4]. Even though *Escherichia coli* has been employed as a representative model microorganism for industrial applications, it has critical weakness for industrial applications in terms of relatively low resistance to chemicals and compounds desired to produce, extreme conditions such as high cell density and toxic compounds, and various external stress conditions. Thus, extensive metabolic engineering and synthetic biology approaches have been used to increase tolerance to extreme conditions [5,6]. For this reason, an extremophilic bacterium, *Deinococcus radiodurans* R1 has received much attention due to extraordinary resistance to extreme stress conditions. *D. radiodurans* R1 equips powerful enzymatic and non-enzymatic system to overcome oxidative stress which could induce severe damage to protein and DNA [5,6]. Moreover, they can efficiently fix shattered chromosomal DNA caused by ionizing radiation (IR) in a few hours [9]. Thus, *D. radiodurans* R1 has been considered as one of the most promising microorganisms for application of bioremediation of radioactive contaminated environments [10,11]. Previously, genetic manipulation tools for *D. radiodurans* R1 had been developed based on non-replicable plasmids containing antibiotic resistance gene flanked by homology to chromosomal DNA [10]. Although this method was widely used in study of *D. radiodurans* R1, it has several limitations on intensive engineering of *D. radiodurans* R1 for industrial application. First, as it was developed based on non-replicable plasmids, repetitive cloning steps and time-consuming processes should be required. Second, it is hardly possible to make multiple knockout strain, because of the absence of versatile tools for rescue antibiotic resistance gene in the genome. Therefore, development of a rapid and efficient multiple knockout method for engineering of *D. radiodurans* R1 is urgently needed. To achieve this goal, the Cre-*lox* system is a promising candidate. Cre recombinase recognizes *loxP* sites and catalyzes site-specific recombination between two *loxP* sites, resulting in the removal of integrated DNA sequences flanked by *loxP* sites [12,13].

Herein, we report the development of a simple and efficient multiple knockout method through Cre-*lox*-based one-step gene inactivation method for engineering of *D. radiodurans* R1 strain. This method allowed us to make a multiple knockout strain more efficiently than currently used nonreplicable plasmids based method. Thus, a wide range of industrial application of *D. radiodurans* R1 followed by massive metabolic engineering has become available.

MATERIALS AND METHODS

1. Bacterial Strains, Plasmids, and Culture Condition

All strains and plasmids used in this study are listed in Table 1. *D. radiodurans* R1 strains were grown in TGY broth (0.5% Tryptone, 0.1% glucose, and 0.3% yeast extract) or on TGY agar plates.

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Table 1. Strains and plasmids used in this study

	Description	Reference
Strains		
D. radiodurans		
R1	wild type (ATCC13939)	ATCC
DrAM1	Δ*dr0053*::*lox66-km*^{r}*-lox71*	This study
DrAM2	Δ*dr0053*::*lox72*, pAM2	This study
DrAM3	Δ*dr0053*::*lox72*	This study
E. coli		
DH5α	Host for plasmid propagation	Lab stock
Plasmids		
pKatAPH3	Kanamycin-resistance gene cassette vector, Km^r	14
pKatCAT5	Chloramphenicol-resistance gene cassette vector, Cm^r	17
pRADZ3	*E. coli-D. radiodurans* shuttle vecter containing the *groES* promotor of *D. radiodurans*, Ap^r, Cm^r	15
p13840	Thermosensitive plasmid for replication in *D. radiodurans*, Spc^r	16
pAM1	A derivative of pKatAPH3 containing *lox66-km*^{r}*-lox71* cassette	This study
pAM2	A derivative of p13840 containing P_{GroES}-*cre*-P_{GroES}-*cm*^{r}	This study

E. coli DH5α strain was used as cloning host and was grown at 37 °C in LB medium (1% tryptone, 0.5% yeast extract, and 1% NaCl). Appropriate antibiotics were added to the culture media as following concentration: 25 μg/ml of kanamycin and 3 μg/ml of chloramphenicol for *D. radiodurans* R1, and ampicillin (100 μg/ml), spectinomycin (40 μg/ml), kanamycin (50 μg/ml) and chloramphenicol (30 μg/ml) for *E. coli*.

2. Construction of the Plasmid

All primers used in this study are listed in Table 2. To construct pAM1 plasmid, 960 bp of the kanamycin resistance gene with two *loxP* sequences was amplified by PCR using Lox66F and Lox71R primers from pKatAPH3 [14] as a template. The amplified DNA fragments were digested with *Eco*RV-*Bam*HI and ligated with *Eco*RV-*Bam*HI digested pKatAPH3 (Fig. 1(a)).

To construct plasmid pAM2, approximately 1 kb of *cre* gene was amplified by PCR using CreF1 and CreR1 primers from artificially synthesized DNA fragment of CRE (Bioneer Corp., Daejeon, Republic of Korea; NCBI accession number: ab449974) as a template. The amplified DNA fragment was digested with *Spe*I and *Not*I and ligated with *Spe*I-*Not*I digested pRADZ3 [15] plasmid. A hybrid DNA fragment containing *groES* promoter of pRADZ3 and *cre* gene was amplified by PCR using CreF2 and CreR2 primer pairs and cloned into p13840 [16] at *Nde*I and *Xho*I restriction sites. The CmF and CmR primers containing *Xho*I restriction site were used for amplification of chloramphenicol resistance gene (*cm*^{r}) from pKatCAT5 plasmid as a template [17]; then the resulting PCR fragments were cloned behind the *cre* gene of p13840 to generate pAM2 (Fig. 1(b)). All plasmids constructed in this study were confirmed by PCR and nucleotide sequencing analysis.

Table 2. Primers used in this study

Primer	Sequence (5′ to 3′)[a]
Lox66F	gcttgatatctaccgttcgtatagcatacattatacgaagttat
Lox71R	tagaggatcctaccgttcgtataatgtatgctatacgaagttat
CreF1	gatcactagtatgtccaatttactgcccgta
CreR1	gatcgcggccgcctaatcgccatcttccagca
CreF2	cgtggcggccgctcggcttggaagcacgtatt
CreR2	gatcctcgagctaatcgccatcttccagca
CmF	catcctcgaggtcgacggtatcgataagct
CmR	gcgactcgaggtcgactctagaggatcctc
DR53-1	tgcctcctcgccggtaaaca
DR53-2	agcttatcgataccgtcgacttccttttcctcctgatgga
DR53-3	tccatcaggaggaaaaggaagtcgacggtatcgataagct
DR53-4	gacttgcaaagaaggcacgccatgcctgcaggtcgactct
DR53-5	agagtcgacctgcaggcatggcgtgccttctttgcaagtc
DR53-6	tattgcggccgctcatttcccctgcatcaacttgga
DR53-7	cacgaatggcgtgcagcgt

[a]Underlining indicates restriction enzyme sites

3. Gene Knockout

The overall procedure for the development of knockout mutant is described in Fig. 2. To demonstrate the method developed here, approximately 1 kb of both upstream and downstream nucleotide sequence of the *dr0053* gene were amplified by PCR from the genomic DNA of *D. radiodurans* R1 using the DR53-1/2 and DR53-5/6 primers, respectively. The kanamycin resistance cassette containing two *loxP* sequences was obtained by pAM1 plasmid using DR53-3/4 primers. All three PCR products were purified by fragment DNA purification kit (Intron biotechnology) and then used as template for fusion PCR. The linear DNA fragment for deletion of the *dr0053* gene was finally obtained by fusion PCR using DR53-1/6 primers. The resulting PCR fragments were transformed into *D. radiodurans* R1 to trigger the homologous recombination event using a typical method as previously described [18] and incubated at 30 °C on 2X TGY agar plate supplemented with 25 μg/ml of kanamycin. The recombinants were verified by colony PCR using DR53-1/7 primers followed by DNA sequencing analysis and designated DrAM1 strain.

To excise the kanamycin resistance gene at the mutation locus, pAM2 was transformed into the DrAM1 strain and incubated at 30 °C on 2X TGY agar plates supplemented with 3 μg/ml of chlor-

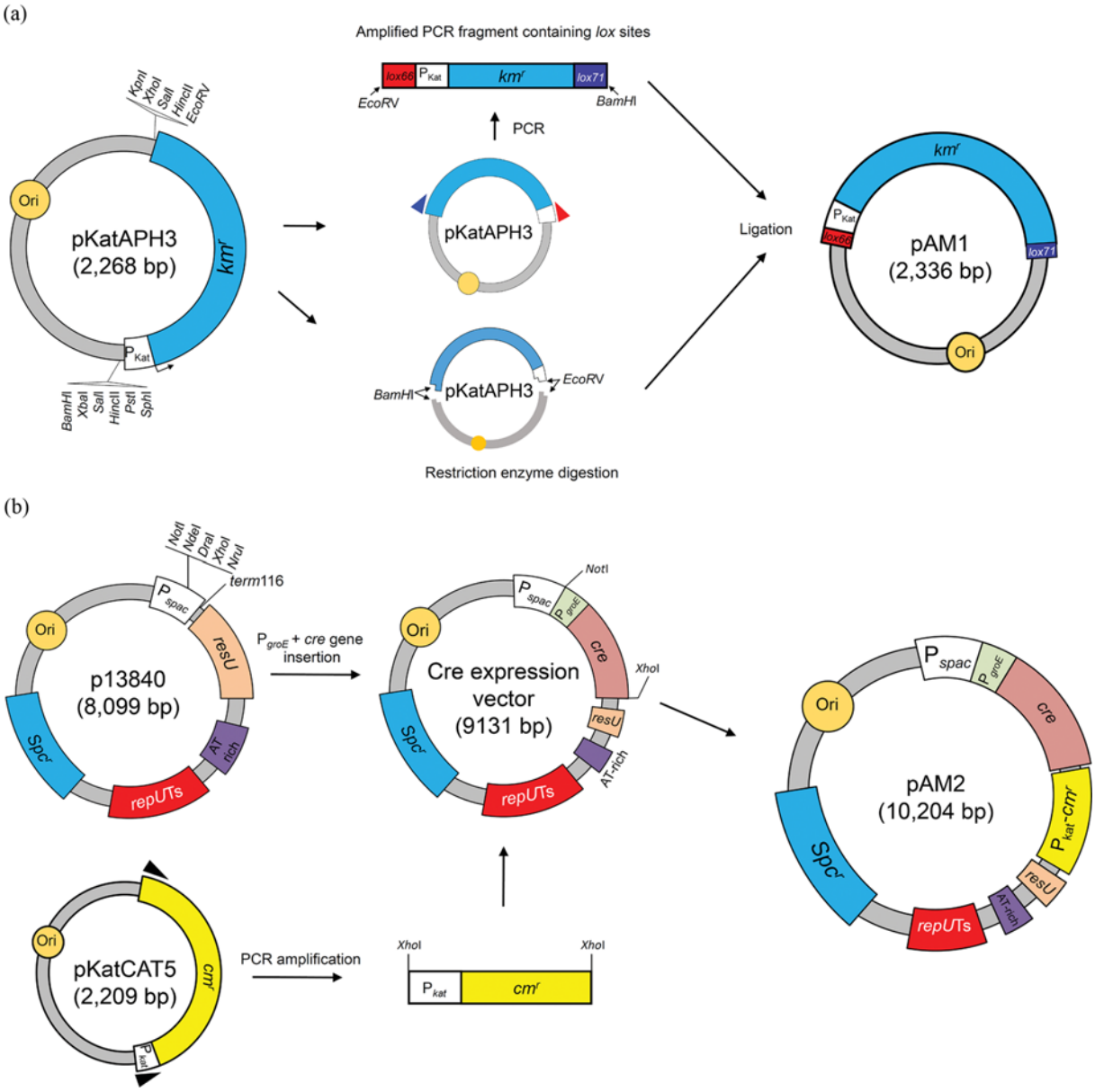

Fig. 1. Schematic representation of the construction of pAM1 (a) and pAM2 (b) vectors. The colored triangle indicates primers for amplification of their corresponding DNA sequences. P_{groES}; *groES* promoter, spc^r; spectinomycin resistance gene, cm^r; chloramphenicol resistance gene, P_{kat}; catalase promoter.

amphenicol. After that, removal of kanamycin resistance gene in the genome was screened at both TGY agar plate containing kanamycin and chloramphenicol. Diagnostic PCR was carried out using DR53-1/7 primer pairs to confirm correct Cre-mediated recombination at the target loci and resulting strain was designated as DrAM2 strain. To make markerless final engineered DrAM3 strain, DrAM2 strain was cultured at antibiotics-free 2X TGY media at 37 °C for 24 h and removal of pAM2 was confirmed at TGY agar plate containing corresponding antibiotics.

4. Biofilm Assay

Biofilm formation was analyzed by 96-well microtiter dishes made of polycinylchloride plastic [19]. The 1 : 100 diluted culture broth of final engineered *D. radiodurans* R1 strain was inoculated in 96 well plate. After inoculation, plates were incubated at 30 °C for two days, then the planktonic cells were removed and the attached cells were washed three times with sterile phosphate-buffered saline (PBS). To measure the amount of biofilm, 200 µl of 0.2% aqueous crystal violet staining solution was added to each well, and the plates were incubated at room temperature for 15 min. The cells were subsequently washed twice with sterilized PBS to wash off the excess crystal violet staining solution. The crystal violet bound to the biofilm was extracted with 200 µl of ethyl alcohol and acetone mixture solution [4 : 1 (vol/vol)] of ethyl alcohol and acetone, and the absorbance of the extracted crystal violet was measured at 595 nm with a vitor X3 (Perkin Elmer, Inc., USA) automatic microplate reader. All biofilm assays were performed in triplicate, with

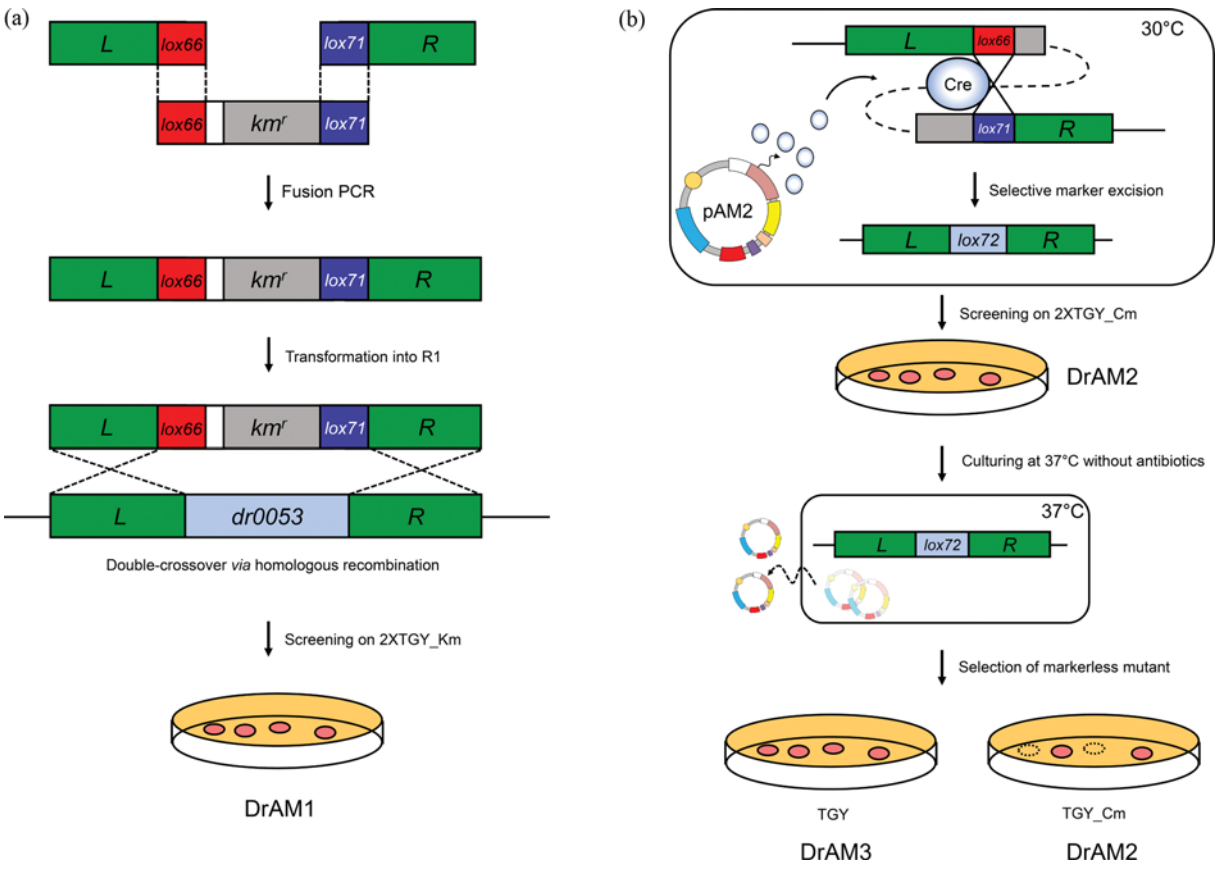

Fig. 2. Schematic representation of Cre-*lox* based knockout system. (a) The procedure of one-step inactivation of the *dr0053* gene by homologous recombination. L, left arm; R, right arm. (b) The excising of antibiotic marker by Cre recombinase and curing of pAM2.

five individual replicates for each strain per assay.

5. Confocal Laser Scanning Microscopy (CLSM)

Biofilm formation was imaged by using confocal laser scanning microscopy (CLSM) as described previous study [20]. Briefly, 0.2 ml of overnight cultured *D. radiodurans* R1 was inoculated in 1.5 ml of TGY broth and incubated for three days at 30 °C. The chamber slides were gently washed three times with PBS to remove loosely attached cells and treated with LIVE/DEAD solution (bacterial viability kit; Molecular proves, Leoden, Netherlands) for 15 min, then the slide was thoroughly washed with PBS. A thin cover slide was mounted over the stained biofilm and imaged by CLSM (Carl Zeiss LSM 800, Germany) equipped with DM IRE 2-inverted microscope (Leica Microsystems, Hessen Wetzlar, Germany).

RESULTS AND DISCUSSION

1. Strategy for Knockout of Specific Gene Using pAM1 and pAM2

To develop efficient and rapid multiple knockout method of *D. radiodurans* R1 strain, the Cre-*lox*-based homologous recombination system, which is currently used for the engineering of microorganisms, was used as a template (Fig. 1). The linear DNA fragment composed of kanamycin resistance gene linked with two *lox* sequences for gene inactivation was generated from pAM1. The pAM2, a derivative of p13840 which contains a temperature sensitive RepU, was constructed for the expression of Cre recombinase to excise integrated selective marker at target locus (Fig. 1(b)). The pAM2 was further modified by replacing P_{spac} promoter to *groES* promoter to allow constitutive expression of Cre recombinase.

The strategy for inactivation of specific gene in *D. radiodurans* R1 consisted of three steps (Fig. 2). First, the linear DNA fragment containing kanamycin resistance gene connected with *lox66* and *lox71* sequences and flanking region of target gene was obtained by fusion PCR and directly transformed to competent *D. radiodurans* R1 cells by standard protocol [21], then positive recombinants were screened by colony PCR analysis. Second, the resistance marker was eliminated by introduction of pAM2 harboring Cre recombinase and screened at TGY agar plate containing chloramphenicol. Finally, pAM2 was cured by increasing temperature up to 37 °C.

2. Construction of DrAM3 Strain (*D. radiodurans* R1 Δ*0053*)

To validate the performance of this method, the *dr0053* gene encoding a DinB-like protein, which is involved in extracellular polysaccharide (EPS) biosynthesis in *D. radiodurans* R1, was selected as an engineering target [7,18]. For the construction of Δ*dr0053* strain, linear DNA fragments containing two flanking regions of the *dr0053* gene, and kanamycin resistance cassette with two *loxP* sequences were constructed and directly transformed to *D. radiodurans* R1 strain. Among colonies grown in kanamycin containing TGY agar plate, eight colonies were subjected to diagnostic PCR and DNA sequencing analysis to find *dr0053::km^r* strain, named

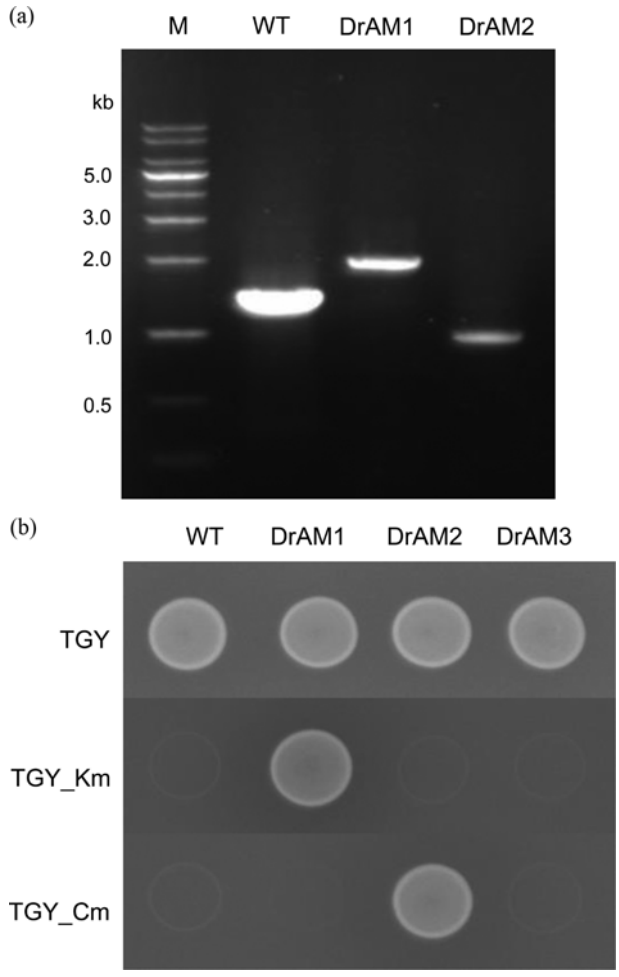

Fig. 3. Confirmation of DrAM strains by gel electrophoresis analysis (a) and spotting assay (b). M, DNA marker; TGY_Km, TGY agar plate containing 25 μg/ml of kanamycin, TGY_Cm; TGY agar plate containing 3 μg/ml of chloramphenicol.

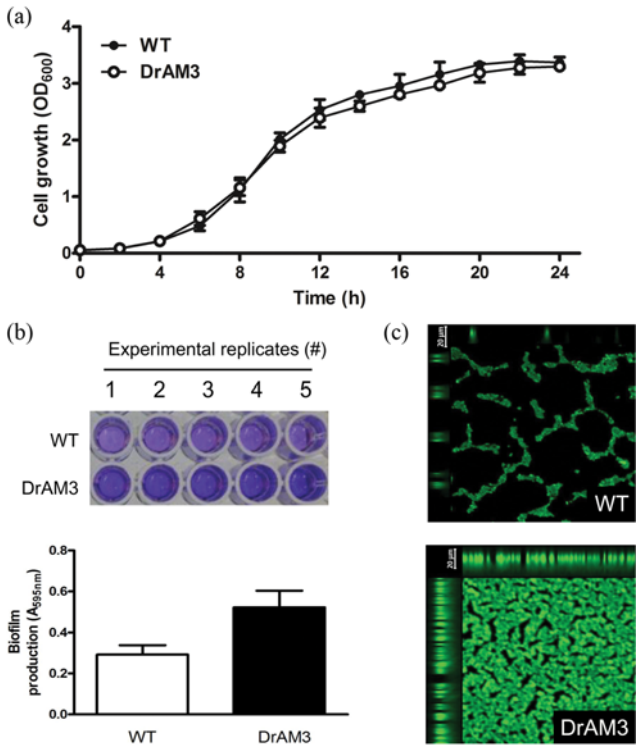

Fig. 4. The comparison of biofilm production in wild-type and DrAM3 strains. (a) Microtiter plate assay. Crystal violet staining of five replicates in wild-type and DrAM3 strains (upper panel). Quantification were determined by the absorbance at 595 nm (lower panel). The quantification data represent the means±standard deviations of three independent experiments, each performed in five technical replicates is shown. (b) CSLM analysis of wild-type and DrAM3 strains. Images of biofilms stained with SYTO9 are shown as digital CLSM images. The thickness of biofilm in each strain is shown at top and left edges (X-Z and Y-Z planes) with scale bar. The data shown are representative microphotographs of two independent experiments.

DrAM1 strain (Fig. 3(a)).

To excise the kanamycin resistance gene flanked by the *loxP* sequence at the targeted locus, pAM2 was introduced to the DrAM1 strain and incubated at 30 °C. Forty colonies grown on chloramphenicol containing TGY agar plate were randomly selected and analyzed by diagnostic PCR followed by DNA sequencing analysis to obtain kanamycin free colony, named DrAM2 strain (Fig. 3(a)) [22]. Then, the curing of pAM2 from DrAM2 strain could be easily achieved by increasing culture temperature up to 37 °C, a temperature not permissive for pAM2 replication. Then, after 24 h DrAM2 was dropped onto three different TGY agar plates containing their corresponding antibiotics to confirm the removal of resistance markers (Fig. 3(b)). The final engineered strain, named DrAM3 strain, was successfully constructed within seven days.

3. Enhanced Production of Biofilm in DrAM3 Strain

Biofilm is an extracellular polymeric substance used in various industrial applications such as water and wastewater treatment and remediation of contaminated environment [23]. There have been a few reports on biofilm formation in *Deinococcus*. *Deinococcus geothermalis* strain, which was isolated on the steel of paper machine, has been known to have ability to synthesize large amount of biofilms [24]. Another study of comparative genomic analysis of *D. radiodurans* R1 also suggested that DinB-like protein encoded by the *dr0053* gene might be also involved in biofilm formation in *D. radiodurans* R1 [7,18]. However, the biochemical study of the *dr0053* gene has not yet been reported. To investigate the role of the *dr0053* gene on biofilm formation, the biofilm produced by wild type and DrAM3 strain was compared by microtiter plate assay. As shown in Fig. 4(a), (b), more than two-fold increased biofilm (dark purple color) was produced in DrAM3 strain compared to wild type strain (light purple color) without growth retardation in individually replicative tests. And to evaluate adherence and thickness, the biofilm produced from both wild-type and DrAM3 strains was further examined using confocal laser scanning microscope (CLSM). As shown in Fig. 4(c), patchy and thick biofilm with the depth of 30±1.5 μm was observed in DrAM3 strain compared to that of wild-type with the depth of 14±2.6 μm.

As mentioned, *D. radiodurans* R1 has great potential as platform microorganism for the bio-based industrial applications due

to the unique properties of its nature such as extraordinary resistance to various extreme stress condition, high content of valued cellular metabolites, and utilization of lignocellulosic biomass [25-27]. Due to the lack of rapid and efficient genetic manipulation tool, which is essential for intensive metabolic engineering approaches, *D. radiodurans* R1 has not been widely used in bioprocess. Thus, we report the Cre-*lox*-based one-step inactivation method for multiple knockout of chromosomal genes in *D. radiodurans* R1. This method will provide efficient and practical ways to overcome various hurdles to make *D. radiodurans* R1 as a platform microorganism for industrial application.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

1. R. R. Kumar and S. Prassad, *Indian J. Microbiol.*, **51**, 403 (2011).
2. S. Singh, S. H. Kang, A. Mulchandani and W. Chen, *Curr. Opin. Biotechnol.*, **19**, 437 (2008).
3. J. Esther, L. B. Sukla, N. Pradhan and S. Panda, *Korean J. Chem. Eng.*, **31**, 1 (2015).
4. C. Roh, C. Kang and J. R. Lloyd, *Korean J. Chem. Eng.*, **32**, 1720 (2015).
5. G. Stephanopoulos, *ACS Synth. Biol.*, **1**, 514 (2012).
6. V. G. Vikramaditya, M. D. Mey, C. G. Lim, P. K. Ajikumar and G. Stephanopoulos, *Metab. Eng.*, **14**, 233 (2012).
7. K. S. Makarova, L. Aravind, Y. I. Wolf, R. L. Tatusov, K. W. Minton, E. V. Koonin and M. J. Daly, *Microbiol. Mol. Biol. Rev.*, **65**, 44 (2001).
8. R. Shashidhar, S. A. Kumar, H. S. Misra and J. R. Bandekar, *Can. J. Microbiol.*, **56**, 195 (2010).
9. J. K. Grimsley, C. I. Masters, E. P. Clark and K. W. Minton, *Int. J. Radiat. Biol.*, **60**, 613 (1991).
10. H. Brim, S. C. McFarlan, J. K. Fredrickson, K. W. Minton, M. Zhai, L. P. Wackett and M. J. Daly, *Nat. Biotechnol.*, **18**, 85 (2000).
11. H. Brim, J. P. Osborne, H. M. Kostandarithes, J. K. Fredrickson, L. P. Wacklett and M. J. Daly, *Microbiology*, **152**, 2469 (2006).
12. K. Abremski, R. Hoess and N. Sternberg, *Cell*, **32**, 1301 (1983).
13. R. Hoess and K. Abremski, *J. Mol. Biol.*, **181**, 351 (1985).
14. H. Ohba, K. Satoh, T. Yanagisawa and I. Narumi, *Gene*, **363**, 133 (2005).
15. R. Meima and M. E. Lidstrom, *Appl. Environ. Microbiol.*, **66**, 3856 (2000).
16. H. H. Nguyen, C. B. de la Tour, M. Toueille, F. Vannier, S. Sommer and P. Servant, *Mol. Microbiol.*, **73**, 240 (2009).
17. K. Satoh, Z. Tu, H. Ohba and I. Narumi, *Plasmid*, **62**, 1 (2009).
18. D. Appukuttan, H. S. Seo, S. W. Jeong, S. H. Im, M. H. Joe, D. S. Song, J. J. Choi and S. Y. Lim, *PLoS One*, **10**, e0118275 (2015).
19. G. A. O'Toole, L. A. Pratt, P. I. Watnick, D. K. Newman, V. B. Weaver and R. G. Kolter, *Method Enzymol.*, **310**, 91 (1999).
20. B. R. Boles and A. R. Horswill, *PLoS Pathog.*, **4**, e1000052 (2008).
21. B. E. Moseley and J. K. Setlow, *Proc. Natl. Acad. Sci. U.S.A.*, **61**, 176 (1968).
22. J. M. Lambert, R. S. Bonger and M. Kleerebezem, *Appl. Environ. Microbiol.*, **73**, 1126 (2007).
23. R. Dixit, Wasiullah, D. Malaviya, K. Pandiyan, U. B. Singh, A. Sahu, R. Shukla, B. P. Singh, J. P. Rai, P. K. Sharma, H. Lade and D. Paul, *Sustainability,*, **7**, 2189 (2015).
24. M. Kolari, J. Nuutinen and M. S. Salkinoja-Salonen, *J. Ind. Microbiol. Biotechnol.*, **27**, 343 (2001).
25. D. Slade and M. Radman, *Microbiol. Mol. Biol. Rev.*, **75**, 133 (2011).
26. L. Lemee, E. Peuchant, M. Clerc, M. Brunner and H. Pfander, *Tetrahedron*, **53**, 919 (1997).
27. E. Gerber, R. Bernard, S. Castang, N. Chabot, F. Coze, A. Dreux-Zigha, E. Hauser, P. Hivin, P. Joseph, C. Lazarelli, G. Letellier, J. Olive and J.-P. Leonetti, *J. Appl. Microbiol.*, **119**, 1 (2015).