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An Efficient Electro-Competent Cells Generation Method of *Xanthomonas campestris pv. campestris*: Its Application for Plasmid Transformation and Gene Replacement

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Abstract

A simple and rapid method to prepare efficient electro-competent cells of *Xanthomonas campestris pv. campestris* was generated, with up to 100-fold transformation efficiencies over the existing procedures. The overnight cultures were treated with sucrose solution and micro-centrifuged at room temperature; the entire electro-competent cells generation process can be completed in 15 minutes. It overcomes the complication and time-consuming shortcomings of the traditional conjugation or electro-transformation methods in this strain. Both the replicative plasmids and non-replicative plasmids could be transformed or integrated efficiently using this method. And the DNA concentration, cells growth stage, field strength and recovery time all had influences on the transformation efficiency. In the optimal conditions, the transformation efficiency for the replicative plasmids was 10^9 transformants per microgram DNA, and for non-replicative plasmids was 150 transformants per microgram DNA. Further with the homology sequences, two chromosomal target genes were deleted efficiently and the knockout strains were obtained easily.

Keywords

***Xanthomonas*, Electro-Competent Cells, Electro-Transformation, Gene Replacement, Integration**

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1. Introduction

Xanthomonas campestris is a gram-negative, pathogenic bacterium belonging to the γ-subdivision of Proteobacteria. The variant *X. campestris pv. campestris* (*Xcc*) generally invades and multiplies in cruciferous plant vascular tissues, resulting in the characteristic "black rot" symptoms of blackened veins and V-shaped necrotic lesions at the foliar margin [1]. It can invade into plant tissues through hydathodes, stomates, roots, or wounds, and infect a wide range of plants in the crucifer family (*Brassicaceae*), including broccoli, cabbage, cauliflower, radish, and the model plant *Arabidopsis thaliana*. Thus, this bacterium is considered as one of the important plant pathogenic bacteria [1]. Developing novel genetic tools for DNA transformation, gene replacement and chromosome modification in the bacterium can be helpful for the understanding its pathogenic mechanism and improving its preventive treatment.

As *Xanthomonas* species can secret xanthan gum, extracellular polysaccharide (EPS) and many extracellular enzymes, it is not sensitive to chemical treatments that induce cell competence necessary for transformation [2] [3]. Therefore, electroporation is widely used for plasmids DNA transfer in *Xanthomonas* [4] [5]. Although electro-transformation can work, its efficiency is much lower than that in *E. coli*, which may relate to the difficulty in removing the heavy out-membrane secretion protein and polysaccharide in the competent cells preparation steps. On the other hand, the gene replacement in *Xanthomonas* is always achieved through conjugation with certain *E. coli* strain to perform the non-replicative plasmids integration and homologous recombination [6] [7]. The process is complicated, time-consuming and cumbersome, especially for multiple genes replacement.

To simplify the DNA transfer and gene replacement procedures in *Xanthomonas*, we construct a fast method to generate high competent cells. Combining with the optimized electro-transformation conditions, the replicative plasmids DNA and the non-replicative plasmids DNA could be efficiently transformed into *Xcc* 8004; further with this method, two chromosomal genes were deleted and two knockout mutant strains were generated successfully.

2. Materials and Methods

2.1. Strains, Plasmids, Enzymes and Chemicals

All strains and plasmids used in this work were listed in **Table 1**. Luria-Bertani (LB) medium was used for strains culture. *E. coli* strains were grown at 37˚C and *Xcc* 8004 strain was grown at 28˚C. For the transformants selection, 10 μg/ml tetracycline (Tc), 10 μg/ml gentamycin (Gm), 10 μg/ml kanamycin (Kan) and 10 μg/ml rifampicin (Rif) were used. *KOD plus* DNA polymerase was obtained from Toyobo Co., Ltd. (Japan); the restriction enzymes, T4 DNA ligase and other enzymes were all purchased from Thermo Fisher Scientific Inc. (USA). All other reagents and chemicals were of analytical grade.

2.2. DNA Manipulation and Plasmid Construction

The oligonucleotides were synthesized and sequenced by Invitrogen Ltd. (Shanghai, China), and their sequences were listed in **Table 2**. PCR amplification was performed with *KOD plus* DNA polymerase (TOYOBO, Japan) according to the manufacturer's protocol. Plasmid DNAs were isolated using the QIA Prep Mini-spin Kit (Qiagen, Shanghai, China) and the genomic DNA were obtained by QIA Amp DNA Mini Kit (Qiagen, Shanghai, China). DNA fragments were purified utilizing the QIA Quick Gel Extraction Kit (Qiagen, Shanghai, China). The restriction enzyme manipulation, molecular cloning, and agarose gel electrophoresis were carried out with the standard protocols.

2.3. Construction of Two Non-Replicative Plasmids

Two non-replicative plasmids were constructed from plasmid pK18mobsacB [8]. Two glucose dehydrogenase genes *XC*1075 and *XC*2659 were selected as targets to be deleted. First, the upstream 500 bp fragment (U500) and the downstream 500 bp fragment (D500) of the two genes were amplified with the corresponding primer pairs (U-F/U-R and D-F/D-R), respectively (**Table 2**). Then the gentamycin encoding gene *accC*1 was amplified from plasmid pEX18Gm [9], flanking with 20 bp homologies to the fragments U500 and D500. Next, the fusion fragment was PCR generated using the fragments U500, *accC*1 and D500 with primer pairs U-F/D-R. Finally, the two fusion fragments were cloned into plasmid pK18mobsacB with restriction enzymes *Eco* RI and *Hind* III,

Table 1. Strains and plasmids used in this work.

Strains and plasmids	Genotype or description	Source
Strains		
E. coli DH5*α*	*SupE*44 *DlacU*169 (Φ80*lacZ*ΔM15) *hsd*R17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *rel*A1	[13]
E. coli S17-1(*λ*pir)	*recA*, *pro*, *hsdR*$^-$M$^+$RP4: 2-Tc:Mu: Km Tn7 *λ*pir (ATCC 47055)	[14]
Xcc 8004	A rifampicin-resistant strain derived from *Xcc* NCPPB No. 1145 (isolated from infected cauliflower, B. oleraceavar. botrytis, in Sussex, UK, 1958)	[15]
Plasmids		
pDN19	7.8 kb, IncP, oriT, Tc^r	[16]
pUFR027	9.3 kb, IncW, MobP, Ap^r, Kan^r	[17]
pRKaraRed	10.9 kb, IncP, oriT, Tc^r	[18]
pK18mobsacB	5.7 kb, RP4 oriT, Kan^r	[19]
pEX18Gm	Gene replacement vector, oriT, Ap^r	[9]
pK18mobsacB-Xc1075	7.2 kb, pK18mobsacB with the upstream 500 bp fragment of *XC*1075, *accC*1 and the downstream 500 bp fragment of *XC*1075, Kan^r	This work
pK18mobsacB-Xc2659	7.2 kb, pK18mobsacB with the upstream 500 bp fragment of *XC*2659, *accC*1 and the downstream 500 bp fragment of *XC*2659, Kan^r	This work

Table 2. Sequences of oligonucleotides.

Name	Sequences	Description
*XC*1075-U-F	5'-GTAGGAATTCatgactgatcaatcctctaaacgcggg-3'	Amplification primers for the upstream 500 bp fragment of *XC*1075
*XC*1075-U-R	5'-ctgaacggcgttgccgcatc-3'	
*XC*1075-D-F	5'-gcagcaagaccaagggctacatg-3'	Amplification primers for the downstream 500 bp fragment of *XC*1075
*XC*1075-D-R	5'-GTAGAAGCTTttatttggcgctggcggcc-3'	
*XC*2659-U-F	5'-GTAGGAATTCatgtcgacattgcttcgtccctccc-3'	Amplification primers for the upstream 500 bp fragment of *XC*2659
*XC*2659-U-R	5'-gggcgattgaccacacctgcg-3'	
*XC*2659-D-F	5'-gtggcgatgccggtggtgc-3'	Amplification primers for the downstream 500 bp fragment of *XC*2659
*XC*2659-D-R	5'-GTAGAAGCTTttaccgctgcggcaacgcgtag-3'	
*accC*1-1075-F	5'-GATGCGGCAACGCCGTTCAGatgttacgcagcagcaacgatgt-3'	Amplification primers for the *accC*1 fragment for fusion with two flanking fragments of *XC*1075
*accC*1-1075-R	5'-GTAGCCCTTGGTCTTGCTGCttaggtggcggtacttgggt-3'	
*accC*1-2659-F	5'-GCAGGTGTGGTCAATCGCCCatgttacgcagcagcaacgatgt-3'	Amplification primers for the *accC*1 fragment for fusion with two flanking fragments of *XC*2659
*accC*1-2659-R	5'-GTGGCGATGCCGGTGGTGCAttaggtggcggtacttgggt-3'	

The oligonucleotides were named according to gene name and were listed from 5' to 3'. F and R primers represented forward and reverse primers, respectively. Lowercase bases matched the template; the capitalized bases in primers *accC*1-1075-F/R and *accC*1-2659-F/R indicated sequences for overlap PCR; and the capitalized and lined bases in the other primers were sequences of restriction enzyme sites.

producing plasmids pK18mobsacB-Xc1075 and pK18mobsacB-Xc2659 (**Table 1**). The constructs was verified by DNA sequencing.

2.4. Preparation of Electro-Competent Cells

The *Xcc* 8004 electro-competent cells were made based on a micro-centrifugation procedure. Single colony of *Xcc* 8004 was inoculated into 10 ml LB medium (in 50 ml bottle) and cultured overnight at 28˚C with 200 rpm

shaking. The overnight culture was equally distributed into four 5 ml centrifuge tubes, and cells were harvested by centrifugation at 8000 rpm for 5 min at room temperature. The cell pellets in each tube were re-suspended with 1 ml of 250 mM sucrose, and transferred into 1.5 ml Eppendorf tubes. Centrifugations and re-suspensions with 1 ml of 250 mM sucrose were repeated three times at 12,000 rpm for 2 min at room temperature. Finally, cells in four tubes were re-suspended with total 100 μl of 250 mM sucrose, and the final cell concentration was about 10^9 ~ 10^{10} colony forming units (CFU)/ml. 50 μl aliquot of the competent cells was used for one electroporation experiment.

2.5. Electro-Transformation of Replicative and Non-Replicative Plasmids

Cells cultured at different stages (OD_{600} = 0.4 ~ 1.2) were used to prepare the electro-competent cells as described above. Electroporation was carried out with 50 μl of competent cells and no more than 10 μl of plasmids DNA. Electro-transformation was performed in a 0.1 cm ice-cold electroporation cuvette, on a Bio-Rad Gene-Pulser II. The electric field strength varied from 10 KV/cm to 20 KV/cm. The concentrations of plasmids DNA ranged from 50 ng to 1 μg. Cells without plasmids DNA were used as negative controls. After the pulse, immediately added 1.0 ml LB broth into the electroporation cuvette, and transferred into a 17 × 100 mm round-bottom, sterile glass tube. The electroporated cells were incubated at 28˚C for 0 ~ 3 hours. Either transformation mixture dilutions (for replicative plasmids) or the entire transformation mixtures (for non-replicative plasmids) was screened on antibiotic-imbued plates and incubated at 28˚C until colonies appeared (usually 48 ~ 72 h). Counted the number of colonies and extracted the plasmids or chromosomal DNA for analysis. For the transformation groups of non-replicative plasmids, the colonies were screened on LB plates with Gm and LB plates with Kan to distinguish double cross-over events from single cross-over events.

To analyze the feasibility of this method in DNA transfer and gene modification, this method was compared with other two transformation methods, one electroporation method and one conjugation method using *E. coli* S17-1 (λpir) [10] [11].

3. Results

3.1. Transformation of Several Replicative Plasmids and Parameters Effects on the Transformation Efficiency

Three plasmids, pUFR027, pDN19 and pRKaraRed was selected as target plasmids to test the efficiency of this method, because the broad host-range vectors with the RK-2 origin or RK-6 origin could replicate in *Xanthomons* (**Table 3**) [5] [11] [12].

Table 3. Transformation efficiencies of plasmids DNA in *Xcc* 8004.

Plasmids	Replicative	Size (kb)	Amount (ng)	Selection	Transformation efficiency (transformants/μg DNA)			
					Recovery time (h)			
					0	1	2	3
pDN19	Yes[a]	7.8 kb	200	Tc^r	2.3×10^3	1.7×10^7	9.6×10^7	6.3×10^7
pRKaraRed	Yes[a]	10.9 kb	200	Tc^r	1.4×10^3	4.0×10^6	6.8×10^7	3.4×10^7
pUFR027	Yes[a]	9.3 kb	200	Kan^r	1.9×10^3	5.6×10^6	3.5×10^7	4.9×10^7
pK18mobsacB-Xc0751	No[b]	7.2 kb	1000	Kan^r	6	64	151	136
pK18mobsacB-Xc2728	No[b]	7.2 kb	1000	Kan^r	8	79	138	142

In all cases, 50 μl aliquots of electro-competent cells were transformed with the indicated amounts of DNA. 1 ml LB medium was added and the cells were either immediately plated on selective plates (0 h) or after 1 - 3 h shaking at 28˚C. Selective plates consisted of LB with 10 μg/ml Tc, 10 μg/ml Kan or 10 μg/ml Gm. Transformants were counted after about 48 ~ 72 h incubation at 28˚C. [a]After incubation for the indicated amount of time, cells were diluted up to 10^5-fold in LB medium and then plated on selective plates to yield single colonies. The numbers shown are the averages from three separate experiments; [b]Since these pK18mobsacB-based plasmids do not replicate in *Xanthonomas*, antibiotic resistant colonies after transformation were the result of either plasmid-integration (merodiploid formation) into the chromosome via a single or double cross-over event. After incubation for the indicated amount of time, the entire mixture was screened on a single LB plate with 10 μg/ml Gm. Colonies were re-selected on LB added 10 μg/ml Gm and LB added 10 μg/ml Kan plates to distinguish double from single cross-over events. Data shown are the total numbers of Gm^r colonies including two cross-over events.

First we analyzed whether the three plasmids could be transformed into *Xcc* 8004. According to previous work, we collected cells of *Xcc* 8004 at OD_{600} about 0.8 and treated cells with sucrose solution and micro-centrifugation to generate the competent cells. Then we electro-transformed 200 ng of plasmids DNA into these cells with following electrical parameters: electrodes of 0.1 cm gap, 14 KV/cm, 2 h recovery time. Results showed that all three plasmids could be transformed into *Xcc* 8004 and the transformants were around 10^9 CFU per microgram DNA (**Table 3**). Although we only tested plasmids within a relatively small size range, the size of input DNA seemed have no obvious effect on transformation efficiencies (**Table 3**). Comparing with other transformation method described before, up to 100-fold increase in the transformation efficiency was obtained using this method (**Table 4**) [11].

Then we analyzed whether the effect of DNA concentration on the transformation efficiency. We found increasing DNA concentration could enhance the transformation efficiency from 1.5×10^6 to 2.7×10^8, when the concentration of plasmid DNA was changed from 50 ng to 1 μg per 50 μl competent cells (**Figure 1(a)**). A linear relationship existed between the transformants number and the DNA concentration. Even at the highest plasmid DNA concentration, a saturation level was not observed. In most conditions, 50 - 100 ng replicative plasmids DNA seemed to be adequate for efficient transformation.

Also the influence of cell growth stage to the transformation efficiency was detected and 200 ng of plasmid pUFR027 was used. The highest transformation efficiency was observed when cells were at the late exponential phase to the early stationary phase (OD_{600} = 0.6 ~ 1.0), about 16 ~ 20 hours (**Figure 1(b)**). If the cells entered into mid or late stationary phase, the transformation efficiency will decrease obviously; it is probably because the large amount of EPS and extracellular enzymes were produced and very hard to be removed, thus the exogenous DNA is very difficult to enter the cells.

Further we analyzed the effect of electric intensity and 14 ~ 18 KV/cm field strengths were found to be optimal (**Figure 1(c)**). The pulse length of the apparatus was all about 5 ms and the ratio of survival cells had little difference at different field strength (data not shown). It was in accord with previous reports that 10 ~ 18 KV/cm field strengths and 5-ms pulse duration are all applicable for the DNA electro-transformation in *Xanthomonas* [4] [10].

In addition, the recovery time also had great impact on the transformation efficiency. When cells were screened on the selective plate immediately after electroporation, the transformation efficiency was only about 2×10^3 per microgram input DNA; however, one- or two-hour recover could improve it significantly by three to four logs (**Table 3**). Therefore, at least one hour recovery time was needed and two hours was enough for efficient transformation.

In a word, this method was feasible to generate efficient electro-competent cells; combining with optimized electroporation parameters, the plasmids DNA could be transformed into *Xcc* 8004 efficiently.

3.2. Transformation of Non-Replicative Plasmids and Generation of *XC*1075 and *XC*2659 Knockout Mutants in *Xcc* 8004

Non-replicative plasmids are widely used in gene deletion and modification by single or double recombination events. In *Xanthomonas*, gene replacement was performed based on the conjugation with *E. coli* S17-1 (λpir) [10].

To test the feasibility of this method for non-replicative plasmid integration and gene deletion, two glucose dehydrogenase genes *XC*1075 and *XC*2659 were selected and we constructed two plasmids pK18mobsacB-Xc1075

Table 4. Transformation efficiencies in *Xcc* 8004 with different methods.

CFU \ Plasmids	pDN19	pRKaraRed	pUFR027	pK18mobsacB-Xc0751	pK18mobsacB-Xc2728
This Method[a]	1.1×10^8	7.8×10^7	3.9×10^7	147	133
Method 1[b]	3.7×10^6	8.6×10^5	1.6×10^6	ND	ND
Method 2[c]	ND	ND	ND	27	19

In all cases, 50 μl aliquots of electro-competent cells were transformed with 200 ng of replicative DNA or 1000 ng of non-replicative DNA. ND was not detected. [a]The electroporation parameters were cells of OD_{600} = 0.8, electrodes of 0.1 cm gap, 14 KV/cm, and 2 h recovery time. Transformants were screen on the selective plates and counted after about 48 ~ 72 h incubation at 28˚C. The numbers shown are the averages from three separate experiments; [b]Method 1 was the electroporation performed as described previously [13]; [c]Method 1 was the conjugation method performed using *E. coli* S17-1(λpir) [11].

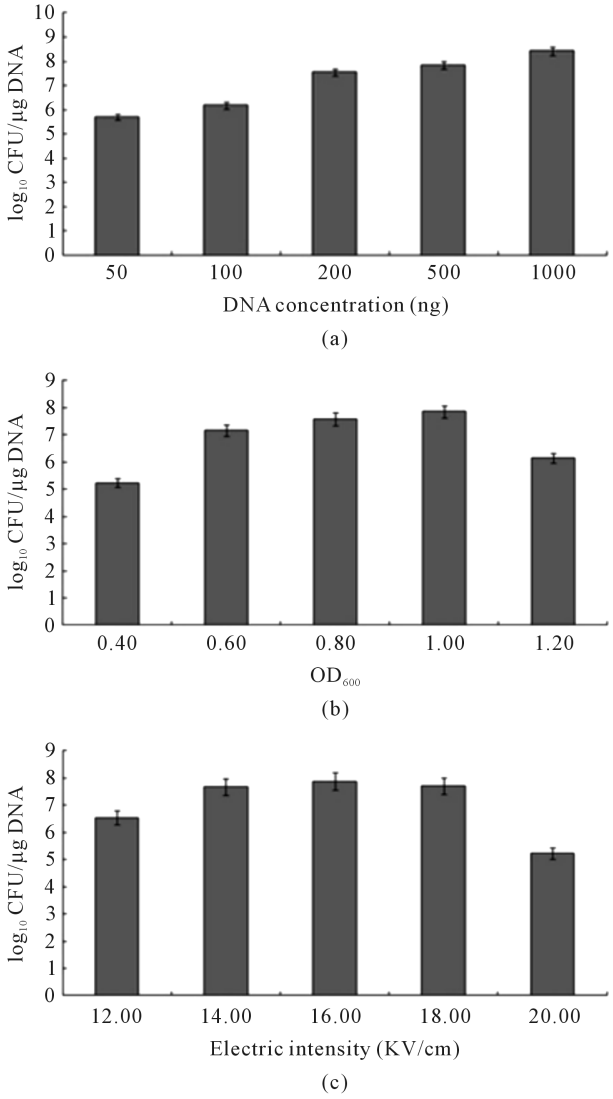

Figure 1. Effects of different parameters on the electro-transformation efficiency in *Xcc* 8004. Replicative plasmid pUFR027 was used in these experiments. (a) Effect of DNA concentration. The DNA concentration ranged from 50 ng to 1000 ng. Other parameters were cells of OD_{600} = 0.8, electrodes of 0.1 cm gap, 14 KV/cm, and 2 h recovery time. (b) Effect of cells growth stage. Cells were collected at different stages to generate competent cells, ranging from OD_{600} = 0.4 - 1.2. 200 ng of plasmid pUFR027 was used and other parameters were same as above. (c) Effect of field strengths. The electric intensity ranged from 12 KV/cm to 20 KV/cm. 200 ng of plasmid pUFR027 was used and other parameters were same as above. All experiments were repeated three times.

and pK18mobsacB-Xc2659, containing gentamycin encoding gene, the upstream 500 bp and the downstream 500 bp chromosomal flanking regions. Since pK18mobsacB-based plasmids cannot replicate in *Xanthonomas*, antibiotic resistant colonies after transformation were the result of either plasmid-integration (merodiploid formation) into the chromosome via single or double cross-over event. Results indicated that in the optimal electroporation conditions, each plasmid yielded about 130 - 150 transformants per microgram of input DNA (**Table 3**). It was much higher than that from the traditional conjugation method with *E. coli* S17-1 (*λ*pir) strain (**Table 4**). Further determination showed that about 66% transformants for plasmid pK18mobsacB-Xc1075 and 61% transformants for plasmid pK18mobsacB-Xc2659 were single integration events; and others were double cross-over events. Double homologous recombination between the homologous sequences deleted the two chromosomal target genes efficiently and two knockout mutant strains of *XC*1075 and *XC*2659 were obtained (**Figure 2**). It should be noted that at least 500 ng of non-replicative plasmids DNA were required for effective

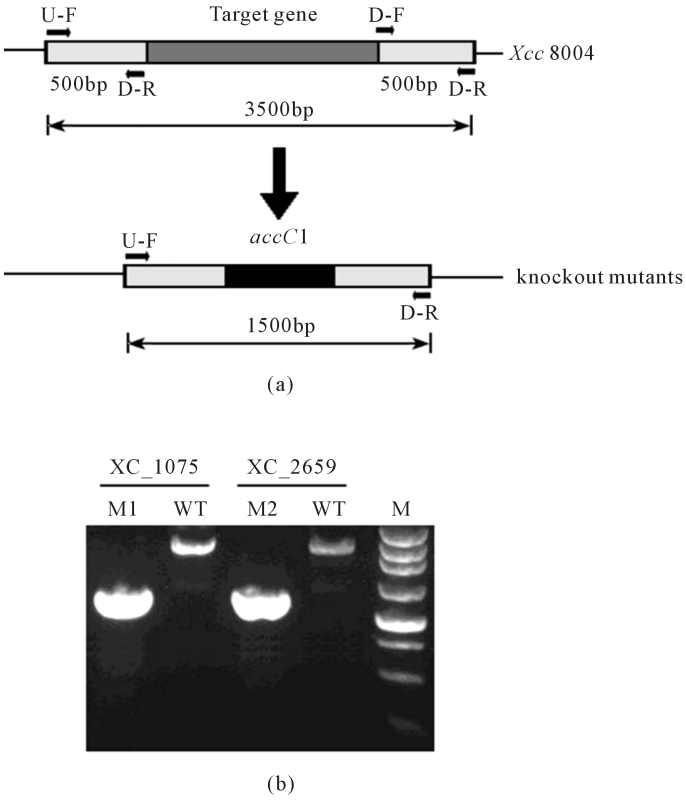

Figure 2. Construction and verification of the two knockout mutant strains. (a) Schematic description of the chromosomal DNA of *Xcc* 8004 and that of mutant strains. (b) PCR detection results of *Xcc* 8004 strain and the mutant strains with primer pairs U-F/D-R. WT represented the fragment amplified from *Xcc* 8004; M1 and M2 represented the fragment amplified from knockout mutant strains of *XC*1075 and *XC*2659. M is a 250 bp DNA Marker (Takara, 250 bp, 500 bp, 750 bp, 1000 bp, 1500 bp, 2250 bp, 3000 bp, and 4500 bp).

integration and recombination; increased amounts of plasmids DNA could improve the efficiency (data not shown).

In brief, it is possible to using this method to transform non-replicative plasmids into *Xanthomonas* directly to induce plasmid integration; and homologous recombination between chromosome and plasmids could generate knockout mutants.

4. Discussion

Electroporation is widely used in many organisms because of its high efficiency. In *Xanthomonas*, electro-transformation is the major plasmid DNA transfer method as *Xanthomonas* is insensitive to the chemical treatment [4]-[6] [10]. But the electroporation efficiency in this bacterium is far from *E. coli* possibly because its heavy EPS and secreted enzymes are quite difficult to be removed, which directly influences the entering of input DNA. This requires the optimization or modification in the competent cells preparation procedure. On the other hand, gene replacement in *Xanthomonas* is still mainly dependent on the conjugation with *E. coli* strain, which is time-consuming, complicated, and with high false positive ratio, hindering further mechanism study greatly [11].

The main advantages of this method for preparation of electro-competent *Xcc* 8004 cells described here are its simplicity and speed, without compromising efficiency. The entire procedure can be performed at room temperature with overnight cultures in a microcentrifuge, simply using 250 mM aqueous sucrose solution and taking less than 15 minutes to complete the steps. It is in contrast to traditional procedures which require time-con-

suming centrifugation steps, vessels and sometimes complex buffers that need to be refrigerated, and take hours to complete. We also tried to use wide-used 10% glycerol to generate competent cells, and found higher efficiencies can be obtained with sucrose solution, probably because of higher cells sensitivities in the latter treatment (data not shown). The entire procedure, from start (preparation of plasmid DNA) to finish (screening on selective media), takes about 3 hours, and colonies are ready for test in about 2 - 3 days. Despite its speed and simplicity, the transformation efficiencies of this method are comparable to or exceed those of other two methods, sometimes up to 100-fold (**Table 4**). The knockout mutants of target chromosomal genes could be easily obtained through the non-replicative plasmids integration.

Therefore, this method can accelerate the speed in routine plasmid DNA transfer and gene replacement experiments in *Xcc* 8004; and the observed transformation efficiencies are sufficient for further analysis. It can be more powerful if conjunction with other marker excision methods (Flp/*FRT*), which allows recycling of the same selection marker in the same strain for construction of mutants to contain multiple lesions in the same chromosome.

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Conflict of Interest

All authors have no conflict of interest to declare.

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