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An easy method for generating deletion mutants in *Agrobacterium tumefaciens* using a simple replacement vector

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ABSTRACT: We have developed a method to make precise mutations in the *A.tumefaciens* genome at frequencies high enough to allow direct identification of mutants by PCR or other screening methods rather than by selection. This method utilized a novel pUC18-based gene replacement vector that was used as a donor plasmid carrying the desired mutation in the target cell. Two sites of single-crossover occurred resulting in efficient replacement of the wild type allele on the chromosome with the modified sequence. The usefulness of this method was demonstrated by making deletion mutants in citrate synthase genes of *A. tumefaciens.*

KEYWORDS: *A. tumefaciens,* deletion mutants, citrate synthase gene.

INTRODUCTION

Agrobacterium tumefaciens is a gram-negative plant pathogenic bacterium. It is capable of transferring DNA from the tumor-inducing (Ti) plasmid into the nucleus of a higher plant cell, where the DNA can be integrated into the host chromosomal genome and expressed. *Agrobacterium* cells have to invest energy during the process of virulence to get the plant metabolites needed for the survival of bacteria in the host-plant environment. In this situation, the citric acid cycle (TCA) may play an important role for effective virulence of the bacterium. However, little is known about the role of the TCA cycle enzyme in the virulence of *A. tumefaciens*. In the *A. tumefaciens* genome database, there are 4 genetically distinct citrate synthase (CS) encoding genes which are located both on the bacterial chromosome, *cisY* (Atu 1392) and *cisZ* (Atu 4851), and crypic plasmid, *cis* (Atu 5306) and *cisZ* (Atu 5307), respectively. In order to examine the functions of these genes, construction of CS deleted mutants is required.

Up to date, the complete genome sequences of many organisms are available, which has opened many new experimental avenues. The ability to make precise genetic modifications to the bacterial chromosome and then to study the resulting phenotypic behavior is very important for functional studies. Because of recent technical advances, several methods have been produced to introduce a desired mutant sequence, synthesized *in vitro* into the microorganism chromosome without leaving behind any unwanted sequence changes from the insertion process. Some of these established techniques include utilization of a recombination system such as in the " gene gorging" method and a broad-host – range Flp-FRT. In the " gene gorging" method[1], a donor plasmid carrying the desired mutation in the target cell is linearized by *in vivo* expression of the meganuclease I-Sec I, providing a DNA substrate for λ Red mediated recombination which results in efficient replacement of the wild type allele on the chromosome with the modified sequence.

In the " Flp-FRT recombination" system, which has been used for isolation of unmarked *Pseudomonas aeruginosa* mutants[2], the FRT cassette contains a Gm^R marker next to the green fluorescent protein structural gene, with both markers being flanked by Flp recombinase target (FRT) sites. This cassette was used to insertionally inactivate the chromosomally located gene. After transfer into the host organism, plasmid integrants were selected, and deletion of unwanted DNA sequence was promoted by sucrose counterselection. The FRT cassette was excised from the chromosome after transfer of a Flp-recombinase-expressing plasmid, resulting in unmarked mutants.

Both methods include several complicated steps, including multiple cloning steps involving specialized vectors, multiple transformations by several plasmid vectors and require strong genetic selections to achieve the goal (useful frequencies).

In this paper, an easier and efficient method for generating a desired mutation was developed. This method utilized a novel pUC18-based gene replacement

vector, the pEX18Gm vector. This vector incorporates (i) the counter-selectable sacB marker, (ii) a lacZ±-allele for blue-white screening, (iii) the pUC18 vector's multiple cloning site with 10 unique restriction sites, (iv) an oriT for conjugation-mediated plasmid transfer and (v) gentamicin (Gm) selectable markers. The method was illustrated by introducing deletions in citrate synthase genes: *cisY* (Atu 1392), *cisZ* (Atu 4851), *cis* (Atu 5306) and *cisZ* (Atu 5307) located on the *A. tumefaciens* genome.

Materials and Methods

Bacterial Strains and Plasmids

Table 1 lists the plasmids as well as bacterial strains used in this study. All cloning was performed using standard protocols[3], and all enzymes were used as recommended by manufacturers.

Culture Media, Growth Conditions and Chemicals

Strains of *Escherichia coli* were grown in Luria-Bertani media (LB) containing the appropriate antibiotics at 37°C. *A. tumefaciens* strains were grown at 28°C in mannitol glutamate luria salts medium (MG/L). Gentamicin was filter-sterilized and added to *E. coli* cultures at 25 μg/mL and 50 μg/mL of cultures. Sucrose was added to media at a concentration of 5% to select against plasmid carrying the *Bacillus subtilis* levansucrase gene. X-gal was obtained from Research Product Internation Corp. (IL 60056) and used at a concentration of 50 mg/mL.

DNA Manipulations

Restriction enzymes, T4 DNA ligase from Invitrogen (Carlsbad, CA, USA) and Ex-Taq from Takara (Tokyo, Japan) were used according to the manufacturers' instructions. DNA amplification primers and sequencing primers were from QIAGEN (Hilden, Germany). All primers used in this study are listed in Tables 2-4. DNA was prepared and manipulated by standard procedures[3]. Plasmids were isolated by using the QIAprep^R Spin Miniprep Kit from QIAGEN. PCR products were cleaned with the QIA quick^R PCR purification kit.

Generation of Donor Plasmid (Deletion Mutant Alleles)

The donor plasmids pMC4851M, pMC1392M, pMC5307M and pMC5306M were used to generate deletions of the *cisZ* (Atu 4851), *cisY* (Atu1392), *cisZ* (Atu 5307) and *cis* (Atu 5306) genes in the *A. tumefaciens* strain C58 via homologous recombination. To construct these plasmids, PCR was first used to amplify 1.5 kb regions the upstream and the downstream of the 5' and 3' ends of the *cisZ* (Atu4851), *cisY* (Atu1392), *cisZ* (Atu5307) and *cis* (Atu5306) genes from genomic DNA of *A. tumefaciens*. DNA amplification was carried out as previously described[4]. The following primers were used: primers 4851Δ*cisZ* Up-5' and 4851Δ*cisZ* Up-3'for the upstream region of *cisZ* (Atu 4851), primers 4851Δ*cisZ* Down-5' and 4851Δ*cisZ* Down-3'for the downstream region of *cisZ* (Atu 4851), primers 1392Δ*cisY* Up-5' and 1392Δ*cisY* Up-3'for the upstream region of *cisY* (Atu

Table 1. Strains and plasmids used in this study.

Strain/Plasmid	Relevant Features	Reference or Source
Agrobacterium strains		
C58	Wild- type *A. tumefaciens* strain. Carries pTiC58 and pAtC58	Lab collection
C58Δ1392	C58 with *cisY* deletion generated by pMC1392M	This study
C58Δ4851	C58 with *cisZ* deletion generated by pMC4851M	This study
C58Δ5306	C58 with *cis* deletion generated by pMC5306M	This study
C58Δ5307	C58 with *cisZ* deletion generated by pMC5307M	This study
E. coli strains		
DH5α	F^-ϕ80lacZΔM15Δ(lacZYA-argF)U16 recA1endA1 hsdR17(rk^-,mk^+) phoA supE44 thi-1 gyrA96 relA1 λ^-	Invitrogen
Plasmids		
pEX18Gm	Gm^R;$oriT^+$ $sacB^+$,gene replacement vector with MCS from pUC18	2
pMC4851M	construct for deletion of *cisZ* (Atu 4851)	This study
pMC1392M	construct for deletion of *cisY* (Atu 1392)	This study
pMC5306M	construct for deletion of *cis* (Atu 5306)	This study
pMC5307M	construct for deletion of *cisZ* (Atu 5307)	This study

Table 2. List of oligonucleotides used to amplify the upstream and downstream regions of citrate synthase genes for mutagenesis in this study. (Underlined portions indicated restriction sites).

Oligonucleotides	sequence and restriction sites
4851Δ*cisZ* Up-5'	5'GC**TCTAGA**CGATAGCACGGGTGTGAGCGAAAAGAAGG3'
4851Δ*cisZ* Up-3'	5'AA**CTGCAG**TAGCGGTGACTGCGGGCCGAACGACCCCG3'
4851Δ*cisZ* Down-5'	5'AA**CTGCAG**CCCACGATCTTGGGCAGAGCTAGGAAAAATC3'
4851Δ*cisZ* Down-3'	5'AAAAAA**AAGCTT**ATGATATCAGGTCATCGCCAGCCACAG3'
1392Δ*cisY* Up-5'	5'CG**GGATCC**ATGACCAGAGCGGACAATGCAACATTC3'
1392Δ*cisY* Up-3'	5'GC**TCTAGA**ACTGATCCTCCGAGAACATGCGGGTG3'
1392Δ*cisY* Down-5'	5'GC**TCTAGA**GCCACGAAACCCTGTAAGATCAAAG3'
1392Δ*cisY* Down-3'	5'AAAAAA**AAGCTT**TTGCTGGTCATGGGCGGCAACCTC3'
5307Δ*cisZ* Up-5'	5'CG**GGATCC**AGGGGCTAAGGCTGGAATTTCTACTG3'
5007Δ*cisZ* Up-3'	5'GC**TCTAGA**GAACCCGGCTCCCGGCCTTGATGATG3'
5307Δ*cisZ* Down-5'	5'GC**TCTAGA**GCGCTCACTACAAAAACGCTGGAGC3'
5007Δ*cisZ* Down-3'	5'AA**CTGCAG**GTTCGTTACAGGATGCTGCCTATAC3'
5306Δ*cis* Up-5'	5'CG**GAATTC**TGGCGATTTCGGTCATGCGTCTTGC3'
5306Δ*cis* Up-3'	5'GC**TCTAGA**GATTTCGCGCAGCAGTGGAGAACAG3'
5306Δ*cis* Down -5'	5'GC**TCTAGA**AGTTTCGGCGGCAACGACATCATCAAG3'
5306Δ*cis* Down -3'	5'AAAAAA**AAGCTT**ATTAGGTTGGCGCGGCTCGCCAAACCG3'

Table 3. List of oligonucleotides used for PCR analysis of mutants.

Oligonucleotides	sequence
For selecting C58Δ5306 mutant	
5306 left	5'GACCAATCTCAACGTGTTAGCTTATG3'
5306 right	5'ACTCGGCCGCAGCGCGATCGGCGGTC3'
For selecting C58Δ5307 mutant	
5307 left	5'CGGGATCCAGGGGCTAAGGCTGGAATTTCTACTG3'
5307 right	5'AACTGCAGGTTCGTTACAGGATGCTGCCTATAC3'
For selecting C58Δ1392 mutant	
1392 left	5'TTTCATGCATGAGGGCAGGG3'
1392 right	5'TTTCCTTTAACCCTGTGTTG3'
For selecting C58Δ4851 mutant	
4851 left	5'CGCCCACCCACGAACTCACC3'
4851 right	5'ATGACATTTTACGATGGTCC3'

1392), primers 1392Δ *cis Y* Down-5' and 1392Δ *cisY* Down-3'for the downstream region of *cisY* (Atu 1392), primers 5307Δ*cisZ* Up-5' and 5307Δ*cisZ* Up-3'for the upstream region of *cisZ* (Atu 5307), primers 5307Δ*cisZ* Down-5' and 5307Δ*cisZ* Down-3'for the downstream region of *cisZ* (Atu 5307), primers 5306Δ*cis* Up-5'and 5306Δ*cis* Up-3'for the upstream region of *cis* (Atu 5306), and primers 5306Δ*cis* Down-5'and 5306Δ*cis* Down-3'for the downstream region of *cis* (Atu 5306). The reaction mixtures were then subjected to three steps of amplification (on a Perkin-Elmer/Cetus DNA thermal cycler). The first step was one cycle at 95°C for 5 min. The second step of 35 cycles was set up as either 95°C for 1 min, 62°C for 1 min and 72°C for 2 min 20s for amplification of *cis Y* (Atu 1392) and *cisZ* (Atu 4851) genes, or 95°C for 1 min, 65°C for 1 min and 72°C for 2 min 20s for amplification of *cisZ* (Atu 5307) and *cis* (Atu 5306) genes. The final step was one cycle at 72°C for 10 min. The amplified fragments were digested with primer specific restriction enzymes as follows: *Xba*I

and *Pst*I for the *cisZ* (Atu 4851) the upstream PCR product, *Pst*I and *Hin*dIII for the *cisZ* (Atu 4851) the downstream PCR product, *Bam*HI and *Xba*I for the *cisY* (Atu 1392) the upstream PCR product, *Xba*I and *Hin*dIII for the *cisY* (Atu 1392) the downstream PCR product, *Bam*HI and *Xba*I for the *cisZ* (Atu 5307) the upstream PCR product, *Xba*I and *Pst*I for the *cisZ* (Atu 5307) the downstream PCR product, *Eco*RI and *Xba*I for *cis* (Atu 5306) the upstream PCR product, *and Xba*I and *Hin*dIII for the *cis* (Atu 5306) the downstream PCR product. After digestion, they were ligated directionally into pEX18-Gm that was simultaneously digested with the corresponding enzymes, resulting in the donor plasmid: pMC4851M, pMC1392M, pMC5307M and pMC5306M. Clones with the deletion alleles were confirmed by restriction mapping and PCR analysis.

Donor Plasmid Transformation

Chemically competent cells of *E. coli* DH5± were from Invitrogen and plasmid transformations were performed according to the manufacturer's protocol. Cells were allowed to recover in SOC medium for 1 h at 37°C and dilutions were plated on LB media containing gentamicin and X-gal.

Table 4. List of oligonucleotides used for sequencing analysis of mutants.

Oligonucleotides	sequence
Δ4851f1L	5'TCTCGCAGAGAACCTCAATGCGCGG3'
Δ4851f1R	5'ATGATTTCTTAAACGAACTCTATCG3'
Δ4851f2L	5'GAGGAACTGTAATATTTCGATAGAG3'
Δ4851f2R	5'GTTTTCCATTCATGCTAATCGGCA3'
Δ4851f3L	5'CGGTCACTACGTAATCCCTATTTG3'
Δ4851f3R	5'TAGGCGCAGCGCTCGATATCGCTTG3'
Δ1392f1L	5'TGGTCTCCACCGTCATCTATCTTGTG3'
Δ1392f1R	5'TGGAAGATCATACCGACCTTCAGCCG3'
Δ1392f2L	5'GATTTCGGCCGGTTCCATATCGGTC3'
Δ1392f2R	5'CGGCGAGGCAGGCGGTGATTTTCTG3'
Δ1392f3L	5'GAAACCGCAAGCTGCCGTTATCCAG3'
Δ1392f3R	5'TCTGGAAGCTCGGCCCCATGATTTC3'
Δ5306f1L	5'TCCGCATGCCAGCTGTAACGGTTGC3'
Δ5306f1R	5'CGCATTCTTGGTAGTTCTATTTTGTC3'
Δ5306f2L	5'TACAAAGTGAAAGCCAGCGACACCTC3'
Δ5306f2R	5'AGCGTTGCACGGCTGATGCCCAACTC3'
Δ5306f3L	5'ACATGCTTGAGGATGCTGTGGTACAC3'
Δ5306f3R	5'CTCAGCGCGGAAAGCCCCGCATTCAG3'
Δ5306f4L	5'TCTCATTCGCCGGGCCCTGGTTCTAC3'
Δ5306f4R	5'CATATTCCAGATCTGAGATAATGGAC3'
Δ5307f1L	5'TGACCAAGACGTTCTCCTCCGGAGTG3'
Δ5307f1R	5'GCTTTGCGCCTTGAAGGGACCACTTC3'
Δ5307f2L	5'TCTCCGCGCCCAAGCGCATCCAGCAG3'
Δ5307f2R	5'ATTCCATCAGGCTCTCATTCCTATCG3'
Δ5307f3L	5'ATTATCTCAGATCTGGAATATGATCG3'
Δ5307f3R	5'GCAAGGCTACGCGCGATCCGCATTAG3'
Δ5307f4L	5'ATGAACGCCGGATCAAACGTTACCTC3'
Δ5307f4R	5'TCGGCATCTTCGGTGGTTGCCGTACC3'

Preparation of *A. tumefaciens* Competent Cells

Strains were innoculated into 5 mL of MG/L and grown overnight. Then 1% was subcultured into 25 mL of MG/L and incubated with shaking at 28°C for 5 h Cells were spun down and washed three times in sterile distilled water. The pellet was resuspended in 2 mL of water. An aliquot of 50 μL was used for each electroporation.

Electroporation of Donor Plasmids into *A. tumefaciens*

Plasmids were introduced into *A. tumefaciens* by electroporation at 400 Ω and 2.5 V using a Bio-Rad electroporator. Cells were recovered in SOC medium for 2 h at 28°C and dilutions were plated on MG/L media containing gentamicin.

Construction of Deletion Mutants by Unmarked Homologous Recombination

To generate gene-deleted mutants, the donor plasmid was introduced into the C58 strain by electroporation, and single recombinants were selected based on resistance to gentamicin and sensitivity to sucrose. These colonies were selected and cultured in 5 mL of MG/L for 3-6 h Appropriate aliquots were plated on LB media supplemented with 5% sucrose and colonies with a second single crossover event in which the gene was deleted were identified as gentamicin-sensitive and sucrose-resistant colonies. These colonies were randomly selected for further verification by PCR and confirmed by sequencing.

PCR Analysis of Mutant

The genomic DNA from the potential mutant strains were extracted and used as template for PCR. To prepare the template, the boiling lysis method was employed. One colony was grown overnight at 28°C in 5 mL MG/L. Aliquots of 500 μL culture were pelleted and resuspended in 50 μL of sterile distilled water. The cell suspension was incubated in 100°C for 5 min and centrifuged to pellet the cell debris. The supernatant was collected and used as template for PCR analysis. DNA amplification was carried out as previously described. The primers used for selecting deleted mutant strains, which will generate 2 types of DNA fragment size: 400 bp for mutant strains and 400 bp plus gene size for wt strains are listed in Table 3. The reactions were subjected to 37 cycles for amplification of *cisZ* (Atu 4851), *cisY* (Atu 1392), and *cis* (Atu 5306). The first cycle was at 95°C for 5 min followed by 35 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min 20s, and the last cycle at 72°C for 10 min. For amplification of *cisZ* (Atu 5307), the reaction conditions were 95°C for 5 min followed by 35 cycles at 95°C for: 1 min, 60°C for 1 min, 72°C for 2 min 30s, and the last

cycle at 72°C for 10 min.

DNA Sequencing and Sequence Analysis

The region of the citrate synthase genes and their borders in the genome of *A. tumefaciens* mutants were sequenced with the ABI Prism BigDye™ Terminator Cycle Sequencing Ready Reaction Kit using the synthetic primers listed in Table 4. The gel analyses were performed by the Seattle Biomedical Research Institute (SBRI). Sequence analysis and database searches were carried out with the GeneJockeyII alias program and BLAST at the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST/).

Results

Construction of Deletion Mutant Allele

The goal was to construct citrate synthase gene deletion alleles. Each deletion mutant was constructed by designing a pair of primer to amplify approximately

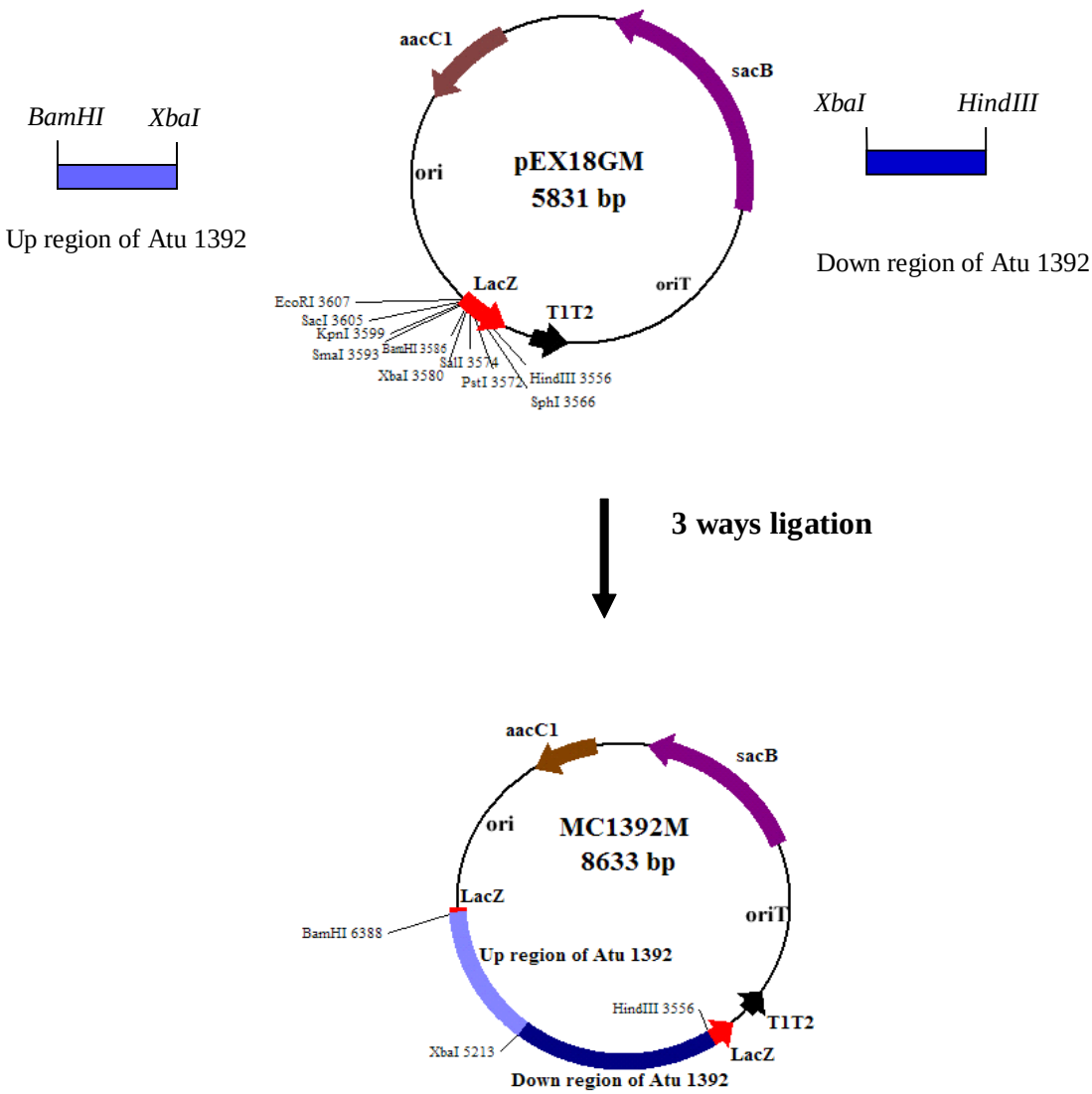

Fig 1. Construction of donor plasmid. Plasmids are drawn to scale. The location of genes and their transcriptional orientation are shown; aacC1, represents the gentamicin resistance genes; ori, the pMB1-based origin of replication[5]; oriT, the origin of transfer from pEM5[6]; *sacB*, the levansucrase-encoding gene from pMOB2[7]; T, the double transcriptional terminators T_1 and T_2 and 5S rRNA gene from pTT3ÄS[8]; Up region of Atu 1392, a 1.5 kb DNA fragment from upstream of the 5' end of *cisY* gene; and Down region of Atu 1392, a 1.5 kb DNA fragment from downstream of the 3' end of *cisY* gene from *A. tumefaciens* genome.

1-1.5 kb the upstream and the downstream regions of 5' and 3' end of the citrate synthase gene. These amplified fragments were digested with primer specific restriction enzymes. The pEX18Gm vector was also digested with the same restriction enzymes compatible to the fragments. The DNA fragments were ligated directionally into pEX18Gm, thus generated the donor plasmids pMC4851M, pMC1392M, pMC5306M, pMC5307M for deletion of the *cisZ* (Atu 4851), *cisY* (Atu1392), *cisZ* (Atu 5307) and *cis* (Atu 5306) genes, respectively, as illustrated for pMC1392M in Fig 1. The presence of the upstream and the downstream regions of the citrate synthase gene inserted into pEX18Gm was verified by restriction mapping and by PCR analysis (Fig 2 and 3, respectively).

Mutagenesis of theCitrate Synthase Gene

To illustrate the usefulness of this method, the donor plasmid was electroporated into *A. tumefaciens* cells. The donor plasmid was then incorporated into the *A. tumefaciens* chromosome as described in Fig 4. Successful gene deletion was monitored by PCR analysis of genomic DNA (Fig 5). When a primer pair which anneal at 200 bp the upstream and the downstream of the citrate synthase gene was used to amplify the chromosomal DNA, the expected 400 bp PCR product and 400 bp plus a gene size PCR product were observed in mutant (lane 1-4) and wt (lane 5), respectively. Nucleotide sequencing result also showed that there was no citrate synthase gene sequence in the chromosome of the mutant strain (Fig 5). The frequencies of generating mutants in the screened population ranged from 1.05-3.45 % for the four citrate synthase genes which were tested (Table 5).

The results for these four genes show that the method developed is generally applicable to generate mutants at relatively high frequencies without direct selection.

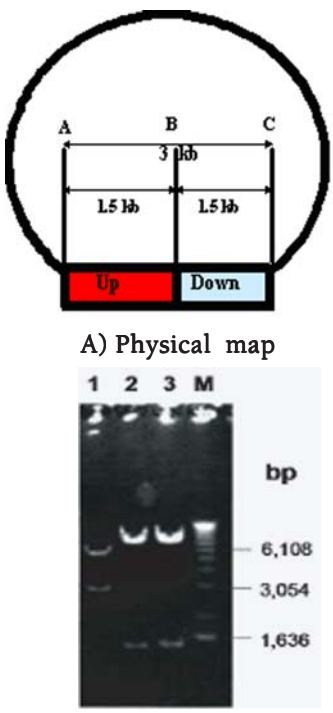

Fig 2. Analysis of donor plasmid by restriction mapping. A) Physical map of the up and down regions of *cisY* gene inserted in pMC1392M. The position of the *Bam*HI(A), *Xba*I(B) and *Hin*dIII (C) sites, as well as the lengths of DNA fragment expected after digestion of donor plasmid pMC1392M are shown. B) Restriction mapping of pMC 1392M, DNA bands in lanes 1,2,3 correspond to fragments obtained after digestion with *Bam*HI/ *Hin*dIII, *Bam*HI/ *Xba*I and *Xba*I/ *Hin*dIII, respectively. DNA size markers (1 kb DNA ladder, New England Biolabs) in bp are indicated at the right.

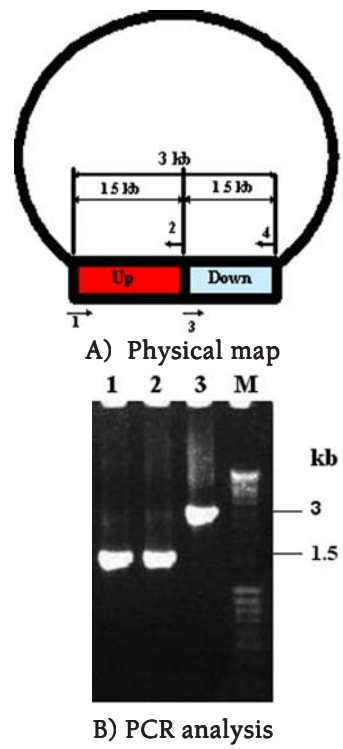

Fig 3. Verification of donor plasmid by PCR analysis. A) Physical map of Up and Down regions of *cisY* gene in pMC1392M. The annealing position of primers as well as the lengths of DNA fragments expected after PCR amplification of the donor plasmid pMC1392M are shown. B) PCR analysis of pMC1392M, DNA bands in lanes 1,2 and 3 correspond to PCR products when using the pairs of primer 1 and 2; 3 and 4; and 1 and 4, respectively. DNA size markers (Mass Ruler™ DNA Ladder, Low Range, ready-to-use, Fermentas) in kb are indicated at the right.

Discussion

The method developed here is based on the previously established mutagenesis method, the " in-out" method[9,10,11,12,13]. In this method, a desired mutant sequence is introduced into the cell on a multicopy circular plasmid. A Rec mediated single homologous crossover results in cointegration of the whole circular plasmid into the genome at the target site with the plasmid vector between a wild type and mutant copy of the target sequence in the "in" step. The cointegrated vector was resolved by a second single crossover in the

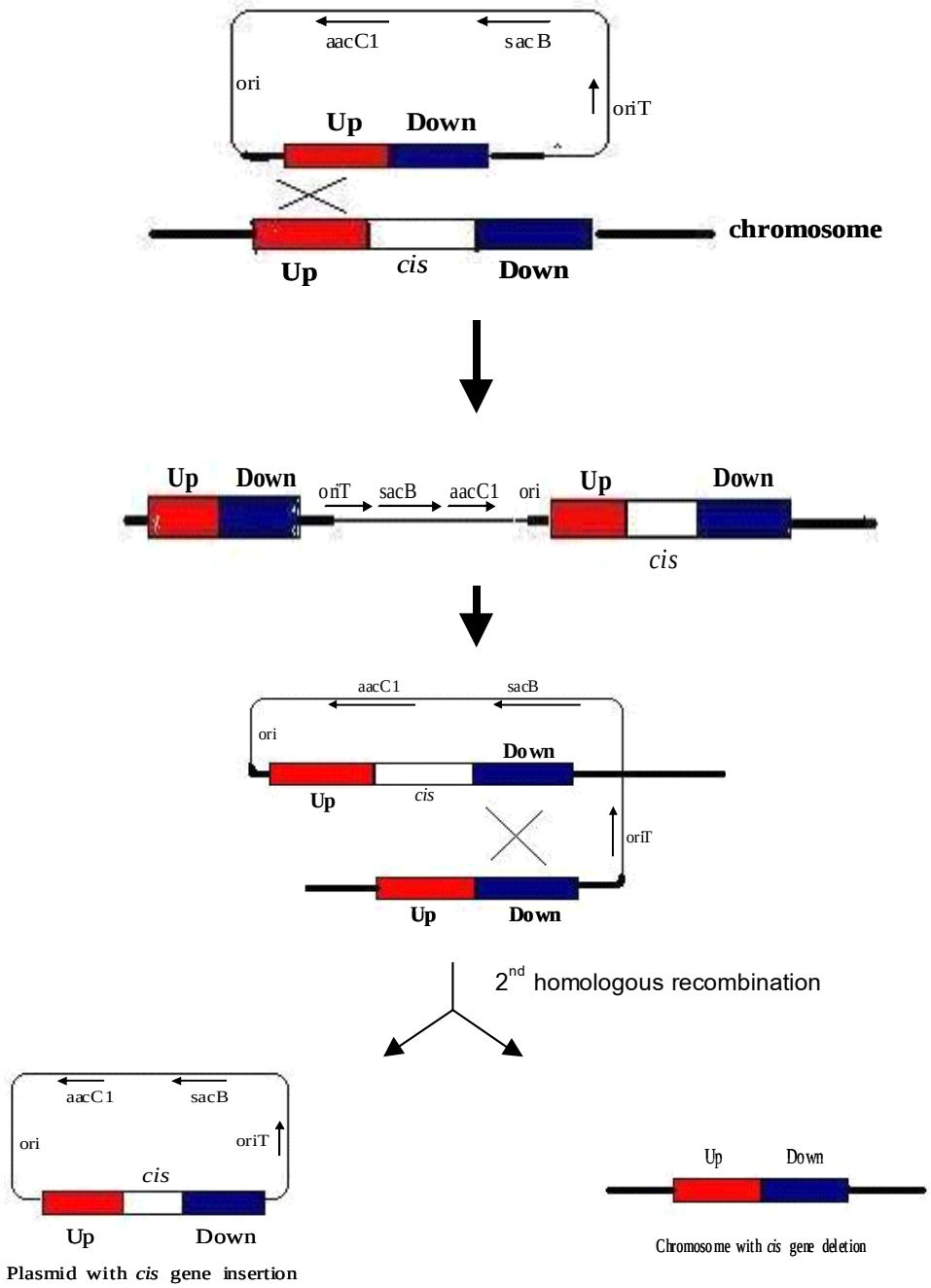

Fig 4. Gene replacement at the *cis* locus of *A. tumefaciens*. Step 1 illustrates the integration of a donor plasmid into the homologous chromosomal region of the wild type strain C58 via recombination. The resulting merodiploid strain is Gm^R and Suc^S. Step 2 depicts the excision of unwanted DNA sequences promoted by selecting Suc^R via homologous recombination. In the resulting mutant strain, the wt *cis* gene is deleted from the chromosome.

Table 5. Gene replacement data.

Gene	Size (bp)	Size of upstream & downstream region	Donor plasmid	Total no. of mutants/ no. of screened colonies	% mutants
cisY (Atu1392)	1290	1.5 kb	pMC1392M	4/380	1.05
CisZ(Atu4851)	1008	1.5 kb	pMC4851M	2/58	3.45
Cis(Atu5306)	1029	1.5 kb	pMC5306M	1/ 78	1.28
CisZ(Atu5307)	1305	1.5 kb	pMC5307M	1/65	1.54

"out" step. When the "in" and " out" crossovers span the mutant site, the desired mutation is transferred to the genome, which results in deletion of the citrate synthase gene in our case. Since a circular DNA molecule sharing homology with a unique region of the bacterial chromosome can become integrated into chromosomal DNA by a Campbell-like mechanism[14], the donor plasmid can be inserted into the *A. tumefaciens* genome by this process.

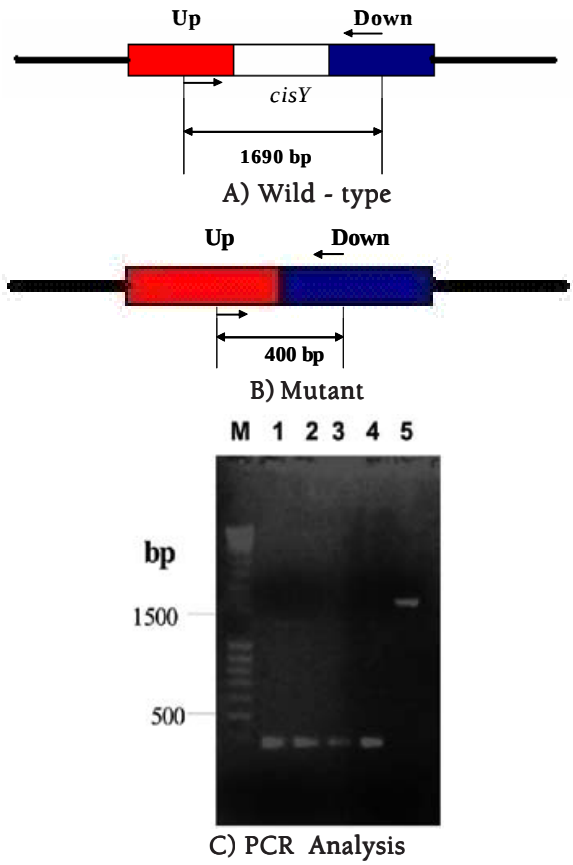

Fig 5. PCR analysis of a mutant strain. A) and B) Physical map of *cisY* region and annealing position of primer in wt and deletion mutant, respectively. The lengths of DNA fragments expected after PCR amplification of the respective chromosomal DNAs are shown. (C) PCR analysis. DNA bands in lanes 1,2,3 and 4 correspond to PCR products from mutant strains (B) and lane 5 corresponds to PCR products from the wild type strain(A). DNA size markers (Mass Ruler™ DNA Ladder, Low Range, ready-to-use, Fermentas) in bp are indicated at the right.

The previously established methods with high efficiency and precision for generating mutants used at least two plasmids (a donor plasmid and a mutagenesis plasmid). Our method can generate chromosomal modification at the precise target site in a convenient way by using only a single replacement vector. Furthermore, in the new method presenting here, the efficiency of gene replacement is high enough to make selection of recombinants unnecessary. Replacement occurred at relatively high levels in the cell population, making it possible to identify mutants by PCR of individual colonies or other means of direct screening.

In this study, the efficiency of gene replacement varied among the four genes tested. Reasons for this variability are unknown. It does not seem to correlate with ORF sizes. In this experiment, glutamate was added in the solid medium used for selecting the mutant clones in the cases of the *cisZ* (Atu 4851), *cis* (Atu 5306) and *cisZ* (Atu 5307) genes. It is possible that, for mutations that have a deleterious effect on growth, it may be an advantage to supplement with the necessary nutrient needed for survival of bacteria, to enhance growth of mutants before growth of wild type cells reduces the relative yield of mutants.

It is noteworthy that the size of the DNA fragment from the upstream region of the 5' end and the downstream region of the 3' end of the gene used to construct the donor plasmid seems to affect the efficiency of gene replacement. In our study, a length of 1 kb of the upstream 5' end and the downstream 3' end regions was first used to construct the donor plasmid. No mutant was detected. After increasing the length up to 1.5 kb, the frequency of gene replacement was high enough to be detected. The explanation for the occurrence of recombination events for the longer inserts may be related to the chi sites, which needs further investigation.

The replacement vector, pEX18Gm, also possesses some unique feature which make it easy to use for routine one-step allele replacement procedures. This plasmid is based on the well-established pUC vectors with a stable replicon and consistent lacZα expression,

which allows for reliable blue/ white screening of recombinants. Furthermore, this plasmid can replicate well in selective media. It was based on a high-copy number vector. The efficiency of gene replacement is high enough to make it feasible to identify mutants by direct PCR screening of individual colonies or other means of direct screening. Furthermore, this method can introduce a mutant sequence synthesized in vitro into bacterial chromosome without leaving behind any 'scars' or unwanted sequence changes from the insertion process. It is desirable to avoid scars such as introduced drug marker genes because they can have unanticipated effects on cell physiology and preclude the use of the same marker in further manipulations.

The method have been successfully used by our group and others[15] to construct various mutants in *A. tumefaciens*. Since this method uses sac B-bearing plasmids, it is imperative to monitor their Suc^S phenotype at each step during transformations. This is best done by streaking single colonies on Suc– containing medium. If this phenotype is not checked, mutations leading to spontaneous Suc^R may accumulate in the process.

Although not explicitely explored in this study, the tools described here should be widely applicable to other pathogenic and non-pathogenic bacteria provided that (i) they do not support replication of pMB1-based plasmids (ii) they are recipients of oriT-mediated conjugation and (iii) they are Suc^S upon expression of sacB[16].

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