

# A Deletion Mutant Generating by Replacement Construct in Sorbitol Dehydrogenase of *Ralstonia solanacearum* Bacterial Wilt Strain

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## ABSTRACT

A method for making precise mutations in the *Ralstonia solanacearum* genome at frequencies high enough to allow direct identification of mutants by PCR or other screening methods rather than by selection was developed. This method utilized a novel pBR322-based gene replacement vector that acted as a donor plasmid carrying the desired mutation into the target cell. Single-crossover occurred resulting in efficient replacement of the wild type allele on the chromosome by the modified sequence. The precision of this method was demonstrated by making deletion mutants in sorbitol dehydrogenase gene of *R. solanacearum*. Sorbitol dehydrogenase (*polS*) of *R. solanacearum* biovar 3 race 1 was cloned and further mutated by site direct mutagenesis by overlapping PCR to eliminate NADP binding and active sites. The deleted *polS* construct ( $\Delta polS$ ) was introduced to *R. solanacearum* by triparental mating. Homologous recombination mutants were screened based on antibiotic resistance and biochemical properties.

**Key words:** *Ralstonia solanacearum*, deletion mutants, overlapping PCR, sorbitol dehydrogenase gene

## INTRODUCTION

*Ralstonia solanacearum*, the causal agent of bacterial wilt disease, is a gram-negative, soil borne bacteria, and has a rod shape. It causes vascular wilt in more than 200 plant species in the tropics, subtropics, and temperate regions around the world. Its primary hosts include tomato, potato, tobacco, peanut, and banana (Hayward, 1995). *R. solanacearum* has been sub-classified into different biovars based on biochemical tests and host-dependent races. The pathogenicity that

causes wilt symptom is attributed mainly to its production of a high molecular mass acidic extracellular polysaccharide (EPS I), which can obstruct vascular tissues and prevent water flow (Shell, 1996). Extracellular proteins also play a major role in pathogenesis (Kang *et al.*, 1994), but individual cell wall-degrading enzymes (e.g., polygalacturonase [PGL] and endoglucanase [EGL]) are not essential, because inactivation of each individual gene only decreases the rate of wilting (Denny *et al.*, 1990). In other cases, colonization on plants by *Erwinia amylovora*, a

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pathogen of fire blight, involves in the sugar gene metabolism such as sorbitol (Aldridge *et al.*, 1997), galactose (Metzger *et al.*, 1994) and sucrose (Bogs and Geider, 2000). These sugars are required for the efficient production of exopolysaccharide amylovoran which is essential for *E. amylovora* to escape plant defense mechanisms and to cause wilt symptoms. Disruption of these sugar utilizing genes affects not only the capsule synthesis but also virulence of *E. amylovora*. Specific gene disruption mutants are crucial and necessary for identification and study of the mechanisms involved in different processes especially pathogenicity. Several methods have been used to produce mutants; insertion-duplication mutagenesis (IDM), allelic exchange (AE), and transposon mutagenesis. The preferable method is the one that can directly inactivate specific genes, which is achieved by homologous recombination (IDM and AE). Cloning and sequencing of partial sorbitol dehydrogenase gene (*polS*) of *R. solanacearum* strain TO264 were

previously reported (GenBank Ay 946241). The aim of this study was to investigate the disruption of *polS* by homologous recombination involving one crossover (IDM) and allelic exchange (AE) and utilize it as a tools to produce mutants in *R. solanacearum* by site-directed gene disruption.

## MATERIALS AND METHODS

### Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *R. solanacearum* strain TO264 isolated from tomato was used as a recipient strain in conjugation assays, whereas *Escherichia coli* S17-1 containing a derivative of the conjugative IncP plasmid RP4 served as a donor. *E. coli* S17-1 harboring plasmid pKS and helper *E. coli* HB101 harboring pRK2013 were grown on LB agar medium containing kanamycin (50 mg/ml) and incubated at 37°C for 18 h. Strain TO264 of *R. solanacearum* was grown on YP

**Table 1** Bacterial strains and plasmids used in this study.

Destination	Relevant characteristics	Source of reference
<b><i>E. coli</i></b>		
HB101	galK2 recA13 hsdS20 (r <sub>B</sub> r <sub>B</sub> <sup>-</sup> ) <i>rpsL20 proA2 xyl-5 mtl-1 supE44 ara14 lacY1</i>	(Maniatis <i>et al.</i> , 1982)
S17-1	mobilizing donor strain; <i>thi pro hsdR</i> <sup>-</sup> <i>hsdM<sup>+</sup> recA tra<sup>+</sup></i> from RP4 integrated in chromosome	(Simon <i>et al.</i> , 1989) (ATCC 47055)
DH5 $\alpha$	F <sup>-</sup> <i>RecA endA gyrA96 thi-1 hsdR17</i> $\Delta$ ( <i>lacZYA-argF</i> )	(Maniatis <i>et al.</i> , 1982)
<b><i>R. solanacearum</i></b>		
TO264	Wild-type	This work
TO264N	Nal <sup>r</sup>	This work
<b>Plasmid</b>		
pGEM-T easy	pGEM5Z'f, thymidylated <i>EcoRV</i> site, 3 kb, Ap <sup>r</sup>	Promega
pRK2013	ColE1 replicon, <i>Tra<sup>+</sup></i> -mobilizing plasmid, Km <sup>r</sup>	(Ditta <i>et al.</i> , 1980)
pK18mob	3.8 kb; <i>E. coli</i> mobilizable vector (Mob of plasmid RP4); <i>pBR322</i> replicon; Km <sup>r</sup> <i>LacZ</i>	(Schafer <i>et al.</i> , 1994) (ATCC 87095)
pKS	a 523 bp <i>EcoRI</i> fragment which is a truncate <i>polS</i> gene from pGEM-T easy cloned in pK18mob, 3.8 kb, Km <sup>r</sup>	This work

medium and incubated at 30°C for 24 h. Spontaneous nalidixic acid resistant (Nal<sup>r</sup>) colonies of *R. solanacearum* were obtained by culturing strain TO264 in YP medium containing 50 or 100 mg/ml of nalidixic acid by shaking at 28-30°C for 24 h. One hundred microliter of cell culture was spread on nalidixic medium agar and incubated at 28-30°C for 48-72 h. Nal<sup>r</sup> colonies were transferred to YP containing 100 mg/l (2x concentrated) to confirm the Nal<sup>r</sup> mutant. Antibiotics used for plasmid selection were kanamycin (50 µg/ml), and ampicillin (100 µg/ml).

### Gene replacement

The pKS mutant allele cloned into the pK18mob gene replacement vector was conjugated into *R. solanacearum* strain TO264 by the following method. The culture of the donor strain of *E. coli* S17-1 harboring pKS plasmid was mixed with culture of recipient strain of *R. solanacearum* TO264 Nal<sup>r</sup>, and helper *E. coli* HB101 harboring pRK2013 in microcentrifuge tubes with equal volume. The mixed culture was centrifuged at 4°C, 1,075 × g for 10 min. Supernatant was discarded and the pellet was resuspended in 50 µl of YP medium. The 50 µl of mixed suspension was spotted on a filter membrane (diameter 25 mm, pore size 0.45 mm), placed over YP medium and, then incubated at 30°C for 3 days before washing off the bacteria into water. Bacterial suspension of mating cells were spread on YP agar medium containing nalidixic acid and kanamycin, and incubated at 30°C for 48-72 h. Non-sorbitol utilizing mutant clones were selected and cultured in sorbitol medium (mineral medium supplemented with peptone, agar, a pH indicator, and 1% sorbitol) (Hayward, 1964). Cultures tubes were incubated at 28-30°C and the reaction was recorded for up to 30 days.

### Polymerase chain reactions

All PCRs were performed in a Perkin-

Elmer 9600 (Applied Biosystem) thermal cycler. DNA amplification was performed in a final volume of 50 µl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM of each deoxynucleoside triphosphate, 1 µM of each primer, 50 ng of DNA template, and 2.5 units of Taq DNA polymerase (Promega). The PCR mixture was incubated in the thermocycler for 5 min of initial denaturation at 94°C, followed by 30 cycles of amplification with denaturation at 94°C for 30 s annealing at 55°C for 30 s and DNA extension at 72°C for 30 s and a final extension for 7 min at 72°C. PCR products were analyzed by 0.8 % agarose gel electrophoresis in TBE buffer and viewed under UV-transilluminator.

### Generation of donor plasmid (deletion mutant alleles)

The donor plasmid pKS was used to generate deletions of *polS* genes in the *R. solanacearum* strain TO264 via homologous recombination. To construct the deletion of *polS* by overlapping PCR, sequences of the oligonucleotide primers were as follows; sorF: 5' GGC ACG CTC GCG CAA CTC ATC GA 3'; sorM: 5'GCC TGC GAA GCC ATG TTG ATG 3'; DsorM22: 5' CAT CAA CAT GGC TTC GCA GGC AAG ATC AAC GTG AAC GGG ATC G 3'; sorR: 5' AGC GTC TGA GCG GTG ATG TAA TC 3'. Overlapping PCR deletion products were constructed in two steps. In the first deletion step, two different asymmetric PCRs (sorF-sorM and ΔsorM22-sorR) were used to generate fragments from genome DNA of *R. solanacearum* strain TO264 to the left and right of the sequences targeted for deletion, respectively. The PCR conditions were as described above except that the Deep Vent DNA polymerase (Biolabs) was used instead. In the second step, the left and right fragments were joined on overlapping region and amplified by PCR as a single fragment using sorF and sorR (end). Briefly, 1 µl of each of the two asymmetric PCR mixtures and 1 µM each of the

end primers were mixed together and PCR was amplified by using Taq DNA polymerase. The construction of pKS was followed by ligating fused PCR products into pGEM-T easy. The fusion product was digested and ligated into the *Eco* RI site of pK18mob. The 523 bp deleted fragments of *polS* inserted into pK18mob was verified by restriction mapping.

### PCR analysis of mutant

The genomic DNA from the potential mutant strains was extracted and used as a template for PCR. The integration process of recombinant plasmid was identified using long PCR to amplify 5.4 kb DNA fragment with forward primer claF; 5' TGA CCG ACG CAG CCCGCATAGG 3' and reverse primer claR; 5' TGT AAG GGG CAT TCG CTC GCA TCT 3'. DNA amplification was performed in a 50  $\mu$ l reaction containing 60 mM Tris-sulfuric acid pH 9.1, 2 mM (NH)<sub>2</sub>SO<sub>4</sub>, 2 mM MgSO<sub>4</sub>, a set of 0.2 mM dNTP, 1  $\mu$ M of each primer, 50 ng of DNA template, and 1  $\mu$ l of mixed Taq : Deep Vent (Biolab) in the ratio 20:1. PCR condition was 50 s at 94°C, 50 s at 60°C, and 4 min at 72°C. All experiments used 30 cycles of amplification including an initial denaturation at 95°C for 5 min and a final extension at 72°C for 7 min. The  $\Delta polS$  inserted mutants derived from double crossing gene replacement event were screened by PCR using sorS and sorR primers to generate 2 types of PCR fragment, 523 bp for deletion mutant strains and 623 bp fragment for wild-type strains. DNA amplification was done as previously described.

### Preparation and manipulation of DNA

Plasmid DNA was purified by alkaline-lysis method (Birnboim and Doly, 1979). Isolation of chromosomal DNA of *R. solanacearum* was done using the Puregene P kit (Invitrogen Inc., Minneapolis, MN) as described by the manufacturer. For restriction endonucleases, T<sub>4</sub> DNA ligase, and modification enzymes were

used as recommended by the manufacturers. DNA restriction fragments were isolated from 0.5% agarose gels by using NaI/SiO<sub>2</sub> method (Yue and Orban, 2001). Transformation of *E. coli* strains was carried out by the calcium chloride method (Hanahan, 1983).

### Hybridization experiments

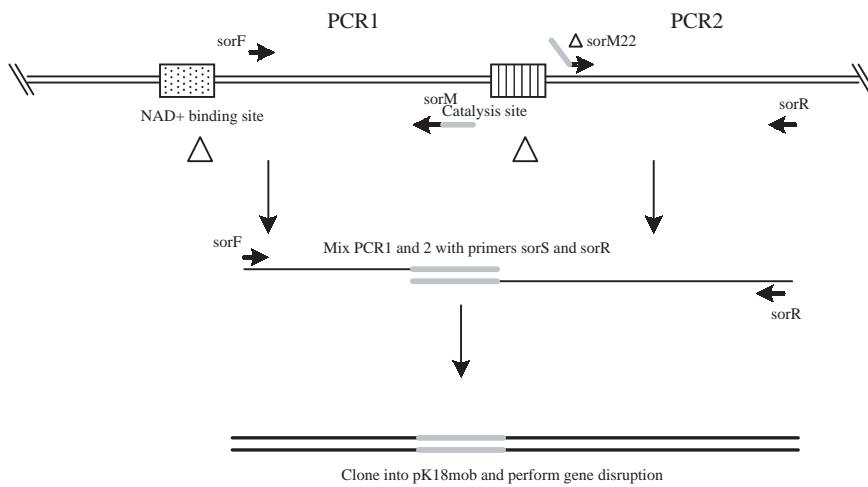
To confirm the integration of the mobilizable plasmids into genome, total DNAs of transconjugant clones were isolated and digested with selected restriction enzymes. Restriction fragments were separated by 0.5% agarose gel electrophoresis and transferred to a nylon membrane. Labeling and hybridization of DNA were performed with the nonradioactive digoxigenin-11-dUTP (Roche). DNA labeling and detection kit (Roche) were used according to the manufacturer's manual. The signal was detected with chemiluminescence (CDP Star) according to the supplier's recommendation.

## RESULTS AND DISCUSSION

Construction of plasmid, (pKS) for insertion-duplication mutagenesis (IDM) *R. solanacearum* was generated. The integrating plasmid pKS was obtained by cloning the 523 bp *Eco*RI fragment corresponding to truncated fragment of *polS* gene into pK18mob containing oriT which was necessary for plasmid mobilization (Table 1). The method for creating precisely engineered deletion of *polS* gene was achieved by overlapping PCR (Horton *et al.*, 1990) (Figure 1A). An NAD binding site, G-X-X-X-G-X-G and the catalytic site motif at Y-X-X-X-K of enzyme function were deleted therefore resulted in nonsense mutation (Figure 1B).

Recombinant plasmids used for conjugation in this work carried the kanamycin resistant gene (Km<sup>r</sup>) and a truncated copy of a *polS* gene for disruption. Thus, there was a potential integration site in the endogenous *polS* gene of *R.*

(A)



(B)

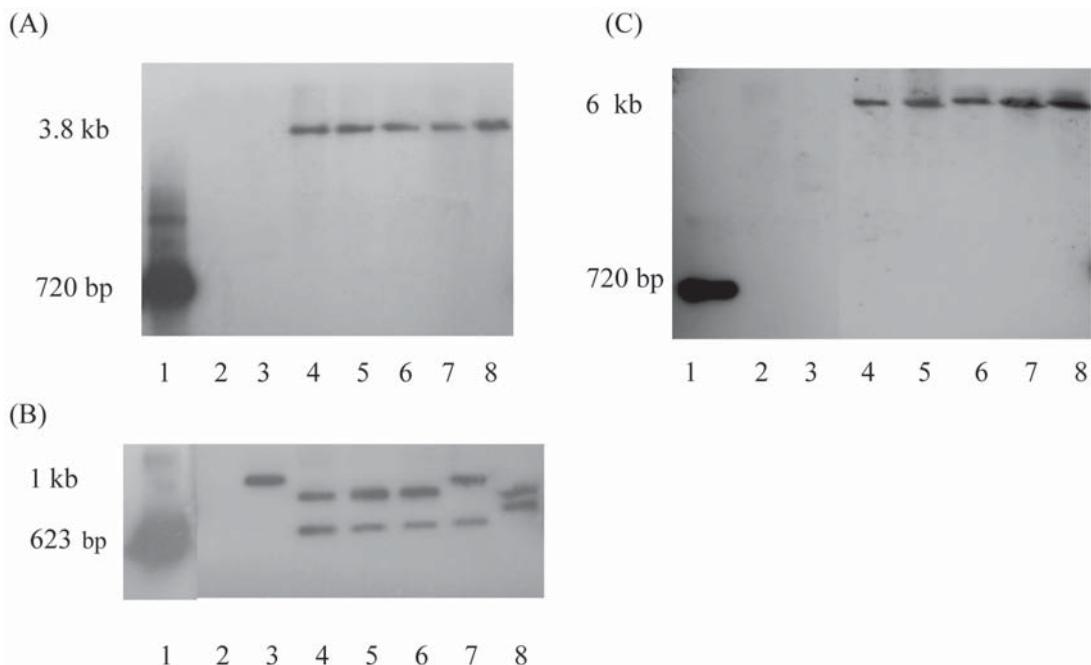
**NAD<sup>+</sup> binding site**

1	L Q D K V A I L T G A A S G I G E A V A Q R Y L E
	TTGCAGGACAAGTCGCGATCCTGACAGGGCGAGCCAGCGGCATCGCGAAGCGGTGCGCAACGCTATCTGGAA
76	A G A R C V L V D L K P A G G T L A Q L I E T H P
	CGGGGCCACGCTGCGTCTCGATTGAAACCCGGCGGCAAGCTCGCGCAACTCATCGAAACGCATCCC
151	D R A F A L S A D V T K R D D I E R I V S A A V E
	GACCGTGCCTCCGCTGTCGGCACGTCAACGAAACGTGACGATATCGAGGCCATCGTCTCGCTGCGTCGAG
226	R F G G I D I L F N N A A A F D M R P L L D E A W
	CGTCTGGCGGACATCGACATCTGTTCAACACGCGGCCGTTGACATGCCCTCGTGTGCTCGACGAAGCTGG
301	E V F D R L F A V N V K G M F F L M Q A V A Q R M
	GAGGTGTCGACCGCTGTTCGCGTCAACGTAAGGGCATGTTCTCTGATGCAGGGTTGCACAGCGGATG
376	A A Q G R G G K I I N M A S Q A G R R G E A L V S
	GGCGCAGGGACGGCGGCCGCAAGATCATCACCATGGCTTCGAGGCCGGCGCGCGAGGGCTGGTGTGCG
451	H Y C A T K A A V I S Y T Q S A A L A L A P Y K I
	<b>CACATTGCGCACGAAGCGGGCGTCATCACGTATACGCACTCGGCTGCACTGGCTCGCGCGTACAAGATC</b>
526	N V N G I A P G V V D T P M W E Q V D A L F A R Y
	AACGTGAACGGGATCGCGCCGGCGTCGTCGACACGCCGATGTTGGGGAGCAGGTCGACGCCGCTGTCGCGCGCTAT
601	E N R P L G E K K R L V G E A V P L G R M G V P A
	GAGAAATGCCCACTGGTGAAGAGAGCGTCTGGTGGTGGAGCGCTGGCGGTACCGCTGGCCGGATGGGGTGGCG
676	D L T G A A L F L A S T D A D Y I T A Q T L N V D
	GACCTGACGGCGCCGCGCTGTTCTCGCGTCAACGACGCTGATTACATCACCGCTCAGACGCTGAACGTCGAC
751	G G N W M S *
	GGCGCAACTGGATGAGCTGA

**Figure 1** Schematic chart for generating deleted *polS* constructs (A). The top line represents a region of the *polS* gene on the chromosome. The two PCRs used to generate fragments (PCR1 and PCR2) to form deletion of catalytic site and NAD<sup>+</sup> binding site when fused. The PCR primers sorM and ΔsorM are complementary over 22 nucleotides (represented by the light gray lines) so that when the two PCR products are mixed, the complementary regions anneal and prime at the 3' overlapping region for a 3' extension of the complementary strands. In the second line, the fused molecule is amplified by PCR with primers sorF and sorR. The nucleotide sequence of endogenous *polS* gene was deleted by overlapping PCR method (shown as bold letters) generating a nonsense mutation (B). Bold letters with a dotted underline represent catalytic sites deleted in mutant. Underlined sequences are sorF and sorR primers.

*solanacearum* chromosome by homologous recombination. Screening for transconjugants occurring integration process was done in two steps; first by kanamycin resistance that was introduced into the chromosome by the plasmid and second by Southern blot hybridization to confirm integration of the plasmid into the chromosome by using *kan* or *sorS* probe that were designed from  $\text{Km}^r$  and *polS* gene region. The use of *kan* probe, a 3.8 kb *Bam*HI-*Eco*RI fragment which was the size of pK18mob plasmid without truncated *polS* gene insertion was observed (Figure 2A). Similarly, the 6 kb of DNA extracted from transconjugants and partially digested with *Cla*I corresponded to the *kan* gene in the plasmid (Figure 2C). When genomic DNA of transconjugant clones and wild type were digested with *Bam*HI

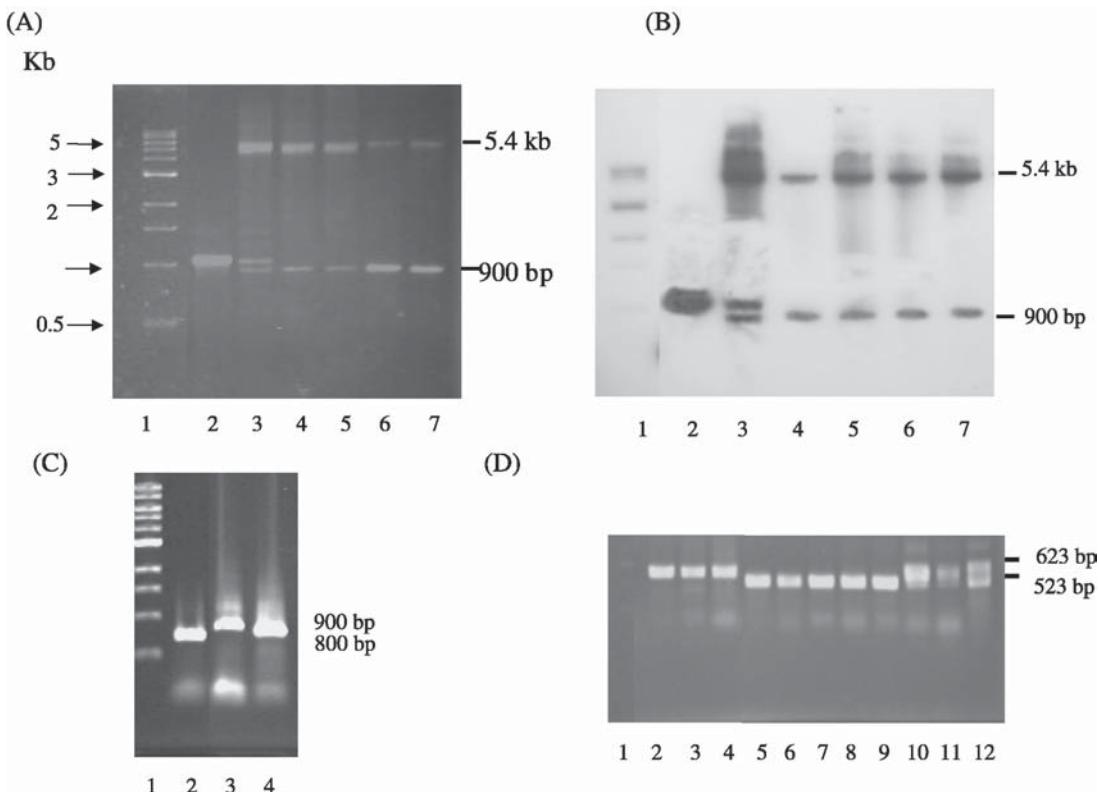
and *Eco*RI, the use of *sorS* probe could detect two fragments of 600 bp and 800 bp (Figure 2B lanes 4-6), 600 bp and 900 bp (Figure 2B lane 7), and 700 bp and 800 bp (Figure 2B lane 8) while a 1 kb fragment was derived from wild-type (Figure 2B lane 3). These results proved that the integration of entire plasmid into chromosome occurred at various sites of *polS* gene regions. The integration process was also confirmed by using specific primer *claF* and *claR* which located at upstream and downstream of *polS* gene. The 1 kb fragment was observed in the wild-type (Figure 3A lane 2), while a 5.4 kb fragment was detected from transconjugant clone (Figure 3A). The 600 and 800 bp fragments were amplified by two sets of primers as *claF-mobR* and *claR-mobF*, respectively when using the 5.4 kb fragment as a



**Figure 2** Southern blot hybridization between the *kan* probe (A) and *sorS* probes (B) with *Bam*HI-*Eco*RI- partially digested DNA extracted from the wild type strain and transconjugant clones; *kan* probe and *sorS* probe (lane1), PO1155 (negative strain; lane2), TO264 (positive strain; lane3), and transconjugant clones (lanes 4-8). (C) Southern blot hybridization between *kan* probe (lane 1) and *Cla*I partially digested DNA extracted from the wild type strain and transconjugant clones as described in (A).

template (Figure 3C). These data indicate that the entire plasmid was integrated into the chromosome by homologous recombination at the *polS* gene. This event demonstrated that the insertion-duplication mutagenesis (IDM) occurred at a

specific *polS* gene (Figure 4A). Mutagenesis involves circular integration, by a single crossover event, between the targeted chromosomal gene and a truncated variant cloned in a transient suicide or a replicative plasmid, resulting in an integration

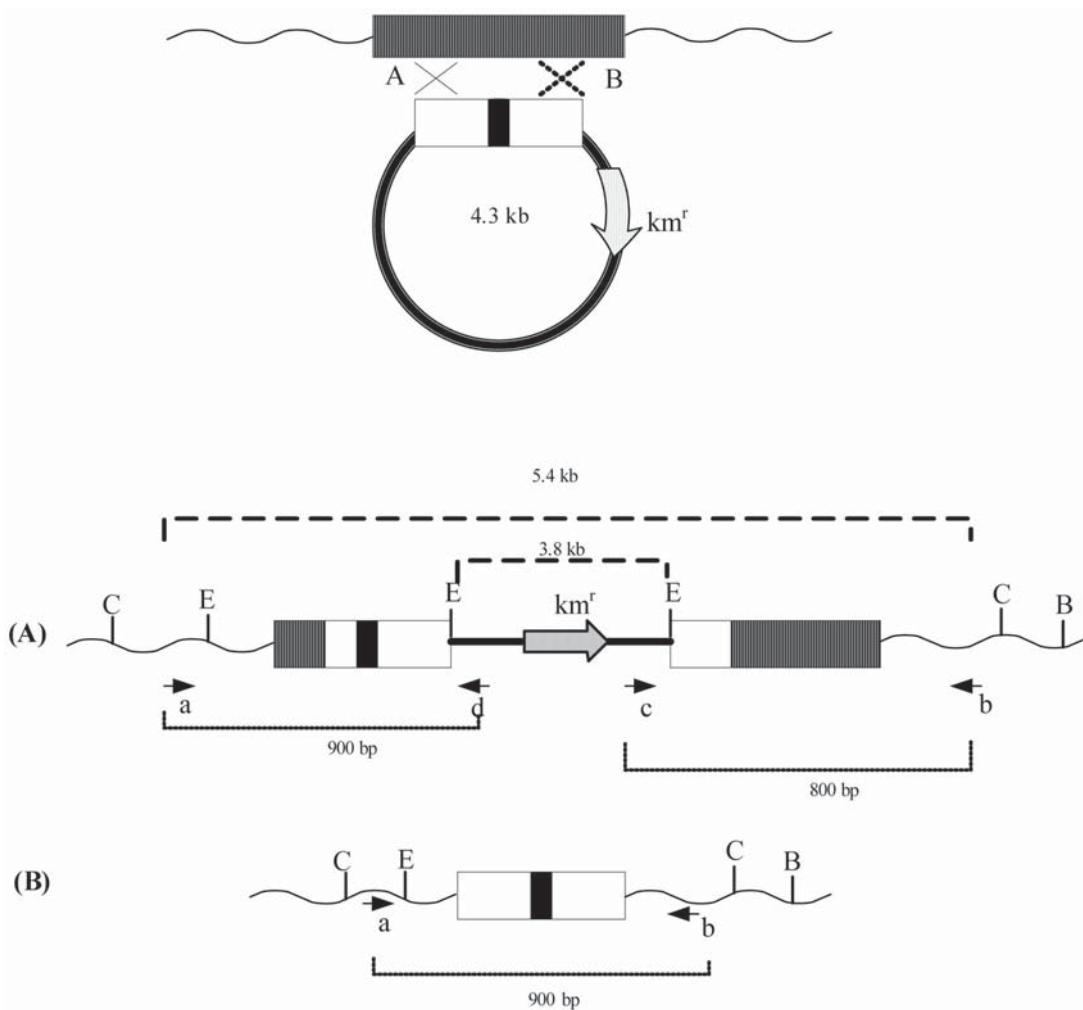


**Figure 3** Agarose gel electroporesis of PCR products amplified by various primers are located upstream and downstream of endogenous *polS* gene (claF and claR) and are located on plasmids (mobF-mobR and kmF-kmR). Long PCR amplification of the wild type (lane 2) and transconjugant clones (lanes 3-7) using primers claF and claR (A) and Southern blot hybridization of long PCR products as described in A (B) PCR product amplified by using kmF and kmR primers (lane 2), claF-mobR (lane 3), claR-mobF (lane 4) when 5.4 kb fragment was used as template (C). Sequences of primers are as follows: kmF: (5' GAT GGA TTG CAC GCA GGT TCT C 3') and kmR: (5' GTA AAG CAC GAG GAA GCG GTC AG 3') used to amplify kanamycin resistance gene, mobF (5' ATG CTT CCG GCT CGT ATG TTG TGT 3') and mobR (5' GCT GGC GAA AGG GGG ATG TGC T 3') are located on pK18mob vector. (D) PCR product amplified by using sorF and sorR located on *polS* gene. The 623 bp internal fragment of *polS* gene (nucleotides 118 to 740) were amplified from the wild type strain TO264, Toud3 and Toud3-N indicated as biovar 3, respectively (lanes 2-4). The 523 bp of deleted construct fragments were amplified in non-oxidizing sorbitol clones (lanes 5-9). Both 623 and 523 fragments of the PCR product were obtained in oxidizing sorbitol clones (lanes 10-12).

of the entire plasmid and a duplication of the target sequence. IDM has been used to disrupt genes in a variety of other organisms, such as *Mycobacterium smegmatis* (Baulard *et al.*, 1996), *Neisseria gonorrhoeae* (Hamilton *et al.*, 2001), *Streptococcus pneumoniae* (Lee *et al.*, 1989), and *Lactobacillus sake* (Leloup *et al.*, 1997).

The PCR fragment from the oxidizing

sorbitol transconjugant showed the 5.4 kb, 1 kb, and 900 bp fragments when amplified with *claF* and *claR* primers (Figure 3B lane 3). Although IDM occurred (5.4 kb), all transconjugant clones could still oxidize sorbitol. This event suggested that the endogenous *polS* located in the 1 kb fragment was still functional. Transconjugant clones that did not oxidize sorbitol showed the 5.4



**Figure 4** Generation of a non-polar insertion mutation. IDM resulted in a single crossover recombination event, insertion of the plasmid into the chromosome, and duplication of homologous sequences (A). AE resulted in the replacement of the endogenous gene by its copy disrupted by an overlapping PCR (B). The position of primers for PCR are shown as a; *claF*, b; *claR*, c; *mobF*, d; *mobR*; thick line indicated vector, vertical line in box is endogenous gene, dark box is deletion site, E; *Eco*RI, C; *Clal*, B *Bam*HI.

kb and 900 bp fragments were located on a truncated *polS* gene (Figure 3B lane 4-7). The amplification of long PCR products using *claF* and *claR* primer clearly elucidated that the allelic exchange (AE) occurred resulting in the replacement of the endogenous *polS* gene by the deleted construct (900 bp). For this event to occur homologous recombination with double crossovers was required (Figure 4B).

Non-oxidizing sorbitol mutants produced only the 5.4 kb and 900 bp from PCR deletion (Figure 3A lanes 4-7). This AE event was confirmed by sequencing of 900 bp fragment containing deleted regions of the *polS* gene (Figure 1B). Southern blot hybridization of the PCR product hybridized with *sorS* probe also confirmed the occurrence of AE (Figure 3B lanes 4-7). When 5.4 kb, 1 kb and 900 bp fragments were probed with *sorS* (Figure 3B lane3), the 1 kb disappeared. This 1 kb fragment is believed to have contained *polS* and thus disappeared after AE took place (Figure 3B lanes 4-7). The AE process was also confirmed by PCR, 623 bp products were observed in the wild type strain while 523 bp products were observed in the non-oxidizing sorbitol mutants when *sorF* and *sorR* primers were used. On the other hand, the oxidizing sorbitol transconjugant showed both PCR products (Figure 3D). Therefore, the sizes of these fragments (5.4 kb and 900 bp fragments from long PCR and 523 bp from PCR) indicated that the mutants were heterogeneous, comprising of cells containing the *polS* gene disrupted by IDM and cells with the *polS* gene disrupted by AE (Figure 4). These results clearly showed the occurrence of a double crossover event in *R. solanacearum* and suggested that it was possible to produce mutants through specific gene disruption by AE in this bacterium. From this experiment, the frequency of the second crossover event seemed to be very low. A high selective pressure using the *sacB* gene (lethal on sucrose containing media) might improve the frequency of double crossover in *R. solanacearum*.

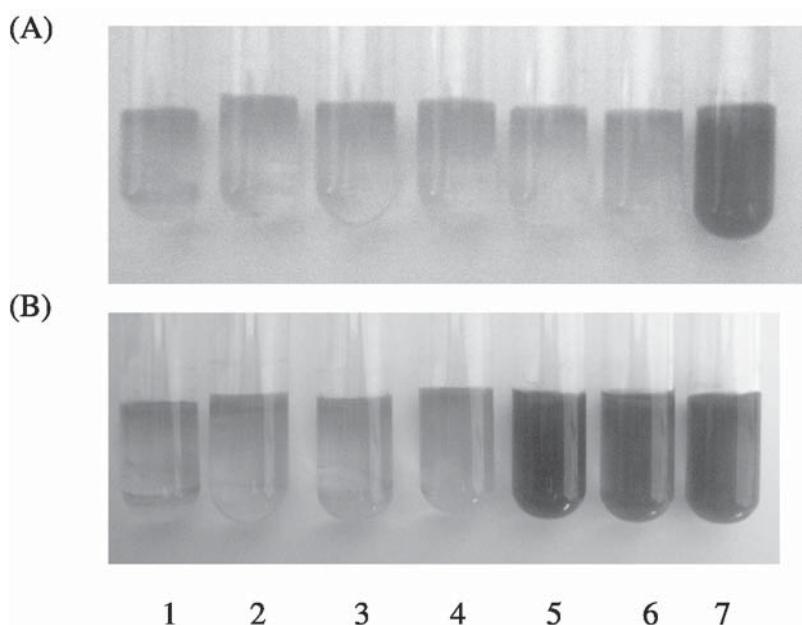
Isolation of defined deletion mutants is still a relative tedious process. It involves construction of deletion alleles, most often tagged with an antibiotic resistant gene, in a suicide plasmid, followed by recombination of the plasmid-borne deletions into the chromosome, usually after conjugal transfer of the suicide plasmid (Schweizer and de Lorenzo, 2004). The AEs arising from chromosomal integration of the suicide plasmid are resolved by utilization of counter-selectable markers, most often *Bacillus subtilis* *sacB* (sucrose counterselection) (Schweizer, 1992) or less frequently, *rpsL* (streptomycin counter-selection) (Stibitz, 1994). In the case where antibiotic selection markers are flanked by site specific recombination sites, e.g., the Flp recombinase target (*FRT*) (Hoang *et al.*, 1998) or Cre recombinase (*loxP*) site (Quenee *et al.*, 2005), they can subsequently be deleted from the chromosome resulting in deletion mutants.

A total of 1,000 transconjugants were screened on 1% sorbitol medium and only 1 % were non-oxidizing sorbitol mutants. The efficiency of this experiment was low because the tri-parental mating method only transferred low copy of the deleted construct plasmid. If the deleted construct plasmids were electroporated directly to the host cell, the efficiency of gene replacement would be high enough to make it feasible for identification of mutants by direct PCR screening of individual colonies or other means of direct screening. The replacement vector pK18mob possesses some unique features which make it easy to use for routine one-step allele replacement procedures. This plasmid is based on the well-established pBR322 vectors with a stable replicon and consistent *lacZα* expression which allows for reliable blue/white screening for recombinants. Furthermore, this plasmid can replicate well in selective media and produce high-copy number vectors.

In order to elucidate that the *polS* gene was disrupted by insertion of the recombinant

plasmid and thus eliminated its oxidizing function, the clones were tested for their ability to oxidize three disaccharides: lactose, maltose, and galactose; and three hexose alcohols: mannitol, sorbitol, and dulcitol. The mutant was unable to oxidize not only sorbitol, but also dulcitol, whereas other sugars were not affected as shown in (Figure 5). From these data, the IDM could affect gene function because insertion of the entire plasmid might disrupt *polS* and other genes downstream. Similarly, AE generated mutation by deleted catalytic site and NAD binding site and therefore, these could disrupt endogenous *polS* gene function as well. The phenotype data showed that site direct mutagenesis by overlapping PCR was capable of creating a mutation at specific sorbitol gene and also disrupted the gene function. The mutations affected oxidizing property of not only sorbitol but also dulcitol. This might imply that sorbitol dehydrogenase could oxidize both sugar but not mannitol.

In these experiments, specific gene replacement mutation with two crossovers event was achieved at a very low rate but function disruption was clearly demonstrated. This method was successfully used in the study to construct mutants in *R. solanacearum* and other microorganisms such as *Xylella fastidiosa* (Gaurivaud *et al.*, 2002), *Xanthomonas campestris* pv. *campestris* (Katzen *et al.*, 1999), *Agrobacterium tumefaciens* (Suksomtip and Tungpradabkul, 2005). Although not explicitly explored in this study, the tool described here should be widely applicable to other pathogenic and non-pathogenic bacteria. The complete genome sequence of *R. solanacearum* is now available, which opens many new experimental avenues (Salanoubat *et al.*, 2002). The ability to make precise genetic modifications to the bacterial chromosome and to study the resulting phenotypic behaviors is very important for functional studies. This study presented, for the first time, a method



**Figure 5** Biovar test between wild type strain (TO 264) and mutant. For disaccharide sugars, lactose, maltose, and cellobiose were used in tube 1-3, respectively (A). For hexose alcohol sugar, mannitol, sorbitol, and dulcitol were used in tube 4-6, respectively, biovar medium without any sugar was used as control (tube 7; B).

to introduce a desired mutant sequence, synthesized *in vitro*, into a microorganism's chromosome without leaving behind unwanted sequence changes from the insertion process. It is therefore a model to study other plant pathogenicity genes in *R. solanacearum*.

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