4. Construction of Plasmid for Insertion-Duplication Mutagenesis (IDM)

Construction of plasmid (pKS) for insertion-duplication mutagenesis (IDM) of *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 mobilizing as shown in Table 2. The method for creating precisely engineered deletion of *polS* gene known as overlapping PCR (Figure 8A). About 100 bp were deleted from the 623 bp fragment which was amplified by using sorF and sorR primers. A NAD binding site, G-X-X-X-G-X-G and the active site motif at Y-X-X-X-K of enzyme function were eliminated therefore generating nonsense mutation as shown in Figure 8B.

5. Conjugation pKS into *R. solanacearum* and Detection for IDM and AE by PCR and Hybridization Analysis

Plasmid pKS containing truncated *polS* gene which deleted a coenzyme (NAD) binding and catalytic site was transconjugated into *R. solanacearum* To-Ud3 wild type strain that showed highly virulence to susceptible tomato. The mutation by IDM process involved circular integration, by single crossover event, between the targeted chromosomal gene and a truncated copy of this gene cloned in a transient suicide or replicative plasmid, resulting in integration of the entire plasmid and duplication of the target sequence as showed in Figure 11A and AE processes results in the replacement of the endogenous gene by its copy disruption. This event involves homologous recombination with two crossovers (Figure 11B).

Recombination plasmid used for conjugation in this work carried the kanamycin (Km^r) selectable marker plasmid and a truncated copy of a *polS* gene for disruption. Thus, there is a potential integration site in the endogenous *polS* gene of *R. solanacearum* chromosome by homologous recombination. Screening of transconjugants resulting from integration process was initially selected by their resistance to a kanamycin marker introduced into the chromosome from the plasmid,

and then Southern blot hybridization was performed to confirm recombination events using *kan* or *sorS* probe.

The Use of *kan* probe, the 3.8 kb *Bam*HI-*Eco*RI fragment which is the size of pK18mob without truncated *polS* gene insertion was observed (Figure 9A). Similarly, the 6 kb of DNA fragment derived from transconjugants genomic DNA which partially digested with *Cla*I hybridized to the *kan* probe (Figure 9C). The *sorS* probe and chromosome of transconjugant clones restricted with *Bam*HI-*Eco*RI, these two fragments as proximately 800 bp and 600 bp (Figure 9B lane 4-6), 900 bp and 600 (Figure 9B lane 7), and 800 and 700 (Figure 9B lane 8) were observed by comparing with the 1 kb fragment derived from wild-type (Figure 9B lane3). These results elucidated that the integration event of entire plasmid into chromosome occurred at various site 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 wild-type (Figure 10A lane 2), in contrast to transconjugant clones, the 5.4 kb fragment was detected as shown in Figure 10A. The 800 and 600 bp fragments were amplified by two sets of primer as claF-mobR and claR-mobF (mobF and mobR located on plasmid), respectively when using 5.4 kb fragment as template (Figure 10C). These data indicated that the entire plasmid is integrated into the chromosome by homologous recombination at *polS* gene. This event demonstrated that insertion-duplication mutagenesis (IDM) occurred at specific *polS* gene (Figure 11A). This mutagenesis involves circular integration, by single crossover event, between the targeted chromosomal gene and a truncated copy of this gene cloned in a transient suicide or replicative plasmid, resulting in integration of the entire plasmid and duplication of the target sequence.

The PCR fragment from oxidizing sorbitol transconjugant showed the 5.4 kb, 1 kb, and 900 bp fragments when amplifying with claF and claR primers (Figure 10A lane 3). Although IDM occurred (5.4 kb), all transconjugant clones can oxidize sorbitol. This event could explain that endogenouse *polS*, located at 1 kb fragment, is

still function. However, some transconjugant clones showed non-oxidizing sorbitol observed only 5.4 kb and 900 bp fragments which verified the truncated *polS* gene (Figure 10A lane 4-7). The amplification of long PCR product using claF and claR primers clearly indicated that the allelic exchange (AE) was generated resulting in the replacement of the endogenous *polS* gene by the deleted construct (900 bp), resulting from homologous recombination with two crossovers (Figure 11B).

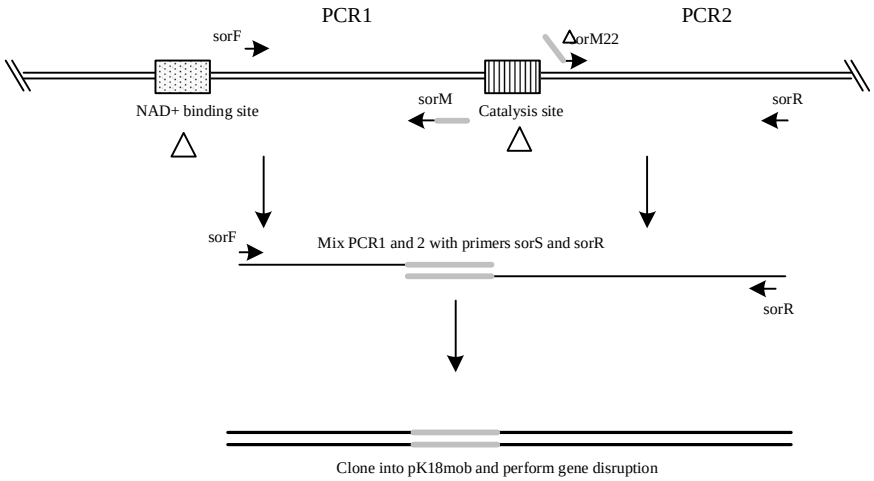
Non-oxidizing sorbitol mutants produced only the 5.4 kb and 900 bp from PCR detection (Figure 10A lane 4-7). This AE event was confirmed by sequencing of 900 bp fragment containing deletion regions of *polS* gene (Figure 8B). The Southern blot hybridization of PCR product hybridized with *sorS* probe was confirmed AE (Figure 10B lane 4-7). The 5.4 kb, 1 kb and 900 bp fragments were probed with *sorS* (Figure 10B lane3) and 1 kb which may contain endogenous *polS* gene was absent after AE occurring (Figure 10B lane 4-7). The AE process also was confirmed by PCR reaction, 623 bp fragments of PCR products were observed in wild type strain but in non-oxidizing sorbitol mutants observed 523 bp fragments deleted construct when using sorF and sorR as primers. However, oxidizing sorbitol transconjugant showed both PCR products (Figure 10D) because of its existence of endogenous *polS* gene.

Therefore, the size of these fragments (5.4 kb and 900 bp fragments from long PCR and 523 bp from PCR) indicated that the mutants have a heterogeneous population with cells containing the *polS* gene disrupted by IDM and cells with the *polS* gene disrupted by AE as concluded in Figure 11. These results clearly showed the occurrence of a double crossover event in *R. solanacearum* and that it is possible to produce mutants through specific gene disruption by AE in this bacterium.

Figure 8 (A) The creation of deletion constructs. The top line represents a region of the *polS* gene on chromosome. The two PCRs used to generate fragments (PCR1 and PCR2) which will form deletion of catalysis site and NAD^+ binding site when fused. The PCR primers sorM and • sorM22 were complementary over 22 nucleotides (represented by the light gray lines) so that when the two PCR products were mixed, the complementary regions anneal and prime at the 3*f*overlapping region for a 3*f* extension of the complementary strand. In the second line, the fused molecule was amplified by PCR with primers sorF and sorR.

(B) The nucleotide sequence of endogenous *polS* gene was deleted by overlapping PCR method showed as bold letter generating nonsense mutation. Bold letters with dot underline represented catalysis site which was deleted in mutant. Sequences with underline were sorF and sorR primers, respectively.

(A)



(B)

NAD+binding site

```
      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
1     TTGCAGGACAAGGTCGCGATCCTGACAGGGGCAGCCAGCGGCATCGGCGAAGCGGTCGCGCAACGCTATCTGGAA
      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
76    GCGGGCGCACGCTGCGTGCTCGTCGATTTGAAACCCGCGGGCGGCACGCTCGCGCAACTCATCGAAACGCATCCC
      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
151   GACCGTGCGTTCGCGCTGTCCGCCGACGTCACGAAACGTGACGATATCGAGCGCATCGTCTCGGCTGCGGTCGAG
      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
226   CGTTTCGGCGGCATCGACATCCTGTTCAACAACGCGGCCGCGTTCGACATGCGTCCGTTGCTCGACGAAGCCTGG
      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
301   GAGGTGTTCGACCGGCTGTTCGCGGTCAACGTGAAGGGCATGTTCTTTCTGATGCAGGCGGTTGCACAGCGGATG
      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
376   GCGGCGCAGGGACGCGGCGGCAAGATCATCAACATGGCTTCGCAGGCCGGCCGGCGCGGCGAGGCGCTGGTGTCG
      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
451   CACTATTGCGCGACGAAGGCGGCGGTCATCAGCTATACGCAGTCGGCTGCACTGGCGCTCGCGCCGTACAAGATC
      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
526   AACGTGAACGGGATCGCGCCGGGCGTCGTCGACACGCCGATGTGGGAGCAGGTCGACGCGCTGTTCGCGCGCTAT
      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
601   GAGAATCGCCCACTCGGTGAGAAGAAGCGTCTGGTCGGTGAGGCGGTACCGCTCGGCCGGATGGGGGTGCCGGCT
      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
676   GACCTGACGGGCGCCGCGCTGTTTCTCGCGTCGACCGACGCTGATTACATCACCGCTCAGACGCTGAACGTCGAC
      G  G  N  W  M  S  *
751   GGCGGCAACTGGATGAGCTGA
```

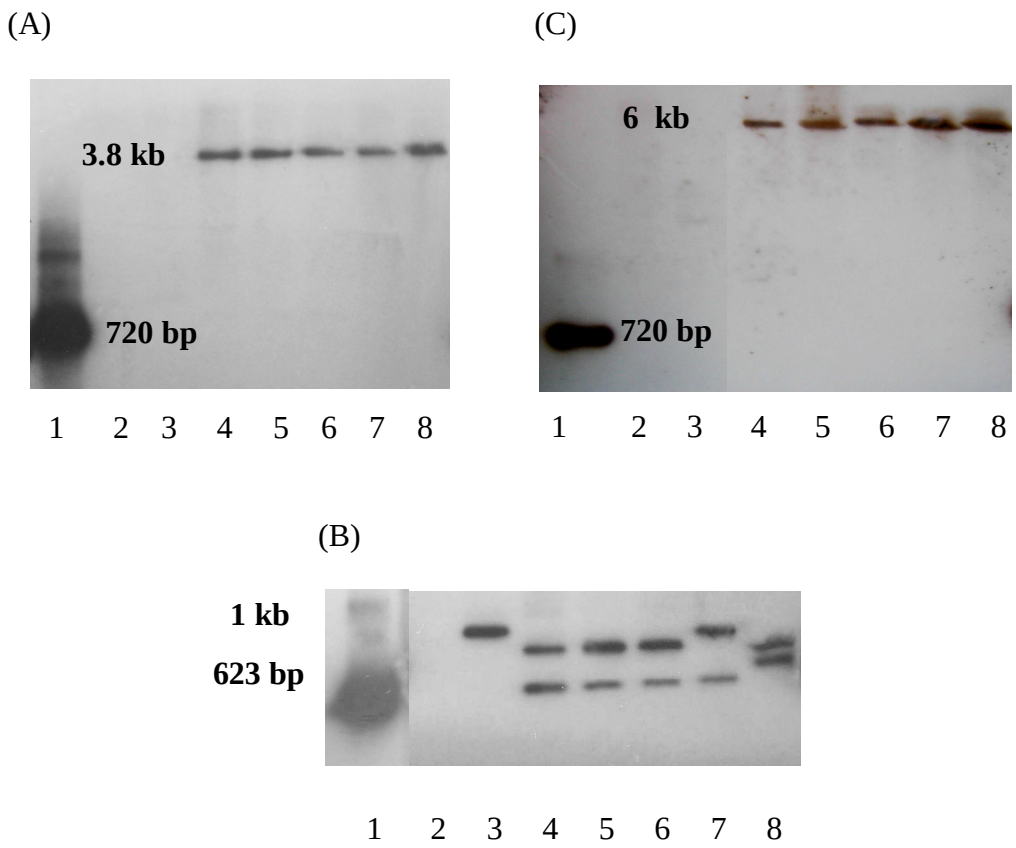


Figure 9 Southern blot hybridization between *kan* probe (A) and *sorS* probe (B) with *Ba*mHI-*Eco*RI- partially digested DNA extracted from wild type strain and transconjugant clones; *kan* probe and *sorS* probe (lane1), PO1155 (negative strain) (lane2), To-Ud3 (positive strain) (lane3), and transconjugant clones (lanes 4-8). (C) Southern blot hybridization between *kan* probe (lane 1) and *Cla*I partially digested DNA extracted from wild type strain and transconjugant clones as described above in (A).

Figure 10 Electroporesis gel of PCR product amplified by various primers located upstream and downstream of endogenous *polS* gene (claF and claR), located on plasmid (mobF-mobR and kmF-kmR) and located on *polS* gene (sorF and sorR)

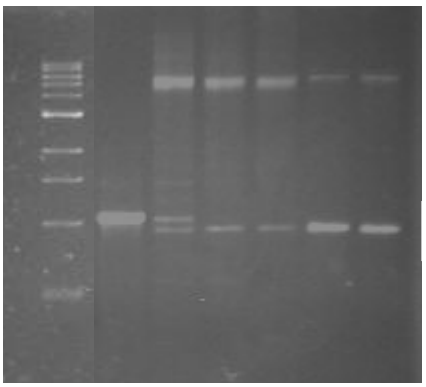
(A) Long PCR amplification of wild type (lane2) and transconjugant clones (lanes 3-7) using primers claF and claR

(B) Southern blot hybridization of long PCR product as described in (A).

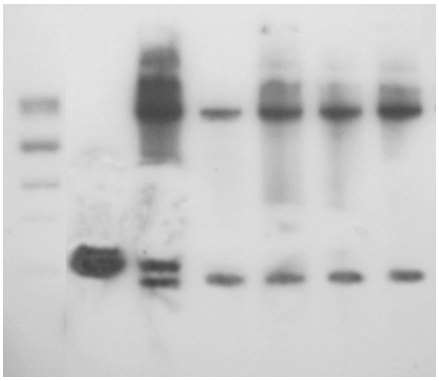
(C) Gel electrophoresis of PCR product was amplified by kmF and kmR primers (lane 2), claF-mobR (lane 3), claR-mobF (lane 4) when 5.4 kb fragment was used as template. The kmF and kmR primers were used to amplify kanamycin resistance gene, mobF and mobR primers were located on pK18mob vector.

(D) Gel electrophoresis of PCR product PCR product was amplified by sorF and sorR primers which located on *polS* gene. The 623 bp internal fragment of *polS* gene (nucleotides 118 to 740) were amplified from wild type strain To264, To-Ud3 and To-Ud3-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 PCR product were obtained in oxidizing sorbitol clones (lanes 10-12).

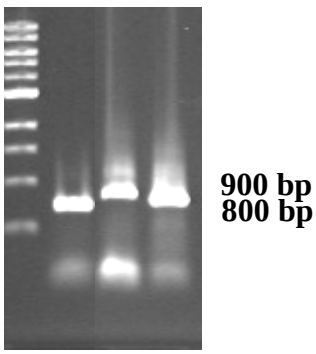
(A)


5.4 kb
900 bp
1 2 3 4 5 6 7

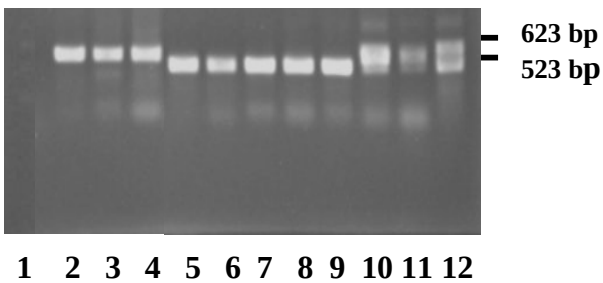
(B)


1 2 3 4 5 6 7

(C)


900 bp
800 bp
1 2 3 4

(D)


623 bp
523 bp
1 2 3 4 5 6 7 8 9 10 11 12

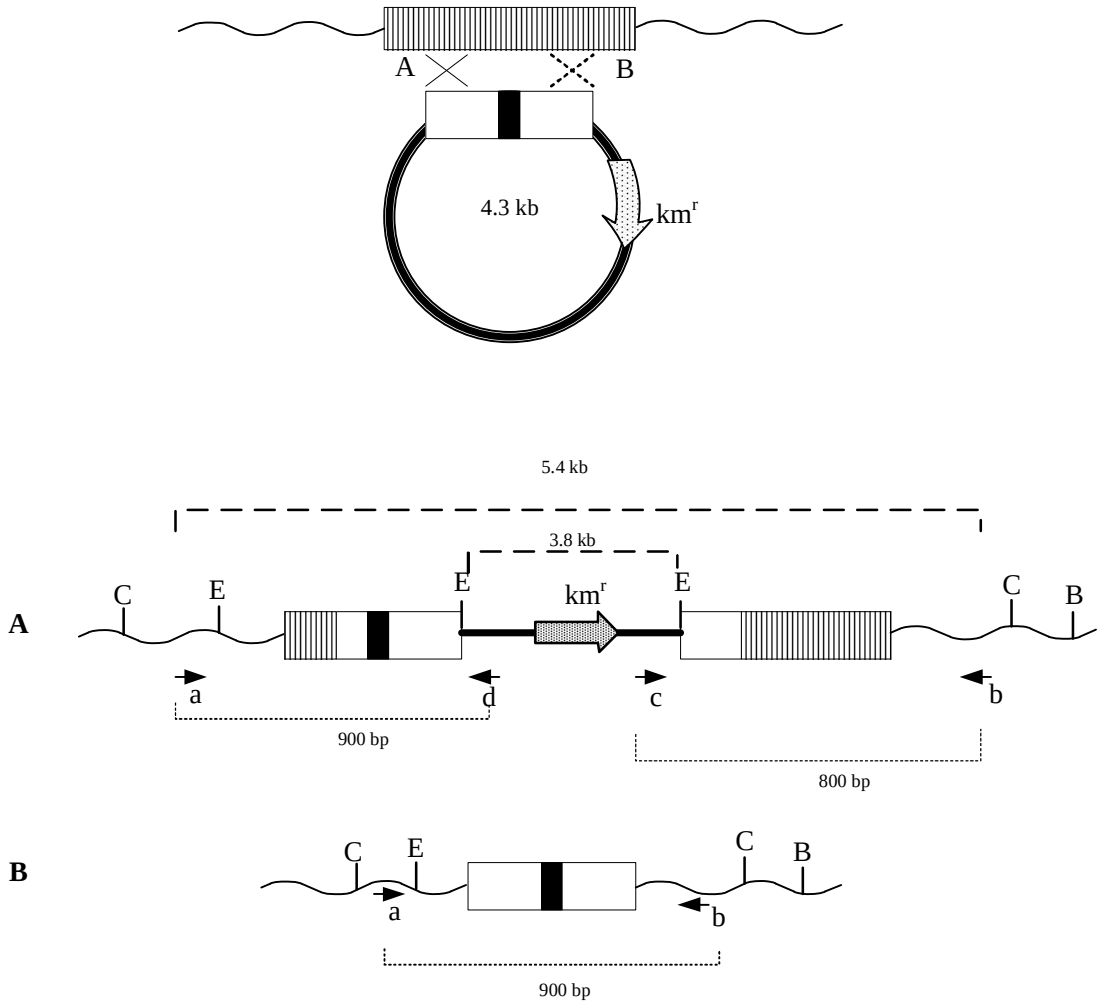


Figure 11 Generation of a non-polar insertion mutation.

(A) Insertion-duplication mutagenesis resulted in a single crossover recombination event, insertion of the plasmid into the chromosome, and duplication of homologous sequences.

(B) Allelic exchange resulted in the replacement of the endogenous gene by its copy disrupted by overlapping PCR. The position of primers for PCR are shown as a; claF, b; claR, c; mobF, d; mobR; thicken line indicated vector, vertical line in box is endogenous gene, dark box is deletion site, E; *Eco*RI, C; *Cla*I, B *Bam*HI

6. Biovar Test and Enzyme Activity Assay for Mutation

In order to elucidate that *polS* gene was disrupted by insertion of the recombinant plasmid and eliminated oxidizing property, the clones were tested for ability to oxidize three disaccharides (lactose, maltose, and galactose) and three hexose alcohols (mannitol, sorbitol, and dulcitol) for three times.

The mutant was unable to oxidize not only sorbitol but also dulcitol whereas other sugars were not affected as shown in Figure 12. From these data, IDM could affect gene function because insertion of entire plasmid might disrupt *polS* and other genes downstream. Similarly, AE generated mutation by deleted active site and NAD binding site construct; therefore, these could disrupt endogenous *polS* gene function.

The sorbitol dehydrogenase activity from wild type and non-utilizing sorbitol mutants was carried out by assaying sorbitol activity in crude extract to confirm biovar test result. Sorbitol dehydrogenase can oxidize sorbitol to D-fructose in the presence of NAD^+. The absorbance of NAD^+ to NADH was detected at OD_{340} nm. When crude extract from wild type was added into reaction mixture, the absorbance was gradually increased whereas crude extract from mutants were slowly increased as shown in Figure 13. Comparing with wild type, specific activity was largely reduced about sixty folds in mutants. Enzyme activity of wild type was higher than biovars 1 and 2 at thirty folds (Table 6). It was the same result obtaining from biovar test that biovars 1 and 2 were unable to oxidizing sorbitol sugar.

From enzyme assay, it was postulated that mutation by homologous recombination at specific *polS* gene can abolish gene function of wild type.

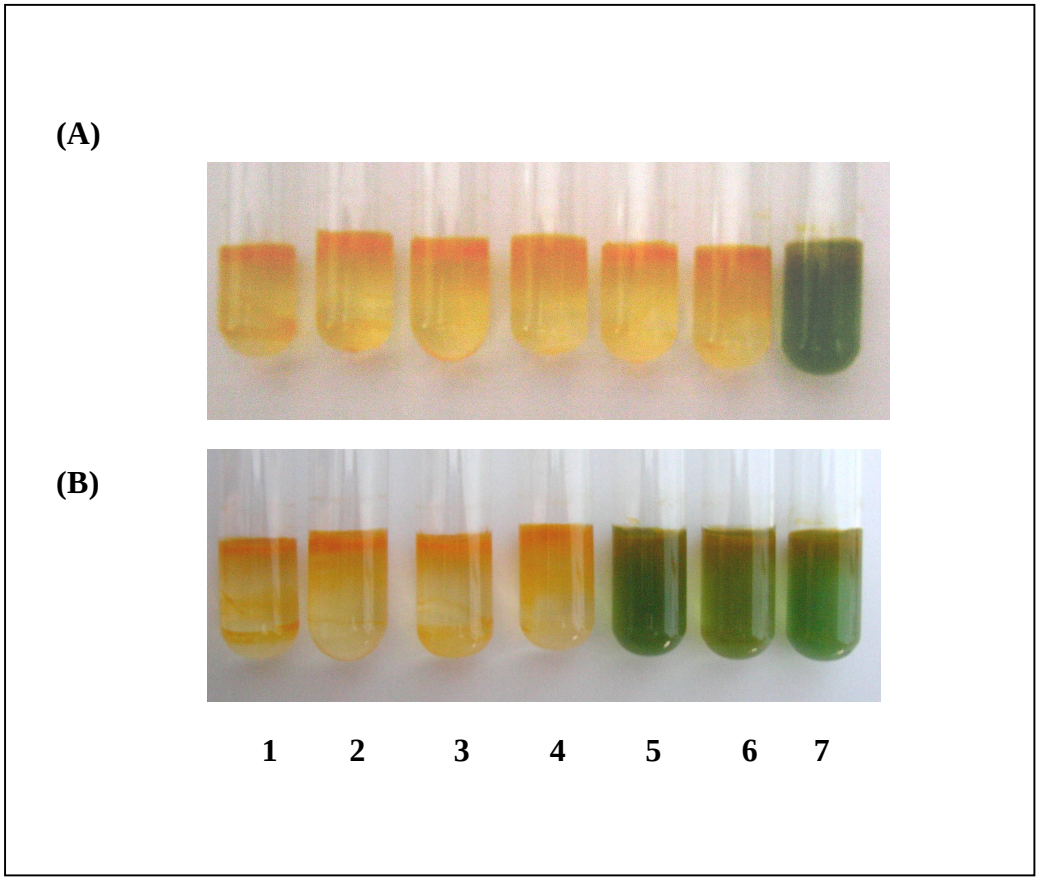


Figure 12 Comparison of biovar test between *Ralstonia solanacearum* wild type strain (To-Ud3) (A) and mutant (B). Tubes 1-3, contain disaccharide sugars indicating as lactose, maltose, and cellobiose, respectively; tubes 4-6 contain hexose alcohol sugar indicating as mannitol, sorbitol, and dulcitol, respectively; and tube 7 contain biovar medium without any sugar was used as control.

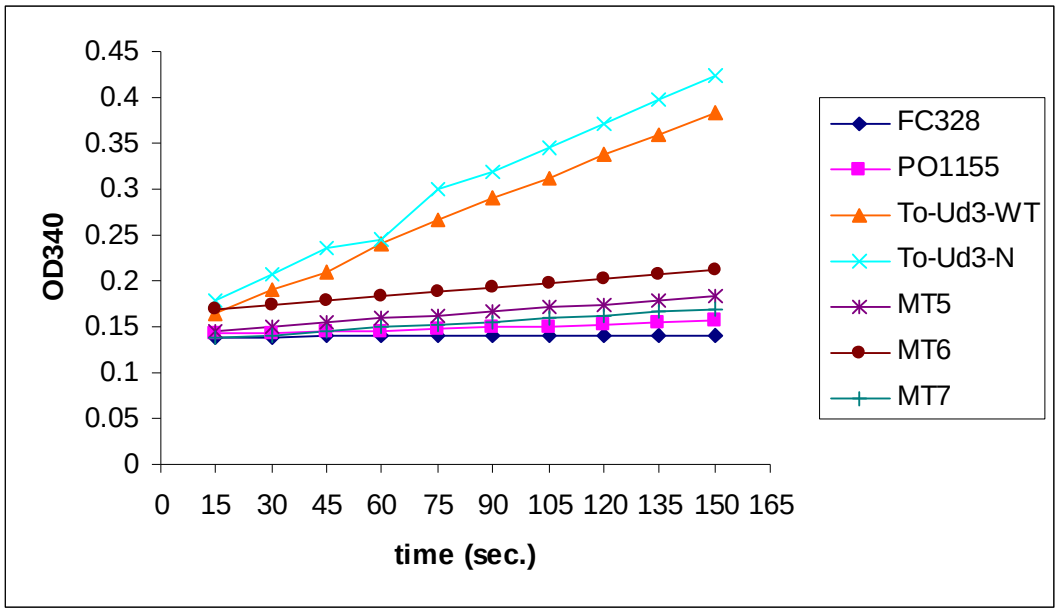


Figure 13 Enzyme activity assay for conversion of sorbitol by the sorbitol dehydrogenase from *Ralstonia solanacearum* wild type (To-Ud3-WT, To-Ud3-N and mutants (MT5, MT6, MT7). The absorbance of crude extract from biovars 1 (FC328) and 2 (PO1155) were measured as a control. The change in NADH absorbance at 340 nm was recorded at 15 sec intervals.

Table 6 Enzyme activity of crude extract from *Ralstonia solanacearum* sorbitol dehydrogenase

Sample From *R. solanacearum*	Enzyme activity (units/ml)	Protein (mg/ml)	Specific activity (units/mg)
To-Ud3-WT	0.097	0.412	0.235
To-Ud3-N	0.11	0.600	0.183
MT5	0.016	1.184	0.014
MT6	0.019	1.158	0.016
MT7	0.013	0.922	0.014
PO1155	0.0048	0.612	0.0078
FC328	0.0038	0.990	0.0038

Enzyme activity was calculated from the equation as shown below:

$$\text{Activity (U)} = \frac{\Delta OD \times v}{\varepsilon \times t} \quad \mu mol/min$$

ΔOD = value obtained from absorbance curve

t = reaction time between enzyme and substrate, usually 1 min

v = volume of reaction solution

ε = 6.3 ml μmol^{-1} cm^{-1}

7. Pathogenicity, HR Test and EPS Quantification of Non-oxidizing Sorbitol Mutant

Hypersensitive reaction (HR) was confirmed in the mutant that could induce Hrp function or pathogenesis. Both wild type and mutant demonstrated HR in tobacco plant (Figure 14D). Therefore, mutant still has pathogenesis function unlike saprophytic bacteria.

To determine the capability of non-oxidizing sorbitol mutant to cause or delay wilt symptom, the severity of wilting was carried out by Winstead and Kelman's (1954) method (Figure14A). Non-oxidizing sorbitol mutant was tested for pathogenecity and severity of wilting comparing with wild type. At one week post inoculation, tomato plants inoculated by mutant showed mild wilt symptom similar as nalidixic resistance strain. In contrast, tomatoes inoculated by wild type showed moderate wilt symptom (Figure 14 B and C). At least 2 weeks post inoculation, wild type strain caused severe wilt symptom in tomato plant will die with in 2-3 weeks after infection. Whereas at 3^{rd} and 4^{th} weeks post inoculated, mutant still caused wilt symptom in moderate level (Table 7).

From this experiment indicated that non-oxidizing sorbitol mutant is still able to cause wilt symptom but it showed lower severity of wilting than wild type. The deleayed symptom or pathogenisis may affect from a prolonged cultivation on synthetic medium supplement with antibiotic. However, the virulence was recovered in mutant or nalidixic resistance strain by re-inoculation these strain in tomato host.

In order to evaluate the amount of EPS from virulence strain is related to sorbitol utilization and important factor to cause wilt symptom, the modified method of Elson and Morgan (Herbert *et al.*, 1971) was performed to comfirm the effect of sorbitol utilization to amoumt of EPS produced by mutant. The quantification of EPS from mutant was calculated and compared with wild type as shown in Table 8. The amount of EPS from wild type and mutant was significantly differrent however it was

not significantly different between nalidixic resistance and mutant. It is probably due to the growth rate of wild type was better than nalidixic resistance and mutant strains that antibiotic resistant and non-oxidizing sorbitol mutant phynotypes might affect to the growth rate. It indicated that the efficiency of cell production enhanced increasing of EPS production. However, the capable of EPS production by *R. solanacearum* To-Ud3 wild type and mutant strains was equally about 1000 µg per mg protein. The re-isolation and re-inoculation of mutant recovered the severity of wilt equally as wild type. Therefore, this result prostulated that sorbitol metabolism was not involved in EPS production *in vitro* by *R. solanacearum*.

Table 7 Pathogenecity tests of *Ralstonia solanacearum* wild type (To-Ud3-WT), nalidixic marker (To-Ud3-N) and non-oxidizing sorbitol (MT5) strains on tomato host (L390)

R. solanacearum strain	Severity of wilting[a]			
	1 week	2 weeks	3 weeks	4 weeks
To-Ud3-WT	3.8 (M)	4.5 (H)	5 (H)	5 (H)
To-Ud3-N	2.2 (L)	3.2 (M)	3.8 (M)	3.8 (M)
MT5	1.5 (L)	3.0 (M)	3.5 (M)	3.9 (M)

[a]Ratings expressed as average disease indices of 5 plants: H = high (4.1-5.0), M = medium (2.6-4.0), L = Low (1.1-2.5), and 0 = none (0) (Prior and Steva, 1990) and (Winstead and Kelman, 1952), 0 = no symptom, 1 = one leaf wilted or partially wilted, 2 = two or three leaves wilted or partially wilt, 3 = all except the top two or three leaves wilted, 4 = all leaves wilted and 5 = plant death.

Table 8 The amount of EPS produced by *Ralstonia solanacearum* wild type (To-Ud3-WT), nalidixic marker (To-Ud3-N) and mutant stains in rich medium.

R. solanacearum Strains	EPS (μg/ml)	Protein (mg/ml)	EPS / Protein (μg/ mg)*
To-Ud3-WT	980.98 [a]	0.907 [a]	1080.82 [a]
To-Ud3-N	842.84 [b]	0.842 [b]	1006.91 [b]
MT5	828.27 [b]	0.822 [b]	1001.28 [b]
F-test	**	**	**
C.V. (%)	2.60	1.59	2.90

* EPS was recovered from BG broth cultures. The data are hexosamine concentrations adjusted for milligrams of total cell protein. Results are the means from three replications.

Means followed by the same letter within a column are not significantly different ($P< 0.05$) by Duncan's multiple range test..

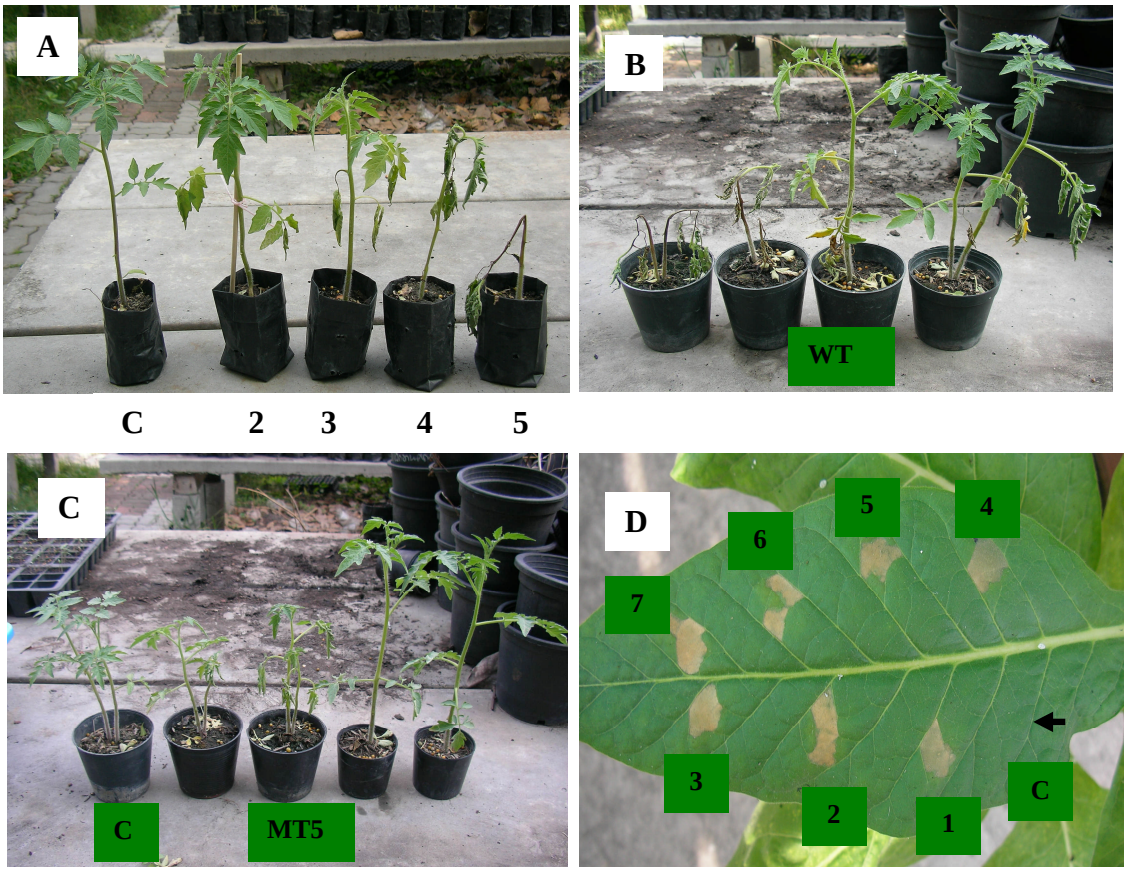


Figure 14 Bacterial wilt symptom and hypersensitive reaction (HR) test caused by *Ralstonia solanacearum* strain To-Ud3 and non-oxidizing sorbitol mutant

A) The severity on plants was scored as followed: 0 = no symptom, 1 = one leaf wilted or partially wilted, 2 = two or three leaves wilted or partially wilt, 3 = all except the top two or three leaves wilted, 4 = all leaves wilted and 5 = plant dead.

B) The wilt symptom of plant infected by wild type strain To-Ud3 at 1 week.

C) The wilt symptom of plant infected by non-oxidizing sorbitol strain at 1 week.

D) HR test of wild type strain To-Ud3 and non-oxidizing sorbitol strain. 1 and 2, wild type To-Ud3; 3 and 4, To-Ud3-N; 5, 6 and 7, non-oxidizing sorbitol strain; C, control (water)

DISCUSSION

Sorbitol dehydrogenase (glucitol, polyol, or L-iditol dehydrogenase) (EC.1.1.1.14) catalyzes the oxidation of sorbitol to fructose with NAD as a cofactor. In *R. solanacearun,* this partial of *polS* gene was identified by using polSF and polSR primer. The 771 nucleotide coding sequence encoded for sorbitol dehydrogenase enzyme (GeneBank accession no. AY946241**).** Biovar 3 and 4 can be differentiated from biovar 1 and 2 by hydrolyzing sorbitol reaction base on biochemical property. PCR technique facilitating with specific primers was capable of classification faster than classical biochemical method. We applied novel primers (polF/polR) specific for sorbitol dehydrogenase gene to us with PCR method as a rapid tool to exclude biovar 3 and 4 from 1 and 2 instead of classical biovar determination of Haward (1964).

This PCR classification result corresponds with the data from the molecular characterization and genetic diversity of *R. solanacearum.* Two clusters within strains of *R. solanacearum* have been reported based on RFLP (Cook and Sequeira, 1991; Cook *et al.*, 1989) and 16S rDNA (Li *et al.*, 1993; Taghavi *et al.*, 1996). The near completed 16S rRNA gene sequences of *R. solanacearum*, *P. cerebensis* (blood disease bacterium) and *P. syzygii* are available. A dendrogram derived from the sequence data resulted in two divisions corresponding to that of Li *et al.,* (1993). Division 1 contains biovar 3, 4 and 5 and some aberrant biovar 2 strains, while division 2 contains biovar 1, 2 and N2 including the blood disease bacterium (BDB) and *P. syzygii*. The level of nucleotide similarity of 16S rDNA ranged from 99.8 to 100% in division 1 and 99.1 to 100% in division 2 (Taghavi *et al.*, 1996). Sequencing of the 16S-23S rRNA gene results in three distinct groups; *R. solanacearum* strains of biovars 1, 2 and N2 from Indonesia formed a cluster (16S subdivision 2b) with *P. syzygii* and the BDB. The other strains of biovar 1, 2 and N2 form a cluster (16S division 2a) while strains of biovars 3, 4 and 5 from another discrete cluster (16S division 1). These results agree with the polygalacturonase and endoglucanase gene sequence cluster (Fegan *et al.*, 1998). From the PCR productions of primers 759f/P760r digested with *Hae* II and *Msp*I restriction, *R. solanacearum* was separated

into biovar 3 and 4 from Asia and Australia, and biovar 1 and 2 from America (Roncal *et al.*, 1999).

From genome sequencing, we also designed other primer for amplification putative D-arabinitol dehydrogenase (*dalD*) accession NC_003295 which involved in mannitol oxidizing gene. Unfortunately, we got non-specific PCR band using these primer therefore, polS primer was an alternative primer of hexose alcohol group for excluding biovar 3 and 4 from 1 and 2. To identify specific primer for disaccharide group, we tried detection gene involving in lactose hydrolyzing. Firstly, β-galactosidase was assaied for detecting enzyme activity. We found that β-galactosidase activity was absent in crude extract from *R. solanaceaum*. It indicated that *R. solanacearum* had another pathway for conversion of lactose. This event correlated with data of Denny and Hayward (2000) that disaacharides group in *R. solanacearum* are oxidizied to bionic acids such as lactoic acid, maltoic acid but are not utilizied as a source of carbon and enery. For differentiation between biovar 1 from 2 or 3 from 4, we should find the key enzyme for conversion disaccharide into bionic acid for generating primer sets used for rapid classification *R. solanacearun* into biovar.

In preliminary experiment, we tried to identify gene involving sugar utilization of *R. solanacearum* by Tn5 mutagenesis to further study the effect of sugar utilizing mutant to pathogenicity. It is difficult to get a result because no transposable elements completely random insert into target DNA. Most transposable elements showed some target specificity, hopping into some sites more often than into others. Even Tn5, which is famous for hopping almost at random, does prefer some site to others. Fortunately, data from genome sequence indicating *polS* gene encoded for sorbitol dehydrogenase. This data is very adventage for us to generate a site directed mutagenesis by homologous recombination which is a better way for mutation at specific gene. The site directed mutagenesis by homologous recombination is rapid and easy to get a mutant that mutated only at designed specicific gene. It also avoided the polar effects that could confuse the assignment of a mutant phenotype to the disrupted gene. Therefore, we amplified a partially sorbitol dehydrogenase *polS* to

generate site direct mutagenesis by homologous recombination at *polS* gene in *R. solanacearum* wild type To-Ud3.

The ORF of partial *polS* gene sequence encodes a protein consisting of 256 amino acid residues with a predicted molecular mass of 27,314 Da. When sorbitol dehydrogenase (SDH) was overexpressed as a glutathione-S-transferase (GST) fusion protein in *E. coli*, the molecular mass of this fusion protein, GST-SDH, is estimated to be approximately 52,000 Da (25,000 Da GST + 27,000 Da SDH) by SDS-PAGE (Figure 6). The molecular mass of the polypeptide was in good agreement with the molecular mass obtained by SDS-PAGE (27,000 Da). This result was in consistent with the molecular mass (29,000 Da) which had been determined for one subunit of purified SDH from *R. sphaeroides* M22. SDH is a homodimeric enzyme with a subunit molecular mass of 29,000 Da. Although the enzyme is active on sorbitol and galactitol, sorbitol had been demonstrated to be a better substrate. The *smoS* gene encoding for SDH from *R. sphaeroides* Si4 had been reported to locate 55 nucleotides upstream from the mannitol dehydrogenase gene (*mtlK*) (Stein *et al.*, 1997). It consisted of 256 amino acid residues with predicted molecular mass of 27,012 Da. The *smoS* had been subcloned into the expression vector pET-24a and the overproduced in *E. coli* BL21 (DE3). The yield of enzyme obtained using pET expression vector is approximately 270-fold higher than that of the native host, *R. sphaeroides*. Unfortunately, we were unable to subclone the *polS* into pQE81L (Qiagen), lac based promoter. Eventhought, this gene is expressed efficiently as a GST fusion protein with the pGEX-2T vector, the reason why is expressed inefficiently with pQE81L vector is unclear.

Sequence comparison analysis revealed significant homologies between the deduced amino acid sequences of *polS* and proteins of the short chain alcohol dehydrogenase/ reductase family (SDR) (Jornvall *et al.*, 1995). SDR protein is a very large family of enzymes, most of which are known to be NAD- or NADP-dependent oxidoreductases. The first member of this SDR family had been characterized was Drosophila alcohol dehydrogenase. This family commonly is referred to as the 'insect-type' or 'short-chain' alcohol dehydrogenase (Persson *et al.*, 1991). Most

member of this family are proteins of about 250 to 300 amino acid residues. The overall identity between *polS* gene and SDR protein was about 30% (data not shown). The similarity of *polS* gene to SDR protein also extends to the predicted secondary structures, which predicted locations of helices and sheets being remarkably similar among these enzymes (Ghosh *et al.*, 1994). Although SDR enzymes typically show overall amino acid residue identities between 15 to 30%, two regions (GGGG and YKSP) are highly conserved among these proteins (Figure 4). Significantly, half of the conserved residues are glycines, which is typical of distantly related proteins with a conserved fold (Jornvall *et al.*, 1995). The highly conserved glycine-rich region had been shown to involve in coenzyme binding and is located at N-terminal. The invariant glycine residues (G) of the G-X-X-X-G-X-G segment is a characteristic of the coenzyme binding fold in dehydrogenases. The invariant tyrosine (Y) and lysine residues (K) of the consensus sequence Y-X-X-X-K had been demonstrated to be functionally importance for catalysis (Chen *et al.*, 1993) and are located in C-terminal (Ghosh *et al.*, 1995). As with other dehydrogenase, the deduced amino acid sequence of *polS* gene also contained glycine box, GAASGIG, at positions 13 to 19. Structural prediction using NCBI conserved domain search (CDD. V2.04) (Marchler-Bauer and Bryant, 2004) suggested that the N-terminal region around the glycine box processed an βαβ structure and that 3D structure of *polS* was similar with SDR protein family.

This protein family includes a large number of highly diverse enzymes, most of which have homodimeric structures like SDH and do not require metals as a cofactor for catalytic activity (Jornvall *et al.*, 1995). The SDH of *R. sphaeroides* differed from that of *B. subtilis* which is a tetrameric zinc metallo-enzyme with M_r of 150,000 (Ng *et al.*, 1992) and *Cephalosporium chrysogenus* which is a 10 subunit enzyme with M_r 3,000,000 (Birken and Pisano, 1976). However, the *R. sphaeroides* SDH resembles the SDH from *Pseudomonas* sp. (Mr 65,000) with respect to size, subunit composition, the absence of a metal requirement and some kinetic properties (Schneider and Giffhorn, 1991). The mammalian SDHs generally require zinc as cofactor for catalytic activity and share a high degree of structural similarity (Karlsson and Hoog, 1993). However, microbial SDHs are much more diverse. On the basis of sequence data, mammalian SDHs have been assigned to the group of zinc-containing

medium-chain alcohol dehydrogenases (Jornvall *et al.*, 1987). Only the SDH of *B. subtilis* (Ng *et al.*, 1992) and *Saccharomyces cerevisiae* (Sarthy *et al.*, 1994) have been included in the same enzyme family and share 36 and 42% homology with the sequences of the mammalian SDHs, respectively. However, other microbial polyol dehydrogenases including *polS* of *R. solanacearum* have been classified on the basis of sequence data as members of the short chain alcohol dehydrogenase family (SDR) which comprises a group of relatively small enzymes exhibiting no metal requirements (Persson *et al.*, 1991). This enzyme family include RDH from *Enterobacter aerogenes* (Dothie *et al.*, 1985), arabinitol dehydrogenase from *Candida albicans* (Wong *et al.*, 1993) and D-glucitol-6-phosphate dehydrogenase from *Klebsiella pneumoniae* (EMBL accession no. S23835).

Nucleotide and amino acid sequences alignment showed that *R. solanacearun* strain TO264 is closely related to *R. solanacearun* strain GMI1000, *B. ceapacia* and *B. fungorum* at 99%, 90%, 85% similarity, respectively but is distantly related from *P. syringae* pv. *syringae, R. sphaeroides, Sinorhizobium meliloti* and *Mesorhizobium* sp. (60% similarity) (Figure 6). A phylogenetic analysis indicated *polS* joined *the Burkholderia cepacia* sequence as sister to the *R. solanacearum* pair (Figure 5). This result clearly concluded that *R. solanacearun* is closely related to *B. ceapacia* and *B. fungorum* by comparing with sorbitol dehydrogenase gene sequence.

To study factors controlling host specificity, mutation at specific *polS* gene has been generated. The two methods were used to produce mutants: insertion-duplication (IDM) and allelic exchange (AE). The directly inactivation of specific genes was achieved by homologous recombination (IDM and AE). We demonstrated the first successful used of site direct mutation in *R. solanacearum*. Using small integrating vectors, we constructed a specific sequence contained 523 bp corresponding to truncate fragment of *polS*. The deletion of *polS* gene fragment was generated by overlapping PCR (Horton *et al.*, 1990). An NAD binding site, G-X-X-X-G-X-G and the active site motif at Y-X-X-X-K of enzyme function were deleted therefore generated nonsense mutation.

We screened for non-oxidizing sorbitol mutant. Only 1 % of non-oxidizing sorbitol mutant was obtained. All non-oxidizing sorbitol mutants showed both IDM and AE event when they were detected by long PCR and Southern blot hybridization. This event explained that after transconjugation, hybrid plasmid was transferred and integrated into chromosome at homologous site by a single crossover known as IDM. We observed the larger fragment from hybridization at 6 kb fragment corresponded with *kan* probe and long PCR at 5.4 kb fragment (Figure 9C and 10A). Integration by entire plasmid might affect downstream gene expression in addition to the gene targeted for inactivation. IDM has been used to disrupt genes in various 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).

Another event, the double crossover or AE occurred in *R. solanacearum* and that it is possible to produce mutants through specific gene disruption in this bacterium as shown by shorter 900 bp PCR fragment comparing with wild type and 523 pb fragement of deleted construct from DNA amplification (Figure 10A and 10D). However, the frequency of the second crossover event seems to be very low. A higher pressure of selection system using the *sacB* gene, might improve the frequency of double crossover in *R. solanacearum*. Identification of replacement of deletion mutant involves tedious work since the frequency of double-homologous crossover to single crossover events is very low, depending on the organism type strain. The double crossover arising from chromosomal integration of the suicide plasmid are resolved by utilization of counter selectable marker, most often *Bacillus subtilis sacB* (sucrose counterselection) encoding for the levansucrase enzyme, whose expression in a sucrose-containing medium leads to a lethal phenotype in some organism (Schweizer, 1992) or less frequently, *rpsL* (streptomycin counterselection) (Stibitz, 1994). In cases where the antibiotic selection markers are flanked by site specific recombination, e.g., the Flp recombinase target (*FRT*) (Hoang *et al.*, 1998) or Cre recombinase (*loxP*) (Quenee *et al.*, 2005) site, they can subsequently be deleted from the chromosome, resulting in deletion mutants.

The mutation efficiency of this experiment was considerably low because tri-parental mating method might be transferred a low copy of deletion construct plasmid. If the deleted construct plasmids were electroporated directly to host cell, the efficiency of gene replacement will be high enough to make it feasible to identify mutants by direct PCR screening of individual colonies or other means of direct screening. Because of the integration vector, pK18mob, processes some unique feature which makes it easy to use for routine allele replacement procedures. This plasmid is based on the well-established pBR322 vectors with a stable replicon and consistent lacZα expression which allowed for reliable blue/white screening of recombinants. Furthermore, this plasmid can replicate well in selective media and produce high-copy number vector.

The mutant is unable to oxidize not only sorbitol but also dulcitol whereas other sugars were not affected as shown in Figure 12. From these data, IDM could affect gene function because insertion of entire plasmid might disrupt *polS* and other genes downstream. Similarly, AE generated mutation by deleted active site and NAD binding site construct; therefore, these could disrupt endogenous *polS* gene function. The phenotype data showed that site direct mutagenesis by overlapping PCR capable of mutation at specific sorbitol gene and also disrupted gene function. The mutations affected oxidizing property not only sorbitol but also dulcitol sugar alcohol. This might imply that sorbitol dehydrogenase processed to oxidizing both sugar but not mannitol even.

Sorbitol dehydrogenase was measured for enzymatic activity to confirm enzyme function in mutants. Enzyme activity of mutants was reduced about sixty folds. It means that endogenous *polS* gene was disrupted by homologous recombination. Although, specific gene replacement mutation with two crossovers event was very low possibility, functional disruption was clearly elucidated. From enzymatic activity assay, biovar 1 and 2 did not able to hydrolyzing sorbitol the same as mutant because these biovars did not present of *polS* gene in chromosome. The *polS* gene was confirmed by hybridization data as shown in Figure 2. This is

probably evidence of genetic rearrangement in strain GMI1000 was observed on the megaplasmid, where a perfect tandem duplication of a 31 kb region flanked by insertion sequences was found (Salanoubat *et al.*, 2002). Genomic instability is a well known phenomenon in *R. solanacearum* (Boucher *et al.*, 1988; Kelman, 1954) and this flexibility could be responsible for the genomic diversity of the species, examplified in terms of host range or existence of biovars (Genin and Boucher, 2002). For another example, *Yersinia pestis*, one of the most devastating diseases in human history, has been divided into three biovars based on the ability to ferment glycerol and arabinose, and to reduce nitrate. Biovar assignment is based on metabolic variations that do not seem to correlate with the virulence. The genetics of metabolic variations is affected from gene loss and pseudogene distribution (Zhou *et al.*, 2004).

The replacement mutation method has been successfully used by our group to construct mutants in *R. solanacearum* and other microorganism 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. Up to date, the complete genome sequences of *R. solanacearum* are available, which has opened many new experimental avenues (Salanoubat *et al.*, 2002). 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.

Non-oxidizing sorbitol mutant showed virulence to susceptible tomato var. L390. The severity of wilt symptom from non-oxidizing sorbitol mutant is lower level than wild type strain. It probably that sorbitol might not a precursor to synthesize EPS I production and/or important factor to determine host specificity for the wilt pathogen. To answer this question, the amount of EPS from *R. solanacearum* wild type and non-oxidizing sorbitol mutant was carried out *in vitro*. EPS from non-oxidizing mutant produced undifferently to wild type therefore it still caused wilting symptom to tomato. It meaned that, *in vitro*, sorbitol metabolism used as a carbon source did not influence to EPS production and might not promote colonization of this

wilt pathogen in plant cell. In contrast, *Erwinia amylovoran* used sorbitol sugar for efficient production of exopolysaccharide amylovoran to escape plant defense mechanisms and to cause wilt symptoms (Bellemann and Geider, 1992; Bernhard *et al.*, 1993). The host plants of *E. amylovoran* all belong to the family of Rosaceae and contain high levels of sorbitol (Zimmermann and Ziegler, 1975). The mutants with a sorbitol deficiency were still virulent on slices of immature pears, but were unable to cause significant fire blight symptoms on apple shoots. This indicates that the capability of *E. amylovoran* to used sorbitol may be an important factor in determining host specificity for the fire blight pathogen (Aldridge *et al.*, 1997).

In this experiment, the production of EPS was measured only *in vitro* but not *in planta*. However, sorbitol did not involve in EPS pathway *in vitro*, it might affect differently *in planta*, for example *hrp* mutant cluster do not apparently affect the structure or reduce significantly the amount of EPS produced *in vitro*. It concluded that this gene cluster is not involved in the biosysthesis of EPS (Denny *et al.*, 1993) but EPS production of GMI1353 (*hrp* mutant) was not detected *in planta*, probably because the invasiveness of strain GMI1353 is rather poor compared with the wild type strain (Trigalet and Trigalet-Demery, 1990). Dispite being unable to cause wilt symptoms, *hrp* mutants still invade unwounded tomato roots and spread into the lower stem, albeit at bacterial densities at least 1,000 fold lower than the wild type. The production of *R. solanacerum* EPS is also controlled by PhcA which is a complex regulatory network that responds to multiple signals (Schell, 2000). PhcA controlled life cyle of *R. solanacearum* between PC-type which is low cell density form to adapt for survival in soil and wild type which is high cell density form in plant cell. It both activates a set of virulence genes; EPS biosynthesis, Pme and Egl exoprotein and represses others those involved in motility, polygalacturonase and sideropore production, *hrp* gene.

From some reseaches, the amount of EPS produced by *R. solanacearum* dependens on several genes function which have been identified. Structural gene clusters include *opsI* (Cook and Sequeira, 1991; Kao and Sequeira, 1992), *opsII* (McWilliams *et al.*, 1995), *rgnII* (Denny and Baek, 1991), and *epsI* (Denny and Baek,

1991). The *opsI* and *opsII* gene clusters are important for both EPS and lipopolysaccharide syntheses since mutations in them affect the production of both macromolecules. The *rgnII* cluster is largely uncharacterized since it is required for EPS production only in culture but not in plants (Denny and Baek, 1991). Mutational analyses of *epsI* suggest that it encodes proteins responsible for synthesis of the acidic component of EPS, which is absolutely required for *R. solanacearum* infection of plants (Denny and Baek, 1991; Kao *et al.*, 1992; Orgambide *et al.*, 1991).

These researchs showed many gene clusters and factors controlling EPS production and pathogenesis of *R. solanacearum* in both culture and/or *in planta* conditions. In our experiments, we study only the effect *polS* mutant to EPS production *in vitro* but did not detect *in planta*. So further experiment, pathogenicity of *polS* mutant *in planta* should be investigated.

The overall experiments can not elucidate the correlation between sugar metabolism and pathogenicity. Therefore, we can not conclude that sugar metabolism such as sorbitol and/or dulcitol involving in pathogenicity. The pathway of EPS synthesis and sugars metabolism in *R. solanacearum* should intensively investivate by radio active labeling in various precursor sugars to understrand what sugar precursor enhanced producing of EPS virulence factor.

CONCLUSION

Sorbitol dehydrogenase gene (*polS*) GeneBank accession no. AY946241 was identified from *R. solanacearum* by primer set designed from *polS* gene. The enzyme processes for converting sorbitol into fructose using NAD as a cofactor. The set of sor primer designed from *polS* gene (at nucleotide 118 to 718) can be separated biovar 1 and 2 from biovar 3 and 4. Therefore, 623 nucleotides were amplified in biovar 3 and 4 but not in biovar 1 and 2. This primer can be used for identification and divide of *R. solanacearum* biovar 1 and 2 from biovar 3 and 4 which are predominated biovars in Thailand and Asia countries. This PCR reaction with set of sor primer can be used replacement of biochemical test which used long time to identify biovar.

The 771 nucleotides of partial *polS* encoded for 256 amino acids and estimated molecular weight as 27 kDa. Analysis of the deduced amino acid sequence revealed homology to enzymes of the short-chain dehydrogenase/reductase protein family. The eight amino acid residues are conserved in most of these proteins. These residues include the almost invariant tyrosine (Y) and lysine (K) residues of consensus sequence Y-X-X-X-K, which are essential for catalysis and are located in the active site in C-terminal whereas the glycine (G) residues of the G-X-X-X-G-X-G segment are characteristic of the NAD^+ binding domain in the N-terminal region.

The precise mutation in the *R. solanacearum* at *polS* gene was mutated by using homologous recombination. The method for creating precisely engineered deletion of *polS* gene known as overlapping PCR. An NAD binding site, G-X-X-X-G-X-G and the active site motif at Y-X-X-X-K of enzyme function were deleted.

The processes of homologous recombination to mutate at *polS* gene were generated by homologous type in IDM and AE event. IDM affected to gene function because insertion of entire plasmid might disrupt *polS* and other genes downstream. Similarly, AE generated mutation by gene replacement in which deleted active site

and NAD binding site construct was replaced endogenous gene; therefore, these could disrupt endogenous *polS* gene function.

Non-oxidizing sorbitol mutant is unable to oxidize not only sorbitol but also dulcitol whereas other sugars were not affected. Biochemical test showed that same result as enzyme activity assay when sorbitol was used as substrate since enzyme activity of mutant was abolished by homologous recombination. These results revealed that site direct mutagenesis by overlapping PCR was capable mutation at specific sorbitol gene and also disrupted gene function.

Non-oxidizing sorbitol mutant showed virulence to susceptible tomato var. L390. The severity of wilt symptom from non-oxidizing sorbitol mutant is lower than wild type strain. However, mutant strain re-isolated and re-inoculated showed high severity equal to wild type. The production of EPS from non-oxidizing mutant did not differ to wild type therefore it still caused wilting symptom to tomato. It meaned that sorbitol metabolism used as a carbon source did not influence to EPS production *in vitro*.

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APPENDIX

Differentiation of Biovars

The mineral medium of Ayers *et al.* (1919) is supplemented with peptone, agar, and a pH indicator.

	per L
$NH_4H_2PO_4$	1.0 g
KCl	0.2 g
$MgSO_4.7H_2O$	0.2 g
Difco Bacto peptone	1.0 g
Agar	3.0 g
Bromothymol blue	80 mg

The pH is adjusted to 7-7.1 (an olivaceous green color) by dropwise addition of 40% sodium hydroxide solution. The medium is heated to melt the agar, dispensed into bottles or tubes, steriled by autoclaving at 121 °C for 20 to 30min, and cooled to 55 to 60 °C.

Prepare 10% aqueous solutions of the test carbohydrates. Sterilize dulcitol by autoclaving at 110 °C for 20 min. Filter sterilize the other carbohydrates. Sufficient carbohydrate solution is added to the warm basal medium to give a final concentration of 1%. After mixing, about 2 ml of the molten medium is dispensed into sterilized culture tubes and allowed to solidify.

Inoculum is prepared by adding several loopfuls of bacteria from 24 to 48 h old cultures on TTC plates to 3 t0 5 ml sterile distilled water to make a suspension containing about 10^8 CFU/ml. Add about 20 µl of the bacterial suspension to the surface of the medium in each tube and incubate the inoculated tubes at 28-32 °C. Examine the tubes at 3, 7, 14, and 28 days after inoculation for change in pH (indicated by a color change; examine from the top of the medium downward).

With dextrose and hexose alcohols, a change to yellow (acid pH <6) indicating oxidation of the carbohydrate occurs within 3-5 days; those biovars capable of oxidizing the disaccharides could take a few days longer to give a clear positive result. The inoculated tubes should be compared with a noninoculated control tube to

observe the change in color (in some cases there could be a slight change to alkaline pH in tubes containing carbohydrates that are not oxidized).

Purification DNA by Silica

Preparation of Silica

Silica was obtained from Sigma (St. Louis, Mo., Cat. No. S-5631) and prepared according to Boom *et al*., (1990) with slight modification. The protocol was as follows: 10 g of silica was put into 100 ml of sterile distilled water, then shaken vigorously overnight. Silica was then allowed to settle for 10 to 12 hours, then supernatant was removed by pipetting or decanting, then silica was resuspended in 10 ml of 6 M sodium-iodide (approx. concentration 100%, wt/vol). The treated silica could be stored in dark at room temperature for at least 3 months.

DNA Purification

DNA or plasmid was extracted by standard method. To purification of DNA extracted, the supernatant of DNA (approx. 200 µl) was added with 600 µl and 8 µl 100% (wt/vol) to each tube, they were vortexed for 5 sec, followed by slight shaking for 2 min. The tubes were then briefly spun for 5 sec, and supernatant was removed using a pipette. The 1 ml of wash solution (10 mM Tris (pH 7.5), 1 mM EDTA (pH 7.5), 100 mM NaCl, and 50% ethanol) was added to each tube, followed by vortexing for 10 sec. Tubes were then centrifuged at 10,000 *g* for 30 sec. The supernatant was removed, and the silica-bound genomic DNA was dried at 37 °C for 5 min. The DNA was then eluted by adding 40 µl of distilled water or 1x TE buffer and centrifugation at 10,000 *g* for 1 min. The supernatant (approx. 40 µl) containing genomic DNA was transferred into new tubes.

Standard Curve of BSA

Standard protein assay was measured by Bradford method (1976). Prepare a dilution series of BSA standards in a five tubes according to the following scheme.

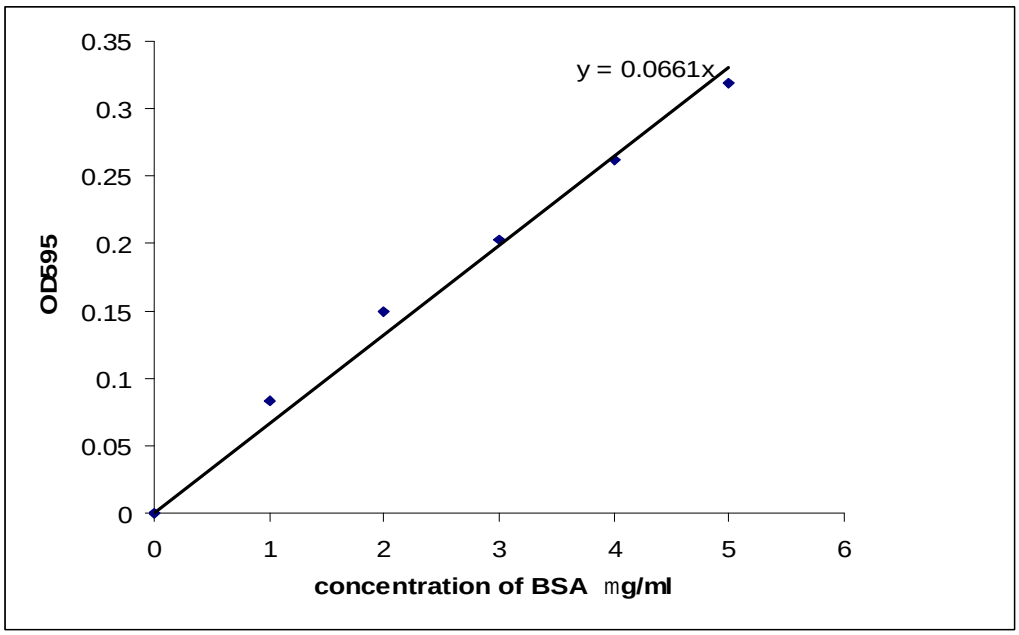
Appendix Table 1 Dilution series of BSA standards

Stock tube	Dilution	Protein concentration
1	10 µl of stock (0.1 mg/ml) + 90 µl of H_2O	1 µg/ml
2	20 µl of stock (0.1 mg/ml) + 80 µl of H_2O	2 µg/ml
3	30 µl Of stock (0.1 mg/ml) + 70 µl of H_2O	3 µg/ml
4	40 µl Of stock (0.1 mg/ml) + 60 µl of H_2O	4 µg/ml
5	50 µl Of stock (0.1 mg/ml) + 50 µl of H_2O	5 µg/ml

Standard curve of BSA was made by using BSA 1-5 µg/ml. Absorbance value was measured at 595 nm according to the method of Bradford method (1976). A 100 µl of standard BSA dilution series was mixed with 900 µl of Bradford reagent and leaf at room temperature for 10 min, then the mixer was measured at OD_{595}. Standard curve was made by plotting between different concentration of standard BSA against their absorbance value as shown in table 2 and figure 1. The absorbance showed linear line with all concentrations used. The protein of samples was determined from this BSA standard curve.

Appendix Table 2 The correlation between final concentration of standard BSA and their absorbance value at 595 nm

BSA concentration (μg/ml)	Absorbance value at 595 nm
0	0.000
1	0.084
2	0.149
3	0.203
4	0.262
5	0.319



Appendix Figure 1 The standard curve of BSA (μg/ml) and their absorbance value at OD_{595}

Standard curve of *N*-acetyl-D-glucosamine

Standard curve of *N*-acetyl-D-glucosamine was measured by modified Elson-Morgan method described by Herbert *et al*. (1971). Prepare a dilution series of *N*-acetyl-D-glucosamine standards in a four tubes according to the following scheme.

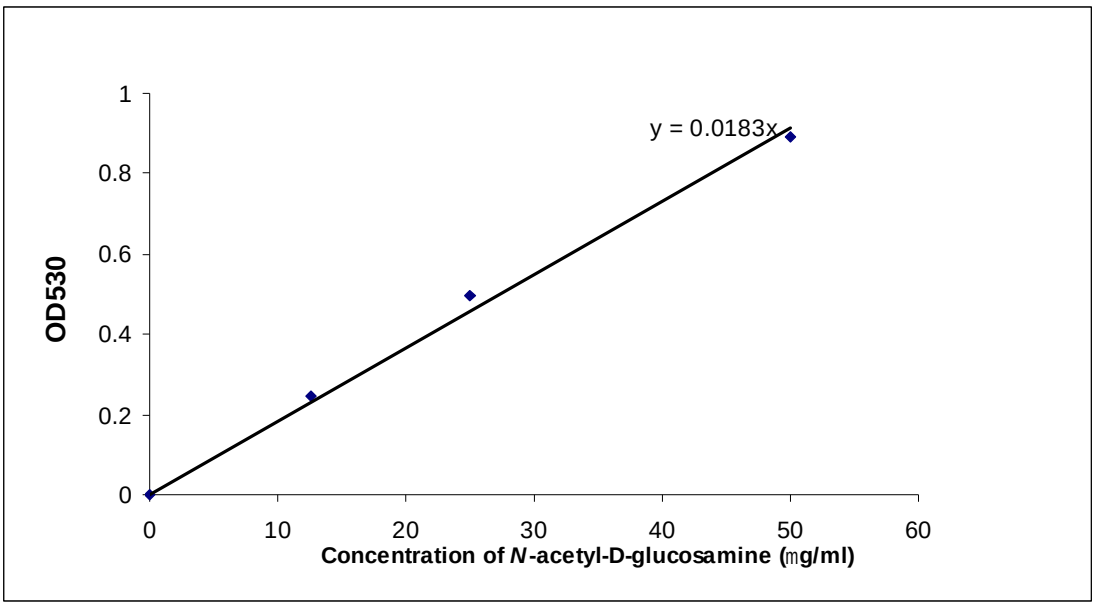
Appendix Table 3 Dilution series of *N*-acetyl-D-glucosamine standards

Stock tube	Dilution	*N*-acetyl-D-glucosamine concentration (μg/ml)
1	12.5 μl of stock (1 mg/ml) + 112.5 μl of H_2O	12.5
2	25 μl of stock (1 mg/ml) + 100 μl of H_2O	25
3	50 μl Of stock (1 mg/ml) + 75 μl of H_2O	50
4	62.5 μl Of stock (1 mg/ml) + 62.5 μl of H_2O	62.5

Standard curve of *N*-acetyl-D-glucosamine was made by using *N*-acetyl-D-glucosamine 10-60 μg. Absorbance value was measured at 530 nm according to the method of modified Elson-Morgan method described by Herbert *et al*. (1971). A 125 μl of standard *N*-acetyl-D-glucosamine dilution series was mixed with freshly prepared 125 μl acetylacetone reagent, and the solutions were boiling for 20 min. Acetylation was terminated by cooling the samples in melting ice. Subsequently, 625 μl 99.5% (v/v) ethanol followed by 125 μl Ehrlich was added. The tubes (uncapped to release carbon dioxide build-up) were placed at 65°C for 10 min before being cooled to room temperature. The absorbance of coloured chromogens developed was measured at 530 nm. Standard curve was made by plotting between different concentration of *N*-acetyl-D-glucosamine against their absorbance value as shown in Table 4 and figure 2. The absorbance showed linear line with all concentrations used. The protein of samples was determined from this BSA standard curve.

Appendix Table 4 The correlation between final concentration of standard *N*-acetyl-D- glucosamine and their absorbance value at 530 nm

N-acetyl-D-glucosamine concentration (μg/ml)	Absorbance value at 530 nm
0	0.000
12.5	0.245
25	0.494
50	0.893
62.5	1.080



Appendix Figure 2 The standard curve of *N*-acetyl-D-glucosamine (μg/ml) and their absorbance value at OD_{530}

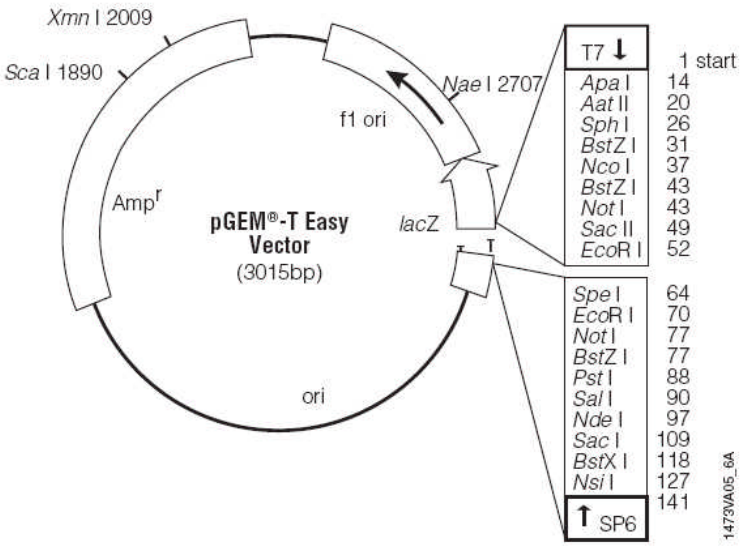
DNA Southern Blotting and Hybridization Required Solutions and Buffer

1	Transfer Buffer	(0.4 N NaOH)		
		5N NaOH	80	ml
		Distill water	920	ml
2	Depurination solution (0.25N HCl)			
		6N HCl	12.5	ml
		Distill water	287.5	ml
3	Washing Buffer	(0.1 M maleic acid + 0.15 M NaCl + 0.3 % (w/v) Tween20)		
		Maleic acid	13.81	g/l
		NaCl	8.76	g/l
		Tween20	3	ml
		Adjust pH to 7.5 with NaOH		
4	Washing I			
		20X SSC	100	ml
		20% SDS	5	ml
		Distill water	895	ml
5	Washing II			
		20X SSC	5	ml
		20% SDS	5	ml
		Distill water	990	ml
6	20X SSC	(3M NaCl, 0.3 M Tri-sodium citrate, pH 7)		
		NaCl	175.3	g/l
		Tri-sodium citrate	88.22	g/l
		Adjust pH to 7 with NaOH		

7	Hybridization solution	(200 ml)		
		20X SSC	50	ml
		10% laurylsarcosine	2	ml
		20% SDS	0.2	ml
		Blocking reagent	2	g
		Distill water	147.8	ml
8	1% blocking buffer	(100 ml)		
		Blocking reagent	1	g
		Maleic acid buffer	100	ml
		heat solution at 65 °C		
9	Detection solution	(400 ml)		
		1 M Tris-HCl, pH 9.5	40	ml
		5 N NaCl	8	ml
		Distill water	352	ml
10	1 M Tris-HCl pH 9.5	(500 ml)		
		Tris-HCl	60.55	g
		Adjust pH to 9.5		

Map and DNA Sequence of Cloning Vector and Expression Vector

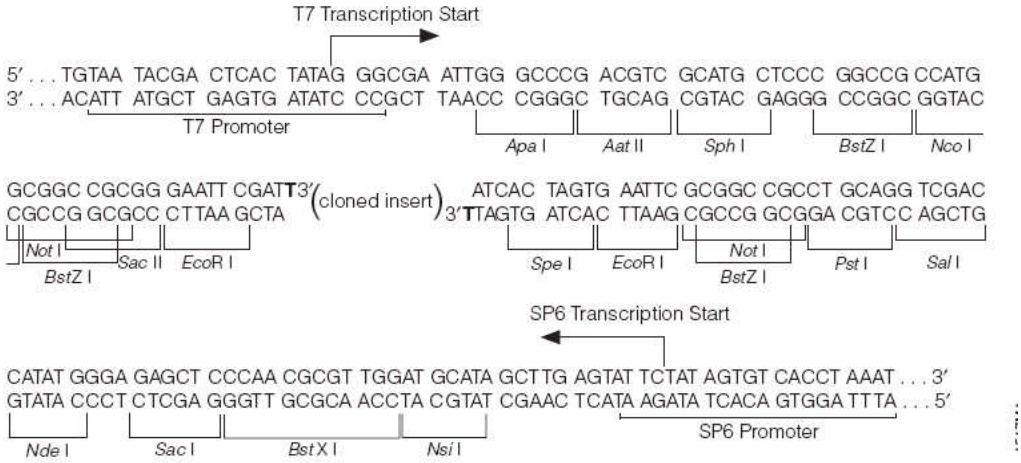
1 pGEM®-T Easy Vector



pGEM®-T Easy Vector sequence reference points:

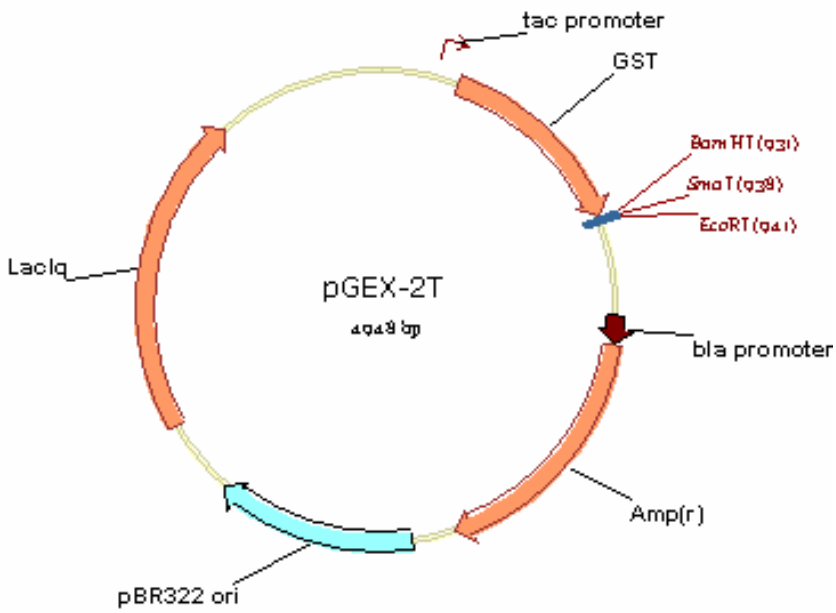
T7 RNA polymerase transcription initiation site	1
multiple cloning region	10–128
SP6 RNA polymerase promoter (–17 to +3)	139–158
SP6 RNA polymerase transcription initiation site	141
pUC/M13 Reverse Sequencing Primer binding site	176–197
*lac*Z start codon	180
lac operator	200–216
β-lactamase coding region	1337–2197
phage f1 region	2380–2835
lac operon sequences	2836–2996, 166–395
pUC/M13 Forward Sequencing Primer binding site	2949–2972
T7 RNA polymerase promoter (–17 to +3)	2999–3

Appendix Figure 3 pGEM®-T Easy Vector circular map and sequence reference points.

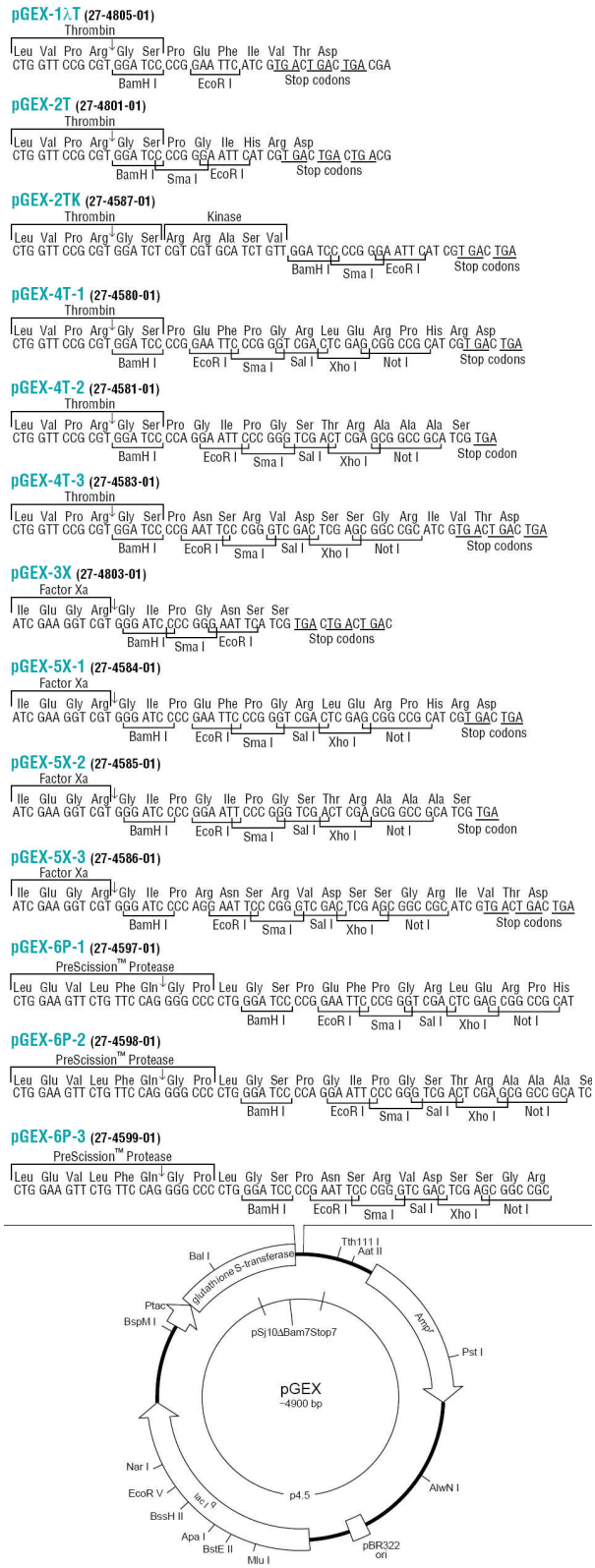


Appendix Figure 4 The promoter and multiple sequence of the pGEM®-T Easy Vector. The top strand of the sequence shown responds to the RNA synthesized by T7 RNA polymerase. The bottom strand corresponds to the RNA synthesized by SP6 RNA polymerase.

2 pGEX-2T Expression Vector

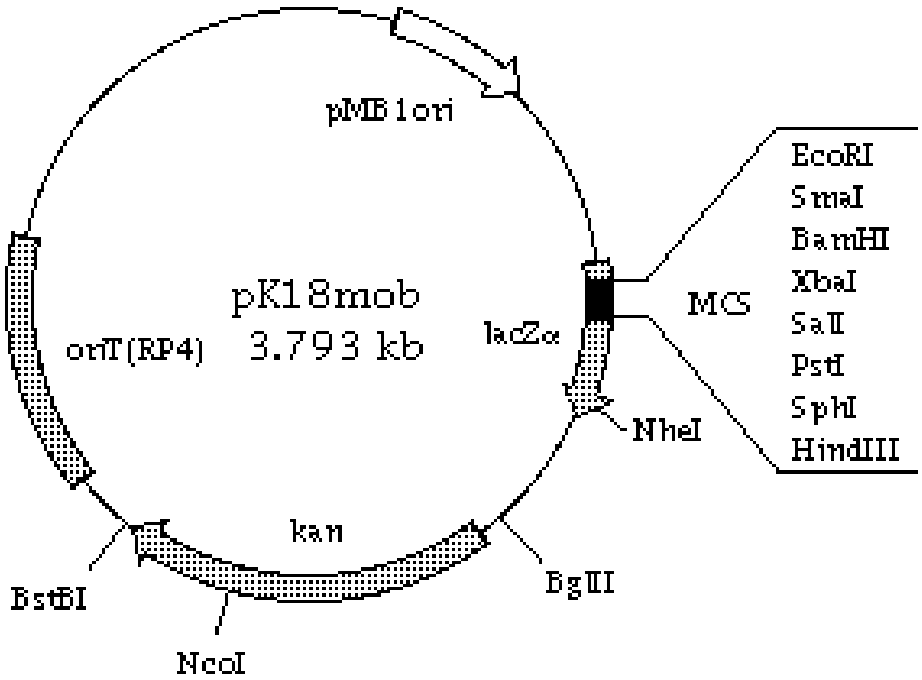


Appendix Figure 5 pGEX-2T Expression Vector circular map and multiple cloning site.



Appendix Figure 5 (Con't) pGEX-2T Expression Vector circular map and multiple cloning site

3 pK18mob



Appendix Figure 6 pK18mob Integrating Vector circular map and multiple cloning sites.

CURRICULUM VITE

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EDUCATION :

YEAR	INSTITUTION	DEGREE/DIPLOMA
1994-1997	Prince of Songkla Univ.	B.Sc. (Biotechnology)
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SCHOLARSHIP : University Development Committion (UDC) Scholarship from Prince of Songkla University (2002-2004)