IDENTIFICATION OF GENES INVOLVING IN SUGAR UTILIZATION OF *RALSTONIA SOLANACEARUM*

INTRODUCTION

Ralstonia solanacearum is a devastating bacterium caused vascular wilt in more than 200 plant species in the tropics, subtropics, and warm temperature regions around the world (Hayward, 1991). Its agronomically important hosts include tomato, potato, tobacco, peanut, and banana. This species has been sub-classified into biovars based on six sugars utilization and host-dependent races.

This pathogen's ability to cause wilt has been attributed mainly to its production of a high molecular mass acidic extracellular polysaccharide (EPS I), which can occlude vascular tissues and prevent water flow (Shell, 1996). Extracellular proteins also have a major role in pathogenesis (Kang *et al.*, 1994), but individual cell wall-degrading enzymes (e.g., polygalacturonase [PGL] and endoglucanase [EGL]) appear to be nonessential, because inactivation of single genes only decreased the rate of wilting (Denny *et al.*, 1990).

All virulent strains produce large amounts of an unusual extracellular polysaccharide (EPS) slime in which the major portion of this slime is EPS I, a> 1,000 kDa acidic, unbranched polymer of *N*-acetylgalactosamine, *N*-acetygalactosaminuronic acid, and N-acetylbacillosamine decorated with 3-OH butyric acid (Orgambide *et al.*, 1991). Early *in vitro* studies (Husain and Kelman, 1958) and recent *in planta* studies of EPS-deficient mutants (Denny and Baek, 1991; Kao and Sequeira, 1992) suggest that at least one major function of EPS I is to cause wilting of defected plants, probably by blocking water flow in the xylem (Denny *et al.*, 1990); however, It is not required for growth *in planta*. Production of EPS I requires the 18-kb *eps* gene cluster, which encodes several membrane-associated and soluble polypeptides involved in its biosynthesis and export (Denny and Baek, 1991; Schell *et al.*, 1994). Synthesis of EPS I and O antigen of *R. solanacearum* also involves portion of the *ops* gene cluster, which may encode synthesis of a common

sugar precursor (Cook and Sequeira, 1991; Kao and Sequeira, 1992; Kao and Sequeira, 1994).

From many researches, genes involving sugars metabolism were exclusively studied and influenced on bacterial virulence of *Erwinia amylovora* such as galactose, sucrose, and sorbitol (Aldridge *et al.*, 1997; Bogs and Geider, 2000; Metzger *et al.*, 1994). These carbohydrates have been shown to be required for the efficient production of exopolysaccharide (EPS) amylovoran by *E. amylovora* (Bellemann and Geider, 1992; Bernhard *et al.*, 1993; Steinberger and Beer, 1988). Since Rosaceous plant contains sorbitol and sucrose as storage and transport carbohydrates. In addition, galactose is essence for producing amylovoran which consists of a glucuronic acid residue and four various linked galactose residues (Nimtz *et al.*, 1996) and their biosynthesis mainly depends on UDP-galactose as a precursor. These carbohydrates are important for colonization on plants by *E. amylovora*. Reduce virulence of these carbohydrates mutants could be due to level of nutrients in xylem vessels, requiring access to these carbohydrates for colonization of the host plant by *E. amylovora.* Since sugar metabolism can divide *R. solanacearum* into 5 biovars, some sugar metabolism may influence polysaccharide biosysthesis and virulence of *R. solanacearum.*

The objectives of this study are:

1. To clone, sequence, and characterize sorbitol dehydrogenase gene of *Ralstonia solanacearum* which involves in biovar classification.

2. To study the effect of mutation at sorbitol dehydrogenase gene on relation of pathogenicity of *Ralstonia solanacearum*

LITERATURE REVIEWS

1. Biology and Characteristic of *Ralstonia solanacearum*

Ralstonia solanacearum (Yabuuchi *et al.*, 1995), a soil borne bacteria and the causal agent of bacterial wilt disease, was originally described by E.F. Smith as *Bacillus solanacearum* and later as *Pseudomonas solanacearum* (Smith, 1986). In 1992, Yabuuchi *et al.* proposed a new genus *Burkholderia* with 7 species of the genus *Pseudomonas*, including *Pseudomonas solanacearum* based on rRNA homology data. The organism was then placed into the genus *Ralstonia* based on the results of phenotypic characterization, fatty acid analysis, 16S rDNA nucleotide sequences and rRNA-DNA hybridization (Yabuuchi *et al.*, 1995). The chronology of name changes of *Ralstonia solanacearum* are as follow:

Bacillus solanacearum E.F. Smith (1896)

Pseudomonas solanacearum (Smith) Smith (1914)

Burkholderia solanacearum (Yabuuchi *et al.*, 1992)

Ralstonia solanacearum (Yabuuchi *et al.*, 1995)

Bacteria that are taxonomically related and often misidentified as *Ralstonia solanacearum* include *Pseudomonas syzigii*, the causal agent of Sumartra disease of cloves (*Zysigium aromaticum*) in Java and Sumatra, and *Pseudomonas celebensis*, the blood disease bacterium (BDB) of Musaceae in Indonesia (Hayward, 2000; Taghavi *et al.*, 1996).

Ralstonia solanacearum is a gram-negative rod shaped, 0.5-0.7 x 1.5-2.0 micrometer and motile by one to four polar flagella. It is aerobic, catalase, oxidase and nitrate reduction positive. The organism is negative for levan production, starch hydrolysis, indole production and hydrogen sulfide hydrolysis (Denny and Hayward, 2000).

It is non-fluorescent on King *et al*'s medium B, accumulates poly-B-hydroxybutyrate, and the G+C content of its genomic DNA 66.5-68% (Goto, 1992).

Vilulent and avirulent colonies of the pathogen can be differentiated by growth on tetrazolium chloride medium (TZC) (Kelman, 1954). White fluidal colonies with a pink color at center are normally virulent whereas red, round and convex colonies are typically avirulent.

R. solanacearum is devided into 5 biovars (Table1) according to its ability to oxidize six sugars containing three disaccharides (maltose, lactose and cellobiose) and three hexose alcohols (mannitol, sorbitol and dulcitol) (Hayward, 1964).

Table 1 Differentiation of *Ralstonia solanacearum* biovars based on utilization and oxidation of sugar as positive (+) and negative (-)

Carbohydrate source	Biovars*				
	1	2	3	4	5
Oxidation of :					
Lactose	-	+	+	-	+
Maltose	-	+	+	-	+
D(+) cellobiose	-	+	+	-	+
Utilization of :					
Mannitol	-	-	+	+	+
Sorbitol	-	-	+	+	-
Dulcitol	-	-	+	+	-

* Biovar 1, 2, 3, and 4 according to Hayward (1964) and biovar 5 of He *et al.* (1983)

2. Pathogenicity and Host Range of *Ralstonia solanacearum*

Ralstonia solanacearum caused wilt diseases on several hundred-plant species in over 50 families in tropics subtropics and warm temperate region of the world (Hayward, 1991). The most host plants are dicotyledons in the family Solanaceae including tomato, potato, tobacco, eggplant and pepper. Bacterial wilt also occurs in monocotyledons plants such as banana, bird of paradise (*Strelitzia reginae* Banks), *Heliconia* spp., including ginger and related plants of Zingiberaceae family (Hayward, 1994), also. In addition, various woody, perennial plants of economic importance have been recorded as hosts (Hayward, 2000).

R. solanacearum often enters host through the roots, either at wounds or site of secondary root emergence, penetrate through the cortex, and then spreads throughout the vascular system (Vasse *et al.*, 1995). The colonization of approximately 25% of xylem vessels in each vascular bundle above the collar zone is sufficient to induce partial wilting of tomato and ultimately leads to plant death.

Strains of *R. solanacearum* causing Moko disease of banana in Central and South America and the Buktok disease of planta in the Philippines are spreaded into two broad groups; group A strains which infect raceme via insect vectors and group B that are mechanically transmitted (Sequeira, 1998).

Strains of *R. solanacearum* can be differentiated into 5 races based on pathogenicity on different hosts as follows: race1 affecting tobacco, tomato, many solanaceous and other weeds, and certain diploid bananas; race2 causing wilt of triploid bananas (moko disease) and *Heliconia* sp.; race3 infecting potatoes and tomatoes, but no other solanaceous crops (Buddenhagen *et al.*, 1962); race4 infecting ginger (Pegg and Moffet, 1971) and race5 infecting mulberry (He *et al.*, 1983). In contrast, Hayward recognize only 3 races for *R. solanacearum*: race1 pathogenic on Solanaceous hosts, diploid bananas, and a variety of non-Solanaceous hosts; race2 strains restricted to triploid banana and *Heliconia* spp.; and race3 strains primarily affecting potato, occasionally tomato, and a few weed hosts (Hayward, 2000).

Bacterial wilt disease in Thailand has been destroyed many economic crops such as tomato, potato, ginger, pepper, tobacco and sesame including marigold and patumma (*Curcuma alismatifolia* Gagnep.), an exported ornamental plant. The phenotypic characterization of those *R. solanacearum* strains was typed mostly into race 1 (Thammakijjawat *et al.*, 1997; Thaveechai *et al.*, 1997) excepted few strains of race3 from potato grown in highland of the north part of Thailand (Boonsuebsakul, 1994).

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3. Molecular Characterization and Genetic Diversity of *Ralstonia solanacearum*

The classification into species of *R. solanacearum* is a complex taxonomic unit in which strains display an important diversity at different levels (physiological, serological, genetic characteristics, and host range). In order to describe this intraspecific variability, several systems of classification have been proposed. Thus, the species was subdivided into five races according to its host range (Buddenhagen *et al.*, 1962; He *et al.*, 1983) and into five 5 biovars based on the utilization of three disaccharides and three hexose alcohols (Denny and Hayward, 2000; Hayward, 1964). Fatty acid analysis (Janse, 1991; Stead, 1993) and protein profiling (Dianese and Dristig, 1994) were also performed but did not further clarify the relationships among *R. solanacearum* strains.

Molecular characterization was firstly described by Cook *et al*. (1989) using the RFLP technique with probes encode information required for virulence and hypersensitive response genes. When southern blots of *Eco*RI or *Ec*oRI and *Bam*HI digested genomic DNA are probed with nine *hrp* gene cloned fragment. The RFLP data suggested dividing the organism into two divisions. Division 1 contains all members of race 1 biovar 3 and 4, designated “Asiaticum” strain and division 2 contains all members of race 1 biovar 1, races 2 and race 3 biovar 2 designated “Americanum” strain.

The phylogenetic relationships with tRNA consensus (Seal *et al.*, 1992), 16SrRNA sequencing (Seal *et al.*, 1993; Taghavi *et al.*, 1996), 16S-23S rRNA gene intergenic spacer region, polygalacturonase gene, and the endoglucanase gene (Fegan

et al., 1998), confirmed the two division and subdivided division 2 into subdivision 2a and 2b.

4. A Model System for the Study of Bacterial Pathogenicity

Many gene products are required by *R. solanacearum* for successful infection of its host. Three strains in particular, K60 (Kelman, 1954), GMI1000 (Boucher *et al.*, 1985) and AW (Schell, 1987) have been the subject of intense study at the molecular level in order to characterize these pathogenicity factors. Insights from these studies have greatly enhanced our understanding of the disease process used by this bacterium.

4.1 Exopolysaccharide and Extracellular Enzyme Production

R. solanacearum produces a variety of extracellular products that contribute to it ability to colonize host plants and cause disease symptoms. One of the most important of these is an acidic, high molecular mass extracellular polysaccharide (EPS I). *In planta* studies of EPS I-deficient mutants (Denny and Baek, 1991; Kao and Sequeira, 1992) suggest that EPS I is the cause of wilting in infected plants, as it blocks the vascular system and thereby alters water movement. Although none of these EPS I-deficient mutants was totally non-pathogenic, recent studies showing that these mutants poorly colonize the stem of infected plants suggest that EPS I may contribute to minimizing or avoiding the recognition of bacterial surface structures by plant defense mechanisms (Araud-Razou *et al.*, 1998; Saile *et al.*, 1997).

In addition to EPS, *R. solanacearum* secretes several plant cell wall-degrading enzymes. These includes three polygalacturonases PglA, PehB, and PehC (Huang and Allen, 1997; Schell *et al.*, 1988), an endoglucanase, Egl (Roberts *et al.*, 1988). However, gene disruption analysis revealed that the roles of individual cell wall-degrading enzymes in bacterial wilt disease are relative minor.

4.2 *hrp* Genes: Delivery of Pathogenicity Effector Proteins into Plant Cells

The *R. solanacearum hrp* cluster is required for pathogenicity on host species and induction of the hypersensitive response in non host plant (Boucher *et al.*, 1987) and encodes components of a type III protein secretion pathway, TTSP (Van Gijsegem *et al.*, 1993). TTSPs have a central role in pathogenesis of many bacterial pathogens of plants and animals and have been intensively characterized at the molecular level (Cornelis and Van Gijsegem, 2000). Type III secretion in *R. solanacearum* requires the production of Hrp pilus, whose structural protein is encoded by *hrpY* gene (Van Gijsegem *et al.*, 2000), and which is speculated to direct protein translocation across the plant cell membrane.

Three other proteins have been shown to be secreted in the extracellular milieu by the *R. solanacearum* secretion system: PopA, PopB, and PopC, which are encoded by genes located on the left-hand border of the *hrp* cluster and regulated by the transcriptional regulator HrpB. PopA causes a hypersensitive-like response when infiltrated into plant tissue at high concentration, similarly to the harpins of other phytopathogenic bacteria (Arlat *et al.*, 1994). A recent study showing that the *Pseudomonas syringae* pv. *phaseolicola* harpin forms pores in lipid bilayer membranes suggests that this class of proteins may allow nutrient acquisition *in plant* and/or the delivery of effector proteins into plant cells (Lee *et al.*, 2001). PopB and PopC have structural features which are usually found in eukaryotic proteins or in some type III-dependent effectors from other bacterial pathogen. PopB contains a bipartite nuclear localization signal which promote its transport to the plant cell nucleus and PopC carries 22 leucine-rich repeats analogous to those found in some plant resistant genes products (Gueneron *et al.*, 2000). However, mutants deficient for PopA, PopB or PopC have normal virulence, probably because of functional redundancy. Alternatively these proteins might be required to infect plants that have not been test (Gueneron *et al.*, 2000).

R. solanacearum regulates its Hrp typeIII secretion pathway by a complex signal transduction cascade that responds to at least two environmental signals: the

first one is detected when bacteria are grown in an ‘apoplast-mimicking’ minimal medium, and the second one is a specific including signal perceived in presence of plant cells (Brito *et al.*, 2002; Marenda *et al.*, 1998; Schell, 2000). Fluorescence microscopy of *R. solanacearum* cell containing a *hrpB-gfp* fusion shows that expression of the regulatory gene *hrpB* is maximally induced in response to physical contact of bacteria with plant cell or cell wall debris (Aldon *et al.*, 2000). Such a contact-dependent activation of *hrpB*-regulated genes could ensure the translocation of effector proteins into plant cells at the appropriate time and place. Since this plant cell contact-dependent signal is superimposed on the ‘minimal medium’-derived signal, it is also possible that this could lead to a differential control of gene expression thus producing a temporal hierarchy in the type III secretion process.

The prime candidate receptor for this non-diffusible hrp-inducing compound from the plant cell wall is the outer membrane protein PrhA (Aldon *et al.*, 2000; Marenda *et al.*, 1998). Prhl and PrhR, form a ′3-compartment signal transduction system′ that allow the modulation of *hrp* gene activation in response to a stimulus from the bacterial cell surface, providing a way for the bacterium to recognize a target plant cell (Brito *et al.*, 2002).

The nature of the plant-derived inducer is not known. However, since this *hrp*-inducing signal is resistant to heat and protease treatments, it seems likely to involve a ubiquitous carbohydrate molecule present in the pectic/cellulosic fraction, in agreement with the observation that cell wall material from both host and non-host plants are potent inducers (Aldon *et al.*, 2000).

4.3 Phenotypic Conversion and Regulation of Virulence

The production of *R. solanacearum* virulence determinants is controlled by a complex regulatory network that responds to multiple signals (Schell, 2000). The central player of this regulatory network is the transcriptional regulator, PhcA (Brumbley *et al.*, 1993), involved in the Phc cell density-sensing system. PhcA activates both a set of virulence genes (EPS biosynthesis, Pme and Egl exoproteins) and represses others (those involved in motility, polygalacturonase and siderophore

production, *hrp* genes). The levels of active PhcA protein are controlled by an endogenous signal molecule, 3-hydroxypalmitic acid methyl ester (3-OH PAME). Only when extracellular 3-OH PAME accumulates above 5 nM (i.e. at high cell density in a restricted space, such as the plant vascular system), repression of PhcA production relieved, resulting in activation (or repression) of the appropriate target genes (Flavier *et al.*, 1997a). This model therefore implies a distinction between 'early' virulence functions (expressed at low bacterial population density when PhcA is inactive) and 'late' virulence functions (expressed at high bacterial population density when PhcA is active).

The picture is even more complex since it is known that PhcS also positively regulates production of acylhomoserine lactone, a second quorum sensing molecules (Flavier *et al.*, 1997b). This additional regulatory system is mediated by the Soll-SolR regulators, which probably activate genes operating in the terminal stages of the disease after virulence factors have largely performed their function (Flavier *et al.*, 1997b; Schell, 2000). Currently, a single gene of unknown function (*aidA*) is known to be regulated by Soll-SolR in strain AW (curiously, this gene appears to be a strain-specific gene since it is absent from the genome of strain GMI1000).

Spontaneous or induced mutations in *phcA* result in the pleiotropic morphological changes called 'phenotype conversion' (PC). PC-type mutants correspond to the frequent spontaneous avirulent mutants originally described by Kelman (1954). There is now evidence that spontaneous PC-type mutants can be the result of distinct mutational events within *phcA* (Brumbley *et al.*, 1993; Jeong and Timmis, 2000), even some are reversible. It has been speculated that bacterium may shift between two different physiological states, one adapted for saprophytic survival ; PC-type and the other for pathogenesis; wild-type (Denny *et al.*, 1994).

5. Insitghts from the *R. solanacearum* Genome Sequence

The complete genome sequence of *R. solanacearum* strain GMI1000 has been recently determined and annotated (Salanoubat *et al.*, 2002), therefore providing a starting point for an exclusive functional analysis of pathogenicity determinants.

5.1 A Bipartite Genome Structure

The genome of the *R. solanacearum* strain GMI1000 has a size of 5.8 Mb with a high G+C content (average value of 67%) and a coding potential for approximately 5120 proteins. It is organized in two replicons : a 3.7 Mb chromosome and a 2.1 Mb megaplasmid. The nature of the origin of replication and the repartition of the genes on these two replicons supports the view that the smaller replicon is indeed a megaplasmid (Salanoubat *et al.*, 2002). The megaplasmid appears to encode numerous genes that might play a role in the overall fitness of the bacterium or that may provide advantages in diverse environments for example; flagellum biosysthesis, many essential pathogenicity genes, catabolism of aromatic compounds, copper and cobalt-zinc-cadminm resistance gene clusters.

However, this replicon also carries duplications of some important genes, such as a rDNA locus, three tRNAs, a second σ54 factor genes (*rpoN2*) and a second subunit α of DNA polymerase III (*dnaE2*). Interestingly, several enzymes involved in the metabolism of small molecule (such as methionine biosynthesis) are also encoded on megaplasmid, with no counterpart on the chromosome.

5.2 Alternate Codon Usage Regions: The evolutome

One of the most striking features of the *R. solanacearum* genome is its complexity and potential for plasticity. This illustrated both by the high number of transposable elements and by the presence of alternate codon usage regions (ACURs) which correspond to approximately 7% of the genome (Salanoubat *et al.*, 2002). Ninety-three ACURs spanning 1 to over 20 kb were defined in the entire genome, with G+C variations ranging from 50% to 70%. ACURs are often associated with transposable elements or prophage sequence and contain (i) a high number of genes with a G+C % significantly lower than the 67% average, (ii) several potential pathogenicity genes (such as those encoding effector proteins translocated through the typeIII secretion pathway, large haemagglutinin-related proteins, etc), and (iii) numberous duplicated ORFs of unknown function (Genin and Boucher, 2002).

These observations suggest that some ACURs may be 'pathogenicity islands (PAI) acquired through horizontal transfer and that ACURs may be involved in some duplication/evolution process, therefore contributing to generate genomic variation by catalyzing acquisition, loss and alteration of genetic material. Therefore, ACURs regions can be viewed a potential 'evolutome'. A global analysis of the ACURs gene content in the genome of other *R. solanacearum* strains, along with the conservation and the repartition of these ACURs, will probably provide additional clues about the roles of these regions in the bacteria adaptation/evolution process (Genin and Boucher, 2002).

6. Possible Roles for Polyols in Plant Pathogen

In a large number of plants (such as celery, parsley, snap-dragon, etc) the sugar alcohol such as mannitol serves as a carbon and energy storage compound as well as a compatible solute and regulator of osmotic potential (Stoop *et al.*, 1996). In these plants mannitol accumulation is at least partly modulated by the expression of the mannitol catabolic enzyme mannitol dehydrogenase (MTD), a 1-oxidoreductase, catalyzes the direct conversion of mannitol to mannose. MTD also appears to be a pathogenesis-related (PR) protein in celery, as it has greater than 90% amino acid similarity to the elicitor-induced ELI-3 proteins of parsley and *Arabidopsis*, and its expression is induced by salicylic acid (SA), an inducer of many plant defense proteins (Williamson *et al.*, 1998; Williamson *et al.*, 1995; Zamski *et al.*, 2001). Mannitol, in addition to being a versatile metabolite and osmoprotectant, is also an antioxidant. Mannitol has been recognized as a potent scavenger of hydroxyl radical (OH) *in vitro* (Smirnoff and Cumbes, 1989), as well as an effective antioxidant *in vivo* (Shen *et al.*, 1997a; Shen *et al.*, 1997b). This antioxidant activity might be significant in the context of bacterial or fungal pathogenesis, because reactive oxygen species (ROS) play a central role in plant responses to pathogen attack (Alvarez *et al.*, 1998; Lamb and Dixon, 1997; Mehdy *et al.*, 1996).

Because ROS serve such a central role in plant responses to pathogen attack, successful pathogen must be able to evade or suppress ROS-mediated defenses. Phytopathogenic bacteria, for instance, are believed to secrete ROS-scavenging

enzymes such as catalases and superoxide dismutases (Klotz and Hutcheson, 1992). The consequent removal of either O^{-}_{2} or H_2O_2 would also prevent the production of OH. Instead of secreting enzymatic ROS scavengers, most fungi, including many plant pathogenic ones, produce the antioxidant mannitol (Bieleski, 1982; Jennings, 1984). Mannitol originally was believed to function in fungi primarily as a storage metabolite or in NAD(P)H recycling. However, mannitol production also appears to be essential for pathogenicity in at least some fungi. For example, production and secretion of mannitol by the tomato pathogen *Cladosporium fulvum* is required for pathogenicity (Joosten *et al.*, 1990). Similarity, the fungal animal pathogen *Cryptococus neoformans* appears to secrete mannitol to quench ROS produced by defense cells in the blood; neutrophils (Chaturvedi *et al.*, 1996a; Chaturvedi *et al.*, 1996b).

The pathogen secretes mannitol to quench ROS-mediated defense responses, then plants with high levels constitutive MTD expression might be more resistant to pathogen, as they have an enhanced ability to metabolize mannitol to the non-quenching sugar mannose. On the other hand, constitutive expression of MTD would not be expected to protect against pathogens that do no secrete mannitol, such as *Cercospora nicotianae.* For example, transgenic plants constitutively expressing the celery MTD displayed enhanced resistant to the mannitol-secreting phytopathogenic fungus *Alternaria alternata* (Jennings, 2000). In *Erwinia amylovora*, causes the devastating disease known as fire blight in some Rosaceouse plant like apple, pear, quince, raspberry and several ornamentals. Rosaceae contains high levels of sorbitol (Zimmermann and Ziegler, 1975). This sugar alcohol has also been shown to be required for the efficient production of amylovoran which is characterized as virulence factors of *E. amylovora* (Aldridge *et al.*, 1997). All mutations in *srl* operon of *E. amylovora* affected symptom formation on apple seedlings. 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. Since sorbitol facilitates carbohydrate transport in host plants and is used for colonization of plants by *E. amylovora*, this sugar alcohol may be an important factor in determining host specificity for the fire blight pathogen.

MATERIALS AND METHODS

1. Bacterial Strain and Growth Condition.

The bacterial strains and plasmids used in this study are listed in Table 2. *R. solanacearum* To264 and To-Ud3 were isolated from wilted tomato in Department of Plant Pathogen Laboratory and grown at 28-30^{o}C on TZC (Kelman, 1954) (1 g casein hydrolysate, 10 g peptone, 5 g glucose, 15 g agar, and 2,3,5-triphenytetrazolium chloride (50 μg/ml) in 1 liter distill water) and TTC medium is TZC without 2,3,5-triphenytetrazolium chloride. For extraction of genomic DNA, *R. solanacearum* was cultured in liquid YP broth (3 g yeast extract and 5 g peptone in 1 liter distill water) at 30^{o}C with agitation. YP broth containing 1% sorbitol was used for sorbitol dehydrogenase assay in *R. solanacearum* wild type and mutant. *Escherichia coli* DH5α which was used as competent cell for transformation was grown on LB (10 g tryptone, 5 g yeast extract, 5 g NaCl, and 15 g agar in 1 liter distill water) supplemented with an appropriated antibiotic at 37^{o}C with agitation. Antibiotics used for plasmid selection were kanamycin at concentration of 50 μg/ml, and/or amplicilin at 100 μg/ml.

Table 2 Bacterial strains and plasmids used in this study

Destination	Relevant characteristics	Source of reference
Escherichai coli HB101	galK2 recA13 hsdS20 (r_B r_B^-) *rpsL20 proA2 xyl-5 mtl-1 supE44 ara14 lacY1*	(Maniatis *et al.*, 1982)
S17-1	mobilizing donor strain; *thi pro hsdR$^-$ hsdM$^+$ recA tra$^+$* from RP4 integrated in chromosome	(Simon *et al.*, 1989) (ATCC 47055)
DH5α	F$^-$ RecA *endA gyrA96 thi-1 hsdR17 • (lacZYA-argF)*	(Maniatis *et al.*, 1982)
Ralstonia solanacearum		
To264	Wild-type	This work
To-Ud3	Wild-type	This work
To-Ud3-N	Nal^r	This work
Plasmid		
pGEM-T easy	pGEM5Z'f, thymidylated *Eco*RV site, 3 kb, Ap^r	Promega
pGEX-2T	pBR322 ori, Ap^r, tac promoter, 4.9 kb	Amersham
pRK2013	ColE1 replicon, Tra$^+$-mobilizing plasmid, Km^r	(Ditta *et al.*, 1980)
pK18mob	3.8 kb; *E.coli* mobilizable vector (Mob of plasmid RP4); pBR322 replicon; Km^r *LacZ*	(Schafer *et al.*, 1994) (ATCC 87095)
pKS	a 523 bp *Eco*RI fragment which is a truncate *polS* gene from pGEM-T easy cloned in pK18mob, 3.8 kb, Km^r	This work

2. Pathogenicity Tests and Biovar Classification

Host plant for pathogenicity tests is tomato var. L390 (*Solanum tuberosum*). Seedings were grown in plastic bags for 4 weeks under greenhouse condition. Day and night temperature ranged from 28-34 °C and 23-26 °C, respectively. Bacterial suspensions were prepared from a 24 hr on NGA liquid culture and adjusted to 0.2 OD at wavelength of 600 nm to obtain about 10^8 CFU/ml. Inoculations were done by leaf clipping method (Thaveechai, 1989). Symptoms were observed weekly for 4 weeks. The severity was based on (Winstead and Kelman, 1952) scales as follows: 0 = no symptom, 1 = one leaf wilted or partially wilted, 2 = two to three leaves wilted or partially wilt, 3 = all except the top two or three leaves wilted, 4 = all leaves wilted and 5 = plant dead. Five plants were used for each treatment.

Biovar classification was carried out using mineral medium supplemented with peptone, agar, and a pH indicator (Hayward, 1964). *R. solanacearum* was tested for utilization of disaccharides (lactose, maltose, and cellobiose) and hexose alcohol (mannitol, sorbitol and dulcitol), as described by Hayward (1964). The mineral medium tests (1 g of $NH_4H_2PO_4$, 0.2 g of KCl, 0.2 g of $MgSO_4$, 1 g bacto-peptone, 3 g agar in 1 liter distilled water) are consisted of sugars in which its final concentration in mineral medium is 1%. Filter sterilization of these sugars will be carried out instead of autoclave to avoid break down of sugar molecules. Bromthymol blue at final concentration of 0.008% is included in the medium as indicator to detect acid production by changing color from green to yellow. Mineral medium without any of these sugars will be used as control. Tubes are incubated at 28-30 °C and the reaction was recorded for up to 30 days.

3. DNA Amplification

DNA amplification was performed in final volume of 50 µl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate, 50 pmole of each primer, 2.5 Unit of Taq DNA polymerase (Promega, USA), and 50 ng of genomic DNA from *R. solanacearum* strain To264.

The condition used in PCR amplification was 5 min of denaturation at 94 ^{o}C, followed by 30 cycles of amplification with denaturation at 94 ^{o}C for 45 sec, annealing at melting temperature of each primer pairs for 30 sec as shown in table 3 and table 4, and DNA elongation at 72 ^{o}C for 30 sec and a final elongation for 7 min at 72 ^{o}C using thermocycler (Perkin-Elmer 9600/Applied Biosystem). The PCR products were separated by 0.8 % agarose gel electrophoresis in TBE buffer and viewed under UV-transilluminator.

Table 3 List of oligonucletides used to amplify internal and whole *polS* gene regions for identification and mutagenesis in this study. (Underlined portions indicated restriction site).

Oligonucleotides	Sequence and restriction sites	Tm (°C)
sorF	5’ GGC ACG CTC GCG CAA CTC ATC GA 3’	65
sorR	5’GAT TAC ATC ACC GCT CAG ACG CT 3’	65
polSF	5’GTC AAG GGA TCC GTT GCA GGA CAA GGT CGC GAT CCT 3’	60
polSR	5’ CTA CAT GAA TTC GCT CAT CCA GTT GCC GCC GTC 3’	60
sorM	5’GCC TGC GAA GCC ATG TTG ATG 3’	60
ΔsorM22	5’ CAT CAA CAT GGC TTC GCA GGC AAG ATC AAC GTG AAC GGG ATC G 3’	55

Table 4 List of oligonucleotides used for PCR analysis of mutants

Oligonucleotides	Sequence and restriction sites	Tm (°C)
claF	5′ TGA CCG ACG CAG CCC GCA TAG G 3′	60
claR	5′ TGT AAG GGG CAT TCG CTC GCA TCT 3′	60
kmF	5′ GAT GGA TTG CAC GCA GGT TCT C 3′	55
kmR	5′ GTA AAG CAC GAG GAA GCG GTC AG 3′	55
mobF	5′ ATG CTT CCG GCT CGT ATG TTG TGT 3′	55
mobR	5′ GCT GGC GAA AGG GGG ATG TGC T 3′	55

4. Enzyme Activity Assay

Sorbitol dehydrogenase activity was determined by spectrophotometer using the absorbance of NADH at 340 nm (ε = 6.3 ml $\mu mol^{-1}cm^{-1}$) at 30^{o}C. The absorbance was monitored by using a UV-1601 SHIMADZU spectrophotometer. Each assay was performed with 1 ml of reaction mixture containing 100 mM Tris-HCl (pH 9.0), 50 mM sorbitol, 2 mM NAD, and 10-100 µl of enzyme extract. One unit is defined as the amount of enzyme needed to generate 1 µmol of NADH/min. *R. solanacearum* wild type and mutant culttured in YP broth at 30 ^{o}C were inoculated in 30 ml of YP broth containing 1% sorbitol and agitatated overnight at 30 ^{o}C. Cells were harvested by centrifugation at 8,000 rpm, 10 min and resuspended in 30 ml of cell breakage buffer (20 mM sodium phosphate buffer pH 7.0) mixed well by vortex and then centrifuged at 8,000 rpm, 10 min. Cell pellet was adjusted to a final volume of 1 ml with the cell breakage buffer. Lysozyme was added in cell suspension and then cells were broken by freezed thawing and passing through a syring ineedle several times. Cell debris was removed by centrifugation at 10,000 rpm, 10 min at 4 ^{o}C. Protein concentrations were determined by using bovine serum albumin (Bradford, 1976) as a protein standard as shown in appendix.

5. Chromosomal DNA and Plasmid Preparation

5.1 Chromosomal DNA Preparation

Culture of *R. solanacearum* was streaked on TZC agar medium, and a single virulent colony was used to purify on TTC agar medium. A virulent, single fluidal colony was used to seed 5 ml of modified YP broth. After growing at 28 ^{o}C on a rotary shaker overnight, 2 ml of culture were used to isolated DNA using the Puregene kit (Invitrogen Inc., Minneapolis, MN) as described by the manufacturer with minor modifications. Culture broth of each strain was pipetted into microcentrifuge tube and centrifuged at 10,000 rpm, 3 min. The supernatant was discarded and re-suspended the pellet in 0.85% sterile saline and centrifuged at 10,000 rpm, 3 min. This was repeated 3 times to reduce the amount of bacterial exo-polysaccharide. After discarding the supernatant, an aliquot of 600 µl of cell lysis solution was added to the pellet and mixed slowly by pipetting up and down. Samples were incubated at 80 ^{o}C for 5-7 min to lyse the cells, 3 µl of RNaseA solution was added, then tubes were mixed by inverting 25-30 times. Samples were incubated in a water bath at 37 ^{o}C for 50-60 min followed by five min at room temperature. A portion of protein precipitation solution of 200 µl was added, vortexed vigorously for 20 sec and centrifuged at 10,000 rpm for 3 min. The supernatant was transferred into clean tube and an aliquot of 25 µl of protein precipitation solution was added, vortexed and centrifuged at 10,000 rpm for 3 min. The supernatant was then transferred into a new tube containing 300 µl isopropanol. To pellet DNA, tubes were inverted gently 5-10 times and centrifuged 10,000 rpm, 2 min. The supernatant was carefully decanted and washed twice with 70% alcohol. The DNA pellet was dried and re-hydrated with 50-100 µl of either a DNA hydration solution or sterile distilled water.

5.2 Plasmid Preparation

Plasmid DNA from *E. coli* was prepared by rapid alkaline method (Birmboim and Doly, 1979). Culture was grown in 5 ml LB broth supplemented with appropriate antibiotic at 37^{o}C with shaking until cell growth reached log phase. Cell culture was centrifuged and re-suspended with 100 µl of solution I (50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, pH 8.0) mixed by vortexing and then 200 µl of solution II or lysis solution (0.2 N NaOH, 1% (w/v) SDS) were added and mixed well by inverting. The 150 µl of solution III (60% (v/v) 5 M Potassium acetate, 11.5% (v/v) Glacial acetic acid, and distill water 28.5% (v/v)) were added and mixed well by inverting. The mixture was centrifuged 5 min and supernatant was collected by centrifugation. Two volumes of absolute ethanol were added, mixed and kept at -20 ^{o}C for 1 hr. The DNA was collected by centrifugation, then washed twice with 70% ethanol, dried and re-suspended with sterile distilled water.

6. DNA Manipulation

6.1 Chromosome and Plasmid Digestion

Chromosome and plasmid digestion or modification with various restriction and modifying enzymes in this study were performed by using buffer provided with the enzymes. Conditions of digestion, and incubation period were performed as recommended by manufacturers.

6.2 Ligation of DNA Fragments into a Vector

The DNA fragments were digested with various enzymes and mixed with linearized vector also cut with the same restriction enzyme. In general, the molar ratio of insert to vector of 5:1 will be used. A 5x ligation buffer and TE were added to make final concentration of 1x before addition of 1-2 units of T4 DNA ligase and incubated at 4°C overnight. For DNA fragment of PCR products, the fragments were

cloned into a T-tailed vector using GEM-T Easy cloning kit (Promega) as described by manufacturer and then the recombinant plasmid was transformed to *E. coli* DH5α by standard $CaCl_2$ method (Sambrook *et al.*, 1989).

6.3 Recovery of a DNA Fragment from Agarose Gel

The method for recovery a DNA fragment from gel was followed by Yue and Orban (2001). Agarose gel electrophoresis of DNA fragment was performed using 7% gel in TAE buffer. DNA fragment was excised from agarose gel by using a clean razor blade. The gel was put into microcentrifuge tube and added 6 M NaI solution 3 volume of gel weight. The tube was heat at 65 °C for melting gel 5 min and then the solution was passed through silica column (Promega) by briefly spining for 5 sec. The volume of 600 µl wash solution (10 mM Tris, 1 mM EDTA, 100 mM NaCl, and 50% ethanol, pH 7.5) was added to silica column and centrifugation for 1 min to wash DNA which was bounded with silica. This step was repeated for 2 times. Silica column was placed in a new tube and then DNA was eluted from silica by adding 50-100 µl of distilled water incubated for 1 min at RT and centrifuged at 10,000 rpm for 1 min.

6.4 Preparation of Cemically Treated Competent *E. coli* Cells

The method described by Hanahan, (1983) was followed. The single colony of *E. coli* strain DH5α was selected and inoculated to LB broth, and incubated at 37 ^{o}C for overnight with shaking. A 1 ml of culture cells was inoculated to new LB broth (100 ml) and shaken at 37 ^{o}C for 3-4 hr O.D. 0.25-0.3 at 600 nm. The culture was harvested by centrifugation. The supernatant was removed and the pellet was re-suspended with ice cold RFI buffer (100 mM RbCl; 50 mM $MnCl_2.4H_2O$; 30 mM potassium acetate; 10 mM $CaCl_2.2H_2O$; 15% glycerol, pH adjusted to 5.8 with and filter steriled with a 0.2 micron filter membrane for sterilization). The mixture was placed on ice for 5 min and centrifuged. The pellet was gently re-suspended in RFII buffer (10 mM MOPS; 10 mM RbCl; 75 mM $CaCl_2$; 15% glycerol, adjusted pH to 6.8

with 0.2 M acetic acid and steriled as RFI). One hundred µl were aliquoted into microcentrifuge tubes and frozen immediately with liquid nitrogen and stored at -80 °C.

6.5 Transformation and Selection

Transformation was performed according to the method described by Sambrook *et al*. (1989). The stored competent cell was thaw on ice. The competent cells were transferred to ligated product, thoroughly mixed, and incubated on ice for 30 min. The mixture was heat-shocked at 42 ^{o}C for 90 sec, chilled on ice immediately for 2 min, and added 1 ml LB broth to tube. The transformed cells were incubated at 37^{o}C with shaking, and then collected the transformed cells by centrifugation and re-suspended with 200 µl LB broth. The 50 µl of cell suspension were spreaded on LB agar containing antibiotic. In order to reduce formamide toxicity, agar plates must be pre-spreaded with 50 µl of X-gal (20 mg/ml in formamide) at least 30 min before spreading the transformed cells. The agar plate was further incubated at 37 ^{o}C for overnight. The white colonies were picked and replicated on new antibiotic plate for further plasmid isolation and analysis.

7. Construction of Expression *polS* in Plasmid pGEX-2T

The *polS* gene was amplified using two sets of specific primers. For amplification of 600 bp fragment of the *polS* gene, the forward primer used was sorF (5’ GGC ACG CTC GCG CAA CTC ATC GA 3’) and reverse primer sorR (5’GAT TAC ATC ACC GCT CAG ACG CT 3’). For amplification of 800 bp fragment, the forward primer used was polSF (5’GTC AAG GGA TCC GTT GCA GGA CAA GGT CGC GAT CCT 3’ where underline indicated *Bam* HI site) and reverse primer polSR (5’ CTA CAT GAA TTC GCT CAT CCA GTT GCC GCC GTC 3’ where underline indicated *Eco*RI site). DNA amplification of *polS* gene was performed as described in DNA amplification. The approximately 800 bp fragment containing the complete coding sequence of *polS* was subsequently cloned in pGEM-T. Clone

containing *polS* insert, pGEM-*polS*, was isolated and double digested with *Bam*HI and *Eco*RI. The recombinant expression plasmid, pGEX- *polS*, was constructed by ligation of the *Bam*HI- and *Eco*RI- digested *polS* gene into plasmid pGEX-2T (Amersham), which pre-digested with the same restriction enzymes.

Sequences were analyzed by Lasergene 4.03 software package (DNASTAR, USA, WI; **http://www.dnastar.com/**). *Seqman* was used to assemble contigs and *MegAlign* was used to compare sequences, assess their similarity. Phylogenetic trees was constructed using Clustal W (Thompson *et al.*, 1994).

8. Expression of Recombinant SDH

E. coli DH5α culture harboring pGEX- *polS* was inoculated in 10 ml of LB broth containing ampicillin (100 μg/ml) and shaken at 37 ^{o}C. After 2 hr of cell growth, which designated as T_0, the expression of the enzyme was induced by addition of IPTG to cell culture at final concentration of 1 mM. At 2, 4, and 6 hr after induction, which is designated as T_2, T_4, T_6, respectively, cells were harvested by centrifugation. All cultures collected from different time intervals were harvested for cell pellet to determine optimum time for induction by SDS-PAGE analysis

9. Protein Analysis on SDS-PAGE analysis

SDS-gel system of (Laemmli, 1970) was used to analyze protein extracted from *E. coli* transformants.

9.1 Gel Preparation

The 15% of separating gel was prepared by mixing 5 ml of solution A (29.2% (w/v) acrylamide, 0.8% (w/v) N, N′-methylene bis acrylamide in distilled water, filtered, and kept at 4^{o}C in dark bottle), 2.5 ml of solution B (1.5M Tris-HCl buffer, pH 8.8), 2.3 ml of distilled water, 100 μl of solution C (10% (w/v) ammonium persulfate freshly prepared), and 100 μl of solution D (10% (w/v) SDS). The solution

mixture was added with 4 µl of N,N,N′,N′- tetramethylenediamine (TEMED), and mixed thoroughly. This mixture was immediately poured into the space between glass plates of slab gel electrophoresis set. The small amount of distilled water was slowly added to cover the surface layer of separating gel. The acrylamide was allowed to polymerize at least 30 min and gel surface was flushed several times with distilled water to remove unpolymerized acrylamide before addition of stacking gel. The 5% of stacking gel solution consisted of 0.83 ml of solution A, 0.63 ml of solution D (1 M Tris-HCl buffer, pH 6.8), 3.4 ml of distilled water, 50 µl of solution C, 50 µl of solution D, and 5 µl of TEMED was poured over separating gel, followed by insertion of comb into the gel surface and allowed the gel to polymerize for at least 40 min. The comb was removed and all the wells were flashed several times with distilled water to eliminate unpolymerized acrylamine. The slab gel was set into the electrophoresis apparatus. Freshly prepared electrophoresis buffer (25 mM Tris-HCl buffer, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3) was added to cover the well before applying samples and pre-electrophoresed at constant voltage (25 mA) for 30 min at 4^{o}C or room temperature.

9.2 Sample Preparation and Electrophoresis Operation

The protein samples were prepared by mixing 50 µl of cell suspension of *E. coli* transformants with 50 µl of 2x sample solubilizing buffer (125 mM Tris-HCl pH 6.8, 4% (w/v) SDS, 5% (v/v) β-mercaptoethanol, and 0.02% (w/v) bromophenol blue, 15% (v/v) glycerol). The mixtures were boiled in boiling water for 10 min and cool on ice then, centrifuged for 1 min before loading into each well. An aliquot of 12 µl of cell extracts was applied to the gel and compared to protein standard marker from Biolab (New England, UK). Electrophoresis was performed until the tracking dye reached the bottom of the gel (3 h) at constant current of 30 mA.

9.3 Determination of Molecular Weight of Protein in SDS-PAGE.

After complete electrophoresis, the gel was stained in staining solution (0.25% (w/v) Coomassie brilliant blue R-250) in fixing solution (40% (v/v) methanol, and 7% (v/v) acetic acid) for a few hours at room temperature. The excess stain was removed by placing the stained gel in destaining solution (5% (v/v) methanol and 7% (v/v) acetic acid in distilled water) until the gel size was reversed back to the normal size. The gel was soaked in this fixing solution with a few changes till the protein bands were clearly seen. Molecular weight (M.W) of protein was determined using standard protein marker Broad Range from Biolabs (cat. P7702S).

10. Construction for IDM Plasmid for Gene Disruption by Homologous Recombination

10.1 Generating of Donor Plasmid (Deletion Mutant Alleles)

The donor plasmids pKS was used to generate deletions of *polS* genes in the *R. solanacearum* strain To-Ud3 virulence strain via homologous recombination. To construct the deletion of *polS* by overlapping PCR, sequences of the oligonucleotide primers are as follows; sorF: 5′ GGC ACG CTC GCG CAA CTC ATC GA 3′; sorM: 5′GCC TGC GAA GCC ATG TTG ATG 3′; ΔsorM22: 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 into 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 (Biolab) was used instead. In the second step, the left and right fragments were annealed at their overlapping region and amplified by PCR as a single fragment, using the outer primers (sorF and sorR). Specifically, 1 µl of each of the two asymmetric PCR mixtures and 200 µM each of

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

10.2 Gene Replacement

Mutant allele cloned into the pK18mob gene replacement vector (pKS) was conjugated into *R. solanacearum* To-Ud3 by following method. *E. coli* S17-1 harboring plasmid pKS and helper *E. coli* HB101 harboring pRK2013 were grown on LB agar medium containing kanamycin (50 μg/ml) and incubated at 37 °C for 18 hr. Strain To-Ud3 of *Ralstonia solanacearum* was generally grown on YP medium and incubated at 30 °C for 24 hr. Spontaneous nalidixic acid (Nal^r) colonies of *R. solanacearum* were obtained by culturing strain To-Ud3 in YP medium containing 50 or 100 μg/ml of nalidixic acid by shaking at 28-30°C for 24 hr. The 0.1 ml of cell culture was spread on nalidixic medium and incubated at 28-30 °C for 48-72 hr. Suspected Nal^r colonies were transferred to YP containing nalidixic acid for two times to confirm the Nal^r mutant.

For bacterial mating, culture of donor strain of *E. coli* S17-1 harboring pKS plasmid was mixed with culture of recipient strain of *R. solanacearum* To-Ud3 Nal^r and helper *E. coli* HB101 harboring pRK2013 in microcentrifuge tube with an equal volume. The mixed culture is centrifuged at 4 °C, 4000 rpm for 10 min. Supernatant was discarded and then 50 μl of YP medium was added to resuspend the cell pellet. The 50 μl of mixed suspension is spotted on nylon membrane filter (diameter 25 mm, pore size 0.45 μm) and placed over YP medium then incubated at 30 °C for 2-3 days before washed off the bacteria into water. Bacterial suspension of mating cells will be spread over the surface of YP agar medium containing nalidixic acid and kanamycin, and incubated at 30 °C for 48-72 hr. Non-utilizing sorbitol mutant clones, colonies were selected and cultured in sorbitol medium; mineral medium supplemented with

peptone, agar, a pH indicator, and 1% sorbitol (Hayward, 1964). Cultures tubes are incubated at 28-30 °C and the reaction was recorded for up to 30 days.

10.3 PCR Analysis of Mutant

The genomic DNA from the potential mutant strains were extracted and used as template for PCR. The integration process of recombination plasmid was identified using long PCR to amplify 5.4 kb DNA fragment with forward primer claF; 5′ TGA CCG ACG CAG CCC GCA TAG G 3′ and reversed primer claR; 5′ TGT AAG GGG CAT TCG CTC GCA TCT 3′. DNA amplification was performed in final volume of 50 µl containing 60 mM Tris-Sulfuric acid pH 9.1, 2 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, the set of 0.2 mM dNTP, 1 , M of each primer, 50 ng of DNA template , and the 1 µl of mixing Taq : Deep Vent (Biolab) in the ratio 20:1 (Unit). The thermal cycles profile was 50 sec at 94^{o}C, 50 sec at 60^{o}C, and 4 min at 72^{o}C. All experiments used 30 cycles and a final 7 min at 72^{o}C extension step. For selecting deleted mutant strains by PCR reaction, sorF and sorR primers were used to generate 2 type of DNA fragment size: 523 bp fragments for deletion mutant strains and 623 bp fragments for wild-type strains. DNA amplification was previously described above.

11. Southern Blot Hybridization

11.1 Preparation of Digoxigenin-Label DNA Probe

Two sets of primer pair, sorF-sorR and kmF-kmR, were used to amplify *sorS* and *kan* probe by DNA amplification, respectively. The DNA amplification was the same as mention above except digoxigenin-11-dUTP was added in the ratio of Dig-11- dUTP : dTTP at 1: 9.

11.2 Southern Blotting

The chromosomal DNA of *R. solanacearum* wild type and mutant were completely digested with various enzymes and DNA fragments were resolved by

agarose gel electrophoresis. After taking a photograph, depurination of DNA in gel was performed by soaking gel in 0.25 M HCl. The DNA in gel was denatured by soaking in 0.4 N NaOH. The DNA from agarose gel was ascending transferred onto a nylon membrane (High bond N^+ from Roche) by capillary blotting technique using 0.4 NaOH as transfer buffer for overnight at room temperature. After Southern blotting, the membrane was rinsed with 0.2xSSC to remove any impurities. The DNA on the membrane was covalently fixed by heating at 120 °C for 30 min and subjected immediately to hybridization process.

11.3 Hybridization with DIG-Labeled Probe

The pre-hybridization experiment was performed by soaking the blotted nylon membrane in a 10 ml pre-hybridization solution (5xSSC, 0.1% N-laurylsacosine, 0.02% SDS, 2% blocking reagent, Roche) and incubated in hybridization oven (SL, SHELLAB) at 68 °C for at least 1 hr. The prehybridization solution was discarded and replaced with a 10 ml hybridization solution. The hybridization solution was the same as pre-hybridization solution except that denatured DIG-labeled DNA probe was also included. Hybridization procedure was continued at 68°C for 12-16 hr. The hybridized membrane was washed twice in a washing solution I (2x SSC, 0.1%SDS) and further washed twice in a washing solution II (0.1x SSC, 0.1%SDS) at 68 °C, then the detection step was performed by rinsing the membrane with washing buffer solution (100 mM maleic acid, 150 mM NaCl, pH 7.5). The membrane was incubated for 1 hr in 1% blocking reagent in washing buffer solution and then incubated for 45 min in 1% blocking reagent in washing buffer solution containing anti-DIG-AP fab fragments at RT. The membrane was re-washed twice in washing buffer solution and submerged into detection buffer (100 mM Tris-HCl, 100 mM NaCl; pH 9.5). Finally, chemiluminescent detection step was performed by addition 1µl of CDP star (Roche Diagnostics) in 500 µl of detection buffer. The detection buffer with CPD star was spreaded on surface of membrane for 3 sec and then the detection solution with CDP star was discarded. The signal was detected by using X-ray film (Kodak, Green 400 screen).

12. Quantification of EPS

Cultures of wild type and non-oxidizing sorbitol mutant were grown for 72 hr in BG (1% Bacto-Peptone, 0.1% Casamino acids, 0.1% yeast extract, 0.5% glucose) broth at 30 °C. Samples (1 ml) were centrifuged at 13,000 rpm at 4 °C, and the supernatants were carefully removed and stored at 4°C. Cell pellets were washed twice with 0.1 M NaCl, suspended in 1 ml of water, and stored at -20 °C. To recover EPS, culture supernatants were adjusted to 0.1 M NaCl and 4 volumes of acetone were added. After incubating overnight at 4 °C, precipitated material was recovered by centrifugation at 13,000 rpm at 4 °C, 10 min, dissolved in 200 µl of H_2O, heated at 65 °C for 10 min, and centrifuged for 5 min to remove insoluble material. The concentration of hexosamines, which is a reliable indication of EPS content, was estimated with modified Elson and Morgan reaction (Herbert *et al.*, 1971) and *N*-acetyl-D-glucosamine standard curved was explained in appendix.

The following reagents were used in developing the modified procedure for the determination of hexosamines. Buffer: eaqual volumes of 1 M $NaHCO_3$ and 1 M Na_2CO_3, pH 9.6; acetylacetone reagent: 4.8% (v/v) acetylacetone in buffer (prepared fresh); and Ehrlich reagent: 0.8 g *p*-dimethylaminobenzaldehyde dissolved in 30 ml 99.5% (v/v) ethanol and 30 ml conc. (35%) HCl, (stored at -20 °C) were prepared.

The 200 µl of EPS solution were placed in microcentrifugation tubes. The 200 µl HCl (12 M) were added slowly to dissolve the polymer. The hydrolysis was carried out by boiling for 1 hr. To terminate the hydrolytic reaction, the samples were cooled in melting ice and neutralized with an equal volume of 6 NaOH (400 µl).

Analysis for hexosamine was based on a modification of the Elson-Morgan method as described below. The 25 µl salt corrected aliquots of neutralized samples diluted with 100 µl of distilled water were pipetted into microcentrifuged tubes. Freshly prepared 125 µl acetylacetone reagent was added to the samples, 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 were 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. The amounts of total hexosamines in EPS liberated during hydrolysis were calculated from standard plot between A530 and *N*-acetyl-D-glucosamine concentration as explained in appendix.

To assay for total cell protein, 25 µl samples of the washed cell suspensions were lysed by mixing with 75 µl of a 1.33% (w/v) sodium dodecyl sulfate-0.133 N NaOH solution and heated 10 min at 65 °C. The protein in solubilized samples was quantified as described by Bradford (1976).

RESULTS

1. Amplification of *polS* Gene by Specific Primers and Hybridization of *polS* Gene.

From whole genome sequencing data of *Ralstonia solanacearum* GMI1000, encoded for sorbitol dehydrogenase *polS* gene was reported (GI 17544719). Sorbitol dehydrogenase (SDH), also known as glucitol, polyol, or L-iditol dehydrogenase (EC.1.1.1.14), catalyzes the oxidation of sorbitol to fructose with NAD as a cofactor. From this sequence, we designed two set of specific primers. First, sorF and sorR located in *polS* gene were used to amplify 600 bp fragment of the *polS* gene. This primer can be confirmed presence or absence of endogenous sorbitol dehydrogenase gene in all biovars. Second, polSF and polSR were designed to amplify 800 bp fragment of structural *polS* gene.

Using sorF and sorR as primers and genomic DNA of *R. solanacearum* as template, PCR amplification resulted in a single band with size approximately 600 bp (Figure 1A). This 600 bp of PCR product was observed in biovars 3 and 4 which able to oxidize sorbitol as a sole carbon source (Figure1A; lane 6-7 and 8-9), but not in biovars 1 and 2 which was unable to oxidize sorbitol (Figure1A; lane 2-3 and 4-5).

To investigate that *polS* gene is present in biovars 3 and 4, Southern blot analysis of DNA isolated from all biovars and partially digested with *Cla*I was carried out. Hybridization DNA isolated from biovars 3 and 4 to the *sorS* probe showed a 1.8 kb fragment corresponded to the endogenous *polS* gene (Figure 2; lane 4-6). However, this fragment is not observed in biovar 1 (Figure 2; lane3).

To further confirm that biovars 3 and 4 contained *polS* gene, the activity of sorbitol dehydrogenase was determined in all biovars. When crude extract from all biovars were assayed for sorbitol dehydrogenase activity, no activity was detected in biovars 1 and 2 (data not show).

Therefore, results from PCR amplification, Southern blot analysis, and measuring sorbitol dehydrogenase activity indicated that biovars 3 and 4 contained *polS* gene in which they can utilize sorbitol as carbon source. However, it is presumed that biovars 1 and 2 do not possess sorbitol utilizing gene. Furthermore, no PCR product was observed when polSF was used as forward primer and polSR as reverse primer in the amplification of the DNA isolated from biovars 1 and 2 (Figure 1B, lane 2-3 and lane 4-5, respectively).

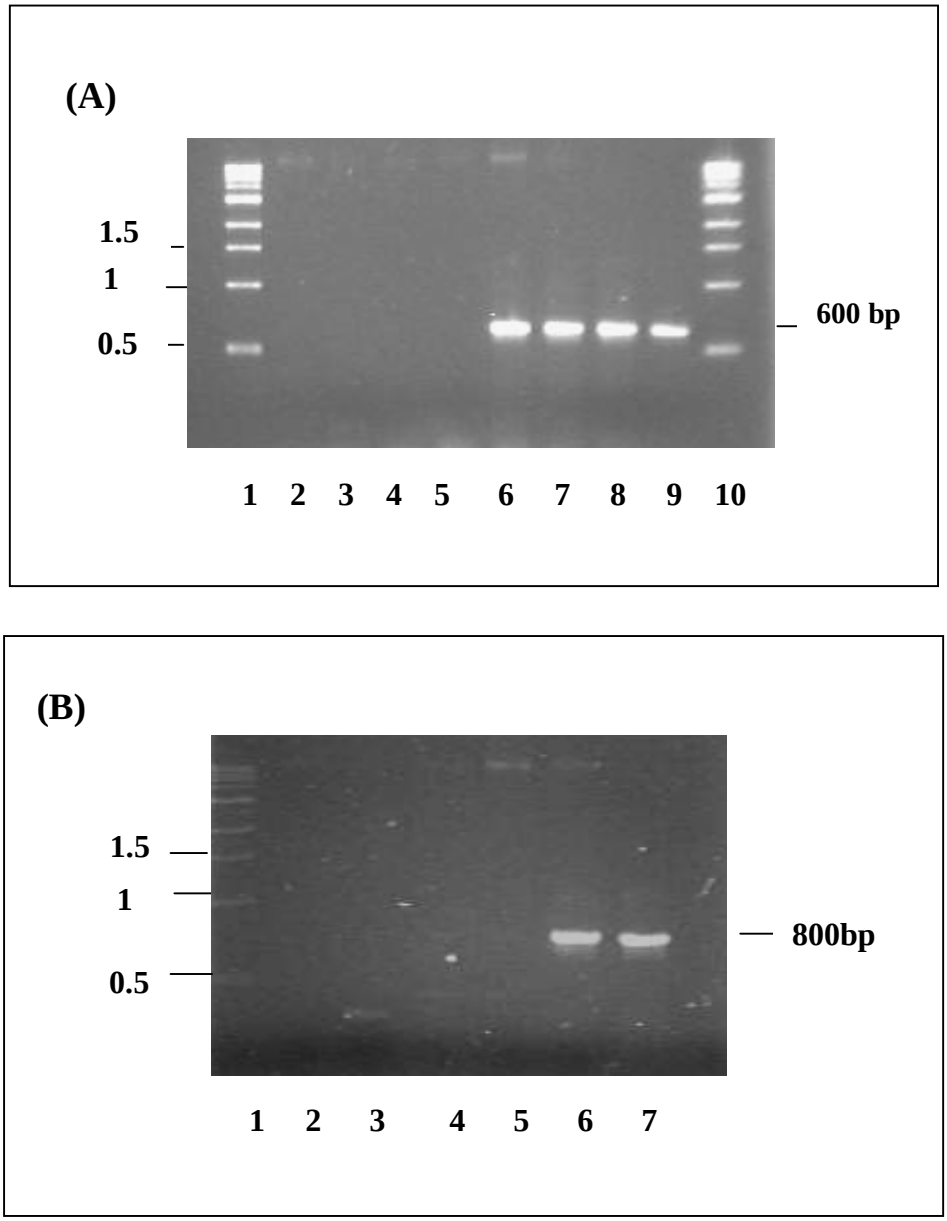

Figure 1 Agarose gel electrophoresis of (A) 600 bp fragment amplified with sorF and sorR primers using DNA extracted form *Ralstonia solanacearum* as a template. Lanes 1 and 10, 1 kb ladder marker; lanes 2-3, biovar 1; lanes 4-5, biovar 2; lanes 6-7, biovar 3; lanes 8-9, biovar 4; (B) 800 bp fragment amplified with polSF and polSR primers using DNA extracted form *R. solanacearum* as a template. Lane 1, 1 kb ladder marker; lanes 2-3, biovar 1; lanes 4-5, biovar 2, lane 6, biovar 3 (strain To264); lane 7, biovar 4 (strain PB41-4).

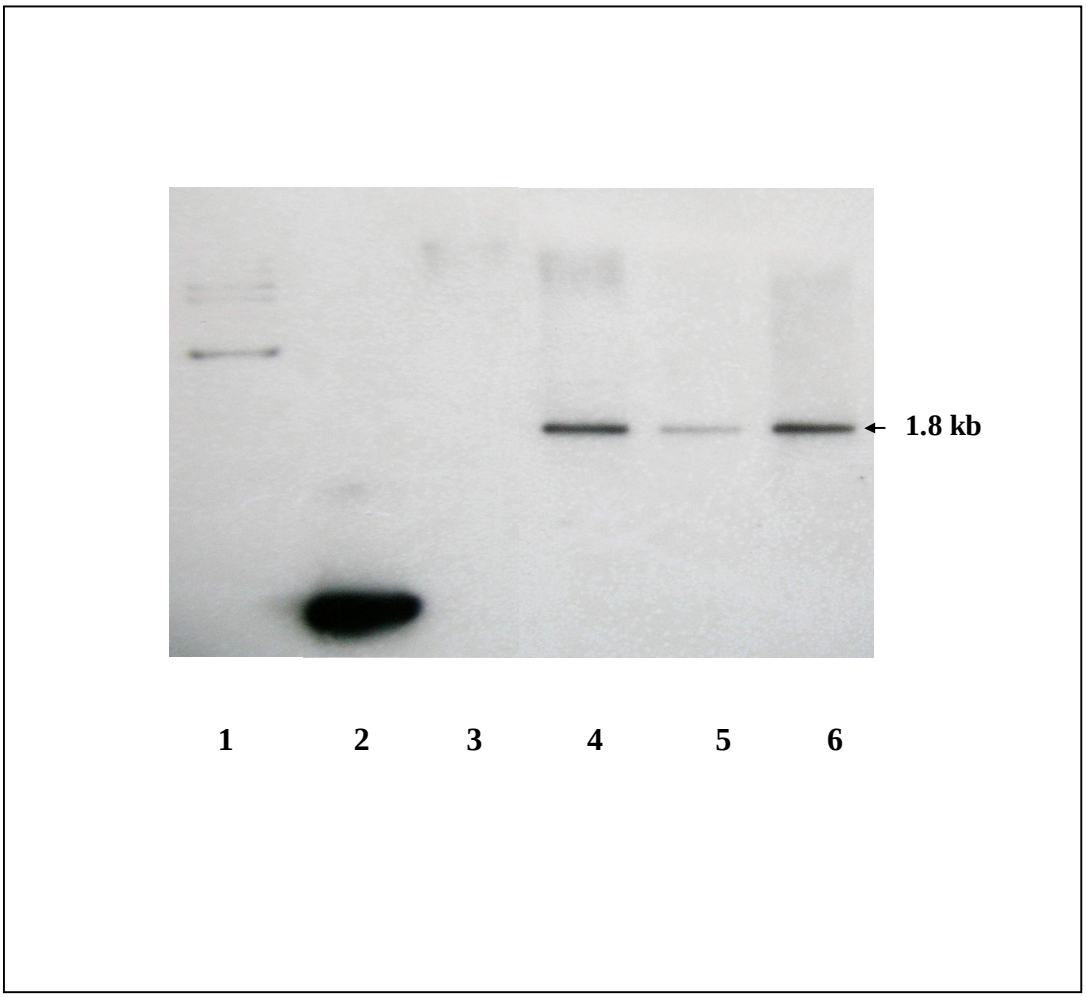

Figure 2 Southern blot analysis between *sorS* probe and DNA extracted from different biovars of *Ralstonia solanacearum* and partially digested with *Cla*I showing specific 1.8 kb DNA product in biovars 3 and 4. Lane 1, 1 kb ladder marker; lane 2, *sorS* probe; lanes 3-6, *R. solanacearum* stain FC 325 (biovar1), strain To264 (biovar3), strain Pe109 (biovar3) and strain PB41-4 (biovar 4), respectively.

2. Nucleotide Sequencing and Analysis of *polS* Gene.

To dentify sorbitol dehydrogenase gene from *R. solanacearum*, polSF and polSR primers were used to amplify the structural *polS* gene. Using polSF and polSR as primers and genomic DNA as template, PCR amplification resulted in a single band with approximately size of 800 bp (Figure 1B).

To determine the nucleotide sequence, the 800 bp fragments from *R. solanacearum* strain To264 was cloned and sequenced. The obtained 771 nucleotide sequences (GeneBank accession no. AY946241) and deduced amino acid sequences were shown in Figure 3. Nucleotides sequence and deduced amino acid sequence initiated from TTG encoded for leucine to TGA defined as a stop codon. When the 771 nucleotide sequence and dedued amino acid was analysed in Gene Bank, it was similar to sorbitol dehydrogenase *polS* gene of *R. solanacearum* GMI1000. This data elucidate that the 771 nucleotide sequence was partial sorbitol dehydrogenase *polS* gene. The ORF of partial *polS* encoded a protein consisting of 256 amino acid residues with predicted molecular mass of 27,314 Da.

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). The eight amino acid residues conserved among these short chain dehydrogenase/reductase protein family. They contained three of glycine residues (G) involved in coenzyme binding, the functionally important residues tyrosine (Y) and lysine (K), and three further residues glycine (G), serine (S), and proline (P) (Figure 4).

Nucleotide and amino acid sequences alignment showed highly conserve between sorbitol dehydrogenase gene from *R. solanacearum* strain To264 and sorbitol dehydrogenase which are proteins of the short chain alcohol dehydrogenase/ reductase family (SDR) from other bacteria. *R. solanacearun* strain To264 is closely related to *R. solanacearun* strain GMI1000 at 99% similarity. Similarly, it is related to *Burkholderia ceapacia* and *Burkholderia fungorum* at 90%, 85% similarity,

respectively but is distantly related from *Pseudomonas syringae* pv. *syringae*, *Rhodobacter sphaeroides*, *Sinorhizobium meliloti* and *Mesorhizobium* sp. (60% similarity) (Figure 6). A phylogenetic analysis indicated that *polS* joined *the Burkholderia cepacia* sequence as sister to the *R. solanacearum* pair as shown in Figure 5.

3. SDS-PAGE of Expression Sorbitol Dehydrogenase

The structural *polS* gene was amplified by specific primers polSF and polSR and then PCR product was cloned in cloning vector and subcloned into expression vector GEX-2T. Molecular weight of sorbitol dehydrogenase was estimated by SDS-PAGE.

The sorbitol dehydrogenase (SDH) was over-expressed as a glutathione-S-transferase (GST) fusion protein in *E. coli*. The molecular mass of this fusion protein, GST-SDH, was estimated to 52,000 Da (25,000 Da GST + 27,000 Da SDH) by SDS-PAGE (Figure 7). The molecular mass of the polypeptide was in good agreement with the molecular mass obtained by SDS-PAGE (27,000 Da). After six hours of induction, protein synthesis increased more than initial stage of induction (2 hr).

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        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
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Figure 3 Partial nucleotide and deduced amino acid sequences of sorbitol dehydrogenase (*polS*) of *Ralstonia solanacearum* strain To264. The two sets of primers, polSF-polSR and sorF-sorR, were marked with underline and dotted-line, respectively. A total of 771 bp and 256 amino acid residues were submitted to Genbank (Accession no.AY946241)

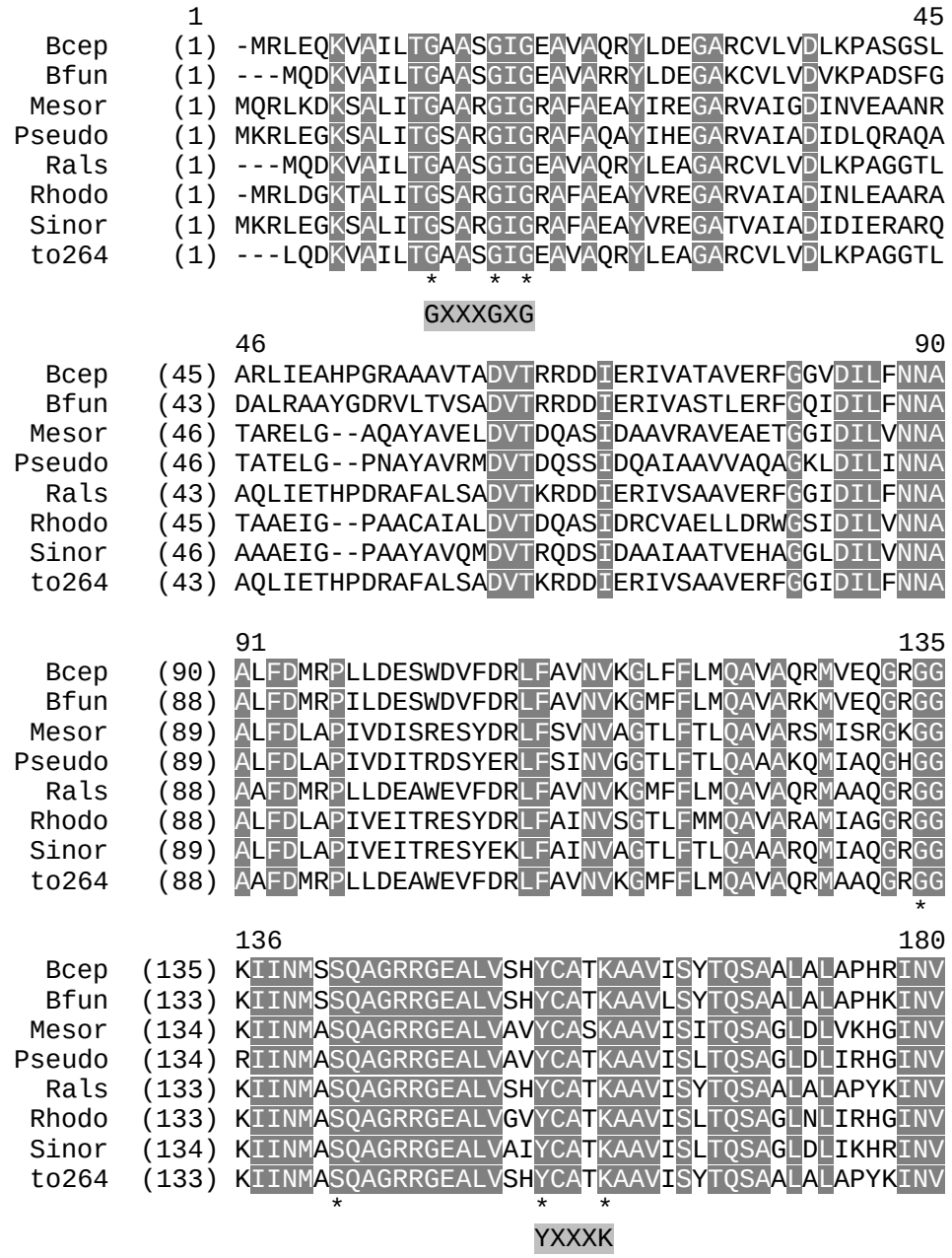

Figure 4 Multiple sequence alignment of the SDR proteins. Sources of sorbitol dehydrogenase protein sequence were from other bacteria shown in Table 5. Conserved amino acids of SDR protein were marked with asterisk. The solid block denoted identical amino acids. The two conserved NAD^+ binding domain and catalytic domain were shaded with gray block GXXXGXG and YXXXK.

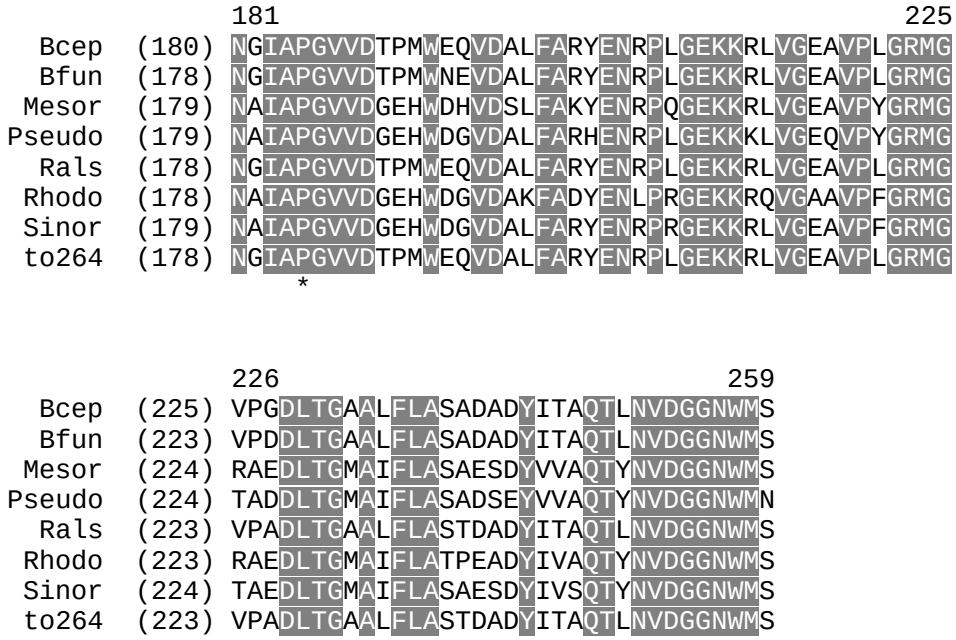

Figure 4 (cont'd) Multiple sequence alignment of the SDR proteins. Sources of sorbitol dehydrogenase protein sequence were from other bacteria shown in Table 5. Conserved amino acids of SDR protein were marked with asterisk. The solid block denoted identical amino acids. The two conserved NAD^+ binding domain and catalytic domain were shaded with gray block GXXXGXG and YXXXK.

Table 5 Sources of sorbitol dehydrogenase protein sequences from different strains of *Ralstonia solanacearum* and other bacteria

Bacteria	Accession
Bcep: *Burkholderia cepacia*	(NZ_AAAJ03000006)
Mesor: *Mesorhizobium* sp. BNC1	(NZ_AAED01000002)
Pseudo: *Pseudomonas syringae* pv. *syringae* B728a	(NZ_AABP02000006)
Rals: *Ralstonia solanacearum* GMI1000	(NC_003295)
Rhodo: *Rhodobacter sphaeroides*	(Q59787)
Sinor: *Sinorhizobium meliloti* 1021	(NC_003047)
To264: *Ralstonia solanacearum* To264.	(AY946241)

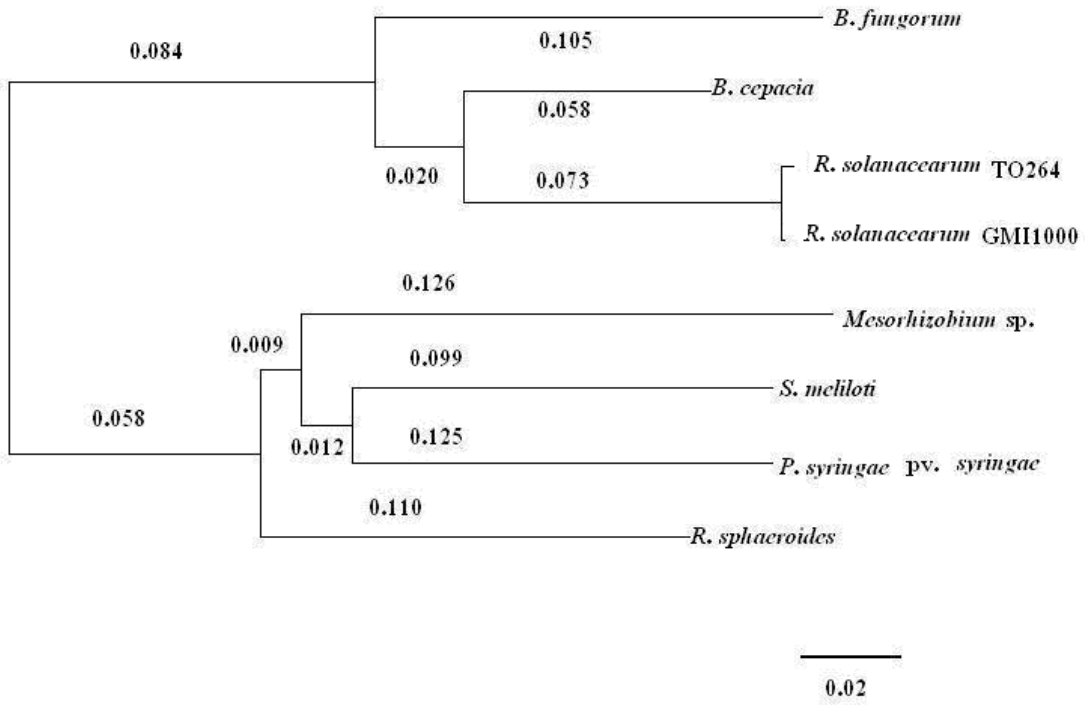

Figure 5 Phylogenetic tree of sorbitol dehydrogenase protein sequences from seven bacteria. Neighbor joining method of ClustalW program was used to construct phylogenetic trees.

Percent nucleotide sequence similarity

1	**2**	**3**	**4**	**5**	**6**	**7**	**8**		**Bacteria**
	84.8	76.0	56.0	55.8	99.6	59.0	57.1	**1**	*R. solanacearum* To264
89.1		80.0	59.3	57.6	85.2	61.7	59.4	**2**	*B. ceapacia*
83.7	86.4		55.9	52.1	76.4	56.2	56.7	**3**	*B. fungorum*
57.6	58.5	58.4		71.4	56.3	72.6	73.5	**4**	*Mesorhizobium* sp.
56.0	57.0	56.0	79.8		55.5	70.3	75.8	**5**	*P. syrinegae* pv. *Syringae*
99.6	89.1	83.7	57.6	56.0		59.5	57.3	**6**	*R. solanacearum* GMI1000
57.6	58.4	58.4	77.4	76.7	57.6		75.1	**7**	*R. sphearoides*
58.0	60.5	59.1	79.1	83.7	58.0	80.5		**8**	*S. meliloti*

Percent amino acid sequence similarity

Figure 6 Nucleotide and amino acid sequence similarity of sorbitol dehydrogenase (SDH) and its homologues among seven bacterial species. Percent nucleotide similarity was denoted above the diagonal and percent amino acid similarity was below. Sequence data of bacterials.

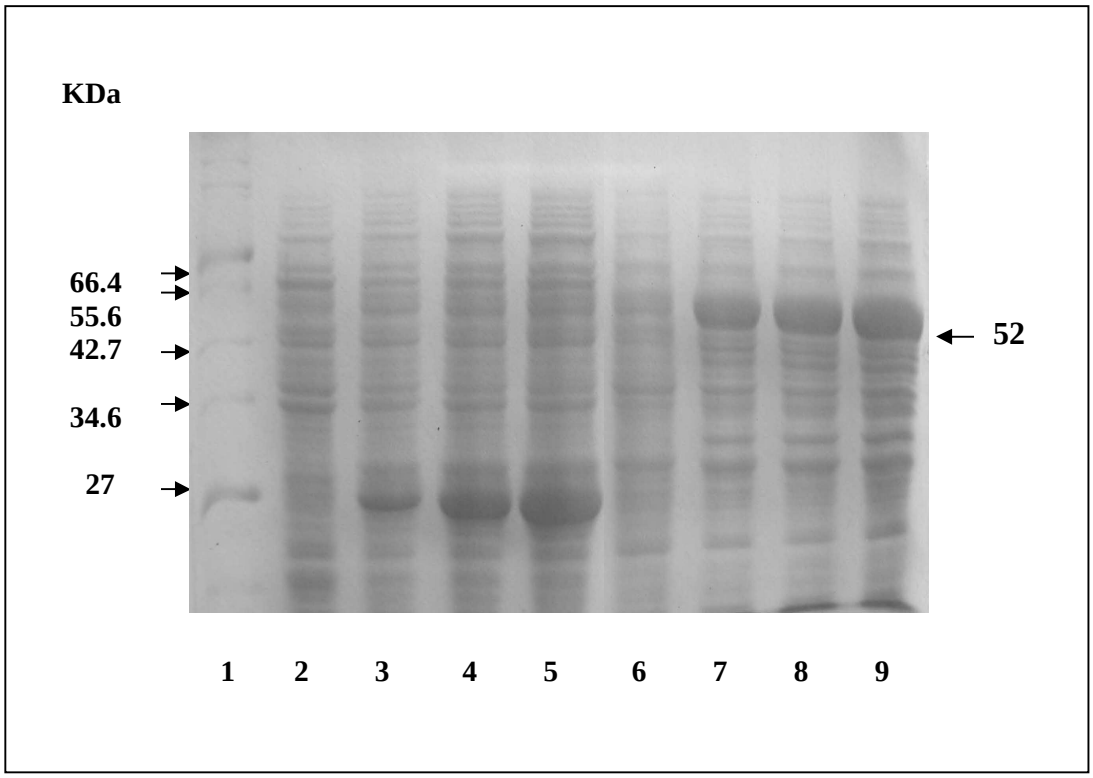

Figure 7 SDS-PAGE of crude extract from *E. coli* harboring pGEX-2T and pGEX-*polS*, respectively. Lane 1, MW marker; lanes 3-5, crude extract of pGEX-2T in which cells were harvested at 2, 4, and 6 hr after induction, respectively; lanes 7-9, crude extract of pGEX-*polS* in which cells were harvested at 2, 4, and 6 hr after induction, respectively; lanes 2 and 6, crude extract of culture cell from *E. coli* harboring pGEX-2T and pGEX-*polS* without induction were used as negative control.