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CHARACTERIZATION OF THREE GENES LOCATED IN A 4.0-KB FRAGMENT
WITHIN THE *MID* GENE CLUSTER OF
RHIZOBIUM SP. STRAIN TAL1145.

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ABSTRACT

Rhizobium sp. strain TAL1145 is a soil bacterium that forms nitrogen-fixing root nodules on the tree-legume, *Leucaena leucocephala*. *Leucaena* produces a toxic non-protein amino acid, known as mimosine, which is also released in root exudates. *Rhizobium* sp. TAL1145 can degrade mimosine and use it as a source of carbon and nitrogen. The genes for mimosine degradation (*mid* genes) in TAL1145 have been recently isolated. The *mid* genes are located in two clusters within a 25-kb DNA fragment in the TAL1145 chromosome. The aim of this investigation is to identify and characterize the genes in a 4.0-kb *EcoRI* fragment nestled between the *mid* and *pyd* gene clusters and to determine their role in mimosine degradation and symbiosis. Sequencing of this fragment showed four open reading frames (ORFs). ORF1 is 503 bp long and it may encode a 17.6-kDa protein of unknown function. The ORF2-encoded protein may be a 1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase), which might be involved in converting ACC sequestered from plants into ammonia and α -ketobutyrate. ACC is a precursor in the synthesis of ethylene by plant roots. By degrading ACC, the enzyme ACC deaminase may reduce ethylene production in the plant root system. The ORF3- and ORF4-encoded proteins have high homology with the small and large subunits of alkyl hydroperoxide reductase of several organisms. These proteins may be involved in protecting *Rhizobium* from oxidative damage during growth in the soil environment. Knockout mutants of TAL1145 were isolated by Tn3Hogus transposon insertion into ORF2 and ORF4. Results of this study indicate that

these genes are not involved in mimosine degradation. However, the role of *Rhizobium* sp. TAL1145 may not be limited to just a symbiotic nitrogen fixer, but may also enhance root development in *Leucaena* by inhibiting ethylene production.

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CHAPTER 1 INTRODUCTION

Rhizobium is a soil bacterium that interacts with leguminous plants and induces their roots to form nodules. Research during the past twenty years in many laboratories has elucidated *Rhizobium*-legume interactions and the nodulation processes (Brewin, 1991; Pueppke, 1996; Mylona et al., 1995; Fischer, 1994). In response to the flavonoids released in the root exudates of legumes, *Rhizobium* bacteria move toward roots, attach to root hairs, and induce root hair deformations. Rhizobia enter the roots through the deformed root hairs and induce the development of the infection thread, a tubular structure that carries the bacteria from the surface of the plant to the plant's inner cortex. Due to specific 'Nod factors' released by these bacteria, nodule formation is also initiated. Once the invading bacteria reach the rapidly dividing cortical cells within the developing nodules, they are released to infect the surrounding cells. The bacteria divide several times within the infected cortical cells and further differentiate into a nitrogen-fixing form known as bacteroids. In the bacteroids, some of the genes that are normally expressed when rhizobia live as free-living microorganisms outside the nodule are turned off and at the same time others that are required for nitrogen fixation are turned on. However most importantly, within the bacteroid, rhizobia convert atmospheric nitrogen (N_2) to ammonia (NH_4^+), a combined form of nitrogen, which is then utilized by the plant as a nutrient source. In return, the plant provides the bacteria with a source of carbohydrate products that are synthesized by photosynthesis. Thus, a mutual

symbiotic relationship between rhizobia and the host legume plant is established in which *Rhizobium* provides fixed nitrogen to the plant and the plant provides fixed carbon to bacteria.

Leucaena (*Leucaena leucocephala*) is a nitrogen-fixing tree legume that grows ubiquitously in tropical and subtropical climates. Over a hundred varieties have been identified and classified into three major types: Hawaiian, Salvador, and Peru (Brewbaker et al, 1983). The Hawaiian type has shown resistance to numerous pests and diseases, and can tolerate a wide range of environmental conditions (National Academy of Sciences, 1977). It has a deep root system, which allows it to be quite tolerant to drought and arid areas where most other tropical plants cannot grow. Although considered a noxious introduced weed in Hawaii, *Leucaena* does have a few desirable characteristics. Throughout tropical areas around the world, *Leucaena* is cultivated and readily used to control soil erosion because of its aggressive growth and is used to improve soil quality by augmenting nitrogen levels in the soil (National Academy of Science, 1977). Several varieties grow large enough to be used as a source of lumber, charcoal fuel and for various wood products (Brewbaker et al, 1983; National Academy of Science, 1977). The foliage of *Leucaena* can also be used as fodder for ruminant livestock animals since leaves are rich in protein and contain several vitamins, minerals and carotene (National Academy of Science, 1977). This last quality also permits *Leucaena* to be an excellent alternative food source for humans. However, there is a single major disadvantage, which is due to the presence of a toxin known as mimosine.

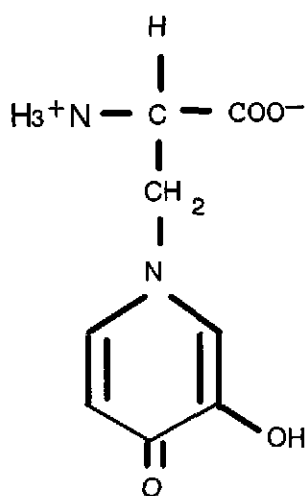


Fig. 1.1. Chemical structure of mimosine

Mimosine [β -N- (3-hydroxy-4-pyridone)- α -aminopropionic acid] (Fig. 1.1) is a toxic non-protein amino acid that is produced throughout *Leucaena*. Mimosine was first isolated from *Mimosa pudica* (Renz, 1939), although its concentrations there are much lower than those in *Leucaena*. The amount of mimosine varies in different plant tissues: 4-5% on a dry weight basis in seeds (Jones and Lowry, 1984); 8-10% mimosine has also been isolated from the leaves, pods, shoots, stems (Soedarjo and Borthakur, 1996; 1998); and 1-1.5% mimosine from roots and root exudates (Matthews and Rai, 1985). Mimosine also possesses antimetabolic properties that inhibit cell division at the late G1 phase by inhibiting replication fork formation during DNA synthesis (Boehme & Lenardo, 1993; Khanna and Lavin, 1993). Its toxic effects also inhibit growth and protein

synthesis in microorganisms (Ebuenga *et al.*, 1979; Serrano *et al.*, 1983). Most single-stomached animals (non-ruminants) are unable to break down mimosine completely when fed a diet of *Leucaena* and often experience hair-loss and development of swollen thyroids (goiters) (Jones *et al.*, 1978). Conversely, some ruminants such as goats, which contain bacteria in their rumen that can effectively degrade and utilize mimosine as a source of nutrients, are virtually unaffected by mimosine toxicity (Jones & Megarrity, 1986). Mimosine also has the ability to chelate iron in the soil with a binding constant of 10^{-6} (Kato *et al.*, 1992). This could have several potential negative effects since the mimosine-iron complex may block several biochemical activities required by the cell by depriving it of available iron.

As mentioned previously, mimosine is toxic to many microorganisms including most strains of *Rhizobium*, *Bradyrhizobium* and *Sinorhizobium* (Soedarjo *et al.* 1994). However, there are some *Leucaena*-nodulating strains of *Rhizobium* that can degrade and detoxify mimosine and thereby overcome its toxicity. Although fast growing strains such as *R. tropicii* and *Sinorhizobium fredii* sp. NGR234 can induce nodulation in *Leucaena*, these strains can neither tolerate nor degrade mimosine. The *Rhizobium* Research Group at the University of Hawaii has identified a *Rhizobium* sp. strain TAL1145 that can degrade mimosine and utilize it as a carbon and nitrogen source. This strain has been observed to effectively nodulate *Leucaena* in a wide range of environments (George *et al.* 1994). Although the ability to catabolize mimosine is not required for nodulation and nitrogen fixation, it provides a competitive nodulation

advantage to mimosine-degrading (Mid^+) *Rhizobium* in the *Leucaena* rhizosphere by providing the bacteria with both a unique and selective source of nutrients and at the same time inhibiting the growth of other microorganisms and rhizobia (Soedarjo & Borthakur, 1998). The Mid^+ *Rhizobium* strain TAL1145 is known to be competitive for nodule occupancy on *Leucaena* (Moawad & Bohlool, 1984).

The genes for mimosine degradation (*mid* genes) in TAL1145 have recently been isolated (Fox & Borthakur, 2001). These genes were located in a cosmid clone, pUHR263, which was isolated from the genomic library of TAL1145 by functional complementation of a mimosine-non-degrading strain TAL182. Borthakur et al. (2002) isolated 5 *mid* genes, *midA*, *midB*, *midC*, *midD* and *midR* involved in mimosine degradation. The first three *mid* genes encoded *midABC* are transporter proteins involved in mimosine uptake while *midD* encodes an aminotransferase required for degrading mimosine into 3-hydroxy-4-pyridone (HP), and *midR* is a regulatory gene encoding a LysR-type transcriptional activator.

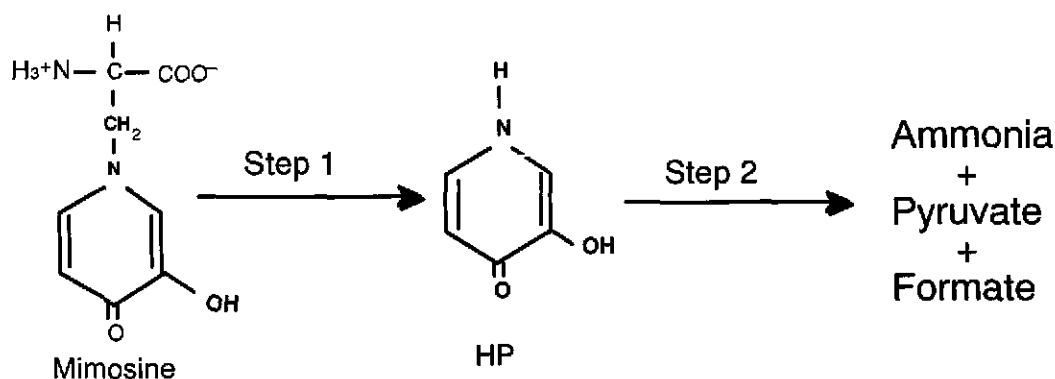


Fig. 1.2. Two major steps in mimosine degradation

The complete degradation of mimosine involves two major steps (Fox and Borthakur 2001) (Fig. 1.2). In the first step, mimosine is converted to HP, which is converted to ammonia, pyruvate, and formate in the second step. Genes for mimosine degradation are located in two clusters within a 25kb DNA fragment cloned in pUHR263. The genes involved in degrading mimosine in the first step, *mid* genes, are grouped together in an approximately 7 kb region of pUHR263 while the genes involved in the second step for the breakdown of HP, *pyd* genes, are clustered together in another region and are located approximately 10 kb apart from the *mid* genes on the same fragment. In between both the *mid* and *pyd* gene clusters is a 4.2-kb *EcoR1* fragment. Genes within this fragment have not yet been studied and characterized. The aim of this investigation is to identify and characterize these genes within this fragment and determine their function.

The specific objectives of this study are:

- (i) Identification of the genes in the 4.2 kb *EcoR1* fragment.
- (ii) Isolation of site-directed mutants of these genes from TAL1145.
- (iii) Characterization of these mutants and determination of their role in mimosine degradation and in *Leucaena-Rhizobium* symbiosis.

CHAPTER 2 REVIEW OF LITERATURE

Mimosine is a toxin that is found throughout various structures of the *Leucaena* tree including its root nodules and the root exudates. The *Leucaena*-nodulating *Rhizobium* sp. strain TAL1145 can effectively degrade mimosine (Mid^+) and utilize it as a source of carbon and nitrogen. Recently, the *mid* genes for mimosine degradation have been isolated and cloned from TAL1145 (Fox and Borthakur, 2001). In the present study, the 4.0-kb DNA fragment adjacent to the *mid* gene cluster was characterized by DNA sequence analysis and characterizing isolated mutants. It is believed that the 4.0-kb fragment may contain additional genes involved in mimosine degradation. However, in the course of this study, it was found that the 4.0-kb fragment contained a gene for 1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase), and two genes encoding the small and large subunits of alkyl hydroperoxide reductase. Therefore, it was considered important to review the literature related to ACC deaminases and alkyl hydroperoxide reductases in addition to discussing the bacterial genes for mimosine degradation.

2.1. Genes involved in mimosine degradation in *Rhizobium*

All work related to mimosine degradation has been reported from our laboratory. Soedarjo et al. (1994) reported that some *Rhizobium* strains isolated from the nodules of several *Leucaena* spp. from different regions around the world can degrade mimosine and are able to utilize it as a source of carbon and nitrogen. Although the ability to catabolize mimosine is not required for

nodulation and nitrogen fixation, it does provide a growth advantage to the mimosine-degrading (Mid^+) *Rhizobium* in the rhizosphere of *Leucaena* by providing a both unique and selective source of nutrients and at the same time inhibiting the growth and proliferation of other microorganisms and rhizobia (Soedarjo and Borthakur, 1996). Soedarjo and Borthakur (1998) demonstrated that mimosine was present not only in the roots and the root nodules of *Leucaena*, but it was also secreted in the root exudates. By conducting competition experiments with Mid^+ and Mid^- mutants for nodulation of *Leucaena* and beans, they have shown that mimosine provides a competitive advantage for the Mid^+ rhizobia in the rhizosphere for nodulation of *Leucaena*. Such advantages were not seen when the competition experiment was done on beans (*Phaseolus*), which do not produce mimosine.

Borthakur and Soedarjo (1999) were the first to pioneer research with mimosine degrading genes in *Rhizobium* sp. strain TAL1145. They isolated pUHR181, a cosmid clone from the genomic library of TAL1145, which contained a cluster of *mid* genes for mimosine degradation. Later Fox and Borthakur (2001) isolated a second cosmid clone, pUHR263, which overlapped pUHR181 and contained additional *mid* genes involved in mimosine degradation. They isolated five classes of Mid^- Tn3Hogus-insertion mutants of TAL1145 on the basis of mimosine-inducible GUS activity. Recently, Borthakur et al. (2002) characterized some of these genes, which are briefly described below.

Sequence analysis of the *mid* gene cluster showed that the *midA*, *midB*, *midC* and *midD* genes are part of an operon. The deduced amino acid

sequences of these genes showed high homology with several amino acid transport proteins. The proteins with which MidA, MidB and MidC showed similarities were all involved in the transport of amino acids, sugars or other small molecules in various bacteria. The characteristics of a number of *Rhizobium* mutants with defects in these genes also indicated that *midA*, *midB* and *midC* are involved in transport functions.

The *midD* gene is located downstream of *midC* and encoded a protein that showed homology with pyridoxal-phosphate-dependent aminotransferase family. Based on these homologies, MidD appears to be an aminotransferase that removes the amino group from the alanyl side chain of mimosine.

The *midABCD* genes operon is regulated by *midR*, which is located 248-bp upstream from *midA* in the opposite direction. The deduced amino acid sequence is homologous with several LysR-type transcriptional activator proteins including NodD1 of *Bradyrhizobium* sp. NC92, NodD2 of *Bradyrhizobium elkanii*, and NodD3 of *Rhizobium leguminosarum* bv. *phaseoli*. Borthakur et al. (2002) showed that *mid* genes are induced by mimosine and induction of these genes by mimosine requires the *midR* gene product. In addition, they have also shown that *mid* genes are expressed in the nodules.

Recently Fox (2002) identified and characterized a cluster of genes involved in HP degradation. The *pydA* and *pydB* genes had homology to a dioxygenase and a hydrolase required for mimosine degradation and *pydCDE* genes show similarities to additional transport proteins. Awaya et al. (2002) isolated two additional genes in the *pyd* gene cluster farther downstream of *pydA*

and *pydB*. These genes are *fba* and *fbp*, encoding fructose-1,6-bisphosphate aldolase (FBA) and fructose-1,6-bisphosphatase (FBP), respectively. TAL1145 mutants with transposon insertions in these genes could catabolize mimosine and formed normal nitrogen-fixing nodules on *Leucaena*, suggesting that the function of these genes is not involved in mimosine degradation and symbiosis. Recently, Awaya, Fox and Borthakur (personal communication) isolated two regulatory genes in the *pyd* gene cluster, which are involved in the regulation of the *pyd* genes. The 4.0-kb *EcoRI* fragment characterized in the present study is located in between *midD* and the *fbp* described above.

2.2. ACC deaminases of bacteria, fungi and plants and their functions

Ethylene is an important phytohormone that is produced in higher plants. It is responsible for eliciting many physiological effects during plant development including: seed germination, leaf and flower abscission, flower sex expression, fruit ripening and plant responses induced by pathogens and environmental stress (Agrios, 1997; Raven et al., 1981). While ethylene is necessary for overall normal plant growth, excess concentrations can have negative impact on root development. In a model proposed by Glick et al. (1998), plant growth promoting bacteria (PGPB) in the plant's rhizosphere can assist plant development by reducing the amount of 1-amino-cyclopropane-1-carboxylate (ACC) within the plant in the root cell (Fig 2.1).

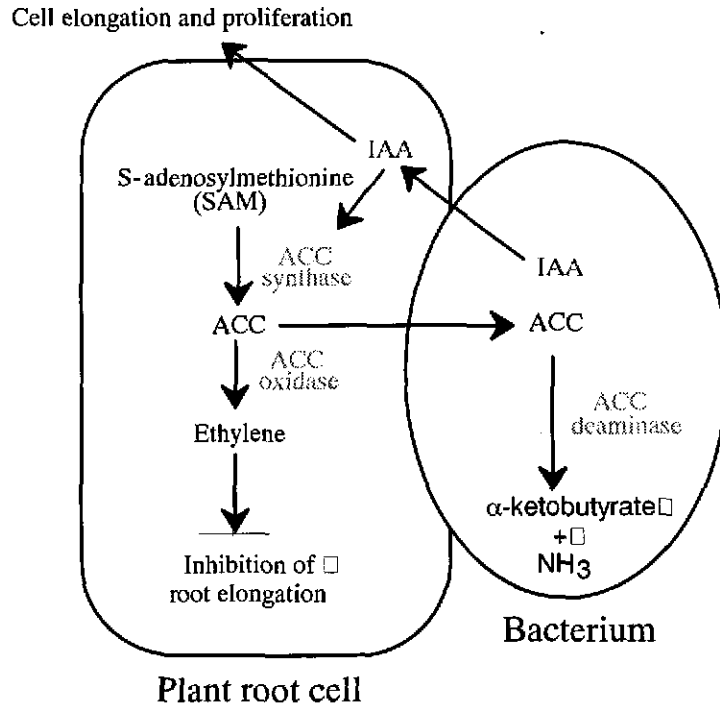


Fig 2.1. Schematic model of ACC deaminase influence on ethylene production and plant root cell development. Adapted from Glick et. al, 1998, *J. Theor Biol.*190: 63-68.

The auxin, indole-3-acetic acid (IAA), is secreted by both the plant and by the surrounding bacteria. IAA influences several changes in the plant cell including cell permeability, elongation and proliferation. It is also responsible for stimulating production of ACC synthase, which is the enzyme responsible for converting S-adenosylmethionine (SAM) to ACC. ACC is the precursor molecule from which ethylene is synthesized by enzymatic conversion by ACC oxidase.

Recent studies have shown (Holguin and Glick, 2001; Penrose et al., 2001; Belimov et al., 2001; and Li et al. 2000) that PGPB can promote plant root development by relieving the plant cells of an accumulation of ACC. ACC

deaminase is a bacterial enzyme that is produced by PGPB. It is responsible for sequestering available ACC from the ethylene biosynthesis pathway when degraded to α -ketobutyrate and ammonia by means of a deamination elimination reaction with water (Walsh et al, 1981, Honma and Shinomura, 1978). From the crystallized structure of ACC deaminase in yeast *Hansenula saturnus*, it has been found that ACC deaminase cleaves the C-C bond between the α carbon and the pro-S methylene carbon of the cyclopropane unit by nucleophilic attack on the pro-S carbon (Yao et al, 2000).

ACC deaminases have been isolated and characterized from a number of microorganisms (Table 2.1) including *Pseudomonas* sp. (Shah et al., 1998; Campbell and Thomson, 1996); *Enterobacter cloacae* (Glick et al., 1995); *Alcaligenes*, *Variovorax*, *Bacillus*, and *Rhodococcus* (Belimov, et al, 2001); in the fungus *Penicillium citrinum* (Jia et al., 1999); and the yeast *H. saturnus* (Sheehy et al, 1991) and even *Arabidopsis* to name just a few. Homology analysis of ACC deaminase genes from two strains of *Pseudomonas* (6G5 and F17) and two strains of *Enterobacter* (CAL2 and UW4) has shown that the genes have at least 85% or higher identity to each other (Shah et al., 1998). When compared to an earlier characterized ACC deaminase gene from *Pseudomonas* sp. strain ACP (Sheehy et al., 1991), results show that the ACC deaminase gene from strain ACP had higher identity homology to ACC deaminase genes in both *E. cloacae* strains than to the aforementioned *Pseudomonas* strains (Shah et al., 1998.) This suggests that while ACC deaminase genes are found in various microorganisms, the homology of the sequences are not conserved even in

related species of bacteria. While there is a substantial amount of sequence information on various ACC deaminases and their relatedness in organisms, unfortunately the same cannot be said for information pertaining to the regulation and expression of ACC deaminase and the functional benefits or consequences of bacterial ACC deaminase to the bacteria themselves.

Table 2.1. ACC deaminase from different organisms.

Organism	Classification	Size of ACC deaminase*	Reference
<i>Rhizobium</i> TAL1145	Bacterium	322	This study
<i>Escherichia coli</i>	Bacterium	360	Blattner et al 1997; Perma et al; 2001
<i>Yersinia pestis</i>	Bacterium	371	Parkhill et al, 2001
<i>Salmonella typhimurium</i>	Bacterium	328	McClelland et al 2001
<i>Bacillus anthracis</i>	Bacterium	331	Read et al, 2002
<i>Pseudomonas fluorescens</i>	Bacterium	410	Klee et al, 1991
<i>Caulobacter crescentus</i>	Bacterium	333	Nierman et al, 2001
<i>Mesorhizobium loti</i>	Bacterium	337	Sullivan et al, 2002
<i>Thermotoga maritima</i>	Bacterium	312	Nelson et al, 1999
<i>Pyrococcus abyssi</i>	Bacterium	333	Heilig R, unpublished
<i>Pyrococcus furiosus</i>	Bacterium	354	Maeder et al, 1999
<i>Pyrococcus horikoshii</i>	Bacterium	328	Kawarabayasi et al, 1998
<i>Burkholderia fungorum</i>	Bacterium	338	NCBI Microbial Genome Annotation Project
<i>Rhizobium leguminosarum</i>	Bacterium	339	Ma W, Guinel FC, and Glick BR, unpublished
<i>Ralstonia solanacearum</i>	Bacterium	338	Salanoubat, 2002
<i>Pseudomonas</i> sp. ACP	Bacterium	338	Sheehy et al, 1991
<i>Schizosaccharomyces pombe</i>	Fungus	338	Wood et al, 2002

<i>Williopsis saturnus</i>	Fungus	341	Yao et al, 2000
<i>Enterobacter cloacae</i>	Bacterium	338	Shah et al 1998

* Size of ACC deaminase amino acid residues

Currently, there is not much literature that has been published on the regulation and expression of ACC deaminase in bacteria. In a study by Gritchko and Glick (2000), the DNA sequence upstream of the UW4 ACC deaminase gene in *E. cloacae* was found to contain several features involved in the transcriptional regulation of the UW4 gene. These include a partial cAMP receptor protein (CRP) binding site, an FNR (fumarate-nitrate reduction regulatory protein) binding site, a leucine responsive regulatory (LRP) protein-binding site, and an LRP-like protein-coding region. Several growth experiments were done under various growth conditions to discern if these two regions influenced bacteria's ability to synthesize ACC deaminase. Conclusive data has shown that ACC deaminase activity is inhibited by (i) the absence of ACC, (ii) initial aerobic growth and induced anaerobic conditions, (iii) the absence of *fnr* gene in anaerobic conditions, (iv) the absence of FNR also under anaerobic conditions, (v) the presence of leucine in aerobic, and lastly, (vi) the absence of LRP protein in aerobic conditions.

In contrast to the little amount of information on ACC deaminase gene regulation and expression, another area of ACC deaminase research where a substantial amount of information is focused upon is the effects of ACC deaminase in promoting root elongation and enhancing improvement of plant development. As previously described, the ACC deaminase gene UW4 of *E.*

cloacae has been used in various studies. In one study, it has been used to transform the bacterium *Azospirillum brasilense* and has been shown to enhance root growth development in canola and tomato, although unsuccessfully in wheat (Holguin and Glick, 2001). In another study involving *E. cloacae* UW4 mutants that are deficient in producing ACC deaminase, significant differences in root elongation and development were also observed in canola seedlings (Li et al., 2000). This area of research offers a lot promise in bioengineering plant growth promoting rhizobacteria (PGPR) that can aid in root development of plants that are used in the phytoremediation of pollutants and heavy metals (Belimov, 2001; Burd et al., 2000).

Finally, since ACC deaminases from several fungi and bacteria have been shown to degrade ACC and consequently reduce ethylene produced in roots, researchers have capitalized on this information and have applied it towards developing plants that can express ACC deaminase in hopes that expression of ACC deaminase throughout the plant may inhibit or at least reduce levels of ethylene that is produced, particularly in fruit development, so that fruit ripening can be delayed and a longer shelf life can be achieved. This already has been successfully accomplished with the development of transgenic tomato plants (Klee et al., 1991). The ACC deaminase gene from soil bacteria was successfully cloned and expressed in tomato plants and as a result, the fruits which underwent a delay in ripening, not only had a longer shelf life but were also firmer and less fragile and thus could better withstand handling during harvesting and transportation.

2.3. Alkyl hydroperoxide reductases and their functions.

Alkyl hydroperoxide reductase is an important enzyme that prevents and limits damage caused by reactive oxygen species (ROS). ROS such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^{\cdot}) are frequently generated by incomplete four-electron reduction of O_2 to water during respiration, lipid metabolism in peroxisomes, exposure to UV light and ionizing radiation (Seis, 1993; Halliwell and Gutteridge, 1989) and other chemicals including herbicides and antibiotics. Normal cellular processes also generate reactive sulfur species (RS, $RSSR^-$ and $RSOO^{\cdot}$) from thiol compounds (Wardman, 1988). Both reactive oxygen and sulfur species are very damaging to the basic biological macromolecules that make up our cells. The specific kinds of damage to the cell include lipid peroxidation (a chain reaction of fatty acid oxidation in lipid membranes), lesions in DNA (breakages or base substitutions) and carbohydrate and protein oxidation (Chae et al, 1994). To protect itself from damage by both ROS and reactive sulfur species, aerobic organisms have evolved several enzymatic defense mechanisms such as cytosolic and mitochondrial superoxide dismutases, cytosolic and peroxisomal catalases, and glutathione peroxidase which reduces both the reactivity and concentration of H_2O_2 and alkyl hydroperoxides. Like the enzymes just mentioned, alkyl hydroperoxide reductase functions like an antioxidant by scavenging the free radicals that are generated by the cell during normal yet inefficient oxidative reduction or by oxidative stress. Alkyl hydroperoxide

reductase converts both organic hydroperoxides and hydrogen peroxide to their corresponding alcohols (Jacobson et al., 1989)

In plants, H₂O₂, organic peroxides and superoxides are frequently generated by the plant hypersensitive defense response to plant pathogens (Agiros, 1997; Levine et al., 1994; Sutherland, 1991). Bacterial plant pathogens must overcome and detoxify the burst in ROS in order to successfully colonize the host plant and initiate disease development. The alkyl hydroperoxide reductase (AhpR) from the plant pathogen *Xanthomonas campestris* pv. *phaseoli* (Loprasert et al., 1997) has been well characterized in other organisms such as *Salmonella typhimurium* (Poole et al., 2000), in yeast (Lee, et al. 1999) and was recently characterized in rat brain (Chae, et al 1994). The basic function of both *Ahp* genes is to protect cells from peroxide toxicity. Early investigations with *S. typhimurium*, *E. coli* and *X. campestris* have yielded evidence that the structures of these alkyl hydroperoxide reductases are fairly conserved and are generally composed of two parts, a large and small subunit. The smaller subunit consists of a homodimer of 2 AhpC (>20-kDa) proteins while the large subunit consists of a single AhpF (44-kDa) protein (Poole and Ellis, 1996). Later, Poole et al., (2000) revised this finding and determined that the large subunit is actually a homodimer and is composed of two AhpF proteins. The larger AhpF subunit catalyzes NADH-dependent reduction of the active site and regenerates the smaller AhpC subunit. The primary function of AhpC is to reduce hydroperoxide substrates to their respective alcohols and water. The smaller AhpC subunit also belongs to a highly conserved family of AhpC/TSA (thiol specific antioxidant)

family of proteins. Further analysis of amino acid sequences of both AhpC and AhpF reveal an astonishing amount of homology with a variety of organisms from each of the major kingdoms including humans, mouse, nematode and a wild grass species, which perhaps is to be expected since alkyl hydroperoxide reductase serves an important biological function and therefore would be highly conserved throughout many different organisms. Interestingly, among the various AhpC/TSA protein homologs mentioned previously, these proteins were segregated into two major types depending on the number (1 or 2) of cysteine residues.

Although somewhat related, another realm of research in which a large amount of interest and information is generated is on alkyl hydroperoxide reductase and tuberculosis resistance research. Researchers have focused much attention towards AhpC in *Mycobacterium tuberculosis* and the mechanisms that allow AhpC to maintain its virulence and resistance from isoniazid (INH) drug resistance. Like the alkyl hydroperoxide reductase described in the previous section, AhpC in *M. tuberculosis* functions by protecting *M. tuberculosis* from ROS. In addition to protecting the bacterium from oxidative stress, the AhpC encoded in *M. tuberculosis* also negates the function of another bacterial enzyme, catalase-peroxidase (KatG). KatG is responsible for activating INH as an anti-tuberculosis drug. It is believed the bacterium overcomes INH susceptibility by suppressing KatG catalase/peroxidase. In doing so, it also loses the protective qualities KatG has as an antioxidant to suppress ROS. To compensate for this loss of function, the *ahpC* gene is up regulated to ameliorate

its vulnerability to ROS. Similarly, the AhpC that is translated does not have the same properties as KatG and is not able to activate INH, thus rendering the drug useless. However, unlike AhpC in other organisms that were described earlier, the encoded AhpC in *M. tuberculosis* possesses two unique and distinguishing features such as: (i) having three cysteine residues instead of one or two like other organisms; and (ii) the large FAD/NADPH-binding subunit AhpF is absent in *M. tuberculosis* unlike *E. coli* and *S. typhimurium* (Chauhan and Mande, 2001).

And lastly, the location and organization of alkyl hydroperoxide reductase genes in most bacteria have displayed a few notable similarities. In both *E. coli* and *S. typhimurium*, regulatory proteins OxyR and SoxRS have been found to regulate the expression of AhpC and AhpF. However in most cases, these genes are usually located within close proximity to each other and are sometimes co-regulated by each other (Loprasert et al., 1979; Ferrante et al., 1995; Antelmann et al., 1996; Basat et al., 1996; Poole 1996; Storz et al., 1989). In *E. coli* and *S. typhimurium*, AhpC is directly upstream of AhpF (Tartalia et al., 1990). In *Bacillus alcalophilus* and *Amphibacillus xylanus*, the ORFs encoding AhpC/TSA homologs were just upstream of genes encoding enzymes related to the AhpF family. Additionally, in *X. campestris* pv. *phaseoli*, the genes for *ahpC*, *ahpF* and *oxyR* are arranged in head-to-tail fashion, each having a strong ribosome binding region preceding the translation initiation codons and the *ahpC* was found to be organized as a monocistronic gene, while the *ahpF* and *oxyR* genes were arranged on an operon (Loprasert et al., 1997).

In *Leucaena-Rhizobium* symbiosis, the role of both ACC deaminase and alkyl hydroperoxide reductase has not yet been determined. Neither is it known whether both of these enzymes are involved in mimosine degradation. However, based on the literature of ACC deaminases and alkyl hydroperoxide reductases in other microorganisms, it may be predicted that both alkyl hydroperoxide reductases may also have a role in protecting *Rhizobium* sp. TAL 1145 from ROS created by oxidative stress and increased hydrogen peroxide concentrations generated via induction by plant–bacteria interactions. In addition to protecting itself, *Rhizobium* sp. TAL 1145 may also contribute in enhancing root development and thereby improving the overall plant development of *Leucaena*.

CHAPTER 3 RESULTS

Identification and characterization of genes in a 4.0-kb DNA fragment in the *mid* gene cluster of *Rhizobium* strain TAL1145

Previously, Fox & Borthakur (2001) isolated a cosmid clone from a genomic library of *Rhizobium* sp. TAL 1145 containing the genes for mimosine degradation. A restriction map of the cosmid pUHR263 is shown in Fig. 3.1. As seen in this figure, there is a 4.0-kb *Eco*R1 fragment between both the *mid* and *pyd* gene clusters. The *mid* genes are involved in degradation of mimosine into HP, while the *pyd* genes are required for complete degradation of HP into ammonia, pyruvate and formate. The 4.0-kb fragment was analyzed by sequencing and transposon insertion mutagenesis. The transposon insertion mutants were characterized for physiological and symbiotic properties. These genes and mutants are described below.

3.1. Sequence analysis

The nucleotide sequence of the 4.0-kb fragment is shown in Fig. 3.2. Sequence analysis of the 4.0-kb fragment yields three complete open reading frames (ORFs) within the fragment in addition to two partial ORFs flanking both ends of the 4.0-kb fragment. At the 3' end of the *mid* gene cluster, the *midD* gene encoding an amino transferase was previously identified and characterized on a 5.0-kb *Pst*I fragment (Borthakur et al., 2002). The 4.0-kb *Eco*R1 fragment overlaps with the 5.0-kb *Pst*I fragment containing the *midABCD* genes by a 0.7-

kb *EcoRI-PstI* segment. The *midD* sequence ends with a TGA stop codon at nucleotide position 508 of the 4.0-kb fragment (Fig. 3.2).

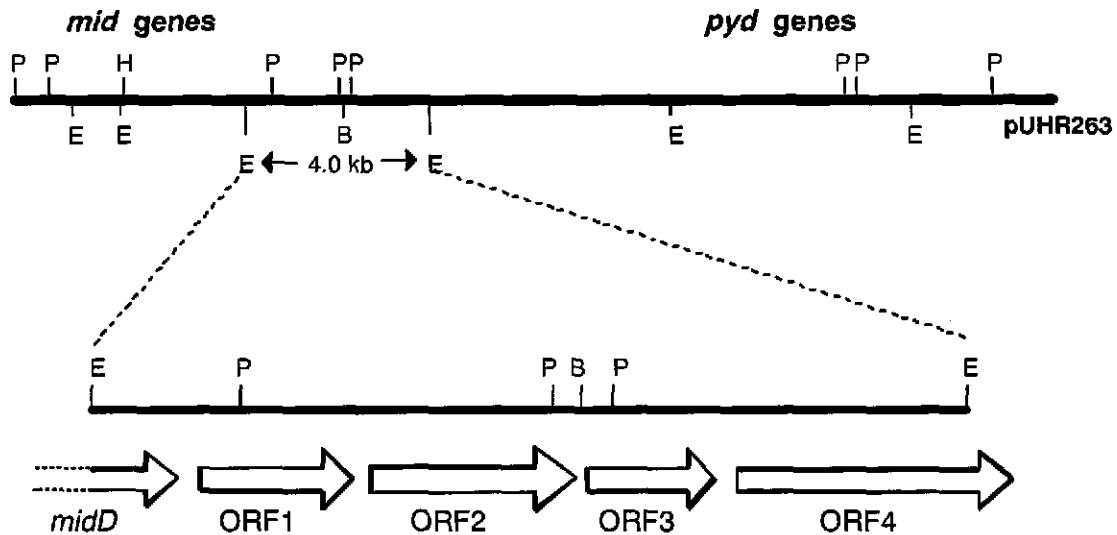


Fig. 3.1. Map of pUHR263 showing the position of the 4.0-kb fragment. The direction and relative positions of the four ORFs are shown by open horizontal arrows. E-*EcoRI*, P-*PstI*, H-*HindIII*, and B-*BamHI*.

ORF 1 is located 64 bp downstream from *midD*. ORF1 is 503 bp long starting at nucleotide position 572 and ending at 1075 with a stop codon TAA. The deduced amino acid sequence of the ORF1 shows that it may encode a protein of size 17,643 Da. It has a Shine-Delgarno sequence GGCGCG 57 bp upstream from the ATG start codon. Comparison of this sequence to the database showed up to 59% identity and 74% similarity with several proteins identified in the database (Fig. 3.3.a). ORF1 also showed 70% similarity with 4sk protein of *Rhizobium* NGR234 and 74% similarity with the translational inhibitor

N	S	V	S	L	L	S	A	S	K	M	F	N	L	T	G	L		17			
g	AAT	TCC	GTG	AGC	CTG	CTT	TCT	GCC	AGC	AAG	ATG	TTT	AAC	CTC	ACG	GGA	CTG	52			
K	H	S	Q	V	I	A	A	N	P	R	L	M	N	E	Y	L	E	35			
AAG	CAC	TCT	CAA	GTC	ATC	GCC	GCT	AAT	CCA	AGG	CTT	ATG	AAT	GAA	TAC	CTG	GAG	106			
G	L	K	R	D	T	S	G	Y	G	G	S	L	F	G	H	V	A	53			
GGG	CTG	AAG	CGC	GAT	ACC	TCG	GGC	TAC	GGC	GGT	TCT	CTG	TTC	GGC	CAC	GTC	GCG	160			
T	E	V	A	Y	R	D	C	D	G	W	L	A	D	L	M	K	Y	71			
ACC	GAG	GTG	GCA	TAC	CGA	GAT	TGC	GAT	GGG	TGG	CTT	GCC	GAT	CTC	ATG	AAG	TAT	214			
V	A	G	N	Y	A	F	A	E	A	F	F	K	E	R	L	P	R	89			
GTT	GCC	GGC	AAC	TAT	GCC	TTC	GCC	GAG	GCT	TTC	TTC	AAG	GAA	AGA	CTC	CCG	CGC	268			
I	R	I	R	P	T	E	S	T	Y	F	M	W	L	D	L	S	D	107			
ATT	CGC	ATA	CGC	CCG	ACA	GAG	TCC	ACC	TAC	TTC	ATG	TGG	CTC	GAC	CTT	TCA	GAT	322			
Y	G	M	S	H	Q	E	M	L	D	L	F	E	T	R	H	G	L	125			
TAC	GGC	ATG	TCG	CAC	CAG	GAG	ATG	CTC	GAT	CTG	TTC	GAA	ACA	AGG	CAT	GGC	CTC	376			
V	L	N	Y	G	E	D	Y	G	P	G	G	E	G	H	I	R	L	143			
GTG	CTC	AAC	TAC	GGC	GAA	GAC	TAT	GGG	CCG	GGC	GGT	GAA	GGA	CAT	ATC	CGT	CTG	430			
N	I	G	T	P	K	S	I	L	A	Q	G	I	E	R	M	A	K	161			
AAT	ATC	GGC	ACG	CCG	AAA	TCG	ATT	CTT	GCT	CAA	GGC	ATC	GAG	CGG	ATG	GCG	AAA	484			
A	L	S	S	H	E	G	*											169			
GCA	TTG	TCC	AGC	CAC	GAA	GGA	TGA	tcttggcatta	aaagatcagttccttcccaggetgacatc								547				
								M	P	T	D	D	P	L	I	F	E	H	R	181	
								ccgacgatctgtgcggcgcgatc	ATG	CCG	ACA	GAT	GAT	CCG	CTC	ATA	TTC	GAA	CAC	CGT	607
D	A	S	H	Q	T	V	T	S	N	S	C	E	E	I	M	E	I	199			
GAT	GCG	TCG	CAT	CAG	ACG	GTC	ACC	TCA	AAC	TCT	TGC	GAG	GAA	ATC	ATG	GAA	ATC	661			
S	T	P	N	A	P	A	A	V	G	P	Y	S	Q	A	R	R	Q	217			
TCC	ACC	CCT	AAC	GCA	CCT	GCA	GCT	GTC	GGT	CCC	TAC	AGC	CAG	GCC	AGA	CGA	CAA	715			
G	N	L	L	F	V	S	G	Q	L	P	L	D	P	K	T	G	E	235			
GGG	AAC	CTG	CTT	TTC	GTC	TCG	GGC	CAA	CTG	CCT	CTC	GAC	CCC	AAG	ACG	GGG	GAA	769			
M	A	S	D	A	E	E	Q	M	R	Q	V	L	A	N	I	E	A	253			
ATG	GCC	AGC	GAT	GCG	GAA	GAG	CAG	ATG	CGT	CAG	GTG	CTG	GCA	AAT	ATC	GAA	GCG	823			
I	V	T	A	A	G	A	K	M	S	D	V	L	K	T	T	V	L	271			
ATC	GTG	ACG	GCG	GCG	GGG	GCA	AAG	ATG	TCC	GAT	GTC	CTG	AAG	ACG	ACC	GTG	CTC	877			
V	T	D	L	S	K	F	K	Q	L	N	E	I	Y	A	A	A	F	289			
GTG	ACC	GAT	CTT	TCG	AAA	TTC	AAG	CAG	CTG	AAT	GAA	ATT	TAT	GCA	GCG	GCT	TTC	931			
P	K	P	C	P	A	R	A	T	Y	Q	V	A	A	L	P	M	G	307			
CCG	AAG	CCA	TGC	CCG	GCA	CGA	GCG	ACC	TAC	CAG	GTC	GCG	GCG	CTG	CCA	ATG	GCG	985			
A	Q	V	E	I	E	A	V	A	V	I	P	G	E	A	D	H	A	325			
GCG	CAA	GTC	GAG	ATC	GAG	GCG	GTC	GCC	GTC	ATT	CCC	GGT	GAA	GCT	GAT	CAC	GCC	1039			
C	K	G	S	C	T	A	A	A	L	S	*							337			
TGC	AAA	GGC	TCC	TGC	ACG	GCG	GCC	GCC	CTG	TCG	TAA	aatcaagaccctcagaggttagc					1098				

D V A D H Y A E L Q R L D V E V Y S	724
GAC GTC GCT GAC CAT TAC GCA GAA CTG CAG CGG CTC GAT GTC GAG GTC TAT TCG	2293
V S T D T H F T H K A W H D S S D T	742
GTA TCG ACC GAC ACG CAC TTC ACG CAC AAG GCG TGG CAT GAC AGC TCC GAT ACG	2347
I R K I E Y T M I G D P T G T V T R	760
ATC AGG AAG ATC GAG TAC ACC ATG ATC GGT GAT CCA ACC GGG ACC GTT ACC CGC	2401
N F E V M R E T E G L A D R G T F I	778
AAT TTC GAG GTC ATG CGG GAA ACG GAA GGC CTT GCC GAC CGT GGA ACT TTC ATC	2455
V T R T D H S G D R N H H R G H R S	796
GTC ACC CGG ACG GAT CAT TCA GGC GAT CGA AAT CAC CAC CGA GGG CAT CGG TCG	2509
K C S R P T A P R K G G A I C P R T	814
AAA TGC AGC CGA CCT ACT GCG CCG CGT AAA GGC GGC GCA ATA TGT CCG CGC ACA	2563
S R R G L P R *	822
TCC CGG CGA GGT TTG CCC CGC TAA atgggaggagggcggaagaccctcgctccgtctttggc	2626
tctcgtcggtaagaattgagcgcaaaagacgagaccccaagcaggttggggtctcattctgtcagagatgct	2698
M L D D A I K S E L S S H	835
tccaggaacaggaatccg ATG CTT GAC GAC GCG ATC AAA AGC GAG CTT TCC TCC CAT	2755
F E K I A R P I E I I A S V D A S P	853
TTC GAG AAA ATT GCC CGA CCG ATC GAG ATC ATC GCA TCG GTC GAC GCA AGC CCG	2809
T S L Q M I D L L T E I A S L S P Q	871
ACA TCT CTC CAG ATG ATC GAT CTC CTG ACC GAA ATC GCC AGC CTG TCG CCC CAA	2863
I T L E Q R H D I G D R A P S F S L	889
ATC ACG CTC GAG CAG CGT CAC GAT ATT GGC GAT CGT GCC CCT TCC TTC TCT TTG	2917
S S P G R D I K L T F A G L S L G H	907
AGT AGC CCA GGA CGC GAT ATC AAA CTG ACC TTT GCT GGC CTT TCG CTC GGC CAC	2971
E F G S L G L A F V A T W G A I A T	925
GAG TTC GGA TCG CTT GGG CTC GCC TTT GTT GCA ACT TGG GGG GCC ATT GCA ACA	3025
L P S E Q G R L V R S K N R F G S L	943
TTG CCG TCG GAA CAA GGG CGG TTA GTT CGA AGC AAG AAT CGG TTT GGG TCG TTG	3079
G K P K L P I F R R K L F R C F C Q	961
GGG AAG CCG AAG TTA CCC ATT TTT CGG AGG AAA TTA TTT CGC TGT TTC TGC CAG	3133
N L P R M S C K R S T L M P I L N P	979
AAC TTG CCC AGA ATG TCG TGC AAG CGC TCA ACC TTA ATG CCC ATT CTC AAC CCC	3187
R I S H V A I E G E A F Q S K V C Q	997
CGC ATC AGC CAT GTC GCG ATC GAG GGG GAA GCC TTT CAG TCG AAG GTG TGC CAG	3241
R Q I M S V P A V Y L N G A L F A Q	1015
CGC CAG ATC ATG TCG GTT CCA GCC GTC TAT TTG AAT GGC GCC TTG TTT GCG CAG	3295
V R M D L Q H I V A K L D S K A D Q	1033
GTG CGC ATG GAC TTG CAG CAT ATC GTG GCA AAG CTG GAT AGC AAG GCG GAC CAG	3349
R A A A K I S N L A T F D L L V G G	1051
CGC GCC GCC GCG AAG ATC AGC AAT CTT GCG ACT TTC GAT CTC CTG GTC GGC GGC	3403

G	P	A	G	A	A	S	A	I	Y	A	A	R	K	G	L	A	V	1069
GGG	CCT	GCC	GGC	GCG	GCG	TCG	GCC	ATC	TAC	GCT	GCA	CGC	AAA	GGG	CTC	GCG	GTC	3457
G	M	V	A	D	R	L	G	G	Q	V	L	E	T	M	L	I	E	1087
GGA	ATG	GTT	GCC	GAT	CGA	CTT	GGC	GGC	CAG	GTG	CTC	GAA	ACG	ATG	CTC	ATC	GAG	3511
N	L	I	S	V	P	Q	T	E	G	P	T	F	R	S	A	L	E	1105
AAC	CTC	ATA	TCC	GTA	CCG	CAG	ACG	GAA	GGC	CCG	ACC	TTC	AGA	TCC	GCG	CTC	GAG	3565
A	H	V	K	E	Y	P	V	E	I	V	K	A	Q	R	V	E	K	1123
GCG	CAT	GTA	AAG	GAA	TAT	CCG	GTC	GAG	ATC	GTA	AAG	GCG	CAG	CGG	GTA	GAG	AAG	3619
L	V	S	T	G	R	A	H	E	L	R	L	A	S	G	G	S	L	1141
CTC	GTA	TCA	ACG	GGG	CGC	GCC	CAC	GAG	CTC	CGA	CTT	GCA	AGT	GGC	GGG	TCC	CTT	3673
R	P	R	R	S	F	L	P	L	A	R	W	R	Q	M	G	V	P	1159
AGG	CCA	AGA	CGG	TCA	TTC	TTG	CCG	CTG	GCT	CGC	TGG	CGC	CAG	ATG	GGG	GTA	CCG	3727
G	E	E	E	Y	R	N	R	G	V	A	Y	C	T	H	C	D	G	1177
GGA	GAG	GAA	GAA	TAC	CGC	AAC	AGA	GGG	GTA	GCC	TAT	TGC	ACG	CAT	TGT	GAC	GGA	3781
P	L	F	K	G	K	R	V	A	V	I	G	G	G	N	S	G	V	1195
CCC	CTG	TTC	AAG	GGC	AAA	CGC	GTC	GCG	GTG	ATC	GGA	GGC	GGC	AAT	TCC	GGT	GTC	3835
E	A	A	I	D	L	S	G	I	V	S	H	V	T	T	D	R	I	1213
GAA	GCG	GCG	ATT	GAT	CTG	TCC	GGA	ATC	GTC	TCC	CAC	GTC	ACC	ACT	GAT	CGA	ATT	3889
C																		3890

Fig. 3.2. DNA sequence of the 4.0-kb *EcoRI* fragment. Amino acid sequences are shown as one-letter symbols above the three-letter codons for DNA sequences. The DNA sequence was translated to amino acid sequences using translation tool at the website: <http://www.infobiogen.fr/services/analyseseq/cgi-bin/forpubout.pl>

protein of *Arabidopsis thaliana*. It also showed 53% identity and 68% similarity with a hypothetical protein from the nematode *Caenorhabditis elegans*.

ORF 2 is located 26 bp downstream from the end of ORF1 starting at nucleotide position 1102 and ending at 2070 with a TGA stop codon (Fig. 3.2). It is 969 bp long and has a Shine-Delgarno-like sequence AGAGG 8 bp upstream of the ATG start site. Analysis of this region shows the gene should encode a deduced protein 34,334 Da. Comparison of this deduced amino acid sequence against known sequences in databases has shown that it has a high homology with 1-aminocyclopropane-1-carboxylate deaminases (ACC deaminase) from several organisms (Fig. 3.3.b). ACC deaminase is found in numerous kinds of bacteria including *E. coli*, *Pyrococcus abyssi*, etc. The deduced ACC deaminase from ORF 2 has 40% identity and 56% similarity with the YedO protein of *E. coli*. Similarly, it also has 39% identity and 54% similarity with the YedO protein of *Salmonella typhimurium*.

ORF3 is located 31 bp downstream from ORF2. It is 486 bp long extending from position 2102 to 2587 and ending with a TAA stop codon (Fig. 3.2). It encodes a protein of molecular mass of 18,177 Da. The encoded protein shows a high homology with AhpC or the small subunit of alkyl hydroperoxide reductase from various bacteria including *Salmonella typhimurium*, *E. coli*, *Bacillus alcalophilus*, *Staphylococcus aureus*, *Microbulbifer degradans* and many other bacteria (Fig. 3.3.c). It shows up to 73% identity and 81% similarities with AhpC-TSA family of proteins from these organisms. However, the homology is

```

At3g20390      MTWSVFRSINTPTLDDLSTALRSTRTPPLVAAGVGCATFAGVSLFRMSSRSPPFASLSVSAS 60
C23G10.2b     -----MRTKVLDM-----AQ 10
ORF1[TAL1145] -----MPTDDPLIFEHRDASHQTVT-----SNS 23
Y4sK[NGR234]  -----

At3g20390      SVKKEVSTEKAPAALGPYSQAIKANNLVFLSGVLGLIPETGKVFSESVEDQTEQVLKNM 120
C23G10.2b     KVTRQIISSANAPGAIGPYSQAVRAGNTIYLSGSLGLDPKTGDLK-EGVVEQTHQSLKNL 69
ORF1[TAL1145] CEEIMEIISTPNAPAAVGPYSQARRQGNLLFVSGQLPLDPKTGEMAS-DAEEQMRQVLANI 82
Y4sK[NGR234]  --MIEPISTNDAPGAVGPYSQAIKVGDLLFVSGQLPIDPATGEFNSANAVEQAEQCLKNL 58
                *: .**.*:***** : .: :*: * : * **.: .. :* .* * *:

At3g20390      GEILKASGADYSSVVKTTIMLADLADFKTVNEIYAKYFPAPSPARSTYQVAALPLNAKIE 180
C23G10.2b     GEVLKAAGADYGNVVKTTVLLQNIADFAAVNEVYGQYFKSPYPARAAQVAALPKGGLVE 129
ORF1[TAL1145] EAIVTAAGAKMSDVLKTTVLVTDLSKFKQLNEIYAAAFPKPCPARATYQVAALPMGAQVE 142
Y4sK[NGR234]  QAIARAAGTDLSKTVKTTVLLTDLGDFADINRVYTGFFSTPYPARACYEVKALPKGAKVE 118
                : *:*:. ...:*****: :...* :*:* * * ***: :* * ** .. :*

At3g20390      IECIATL----- 187
C23G10.2b     IEAVAIAGEIEEVKN----- 144
ORF1[TAL1145] IEAVAVIPGEADHACKGSCATAALS 167
Y4sK[NGR234]  IEAVISLT----- 126

```

Symbol key

- missing sequence
- * amino acid residues conserved in all organisms compared
- : high level of amino acid residue conservation in organisms compared
- . moderate level of amino acid residue conservation in organism compared

Fig. 3.3.a. CLUSTAL W multiple amino acid sequence alignment for ORF1. It shows homology with several proteins in the database including Y4sK of *Rhizobium* sp. NGR234, At3g20390 (translational inhibitor protein) of *Arabidopsis thaliana*, and hypothetical protein C23G10.2b of *Caenorhabditis elegans*.


```

AhpC[Sen]      -MSLINTKIKPFKNQAFKNGEFIEVTEKDTGRWSVFFFYPADFTFVCPTLGDVADHYE 59
Mdeg_p_2068    MQTIINTKILPFKATAFSKGDFIPVSEQDLLGKWSVVMFYFADFTFVCPTLGDMDADHYA 60
ORF3[TAL1145]  ---MSTSRDRHRSQALPS---HQTGIRDEPSARSALLCYPADFTFVCPTLGDVADHYA 53
                :.*.  .:  *:  .  .  :*  .  *.: *****:****

AhpC[Sen]      ELQKLGVDVYSVSTDTHFTHKAWHSSSETIAKIKYAMIGDPTGALTRNFDNMREDEGLAD 119
Mdeg_p_2068    QLQEMGVEVYSVSTDTHFTHKAWHDSSDTIGKINFPMIGDPTGTISRNFVGMIEEDGLAL 120
ORF3[TAL1145]  ELQRLDVEVYSVSTDTHFTHKAWHDSSDTIRKIEYTMIGDPTGTVTRNFEVMRETEGLAD 113
                :*.:.*:*****.**:** **::.*****:*** ** :***

AhpC[Sen]      RATFVVDPOGIIQAIEVTAEGIGRDASDLLRKIKAAQYVAHPGEVCPAKWKEGEATLAP 179
Mdeg_p_2068    RGTFFVINPEGEIKVAEIHDLGIGRSKELVRKIQAQYVAEHDGEVCPAAWQPGEATLAP 180
ORF3[TAL1145]  RGTFFIVTR-----TDHSGDRNHHRGHRSKCSRPTAPRKGGAICPRTSRRG----LP 160
                *,**::      .*.      *  :  .. : * :**  : *      *

AhpC[Sen]      SLDLVGKI 187
Mdeg_p_2068    SLDLVGKI 188
ORF3[TAL1145]  R----- 161

```

Symbol key

- missing sequence
- * amino acid residues conserved in all organisms compared
- : high level of amino acid residue conservation in organisms compared
- . moderate level of amino acid residue conservation in organism compared

Fig. 3.3.c. CLUSTAL W multiple amino acid sequence alignment for ORF3 encoding AhpC or alkyl hydroperoxide reductase small subunit. It shows homology with AhpC of several bacteria including *Salmonella enterica* subsp. *enterica* serovar *Typhi* (Sen), and Mdeg_p_2068 of *Microbulbifer degradans*.

```

AHPF [Xca] -----LDANLKTQLTAYLERVTRP 20
AHPF [Xfa] MRRSFVCGDIFAVDSLYVSPSAICRAATPCAFNPPLQEIVMFDESLSKAQLKAYLDRVIRS 60
ORF4 [TAL1145]-----MLDDAIKSELSSHFEKIARP 20
*: * : * : * : * : * : * : *

AHPF [Xca] IQINASIDDSAGSREMLDLLEELVLLS-DKISLDIHRDDNQRKPSFALTPGQDISLRFA 79
AHPF [Xfa] IHIHASVDESHKSREMLDLLENIVLLSGKKVLEVRDDDQRKPSFGLGTPGESISIRFA 120
ORF4 [TAL1145] IEIIASVDASPTSLQ MIDLLTEIASLS-PQITLQQRHDIGDRAPSFSLSSPGRDIKLTFA 79
* . * * * : * * * * : : . * * : : * : * * . * : * * . * : * *

AHPF [Xca] GLPMGHEFTSLVLA LLQVGGHPSKAAAE LIEQVQHLE-----GDYQFETY-----FSLSCQ 130
AHPF [Xfa] GLPMGHEFASLV LGLLQVGGHPPKVADDLIERVRNLD-----GHYRFETY-----FSVSCQ 171
ORF4 [TAL1145] GLSLGHEFGSLGLAFVATWGAIATLPSEQGR LVRSKNRF GSLGKPKLP IFRRKLFRFCFCQ 139
* * . : * * * * * * * * . : . * . . . : . * : : * . : : : * * *

AHPF [Xca] NCP-DVVQALNLA AVLNPRIKHVAIDGAWFQDEVQARQIMSVP TVYLNGLF DQGRMTLE 189
AHPF [Xfa] SCP-DVVQALNMA AVLNPQIEHVAIDGALFQDEVEARQIMSVP TVYLNGLF GQGRMTLE 230
ORF4 [TAL1145] NLPRMSCKRSTLMPILNPRISHVAIEGEAFQSKVCQRQIMSVP AVYLNGLA LFAQVRMDLQ 199
. * : . : . : * * * : * . * * * : * * . : * * * * * * * * * * *

AHPF [Xca] QIVAKLDTNAAKRDAAKIAAKEAFDVLVVG GGPAGSAAAVYAARKGIRTVGAAERFGGQV 249
AHPF [Xfa] QIVAKLDSGA AKRTAESIQAKAFDVLVVGAGPAGTAAAVYAARKGVRTGLVAERFGGQV 290
ORF4 [TAL1145] HIVAKLDSKADQRAAAKISNLATFDLLVGG-GPAGAASAIYAARKGLAVGMVADRLGGQV 258
: * * * * * : * : * * . * : * * * * * * * * * * * * * * * * * * * * *

AHPF [Xca] LDTMSIENFISVPETEGPKMAAALEQHVRQYDVIDMNLQRAEQ LIPAGADGLIEIKLANG 309
AHPF [Xfa] LDTMTIENFISVPETDGP RMVAALEQHLRQYEV DIRMNLQRVETLVPAGENG FIEVRLASG 350
ORF4 [TAL1145] LETMLIENLISVPQTEGPTFRSALEAHVKEYPVEIVKAQRVEKLVSTG--RAHELRLASG 316
* : * * * * * : * * * * * : * * * * * : * * * * * : * * * * * : * * * * *

AHPF [Xca] ASLKSKTVILSTGARWRQMNVPGEDQYK NKGVA YCPHCDG PLFKGKRVA VIGGGNSGVEA 369
AHPF [Xfa] ASLSSRTVILSTGARWRQMNVPGEDEYRNKGVA YCPHCDG PLFKGKRVA VIGGGNSGVEA 410
ORF4 [TAL1145] GSLRPRRSFLPL-ARWRQMGVPGEEYRN RGVAYCTHCDG PLFKGKRVA VIGGGNSGVEA 375
. * * . : : * . * * * * * . * * * * * : * * * * * * * * * * * * * * * * * * * * *

AHPF [Xca] AIDLAGIVAHVTLVEFDDKLRAD EVLQRKLRSLHNVRIITSAQTTEVLGDGQKVTGLVYK 429
AHPF [Xfa] AIDLAGIASYVTLLEFDTKLRAD EVLQRKLRSMNVEVIMGAQTTEVRGDGKKTGLVYV 470
ORF4 [TAL1145] AIDLSGIVSHVTTDRI----- 391
* * * * * : * * * * * . :

AHPF [Xca] DRTGGDIQHIELEGV FVQIGLLPNTEFLRGTVALSPRGEIIVDDRGT DVPGVFAAGDAT 489
AHPF [Xfa] DRSTGGSFTLAL EGVFVQIGLLPNTEWLKGRVELSPRGEIIVDECGHTSLPGVFAAGDAT 530
ORF4 [TAL1145]-----

AHPF [Xca] TVPYKQIVIAMGEGSKAALS AFDHLIRTSAPATAD SVAQAA 530
AHPF [Xfa] TVPYKQIVIAMGEGSKAALS AFDYLIRNVVEITGIAQAQAA 571
ORF4 [TAL1145]-----

```

Fig. 3.3.d. CLUSTAL W multiple sequence alignment for ORF4 encoding AhpF or alkyl hydroperoxide reductase large subunit or F subunit. It shows homology with AHPF of *Xanthomonas campestris* pv. *phaseoli* (Xca), and *Xylella fastidiosa* (Xfa). Note that the remainder of the TAL1145 AhpF sequence is located in a 5.5-kb fragment adjacent to the 4.0-kb fragment and has determined recently by Jon Awaya (not shown).

mostly present in the first half of the ORF3-encoded protein. The AhpC/TSA family contains proteins related to alkyl hydroperoxide reductase (AhpC) and thiol specific antioxidant (TSA).

ORF4 is an 1174 bp long incomplete gene and is located 129 bp downstream of ORF3 and at the other end of the 4.0 kb *EcoR1* fragment (Fig. 3.2). Comparison analysis of this partial sequence against the database showed that the sequence encodes AhpF or the large subunit of the alkyl hydroperoxide reductase (Fig. 3.3.d). This gene extends up to the adjacent 5.5-kb fragment, which contains the *fb*a and *fb*p genes encoding for fructose-1-6-bis-phosphate aldolase and fructose-1-6-bis-phosphatase (Awaya et al. 2002). The incomplete ORF4 showed up to 50% identity and 65% similarities with AhpF from several organisms.

3.2. Hydrophobicity analyses of the encoded proteins of the three ORFs

Hydrophobicity analysis showed that the ORF1- and ORF3-encoded proteins do not have any hydrophobic residues and are therefore likely to be located in the cytoplasm (Fig. 3.4). On the other hand, the ORF2-encoded protein has four small peaks that extend past the hydrophobic threshold. These regions containing hydrophobic residues suggest that the ORF2 protein may be either loosely associated with the membrane or is a peripheral membrane protein. A topology analysis shows the N-terminus end (143 amino acid residues) of this protein could remain suspended in the cytoplasm while the C-terminus end may be imbedded in the membrane.

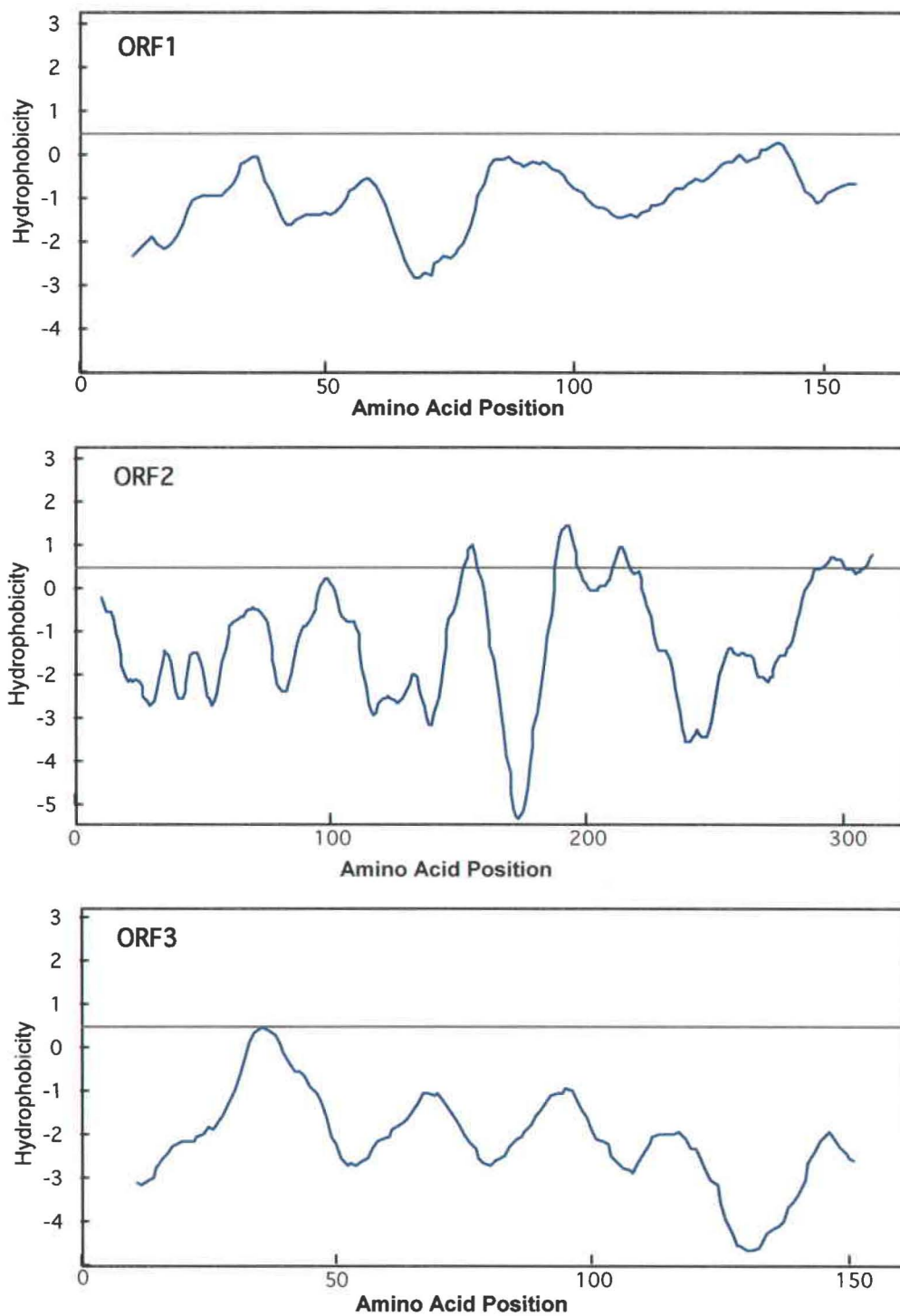


Fig. 3.4. Hydropathy profiles of the proteins encoded by ORF1, ORF2 and ORF3 of *Rhizobium* sp. strain TAL1145. The profiles were made using the TopPred II computer program with a window of 21 amino acids (Claros & von Heijne, 1994).

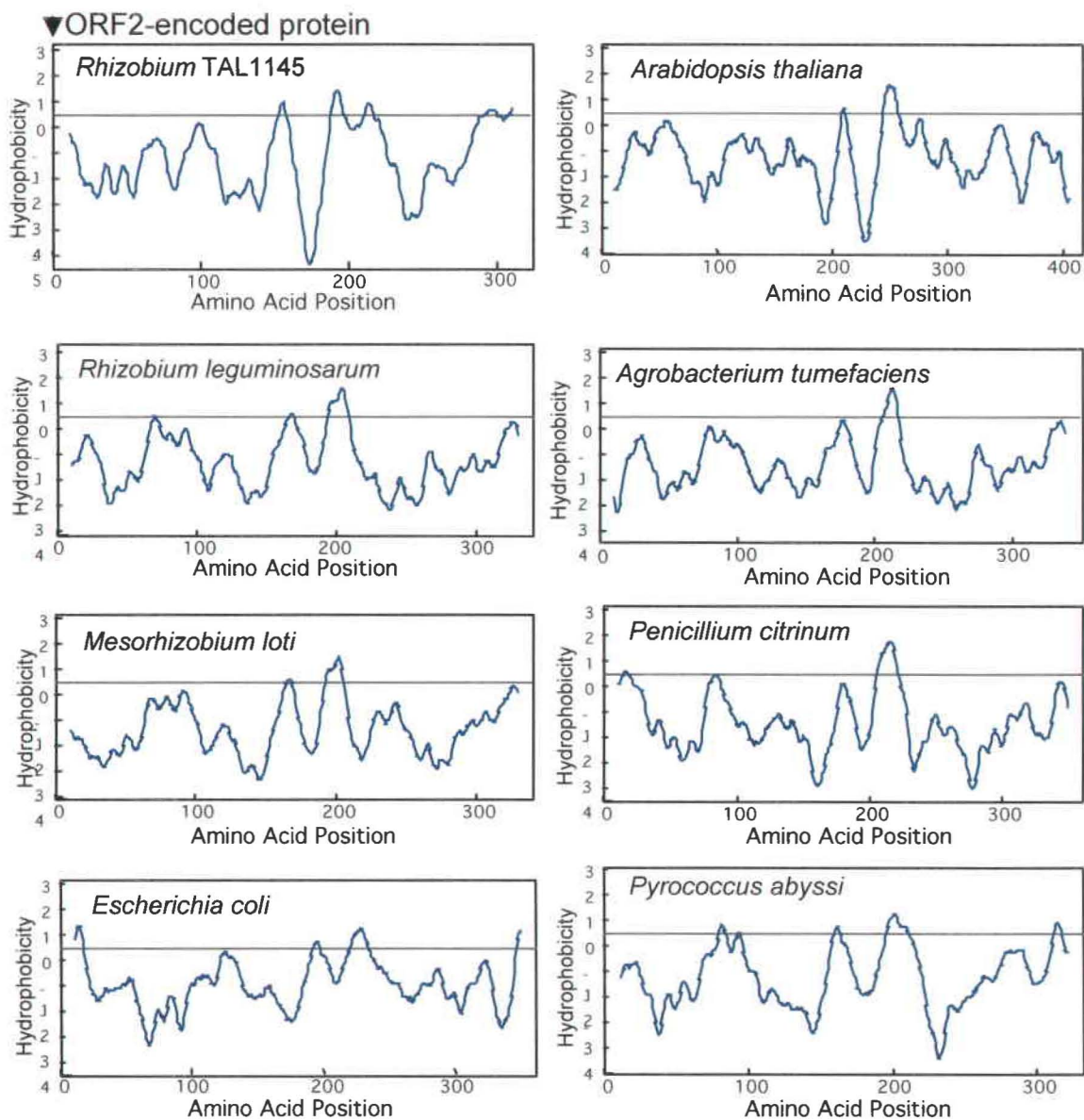


Fig. 3.5. Comparison of hydropathy profiles of ACC deaminase from ORF2-encoded protein of *Rhizobium* sp. strain TAL1145 with those from other organisms including bacteria, fungi, and plant.

3.3. Similarities of the ORF2-encoded protein with ACC-deaminases from both prokaryotes and eukaryotes

Blast analysis showed that the ORF2-encoded protein has homology with ACC deaminases from a diverse group of organisms including many bacteria, fungi and even *Arabidopsis thaliana*. To compare the nature of the ORF2-encoded protein with those from other diverse organisms, hydrophobicity plots for a number of organisms including bacteria, fungi and plants were made (Fig. 3.5). It was interesting to find that the hydrophobicity profiles of several ACC deaminases in a fungus, *Arabidopsis*, three rhizobia and other bacteria including *E. coli* and *Agrobacterium* were relatively similar. However, the length of amino acid residues for each of the ACC deaminases in the compared organisms varied. ACC deaminase from *Arabidopsis* has 414 amino acids compared to 322 amino acids in that from *Rhizobium* TAL1145 and 360 in that from *E. coli*.

3.4. Phylogenetic relationship of ACC deaminase from *Rhizobium* TAL1145 with those from other organisms

To investigate the phylogenetic relationship of ACC deaminase from TAL1145 and those of other rhizobia, bacteria, fungi and plants, the ACC deaminase sequences that showed relatively high scores in blast analyses were downloaded from the NCBI database. The phylogenetic tree was generated from the DNA sequence and results showed that the ACC deaminase from *Rhizobium* TAL1145 is distinct from those of other rhizobia including *Rhizobium leguminosarum* and *Mesorhizobium loti* (Fig. 3.6). It alone forms a single branch on the phylogenetic tree and shows only a distant relationship with ACC

deaminases from both *E. coli* and *Salmonella typhimurium*. Conversely, the ACC deaminases from both *R. leguminosarum* and *M. loti* were far more related to each other and were similar to several other bacteria that occupy the same major branch on the phylogenetic tree than that of TAL1145. Both the *Arabidopsis* and *Cytophaga hutchinsonii* ACC deaminases are located on the farthest branch from that of TAL1145 on the phylogenetic tree. These results indicate that TAL1145 may have acquired ACC deaminase gene from unrelated bacteria in the distant past.

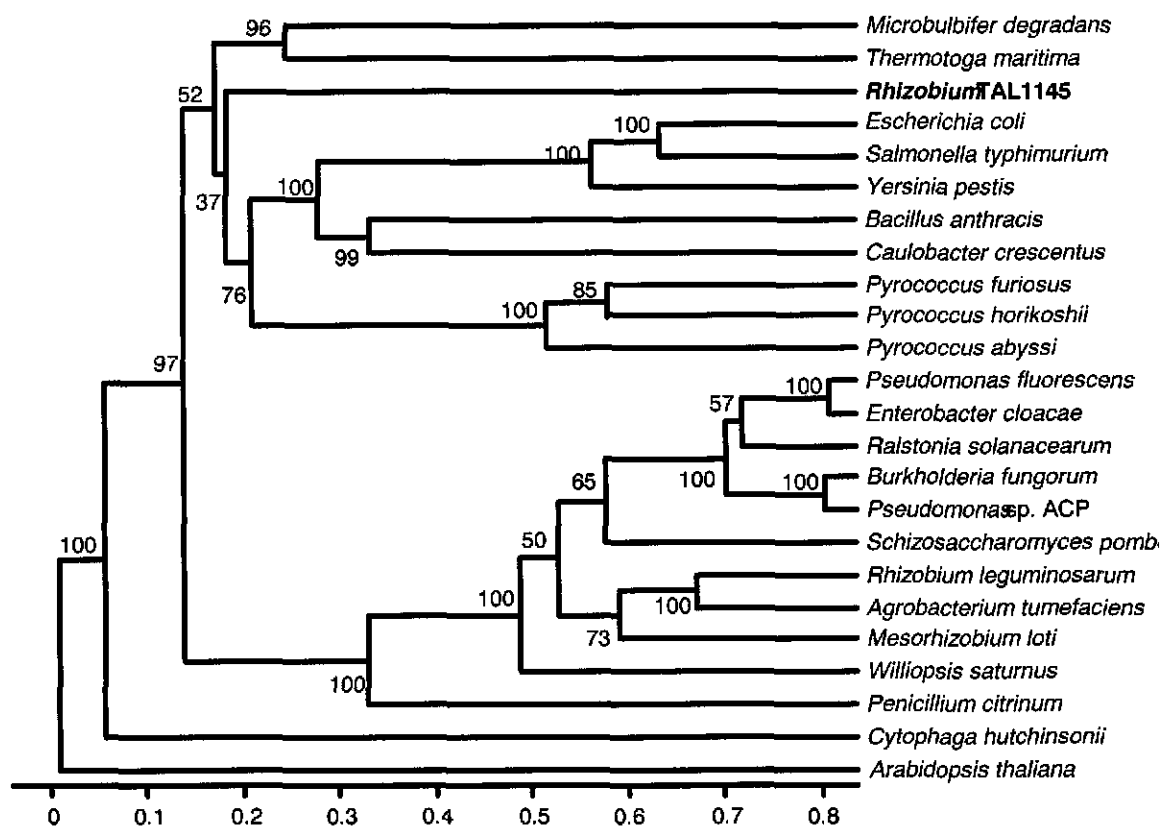


Fig. 3.6. Phylogram showing genetic relationship of the ACC deaminase gene of *Rhizobium* sp. strain TAL1145 with those from different organisms. Includes three *Pyrococcus* spp. (Archaea), *Schizosaccharomyces pombe* (yeast), *Williopsis saturnus*, *Penicillium citrinum* (fungi) and *Arabidopsis thaliana* (plant). The numbers on the phylogram represent bootstrap values supporting the nodes.

3.5. Isolation of mutants of TAL1145 with Tn3 insertion in the 4.0-kb *EcoR1* fragment

To isolate site-directed mutants in the 4.0-kb fragment of TAL1145, the 4.0-kb *EcoR1* fragment was subcloned from the cosmid clone pUHR263 into the wide-host range bacterial vector pRK404A. The resulting plasmid was named pJA42. This plasmid was used to create insertion mutants in *E. coli* using the transposon insertion with Tn3Hogus and described in materials and methods (see chapter 5). Ideally, the resulting pJA42::Tn3Hogus derivatives were expected to contain random Tn3Hogus insertions either in the same or opposite orientation of the genes in the 4.0-kb fragment. These plasmids were transferred to TAL1145 via tri-parental mating, which resulted in the creation of 67 *Rhizobium* transconjugants. These 67 transconjugants were individually spotted on YEM agar containing mimosine and MUG, to select for GUS fusions which may be induced by mimosine. A total of 32 transconjugants were selected based upon GUS activity induced by mimosine and were characterized by their ability to fluoresce either brightly (BS) or moderately (M) under UV light. Fourteen BS and eight M pJA42::Tn3Hogus plasmid derivatives were re-isolated from *Rhizobium* and transformed into *E.coli* from which larger quantities of re-isolated plasmids were screened. The position and direction of the Tn3Hogus derivatives were determined with restriction digestion with several restriction endonucleases. Restriction digestion analyses of a few derivatives are shown in Fig. 3.7. Of the 32 derivatives, only six (BS1, BS3, BS4, BS5, BS6, BS12) out of the fourteen BS pJA42::Tn3Hogus derivatives screened, appeared to contain the transposon

(a)

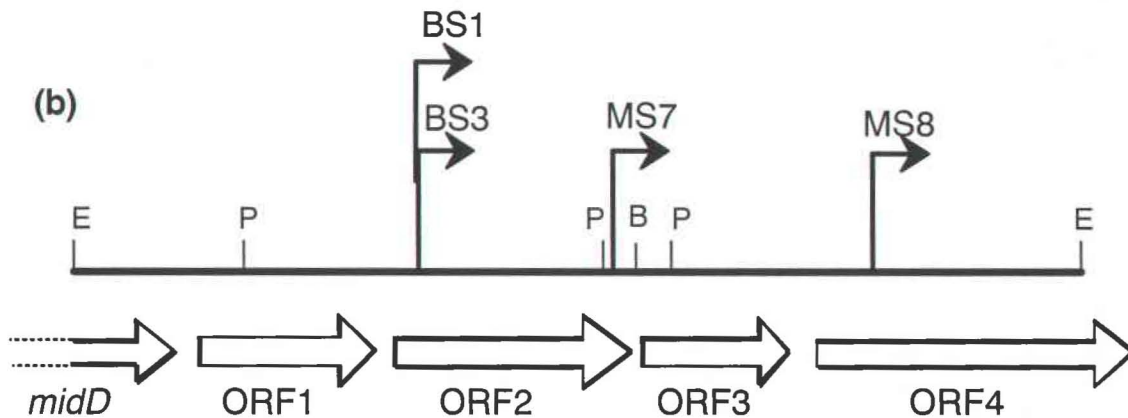
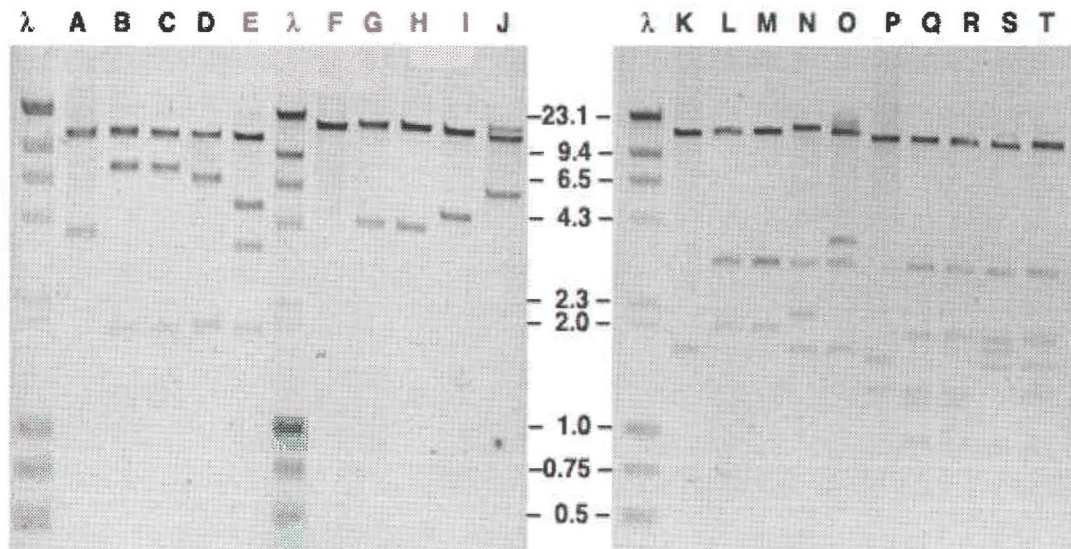


Fig. 3.7 (a) Gel photographs that were used to determine the position and direction of Tn3Hogus insertions in four mutants, BS1, BS3, M7 and M8. Plasmids pJA42 and its derivatives pJA42::BS1, pJA42::BS3, pJA42::M7 and pJA42::M8 were digested with *EcoRI* (lanes A to E), *HindIII* (lanes F to J), *BamHI* (lanes K to O), and *PstI* (lanes P to T). Marker lanes are indicated with λ .

(b) Map of the positions of the Tn3Hogus insertions in the mutants BS1, BS3 and M7 in ORF2 and M8 in ORF4 in the 4.0-kb *EcoRI* fragment. ORF1 shares homology with several hypothetical proteins. Homology analysis yields ORF2 to be an ACC deaminase and both ORF 3 and ORF4 as the small and large subunit for an alkyl hydroperoxide reductase, respectively.

insertion within the 4.0 kb region. Digestion of pJA42::Tn3Hogus plasmid derivatives with *EcoR*I would ideally cleave the plasmid into four fragments: a 10.6 kb pRK404A fragment; 1.95 kb fragment from within transposon construct; and two separate fragments whose combined total size equals approximately 8.5 kb. Since restriction digestion of most of the six BS-type derivatives yielded fragments that were very similar in size, only two, pJA42:: Tn3Hogus-BS1 and pJA42:: Tn3Hogus-BS3, were selected for further analysis. Similarly, three of eight M-type derivatives (pJA42:: Tn3Hogus-M6, pJA42:: Tn3Hogus-M7, and pJA42:: Tn3Hogus-M8) were also chosen for further restriction digestion analysis using the same method as above. These derivatives showed the best potential for having the transposon inserted within the desired 4.0 kb region.

Detailed restriction mapping using four restriction enzymes, *EcoR*I, *Hind*III, *Bam*HI and *Pst*I, showed that the transposons in both pJA42::Tn3Hogus-BS1 and pJA42::Tn3Hogus-BS3 are located at the 5' end of ORF2 encoding a homolog of ACC deaminase, while the location of the transposon in pJA42::Tn3Hogus-M7 was located on the 3' end of the same ORF (Fig. 3.7). The insertion location of pJA42::Tn3Hogus-M8 was mapped on ORF4, encoding the large subunit of alkyl hydrogen peroxide reductase. None of the insertions were located on ORF1 and ORF3.

Mutants alleles in these four constructs, pJA42::Tn3Hogus-BS1, pJA42::Tn3Hogus-BS3, pJA42::Tn3Hogus-M7 and pJA42::Tn3Hogus-M8 were individually transferred to the corresponding homologous positions of the TAL1145 chromosome by double homologous recombination. It is expected that

within these transconjugants the mutant alleles, through double homologous recombination, will recombine with corresponding homologous positions in the TAL1145 chromosome at a very low frequency to yield the mutant allele. To select for such recombinant mutants, another plasmid pPH1JI, which is incompatible with P-group plasmids such as pRK404A, was transferred to the transconjugants from *E. coli* strain 2174. The transconjugants were selected for resistance to gentamycin (conferred by pPH1JI) and kanamycin (conferred by Tn3Hogus). Gentamycin- and kanamycin-resistant colonies were then screened for the loss of the pJA42::Tn3Hogus derivatives while simultaneously selecting for tetracycline sensitivity. In this way, the following *Rhizobium* mutants were successfully created: BS1, BS3, M7, and M8.

3.6. Characterization of Mutants

The phenotype of the mutants when grown on solid YEM media showed slight differences in morphology and appearance. Mutant colonies displayed the same off-white color, formed smaller colonies, grew much slower, and appeared to produce smaller amounts of exopolysaccharides than TAL1145 (Fig. 3.8). Liquid culture growth experiments were performed to characterize the growth characteristics of each mutant in comparison to the wild type TAL1145 in various types of liquid growth media. The density of cells growing in liquid cultures was measured because it is difficult to accurately quantify growth on solid medium. In addition, the wild type normally secretes copious amounts of exopolysaccharides, which may also be wrongly interpreted as increased cell growth.

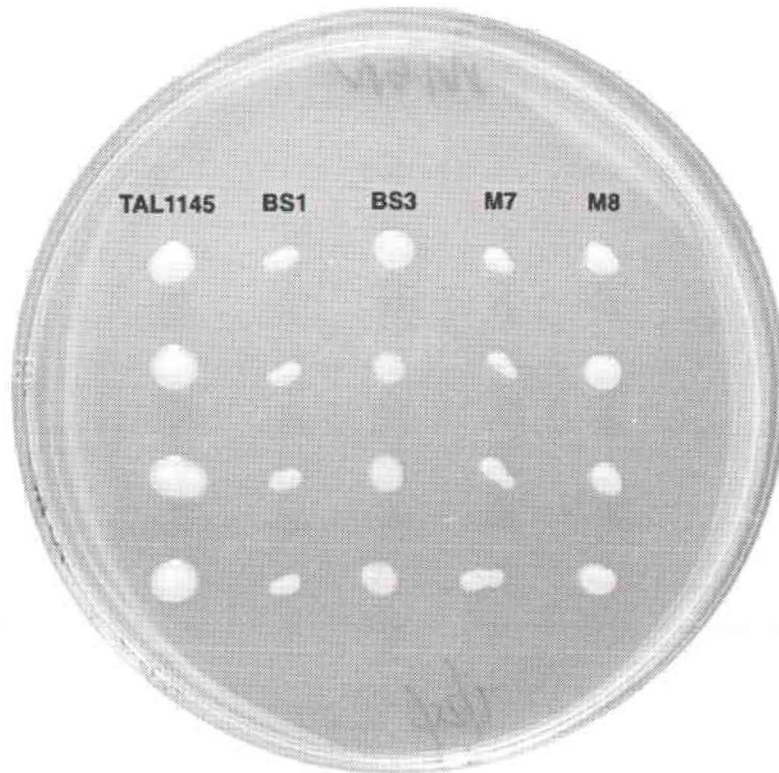


Fig. 3.8. Photograph showing the colony morphologies of TAL1145 and the four mutants, BS1, BS3, M7 and M8 on YEM agar.

Fig. 3.9.a shows growth rate of TAL1145 and the mutants BS1, BS3, M7 and M8 in liquid YEM and minimal medium (MM) containing either of the following additives: 3 mM sodium glutamate (Fig 3.9.b), 3 mM mimosine (Fig. 3.9.c), and 1% mannitol and 3mM ammonium chloride (Fig. 3.9.d). In liquid YEM for the first 55 hours, both the wild type and all four mutants were observed growing at the same rate. However at 60 hours an abrupt change in the growth rates of all four mutants was observed, which reflected a total growth difference at 96 hours where the cell densities of the mutants were much reduced compared to the wild type (Fig. 3.9.a). In minimal medium containing 3 mM

sodium glutamate, the wild type entered log phase at 24 hours as opposed to 60 hours for YEM whereas the mutants grew at a much reduced rate than the wild type, although they reached almost the same cell densities as the wild type at 108 hours (Fig. 3.9.b). In minimal medium containing 3 mM mimosine (Fig. 3.9.c), the overall growth of all the strains was inhibited, reading 0.3 -0.4 in cell density at approximately 60 hours, with the exception of M7 which had a lower cell density of approximately 0.25. The growth rates of all the mutants in the first 24 hours grew like that of the wild type and achieved slightly greater cell densities than that of TAL1145 in 72 – 120 hours. This suggests that the mutants, like the wild type, are able to grow and utilize mimosine though the overall growth (both growth rate and density) is inhibited, they are not defective in mimosine degradation. In minimal medium containing 1% mannitol and 3 mM ammonium chloride (Fig. 3.9.d.), all strains again experienced growth inhibition similar to that when grown in minimal medium and mimosine. All strains grew almost at the same rate during the first 24 hours. Afterwards the growth rates of the mutants slowed mid-exponential phase between 24-36 hours, which resulted in lower recorded cell densities than that of the wild type from 48 –120 hours.

3.7. Complementation of the mutants

The plasmid pJA42 was transferred into the four mutants to obtain a complemented derivative of each mutant. The complemented mutants showed the same colony morphologies as the wild type (data not shown). These mutants did not show a significant difference in growth rates on YEM agar. Growth experiments were done in YEM broth to compare the growth of the wild type,

mutant and its complement (Fig. 3.10). Overall, both the complemented transconjugants of BS1 and BS3 achieved a higher cell density than each of their respective mutants. The recorded growth densities of both the BS1 and BS3 complements for the first 96 hours were greater than that of the wild type, though in the last 12 hours of the growth experiment, both cell densities appear to be close to equal to TAL1145. The observed growth rates of complemented mutants BS3 and M7 grew at the same rate as the wild type within 24 – 60 hours, however the growth rate of complemented BS1 mutant increased at 48 hours and conversely, the growth rate of complement M8 decreased at the same time when compared to TAL1145. Both the complemented M7 and M8 mutants did not achieve higher cell densities like the BS-type mutants. In both cases the cell densities of each of the M-type complements between 84-120 hours were close to equal to their respective mutant counterparts, however both densities were still much lower than that of the wild type. These results indicate that the growth characteristics BS-type mutants were restored by complementation. However, the complemented M-type mutants did not grow as well as the wild type, despite having very little morphological difference when compared to the wild type strain when grown on YEM agar.

3.8. Plant inoculation experiment

The mutants, their complemented transconjugants and TAL1145 were used to inoculate *Leucaena* plantlets in modified Leonard jars containing horticultural vermiculite as described in materials and methods. Each Leonard jar assembly ideally contained 5 *Leucaena* plants and 3 jar assemblies were

prepared for each inoculum treatment. The planter assemblies were arranged in randomized block fashion and plants were grown for 7 weeks after inoculation. There were no obvious visual physiological differences in growth between the plants inoculated with the mutants, complemented transconjugants and the wild type. All plants were healthy and produced dark green leaves and normal indeterminate-type nodules indicating that these strains formed effective nitrogen-fixing symbioses. The nodules were harvested and counted for each treatment. Overall, the number of nodules collected from the wild type strain was greater than the number of nodules counted from both mutants and complemented strains. The total fresh weight of the nodules was measured to determine if there were any differences in the size (mass) of nodules produced. However, the results indicate that the differences in the nodule fresh weight among the mutants, their complemented derivatives and the wild type were neither significant nor distinguishing enough to identify a particular growth trend (Table 3.1). Nonetheless, bacteria were isolated from the vermiculite and nodules formed for each treatment and were tested to confirm their antibiotic resistance markers. These results suggest that although these mutants were found not to be defective for nodulation and nitrogen fixation, a better method to determine differences in *Leucaena* nodules should be used.

3. 9. Gus activity of the Tn3Hogus insertion mutants

To determine the level of expression of the genes located in the 4.0-kb fragment, the four *gus* insertion mutants and the four transconjugants of TAL1145 containing different pJA42::*gus* derivatives were assayed for Gus activity (Table 4.2). None of the four mutants showed any detectable levels of Gus activity, indicating that these genes are either not expressed or are expressed at a very low level under the growth conditions used in the experiment. However, the TAL1145 transconjugants containing the plasmid derivatives pJA42::Tn3Hogus-BS1 and pJA42:: Tn3Hogus-BS3 were able to produce detectable levels of Gus activity. The *gus* insertions in BS1 and BS3 are located on the 5' end of ORF2. Expression of multiple copies of these plasmid derivatives in the transconjugants was probably needed to detect Gus activity. This suggests that ORF2::*gus* protein fusion is being transcribed in the transconjugants containing multiple copies of pJA42::Tn3Hogus-BS1 or pJA42::Tn3Hogus-BS3. On the other hand, in transconjugants containing multiple copies of pJA42::Tn3Hogus-M7 or pJA42::Tn3Hogus-M8 derivative insertions, no Gus activity was detected. The addition of mimosine to the growth culture did not induce any significant difference in the Gus activities although in the pJA42::Tn3Hogus-BS1 strain, mimosine lowered Gus expression and in pJA42::Tn3Hogus-BS3 it increased expression a bit, thus suggesting that mimosine is not required for induction of these genes. This also supports the previous conclusion that these genes are not involved in mimosine degradation.

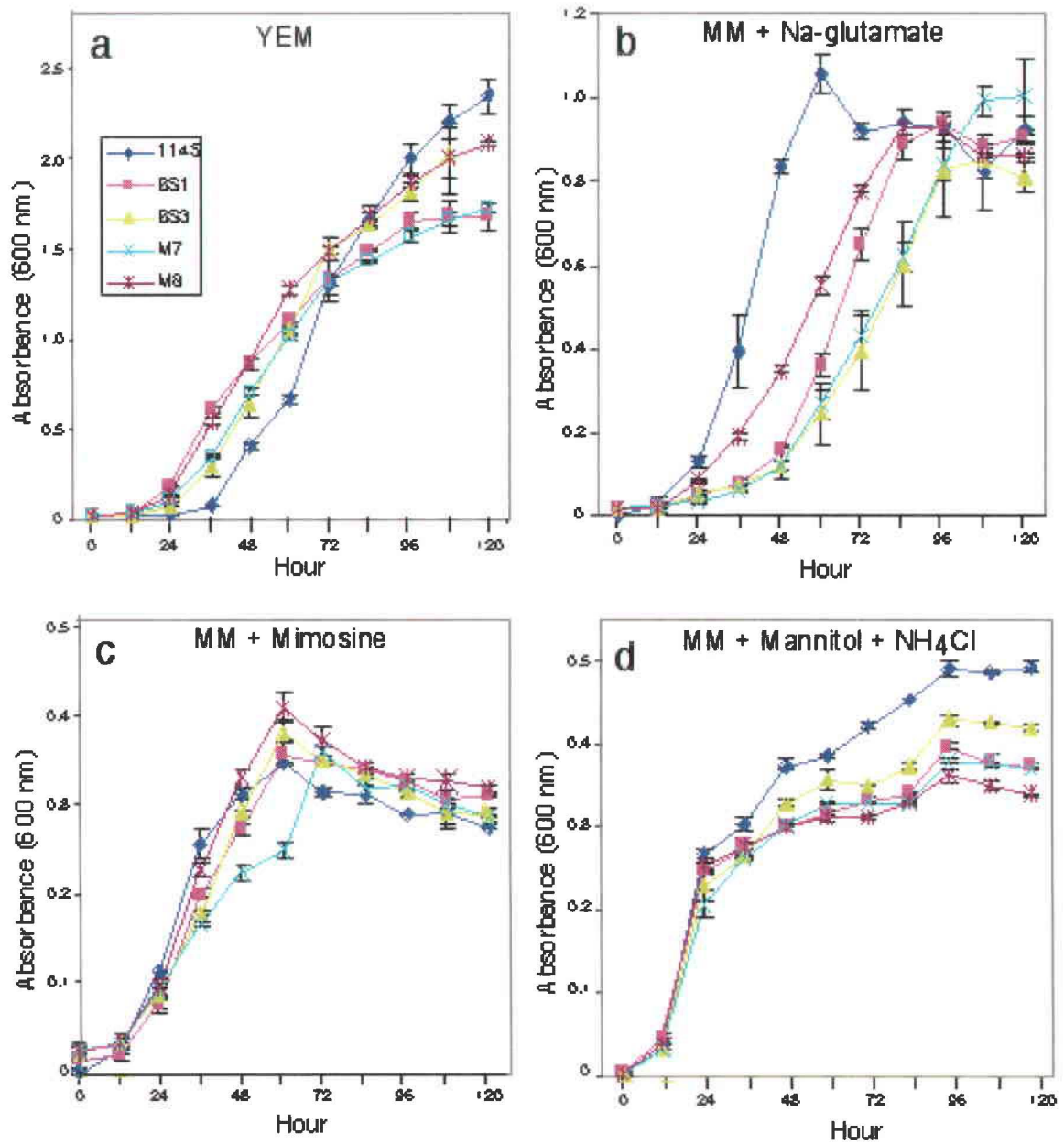


Fig. 3.9. Growth of TAL1145 (dark blue diamond), BS1 (pink square), BS3 (yellow triangle), M7 (blue cross) and M8 (purple star) in liquid YEM (a), MM + 3 mM Na-glutamate (b), MM + 3 mM mimosine (c) and MM + 1% mannitol and 3 mM NH₄Cl (d). MM = Minimal medium.

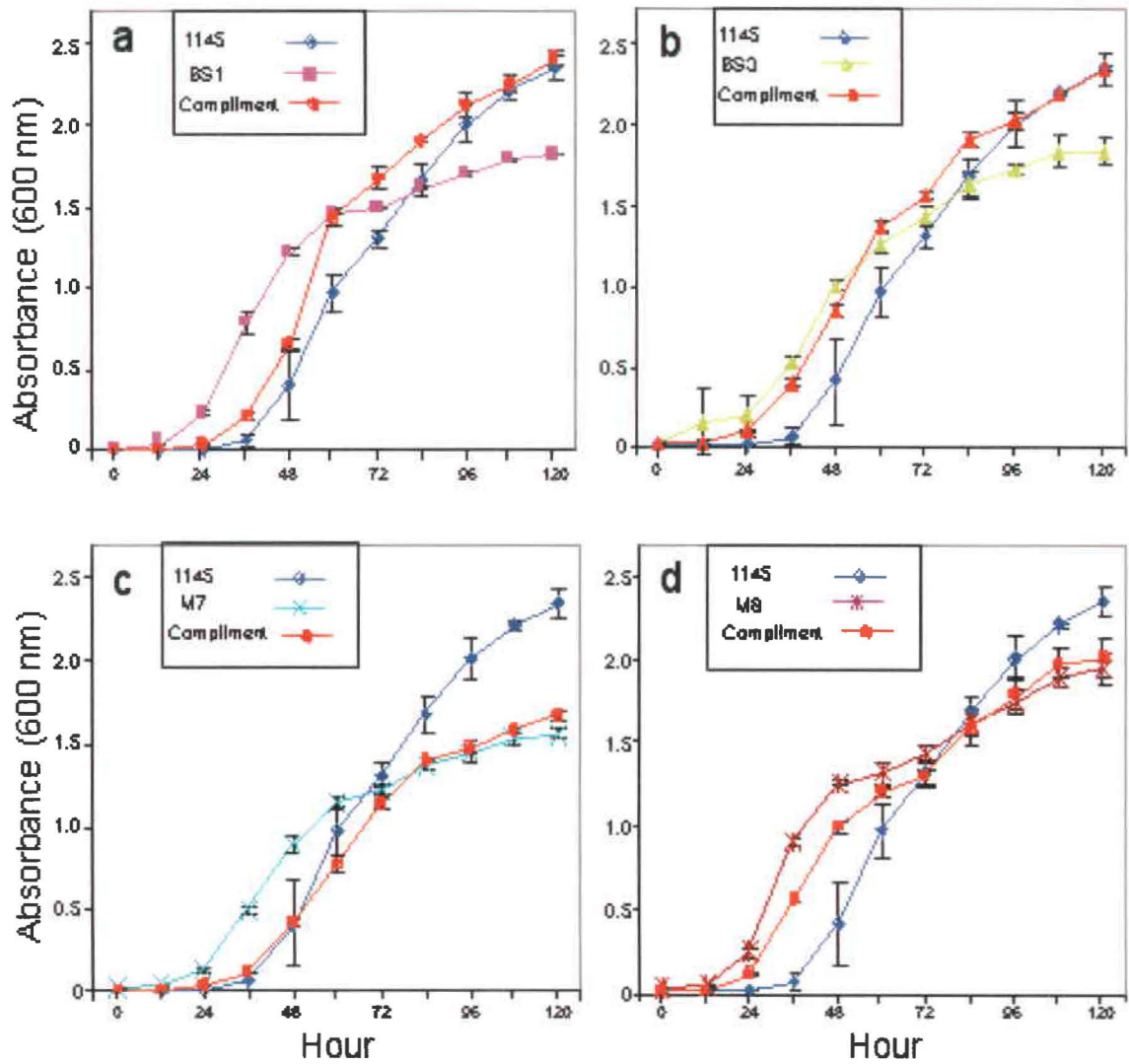


Fig. 3.10. Comparison growth of mutants BS1, BS3, M7, and M8 with that of TAL1145 and complemented derivatives of each mutant. The experiment was done in liquid YEM with three replications.

Table 3.1. Mean number and fresh weight of nodules harvested from *Leucaena* plants inoculated with either of the 4 mutants, their complements or TAL1145.

Strain	Mean Number of Nodules*	Nodule fresh weight* (mg)
TAL1145	53.0 ± 19.67	.807 ± .253
BS1	39.0 ± 5.00	.755 ± .106
BS3	41.7 ± 11.0	.702 ± .032
M7	41.0 ± 6.92	.772 ± .277
M8	50.7 ± 10.6	.861 ± .092
BS1 (pJA42)	32.0 ± 4.36	.605 ± .123
BS3 (pJA42)	51.0 ± 9.84	.834 ± .226
M7 (pJA42)	48.0 ± 22.0	.828 ± .431
M8 (pJA42)	46.3 ± 7.09	.738 ± .070

*The data are mean values ± standard deviations of three replications. The total number of nodules harvested per replication was averaged to obtain the values in the second column. The averaged values in the third column were calculated from the total fresh weight of nodules collected per replicate.

Table 3.2. Gus activity of *Rhizobium* mutants and transconjugants containing Tn3Hogus insertions.

Strain	Copy number of gus	GUS activity (nM of MU ml⁻¹ h⁻¹) (-) mimosine	GUS activity (nM of MU ml⁻¹ h⁻¹) (+) mimosine
TAL1145	0	0	0
BS1	1	0	0
BS3	1	0	0
M7	1	0	0
M8	1	0	0
TAL1145pJA42::BS1	4-7	35,467 ± 15	31,670 ± 15
TAL1145pJA42::BS3	4-7	66,467 ± 17	70,767 ± 10
TAL1145pJA42::M7	4-7	0	0
TAL1145pJA42::M8	4-7	0	0

The data are mean and standard deviations from three replicates.

CHAPTER 4 DISCUSSION

In this study, we have characterized a 4.0-kb fragment from within the *mid-pyd* gene cluster of *Rhizobium* TAL1145, which contains a mixture of complete and fractioned ORFs. We had originally hypothesized that the region between the *mid* and *pyd* gene clusters would contain additional genes involved in the degradation of mimosine. However, based on the analysis of the 4.0-kb fragment and characterization of knock out mutants in ORF2 and ORF4, it is safe to conclude that the genes located in this region are not involved in mimosine transport, degradation, and symbiosis. Knocking out ORF2 and ORF4 had little effect on these processes and it is likely that ORF1 and ORF3, which are part of the same cluster, also do not participate in these processes. In fact, the deduced amino acid sequences of the identified ORFs in the 4.0-kb fragment have been shown to have high homologies to the following proteins: ORF1 to several hypothetical proteins in various organisms, ORF2 to 1-aminocyclopropane-1-carboxylate deaminase or “ACC deaminase” and ORF3 and ORF4 to the small and large subunit of alkyl hydroperoxide reductase. The ACC deaminase gene has been named as *accD* in *M. loti* (Sullivan et al., 2002), *acdS* in *R. leguminosarum* (Ma, W., Guinel, F.C. and Glick, B.R, unpublished), *acd* in *Penicillium citrinum* (Jia et al., 2000) and with various names in other bacteria. Among these names, *acd* corresponds well for 1-aminocyclopropane-1-carboxylate deaminase, and therefore, we name the ACC deaminase gene in *Rhizobium* TAL1145 as *acd*. Similarly, ORF3 and ORF4 in the present study

have high homologies with the *ahpC* and *ahpF* genes for the small and large subunits of alkyl hydroperoxide reductase of *Salmonella typhimurium* and a few other organisms. Therefore, ORF3 and ORF4 in TAL1145 will also be referred to as *ahpC* and *ahpF*, respectively.

Similar analysis of the adjacent 5.5-kb fragment in *Rhizobium* sp. strain TAL1145 reveal there are additional genes, the *fba* and *fbp* genes encoding a fructose-1,6-bisphosphate aldolase and a fructose-1,6-bisphosphatase respectively, also not required for mimosine degradation, although they too are located within the *mid-pyd* gene cluster (Awaya et al, 2002). The location of these genes, genes that are not related to mimosine degradation, raises several questions. For example, why are genes located in the middle of the *mid-pyd* gene cluster, what are their functions, and do these genes have any role in *Rhizobium*-plant interactions. The remainder of this discussion will try to address some of these questions.

4.1. Position of ACC deaminase gene in *mid* gene cluster

It is interesting to note that the *acd* gene is located in between the *mid* and *pyd* gene clusters in TAL1145. Some other rhizobia such as *R. leguminosarum* and *M. loti* also contain homologous genes. It is possible that *Leucaena*-nodulating rhizobia such as TAL1145 acquired *mid* genes relatively late during evolution from other soil bacteria harboring genes for the degradation of aromatic compounds on transmissible plasmids, since the ability to degrade mimosine is a unique quality of TAL1145. The *mid* and *pyd* genes may have

been transferred into a particularly susceptible region of TAL1145 chromosome in two separate events. This may also explain why the integration and current position of the *mid* and *pyd* gene clusters is flanking either side of the preexisting *acd* gene.

Another explanation for the presence of the *acd*, *fba* and *fbp* genes in the middle of the *mid-pyd* gene cluster is that the chromosome of TAL1145 might have undergone several internal recombination and reorganization events. According to this idea, the *mid* and *pyd* gene clusters were once grouped together, while the *acd*, *fba* and *fbp* genes were also clustered together in a different region on the TAL1145 chromosome. During evolution, these genes may have been reshuffled and recombined into their current sequence, consequently separating the *mid-pyd* group into two clusters.

4.2. The effect of ACC deaminase on bacterial growth

We have observed that the ACC deaminase mutants produced less exopolysaccharide and in most cases, the mutants grew to lesser cell densities (Fig. 3.9). This suggests that the ACC deaminase enzyme is involved in some bacterial growth-related biochemical processes. The elucidation of this process would require further investigation, however, we propose the following theory of how ACC deaminase influences the growth of bacteria in the plant rhizosphere.

Like plant cells, rhizobacteria may also endogenously produce small amounts of ethylene, which diffuses into the surrounding environment and could possibly serve as a signal molecule to stop growth and cell proliferation. Several

plant pathogenic bacteria and fungi have been shown to produce ethylene, which causes increased plasticity of plant cell walls and increased permeability to cell membranes (Agrios, 1997; Raven, et al., 1981). While large quantities of ethylene can have a negative effect on plant root elongation and hence development, levels of ethylene are both positively and negatively controlled, respectively, either by IAA or ACC deaminase that is produced by the rhizobacteria. However, unlike in plant cells, the ACC deaminase that is produced by bacteria is perhaps a key enzyme, which also auto-regulates and inhibits bacterial ethylene biosynthesis thereby reducing the levels of ethylene in the surrounding environment and preventing the bacterium from premature aging. In the absence of this “anti-aging” enzyme, such as is the case with the mutants in this study, bacteria seem to reach the stationary phase rather prematurely and as a result fail to achieve a higher cell density.

In the past, associative plant-bacterial symbioses such as rice-*Azospirillum* (Patnaik and Rao, 1994), corn-*Azotobacter* (Monib et al, 1979) and even rice-*Rhizobium* (Yanni et al, 1997; Ueda et al 1995) associations have been investigated. It has been shown that these bacterial associations provide some growth benefits to the plant (Steenhoudt and Vanderleyden, 2000; Skvortsov and Ignatov, 1998; Schloter et al., 1997). Generally, the associative bacteria are also free-living nitrogen fixers (diazotrophs) and thus they provide the plant with a source of fixed nitrogen. These bacteria are also known to produce some amounts of IAA, which stimulates plant growth (Mehnaz et al, 2001; Datta and

Basu 2000; Pattern and Glick, 2002). We believe that like other associative bacteria symbionts, *Rhizobium* sp. TAL1145 may also form associative symbiosis and enhance the growth of plant roots by inhibiting ethylene production through ACC deaminase activity. If this hypothesis is correct, then the *acd* mutants of TAL1145 should produce an underdeveloped root system – as has been demonstrated previously with *E. cloacae* UW4 mutants, which are deficient in producing ACC deaminase and are also unable to promote root elongation in canola seedlings (Li et al., 2000). Likewise such mutants might be additionally defective in nodulation competition.

In our present study, we concentrated only on nodulation, nitrogen fixation and growth characteristics of the inoculated plants and not on root system development since it was initially believed that the genes within the 4.0-kb fragment were involved in mimosine-degradation and would consequently influence the rhizosphere colonization. Moreover, the differences in the root system development due to these bacteria may be small and may not be detectable under the experimental conditions in this present study. For detection of minute differences in root growth, the experiments need to be conducted for a longer period with higher number of replications or perhaps with another model plant. We have also not tested the *acd* mutants for nodule occupancy in competition with the wild-type strain. Nevertheless, experiments in the present investigation open a new avenue in the study of *Rhizobium* as an associative

symbiont of plants. Until now *Rhizobium* has been studied primarily as a symbiont that forms nitrogen-fixing nodules on legumes.

4.3. The effect of ACC deaminase on plant growth

Bacterial ACC deaminase genes may also contribute towards increasing the types of crops targeted for prolonged shelf life. Already the research group of agricultural research giant Monsanto has capitalized on this information with the development of tomatoes expressing ACC deaminase from a soil bacterium (Klee et al, 1991). To date, not much has been published on bioengineering and utilizing ACC deaminase in other plants. Instead, emphasis on controlling ethylene synthesis has been focused on the development of transgenic plants expressing antisense RNA transcripts of enzymes involved in the ripening process (Liu et al, 1998; Ayub et al., 1996). Perhaps the rationale for preferring one method to the other might stem from the fact that one requires the introduction of a foreign gene from an exogenous source, while the other does not. This fact alone supports much of the notoriety and bad publicity genetically modified organisms (GMO) receive, particularly by extreme environmentalists. Nonetheless, more attention should be directed towards this endeavor. Then perhaps biotechnology will broaden the spectrum of plants expressing ACC deaminase so that the shelf life of various fruits and cut foliage may also be prolonged.

4.4. The effect of alkyl hydroperoxide reductase on growth

The ORF3 and ORF4 encoded proteins in the present study show high homologies with alkyl hydroperoxide reductase small and large subunits from various organisms (Chae et al., 1994). Alkyl hydroperoxide reductases are known to be antioxidants that protect the cells from oxygen damage. Under aerobic growth conditions, organisms are subject to oxidative damage due to production of hydrogen peroxide in the cells (Sies, 1993). Protection against the toxic and damaging effects of oxygen is provided by a variety of enzymes. In *E. coli* and *Salmonella typhimurium*, alkyl hydroperoxide reductase, a 57-kDa flavoprotein encoded by *ahpF* and a 21 kDa protein encoded by *ahpC* constitute a peroxidase system for catalysis of NADH- and NADPH- dependent reduction of a wide variety of hydroperoxide substrates to their corresponding alcohols (Niimura et al., 1995). Based on the *ahpF/ahpC* system in other organisms, it is likely that the ORF3 and OR4 encoded proteins in *Rhizobium* TAL1145 are also involved in oxidative protection of the cells in culture under free-living aerobic growth conditions. According to this idea, the ORF4 mutant should show some growth defect due to oxidative damage during the late exponential growth phase. The mutant M8 was observed to have some growth defect, its growth rate in most experiments was slower than that of TAL1145 and its final cell densities were less than that of TAL1145. These differences were more apparent when these strains were grown in minimal medium containing 1% mannitol and 3 mM ammonium chloride (Fig. 3.9.d). In this experiment the mutant M8 grew like

TAL1145 during the first 24 hours, after which the growth rate of the mutant was slowed down, presumably because of oxidative damage.

The *ahpC-ahpF* genes in *S. typhimurium* are transcribed together as part of an operon. It is likely that ORF1 to ORF4 in the present study are transcribed together in a single transcript. In that case the Tn3Hogus insertion in mutants BS1, BS3 and M7 (ORF2) will have a polar effect on the downstream ORF3 and ORF4. If the similar phenotypes of the ORF2 and ORF4 mutants are due to the polar effect of transposon insertion, then the phenotype of these mutants cannot be distinguished without complementation analysis using smaller subcloned fragments. However this has yet to be determined and future experiments tailored towards alkyl hydroperoxide reductase function and activity in TAL 1145, as well as ACC deaminase, will be needed to possibly ascertain this.

**CHAPTER 5
MATERIALS AND METHODS**

***Rhizobium* strains and plasmids**

Rhizobium strains and plasmids used in this study and their properties are listed in Table 5.1.

Table 5.1. *Rhizobium* strains and plasmids used in this study

Strain/plasmid	Characteristics	Reference
<i>Rhizobium</i>		
TAL1145	Wild-type <i>Rhizobium</i> sp. strain that nodulates leucaena, Mid ⁺ .	George et al., 1994
BS1	Mutant of TAL1145 containing a Tn3Hogus insertion on ORF2 of the 4.0-kb <i>EcoRI</i> fragment, Kan ^R , Mid ⁺ .	This study
BS2	Mutant of TAL1145 containing a Tn3Hogus insertion on ORF2 of the 4.0-kb <i>EcoRI</i> fragment, Kan ^R , Mid ⁺ .	This study
M7	Mutant of TAL1145 containing a Tn3Hogus insertion on ORF2 of the 4.0-kb <i>EcoRI</i> fragment, Kan ^R , Mid ⁺ .	This study
M8	Mutant of TAL1145 containing a Tn3Hogus insertion on ORF4 of the 4.0-kb <i>EcoRI</i> fragment, Kan ^R , Mid ⁺ .	This study
Plasmid		
pLAFR3	Wide-host range P1-group cosmid vector, Tet ^R	Staskawicz et al., 1987

pRK404	Wide-host range P1-group cloning vector, Tet ^R	Ditta <i>et al.</i> , 1985
pPH1J1	P1 group plasmid used to eliminate other P1-group recombinant plasmids from <i>Rhizobium</i> strains, Gen ^R .	Beringer <i>et al.</i> , 1978
pUHR181	pLAFR3 cosmid clone isolated from the genomic library of TAL1145, contains <i>mid</i> genes.	Borthakur & Soedarjo, 1999
pUHR263	pLAFR3 cosmid clone isolated from the genomic library of TAL1145, overlaps with pUHR181 and contains <i>mid</i> genes	This study
PJA42	The 4.0-kb <i>Eco</i> RI fragment from pUHR263 cloned in pRK404.	This study
pJA42:: <i>gus</i> -BS1	pJA42orf2:: <i>Tn3Hogus</i> -BS1	This study
pJA42:: <i>gus</i> -BS3	pJA42orf2:: <i>Tn3Hogus</i> -BS3	This study
pJA42:: <i>gus</i> -M7	pJA42orf2:: <i>Tn3Hogus</i> -M7	This study
pJA42:: <i>gus</i> -M8	pJA42orf4:: <i>Tn3Hogus</i> -M8	This study

Wide-host range plasmid vector pRK404A

This P-group plasmid vector was modified from the original plasmid pRK404 (Ditta *et al.*, 1985) by William Buikema at the University of Chicago. The map of the plasmid pRK404A is shown in Fig. 5.1.

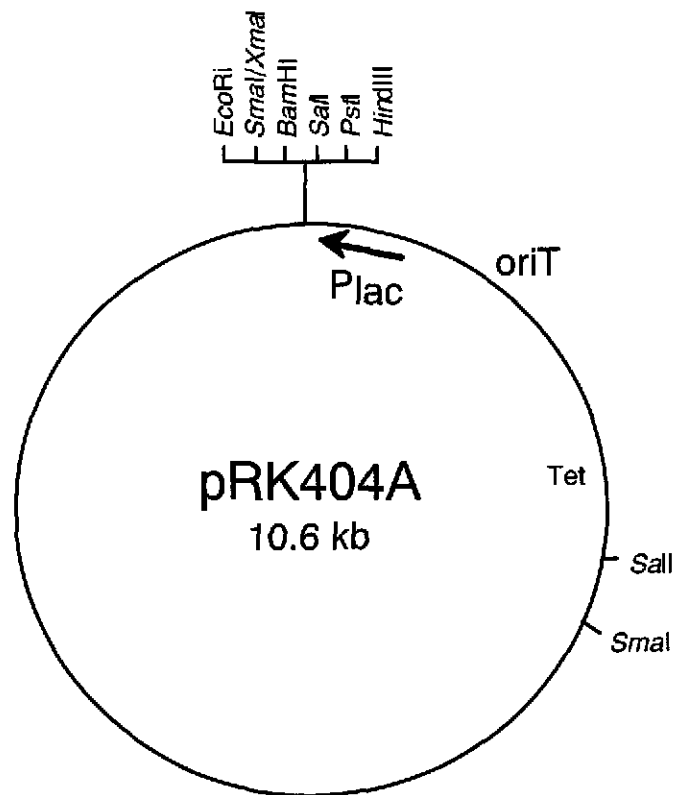


Fig. 5.1 Wide-host range cloning vector pRK404A, which was modified from pRK404 (Ditta et al., 1985) by W. Buikema at the University of Chicago.

Media and growth conditions

All *Rhizobium* strains were grown in yeast extract mannitol (YEM) (Vincent 1970), tryptone yeast extract (TY) or *Rhizobium* minimal medium (MM) (Soedarjo et al. 1994) media at 28°C, while *E. coli* strains were grown in Luria-Bertani (LB), SOB or SOC media at 37°C. All liquid media were prepared according to recipe protocols listed below. Solid media were prepared by adding 15 g of agar per liter of liquid media.

LB medium (Sambrook et al., 1989)

Per liter

To 950 ml of deionized water, add:

Bacto-tryptone	10 g
Bacto yeast extract	5 g
NaCl	5 g

Mix until the solutes are dissolved.

Adjust pH to 7.0 with 5 N NaOH.

Adjust volume to 1 liter.

Sterilize by autoclaving.

SOB medium (Sambrook et al., 1989)

Per liter:

To 950 ml of deionized water add:

Bacto-tryptone	20 g
Bacto-yeast extract	5 g
NaCl	0.5 g

Mix until the solutes have dissolved.

Add 10 ml of a 250 mM solution KCl.

Adjust pH to 7.0 with 5N NaOH.

Adjust volume to 1 liter.

Sterilize by autoclaving.

Just before use, add 10 ml of a sterile solution of 1 M MgCl₂.

SOC medium (Sambrook et al., 1989)

SOC medium is identical to SOB medium, except that it contains 20 mM glucose. Adjust the final volume before autoclaving to 980 ml. After the SOB medium is autoclaved, allow it to cool to 60°C or less. Then add 20 ml of sterile 1 M solution of glucose (18 g glucose in 100 ml volume = 1M solution, sterilize by filtration). Just before use, add 10 ml of a sterile solution of 1 M MgCl₂.

***Rhizobium* minimal medium (MM) (Soedarjo et al., 1994)**

Rhizobium MM contained the following components per liter of deionized water

Per liter:

To 950 ml of deionized water add:

K ₂ HPO ₄	250 mg
KCl	100 mg
Na ₂ EDTA · 2H ₂ O	10 mg
FeCl ₂	8 mg

Mix until solutes have dissolved.

Adjust pH to 6.8. with 1 M HCl.

Adjust volume to 1 liter.

Sterilize by autoclaving.

Add the following filtered stock solutions after autoclaving:

1 M CaCl ₂	0.5 mL
1 M MgSO ₄	1 mL
Micronutrient solution	1 mL
Vitamin solution	5 mL

The micronutrient solution contained 1.5 g MnSO₄·7H₂O, 170 mg CuCl₂·2H₂O, 50mg Na₂MoO₄·2H₂O and 10 mg CaCl₂·2H₂O in 1 liter deionized water until

dissolved. The vitamin solution contained 100mg of biotin, 100 mg of thiamine and 100 mg of DL- pantothenate in one liter of deionized water.

YEM medium (Vincent, 1970)

Per liter

To 950 ml of deionized water, add:

Bacto-tryptone	10 g
Bacto yeast extract	5 g
NaCl	10 g

Mix until the solutes are dissolved.

Adjust volume to 1 liter.

Adjust pH to 7.0 with 5 N NaOH.

Sterilize by autoclaving.

TY medium (Beringer, 1974)

Per liter

To 950 ml of deionized water, add:

Bacto-tryptone	10 g
Bacto yeast extract	5 g
CaCl ₂	0.2 g

Mix until the solutes are dissolved.

Adjust volume to 1 liter.

Sterilize by autoclaving.

Antibiotics

The following list of antibiotics was made for the selection of bacteria harboring plasmids with specific antibiotic resistance genes. Stock solutions of antibiotics were either prepared with the sterilized water or filtered solvents and stored in light-resistant tubes at 4°C for up to one month. Stock solutions of antibiotics were added to media after being cooled to 45°C to obtain the following final concentrations (Table 5.2).

Table 5.2. Antibiotics used in this study

Antibiotic	Stock Conc. (mg/mL)	Solvent	Final Conc.	Supplier
Ampicillin (Amp)	100.0	Deionized water	5.0	Sigma
Chloramphenicol (Cm)	10.0	Ethanol	10.0	USB Corp
Gentamycin sulfate (Gen)	10.0	Deionized water	10.0	Sigma
Kanamycin sulfate (Kan)	25.0	Deionized water	25.0	USB Corp
Streptomycin sulfate (Str)	10.0	Methanol	20.0	Sigma
Tetracycline hydrochloride (Tet)	10.0	Ethanol	5.0	Sigma

Molecular biological techniques used in the cloning

Several of the basic molecular biological techniques that were utilized throughout this study are described. In addition to the molecular methods, the methods used for the growth, plant, and Gus activity experiments are also included.

Procedures for isolation of genomic and plasmid DNA from bacteria:

Small Scale Preparation of Total Genomic DNA from *Rhizobium*

When we need only a small amount (30-40 μ g) of genomic DNA the following method is preferable. This procedure was performed in 1.5-ml microfuge tubes. In this method, guanidine isothiocyanate was used to disrupt the cell membrane and for rapid protein denaturation. Also, the guanidinium cation and the isothiocyanate anion inactivate the ribonucleases by disrupting their tertiary structure.

Solutions and media required for this method include:

- Lysozyme: 10 mg/ml in TE₂₅ (preferably in 25% sucrose, 50 mM Tris, 1 mM EDTA pH 8.0).
- TE : Tris 10 mM, EDTA 1 mM
- 5 M Guanidine isothiocyanate in 0.1 M EDTA pH 7.0
- 7.5 M ammonium acetate
- Isoamyl alcohol-chloroform (1:24 v/v)
- 75% ethanol, 10 mM NH₄Ac
- TY media (described previously)

Procedure:

Liquid YEM was inoculated with a single colony of *Rhizobium* strain and incubated in a rotary shaker for 2 days at 28°C. The culture (1 ml) was used to inoculate 30 ml of TY media. Cells were again grown overnight at 28°C in a rotary shaker. Cultures were grown in TY medium since it does not contain sugars (carbon) and limits the amount of exopolysaccharides produced by

Rhizobium. The following day, aliquots of culture were divided into 1.5-ml microfuge tubes and spun for 2 min at 8,000 rpm to remove the supernatant. Pellets of cells were washed with 1 ml of 1M NaCl and again spun down at 8000 rpm for 1 min to wash cells free of media and to remove supernatant. The pellet of cells was resuspended in 100 μ l of 10 mg/ml lysozyme in 25% sucrose, 50 mM Tris, 1 mM EDTA pH 8.0 and left to incubate at room temperature for 10 min. Following incubation, the cells were lysed by adding 200 μ l of 5 M guanidine isothiocyanate in 0.1 M EDTA pH 7.0 and mixed by inversion. 150 μ l of 7.5 M ammonium acetate and an equal volume (500 μ l) of isoamyl alcohol-chloroform (1:24 v/v) was then added to the mixture then the cell lysate. The mixture was vortexed for a few seconds and spun at 12000 rpm for 4 min to settle the organic phase from the aqueous one. About 350 μ l of the aqueous phase was transferred into a fresh tube and 0.6 volume (210 μ l) of isopropanol was added. A gentle rocking motion was used to mix the solutions and to encourage DNA to precipitate from solution. The DNA was collected from solution by spinning for 10 min and removing the supernatant. The DNA pellet was washed twice with 75% ethanol, 10 mM NH₄Ac and at last dried under vacuum and resuspended in 100 μ l TE.

Alkaline lysis procedure for isolation of recombinant plasmid DNA from *E. coli*

Both the alkaline and the Triton lysis (STET) (described below) preps rely on the fact that extrachromosomal plasmid DNA is much smaller than chromosomal DNA and is present in cells as covalently closed circular

molecules. After the cells are lysed, both protocols utilize centrifugation to remove the bulk of the chromosomal DNA and cellular debris, followed by precipitation of the plasmid DNA. This method was used as a quick and efficient method to harvest plasmid DNA when screening for recombinant vectors.

Solutions and medium required for this method include:

- Solution A: 50 mM glucose, 25 mM Tris, 10 mM EDTA
- Solution B: 0.2N NaOH, 1% SDS
- Solution C: 60 ml 5 M KAc, 11.5 ml glacial acetic acid and 28.5 ml dH₂O
- Equilibrated phenol
- Isoamyl alcohol-chloroform (1:24 v/v)
- 75% ethanol
- LB medium (described previously)

Procedure:

Overnight *E. coli* cultures were started by inoculating 5 ml of LB with a single colony and incubating at 37°C in a shaker. Cells were pelleted in a Beckman centrifuge for 5-10 minutes at 3,000 rpm and liquid media was removed by decanting. Pelleted cells were resuspended in 200 µl of Solution A and incubated at room temperature for 5 minutes. Following incubation, 400 µl of Solution B was added and gentle mixing without vortexing was applied to mix solution. After 5 minutes on ice, 300 µl of Solution C was added followed by vortexing for 2 seconds. After another 5-minute incubation on ice, the solution was spun for 5 minutes at 14,000 rpm to pellet cellular debris. The supernatant was removed into fresh tubes and a half volume of equilibrated phenol was

added to denature and precipitate proteins. The phenol-aqueous solution was vortexed and left for 5 minutes at room temperature. Then a half volume of chloroform/isoamyl alcohol (24:1) was added and the solution was again vortexed and spun for 2 minutes to settle the organic and aqueous layers. The upper aqueous phase was carefully removed and transferred into a new tube. An equal volume of chloroform was added and again the solution was vortexed and spun for 2 minutes to remove traces of phenol. Again the upper phase was transferred into a new tube and 1 volume of isopropyl alcohol and 1/10 volume of 3M Sodium acetate was added to precipitate out the DNA from the solution. Gentle mixing was applied and the tube was kept on ice for 5-10 minutes. The DNA was spun for 10 minutes to concentrate the DNA and remove the supernatant. Following several washes with 70% ethanol, the pellet was either air-dried or vacuum dried and resuspended in 20-50 μ l of TE.

Small Scale Preparation (STET) of recombinant plasmid DNA from *E. coli*

Small recombinant plasmids can be easily isolated from *E. coli* and used for restriction analysis, subcloning, DNA sequencing and other analyses. In the boiling or STET prep method, *E. coli* cell membranes are solubilized using lysozyme and the detergent Triton X-100. The cells in this prep were lysed by boiling to release plasmid DNA into the solution and precipitated by isopropanol or ethanol.

Solutions and medium required for this method include:

- Lysozyme: 10 mg/ml in TE, Tris 10 mM, EDTA 1 mM (TE)

- STET buffer containing 8% sucrose, 0.5% Triton X-100, 50 mM EDTA, 50 mM Tris-HCl, pH 8.0
- Isopropanol
- 70% ethanol

Procedure:

An overnight culture of *E. coli* was grown either in LB broth or LB agar with appropriate antibiotic for the selection of the recombinant plasmid. Cells were collected either by spinning down 0.5 ml culture or resuspending and pelleting a loopful of cells in media. Either way, the supernatant was discarded and 200 μ l of STET buffer was added to resuspend pellet. After vortexing, 20 μ l of lysozyme solution was added, mixed and incubated at room temperature for 5 min. Tubes were then placed in boiling water for 50 sec followed by centrifuging for 5 min at room temperature. With a sterile toothpick or using the suction from a pipette, the gooey residual pellet was removed, and the leftover supernatant was transferred to a fresh tube. An equal volume of isopropanol or 3 volumes of 95% ethanol was added to the solution and gentle mixing was applied to precipitate the DNA out of solution. The solution was then spun for 5 min to pellet the DNA. The supernatant was discarded and the pellet was washed with 75% ethanol. The DNA pellet was dried and resuspended in 20-50 μ l TE.

Procedure for making competent cells

The use of competent cells to grow and harvest plasmid vectors for DNA manipulation is an essential tool used in molecular research. A 2-liter flask with 250 ml SOB was inoculated with 10-12 large *E. coli* colonies and grown at 18° -

28°C with vigorous shaking (200-250 rpm) to an A_{600} of 0.6. Next the flask was placed on ice for 10 min to slow the growth of the cells. The culture was then divided into large centrifuge tubes (200 ml or 500 ml) and spun at 3000 rpm for 10 min at 4°C. The liquid media was carefully removed and the pellet was resuspended in 80 ml of ice-cold transformation buffer (TB). Following incubation on ice for 10 min, cells were again spun down as previously described and the pellet resuspended in 20 ml of ice-cold TB. DMSO was added to a final concentration of 7% and then incubation on ice for 10 min. Cells in TB solution were then dispensed in 0.5-ml cell suspensions into microfuge tubes and immediately chilled by immersion in liquid nitrogen. These were stored frozen in the -80°C freezer and thawed when necessary.

Transformation procedure

A tube of competent cells (previously described) was thawed on ice and 200 μ l of cells were dispensed into a 15 ml polypropylene tube and placed on an ice bath. Plasmid DNA (1-5 μ l) was added into each tube and left to incubate on ice for 30 min. The mixture was then heat shocked for 30 sec at 42°C for 30 sec without agitation and then placed on ice for 1 min. SOC liquid (0.8 ml) was added and cells were subsequently incubated at 37°C with gentle shaking for 1 hour. Cells were then plated on LB agar containing the appropriate antibiotic(s) for selection of the plasmid.

Transfer of plasmids through conjugation

The plasmids used in this study were non-conjugative and lacked the ability for self transfer. Therefore, a helper plasmid was necessary for

conjugation. For this procedure equal proportions (in loopful amounts) of *E.coli* strain pRK2013, *Rhizobium* sp. TAL1145, and the *E.coli* strain containing plasmid of interest (e.g. pJA42) were scraped from agar plates containing the necessary antibiotics and applied to TY (without antibiotics) plate. These strains were mixed thoroughly and incubated for two days at 28°C. After incubation, a loopful of cells was streaked onto a media plate with the appropriate antibiotics for selection.

Restriction digestion and agarose gel electrophoresis of genomic and plasmid DNA

Restriction enzymes recognize specific base pair sequences of double-stranded DNA and cleave the DNA in both strands at specific sites. When a DNA sample from a particular organism is digested with a restriction enzyme, a definite set of restriction fragments are produced. Thus a restriction enzyme cleaves a DNA sample into discrete and reproducible fragments. To clone DNA fragments from an organism into a plasmid vector, both the source DNA and the plasmid must be digested. Restriction enzymes are also called type II restriction endonucleases and *EcoRI* is one of the earliest restriction enzymes to be characterized and isolated from *E. coli*.

Generally, 0.5 to 1 µg of plasmid DNA was treated with 2-3 units of a restriction enzyme at 37°C for 1 hour. In the case of genomic DNA, 2-5 µg of genomic DNA was digested with 5 - 10 units of a restriction enzyme. The resulting fragments were then separated by an agarose gel and visualized by staining the gel with ethidium bromide (EtBr).

Procedure:

The following restriction digestion recipe was carried out in a 1.5 ml tube to digest plasmid or genomic DNA either for restriction mapping or for cloning various plasmid constructs. To a microfuge tube on ice, 0.5 to 3 μg DNA; 2 μl of the appropriate 10X restriction buffer (see recommendations according to enzyme manufacturer); and 0.5 to 1 μl of *EcoR*I, *Hind*III, *Pst*I or *Bam*HI restriction enzyme (2.5 to 10 units); and enough dH_2O was added to a final volume to 20 μl . The reaction was mixed thoroughly with vortexing and pulse spun to concentrate reaction to the bottom of the tube. The following reaction was incubated at 37°C for 1 to 2 hours and stored on ice, then separated via agarose gel electrophoresis. A 0.7-1.5% agarose gel was prepared by dissolving of agarose in TAE buffer in a 250-ml flask and microwaving for 1 to 2 minutes with occasional stirring in between until the agarose completely dissolved. The percentage of agarose gel is dependent on the resolution desired. Generally, the smaller the DNA fragments and the higher the resolution desired, the higher percentage agarose required. After a couple of minutes of cooling, the dissolved agarose was poured into a casting unit up to a thickness of about 5 mm. Well combs were set and the agarose was allowed to cool for about half an hour. Afterwards, the comb was carefully removed from the gel and carefully set inside the gel box. Enough 1x TAE buffer was poured into the electrophoresis unit until the gel was completely covered with 1-3 mm layer of buffer. Next, 2 μl of 10x loading dye was added and mixed into the each tube containing digested DNA

samples. Each agarose well was carefully loaded with 20 μ l of each sample using care to avoid spilling over into neighboring wells (one well was reserved for a digested marker ladder, usually lambda and 1-kb PCR marker). After loading the samples, the gel box was carefully covered and the electrical terminals of the electrophoresis were plugged into the power supply unit. The terminal closest to the wells must be plugged to the negative (cathode) of the power supply since negatively charged DNA will migrate to the anode. The power supply unit was set to 50-80 V to allow DNA to run for 1 to several hours. The progress of DNA separation was monitored by tracking the migration of the loading dye through the gel. The run was terminated when the tracking dye had migrated to about 1-2 cm away from the end of the gel. The gel was then stained for 30 min in EtBr solution (50 μ l of Ethidium bromide in 500 ml of TAE buffer. Caution: EtBr is a powerful mutagen. Wear gloves when handling.). EtBr intercalates in the grooves of double-stranded DNA and fluoresces in UV light. After 20 minutes of de-staining in a water bath, the gel was visualized with an UV transilluminator and photographed using a digital camera.

DNA sequencing

The 4.0-kb fragment was cloned into pUC19 host vector. The plasmid was sequenced in both directions by primer walking technique. Sequencing was done using the automated sequencing facilities at the Molecular Biology and Biotechnology Instrumentation Facilities at the University of Hawaii. DNA that was sent for sequencing was prepared according to specifications outlined by MBBIF. Primers were initially created from pUC19 vector sequences just outside

of the 4.0-kb fragment. Subsequent primers for areas within the 4.0-kb fragment were made from the ends of already known and sequenced fragments.

Computer analysis of DNA sequences

Homologous protein sequences were analyzed using BlastP (Altschul et al., 1990) from <http://www.ncbi.nlm.nih.gov/BLAST/>. Amino acid residue sequence alignments were carried out using the ClustaW Program from <http://www.molbiol.net/>. Phylogenetic analysis was performed using the TreeTop phylogenetic tree prediction tool from <http://www.molbiol.net/>.

Isolation of Tn3Hogus insertion mutants of TAL1145

Plasmid pJA42 was mutagenized by random insertion of the transposon Tn3Hogus. This 6.62-kb transposon contains both ampicillin and kanamycin resistance markers and a promoterless *gus* gene near the left inverted repeat. The Tn3Hogus insertions in the plasmid pJA42 were made following the same method described for Tn3-HoHo1 mutagenesis (Stachel et al. 1985) except that kanamycin was used for selection of the transposon (Fig. 5.2). Chemically competent *E. coli* strain HB101 pTn3Hogus, pShe was transformed with isolated pJA42 plasmid DNA. It is expected that within the transformed *E. coli* strain, the Tn3Hogus (kan) transposon will randomly integrate and insert itself into pJA42 with the help of the expressed transposase from pShe (Cm). *E. coli* transconjugants with plasmid derivatives containing the transposon insertions or pJA42::Tn3Hogus derivatives, were transferred to *Rhizobium* TAL1145 via tri-parental mating with an *E. coli* helper strain containing a plasmid p2013 since *Rhizobium* cells cannot be made competent. *Rhizobium* TAL1145 containing the

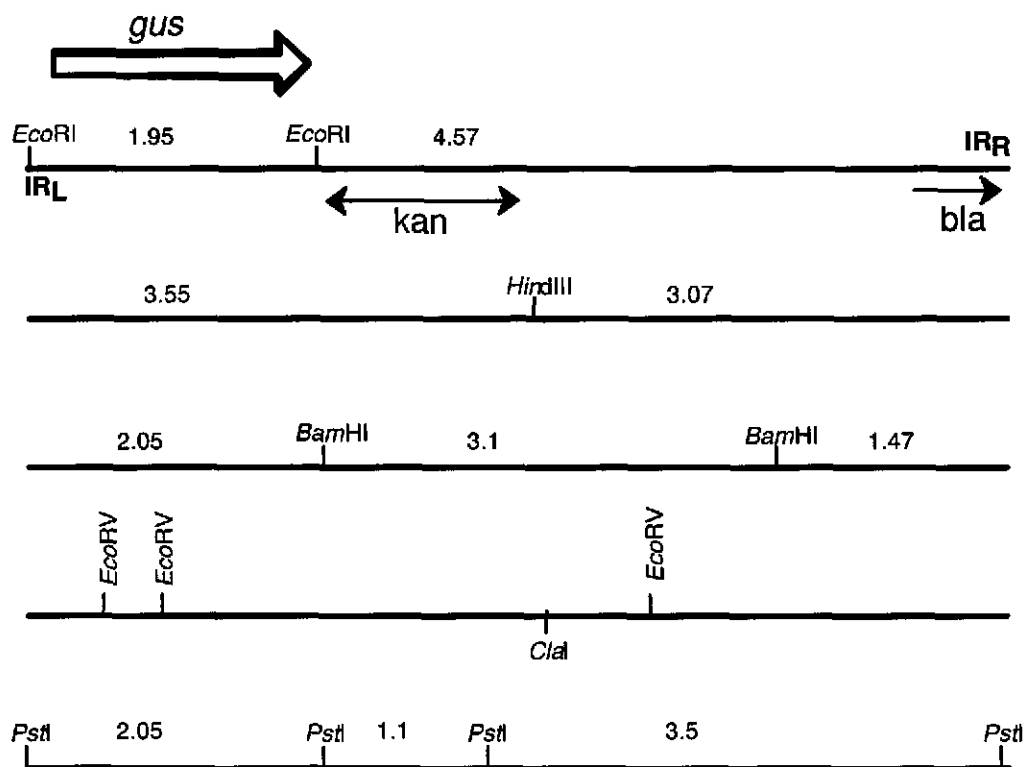


Fig. 5.2. The restriction map of the transposon Tn3Hogus used in this study. This transposon is 6.62-kb in size and it does have a transposase gene. It has a promoterless *gus* gene at the 5' end, which is indicated with an open horizontal arrow. The numbers indicate the size of the restriction fragments. The right and left inverted repeats are indicated as IR_L and IR_R , respectively. It has two antibiotic markers, *kan* and *bla*, which are shown with arrows.

pJA42::Tn3Hogus derivatives were selected by antibiotic screening with kanamycin (conferred by Tn3Hogus), tetracycline (conferred by pJA42), and streptomycin to select for TAL1145. After a few more rounds of screening with GUS and mimosine, the pJA42::Tn3Hogus derivatives were isolated from *Rhizobium* strains and transformed into *E.coli* to generate large quantities of plasmid DNA. The isolated plasmid DNA from these derivatives was digested with *EcoR1* restriction enzymes and mapped. Based on the results of the

restriction mapping, a few pJA42::Tn3Hogus derivatives were found to contain Tn3Hogus insertions in the desired 4.0-kb fragment in pJA42.

The derivatives of pJA42 carrying Tn3Hogus insertions were transferred to the wild-type *Rhizobium* strain TAL1145. The Tn3Hogus insertions in pJA42 were homogenotized to the TAL1145 chromosome by marker exchange using a P1-group incompatible plasmid, pPH1JI (Ruvkun and Ausubel 1981) to finally yield mutants.

Complementation analysis

Restoring the wild type phenotype in mutants by the transfer of cloned fragments of a particular gene confirms the presence of the gene. Therefore, attempts were made to complement the “knocked-out” gene of TAL1145 mutants BS1, BS3, M7 and M8 with whole copies from pJA42. This was accomplished by crossing each of the TAL1145 mutants with *E. coli* strain pJA42 and the helper plasmid pRK2013, as previously described. The transconjugants were identified according to their resistance to tetracycline and kanamycin.

Bacterial growth experiments

The growth characteristics of the *Rhizobium* strains including TAL1145; mutants BS1, BS3, M7 and M8; and their respective complemented transconjugants were determined by growing the strains in YEM medium and in MM containing one of the following three additives: (i) 3 mM mimosine, (ii) 3 mM sodium glutamate and (iii) 1% mannitol and 3 mM ammonium chloride. The mimosine stock solution (40 mg ml⁻¹) was made in 0.4 M NaOH and added to the medium after autoclaving. The pH of the medium was adjusted to 8.4 after

adding mimosine with 1 M HCl. The sodium glutamate, mannitol and ammonium chloride were added before autoclaving and the pH of the medium was adjusted to 6.8 before autoclaving. The growth rates of *Rhizobium* strains were determined by inoculating 25 ml of medium in 250-ml screw-cap bottles with 0.25 ml of *Rhizobium* culture. Cultures were grown at 28° C with shaking, and growth was determined at 10-14 h intervals by measuring the cell density at 600 nm (OD₆₀₀) in a Spectronic 401 spectrophotometer (Spectronic Instruments, Rochester, NY). Each time interval was taken in triplicate and the calculated average cell density was recorded.

Plant experiment

L. leucocephala variety K8 plants were grown for nodulation and nitrogen fixation assays in modified Leonard jar assembly containing nitrogen-free nutrient solution. The modified Leonard jar assembly consisted of two 500-ml plastic containers fitted one on top of the other. A hole was made on the bottom of the top container and a 4-inch cloth wick (from household mop) was inserted through the hole so that solution in the bottom container could be drawn to the planter above. The top planter was filled with horticultural vermiculite and covered with aluminum foil. The entire assembly was autoclaved twice and set aside for planting of sprouted *Leucaena* seedlings.

Seed prep

A handful of *Leucaena* seedlings were prepared by scarifying the seed surface with concentrated HSO₄ for 10 min. The seeds were rinsed several times with sterilized H₂O till free of acid. Undiluted bleach was added to cover

seeds and shaken for 30 min, followed again by several washes of sterilized water. 7-10 volumes of sterilized water were added to cover and imbibe seeds overnight. Swollen floating seeds were removed and again seeds were rinsed several times with sterile H₂O and planted on water agar plates (7.5 g of agar/L of H₂O) and placed in the dark to germinate at room temperature.

N-free nutrient plant solution (Somasegaran and Hoben, 1994)

Per liter

Add 2 ml of each stock solution to 5L of H₂O:

Stock Solution	Chemical	g/liter
1	CaCl ₂ ·2H ₂ O	294.1
2	KH ₂ PO ₄	136.1
3	FeC ₆ H ₅ O ₇ ·3H ₂ O	6.7
	MgSO ₄ ·7H ₂ O	123.3
	K ₂ SO ₄	87.0
	MnSO ₄ ·H ₂ O	0.338
4	H ₃ BO ₃	0.247
	ZnSO ₄ ·7H ₂ O	0.277
	CuSO ₄ ·5H ₂ O	0.100
	CoSO ₄ ·7H ₂ O	0.056
	NaMoO ₂ ·2H ₂ O	0.048

Dilute to 10 liters by adding another 5 liters of water.

Adjust pH to 6.6-6.8 with 1 N NaOH.

Sterilize by autoclaving at 121°C for 30 min.

Five sprouted seedlings (between 3-5 inches in length) were planted in wetted vermiculite in the aforementioned assembly after addition of plant nutrient solution (recipe listed below) to the bottom reservoir and placed under fluorescent lighting set for 12 hrs of illumination until the appearance of first true

leaves. Bacterial inoculum was then prepared by harvesting a plateful of bacteria into 10 ml of MM. Several serial dilutions were made, with the final inoculum concentration at $OD_{600} = 1$. Each plantlet received $\sim 200 \mu\text{l}$ of bacterial inoculum and plants were harvested 7 weeks after inoculation. Vermiculite was carefully removed from the roots of *Leucaena* plants and number of nodules and total fresh weight of nodules from each planter was recorded.

β -Glucuronidase (GUS) activity assay

Fluorometric assay for GUS activity using 4-methylumbelliferyl β -D-glucuronide (MUG) (Sigma) was done according to the modified protocol described by Fox and Borthakur (2001) based on the method of Jefferson *et al.*, (1987). The fluorescent product 7-hydroxy-4-methylcoumarin (MU) is produced through hydrolysis of MUG by GUS. *Rhizobium* cells from a 4-ml overnight culture in TY broth were spun down and resuspended in 4 ml RM medium with or without mimosine and grown for 12 h. The cells were pelleted, washed with 1 M NaCl and resuspended in GUS extraction buffer containing 50 mM Na_2HPO_4 (pH 7.0), 10 mM β -mercaptoethanol, 10 mM EDTA, 0.1% sodium lauryl sarcosine and 0.1% Triton X-100 such that the density of the cell suspension is approximately 1 at 600 nm. Lysozyme was added to a concentration of 1 mg/ml and incubated for 30 min at 37°C . $50\text{-}\mu\text{l}$ aliquots were added to $450\text{-}\mu\text{l}$ volume of MUG assay buffer containing 1 mM of MUG in GUS extraction buffer and incubated at 37°C . $100 \mu\text{l}$ samples were taken after 1 h and the reaction was stopped by adding the sample into $900 \mu\text{l}$ of a buffer containing 0.2 M Na_2CO_3 . Fluorometric measurements were made using a Hitachi F2500 fluorescence

spectrophotometer which was precalibrated with 50, 100, 500, 1000, 3300 nM of MU that corresponded to 140, 312, 1590, 3145 and 9400 fluorescence units, respectively. The wavelength of the spectrophotometer was set to 365 nm for excitation and 455 nm for emission. The fluorescence readings of the samples were automatically converted to nM of MU by the spectrophotometer. The MU concentrations of a few samples were measured at 30 min intervals for 3 h and the enzyme activity was found to be linear. The GUS activity was measured as the amount of MU produced per hour by GUS per ml of cells.

REFERENCES

- Agrios GN.** 1997. *Plant Pathology*. 4th ed. Academic Press, San Diego.
- Antelmann HS, Engelmann S, Schmid R, and Hecker M.** 1996. General and oxidative stress responses in *Bacillus subtilis*: cloning, expression, and mutation of alkyl hydroperoxide reductase operon. *J Bacteriol.* **178**: 6571-6578.
- Awaya J, Fox PM, and Borthakur D.** 2002. Genes encoding a fructose-1,6-bisphosphate aldolase and a fructose-1,6-bisphosphatase are present within the gene cluster for mimosine degradation in *Rhizobium* sp. strain TAL1145. *Plant Soil* (submitted).
- Ayub R, Guis M, Ben Amor M, Gillot L, Roustan JP, Latche A, Bouzayen M, and Pech JC.** 1996. Expression of ACC oxidase antisense gene inhibits ripening of cantaloupe melon fruits. *Nat Biotechnol* **14**:862-6.
- Basat N, Chen L, and Helmann JD.** 1996. Mutation of the *Bacillus subtilis* alkyl hydroperoxide reductase (*ahpCF*) operon reveals compensatory interactions among hydrogen peroxide stress genes. *J. Bacteriol.* **178**: 6579-6586.
- Belimov AA, Safronova VI, and Mimura T.** 2002. Response of spring rape (*Brassica napus* var. *oleifera* L.) to inoculation with plant growth promoting rhizobacteria containing 1-aminocyclopropane-1-carboxylate deaminase depends on nutrient status of the plant. *Can J Microbiol.* **48**:189-199.
- Belimov AA, Safronova VI, Sergeyeva TA, Egorova TN, Matveyeva VA, Tsyganov VE, Borisov AY, Tikhonovich IA, Kluge C, Preisfeld A, Dietz KJ, and Stepanok VV.** 2001. Characterization of plant growth promoting rhizobacteria isolated from polluted soils and containing 1-aminocyclopropane-1-carboxylate deaminase. *Can J Microbiol.* **47**:642-652.
- Beringer JE, Beynon JL, Buchanan-Wollaston AV, Hirsch PR and Johnston AWB.** 1978. Introduction of drug resistance transposon Tn5 into *Rhizobium*. *Nature* **276**: 633-634.
- Blattner FR, Plunkett G 3rd, Bloch CA, Perna NT, Burland V, Riley M, Collado-Vides J, Glasner JD, Rode CK, Mayhew GF, Gregor J, Davis NW, Kirkpatrick HA, Goeden MA, Rose DJ, Mau B, and Shao Y.** 1997. The complete genome sequence of *Escherichia coli* K-12. *Science* **277**:1453-74

- Boehme SA and Lenardo MJ.** 1993. Ligand-induced apoptosis of mature T lymphocytes (propriciodal regulation) occurs at distinct stages of the cell cycle. *Leukemia* **7**: 45-49.
- Borthakur D and Gao X.** 1996. A 150-MDa plasmid in *Rhizobium etli* strain TAL182 contains genes for nodulation competitiveness on *Phaseolus vulgaris* L. *Can J. Microbiol* **42**: 903-910.
- Borthakur D and Soedarjo M.** 1999. Isolation and characterization of a DNA fragment containing genes for mimosine degradation from *Rhizobium* sp strain TAL1145. In Highlights of nitrogen fixation research. Edited by E. Martinez and G Hernández. Kluwer Academic Publishers, New York, pp 91-95.
- Borthakur D, Soedarjo M, Fox PM, and Webb DT.** 2002. The *mid* genes of *Rhizobium* sp. strain TAL1145 are required for degradation of mimosine into 3-hydroxy-4-pyridone and are inducible by mimosine. *Microbiology (in press)*.
- Brewbaker JL, Halliday J, and Lyman J.** 1983. Economically important nitrogen fixing tree species. *Nitrogen Fixing Tree Res.* **1**: 35-40.
- Brewin NJ.** 1991. The development of the legume root nodule. *Annu. Rev. Cell Biol.* **7**:119-226.
- Burd GI, Dixon DG and Glick BR.** 2000. Plant growth promoting bacteria that decrease heavy metal toxicity in plants. *Can. J. Microbiol.* **46**:247-245.
- Campbell BG and Thomson JA.** 1996. 1-Aminocyclopropane-1-carboxylate deaminase genes from *Pseudomonas* strains. *FEMS Microbiol Lett.* **138**: 207-210.
- Chae HZ, Robison K, Poole LB, Church G, Storz G, and Rhee SG.** 1994. Cloning and sequencing of thiol-specific antioxidant from mammalian brain: alkyl hydroperoxide reductase and thiol-specific antioxidant define a large family of antioxidant enzymes. *Proc. Natl. Acad. Sci. USA.* **91**: 7017-7021.
- Chauhan R, Mande SC.** 2001. Characterization of the *Mycobacterium tuberculosis* H37Rv alkyl hydroperoxidase AhpC points to the importance of ionic interactions in oligomerization and activity. *Biochem J.* **354**:209-15.

- Considine PJ, Flynn N, and Patching JW.** 1977. Ethylene production by soil microorganisms. *Appl Environ Microbiol* **33**:977-979.
- Datta C, Basu PS.** 2000. Indole acetic acid production by a *Rhizobium* species from root nodules of a leguminous shrub, *Cajanus cajan*. *Microbiol Res* **155**:123-127.
- Ditta G, Schmidhauser T, Yakobson E, Lu P, Liang XW, Finlay DR and Helinski DR.** 1985. Plasmids related to the broad host range vector pRK290, useful for gene cloning and monitoring gene expression. *Plasmid* **13**:149-153.
- Eubenga MD, Ilag LL, and Mendoza EMT.** 1979. Inhibition of pathogens of field legumes by mimosine. *Philipp. Phytopathol.* **15**:348-349.
- Ferrante AA, Augliera J, Lewis K, and Klibanov AM.** 1995. Cloning of an organic solvent-resistance gene in *Escherichia coli*: the unexpected role of alkyhydroperoxide reductase. *Proc Natl Acad Sci U S A.* **92**:7617-21.
- Fischer HM.** 1994. Genetic regulation of nitrogen fixation in *Rhizobia*. *Microbiol. Rev.* **58**:352-386.
- Fox PM and Borthakur D.** 2001. Selection of several classes of mimosine-degradation-defective Tn3Hogus-insertion mutants of *Rhizobium* sp. strain TAL1145 on the basis of mimosine-inducible GUS activity. *Can J Microbiol.* **47**:488-494.
- George MLC, Young JPW and Borthakur D.** 1994. Genetic characteristics of *Rhizobium* sp. strain TAL1145 that nodulates tree legumes. *Can. J. Microbiol.* **40**:208-215.
- Glick BR, Penrose DM, and Li J.** 1998. A model for lowering the plant ethylene concentrations by plant growth promoting bacteria. *J. Theor. Biol.* **190**:63-68.
- Grichko VP, and Glick BR.** 2000. Identification of DNA sequences that regulate the expression of the *Enterobacter cloacae* UW4 1-aminocyclopropane-1-carboxylic acid deaminase gene. *Can J Microbiol.* **46**:1159-1165.
- Halliwell B and Gutteridge JMC.** 1989. *Free Radicals in Biology and Medicine*, Clarendon Press, Oxford.
- Heilig R.** Unpublished. *Pyrococcus abyssi* genome sequence: insights into archaeal chromosome structure and evolution. *NCBI Microbial Genomes Annotation Project*.

- Holguin G and Glick BR.** 2001. Expression of the ACC Deaminase Gene from *Enterobacter cloacae* UW4 in *Azospirillum brasilense*. *Microb Ecol.* 2001 41:281-288.
- Honma M and Shinomura T.** 1978. Metabolism of enzyme 1-aminocyclopropane-1-carboxylate deaminase. *Agri. Biol. Chem.* 42:1825-1831.
- Jacobson FS, Morgan RW, Christman MF, and Ames BN.** 1989. An alkyl hydroperoxide reductase from *Salmonella typhimurium* involved in the defense of DNA against oxidative damage. Purification and properties. *J Biol Chem.* 264: 1488-1496.
- Jefferson RA, Kavanagh TA and Bevan MW.** 1987. GUS fusions: β -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* 6: 3901-3907.
- Jia YJ, Ito H, Matsui H, and Honma M.** 2000. 1-aminocyclopropane-1-carboxylate (ACC) deaminase induced by ACC synthesized and accumulated in *Penicillium citrinum* intracellular spaces. *Biosci Biotechnol Biochem* 64:299-305.
- Jones RJ.** 1979. The value of *Leucaena leucocephala* as a feed for ruminants in the tropics. *World Animal Rev* 31: 13-23
- Jones RJ, Blunt CG, and Nurnberg BI.** 1978. Toxicity of *Leucaena leucocephala*. The effect of iodine and mineral supplements on penned steers fed a sole diet of *Leucaena*. *Aust Vet J.* 54:387-92.
- Jones RJ and Lowry JB.** 1984. Australian goats detoxify the goitrogen 3-hydroxy-4(1H)pyridone (DHP) after rumen infusions from an Indonesian goat. *Experientia* 40:1435-1436.
- Jones RJ and Megarrity RJ.** 1986. Successful transfer of DHP-degrading bacteria from Hawaiian goats to Australian ruminants to overcome the toxicity of *Leucaena*. *Aust. Vet J.* 60:259-26
- Katoh S, Toyama J, Kodama I, Kamiya K, Akita T, and Abe T.** 1992. Protective action of iron-chelating agents (catechol, mimosine, diferxamine, and Kojic acid) against ischemiareperfusion injury of isolated neonatal rabbit hearts. *Eur Surg. Res.* 24:349-335.
- Kawarabayasi Y, Sawada M, Horikawa H, Haikawa Y, Hino Y, Yamamoto S, Sekine M, Baba S, Kosugi H, Hosoyama A, Nagai Y, Sakai M, Ogura**

- K, Otsuka R, Nakazawa H, Takamiya M, Ohfuku Y, Funahashi T, Tanaka T, Kudoh Y, Yamazaki J, Kushida N, Oguchi A, Aoki K, and Kikuchi H.** 1998. Complete sequence and gene organization of the genome of a hyper-thermophilic archaebacterium, *Pyrococcus horikoshii* OT3. *DNA Res.* **5**:55-76.
- Khanna KK and Lavin MF.** 1993. Ionizing radiation and UV induction of protein by different pathways in ataxia-telangiectasia cells. *Oncogene.* **8**:3307-3312.
- Klee HJ, Hayford MB, Kretzmer KA, Barry GF, and Kishore GM.** 1991. Control of ethylene synthesis by expression of a bacterial enzyme in transgenic tomato plants. *Plant Cell* **3**:1187-1193.
- Lee J, Spector D, Godon C, Labarre J, and Toledano MB.** 1999. A new antioxidant with alkyl hydroperoxide defense properties in yeast. *J Biol Chem.* **274**:4537-44.
- Li J, Ovakim DH, Charles TC, and Glick BR.** 2000. An ACC deaminase minus mutant of *Enterobacter cloacae* UW4 no longer promotes root elongation. *Curr Microbiol.* **4**:101-105.
- Liu C, Tian Y, Shen Q, Jiang H, Ju R, Yan T, Liu C, and Mang K.** 1998. Cloning of 1-aminocyclopropane-1-carboxylate (ACC) synthetase cDNA and the inhibition of fruit ripening by its antisense RNA in transgenic tomato plants. *Chin J Biotechnol* **14**:75-84.
- Loprasert S, Atichartpongkun S, Whangsuk W, and Mongkolsuk S.** 1997. Isolation and analysis of the *Xanthomonas* alkyl hydroperoxide reductase gene and the peroxide sensor regulator genes ahpC and ahpF-oxyR-orfX. *J Bacteriol.* **179**: 3944-3949.
- Ma W, Guinel, FC, and Glick BR.** Unpublished. The effect of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase by *Rhizobium leguminosarum* bv. *viciae*.
- Maeder DL, Weiss RB, Dunn DM, Cherry JL, Gonzalez JM, DiRuggiero J, and Robb FT.** 1999. Divergence of the hyperthermophilic archaea *Pyrococcus furiosus* and *P. horikoshii* inferred from complete genomic sequences. *Genetics.* **152**:1299-305.
- Matthews A and Rai VP.** 1985. Mimosine content of *Leucaena leucocephala* and sensitivity of rhizobium to mimosine. *J. Plant Physiol.* **117**:3:377-382.

- McClelland M, Sanderson KE, Spieth J, Clifton SW, Latreille P, Courtney L, Porwollik S, Ali J, Dante M, Du F, Hou S, Layman D, Leonard S, Nguyen C, Scott K, Holmes A, Grewal N, Mulvaney E, Ryan E, Sun H, Florea L, Miller W, Stoneking T, Nhan M, Waterston R, and Wilson RK.** 2001. Complete genome sequence of *Salmonella enterica* serovar *typhimurium* LT2. *Nature*. **413**: 852-856
- Mehnaz S, Mirza MS, Haurat J, Bally R, Normand P, Bano A, and Malik KA.** 2001. Isolation and 16S rRNA sequence analysis of the beneficial bacteria from the rhizosphere of rice. *Can J Microbiol* **47**:110-117.
- Moawad H and Bohlool B.** 1984. Competition among *Rhizobium* spp. For nodulation of *Leucaena leucocephala* in two tropical soils. *Appl. Environ. Microbiol.* **48**:5-9.
- Monib M, Abd-el-Malek Y, Hosny I, and Fayez M.** 1979. Associative symbiosis of *Azotobacter chroococcum* and higher plants. *Zentralbl Bakteriol Naturwiss* **134**:133-139.
- Mylona P, Pawlowski K, and Bisseling T.** 1995. Symbiotic nitrogen fixation. *The Plant Cell*. **7**:869-885.
- Nagahama K, Yoshino K, Matsuoka M, Sato M, Tanase S, Ogawa T, and Fukuda H.** 1994. Ethylene production by strains of the plant-pathogenic bacterium *Pseudomonas syringae* depends upon the presence of indigenous plasmids carrying homologous genes for the ethylene-forming enzyme. *Microbiology* **140**:2309-2313.
- Niimura Y, Poole LB, and Massey V.** 1995 *Amphibacillus xylanus* NADH oxidase and *Salmonella typhimurium* alkyl-hydroperoxide reductase flavoprotein components show extremely high scavenging activity for both alkyl hydroperoxide and hydrogen peroxide in the presence of *S. typhimurium* alkyl-hydroperoxide reductase 22-kDa protein component. *J. Biol. Chem.* **270**, 25645-25650.
- National Academy of Sciences.** 1977.
- NCBI Microbial Genomes Annotation Project.** Residues 1 to 95851 of *Burkholderia fungorum*. Submitted (18-SEP-2002) National Center for Biotechnology Information, NIH, Bethesda, MD 20894, USA.
- Nelson KE, Clayton RA, Gill SR, Gwinn ML, Dodson RJ, Haft DH, Hickey EK, Peterson JD, Nelson WC, Ketchum KA, McDonald L, Utterback TR, Malek JA, Linher KD, Garrett MM, Stewart AM, Cotton MD, Pratt MS, Phillips CA, Richardson D, Heidelberg J, Sutton GG, Fleischmann**

- RD, Eisen JA, White O, Salzberg SL, Smith HO, Venter JC, & Fraser CM.** 1999. Evidence for lateral gene transfer between Archaea and bacteria from genome sequence of *Thermotoga maritima*. *Nature*.399:323-329.
- Nierman WC, Feldblyum TV, Laub MT, Paulsen IT, Nelson KE, Eisen JA, Heidelberg JF, Alley MR, Ohta N, Maddock JR, Potocka I, Nelson WC, Newton A, Stephens C, Phadke ND, Ely B, DeBoy RT, Dodson RJ, Durkin AS, Gwinn ML, Haft DH, Kolonay JF, Smit J, Craven MB, Khouri H, Shetty J, Berry K, Utterback T, Tran K, Wolf A, Vamathevan J, Ermolaeva M, White O, Salzberg SL, Venter JC, Shapiro L, Fraser CM, and Eisen J.** 2001. Complete genome sequence of *Caulobacter crescentus*. *Proc Natl Acad Sci U S A*. 98:4136-4141.
- Parkhill J, Wren BW, Thomson NR, Titball RW, Holden MT, Prentice MB, Sebahia M, James KD, Churcher C, Mungall KL, Baker S, Basham D, Bentley SD, Brooks K, Cerdeno-Tarraga AM, Chillingworth T, Cronin A, Davies RM, Davis P, Dougan G, Feltwell T, Hamlin N, Holroyd S, Jagels K, Karlyshev AV, Leather S, Moule S, Oyston PC, Quail M, Rutherford K, Simmonds M, Skelton J, Stevens K, Whitehead S, and Barrell BG.** 2001. Genome sequence of *Yersinia pestis*, the causative agent of plague. *Nature*. 413:523-7
- Patnaik GK and Rao VR.** 1994. Influence of ammonium and 2,4-dichlorophenoxy acetic acid on nitrogenase activity in *Azospirillum* species from rice rhizosphere. *Microbios* 80:17-22.
- Patten CL and Glick BR.** 2002. Role of *Pseudomonas putida* indole-acetic acid in development of the host plant root system. *Appl Environ Microbiol*. 68: 3795-3801.
- Penrose DM, Moffat BA, and Glick BR.** 2001. Determination of 1-aminocyclopropane-1-carboxylic acid (ACC) to assess the effects of AA deaminase-containing bacteria on the roots of canola seedlings. *Can. J. Microbiol*. 47:77-80.
- Perna NT, Plunkett G 3rd, Burland V, Mau B, Glasner JD, Rose DJ, Mayhew GF, Evans PS, Gregor J, Kirkpatrick HA, Posfai G, Hackett J, Klink S, Boutin A, Shao Y, Miller L, Grotbeck EJ, Davis NW, Lim A, Dimalanta ET, Potamousis KD, Apodaca J, Anantharaman TS, Lin J, Yen G, Schwartz DC, Welch RA, and Blattner FR.** 2001. Genome sequence of enterohaemorrhagic *Escherichia coli* O157:H7. *Nature*, 409:529-33.

- Poole LB.** 1996. Flavin-dependent alkyl hydroperoxide reductase from *Salmonella typhimurium*. 2. Cystine disulfides involved in catalysis of peroxide reduction. *Biochemistry*. **35**: 65-75.
- Poole LB and Ellis HR.** 1996. Flavin-dependent alkyl hydroperoxide reductase from *Salmonella typhimurium*. 1. Purification and enzymatic activities of overexpressed AhpF and AhpC proteins. *Biochemistry*. **35**:56-64.
- Poole LB, Godzik A, Nayeem A, and Schmitt JD.** 2000. AhpF can be dissected into two functional units: tandem repeats of two thioredoxin-like folds in the N-terminus mediate electron transfer from the thioredoxin reductase-like C-terminus to AhpC. *Biochemistry*. **39**: 6602-6615.
- Pueppke SG.** 1996. The genetic and biochemical basis for nodulation of legumes by rhizobia. *Crit Rev. in Biotech.* **16**:1-51.
- Raven PH and Evert RF.** 1981. *Biology of Plants*. 3rd ed. Woolworth Publishers, Inc. New York, New York.
- Read TD, Salzberg SL, Pop M, Shumway M, Umayam L, Jiang L, Holtzapple E, Busch JD, Smith, KL, Schupp JM, Solomon D, Keim P, and Fraser CM.** 2002. Comparative genome sequencing for discovery of novel polymorphisms in *Bacillus anthracis*. *Science*. **296**:2028-2033.
- Renz J.** 1936. Über das Mimosine *Z. Phys. Chem.* **244**:153-158.
- Ruvkun, GB & Ausubel FM.** 1981. A general method for site-directed mutagenesis in prokaryotes. *Nature* **289**, 85-89.
- Salanoubat M, Genin S, Artiguenave F, Gouzy J, Mangenot S, Arlat M, Billault A, Brottier P, Camus JC, Cattolico L, Chandler M, Choisine N, Claudel-Renard C, Cunnac S, Demange N, Gaspin C, Lavie M, Moisan A, Robert C, Saurin W, Schiex T, Siguier P, Thebault P, Whalen M, Wincker P, Levy M, Weissenbach J, and Boucher CA.** 2002. Genome sequence of the plant pathogen *Ralstonia solanacearum*. *Nature*. **415**:497-502.
- Schlöter M, Wiehe W, Assmus B, Steindl H, Becke H, Hofflich G, and Hartmann A.** 1997. Root colonization of different plants by plant-growth-promoting *Rhizobium leguminosarum* bv. *trifolii* R39 studied with monospecific polyclonal antisera. *Appl Environ Microbiol* **63**:2038-2046.
- Sies H.** 1993 Strategies of antioxidant defense. *Eur. J. Biochem.* **215**:213-219.
- Serrano EP, Ilag LL, and Mendoza EMT.** 1983. Biochemical mechanisms of mimosine toxicity to *Sclerotium rolfsii* Sacc. *Aust. J Biol. Sci.* **36**:445-454.

- Shah S, Li J, Moffatt BA, and Glick BR.** 1998. Isolation and characterization of ACC deaminase genes from two different plant growth-promoting rhizobacteria. *Can J Microbiol.* **44**: 833-43.
- Sheehy RE, Honma M, Yamada M, Sasaki T, Martineau B, and Hiatt WR.** 1991. Isolation, sequence, and expression in *Escherichia coli* of the *Pseudomonas* sp. strain ACP gene encoding 1-minocyclopropane-1-carboxylate deaminase. *J Bacteriol.* **173**:5260-5265.
- Skvortsov IM and Ignatov VV.** 1998. Extracellular polysaccharides and polysaccharide-containing biopolymers from *Azospirillum* species: properties and the possible role in interaction with plant roots. *FEMS Microbiol Lett.* **165**:223-9.
- Soedarjo M and Borthakur D.** 1996. Mimosine produced by the tree-legume *Leucaena* provides a growth advantages to some *Rhizobium* strains that utilize it as a source of carbon and nitrogen. *Plant Soil.* **186**: 87-92.
- Soedarjo M and Borthakur D.** 1996. Simple procedures to remove mimosine from young leaves, pods and seeds of *Leucaena leucocephala* used as food. *Intl J Food Sci and Tech.* **31**:91-103.
- Soedarjo M and Borthakur D.** 1998. Mimosine produced by the tree-legume *Leucaena* provides a nodulation competition advantage to mimosine-degrading *Rhizobium* strains. *Soil Biol. Biochem* **30**: 1605-1613.
- Soedarjo M, Henscheidt TK, and Borthakur D.** 1994. Mimosine, a toxin present in leguminous trees (*Leucaena* spp.) induces a mimosine-degrading enzyme activity in some *Rhizobium* strains. *Appl Environ Microbiol* **60**: 4268-4272.
- Stachel SE, An G, Flores C, and Nester EW.** 1985. A *Tn3lacZ* transposon for the random generation of β -galactocidase gene fusions: application to the analysis of gene expression in *Agrobacterium*. *EMBO J* **4**: 891-898.
- Steenhoudt O and Vanderleyden J.** 2000. *Azospirillum*, a free-living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. *FEMS Microbiol Rev* **24**:487-506.
- Storz G, Jacobson FS, Tartaglia LA, Morgan RW, Silveira LA, and Ames BN.** 1989. An alkyl hydroperoxide reductase induced by oxidative stress in *Salmonella typhimurium* and *Escherichia coli*: genetic characterization and cloning of ahp. *J Bacteriol.* **171**: 2049-2055.

- Sullivan JT, Trzebiatowski JR, Cruickshank RW, Gouzy J, Brown SD, Elliot RM, Fleetwood DJ, McCallum NG, Rossbach U, Stuart GS, Weaver JE, Webby RJ, De Bruijn FJ, and Ronson CW.** 2002 Comparative sequence analysis of the symbiosis island of *Mesorhizobium loti* strain R7A. *J Bacteriol* **184**:3086-3095.
- Tartaglia LA, Storz G, Brodsky MH, Lai A, and Ames BN.** 1990. Alkyl hydroperoxide reductase from *Salmonella typhimurium*. Sequence and homology to thioredoxin reductase and other flavoprotein disulfide oxidoreductases. *J Biol Chem.* **265**: 10535-10540.
- Ueda T Suga Y, Yahiro N, and Matsuguchi T.** 1995. Remarkable N₂-fixing bacterial diversity detected in rice roots by molecular evolutionary analysis of *nifH* gene sequences. *J. Bacteriol* **177**:1414-1417.
- Walsh C, Pascal RA Jr, Johnston M, Raines R, Dikshit D, Krantz A, and Honma M.** 1981. Mechanistic studies on the pyridoxal phosphate enzyme 1-aminocyclopropane-1-carboxylate deaminase from *Pseudomonas* sp. *Biochemistry.* **20**:7509-519.
- Vincent JM.** 1970. A manual for the practical study of root nodule bacteria. IBP handbook no. 15. Blackwell Scientific Publications, Oxford.
- Wardman P.** 1988. in *Glutathione Conjugation*, eds. Sies H and Ketterer B. Academic, New York, pp. 43-72.
- Wood V, Gwilliam R, Rajandream MA, Lyne M, Lyne R, Stewart A, Sgouros J, et al.** 2002. The genome sequence of *Schizosaccharomyces pombe*. *Nature.* 2002. **15**:871-80.
- Yanni YG, Rizk RY, Corich V, Squartini A, Ninke K, Philip-Hollingsworth S, Orgambide G, De Bruijn F, Stoltzfus J, Buckley D, Schmidt TM, Mateos PF, Ladha JK, and Dazzo FB.** 1997. Natural endophytic association between *Rhizobium leguminosarum* bv. *trifolii* and rice roots and assessment of its potential to promote rice growth. *Plant Soil* **194**:99-114.
- Yao M, Ose T, Sugimoto H, Horiuchi A, Nakagawa A, Wakatsuki S, Yokoi D, Murakami T, Honma M, and Tanaka I.** 2000. Crystal structure of 1-aminocyclopropane-1-carboxylate deaminase from *Hansenula saturnus*. *J Biol Chem.* **275**:34557-34565.