1-AMINOCYCLOPROPANE-1-CARBOXYLATE (ACC) DEAMINASE GENES IN RHIZOBIA: ISOLATION AND CHARACTERIZATION

by

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I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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ABSTRACT

A collection of 233 putative Rhizobia strains from 30 different sites across Saskatchewan, Canada was assayed for ACC deaminase activity, with 27 of the strains displaying activity. When all 27 strains were characterized based on 16S rRNA gene sequences, it was noted that 26 strains are *Rhizobium leguminosarum* and one strain is *Rhizobium gallicum*. PCR was used to rapidly isolate ACC deaminase structural genes from the above mentioned 27 strains; 17 of them have 99% identities when compared with the previously characterized ACC deaminase structural gene (*acdS*) from *Rhizobium leguminosarum* bv. *viciae* 128C53K, whereas the other 10 strains are 83% identical compared to the *acdS* of *R. leguminosarum* bv. *viciae* 128C53K. Southern hybridization showed that each strain has only one ACC deaminase gene. Using inverse PCR, the region upstream of the ACC deaminase structural genes was characterized for all 17 strains and shown to encode a leucine responsive regulatory protein. The results are discussed in the context of a previously proposed model for the regulation of bacterial ACC deaminase and facilitates an elaboration of the role of ACC deaminase in nodulation and nitrogen fixation.

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GLOSSARY

ACC	1-aminocyclopropane-1-carboxylic acid
acdS	The ACC deaminase structural gene
acdR	The ACC deaminase regulatory gene
Amp	ampicillin
AOA	aminooxyacetic acid
ATP	adenosine triphosphate
AVG	aminoethoxy-vinylglycine
CRP	cAMP receptor protein
DIG	digoxigenin
FNR	fumarate and nitrate reduction protein
GACC	1-(γ-L-glutamylamino) cyclopropane-1-carboxylic acid
HGT	horizontal gene transfer
Km	kanamycin
Lrp	Leucine responsive regulatory protein
MTA	5'-methylthioadenosine
PGPR	Plant growth-promoting bacteria
SAM	S-adenosyl-L-methionine
Sm	streptomycin
Tc	tetracycline

1 INTRODUCTION

In the symbiotic nitrogen-fixation process, Rhizobia convert atmospheric dinitrogen (N_2) to ammonia (NH_3), which can be effectively utilized by host legume plants. Ethylene, a plant hormone that functions as a regulator of many aspects of a plant's life, inhibits the infection and nodulation by Rhizobia of most legumes (Grobbelaar et al. 1971; Lee and LaRue 1992; Hirsch and Fang 1994; Penmetsa and Cook 1997). Recent studies suggest two different strategies used by Rhizobia to reduce the amount of ethylene synthesized by their legume symbionts. One strategy utilizes the compound through rhizobitoxine which acts to inhibit the enzyme ACC synthase and hence ethylene biosynthesis (Duodu et al. 1999; Yuhashi et al. 2000; Parker and Peters 2001). In addition, the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase which catalyzes the cleavage of ACC to α-ketobutyrate and ammonia decreases ethylene levels in host roots and thereby enhances nodulation (Ma et al. 2003a; Ma et al. 2004).

1.1 Plant Growth Promoting Bacteria

In soil, plant growth is influenced by a number of abiotic and biotic factors. The region around the root is relatively rich in nutrients because as much as 40% of plant photosynthates are lost from the plant roots (Nelson 2004). These nutrients attract bacteria and fungi, which multiply in the rhizosphere to densities up to and exceeding 100 times those in the bulk soil (Lynch and Whipps 1991; VanLoon and Glick 2004). The microorganisms may benefit or inhibit plant growth and it has been first defined by Kloepper and Schroth (1978) that "bacteria that colonize the roots of plants following inoculation onto seed and that enhance plant growth" are plant growth promoting rhizobacteria (PGPR) (Zahir et al. 2004; Nelson 2004). There are two types of bacteria that are beneficial to plants, one that can "form a symbiotic relationship with the plant,

which involves formation of specialized structures or nodules on host plant roots" (Glick 2005), and bacteria that are free-living and interact with the roots in the soil (Glick 1995; Zahir et al. 2004; Glick 2005).

PGPR can promote plant growth and development by indirect or direct means (Glick 1995; Glick et al. 1999; Nelson 2004). "Direct mechanisms can be demonstrated in the absence of plant pathogens or other rhizosphere microorganisms, while indirect promotion of plant growth involves these bacteria reducing the deleterious effects of plant pathogens" (Nelson 2004). There are several ways in which plant growth-promoting bacteria can directly enhance plant growth and development (Glick 1995). For example, they can fix atmospheric nitrogen that is transferred to the plant; produce siderophores that chelate iron and make it available to the plant root; solubilize minerals such as phosphorus; produce phytohormones; and synthesize some less well characterized low molecular mass compounds or enzymes that can modulate plant growth and development (Glick 1995; Brown 1974; Kloepper et al. 1989; Glick 2005).

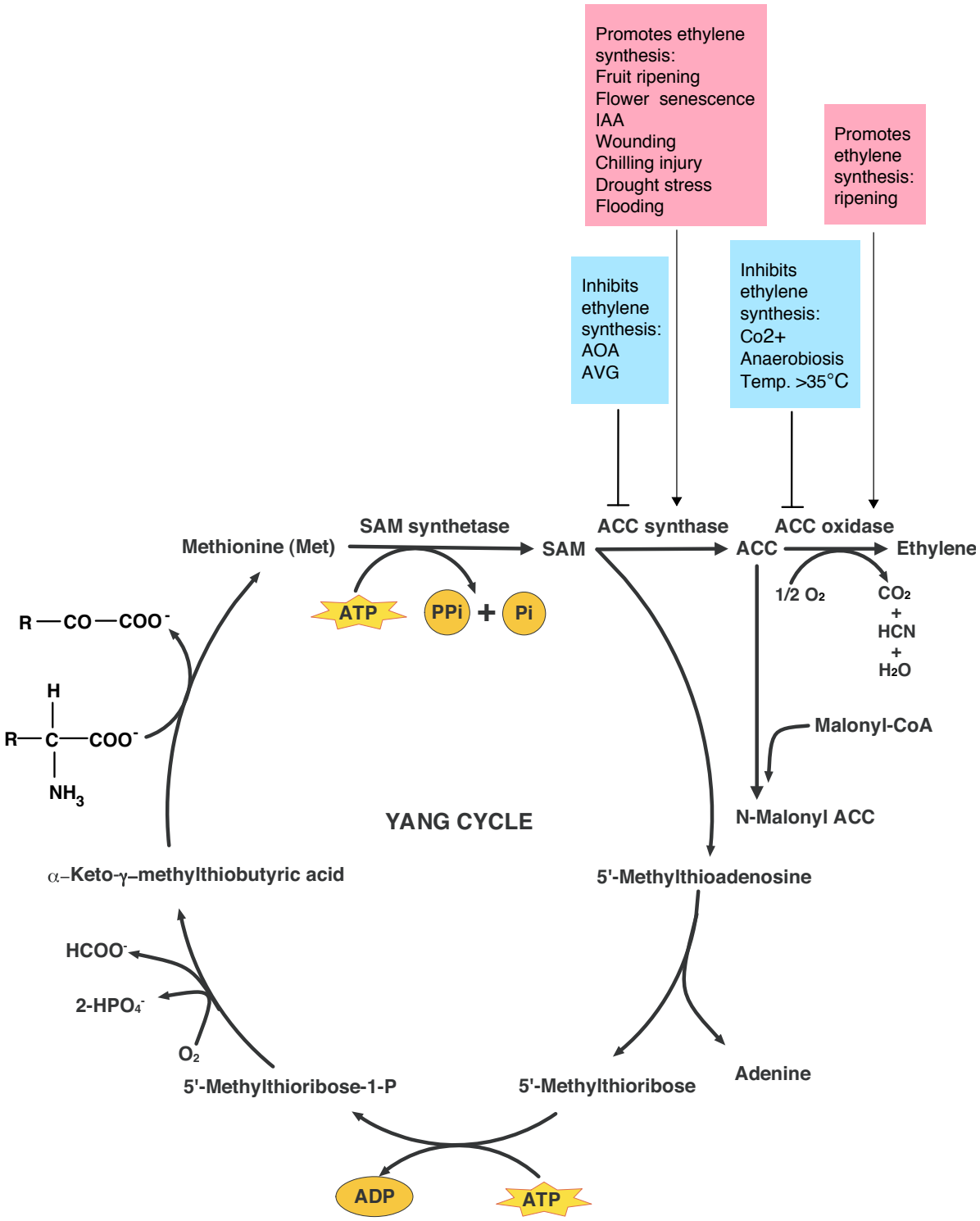
To commercialize PGPR, "effective strategies for initial selection and screening of rhizobacterial isolates are required" (Nelson 2004). One of the key mechanisms that are responsible for many of the direct effects of plant growth-promoting bacteria on plants is the production of phytohormones, including auxins such as indoleacetic acid (Brown 1974; Patten and Glick 1996; Patten and Glick 2002). In addition, a number of plant growth-promoting bacteria contain the enzyme ACC deaminase and this enzyme can cleave ACC, which is the immediate precursor of ethylene in plants, to α-ketobutyrate and ammonia and thereby lower the level of ethylene in a developing or stressed plant (Glick 1995; Jacobson et al. 1994; Glick et al. 1998; Glick 2005).

1.2 Ethylene

The gaseous plant hormone ethylene has been recognized as being involved in a wide range of plant responses and developmental steps including seed germination, tissue differentiation, formation of root and shoot primordial, root elongation, lateral bud development, flowering initiation, anthocyanin synthesis, flower opening and senescence, fruit ripening and degreening, production of volatile organic compounds responsible for aroma formation in fruits, storage product hydrolysis, leaf and fruit abscission and the response of plants to biotic and abiotic stresses (Mattoo and Suttle 1991; Abeles et al. 1992; Frankenberger and Arshad 1995; Spaink 1997).

In higher plants ethylene is produced from L-methionine via the intermediates, S-adenosyl-L-methionine (SAM) and 1-aminocyclopropane-1-carboxylic acid (ACC) (Yang and Hoffman 1984). In vivo experiments showed that plant tissues convert 1-[^{14}C]methionine to [^{14}C]ethylene, and that the ethylene is derived from carbons 3 and 4 of methionine (Fig 1-1) SAM, which is synthesized from methionine and adenosine triphosphate (ATP), is an intermediate in the ethylene biosynthetic pathway, and the immediate precursor of ethylene is 1-aminocyclopropane-1-carboxylic acid (ACC) (Taiz and Zeiger 2006). In general, when ACC is supplied exogenously to plant tissues, ethylene production increases substantially. This observation indicates that the synthesis of ACC is usually the biosynthetic step that limits ethylene production in plant tissues. The enzymes involved in this metabolic sequence are SAM synthetase, which catalyzes the conversion of methionine to SAM (Giovanelli et al. 1980); ACC synthase, which is responsible for the hydrolysis of SAM to ACC and 5'- methylthioadenosine (MTA) (Kende 1989), and ACC oxidase which metabolizes ACC to ethylene, carbon dioxide and cyanide (John 1991).

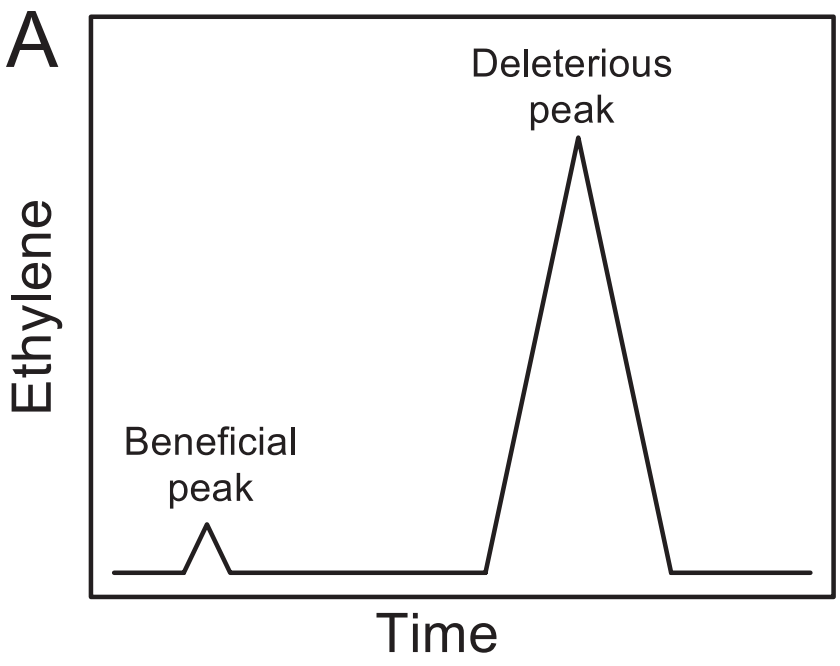
Figure 1-1 Ethylene biosynthetic pathway and the Yang cycle (Taiz and Zeiger 2006, Figure 22.1). ACC=1-aminocyclopropane-1-carboxylic acid; SAM=*S*-Adenosyl-methionine AOA=aminooxyacetic acid; AVG=aminoethoxy-vinylglycine.

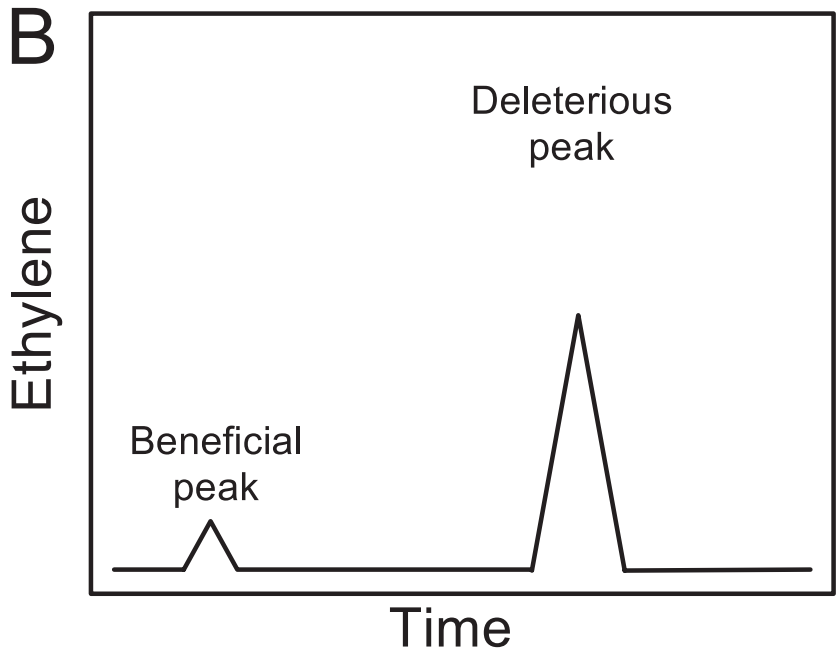

Promotes ethylene synthesis:
Fruit ripening
Flower senescence
IAA
Wounding
Chilling injury
Drought stress
Flooding
Promotes ethylene synthesis: ripening
Inhibits ethylene synthesis:
AOA
AVG
Inhibits ethylene synthesis:
Co2+
Anaerobiosis
Temp. >35°C
Methionine (Met)
SAM synthetase
SAM
ACC synthase
ACC
ACC oxidase
Ethylene
ATP
PPi
Pi
1/2 O2
CO2 + HCN + H2O
Malonyl-CoA
N-Malonyl ACC
YANG CYCLE
α–Keto-γ–methylthiobutyric acid
5'-Methylthioadenosine
HCOO-
2-HPO4-
O2
5'-Methylthioribose-1-P
5'-Methylthioribose
Adenine
ADP
ATP

Not all the ACC found in the tissue is converted to ethylene. Under some conditions ACC can also be converted to a conjugated form, N-malonyl ACC (Amrhein et al. 1981; Martin and Saftner 1995) by ACC N-malonyltransferase or into 1-(γ-L-glutamylamino) cyclopropane-1-carboxylic acid (GACC) (Martin et al. 1995). The conjugation of ACC may play an important role in the control of ethylene biosynthesis, in a manner analogous to the conjugation of auxin and cytokinin (Philosoph-Hadas et al. 1985; Finlayson et al. 1991; Machackova et al. 1997).

Ethylene has long been recognized as a hormone that controls plant responses under growth-limiting conditions or stress (Abeles et al. 1992; Morgan and Drew 1997). “There is a complicated web of interactions between ethylene and other plant hormones that varies somewhat from one plant to another, therefore it is difficult to explain the function of stress ethylene in one simple model” (Glick et al. 2007). It has been proposed that, ethylene, in response to environmental stresses, is produced in two peaks (Stearns and Glick 2003; Pierik et al. 2006). The first peak is small and usually occurs a few hours after the stress. It is thought to “function as a signal to turn on transcription of genes that encode proteins that help to protect the plant against the pathogen” (VanLoon and Glick 2004; VanLoon et al. 1997; Glick 2004). The second peak is much larger and generally occurs one to three days after the stress (Fig 1-2A). The second ethylene peak is often “concomitant with the appearance of visible damage to the plant, such as senescence, chlorosis and abscission” (Glick 2007). Therefore, after a severe infection by pathogens, “a large portion of the damage that occurs to a plant is due to autocatalytic ethylene synthesis and not from direct pathogen action” (VanLoon 1984; Glick 2007). At this point, “the inhibitors of ethylene synthesis or ethylene action can significantly decrease the severity of a fungal or bacterial infection” (Fig 1-2B) (Glick 2007).

Figure 1-2 Plant ethylene production as a function of time following an environmental stress (Glick 2007). A. There is an initial small peak of ethylene at the onset of the stress and then a second much larger peak comes later. B. By using ACC deaminase-containing plant growth-promoting bacteria, a selective decrease in the second but not the first ethylene peak may be achieved.


A
Deleterious
peak
Ethylene
Beneficial
peak
Time


B
Deleterious
peak
Ethylene
Beneficial
peak
Time

1.3 Nodulation of legumes by rhizobia

Nitrogen-fixing root nodules develop as a consequence of a series of interactions between Rhizobia and leguminous host plants (Fisher and Long 1992). The whole nodulation process is highly regulated by complex chemical communications between the plant and the bacteria. The plant signals, flavonoids excreted by the roots, activate the expression of nodulation genes in Rhizobia, produce rhizobial lipochitooligosaccharide signals called Nod factors (Perret et al. 2000). These molecules stimulate the legume root hairs to curl. Rhizobia then invade the root through the root hair tip where they induce the formation of an infection thread (a tube-like structure growing from the root hair tip toward the root cell body) through which the Rhizobia enter, travel down a root hair, and are thereafter released into the dividing cortical cells of legume roots. Rhizobial cells released in the plant cells differentiate into their symbiotic form, nitrogen-fixing bacteroids.

There are several types of legume nodules and most legume species, including *Medicago truncatula*, develop indeterminate nodules. In indeterminate nodules, there are different zones: the distal meristem that delivers new cells to the infection zone where bacteria are internalized, an interzone with amyloplast accumulation and differentiation of bacteroids, a fixation zone with plant cells and a senescent zone (Vasse et al. 1990; Pawlowski and Bisseling 1996; Timmers et al. 1999; Jeroen et al. 2006). Another type of nodule is determinate which is typically found in *Lotus japonicus* and a number of tropical legumes. Determinate nodules have a typical round shape that derived from the cessation of meristem activity after nodule initiation and growth of the nodule mainly by cell expansion (Sprent 2002; Jeroen et al. 2006).

Although legumes form root nodules mainly in response to Nod factors, it has been thought that the plant's perception of endogenous signals, particularly plant hormones, is also important

for the establishment of proper symbiotic interactions between Rhizobia and legumes (Caetano-Anolles and Gresshoff 1991).

1.4 Effect of ethylene on nodulation

It has been known for more than a decade now that ethylene inhibits nodulation in various legumes (Hirsch and Fang 1994). For example, ethylene has been shown to inhibit nodule development in *Medicago sativa* (Peters and Crist-Estes 1989), *Pisum sativum* (Goodlass and Smith 1979; Guinel and Sloetjes 2000; Lee and LaRue 1992; Guinel and Geil 2002) and *Trifolium repens* (Goodlass and Smith 1979) that forms indeterminate nodules. A *Medicago truncatula* hypernodulating mutant, *sickle*, has been demonstrated to be ethylene insensitive (Penmetsa and Cook 1997). On the other hand, determinate nodulators showed inconsistent ethylene effect on nodulation and it is species dependent. For example, ethylene inhibited nodulation in *Phaseolus vulgaris* (Grobbelaar et al. 1971), *Lotus japonicus* and *Macroptilium atropurpureum* (Nukui et al. 2000), but it didn't alter nodulation in *Glycine max* (Hunter 1993; Schmidt et al 1999; Suganuma et al. 1995).

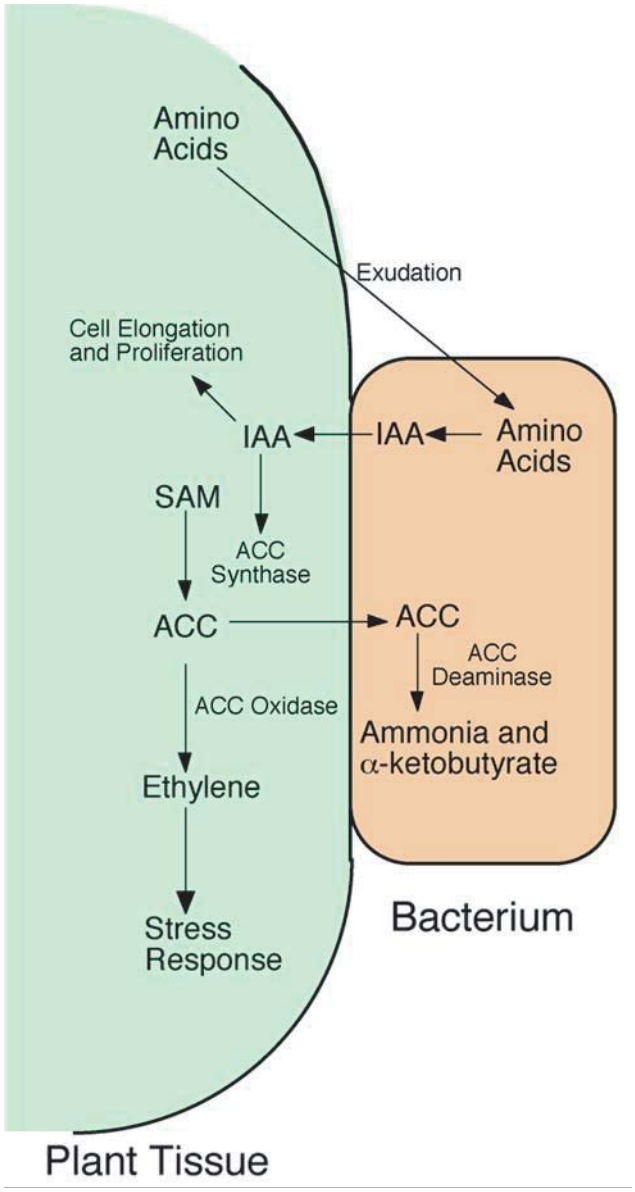
The mechanism of how ethylene controls nodulation is not fully known. Ethylene does not appear to inhibit root colonization because neither the ethylene inhibitors aminoethox increases the number of rhizobial infections with a low-nodulating pea mutant, *brz*, which has a third less rhizobial infections (Guinel and LaRue 1992) than its parent "Sparkle". There is evidence suggesting that exogenous ethylene blocks the invasion of the infection thread in the root cortex of the plant host (Spaink 1997). Because legume root nodulation is dependent on the density of Rhizobia in the root environment (Francoise and Schmidt 1982; Kucey and Hynes 1989; Weaver and Frederick 1974), it is possible that ethylene controls nodulation by limiting rhizobial

proliferation (Tamimi and Timko 2003). Oldroyd et al. (2001) and Tamimi (2003) suggested that ethylene modulates an early step in the Nod factor signal transduction pathway, which indicates that the effect of ethylene on nodulation is not limited to the host plant but also may involve the bacterial symbiont.

1.5 ACC Deaminase

In 1978, an enzyme capable of hydrolyzing ACC, the immediate precursor of ethylene was isolated from *Pseudomonas* sp. strain ACP and from the yeast, *Hansenula saturnus* (Honma and Shimomura 1978; Minami et al. 1998). Since then, ACC deaminase (EC: 4.1.99.4) has been detected in the fungus, *Penicillium citrinum* (Honma 1993), and in a number of other bacterial strains (Klee et al. 1991; Sheehy et al. 1991; Jacobson et al 1994; Glick et al 1995; Campbell and Thomson 1996; Burd et al 1998; Minami et al 1998; Jia et al. 1999; Belimov et al. 2001; Mayak et al. 2001; Babalola et al. 2003; Ghosh et al. 2003; Ma et al. 2003b; Dey et al. 2004; Uchiumi et al. 2004; Belimov et al. 2005; Hontzeas et al. 2005; Blaha et al. 2006; Madhaiyan et al. 2006) including *Rhizobium leguminosarum* bv. *viciae* 128C53, 128C53K, 128C53G and 99A1; *Rhizobium hedysari* (Ma et al. 2003b). Moreover, an ACC deaminase structural gene (*acdS*) was recently found in *Azopirillum* (previously reported as *acdS*$^-$), in 10 species of *Burkholderia* and 6 *Burkholderia cepacia* genomovars (which include PGPR, phytopathogens and opportunistic human pathogens), and in five *Agrobacterium* genomovars (Blaha 2006). Many of these microorganisms were identified by their ability to grow on minimal medium containing ACC as its sole nitrogen source (Honma and Shimomura 1978; Klee et al. 1991; Honma 1993; Jacobson et al. 1994; Glick et al. 1995; Campbell and Thomson 1996; Burd et al. 1998; Belimov et al. 2001; Ma et al. 2003b).

Figure 1-3 A model describing the role of bacterial ACC deaminase in the promotion of plant root elongation.


Amino
Acids
Exudation
Cell Elongation
and Proliferation
IAA
IAA
Amino
Acids
SAM
ACC
Synthase
ACC
ACC
ACC
Deaminase
ACC Oxidase
Ammonia and
α-ketobutyrate
Ethylene
Stress
Response
Bacterium
Plant Tissue

A model describing the role of ACC deaminase in plant growth-promoting rhizobacteria was suggested by Glick et al. (1998) (Fig. 1-3). Briefly, ACC is exuded from plant roots or seeds. Rhizobacteria, attached to the surface of the seeds or roots, can take up some of this ACC and hydrolyze it by the activity of ACC deaminase. The amount of ACC outside the root is thus reduced. To maintain the equilibrium between internal and external ACC levels, more ACC is exuded from seeds or roots, resulting in a reduction in the amount of ACC available for oxidation to ethylene inside the cell. By decreasing the level of ACC inside the plant cell, less ethylene will be synthesized, and its inhibitory effect on root elongation will be reduced.

Since ACC deaminase is usually present in bacteria at a low level before it is induced and the induction of enzyme activity is a slow and complex process, right after environmental stresses, the pool of ACC in the plant is low. Following the induction of ACC oxidase in the plant, the first small peak of ethylene, which will induce a defensive response in the plant occurs. “As the amounts of ACC increases from the induction of ACC synthase, bacterial ACC deaminase is induced, resulting in a second, deleterious, ethylene peak which may be decreased dramatically by ACC deaminase” (Glick 2007).

ACC deaminase enzymatic activity is quantified by monitoring the production of either ammonia or α-ketobutyrate, the products of ACC hydrolysis (Honma and Shimomura 1978). However, at present, monitoring the amount of α-ketobutyrate is more widely used by researchers. To date, ACC deaminase has been detected only in microorganisms; and no microorganism is known to synthesize ethylene via ACC (Fukuda et al. 1993). However, there is strong evidence that the fungus, *Penicillium citrinum*, produces ACC from SAM via ACC synthase, one of the enzymes of plant ethylene biosynthesis, and degrades the ACC by ACC deaminase. It appears that the ACC, which accumulates in the intracellular spaces of this fungus, can induce ACC

deaminase (Jia, et al. 2000). In addition, throughout the many years that plants and microorganisms have been associated with each other, some plants may have obtained microbial ACC deaminase genes. Moreover, some of these genes may be present in the plant as a normal part of the plant genome such as *Arabidopsis thaliana* and poplar plants (McDonnell et al., submitted for publication). However, at the present time, there are no reports of ACC deaminase activity occurring naturally in plants.

ACC deaminase genes have been cloned from a number of different soil bacteria, including *Pseudomonas* sp. strains 6G5 and 3F2 (Klee et al. 1991; Klee and Kishore 1992), *Pseudomonas* sp. strain 17 (Campbell and Thomson 1996), *Pseudomonas* sp. strain ACP (Sheehy et al. 1991) and *Enterobacter cloacae* strain CAL2 and *Pseudomonas putida* UW4 (Glick et al. 1995; Shah et al. 1998), *Pseudomonas brassicacearum* Am3 (Belimov et al. 2007), *Rhizobium leguminosarum* bv. *viciae* (Ma et al. 2003b) as well as from yeast, *Hansenula saturnus* (Minami et al. 1998), and fungi, *Penicillium citrinum* (Jia et al. 1999). In all of these instances the gene that was characterized was directly shown to encode a functional ACC deaminase. Putative ACC deaminase genes have been identified by DNA sequence in *Escherichia coli*, various Archaebacteria and the plant *Arabidopsis thaliana*; however, none of these organisms has been demonstrated to have ACC deaminase activity, and these genes cluster separately from the rhizobacterial and fungal ones. It is possible that as the genomes of additional organisms are sequenced, more putative ACC deaminase genes will be discovered.

When a broad host range plasmid containing the ACC deaminase gene from *Pseudomonas putida* UW4 was introduced into two non-plant growth-promoting bacteria, *P. putida* ATCC 17399 and *P. fluorescens* ATCC 17400, or into *Azospirillum brasilense*, by conjugational transfer, the transconjugants acquired the ability to grow on minimal medium using ACC as the sole

source of nitrogen and to promote the elongation of canola roots (Shah et al. 1998; Holguin and Glick 2001). For the ACC deaminase gene to be expressed in *A. brasilense*, it was necessary to first replace the endogenous *P. putida* UW4 promoter region with another bacterial promoter such as the promoter from the *E. coli lac* operon (Holguin and Glick 2001). Furthermore, to reduce the metabolic load on *Azospirillum brasilense* Cd and improve its fitness, the ACC deaminase gene under the control of a Tet^r gene promoter was constructed. Although the resulting transformants showed lower ACC deaminase activity, IAA synthesis and bacterial growth rate were significantly increased. The transformants showed "increased ability to survive on the surface of tomato leaves and to promote the growth of tomato seedlings" (Holguin and Glick 2003).

The molecular mass and form of ACC deaminases purified from *Pseudomonas* sp. strain ACP (Honma and Shimomura 1978) and partially purified from *Pseudomonas* sp. strain 6G5 (Klee et al. 1991) and *P. putida* GR12-2 (Jacobson et al. 1994) are similar to one another. The enzyme is a trimer (Honma 1985); the size of the holoenzyme is approximately 104 to 105 kDa (Honma and Shimomura 1978; Honma 1985; Jacobson et al. 1994), and the subunit mass is approximately 36,500 Da (Honma and Shimomura 1978; Jacobson et al. 1994). Similar subunit sizes were predicted from nucleotide sequences of cloned ACC deaminase genes from *Pseudomonas* strains ACP (Sheehy et al. 1991) and 6G5 (Klee et al. 1991) and from *P. putida* UW4 (Shah et al. 1997). However, the molecular mass of the holoenzymes and subunits from *Hansenula saturnus* (69 kDa and 40 kDa, respectively) and *Penicillium citrinum* (68 kDa and 41 kDa, respectively) suggest that these ACC deaminsaes are dimers (Minami et al. 1998; Jia et al. 1999).

Km values for the binding of ACC by ACC deaminase have been estimated for enzyme extracts of 12 microorganisms at pH 8.5. These values ranged from 1.5 to 17.4 mM (Honma and

Shimomura 1978; Klee and Kishore 1992; Honma 1993; Hontzeas et al. 2004), indicating that the enzyme does not have a particularly high affinity for ACC (Glick et al. 1998). The optimum temperature for the activity of ACC deaminase is 30°C or 35°C and the optimum pH is 8.5. (Jacobson et al., 1994; Honma and Shimomura 1978; Jia et al., 1999).

The bacterial enzyme activity is localized only in the cytoplasm (Jacobson et al. 1994). ACC deaminase activity has been induced in both *Pseudomonas* sp. strain ACP and *P. putida* GR12-2 by ACC, at levels as low as 100 nM (Honma and Shimomura 1978; Jacobson et al. 1994); both bacterial strains were grown on a rich medium and then switched to a minimal medium containing ACC as its sole nitrogen source. The rate of induction, similar for the enzyme from the two bacterial sources, was relatively slow: complete induction required 8 to 10 h. Enzyme activity increased only approximately 10-fold over the basal level of activity, even when the concentration of ACC increased up to 10,000-fold.

Pyridoxal phosphate is a tightly bound cofactor of ACC deaminase in the amount of approximately 3 mol of enzyme-bound pyridoxal phosphate per mole of enzyme, or 1 mol per subunit (Honma 1985).

1.6 ACC deaminase: A Strategy to reduce ethylene level and promote nodulation

Based on the model described for free-living bacteria, it was suggested that strains of rhizobia that have ACC deaminase activity may have the ability to lower ethylene levels in their host-specific legumes and overcome some of the negative effects of ethylene on nodulation (Ma et al. 2003a; Ma et al. 2004).

In 2003, the first report documenting the presence of ACC deaminase in *Rhizobium* spp. showed that five of thirteen strains of Rhizobia that were tested were found to have active ACC

deaminase in the presence of ACC (Ma et al. 2003b). The ACC deaminase genes from *R. leguminosarum* bv. *viciae* 128C53, 128C53K, 128C53G, and 99A1 appeared to be highly similar to the gene from the well-known plant growth-promoting bacterium, *P. putida* UW4 (Shah et al. 1998). However, all of these strains of Rhizobia had relatively low ACC deaminase activities compared with the activity that is associated with *P. putida* UW4.

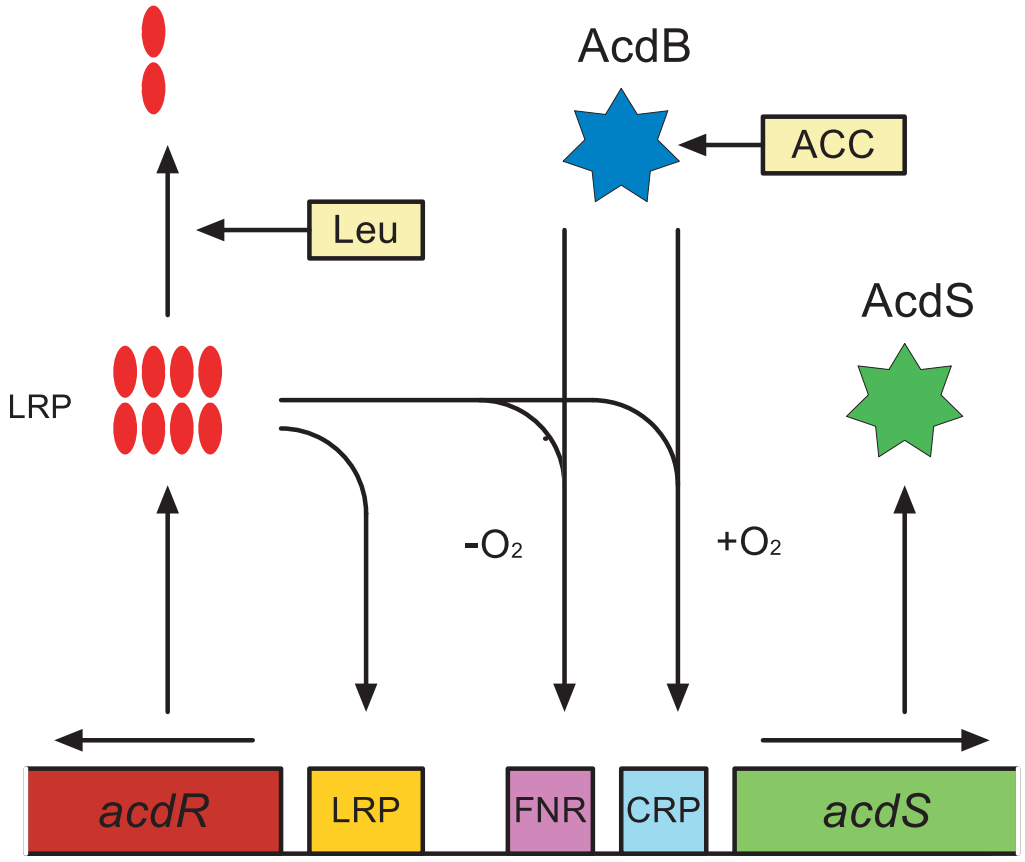
For other Rhizobia strains, such as *R. leguminosarum* bv. *trifolii*, it is possible that it has a silent ACC deaminase gene, similar to the putative ACC deaminase gene in *E. coli* (Itoh et al. 1996), that *R. leguminosarum* bv. *trifolii* has another DNA sequence that is similar to the *P. putida* UW4 ACC deaminase gene, or that the ACC deaminase gene present in this bacterium is transcriptionally activated differently from other known ACC deaminase genes (Ma et al. 2003b).

A further study of the regulation of ACC deaminase in *R. leguminosarum* bv. *viciae* 128C53K revealed that its basal level of expression was very low without ACC but could be induced by ACC concentrations as low as 1 μM (Ma et al. 2003b). It was postulated that *R. leguminosarum* bv. *viciae* 128C53K can lower the ethylene production that occurs in pea roots as a consequence of nodulation through the action of the bacterial ACC deaminase, to obtain an optimal ethylene level which allows progression of the infection threads inside the cortex and the formation of functional nodules (Ma et al. 2003a). It was also shown that after introducing the ACC deaminase gene and its upstream regulatory gene, a leucine-responsive regulatory protein (LRP)-like gene (*acdR*) from *R. leguminosarum* bv. *viciae* 128C53K, to a strain of *Sinorhizobium meliloti*, which does not produce this enzyme, the resulting ACC deaminase-producing *S. meliloti* strain showed 35 to 40% greater efficiency in nodulating *Medicago sativa* (alfalfa) (Ma et al. 2004). Furthermore, this strain was more competitive in nodulation than the wild-type strain (Ma et al. 2004).

The complete genomic sequence of *Mesorhizobium loti* MAFF303099 revealed a gene encoding a putative ACC deaminase (Kaneko et al. 2000) with a deduced amino acid sequence that shows 62% identity at DNA level and 79% similarity at protein level with the ACC deaminsae gene from *Pseudomonas putida* UW4. Previously, ACC deaminase activity was assayed in free-living conditions. But for *M. loti*, the activity was detected only in symbiotic nodules (Uchiumi et al. 2004). ACC deaminase has been identified as a gene product of the symbiosis island R7A of *Mesorhizobium loti* ICMP3153, a microsymbiont of several *Lotus* species (Sullivan et al. 2002). It was predicted that ACC deaminase, the product of gene msi273, which is located in a cluster of genes associated with nitrogen fixation, might be regulated by NifA. A very recent study has proven that expression of the ACC deaminase gene requires the symbiotic nitrogen-fixing regulator gene *nifA2* (Nukui et al. 2006) in *M. loti*. Moreover, upstream of this ACC deaminase gene there is the absence of any of the regulatory elements previously shown to control the expression of ACC deaminase (Grichko and Glick 2000; Li and Glick 2001).

A model (Grichko and Glick 2000; Li and Glick 2001; Cheng et al. submitted for publication) (Fig. 1-4) for the regulation of the ACC deaminase gene from *Pseudomonas putida* UW4 was previously proposed. This complex regulation consists of the regulatory gene *acdR* (encoding the Lrp protein) located 5' upstream of the ACC deaminase structural gene (*acdS*) and in between them there are at least two promoter regions including a possible binding site for the Lrp protein (an Lrp box), a possible binding site for a fumarate and nitrate reduction protein (FNR box) and a possible binding site for a cAMP receptor protein (CRP box). More recently, in this same bacterium, a protein (AcdB) that interacts directly with ACC, the Lrp protein and the region of DNA upstream of *acdS* was identified and characterized (Cheng et al. submitted for publication). The regulatory region of the ACC deaminase gene from some bacteria, such as

Figure 1-4 Model of the transcriptional regulation of ACC deaminase expression in *Pseudomonas putida* UW4 (Glick 2007).


AcdB
ACC
Leu
AcdS
LRP
-O2
+O2
acdR
LRP
FNR
CRP
acdS

Variovorax parrdoxus 5C2 and *Achromobacter xylosoxidans* A551 does not include all of these elements. However the Lrp protein is essential for transcription of the ACC deaminase gene from a number of different bacteria including *V. paradoxus* 5C-2, *A. xylosoxidans* A551 (Hontzeas unpublished data), *P. putida* UW4 (Grichko and Glick 2000; Li and Glick 2001), *R. leguminosarum* bv. *viciae* 128C53K and ACC deaminase-producing *S. meliloti* (Ma et al. 2003a; Ma et al. 2004).

Analysis of the upstream of *R. leguminosarum* bv. *viciae* 128C53K revealed two 15-nucleotide sequences that might contain LRP boxes were found based on the consensus sequence of the LRP-binding sites in *E. coli* (Cui et al. 1995). The experimental results suggest that the 314-nucleotide upstream region of the *acd*S gene, including the two potential LRP-binding sites, is required for its transcription in *R. leguminosarum* bv. *viciae* 128Sm.

1.7 Horizontal gene transfer and Phylogeny of ACC deaminase encoding gene

Natural selection, which Charles Darwin proposed, has been widely accepted for more than a century. According to the concept of natural selection, all life on earth developed gradually over millions of years from a few common ancestors and a population of organisms can change over time resulting in individuals with certain heritable traits leaving more offspring than others. This was achieved by passing more copies of their heritable traits on to the next generation (Lande and Arnold 1983). There are two ways in which the evolution of a species can occur, gradualism and punctuated equilibrium. Species with a shorter evulution evolved mostly by punctuated equilibrium (Eldredge and Gould 1972). However, using the principles of gradualism, small changes over a long period of time can also eventually cause substantial change.

Recently the endosymbiotic theory of the origin of eukaryotic mitochondrial and chloroplast organelles (Margulis 1970; Margulis 1981) provided exceptions to the strictly branching conformation of a universal tree. Evidence of high frequencies of horizontal DNA transfer, particularly in prokaryotes (Smith et al. 1992; Syvanen 1994; Woese 1998; Syvanen 1999; Jain et al. 1999), has made individual gene phylogeny with organismal phylogeny a problem (Doolittle 1999) and seems to challenge the traditional, tree-based view of the evolution of life.

Horizontal gene transfer (HGT) is termed as the transfer of genes between different species where it is integrated into the recipient's genome stably. The process is distinguished from the vertical transfer, which proceeds from parents to descendants. Except for its influences on evolutionary phylogenetics, HGT has important implications in basic and applied biology.

There are three types of HGT: transformation, transduction and conjugation (Salyers et al. 1995; Yin and Stotzky 1997; Ochman et al. 2000). Natural transformation has been well studied in *Bacillus subtilis*, *Streptococcus pneumoniae*, *Neisseria gonorrhoeae*, and *Haemophilus influenzeae* (Redfield 1993; Brautigam et al. 1997; Prudhomme et al. 2006; Hamilton and Dillard 2006). In this method, bacteria acquire DNA from the surrounding environment directly and a living DNA donor is not required. A set of functional genes will be distributed throughout the genome of recipient bacteria (Lorenz and Wackernagel 1994: Friedrich et al. 2001; Mercier et al. 2006). Since DNA is found in all living organisms, therefore natural transformation could occur in soil, water and digestive tract (Lorenz and Wackernagel 1994; Dröge et al. 1998; Nielsen et al. 1998; Friedrich et al. 2001). It may take place in both among the same species bacteria and to phylogenetically less related organisms (Lorenz and Wackernagel 1994; Kriz et al. 1999; Sikorski et al. 2002; Mercier et al. 2006).

Transduction between prokaryotes occurs when bacterial viruses or phages acquire host DNA, then infect another bacterium resulting in DNA transfer from the host bacterium to the infected bacterium (Calendar 1988). It can happen in aquatic and terrestrial environments between prokaryotes and eukaryotes "where viral abundance exceeds that of the prokaryotic biomass by 10 to 1" (Barkay and Smets 2005). Generalized transducing phages, such as P1 phage, can transfer any part of the bacterial genome during the lytic cycle of virulent and temperate bacteriophages, while specialized (restricted) transducing phages can carry only specific portions of the bacterial genome. Subsequent transduction is carried out only by temperate phages that have integrated their DNA into the host chromosome at a specific site.

Conjugation is the direct exchange of genetic material by cell-to-cell contact. It is mediated by a number of genetic elements like plasmids, genetic islands, transponsons and integrons (Mercier et al. 2006; Roberts and Mullany 2006). These genetic elements are "important vectors for transferring genes coding for pathogenic traits, resistance to heavy metals or antibiotics" (Dobrindt and Hacker 1999, 2001; Rowe-Magnus and Mazel 2001; Mercier et al. 2006). In conjugation, one bacterial cell serves as a donor, which carry self-transmissible or mobilisable plasmids, the other acts as a recipient. Physical contact between the donor and the new host is required. Conjugation is frequently associated with plasmids, probably because plasmids can be transferred quickly whereas a whole chromosome can take more than an hour. In addition, transfer of a whole chromosome will rarely be completed since the interbacterial junctions will break down (Thomas and Nielsen 2005). In the lab, conjugative process between two bacteria ranges from 10^{-1} to 10^{-3} under optimal conditions, while in nature, many factors can affect the frequency of bacterial conjugation. For example, soil type, pH and temperature were all considered as factors that may cause a drop in the frequency to 10^{-9} (Rochelle et al. 1989;

Richaume et al. 1989; Richaume et al. 1992; Frischer et al. 1994; Jiang and Paul 1998; Sorensen et al. 2005).

Generally, organisms are much more likely to refuse foreign DNA than accept it. Depending on the gene-transfer mechanisms and the bacterial species involved, a number of barriers limit the transfer, uptake and stabilization of foreign DNA (Thomas and Nielsen 2005), such as "the availability of adaptive DNA in the environment, the DNA uptake specificity" (Thomas and Nielsen 2005). In addition, genes prefer to exchange among organisms sharing similar genome size, genome G/C contents, carbon utilization, and oxygen tolerance (Jain et al. 2003) as well as codon usage (Soto et al. 2004).

There are two main methods of detecting horizontal gene transfer: phylogenetic methods and parametric methods (Azad and Lawrence 2005). Phylogenetic methods are based on the comparison of phylogenetic trees generated from different genes of the genome whereas parametric methods involve the detection of atypical gene in current genomic context. It has been found that different methods detect different sets of transfer events (Ragan 2001; Lawrence and Ochman 2002). The drawback of parametric methods is they can only detect recent transfers of very different composition. Furthmore, the composition of transferred genes changes to match the composition of the new genome, which will cause false negative (Lawrence and Ochman 1997; Koski et al. 2001). Phylogenetic methods may cause false negative results too when the sequences of transferred gene are so divergent that were not included in the analysis (Hamady et al. 2006). New HGT-detection methods have been developed recently. For example, Hamady and colleagues looked for genes that evolve according to a different nucleotide substitution rate matrix that consists of model parameters in the theory of neutral sequence evolution (Kimura 1968;

Kimura 1983). The new techniques discriminate simulated HGT events with an error rate up to 10 times lower than does GC content (Hamady et al. 2006).

A phylogenetic analysis of several *acdS* genes from recently isolated bacteria, such as *Achromobacter xylosoxidans* A551, *Achromobacter xylosoxidans* Bm1, *Variovorax paradoxus* 5C-2, *Variovorax paradoxus* 3P-3, (mostly free-living) suggested that these genes did not evolve strictly vertically but instead may have undergone horizontal gene transfer (Hontzeas et al. 2005). Moreover, phylogenetic analysis of partial *acdS* and deduced *acdS* sequences from 71 proteobacterial strains indicated three main phylogenetic clusters, each gathering pathogens and plant-beneficial strains of contrasting geographic and habitat origins. The *acdS* phylogenetic tree was only partly consistent with the 16S ribosomal RNA tree, and two clusters gathered both *Betaprotobacteria* and *Gammaproteobacteria*, suggesting extensive horizontal transfers of *acdS* (Blaha et al. 2006). At this point, it is not clear whether the *acdR* genes were inherited along with the *acdS* genes since the previous study only looked at *acdR* genes from *Rhizobium leguminosarum* bv. *viciae* 128C53, *Variovorax paradoxus* 5C2 and *Achromobacter xylosoxidans* A551. It is premature to assume that the LRP genes have also undergone HGT since there are only four plant growth promoting bacterial species whose regulatory region has been sequenced and show to contain Lrp genes (Hontzeas unpublished data).

1.8 Objectives

Since the existence and prevalence of ACC deaminase in *Rhizobium* spp. has been discovered, it was postulated that ACC deaminase may play an important role to enable efficient nodulation by decreasing ethylene levels.

Our lab recently obtained 233 uncharacterized Rhizobia strains from Philom Bios Inc. and, following enzyme assays, found that 27 of them showed ACC deaminase activity. This result indicates that ACC deaminase genes are commonly present in *Rhizobia*. The fact that, in these strains, ACC deaminase activity was detected following induction by ACC suggests that regulation of *acdS* probably does not include *nifA* (since enzyme activity was detected in culture and not tested in nodules) but might include *acdR*. In addition, because *acdS* may be on one of the rhizobial plasmids, it will be of interest to identify if lateral gene transfer occurs during the evolutionary process. Thus the objectives of this thesis are:

1. To isolate and sequence all ACC deaminase genes from above mentioned 27 Rhizobia strains.
2. To determine the gene copy number of the ACC deaminase gene in each strain by Southern hybridization.
3. To PCR amplify and sequence the 16S rDNA from each of these Rhizobia strains and then to assign a genus and species to each strain.
4. To isolate and sequence the 5' upstream region for each ACC deaminase gene and analyze these upstream regions for both potential genes and regulatory regions.
5. To develop phylogenetic trees of these strains based on their ACC deaminase gene sequences.

2 Materials and Methods

2.1 Bacterial Strains

Twenty-seven rhizobial strains were used in this study (Table 2-1: Rhizobia strains used in this study, showing ACC deaminase activity, host legume plants and location where they were collected). These strains were collected by Philom Bios Inc. from 12 different sites across Saskatchewan (Fig. 2-1) and from the rhizosphere soil of three native host legume plants: *Vicia americana*, *Vicia cracca*, *Lathyrus venousus* (Fig. 2-2). When a target plant was found, a 6"× 2.5" ×5" soil sample was collected, including the target plant, placed into a plastic bag, packed into a cooler and removed to the company's lab. At the lab the samples were removed from their plastic bag and placed into a large plastic tray. Host plants were removed at the soil line washed and a press mount was made for positive identification. The remaining soil was crushed by hand and sieved through a ¼" screen to remove any roots, twigs or other organic material. Bleached pots and sterile silica sand was mixed with the soil sample (50:50). Pea seeds were surface sterilized with 95% ethanol for 2 min, followed by 2.5% sodium hypochlorite for 15 min, and then washed thoroughly with sterile distilled water and then sown into the medium. After 30 days the plants were harvested and bacterial strains were isolated, each from a single nodule.

Escherichia coli DH5α (Hanahan, 1983) was used as a recipient for recombinant plasmids. This strain and its transformants with different plasmids were grown at 37°C in Luria Broth medium (Difco Laboratories, Detroit, MI), with 100 μg/mL ampicillin.

All of the 27 Rhizobia strains and the control strains, *Rhizobium leguminosarum* bv. *viciae* 128Sm, the *acdS*$^-$ mutant of this strain *Rhizobium leguminosarum* bv. *viciae* 128Sm*acdS*Ω::Km, *Sinorhizobium meliloti* Rm5356, *Rhizobium hegysari* ATCC43676 and *Mesorhizobium loti*

MAFF303099 were grown at 30°C in TY (0.5% Tryptone, 0.3% Yeast Extract, 0.044% $CaCl_2 \bullet 2H_2O$, w/v) or modified M9 minimal medium (5.8 $g \bullet L^{-1}$ Na_2HPO_4; 3 $g \bullet L^{-1}$ KH_2PO_4; 0.5 $g \bullet L^{-1}$ NaCl; 1 $g \bullet L^{-1}$ NH_4Cl; supplemented with 0.25 mM $CaCl_2$; 1 mM $MgSO_4$; 0.15% glucose; and 0.3 $\mu g \bullet mL^{-1}$ biotin). Appropriate antibiotics were added to the medium when necessary.

Antibiotics were used at the following concentrations for both *E. coli* and *Rhizobium* strains (μg/mL): ampicillin (Amp), 100; streptomycin (Sm), 200; tetracycline (Tc), 15; kanamycin (Km), 30.

2.2 ACC deaminase activity assay for Rhizobia strains

To measure ACC deaminase activity, *Rhizobia* cells, including the 27 uncharacterized strains and the controls *Rhizobium leguminosarum* bv. *viciae*, the *acdS*$^-$ mutant of this strain, *Sinorhizobium meliloti* Rm5356, *Rhizobium hegysari* ATCC43676 and *Mesorhizobium loti* MAFF303099, were grown in 5 mL of TY medium at 30°C for 2-3 days until they reached stationary phase. To induce ACC deaminase activity, the cells were collected by centrifugation, washed twice with 0.1 M Tris-HCl (pH 7.5), suspended in 2 mL of modified M9 minimal medium supplemented with 5 mM final concentration ACC, and incubated at 30°C with shaking for another 36-40 hours.

ACC deaminase activity was determined by measuring the production of α-ketobutyrate generated by the cleavage of ACC by ACC deaminase (Honma and Shimomura 1978; Penrose and Glick 2003). The induced bacterial cells were harvested by centrifugation for 10 min at 10,000 × g, washed twice with 0.1 M Tris-HCl (pH 7.5), and resuspended in 200 μL of 0.1 M Tris-HCl (pH 8.5). The cells were labilized by adding 5% toluene (v/v) and then vortexed at the highest speed for 30 seconds. Fifty μL of labilized cell suspension was incubated with 5 μL of 0.5

Table 2-1 Activity of ACC deaminase-containing Rhizobia strains. Location indicates where the soil sample that was the source of the bacterium was obtained.

Strain Number	μmoles/mg/hr	Location	Host legume
2	0.528	Saskatoon, SK	*Vicia americana*
45	0.119	Wakaw, SK	*Vicia americana*
61	0.160	St. Brieux, SK	*Lathyrus venosus*
62	0.123	Borden, SK	*Lathyrus venosus*
126	0.106	Rouleau, SK	*Vicia cracca*
129	0.219	Rouleau, SK	*Vicia cracca*
130	0.118	Rouleau, SK	*Vicia cracca*
131	0.147	Rouleau, SK	*Vicia cracca*
141	0.102	Balgonie, SK	*Vicia americana*
142	0.145	Balgonie, SK	*Vicia americana*
154	0.110	Lipton, SK	*Vicia americana*
161	0.112	Lipton, SK	*Vicia americana*
162	0.112	LeRoss, SK	*Vicia americana*
163	0.170	LeRoss, SK	*Vicia americana*
164	0.162	LeRoss, SK	*Vicia americana*
165	0.183	LeRoss, SK	*Vicia americana*
166	0.179	LeRoss, SK	*Vicia americana*
167	0.128	LeRoss, SK	*Vicia americana*
168	0.152	LeRoss, SK	*Vicia americana*
169	0.140	LeRoss, SK	*Vicia americana*
170	0.211	Mozart, SK	*Vicia americana*
171	0.274	Mozart, SK	*Vicia americana*
172	0.240	Mozart, SK	*Vicia americana*
173	0.118	Mozart, SK	*Vicia americana*
180	0.184	Colonsay, SK	*Vicia americana*
194	0.148	Plunkett, SK	*Vicia americana*
223	0.216	Dalmeny, SK	*Lathyrus* spp.

Figure 2-1 Geographic map of the Rhizobia strains collection. (North-South: 350 Km; East-West: 320 Km)

1. Wakaw, SK;
2. St. Brieux, SK;
3. Borden, SK;
4. Dalmeny, SK;
5. Saskatoon, SK;
6. Colonsay, SK;
7. Plunkett, SK;
8. Mozart, SK;
9. LeRoss, SK;
10. Liption, SK;
11. Balgonie, SK;
12. Rouleau, SK.

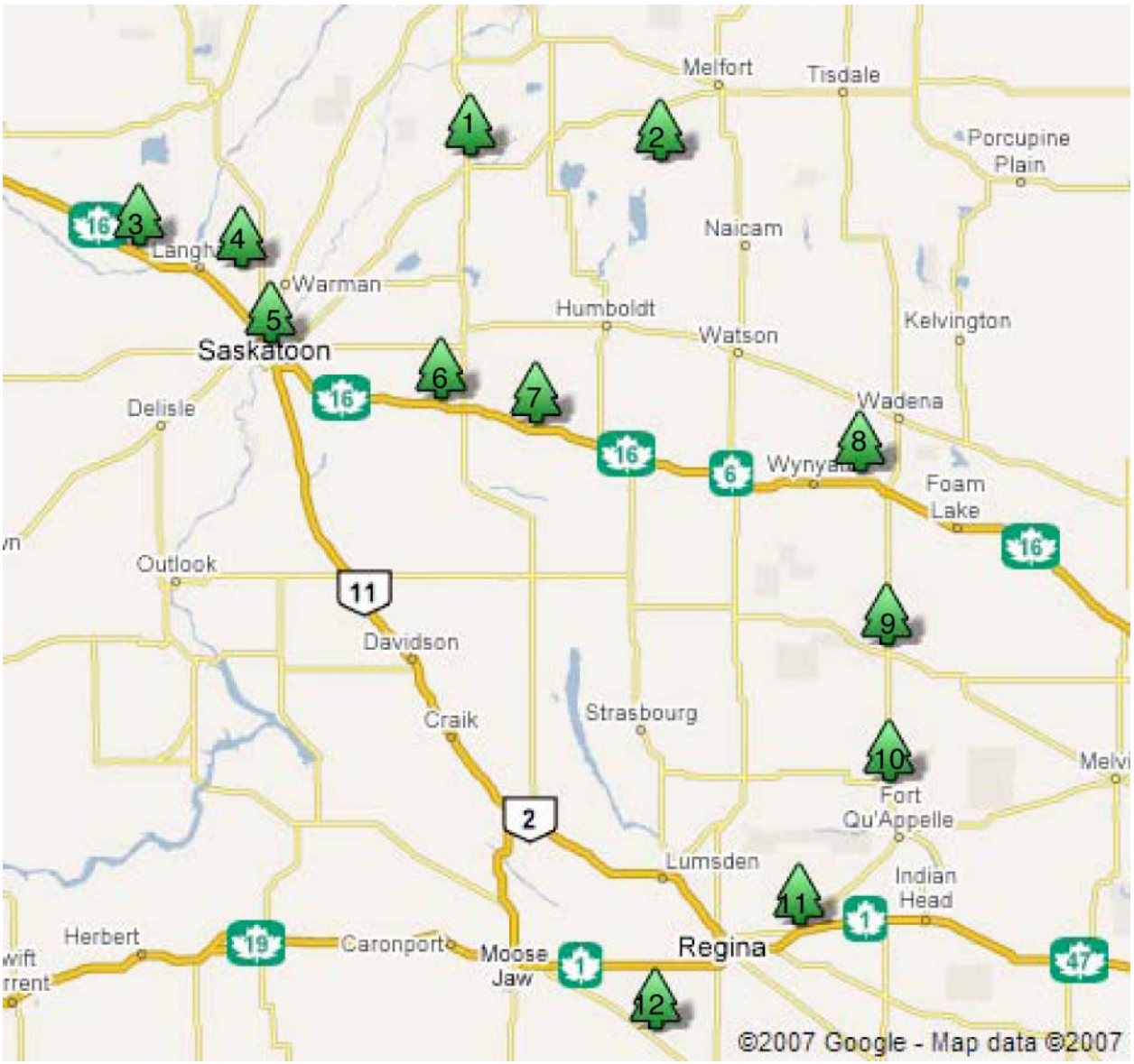

Melfort
Tisdale
Porcupine Plain
Naicam
Warman
Humboldt
Watson
Kelvington
Saskatoon
Delisle
Wadena
Foam Lake
Outlook
Davidson
Craik
Strasbourg
Fort Qu'Appelle
Lumsden
Indian Head
Herbert
Caronport
Moose Jaw
Regina
1
2
3
4
5
6
7
8
9
10
11
12
16
11
2
19
1
6
47
©2007 Google - Map data ©2007

Figure 2-2 Host legume plants of 27 Rhizobia strains.

Vicia americana

Lathyrus venosus

Vicia cracca

M ACC in an Eppendorf tube at 30°C for 30 minutes. The negative control for this assay included 50 μL of labilized cell suspension without ACC, while the blank included 50 μL of 0.1 M Tris-HCl (pH 8.5) with 5 μL of 0.5 M ACC. The samples were then mixed thoroughly with 500 μL of 0.56 N HCl by vortexing, and the cell debris was removed by centrifugation at 20,000 X g (Eppendorf centrifuge 5417c) for 5 minutes. A 500 μL aliquot of the supernatant was transferred to a 13 X 100 mm glass test tube and mixed with 400 μL of 0.56N HCl and 150 μL of DNF solution (0.1 g 2,4-dinitrophenylhydrazine in 100 mL of 2N HCl); and the mixture was incubated at 30°C for 30 minutes. One mL of 2N NaOH was added to the sample before the absorbance at 540 nm was measured.

The concentration of α-ketobutyrate in each sample was determined by comparison with a standard curve generated as follows: 500 μL α-ketobutyrate solutions of 0, 0.01, 0.05, 0.1, 0.2, 0.5, 0.75 and 1 mM were mixed respectively with 400 μL of 0.56 N HCl and 150 μL DNF solution. One mL of 2N NaOH was added and the absorbances at 540 nm were determined as described above. The values for absorbance versus α-ketobutyrate concentration (mM) were used to construct a standard curve.

2.3 Protein concentrations determination

The protein concentration of toluenized cells was determined by the method of Bradford (1976) using the Bio-Rad protein reagent (Bio-Rad Lab., USA) according to the manufacturer's instructions. A 26.5 μL aliquot of the toluene-labilized bacterial cell sample used for the ACC deaminase enzyme assay was diluted with 173.5 μL of 0.1 M Tris-HCl (pH 8.0), and boiled with 200 μL of 0.1 N NaOH for 10 minutes. After the cell sample was cooled to room temperature, the

protein concentration was determined by measuring the absorbance at 595 nm immediately after mixing the solution with 200 μL of Bradford's reagent.

Bovine serum albumin (BSA) was used to establish a standard curve. A stock solution of BSA (10 mg· mL^{-1}) was diluted with distilled deionized water to final concentrations of 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 mg· mL^{-1} respectively. The value of the absorbance at 595 nm versus the BSA concentration was used to construct a standard curve.

2.4 Isolation of genomic DNA from *Rhizobium*

Rhizobium cells were grown in 5 mL of TY medium at 25°C for 2 or 3 days until they reached stationary phase. One mL of the culture was added to a 1.5 mL microcentrifuge tube and centrifuged at 20,000 × g for 5 minutes using an Eppendorf centrifuge 5417c (Hamburg, Germany). Genomic DNA was isolated from all the 27 Rhizobia strains by using the Promega (Mississauga, ON, Canada) Wizard genomic DNA purification system according to the manufacturer's suggested protocol.

2.5 Isolation of 16S rDNA from the 27 Rhizobia strains

PCR primers 1F (5'-AGCGGCAGACGGGTGAGTAATG-3') and FGPS1509R (5'-AAGGAGGGGATCCAGCCGCA-3') (Young et al. 2004) were used to amplify the 16S rDNA coding region. The amplification reactions were performed in a total volume of 25 μl containing 2 μl sample of DNA extracted from bacterial colonies, 2.5 U of KOD hot start DNA Polymerase (Novagen, Mississauga, ON, Canada), 2.5 μl KOD hot start DNA Polymerase 10×buffer (20mM Tris-HCl pH 7.5, 7.5 mM DTT, 50 μg/ml BSA), 2.5 μl dNTP mixtures (2 mM each), 1 μl $MgSO_4$ (25 mM) and 20 pmol of each primer. A few drops of mineral oil (white, light; Mallinckrodt)

were added to cover the reaction mixture in order to prevent evaporation during the PCR reaction. The PCR program was set up as follows: one cycle of 94°C for 1 minute; 30 cycles of 92°C for 40 seconds, 68°C for 40 seconds, 75°C for 1 minute and 30 seconds, and one cycle of 72°C for 5 minutes. The amplified 16S rRNA genes were purified with QIAquick Gel Extraction Kit (QIAGEN Inc., Mississauga, ON, Canada) Purified PCR products were ligated into the pGEM-T Easy vector system (Promega, Mississauga, ON, Canada). The ligation products were transformed into *E. coli* DH5α and plated on LB (Luria Broth)/Ampicillium (100μg/mL final concentration) with 15 μL 100 mM IPTG and 20 μL 50 mg/mL X-Gal spread on top of the plate. White colonies were selected from the plates and streak purified. Plasmid DNA was isolated from the transformants and confirmed with restriction digestions before they were sequenced with T7 and SP6 forward and reverse primers. The sequences obtained were analyzed by comparison with sequences in the GeneBank database.

All the sequence alignments were first done by ClustalW online (Chenna et al. 2003) and further analyzed by Bioedit 7.0.4.1. (Hall 1999).

2.6 Isolation of ACC deaminase genes from the 27 Rhizobia strains

The specific primers (1F: 5'-GGCAAGGTCGACATCTATGC-3' and 1R: 5'-GGCTTGCCATTCAGCTATG-3') were designed to amplify ACC deaminase gene of 16 *rhizobia* strains based on ACC deaminase gene of *Rhizobium leguminosarum* bv. *viciae* 128C53K and were synthesized by Sigma (Oakville, ON, Canada). The 25 μl PCR mixture contained 2.5 μl of KOD hot start DNA Polymerase 10×buffer (20mM Tris-HCl pH 7.5, 7.5 mM DTT, 50 μg/ml BSA), 2.5 μl of 2 mM dNTPs, 2 μl of 20 pmol each primer, 2.5 units of KOD hot start DNA Polymerase (Novagen, Mississauga, ON, Canada) and approximately 100 ng template DNA. The

DNA was amplified in a MJ Instruments PTC-100 thermocycler (Waltham, MA) with the following program: 1 minute and 30 seconds initial denaturation at 94°C, 35 cycles of 1 minute denaturation at 92°C, 50 seconds primer annealing at 58°C, 1 minute of elongation at 72°C. A final elongation step of 5 minutes at 72°C was included.

Another set of primers: 5'-CTCCACGCCTACAAGACCG-3' and 5'-CGAACTCGCCTGATGTCCC-3' were designed to amplify ACC deaminase gene from the other 10 Rhizobia strains based on the sequences of ACC deaminase gene from inverse PCR. The 25 μl PCR mixture contained 2.5 μl of KOD hot start DNA Polymerase 10×buffer (20mM Tris-HCl pH 7.5, 7.5 mM DTT, 50 μg/ml BSA), 2.5 μl of 2 mM dNTPs, 2 μl of 20 pmol each primer, 2.5 units of KOD hot start DNA Polymerase (Novagen, Mississauga, ON, Canada) and approximately 100 ng template DNA. The DNA was amplified in a MJ Instruments PTC-100 thermocycler (Waltham, MA) with the following program: 1 minute and 30 seconds initial denaturation at 94°C, 35 cycles of 1 minute denaturation at 92°C, 40 seconds primer annealing at 62°C, 2 minutes of elongation at 72°C. A final elongation step of 5 minutes at 72 °C was included.

Following PCR, bands were extracted, cloned and sequenced as described earlier.

2.7 Southern hybridization

2.7.1 Labeling the probe with digoxigenin

The purified PCR product was tagged with digoxigenin (DIG) by using a DIG Oligonucleotide 3'–End Labeling Kit (2nd Generation) (Roche Diagnostics GmbH, Mannheim, Germany). 2 μg of template DNA (the purified 1 Kb PCR product of *acdS* gene) was diluted to a total volume of 10 μl with double distilled water. On ice, the following reagents were added to the template and gently mixed and centrifuged briefly: 5 × Reaction buffer, 4 μl; $CoCl_2$-solution, 4 μl

(5 mM); DIG-ddUTP solution, 1 μl (0.05 mM); terminal transferase, 1 μl (20U). The mixture was incubated in a 37°C incubator for one hour and the reaction was stopped by the addition of 2 μl 0.2 M EDTA (pH 8.0).

2.7.2 Preparation of the genomic DNA for Southern Hybridization

Approximately 2 μg of genomic DNA isolated from each rhizobial strain was completely digested with restriction enzyme *EcoR* V (MBI Fermentas, Inc.) overnight at 37°C. In each reaction, the genomic DNA was digested with 10 U *EcoR* V in the reaction buffer provided by the company. The digested DNA was loaded and run on a 1% agarose gel together with a 1 kb DNA ladder (MBI Fermentas, Inc.) as a molecular weight standard. *EcoR* V digested genomic DNA of *Rhizobium leguminosarum* 128Sm was also included and used as a positive control. The gel was stained with 0.3 μg/ml (final concentration) ethidium bromide, visualized under UV light, and saved by photography.

DNA on the agrose gel was nicked by washing the gel in 0.25 M HCl at room temperature for 10 minutes. Then the gel was submersed in denaturation solution (0.5 M NaCl, 150 mM NaOH) twice for 15 minutes. Next the gel was neutralized by washing with 0.5 M Tris-Cl (pH 8.0), 0.5 M NaCl twice for 15 minutes to facilitate DNA transfer. The DNA on the gel was then ready to be transferred to a piece of nylon membrane by capillary action using 10× SSC (1.5 M NaCl; 0.15 M trisodium citrate; pH 7.0) as the transfer buffer (Sambrook and Russel 2001).

A glass dish containing 10 × SSC was used as the reservoir dish for the transfer buffer. A plastic plate was put on the dish and served as a support for the gel; the level of the transfer buffer was just below the surface of the plate. A wick paper (3MM Whatman) was cut to same width as the gel and placed on top of the support plate lying across the dish and tucked under so that the

paper touches the 10 × SSC solution, but not the sides of the dish. Using a Pasteur pipette, the 3MM bridge was wetted with the 10× SSC. The wells of gel were excised and then placed gel face down on the support with the wick paper. A wetted nylon membrane was placed on top of the gel to allow DNA transfer. Three pieces of dry 3MM Whatman paper were put on top of nylon membrane and a ~10 cm stack of paper towels were added placed on top, followed by a glass plate over the paper towels with a weight on top of it to hold it in place. Tape was used to hold the plate down firmly. The dish was wrapped with Saran Wrap to minimize evaporation and transfer overnight.

The nylon membrane was retrieved from the apparatus after the transfer was complete. The DNA was fixed to the membrane by exposing the membrane with the DNA side up to 150 mJoules of UV light in a Gene Linker (Bio-Rad Laboratories, Hercules, CA).

2.7.3 Southern Hybridization

The nylon membrane was placed in a 6.5" ×8" heat sealable bag (Kapak® Corp., Minneapolis, MN) and pre-hybridized in 50 mL DIG Easy Hyb (5×SSC; 0.1% w/v N-laurylsarcosine; 0.02% w/v SDS; 1% w/v) blocking solution (Roche Diagnostics GmbH) for 3 hours at 60°C with gentle agitation. The hybridization solutions were made by adding diluted DIG-labeled probes (100 pmol), either *acdS* gene of strain 45 or *acdS* gene of strain 163, in 50 mL newly preheated DIG Easy Hyb. The membrane was incubated with the hybridization buffer at 60°C with gentle agitation for 16 hours.

After hybridization, the membrane was transferred from the bag to a small plastic container and washed twice at room temperature for 5 minutes in 2×SSC and 0.1% SDS and twice under constant agitation for 15 minutes in 0.5×SSC and 0.1% SDS at 60°C. The membrane

was either used directly for detection of hybridized oligonucleotides or stored air-dried for later detection.

2.7.4 Visualization of DNA in Southern Hybridization

After hybridization and stringency washes, at room temperature, the membrane was rinsed briefly in 20 mL 1×Washing buffer (10×concentration buffer contains Maleic acid buffer (10×concentration) and 3-5% Tween 20 v/v) for 5 minutes, incubated for 30 minutes in 50 mL Blocking solution (Roche Diagnostics GmbH) and 30 minutes in 20 mL Antibody solution (1 μL Anti-DIG-AP in 5 mL of 1×blocking solution: 1:5000 dilution) with gentle agitation, respectively. Then the membrane was washed twice in 50 mL Washing buffer for 15 minutes and equilibrated for 5 minutes in 20 mL 1×Detection buffer (10×concentration buffer contains 1M Tris-HCl, pH 9.5 and 1 M NaCl). The membrane was put into hybridization bag with the face up. About 20 drops (0.5 mL) CDP-Star (Roche Diagnostics GmbH) solution were applied and the membrane was immediately covered with the second sheet of the bag to spread the substrate evenly and without air bubbles over the membrane. This was incubated for 5 minutes at room temperature and then excess liquid was squeezed out and the bag was sealed for chemiluminescence detection.

2.8 Inverse PCR

Inverse PCR was carried out in order to isolate complete ACC deaminase genes and their 5' upstream regulatory regions from the partial fragments obtained. About 100 ng of total bacterial genomic DNA was digested overnight with restriction enzyme *Sal*I. The sample was phenol-chloroform extracted and the aqueous layer was used for self-ligation. Then the sample was

phenol-chloroform extracted again and the aqueous layer was used for PCR. The primers were designed based on the previously determined partial sequence of the ACC deaminase gene. They are: 5'-CCGTGCCGAATTTGTGGTC-3' and 5'-CGAACGCTACCCGCTCACCT-3'. PCR was carried out using the following conditions: one cycle of 94°C for 1 minute; 35 cycles of 92°C for 50 seconds, 62°C for 50 seconds, 72°C for 1 minute; and one cycle of 72°C for 5 minutes. After PCR, bands were extracted, cloned and sequenced as described earlier.

2.9 Phylogenetic analysis

16S rRNA sequences and ACC deaminase nucleotide sequences were aligned using ClustalW (Chenna et al. 2003) and refined by eye. Their GenBank accession numbers are shown in Table 2-2. Phylogenetic analyses were performed using PAUP v4.10b (Swofford 2002) and MrBayes (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). Appropriate evolutionary models were chosed using Modeltest 3.7 (Posada and Crandall 1998). The chosen substitution models were Tamura-Nei TrN+I+G for 16S rRNA sequences and general time-reversible model GTR+G for ACC deaminase nucleotide sequences. Nodal support in Maximum Parsimony (MP) and Neighbor Joining (NJ) was evaluated by 1000 bootstrap pseudoreplications. The topologies of the ML trees were evaluated by calculating posterior probabilities in the Bayesian analysis. Phylograms were generated with TreeView (Page 1996).

2.10 Nucleotide sequence accession numbers

The nucleotide sequences of the 16S rRNA gene, ACC deaminase structural gene (*acdS*) of the 27 Rhizobia strains as well as *acdR* gene of the 17 Rhizobia strains have been deposited in the GenBank database under accession numbers shown in Table 2-3.

Table 2-2 List of bacteria tested for phylogenetic analysis of ACC deaminase and 16S rRNA gene. "-" in the table means the strains don't have a 16S rRNA gene sequence in GenBank.

Strain	Accession number		Reference(s) or source
	16S rRNA gene	ACC deaminase gene	
Achromobacter xylosoxidans A551	AY559500	AY604539	Hontzeas et al. 2005
Achromobacter xylosoxidans Bm1	AY556401	AY604540	Hontzeas et al. 2005
Achromobacter sp. CM1	AF288728	AY604541	Hontzeas et al. 2005
Acidovorax facilis 4P6	AY197008	AY604529	Hontzeas et al. 2005
Agrobacterium tumefaciens d3	-	AF315580	Trott et al. 2001
Azospirillum lipoferum 4B	DQ438996	DQ125242	Blaha et al. 2006
Burkholderia phytofirmans PsJN	AY497470	NZ_AAUH01000001	Copeland et al. 2006
Enterobacter aerogenes CAL3	AY559494	AY604544	Hontzeas et al. 2005
Enterobacter cloacae CAL2	-	AF047840	Shah et al. 1998
Pseudomonas brassicacearum Am3	AY007428	AY604528	Hontzeas et al. 2005
Pseudomonas marginalis DP3	AF311387	AY604542	Hontzeas et al. 2005
Pseudomonas putida Bm3	AF288727	AY604533	Hontzeas et al. 2005
Pseudomonas putida UW4	AY559493	AF047710	Shah et al. 1998
Pseudomonas syringae GR12-2	AY559495	AY604545	Hontzeas et al. 2005
Pseudomonas sp. ACP	-	M73488	Sheehy et al. 1991
Rhizobium leguminosarum 99A1	AY559497	AY604535	Hontzeas et al. 2005
Rhizobium leguminosarum 128C53K	AY559496	AF421376	Ma et al. 2003
Rhizobium gallicum PB2	EF525207	EF525234	This study
Rhizobium leguminosarum PB45	EF525208	EF525235	This study
Rhizobium leguminosarum PB163	EF525220	EF525253	This study
Rhizobium sullae ATCC 43676	AY559498	AY604534	Hontzeas et al. 2005
Rhodococcus sp. 4N4	AY197005	AY604538	Hontzeas et al. 2005
Rhodococcus sp. Fp2	AF288731	AY604537	Hontzeas et al. 2005
Serratia proteamaculans SUD165	AY559499	AY604543	Hontzeas et al. 2005
Variovorax paradoxus 2C1	AY196950	AY604530	Hontzeas et al. 2005
Variovorax paradoxus 5C2	AY197003	AY604531	Hontzeas et al. 2005
Variovorax paradoxus 3P3	AY197002	AY604532	Hontzeas et al. 2005

Table 2-3 Accession numbers of 16S rRNA gene, ACC deaminase structural gene (*acdS*) of the 27 Rhizobia strains and LRP-like protein gene (*acdR*) of the 17 Rhizobia strains. "-" in the table means the corresponding strains don't have a full LRP-like protein gene.

Strain Number	Accession number		
	16S rRNA gene	ACC deaminase gene	LRP-like protein gene
2	EF525207	EF525234	EF525261
45	EF525208	EF525235	EF525262
61	EF525209	EF525236	EF525263
62	EF525210	EF525237	EF525264
126	EF525211	EF525238	EF525265
129	EF525212	EF525239	EF525266
130	EF525213	EF525240	EF525267
131	EF525214	EF525241	EF525268
141	EF525215	EF525242	EF525269
142	EF525216	EF525243	EF525270
154	EF525217	EF525244	EF525271
161	EF525218	EF525245	-
162	EF525219	EF525246	-
163	EF525220	EF525247	-
164	EF525221	EF525248	-
165	EF525222	EF525249	-
166	EF525223	EF525250	-
167	EF525224	EF525251	-
168	EF525225	EF525252	-
169	EF525226	EF525253	-
170	EF525227	EF525254	EF525272
171	EF525228	EF525255	EF525273
172	EF525229	EF525256	-
173	EF525230	EF525257	EF525274
180	EF525231	EF525258	EF525275
194	EF525232	EF525259	EF525276
223	EF525233	EF525260	EF525277

3 Results

3.1 ACC deaminase activity assay of the Rhizobia strains

Our lab obtained 233 Rhizobia strains from Philom Bios, Inc. The ACC deaminase activity assay of these strains was measured by Ms. Susanne Vesely and 27 strains were found to contain ACC deaminase activity (Table 2-1). To confirm the presence of ACC deaminase activity four of these Rhizobia strains, i.e. PB2, PB45, PB163 and PB172 were reassayed for ACC deaminase activity and compared to the control strains. *Rhizobium leguminosarum* bv. *viciae* 128Sm, the *acdS*$^-$ mutant of this strain (*R. leguminosarum* bv. *viciae* 128Sm*acdS*Ω::Km), *Sinorhizobium meliloti* 5356, *Rhizobium hegysari* ATCC43676 and *Mesorhizobium loti* MAFF303099 (From Dr. Trevor Charles's lab, University of Waterloo). All the Philom Bios Rhizobia strains showed low levels of ACC deaminase activity, ranging from 76-235 nmoles/mg/hr. As expected, the negative controls, the *acdS*$^-$ mutant of *R. leguminosarum* bv. *viciae* 128Sm*acdS*Ω::Km, *Sinorhizobium meliloti* 5356, *Mesorhizobium loti* MAFF303099 didn't show any activity under free-living conditions (Table 3-1).

3.2 Isolation and sequence characterization of Rhizobial 16S rDNA

The 16S rDNAs from the above mentioned 27 Rhizobia strains were PCR amplified and the approximately 1400 bp products were then sequenced using the oligonucleotide primers 1F: 5'-AGCGGCGGACGGGTGAGTAATG-3' and FGPS1509R: 5'-AAGGAGGGGATCCAGCCGCA-3' (Young et al. 2004) (Fig. 3-1). Analysis of these 27 sequences by comparison with the sequences in the GenBank database indicated that 26 strains were *Rhizobium leguminasarum* with > 99% identities to the type strain (i.e. 1398/1402 bp),

Table 3-1 Assay ACC deaminase activity of several Rhizobia strains.

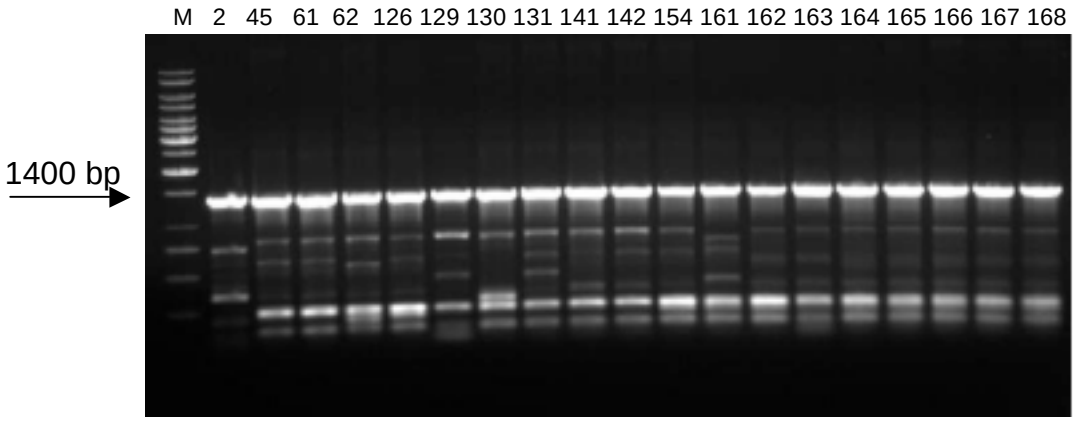
Strains	ACC deaminase activity (nmoles/mg/hr)
Rhizobium gallicum PB2	82
Rhizobium leguminosarum PB45	143
Rhizobium leguminosarum PB163	76
Rhizobium leguminosarum PB172	235
Rhizobium leguminosarum bv. *viciae* 128Sm	177
R. leguminosarum bv. *viciae* 128Sm*acdS*Ω::Km	0
Sinorhizobium meliloti 5356	0
Rhizobium hegysari ATCC43676	200
Mesorhizobium loti MAFF303099	0

while number 2, was > 99% identical (1377/1380 bp) to the type strain of *Rhizobium gallicum*.

3.3 Isolation and characterization of Rhizobia ACC deaminase genes and their 5' upstream sequences

An ACC deaminase structural gene (*acdS*) was PCR amplified and sequenced from each of the 27 Rhizobia strains either by PCR using primers designed from the previously characterized ACC deaminase gene of *Rhizobium leguminosarum* bv. *viciae*, or by using the primers designed from the sequences obtained from inverse PCR experiments. The primers 1F: 5'-GGCAAGGTCGACATCTATGC-3' and 1R: 5'-GGCTTGCCATTCAGCTATG-3' were used to PCR amplify ACC deaminase genes from 17 strains including strains 2, 45, 61, 62, 126, 129, 130, 131, 141, 142, 154, 170, 171, 173, 180, 194 and 223. After PCR, PCR products about 1 kb in size were obtained for these 17 strains (Fig. 3-2). When these 17 sequences were compared with the ACC deaminase gene sequences in the GeneBank database, they showed > 99% identities at the nucleotide level compared with the *acdS* genes of *Rhizobium leguminosarum* bv. *viciae* (Accession number: AF421376) and *Rhizobium leguminosarum* strain 99A1 (Accession number: AY604535) (Fig. 3-3). Subsequently, the 5' upstream regulatory region of each of these 17 *acdS* genes was cloned by inverse PCR and a 1 kb PCR product was obtained for each strain (Fig. 3-4). The sequence alignments of these 17 5' upstream regions show 95% identities (Fig. 3-5) at the nucleotide level and 96% at the protein level (Fig. 3-6) when compared to the 5' upstream sequences of *acdS* of *Rhizobium leguminosarum* bv. *viciae*, leucine-responsive regulatory-like (*acdR*) protein gene (Accession number: AY172673). Furthermore, all 17 strains contain promoters for both *acdS* and *acdR* as described previously in *Rhizobium leguminosarum* bv. *viciae* (Ma et al. 2003a) including two LRP-binding sites (5'-CGAAAAATTACGCCG-3',

Figure 3-1 Agarose gel electrophoresis of the 16S rDNA PCR products of 27 Rhizobia strains. M: 1 kb DNA ladder; numbers shown above each lane are the number of the Rhizobia strains.


M 2 45 61 62 126 129 130 131 141 142 154 161 162 163 164 165 166 167 168
1400 bp

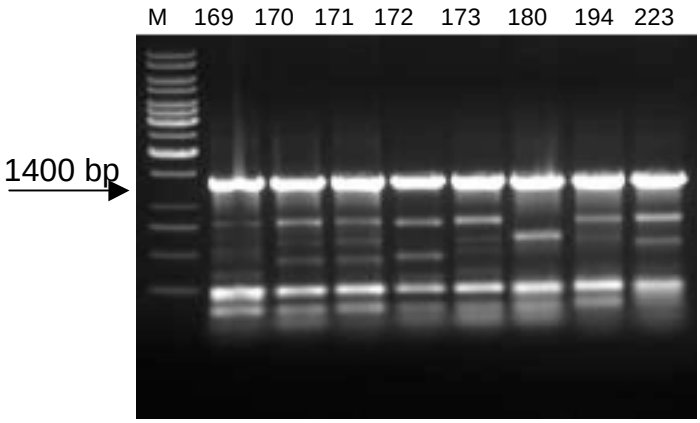

M 169 170 171 172 173 180 194 223
1400 bp

Figure 3-2 Agarose gel electrophoresis of the ACC deaminase gene of 17 Rhizobia strains. M: 1 kb DNA ladder; numbers shown above each lane are the number of the Rhizobia strains.

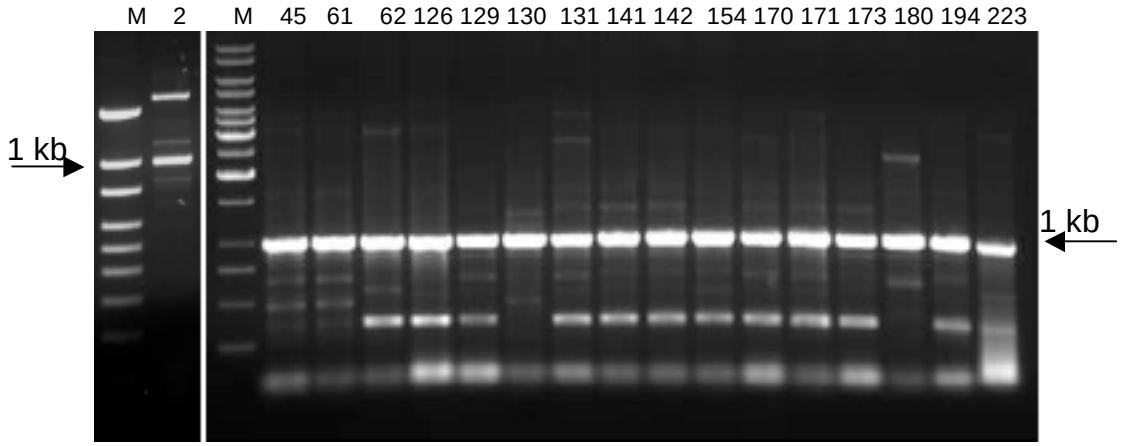
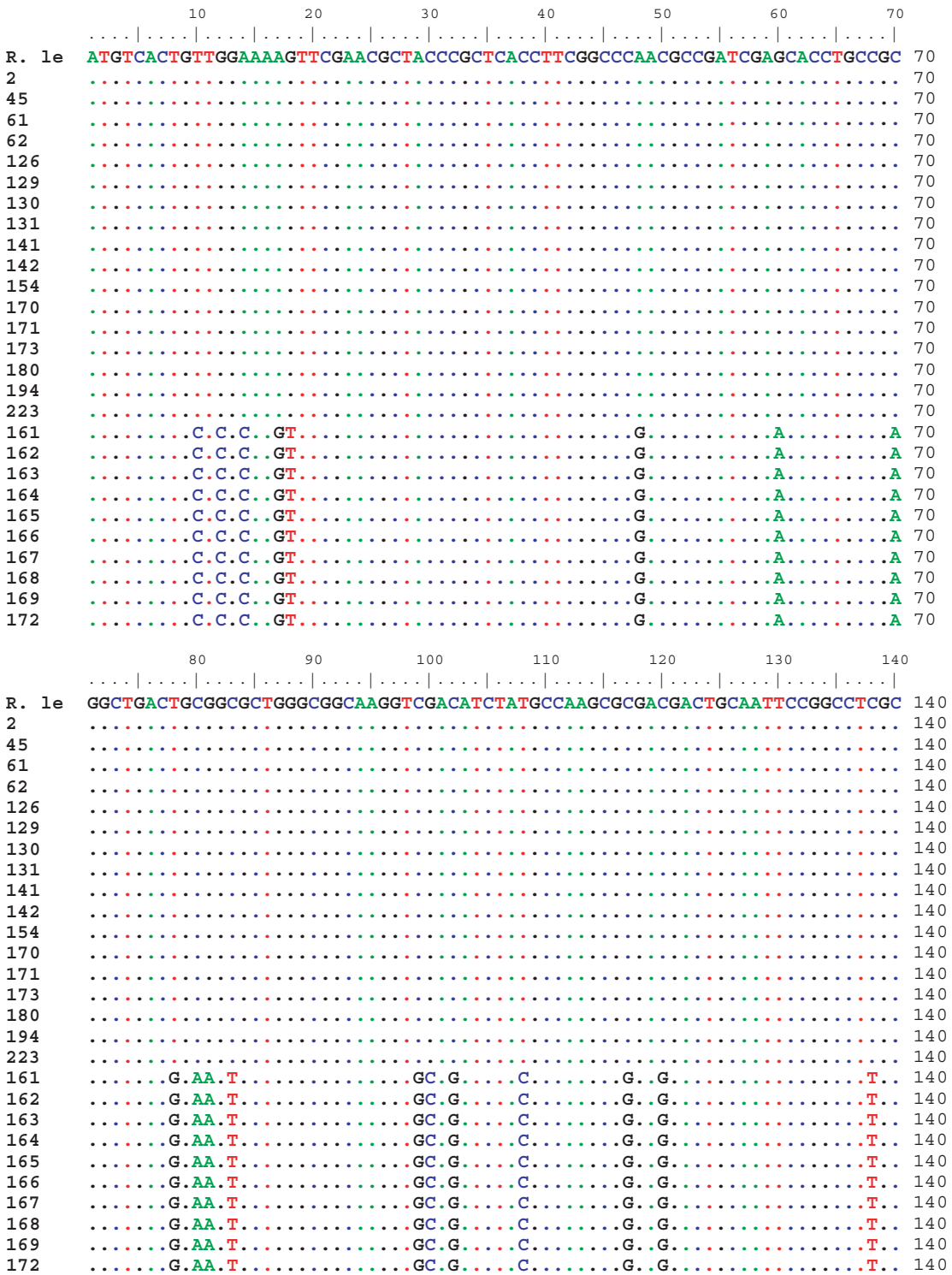

M 2 M 45 61 62 126 129 130 131 141 142 154 170 171 173 180 194 223
1 kb
1 kb

Figure 3-3 Sequence alignments of ACC deaminase gene of 27 Rhizobia strains and the ACC deaminase gene of *Rhizobium leguminosarum* bv. *viciae* (R. le) by ClustalW (Chenna et al. 2003). The alignment was plotted with reference to a standard sequence, i.e. the *acdS* gene of *Rhizobium leguminosarum* bv. *viciae* at the top. Any residues in a column which are identical to the standard at that point are shown as a dot.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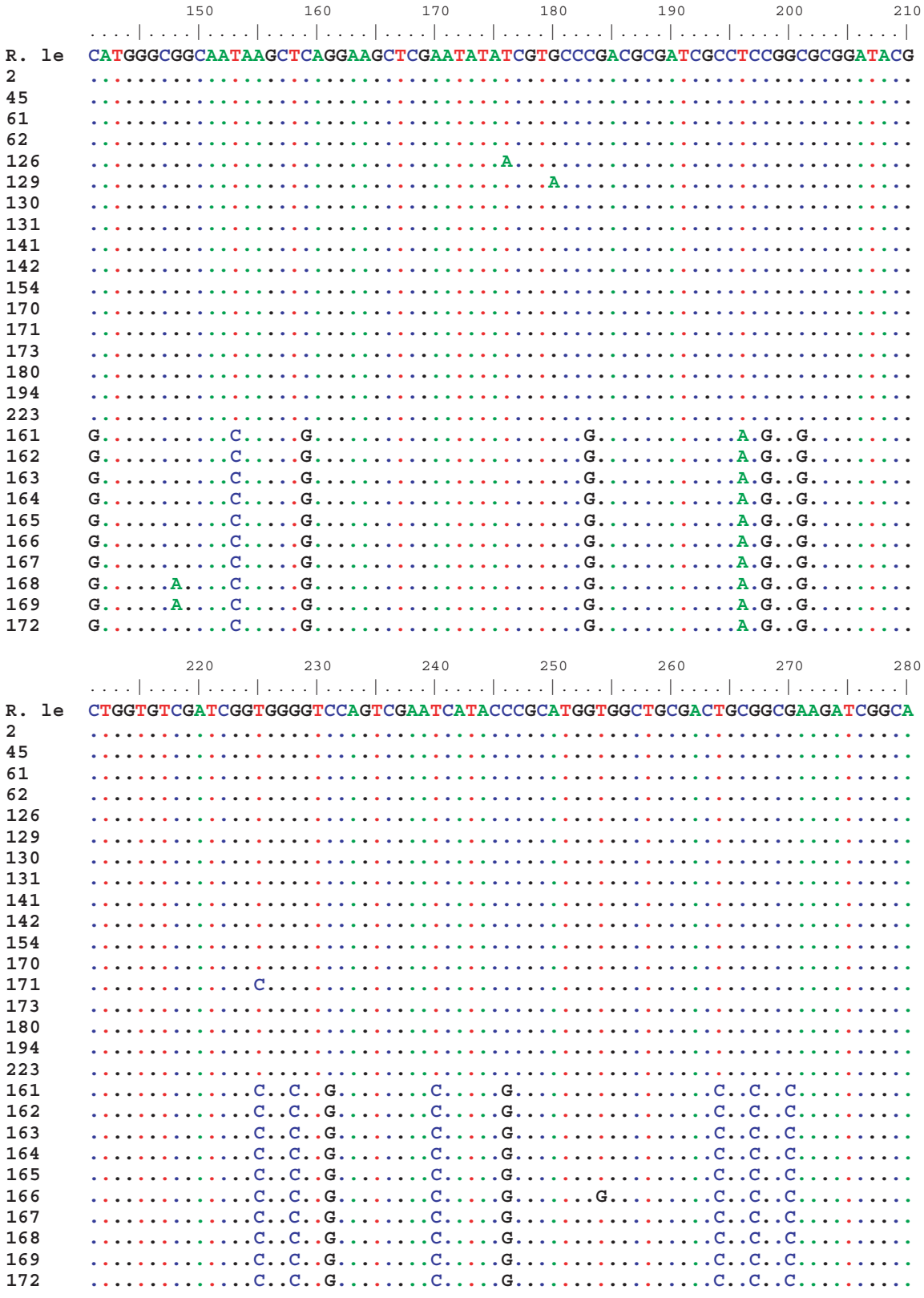
R. le ATGTCACTGTTGGAAAAGTTCGAACGCTACCCGCTCACCTTCGGCCCAACGCCGATCGAGCACCTGCCGC 70
2 70
45 70
61 70
62 70
126 70
129 70
130 70
131 70
141 70
142 70
154 70
170 70
171 70
173 70
180 70
194 70
223 70
161 .....C.C.C..GT.....G.....A.....A 70
162 .....C.C.C..GT.....G.....A.....A 70
163 .....C.C.C..GT.....G.....A.....A 70
164 .....C.C.C..GT.....G.....A.....A 70
165 .....C.C.C..GT.....G.....A.....A 70
166 .....C.C.C..GT.....G.....A.....A 70
167 .....C.C.C..GT.....G.....A.....A 70
168 .....C.C.C..GT.....G.....A.....A 70
169 .....C.C.C..GT.....G.....A.....A 70
172 .....C.C.C..GT.....G.....A.....A 70
80 90 100 110 120 130 140
R. le GGCTGACTGCGGCGCTGGGCGGCAAGGTCGACATCTATGCCAAGCGCGACGACTGCAATTCCGGCCTCGC 140
2 140
45 140
61 140
62 140
126 140
129 140
130 140
131 140
141 140
142 140
154 140
170 140
171 140
173 140
180 140
194 140
223 140
161 .....G.AA.T.....GC.G.....C.....G..G.....T.. 140
162 .....G.AA.T.....GC.G.....C.....G..G.....T.. 140
163 .....G.AA.T.....GC.G.....C.....G..G.....T.. 140
164 .....G.AA.T.....GC.G.....C.....G..G.....T.. 140
165 .....G.AA.T.....GC.G.....C.....G..G.....T.. 140
166 .....G.AA.T.....GC.G.....C.....G..G.....T.. 140
167 .....G.AA.T.....GC.G.....C.....G..G.....T.. 140
168 .....G.AA.T.....GC.G.....C.....G..G.....T.. 140
169 .....G.AA.T.....GC.G.....C.....G..G.....T.. 140
172 .....G.AA.T.....GC.G.....C.....G..G.....T.. 140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R. le CATGGGCGGCAATAAGCTCAGGAAGCTCGAATATATCGTGCCCGACGCGATCGCCTCCGGCGCGGATACG 210
2 210
45 210
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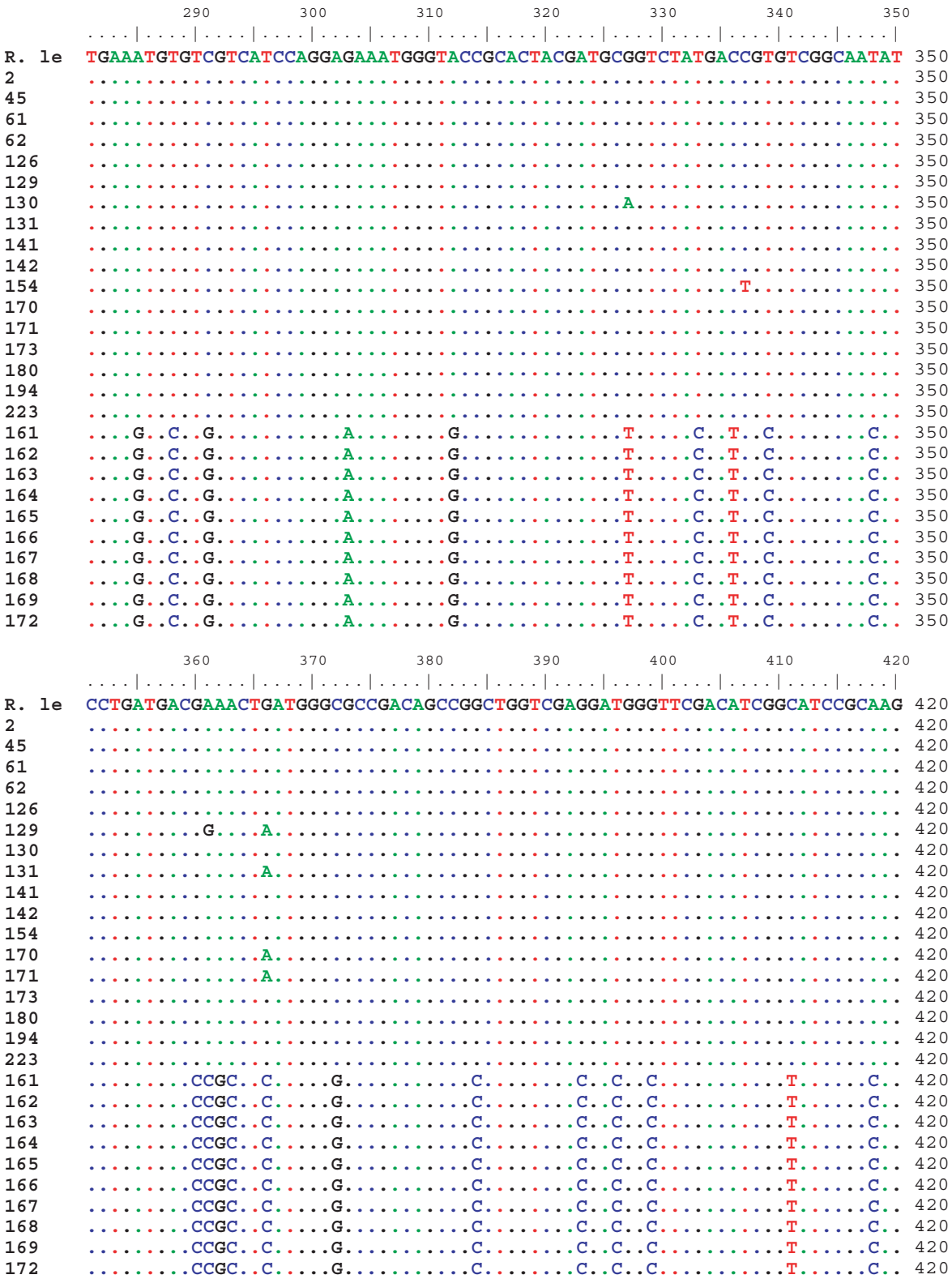
62 210
126 A 210
129 A 210
130 210
131 210
141 210
142 210
154 210
170 210
171 210
173 210
180 210
194 210
223 210
161 G C G G A G 210
162 G C G G A G 210
163 G C G G A G 210
164 G C G G A G 210
165 G C G G A G 210
166 G C G G A G 210
167 G C G G A G 210
168 G A C G G A G 210
169 G A C G G A G 210
172 G C G G A G 210
220 230 240 250 260 270 280
R. le CTGGTGTCGATCGGTGGGGTCCAGTCGAATCATACCCGCATGGTGGCTGCGACTGCGGCGAAGATCGGCA 280
2 280
45 280
61 280
62 280
126 280
129 280
130 280
131 280
141 280
142 280
154 280
170 280
171 C 280
173 280
180 280
194 280
223 280
161 C C G C G C 280
162 C C G C G C 280
163 C C G C G C 280
164 C C G C G C 280
165 C C G C G C 280
166 C C G C G G C 280
167 C C G C G C 280
168 C C G C G C 280
169 C C G C G C 280
172 C C G C G C 280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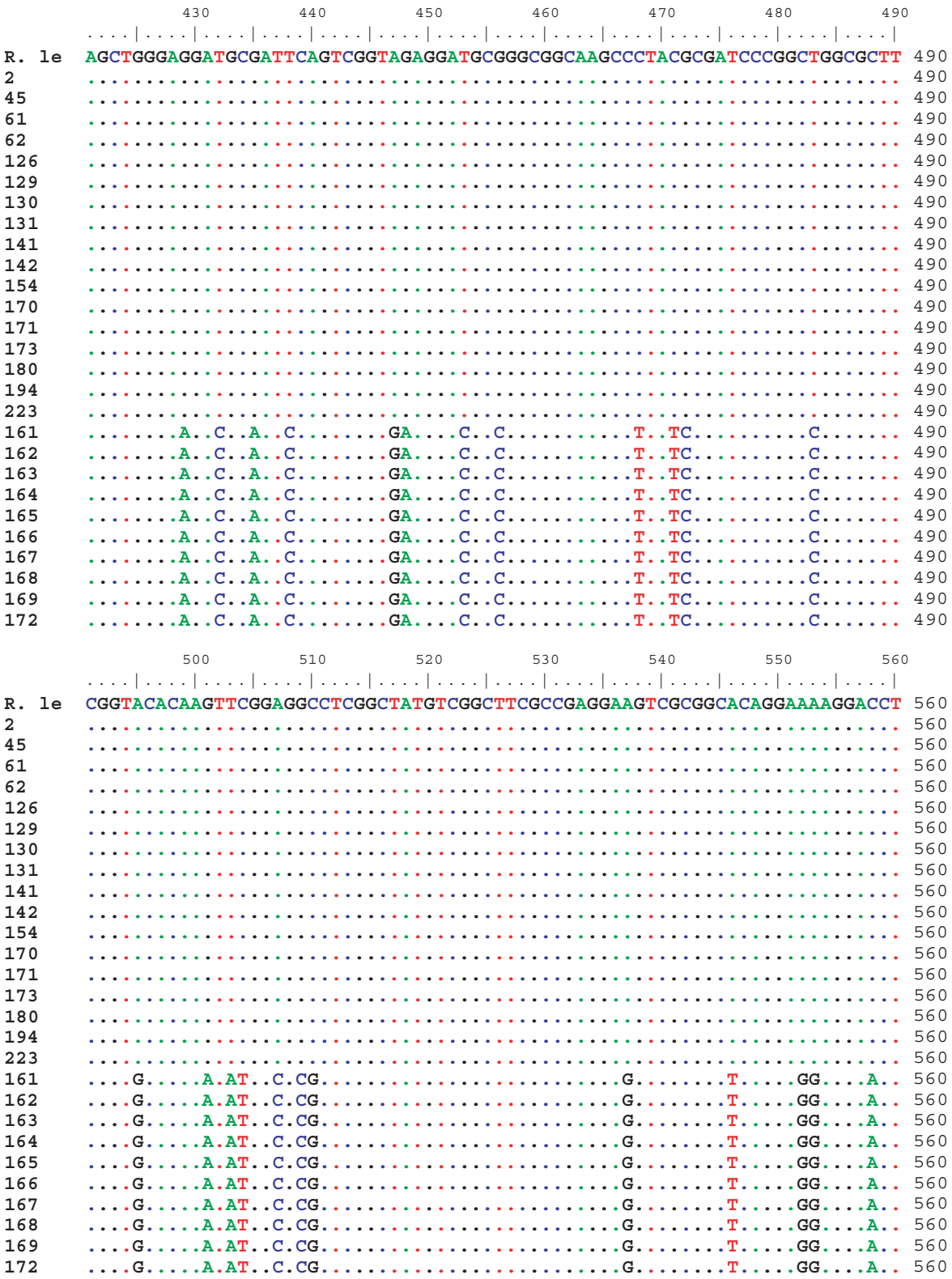
R. le TGAAATGTGTCGTCATCCAGGAGAAATGGGTACCGCACTACGATGCGGTCTATGACCGTGTCGGCAATAT 350
2 350
45 350
61 350
62 350
126 350
129 350
130 A 350
131 350
141 350
142 350
154 T 350
170 350
171 350
173 350
180 350
194 350
223 350
161 G C G A G T C T C C 350
162 G C G A G T C T C C 350
163 G C G A G T C T C C 350
164 G C G A G T C T C C 350
165 G C G A G T C T C C 350
166 G C G A G T C T C C 350
167 G C G A G T C T C C 350
168 G C G A G T C T C C 350
169 G C G A G T C T C C 350
172 G C G A G T C T C C 350
360 370 380 390 400 410 420
R. le CCTGATGACGAAACTGATGGGCGCCGACAGCCGGCTGGTCGAGGATGGGTTCGACATCGGCATCCGCAAG 420
2 420
45 420
61 420
62 420
126 420
129 G A 420
130 420
131 A 420
141 420
142 420
154 420
170 A 420
171 A 420
173 420
180 420
194 420
223 420
161 CCGC C G C C C T C C 420
162 CCGC C G C C C T C C 420
163 CCGC C G C C C T C C 420
164 CCGC C G C C C T C C 420
165 CCGC C G C C C T C C 420
166 CCGC C G C C C T C C 420
167 CCGC C G C C C T C C 420
168 CCGC C G C C C T C C 420
169 CCGC C G C C C T C C 420
172 CCGC C G C C C T C C 420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2 490
45 490
61 490
62 490
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129 490
130 490
131 490
141 490
142 490
154 490
170 490
171 490
173 490
180 490
194 490
223 490
161 .....A..C..A..C.....GA....C..C.....T..TC.....C..... 490
162 .....A..C..A..C.....GA....C..C.....T..TC.....C..... 490
163 .....A..C..A..C.....GA....C..C.....T..TC.....C..... 490
164 .....A..C..A..C.....GA....C..C.....T..TC.....C..... 490
165 .....A..C..A..C.....GA....C..C.....T..TC.....C..... 490
166 .....A..C..A..C.....GA....C..C.....T..TC.....C..... 490
167 .....A..C..A..C.....GA....C..C.....T..TC.....C..... 490
168 .....A..C..A..C.....GA....C..C.....T..TC.....C..... 490
169 .....A..C..A..C.....GA....C..C.....T..TC.....C..... 490
172 .....A..C..A..C.....GA....C..C.....T..TC.....C..... 490
500 510 520 530 540 550 560
R. le CGGTACACAAGTTCGGAGGCCTCGGCTATGTCGGCTTCGCCGAGGAAGTCGCGGCACAGGAAAAGGACCT 560
2 560
45 560
61 560
62 560
126 560
129 560
130 560
131 560
141 560
142 560
154 560
170 560
171 560
173 560
180 560
194 560
223 560
161 ....G.....A.AT..C.CG.....G.....T.....GG....A.. 560
162 ....G.....A.AT..C.CG.....G.....T.....GG....A.. 560
163 ....G.....A.AT..C.CG.....G.....T.....GG....A.. 560
164 ....G.....A.AT..C.CG.....G.....T.....GG....A.. 560
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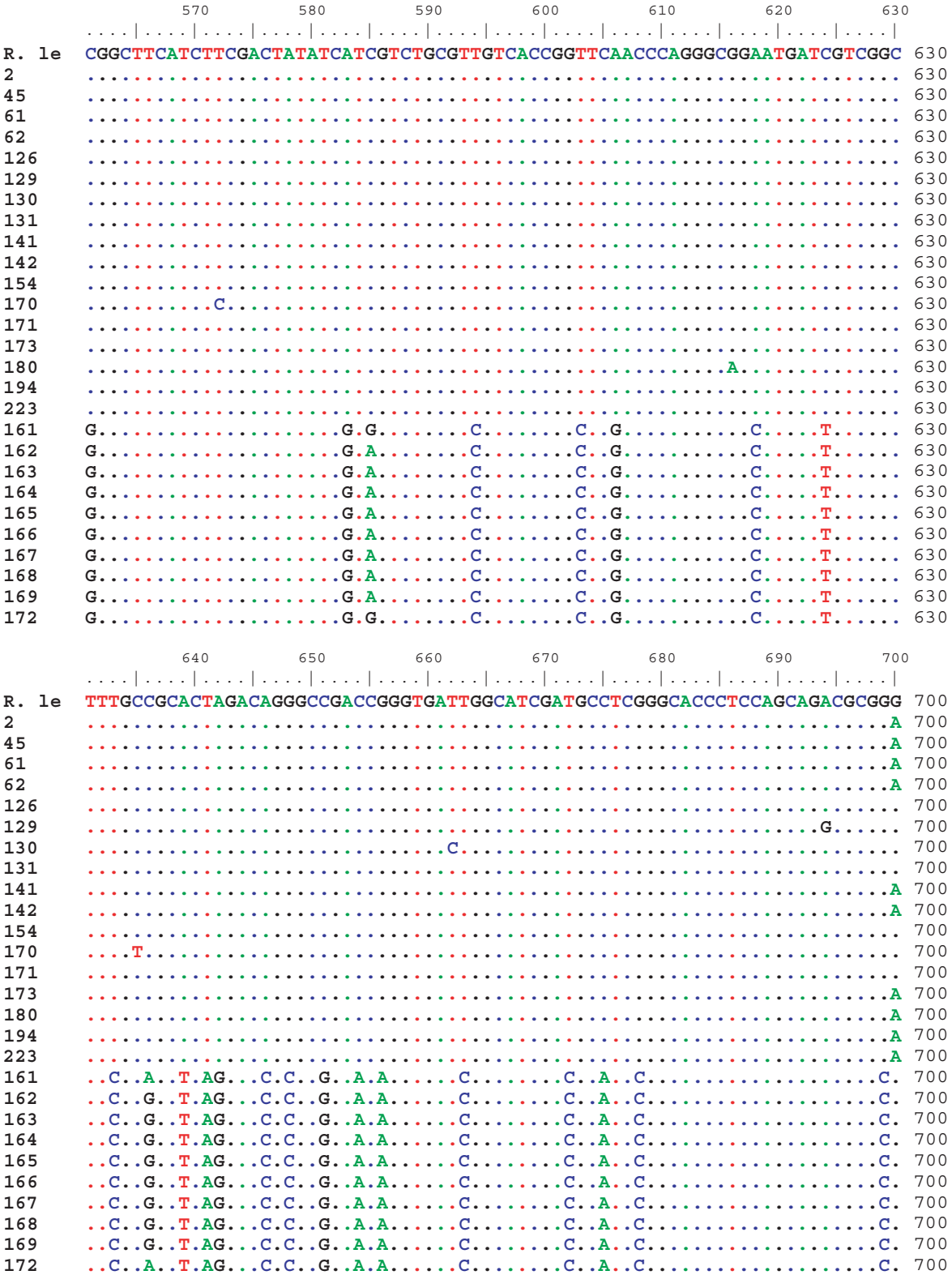
166 ....G.....A.AT..C.CG.....G.....T.....GG....A.. 560
167 ....G.....A.AT..C.CG.....G.....T.....GG....A.. 560
168 ....G.....A.AT..C.CG.....G.....T.....GG....A.. 560
169 ....G.....A.AT..C.CG.....G.....T.....GG....A.. 560
172 ....G.....A.AT..C.CG.....G.....T.....GG....A.. 560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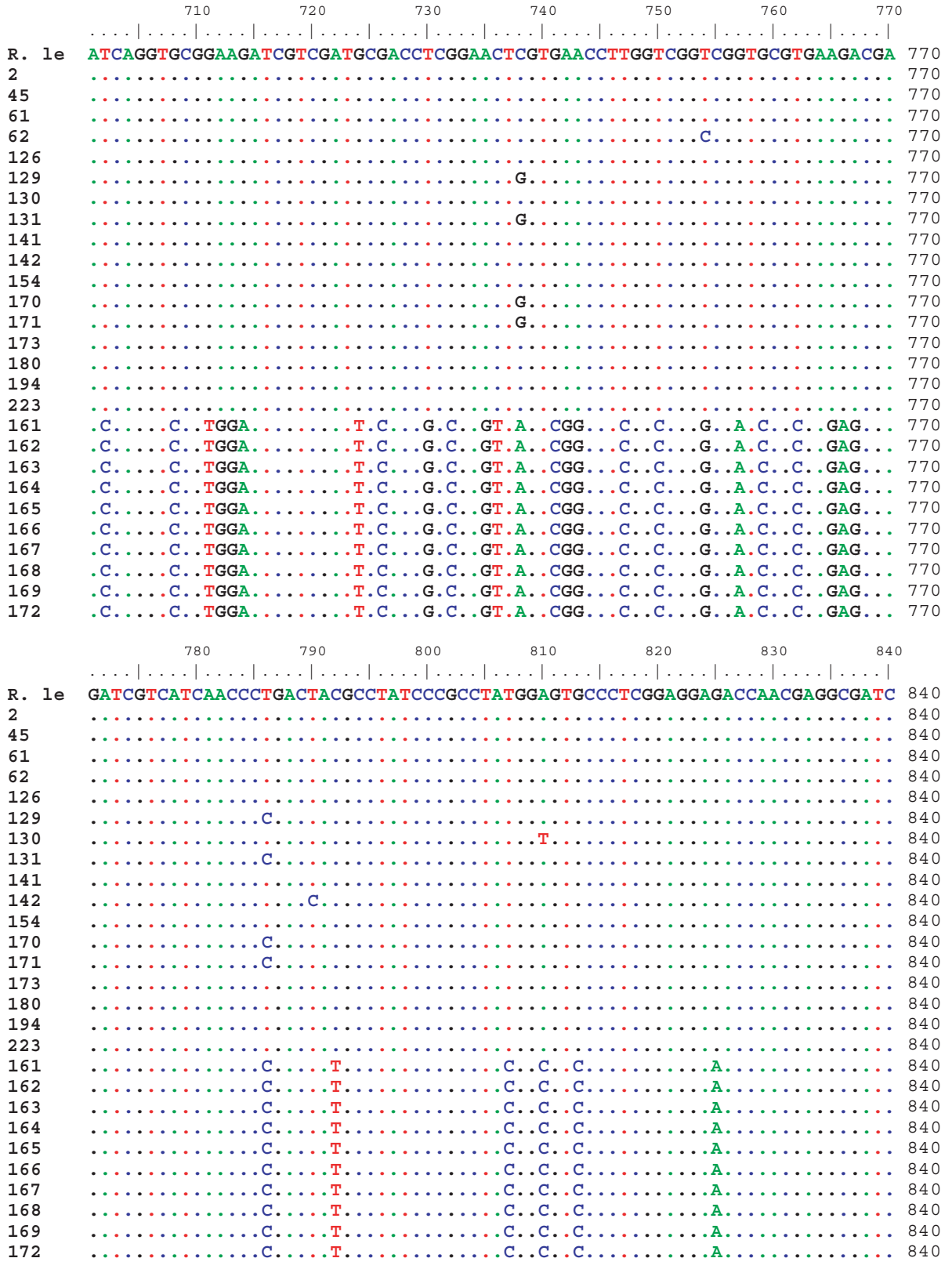
R. le CGGCTTCATCTTCGACTATATCATCGTCTGCGTTGTCACCGGTTCAACCCAGGGCGGAATGATCGTCGGC 630
2 630
45 630
61 630
62 630
126 630
129 630
130 630
131 630
141 630
142 630
154 630
170 630
171 630
173 630
180 630
194 630
223 630
161 630
162 630
163 630
164 630
165 630
166 630
167 630
168 630
169 630
172 630
640 650 660 670 680 690 700
R. le TTTGCCGCACTAGACAGGGCCGACCGGGTGATTGGCATCGATGCCTCGGGCACCCTCCAGCAGACGCGGG 700
2 700
45 700
61 700
62 700
126 700
129 700
130 700
131 700
141 700
142 700
154 700
170 700
171 700
173 700
180 700
194 700
223 700
161 700
162 700
163 700
164 700
165 700
166 700
167 700
168 700
169 700
172 700


710 720 730 740 750 760 770
R. le ATCAGGTGCGGAAGATCGTCGATGCGACCTCGGAACTCGTGAACCTTGGTCGGTCGGTGCGTGAAGACGA 770
2 770
45 770
61 770
62 C 770
126 770
129 G 770
130 770
131 G 770
141 770
142 770
154 770
170 G 770
171 G 770
173 770
180 770
194 770
223 770
161 .C....C..TGGA.....T.C...G.C..GT.A..CGG...C..C...G..A.C..C..GAG... 770
162 .C....C..TGGA.....T.C...G.C..GT.A..CGG...C..C...G..A.C..C..GAG... 770
163 .C....C..TGGA.....T.C...G.C..GT.A..CGG...C..C...G..A.C..C..GAG... 770
164 .C....C..TGGA.....T.C...G.C..GT.A..CGG...C..C...G..A.C..C..GAG... 770
165 .C....C..TGGA.....T.C...G.C..GT.A..CGG...C..C...G..A.C..C..GAG... 770
166 .C....C..TGGA.....T.C...G.C..GT.A..CGG...C..C...G..A.C..C..GAG... 770
167 .C....C..TGGA.....T.C...G.C..GT.A..CGG...C..C...G..A.C..C..GAG... 770
168 .C....C..TGGA.....T.C...G.C..GT.A..CGG...C..C...G..A.C..C..GAG... 770
169 .C....C..TGGA.....T.C...G.C..GT.A..CGG...C..C...G..A.C..C..GAG... 770
172 .C....C..TGGA.....T.C...G.C..GT.A..CGG...C..C...G..A.C..C..GAG... 770
780 790 800 810 820 830 840
R. le GATCGTCATCAACCCTGACTACGCCTATCCCGCCTATGGAGTGCCCTCGGAGGAGACCAACGAGGCGATC 840
2 840
45 840
61 840
62 840
126 840
129 C 840
130 T 840
131 C 840
141 840
142 C 840
154 840
170 C 840
171 C 840
173 840
180 840
194 840
223 840
161 .....C.....T.....C..C..C.....A..... 840
162 .....C.....T.....C..C..C.....A..... 840
163 .....C.....T.....C..C..C.....A..... 840
164 .....C.....T.....C..C..C.....A..... 840
165 .....C.....T.....C..C..C.....A..... 840
166 .....C.....T.....C..C..C.....A..... 840
167 .....C.....T.....C..C..C.....A..... 840
168 .....C.....T.....C..C..C.....A..... 840
169 .....C.....T.....C..C..C.....A..... 840
172 .....C.....T.....C..C..C.....A..... 840

```
                850       860       870       880       890       900       910
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
R. le   CGGCTCGCGGCGCGCACCGAGGCGATGATTACCGACCCAGTCTATGAGGGAAAGTCGATGCAGGGGATGA 910
2       .............................................................. 910
45      .............................................................. 910
61      .............................................................. 910
62      .............................................................. 910
126     ............................T..................................... 910
129     .............................................................. 910
130     ...........................T.................................... 910
131     .............................................................. 910
141     .............................................................. 910
142     .............................................................. 910
154     .............................................................. 910
170     ..............G............................................... 910
171     .............................................................. 910
173     .............................................................. 910
180     .............................................................. 910
194     .............................................................. 910
223     .............................................................. 910
161     ..C.....C..A..T.....A........C.....T..C...........C..A..C..... 910
162     ..C.....C..A..T.....A........C.....T..C...........C..A..C..... 910
163     ..C.....C..A..T.....A........C.....T..C...........C..A..C..... 910
164     ..C.....C..A..T.....A........C.....T..C...........C..A..C..... 910
165     ..C.....C..A..T.....A........C.....T..C...........C..A..C..... 910
166     ..C.....C..A..T.....A........C.....T..C...........C..A..C..... 910
167     ..C.....C..A..T.....A........C.....T..C...........C..A..C..... 910
168     ..C.....C..A..T.....A........C.....T..C...........C..A..C..... 910
169     ..C.....C..A..T.....A........C.....T..C...........C..A..C..... 910
172     ..C.....C..A..T.....A........C.....T..C...........C..A..C..... 910

                920       930       940       950       960       970       980
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
R. le   TCGATCTCGCACGCAAGGGCTTCTTTCCCGAGGGCTCGAAGGTGCTGTACGCCCATCTCGGCGGCGCCCC 980
2       ......................C.C....................................... 980
45      ......................C........................................ 980
61      ......................C........................................ 980
62      ......................C.......................C................ 980
126     ......................C........................................ 980
129     ......................C........................................ 980
130     ......................C........................................ 980
131     ......................C........................................ 980
141     ......................C........................................ 980
142     ......................C........................................ 980
154     ......................C........................................ 980
170     ......................C........................................ 980
171     ......................C........................................ 980
173     ......................C........................................ 980
180     ......................C........................................ 980
194     ......................C........................................ 980
223     ......................C........................................ 980
161     ........A.C............C.....................C..............G....GA.. 980
162     ........A.C............C.....................C..............G..... 980
163     ........A.C............C.....................C..............G..... 980
164     ........A.C............C.....................C..............G..... 980
165     ........A.C............C.....................C..............G..... 980
166     ........A.C............C.....................C..............G..... 980
167     ........A.C............C.....................C..............G..... 980
168     ........A.C............C.....................C..............G..... 980
169     ........A.C............C.....................C..............G..... 980
172     ........A.C............C.....................C..............G..... 980
```

```
                990         1000        1010        1020
        ....|....|....|....|....|....|....|....|
R. le   GGCGCTCAACGGCTATAGCTATTACTACAAGGACGGGTGA 1020
2       ........................................ 1020
45      ........................................ 1020
61      ........................................ 1020
62      ........................................ 1020
126     ........................................ 1020
129     ........................................ 1020
130     ........................................ 1020
131     ........................................ 1020
141     ........................................ 1020
142     ........................................ 1020
154     ........................................ 1020
170     ........................................ 1020
171     ........................................ 1020
173     ........................................ 1020
180     ........................................ 1020
194     ........................................ 1020
223     ........................................ 1020
161     .........T.....C........T...........C... 1020
162     .........T.....C........T...........C... 1020
163     .........T.....C........T...........C... 1020
164     .........T.....C........T...........C... 1020
165     .........T.....C........T...........C... 1020
166     .........T.....C........T...........C... 1020
167     .........T.....C........T...........C... 1020
168     .........T.....C........T...........C... 1020
169     .........T.....C........T...........C... 1020
172     .........T.....C........T...........C... 1020
```

Figure 3-4 Agarose gel electrophoresis of the PCR amplified 5' upstream regulatory region of the ACC deaminase genes from 17 Rhizobia strains. M: 1 kb DNA ladder; numbers shown above each lane are the number of the Rhizobia strains.

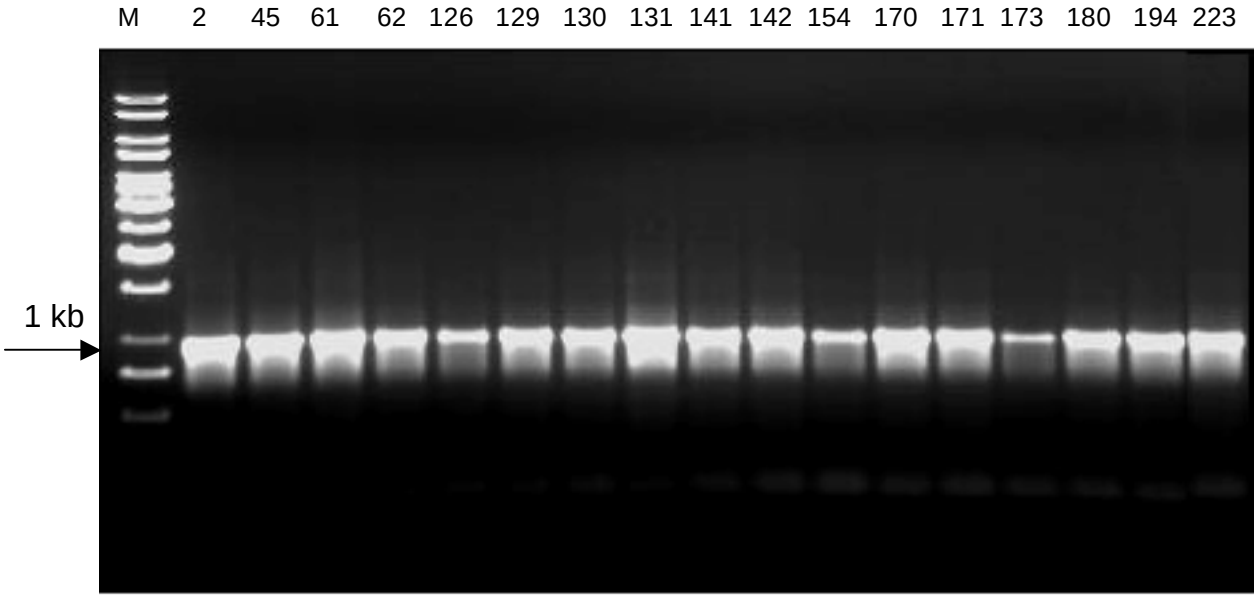
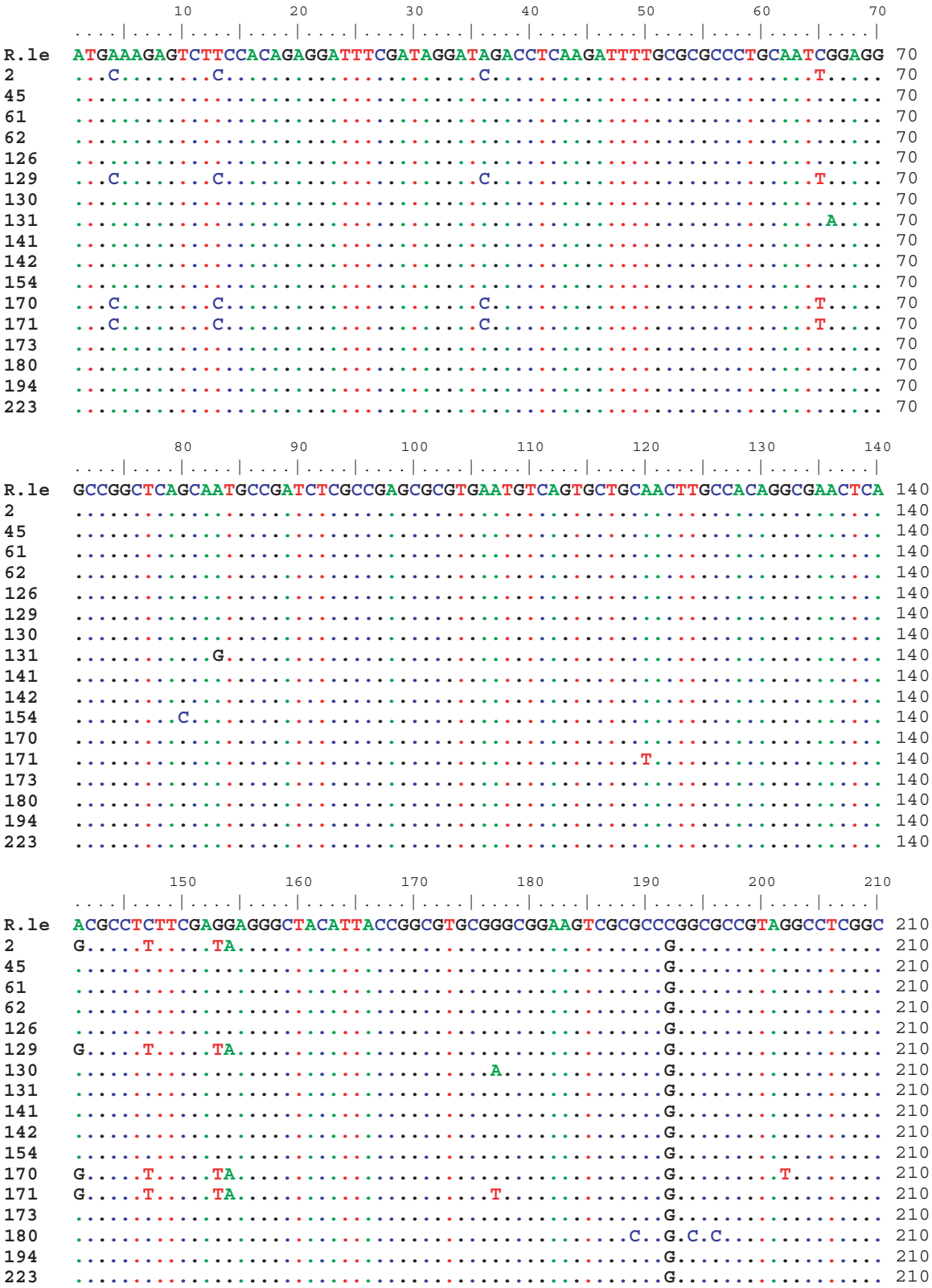
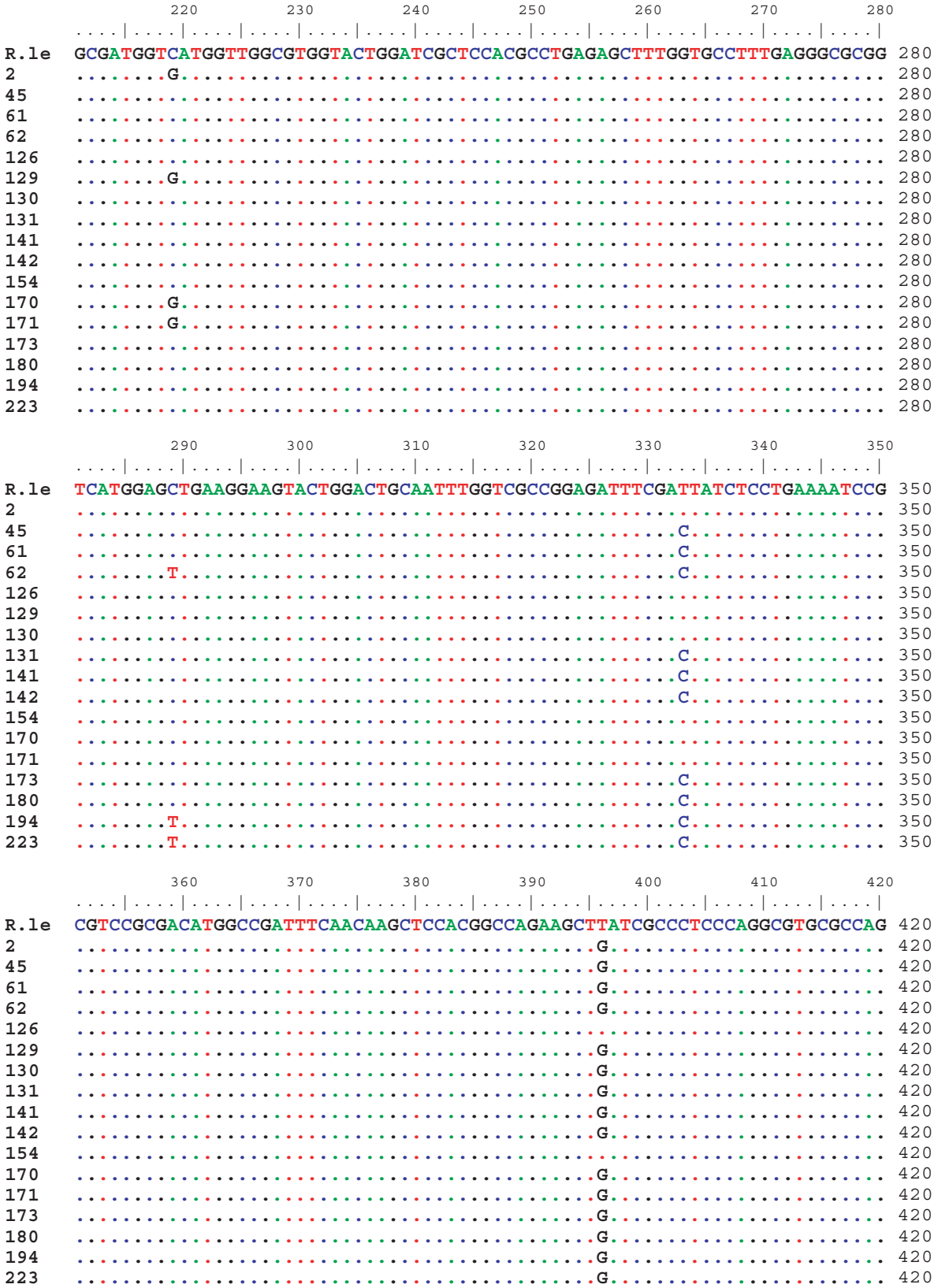

M 2 45 61 62 126 129 130 131 141 142 154 170 171 173 180 194 223
1 kb

Figure 3-5 Sequence alignments of upstream regulatory region of ACC deaminase gene of 17 Rhizobia strains and *Rhizobium leguminosarum* bv. *viciae* (R. le) by ClustalW (Chenna et al. 2003).


10 20 30 40 50 60 70
R.le ATGAAAGAGTCTTCCACAGAGGATTTCGATAGGATAGACCTCAAGATTTTGCGCGCCCTGCAATCGGAGG 70
2 ...C.....C.....C.....T..... 70
45 70
61 70
62 70
126 70
129 ...C.....C.....C.....T..... 70
130 70
131 .....A.... 70
141 70
142 70
154 70
170 ...C.....C.....C.....T..... 70
171 ...C.....C.....C.....T..... 70
173 70
180 70
194 70
223 70
80 90 100 110 120 130 140
R.le GCCGGCTCAGCAATGCCGATCTCGCCGAGCGCGTGAATGTCAGTGCTGCAACTTGCCACAGGCGAACTCA 140
2 140
45 140
61 140
62 140
126 140
129 140
130 140
131 .....G..... 140
141 140
142 140
154 .....C..... 140
170 140
171 .....T..... 140
173 140
180 140
194 140
223 140
150 160 170 180 190 200 210
R.le ACGCCTCTTCGAGGAGGGCTACATTACCGGCGTGCGGGCGGAAGTCGCGCCCGGCGCCGTAGGCCTCGGC 210
2 G.....T.....TA.....G..... 210
45 .....G..... 210
61 .....G..... 210
62 .....G..... 210
126 .....G..... 210
129 G.....T.....TA.....G..... 210
130 .....A.....G..... 210
131 .....G..... 210
141 .....G..... 210
142 .....G..... 210
154 .....G..... 210
170 G.....T.....TA.....G.....T..... 210
171 G.....T.....TA.....T.....G..... 210
173 .....G..... 210
180 .....C..G.C.C..... 210
194 .....G..... 210
223 .....G..... 210


220 230 240 250 260 270 280
R.le GCGATGGTCATGGTTGGCGTGGTACTGGATCGCTCCACGCCTGAGAGCTTTGGTGCCTTTGAGGGCGCGG 280
2 .....G..... 280
45 280
61 280
62 280
126 280
129 .....G..... 280
130 280
131 280
141 280
142 280
154 280
170 .....G..... 280
171 .....G..... 280
173 280
180 280
194 280
223 280
290 300 310 320 330 340 350
R.le TCATGGAGCTGAAGGAAGTACTGGACTGCAATTTGGTCGCCGGAGATTTCGATTATCTCCTGAAAATCCG 350
2 350
45 .....C..... 350
61 .....C..... 350
62 .....T.....C..... 350
126 350
129 350
130 350
131 .....C..... 350
141 .....C..... 350
142 .....C..... 350
154 350
170 350
171 350
173 .....C..... 350
180 .....C..... 350
194 .....T.....C..... 350
223 .....T.....C..... 350
360 370 380 390 400 410 420
R.le CGTCCGCGACATGGCCGATTTCAACAAGCTCCACGGCCAGAAGCTTATCGCCCTCCCAGGCGTGCGCCAG 420
2 .....G..... 420
45 .....G..... 420
61 .....G..... 420
62 .....G..... 420
126 420
129 .....G..... 420
130 .....G..... 420
131 .....G..... 420
141 .....G..... 420
142 .....G..... 420
154 420
170 .....G..... 420
171 .....G..... 420
173 .....G..... 420
180 .....G..... 420
194 .....G..... 420
223 .....G..... 420

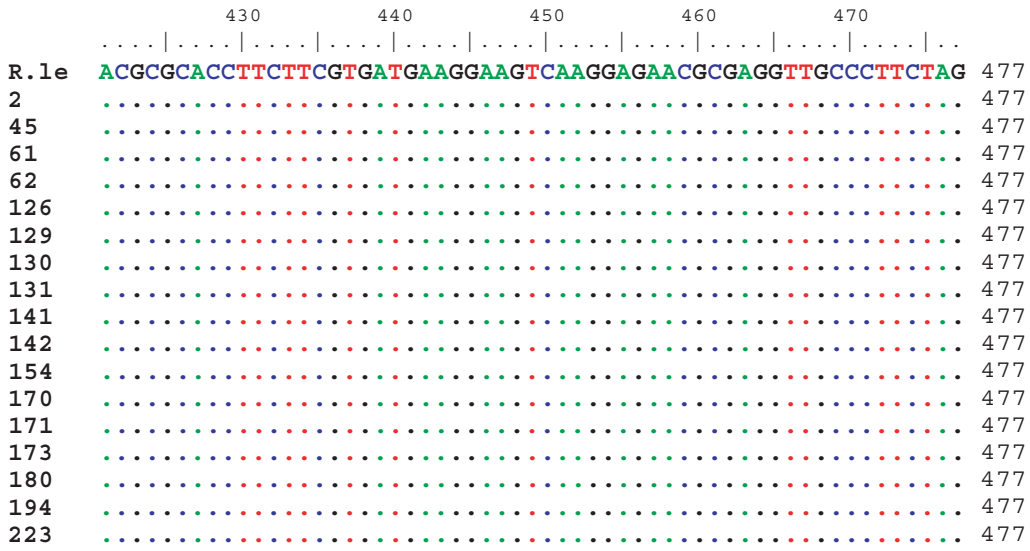
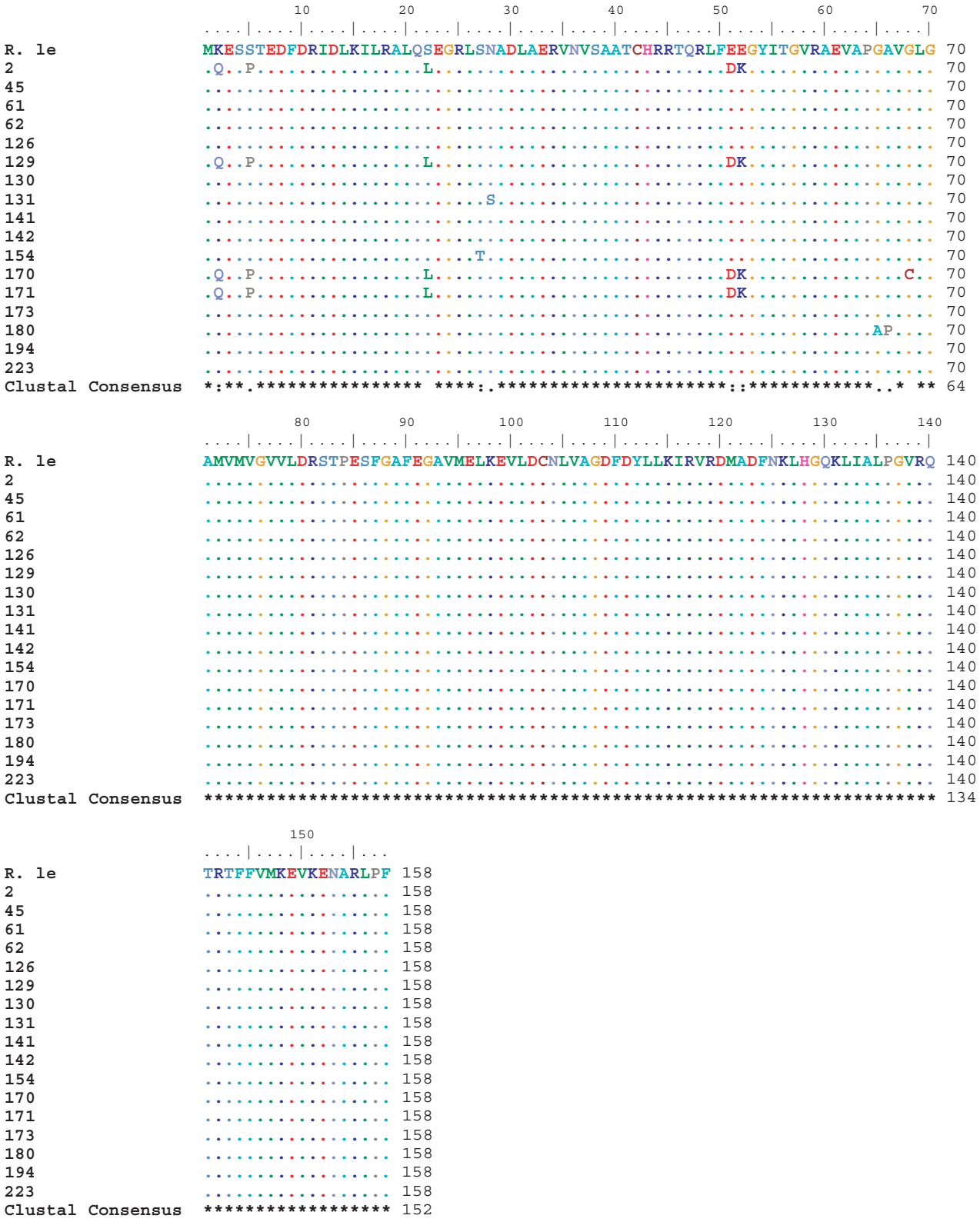

430
440
450
460
470
R.le ACGCGCACCTTCTTCGTGATGAAGGAAGTCAAGGAGAACGCGAGGTTGCCCTTCTAG 477
2 477
45 477
61 477
62 477
126 477
129 477
130 477
131 477
141 477
142 477
154 477
170 477
171 477
173 477
180 477
194 477
223 477

Figure 3-6 Sequence alignments of upstream regulatory region of ACC deaminase gene of 17 Rhizobia strains and *Rhizobium leguminosarum* bv. *viciae* (R. le) at protein level by ClustalW (Chenna et al. 2003). Identities are 96%. Complete conserved residues are indicated by "*". Conserved substitutions and semi-conserved substitutions are indicated by ":" and ".", respectively.


10 20 30 40 50 60 70
R. le MKESSTEDFDRIDLKILRALQSEGRLSNADLAERVNVSAATCHRRTQRLFEEGYITGVRAEVAPGAVGLG 70
2 .Q..P.....L.....DK..... 70
45 70
61 70
62 70
126 70
129 .Q..P.....L.....DK..... 70
130 70
131 .....S..... 70
141 70
142 70
154 .....T..... 70
170 .Q..P.....L.....DK.....C.. 70
171 .Q..P.....L.....DK..... 70
173 70
180 .....AP.... 70
194 70
223 70
Clustal Consensus *:**.**************** ****:.**********************::************..* ** 64
80 90 100 110 120 130 140
R. le AMVMVGVVLDRSTPESFGAFEGAVMELKEVLDCNLVAGDFDYLLKIRVRDMADFNKLHGQKLIALPGVRQ 140
2 140
45 140
61 140
62 140
126 140
129 140
130 140
131 140
141 140
142 140
154 140
170 140
171 140
173 140
180 140
194 140
223 140
Clustal Consensus ** 134
150
R. le TRTFFVMKEVKENARLPF 158
2 158
45 158
61 158
62 158
126 158
129 158
130 158
131 158
141 158
142 158
154 158
170 158
171 158
173 158
180 158
194 158
223 158
Clustal Consensus ****************** 152

G at 3' is located at -93 and 5'-AAGCAAAATTAGA-3', A at 3' is located at -64). DNA sequences between the *acdS* and *acdR* genes of these 17 strains had 96% identities and the changed nucleotides were not found in the conserved binding sites or the predicted promoter regions.

Inverse PCR was performed for the ten remaining 10 Rhizobia strains including strains 161, 162, 163, 164, 165, 166, 167, 168, 169 and 172. An approximately 1.8 kb PCR product was obtained for all 10 strains (Fig. 3-7). There was also a longer band of about 2 kb that was amplified for 8 of the 10 strains, i.e. not for 165 and 167. Subsequent sequence analysis indicated that the 2 kb band was obtained due to nonspecific binding between template and primers. Based on the sequencing result obtained from one strain, i.e. 163, a new set of primers, 5'-CCGTGCCGAATTTGTGGTC-3' and 5'-CGAACGCTACCCGCTCACCT-3', were designed to PCR amplify the upstream regulatory region as well as ACC deaminase gene. The resulting PCR product is approximately 2.4 kb for these 10 strains (Fig. 3-8) and the multiple sequence alignments of these 10 sequences shows that they are 91% identical to each other. When these sequences were compared to the known *acdS* gene sequence of *Rhizobium leguminosarum* bv. *viciae* and the *acdS* gene sequence of the other 17 Rhizobia strains used in this study, the DNA sequences of these 10 genes are 83% identical and are 94% identical at the protein level (Fig 3-3, Fig 3-9), indicating that most changes are third position codon changes. An examination of the upstream region of the *acdS* gene for these 10 strains indicates that they all contain a leucine-responsive regulatory-like protein gene, but only 295 bp are comparable to the *acdR* found upstream of *acdS* of *Rhizobium leguminosarum* bv. *viciae* and the identities are 80%. Although the identity of overall alignment is high, there is one gap where a C (at position 75 of *Rhizobium leguminosarum* bv. *viciae* leucine-responsive regulatory-like protein gene) was deleted in the

sequence alignments for all the 10 strains. The deduced amino acid sequence showed that these 10 strains contain a partial *acdR* (only 26 amino acids). Compared to *acdR* in *R. leguminosarum* bv. *viciae* 128C53K, they have 88% identities and 92% similarities. The deletion of the nucleotide changed the reading frame of the protein (Fig 3-10), suggesting that the *acdR* in the 10 strains might not be functional.

3.4 The *acdS* gene copy number

Southern hybridization was used to estimate the ACC deaminase gene copy number in each of the 27 Rhizobia strains. Rhizobia genomic DNA was digested with *Eco*RV which does not cut within the sequenced *acdS* genes. For the 17 similar strains, the digested genomic DNA was probed with the *acdS* gene from strain number 45. For the other 10 strains, the digested genomic DNA was probed with the *acdS* gene form strain number 163 (Fig. 3-11). The results show that strains 45, 61, 129, 131, 141, 142, 170, 171, 173, 180 and 194 all yielded a single band of about 3500 bp while strains 62, 126, 130, 154 and 223 yielded a single band of about 1500 bp. Strain 2 showed a single band at around 3600 bp, while strains 161, 162, 163, 164, 165, 166, 167, 168, 169 and 172 showed a single band of about 3000 bp.

3.5 Phylogenetic analysis of *acdS* gene

The topology of the 16S rRNA tree (Fig 3-12A) follows a predicted taxonomy. *Rhizobium* spp. which belong to the phylum *Proteobacteria,* class *Alphaproteobacteria* are grouped together. Members of the class *Gammaproteobacteria, Pseudomonas, Enterobacter* and *Serratia* are also grouped together. More distant are the order Burkholderiales including *Variovorax, Acidovorax, Achromobacter* and *Burkholderia*.

Figure 3-7 Agarose gel electrophoresis of the inverse PCR from 10 Rhizobia strains. M: 1 kb DNA ladder; numbers shown above each lane are the number of the Rhizobia strains.

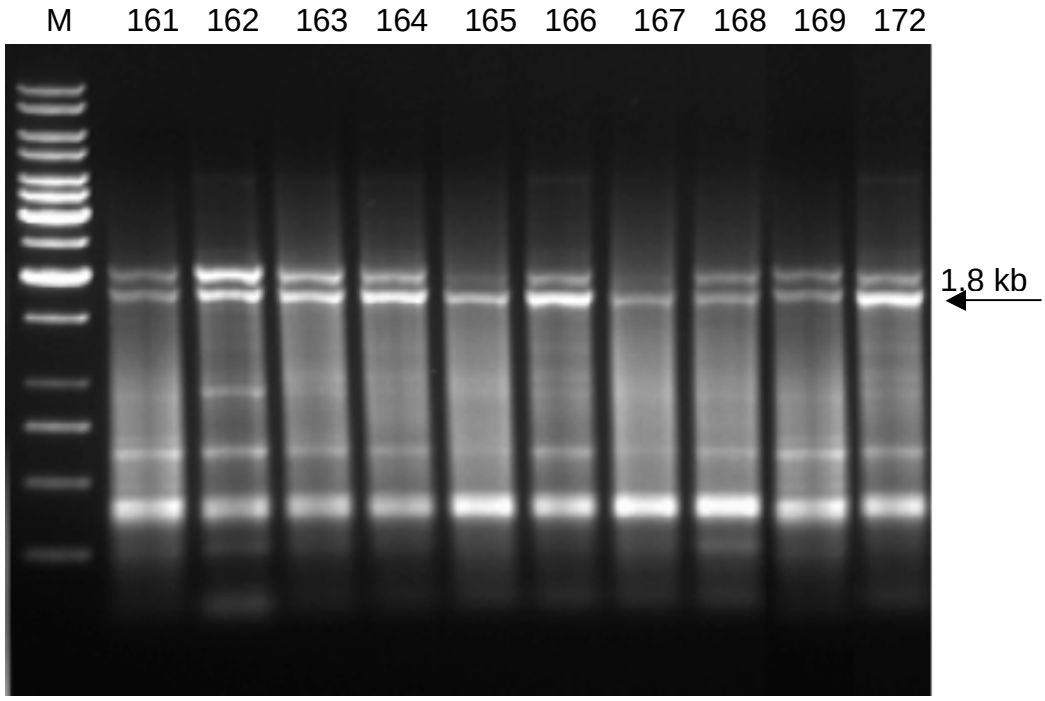

M
161
162
163
164
165
166
167
168
169
172
1.8 kb

Figure 3-8 Agarose gel electrophoresis of the ACC deaminase gene and its upstream regulatory region from 10 Rhizobia strains. M: 1 kb DNA ladder; numbers shown above each lane are the number of the Rhizobia strains.

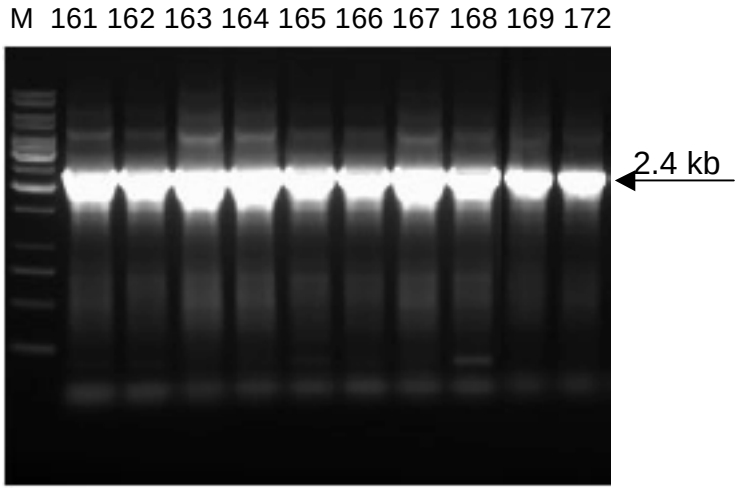
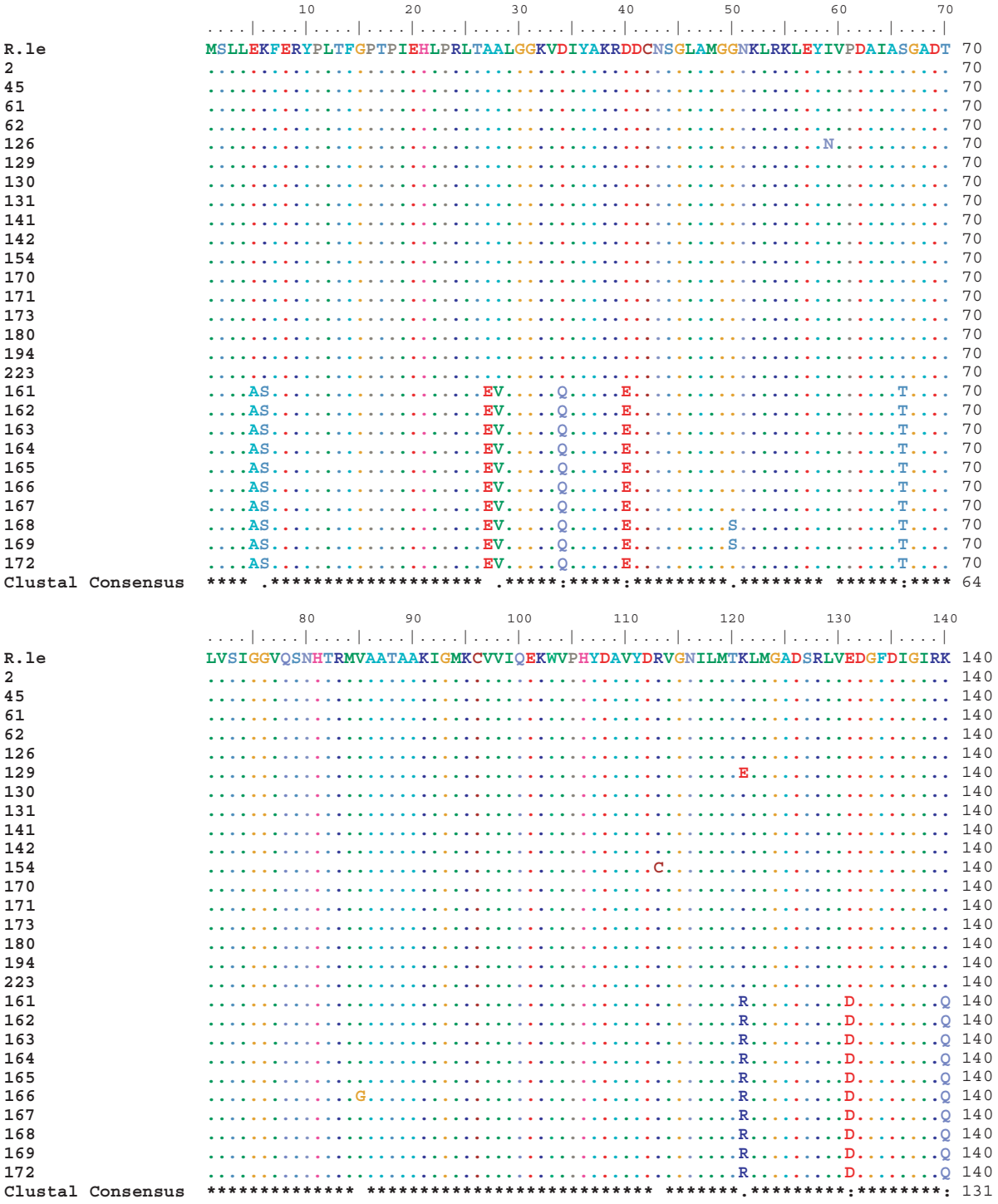
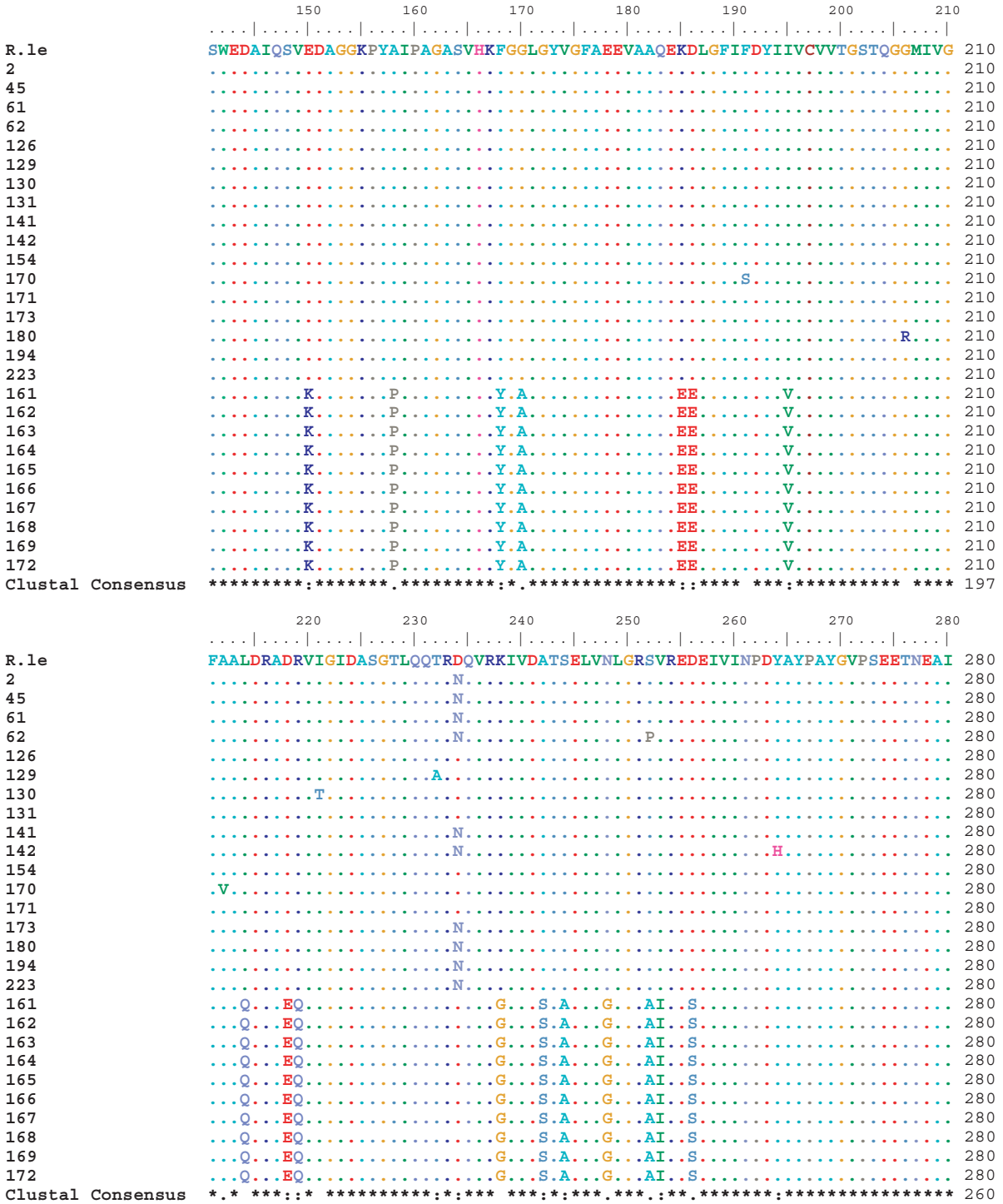

M 161 162 163 164 165 166 167 168 169 172
2.4 kb

Figure 3-9 ACC deaminase amino acid sequence alignments for all 27 strains, using ACC deaminase protein of *Rhizobium leguminosarum* bv. *viciae* as a reference.


10 20 30 40 50 60 70
R.le MSLLEKFERYPLTFGPTPIEHLPRLTAALGGKVDIYAKRDDCNSGLAMGGNKLRKLEYIVPDAIASGADT 70
2 70
45 70
61 70
62 70
126 .....N..... 70
129 70
130 70
131 70
141 70
142 70
154 70
170 70
171 70
173 70
180 70
194 70
223 70
161 ....AS.....EV.....Q.....E.....T.... 70
162 ....AS.....EV.....Q.....E.....T.... 70
163 ....AS.....EV.....Q.....E.....T.... 70
164 ....AS.....EV.....Q.....E.....T.... 70
165 ....AS.....EV.....Q.....E.....T.... 70
166 ....AS.....EV.....Q.....E.....T.... 70
167 ....AS.....EV.....Q.....E.....T.... 70
168 ....AS.....EV.....Q.....E.....S.....T.... 70
169 ....AS.....EV.....Q.....E.....S.....T.... 70
172 ....AS.....EV.....Q.....E.....T.... 70
Clustal Consensus **** .********** .*****:*****:*********.******** ******:**** 64
80 90 100 110 120 130 140
R.le LVSIGGVQSNHTRMVAATAAKIGMKCVVIQEKWVPHYDAVYDRVGNILMTKLMGADSRLVEDGFDIGIRK 140
2 140
45 140
61 140
62 140
126 140
129 .....E..... 140
130 140
131 140
141 140
142 140
154 .....C..... 140
170 140
171 140
173 140
180 140
194 140
223 140
161 .....R.....D.....Q 140
162 .....R.....D.....Q 140
163 .....R.....D.....Q 140
164 .....R.....D.....Q 140
165 .....R.....D.....Q 140
166 ....G.....R.....D.....Q 140
167 .....R.....D.....Q 140
168 .....R.....D.....Q 140
169 .....R.....D.....Q 140
172 .....R.....D.....Q 140
Clustal Consensus ********** ********** ********** ********** ******* ********.*********:*********: 131


150 160 170 180 190 200 210
R.le SWEDAIQSVEDAGGKPYAIPAGASVHKFGGLGYVGFAEEVAAQEKDLGFIFDYIIVCVVTGSTQGGMIVG 210
2 210
45 210
61 210
62 210
126 210
129 210
130 210
131 210
141 210
142 210
154 210
170 210
171 210
173 210
180 210
194 210
223 210
161 210
162 210
163 210
164 210
165 210
166 210
167 210
168 210
169 210
172 210
Clustal Consensus 197
220 230 240 250 260 270 280
R.le FAALDRADRVIGIDASGTLQQTRDQVRKIVDATSELVNLGRSVREDEIVINPDYAYPAYGVPSEETNEAI 280
2 280
45 280
61 280
62 280
126 280
129 280
130 280
131 280
141 280
142 280
154 280
170 280
171 280
173 280
180 280
194 280
223 280
161 280
162 280
163 280
164 280
165 280
166 280
167 280
168 280
169 280
172 280
Clustal Consensus 260

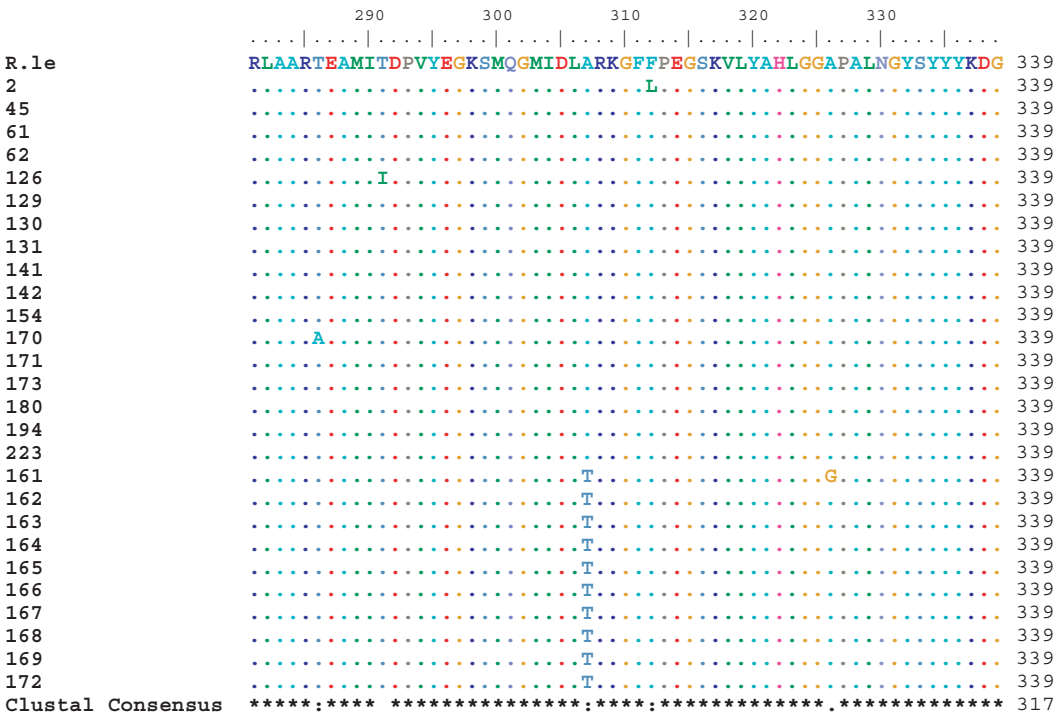
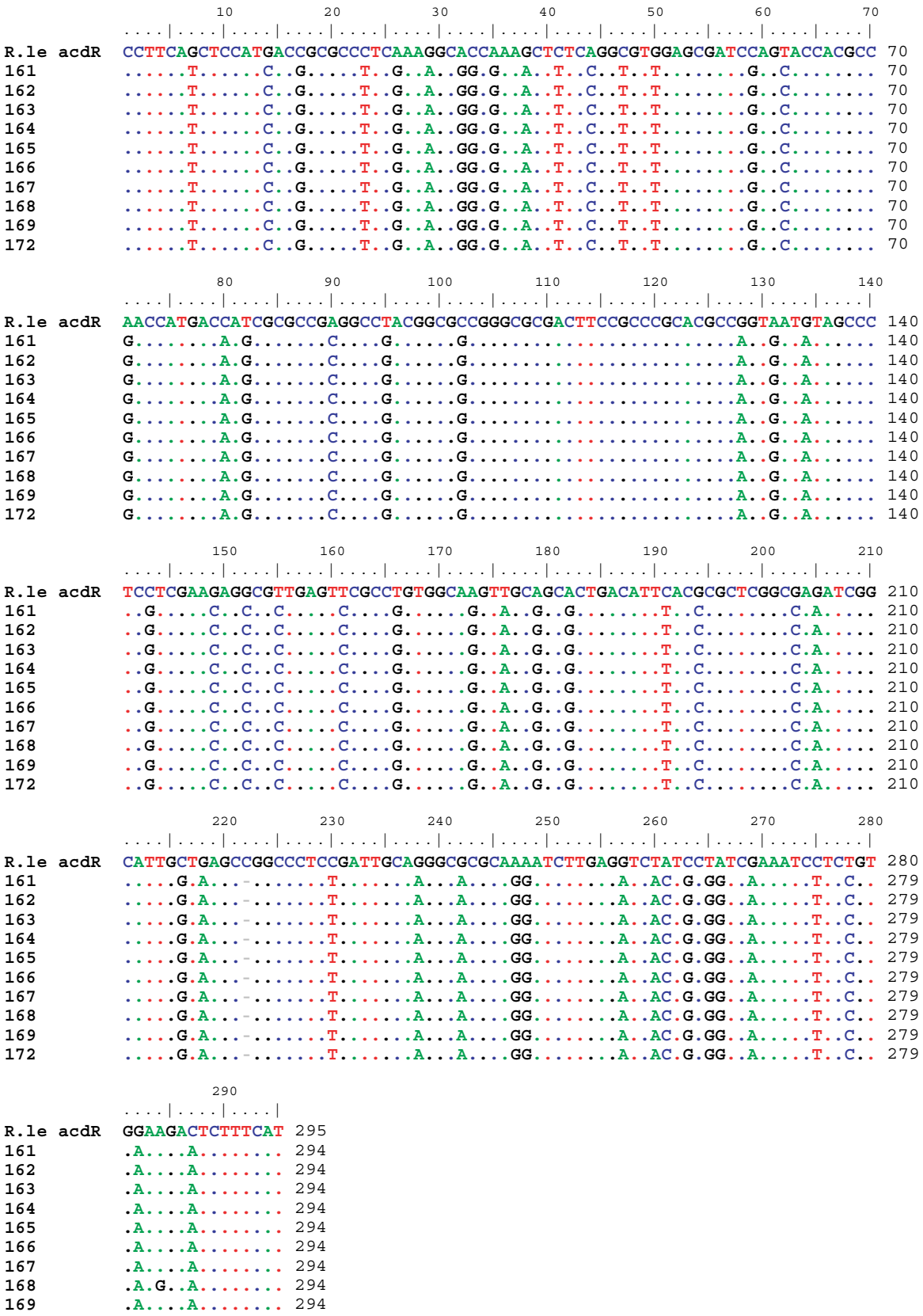

290
300
310
320
330
R.le RLAARTEAMITDPVYEGKSMQGMIDLARKGFFPEGSKVLYAHLGGAPALNGYSYYYKDG 339
2 ..L.. 339
45 339
61 339
62 339
126 .I.. 339
129 339
130 339
131 339
141 339
142 339
154 339
170 .A.. 339
171 339
173 339
180 339
194 339
223 339
161 .T..G.. 339
162 .T.. 339
163 .T.. 339
164 .T.. 339
165 .T.. 339
166 .T.. 339
167 .T.. 339
168 .T.. 339
169 .T.. 339
172 .T.. 339
Clustal Consensus *****:**** ***************:****:*************.************* 317

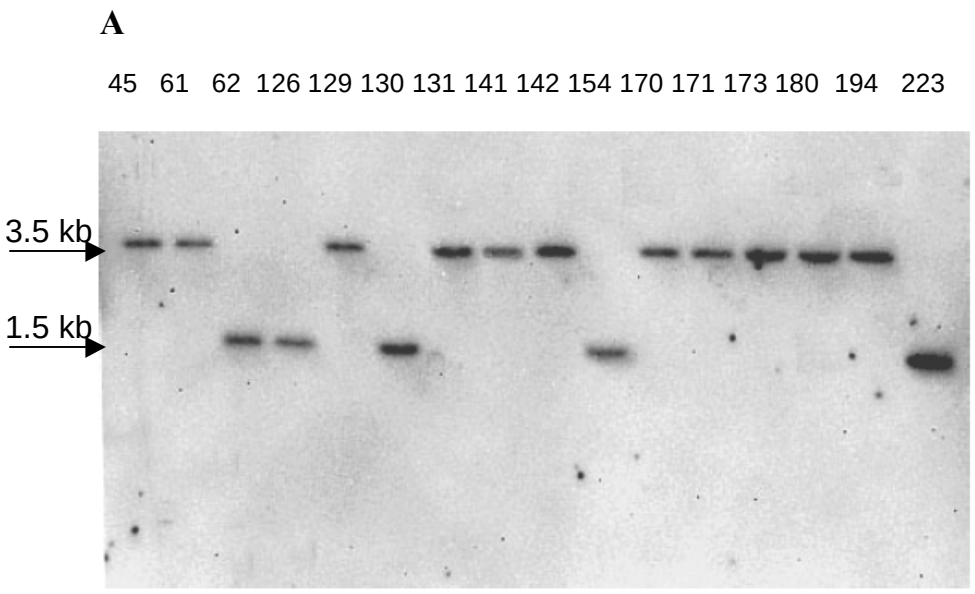
Figure 3-10 Sequence alignments of partial leucine-responsive regulatory-like protein gene that located at 5' upstream of *acdS* gene of the 10 Rhizobia strains, using *acdR* gene of *Rhizobiam leguminosarum* bv. *viciae* as a reference.


10 20 30 40 50 60 70
R.le acdR CCTTCAGCTCCATGACCGCGCCCTCAAAGGCACCAAAGCTCTCAGGCGTGGAGCGATCCAGTACCACGCC 70
161 ......T......C..G.....T..G..A..GG.G..A..T..C..T..T........G..C........ 70
162 ......T......C..G.....T..G..A..GG.G..A..T..C..T..T........G..C........ 70
163 ......T......C..G.....T..G..A..GG.G..A..T..C..T..T........G..C........ 70
164 ......T......C..G.....T..G..A..GG.G..A..T..C..T..T........G..C........ 70
165 ......T......C..G.....T..G..A..GG.G..A..T..C..T..T........G..C........ 70
166 ......T......C..G.....T..G..A..GG.G..A..T..C..T..T........G..C........ 70
167 ......T......C..G.....T..G..A..GG.G..A..T..C..T..T........G..C........ 70
168 ......T......C..G.....T..G..A..GG.G..A..T..C..T..T........G..C........ 70
169 ......T......C..G.....T..G..A..GG.G..A..T..C..T..T........G..C........ 70
172 ......T......C..G.....T..G..A..GG.G..A..T..C..T..T........G..C........ 70
80 90 100 110 120 130 140
R.le acdR AACCATGACCATCGCGCCGAGGCCTACGGCGCCGGGCGCGACTTCCGCCCGCACGCCGGTAATGTAGCCC 140
161 G.........A.G.......C....G......G.........................A..G..A...... 140
162 G.........A.G.......C....G......G.........................A..G..A...... 140
163 G.........A.G.......C....G......G.........................A..G..A...... 140
164 G.........A.G.......C....G......G.........................A..G..A...... 140
165 G.........A.G.......C....G......G.........................A..G..A...... 140
166 G.........A.G.......C....G......G.........................A..G..A...... 140
167 G.........A.G.......C....G......G.........................A..G..A...... 140
168 G.........A.G.......C....G......G.........................A..G..A...... 140
169 G.........A.G.......C....G......G.........................A..G..A...... 140
172 G.........A.G.......C....G......G.........................A..G..A...... 140
150 160 170 180 190 200 210
R.le acdR TCCTCGAAGAGGCGTTGAGTTCGCCTGTGGCAAGTTGCAGCACTGACATTCACGCGCTCGGCGAGATCGG 210
161 ..G.....C..C..C.....C....G......G..A..G..G........T..C........C.A..... 210
162 ..G.....C..C..C.....C....G......G..A..G..G........T..C........C.A..... 210
163 ..G.....C..C..C.....C....G......G..A..G..G........T..C........C.A..... 210
164 ..G.....C..C..C.....C....G......G..A..G..G........T..C........C.A..... 210
165 ..G.....C..C..C.....C....G......G..A..G..G........T..C........C.A..... 210
166 ..G.....C..C..C.....C....G......G..A..G..G........T..C........C.A..... 210
167 ..G.....C..C..C.....C....G......G..A..G..G........T..C........C.A..... 210
168 ..G.....C..C..C.....C....G......G..A..G..G........T..C........C.A..... 210
169 ..G.....C..C..C.....C....G......G..A..G..G........T..C........C.A..... 210
172 ..G.....C..C..C.....C....G......G..A..G..G........T..C........C.A..... 210
220 230 240 250 260 270 280
R.le acdR CATTGCTGAGCCGGCCCTCCGATTGCAGGGCGCGCAAAATCTTGAGGTCTATCCTATCGAAATCCTCTGT 280
161 .....G.A...-.......T.......A...A....GG........A..AC.G.GG..A.....T..C.. 279
162 .....G.A...-.......T.......A...A....GG........A..AC.G.GG..A.....T..C.. 279
163 .....G.A...-.......T.......A...A....GG........A..AC.G.GG..A.....T..C.. 279
164 .....G.A...-.......T.......A...A....GG........A..AC.G.GG..A.....T..C.. 279
165 .....G.A...-.......T.......A...A....GG........A..AC.G.GG..A.....T..C.. 279
166 .....G.A...-.......T.......A...A....GG........A..AC.G.GG..A.....T..C.. 279
167 .....G.A...-.......T.......A...A....GG........A..AC.G.GG..A.....T..C.. 279
168 .....G.A...-.......T.......A...A....GG........A..AC.G.GG..A.....T..C.. 279
169 .....G.A...-.......T.......A...A....GG........A..AC.G.GG..A.....T..C.. 279
172 .....G.A...-.......T.......A...A....GG........A..AC.G.GG..A.....T..C.. 279
290
R.le acdR GGAAGACTCTTTCAT 295
161 .A....A........ 294
162 .A....A........ 294
163 .A....A........ 294
164 .A....A........ 294
165 .A....A........ 294
166 .A....A........ 294
167 .A....A........ 294
168 .A.G..A........ 294
169 .A....A........ 294
172 .A....A........ 294

The nucleotide sequence of *acdS* genes from three Philom Bios Rhizobia strains, PB2, PB45 and PB163 were compared to those from other organisms. A ML tree was shown in Fig 3-12B. In this tree, *Enterobacter* sp., *Pseudomonas* sp. and *Achromobacter* sp. ACC deaminase sequences are distributed throughout the tree which, as was previously pointed out, was unexpected if one assumes that "a vertical transmission of the genes mirroring the bacterial 16S rRNA tree" (Hontzeas et al. 2005). Moreover, in this tree, Rhizobial ACC deaminase genes of Philom Bios strains 2 (PB2) and 45 (PB45) were closely grouped with *R. leguminosarum* 128C53K and *R. leguminosarum* 99A1 whereas Philom Bios strain 163 (PB163) displayed further distance with them. This tree is generally in agreement with the tree that was constructed using ML method and published by Hontzeas et al. (2005) with minor topological differences.

Figure 3-11 Southern hybridization of ACC deaminase genes from 27 different Rhizobia strains. Genomic DNA from each strain was isolated and digested with *Eco*RV, and then probed with an *acdS* gene, either from strain 45 (panel A) or strain 163 (panel B).

A


45 61 62 126 129 130 131 141 142 154 170 171 173 180 194 223
3.5 kb
1.5 kb

B

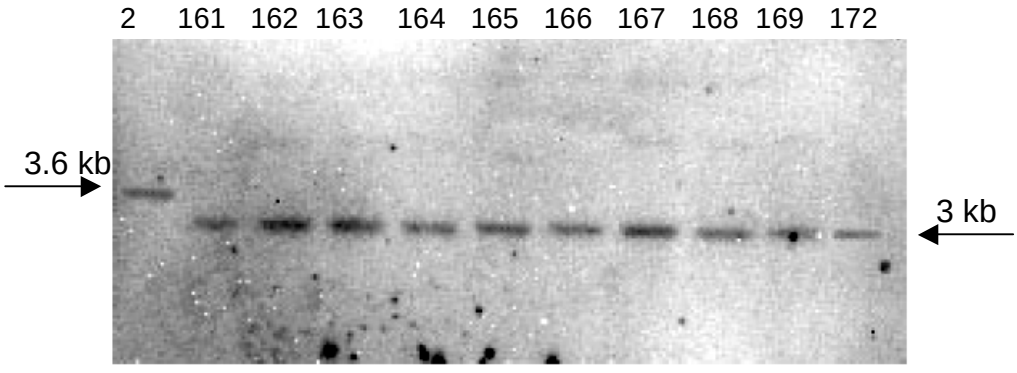
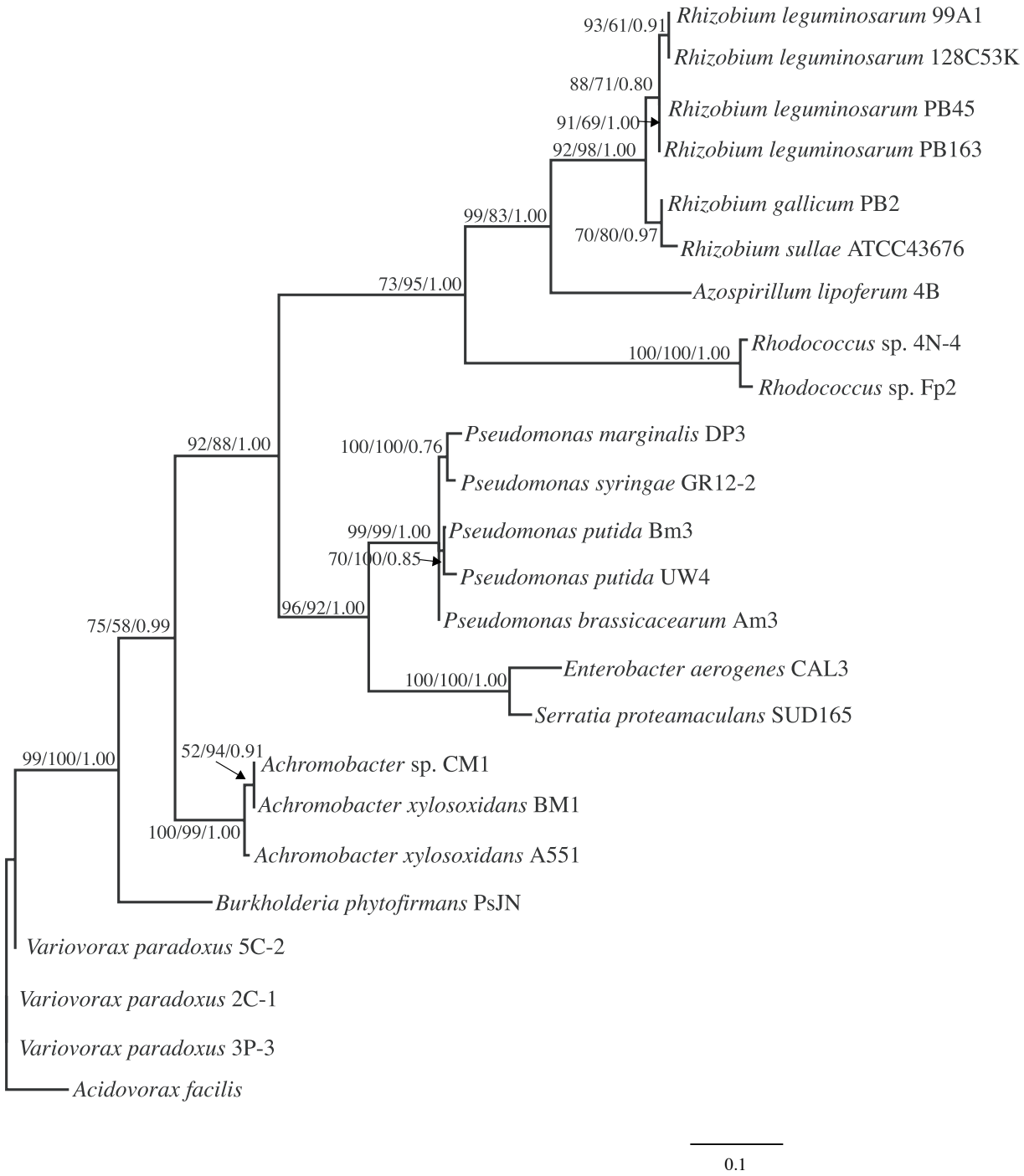
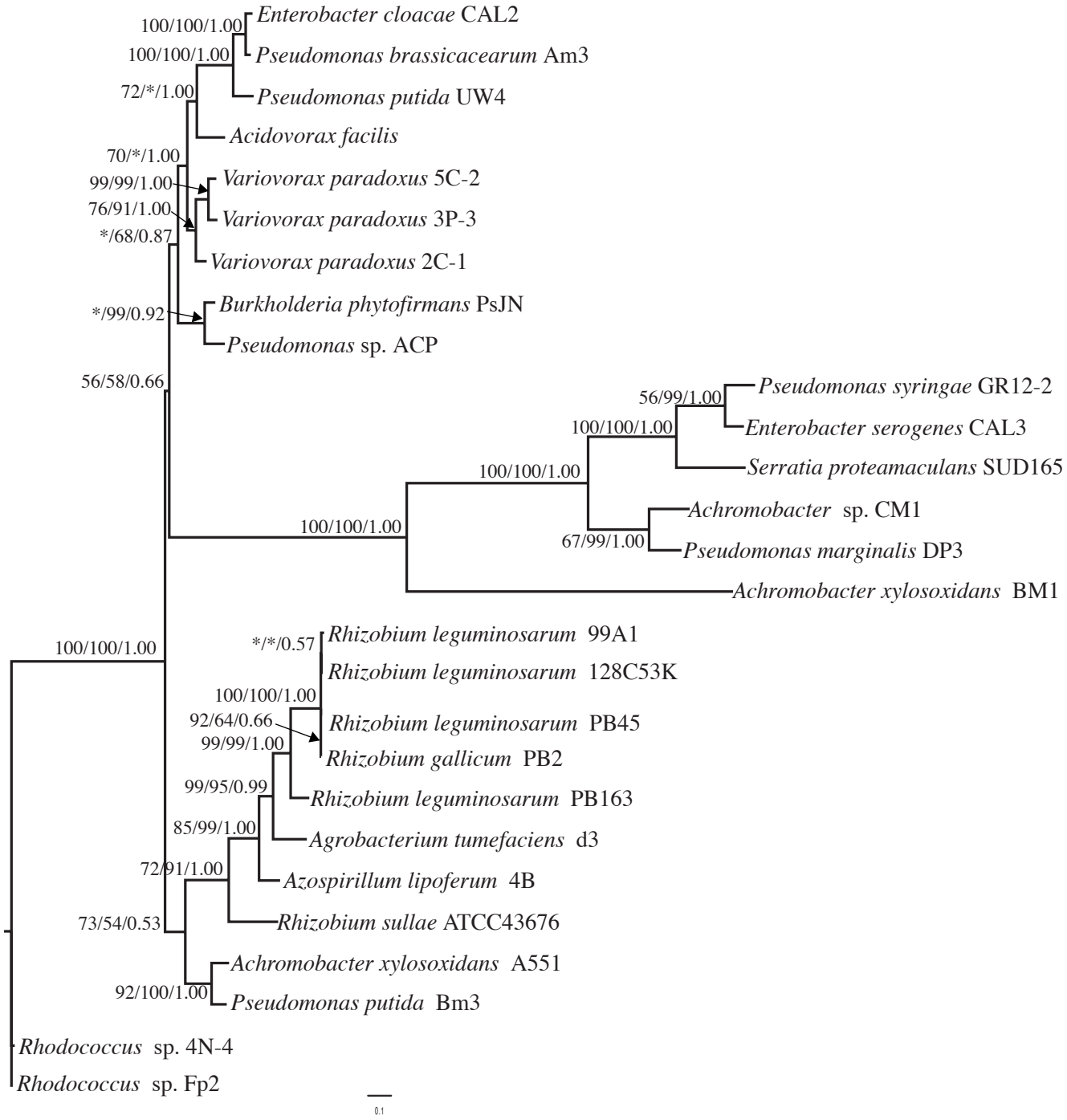

2 161 162 163 164 165 166 167 168 169 172
3.6 kb
3 kb

Figure 3-12 A. Maximum likelihood phylogenetic tree of 16S rRNA gene sequences. B. Maximum likelihood phylogenetic tree of the ACC deaminase nucleotide sequences. Numbers above branches represent bootstrap values (1000 replicates) using Neighbor-joining (first number), bootstrap values using maximum parsimony (second number) and Bayesian posterior probabilities (third number). Branches with an asterisk had less than 50% support in that analysis.

A


93/61/0.91
Rhizobium leguminosarum 99A1
Rhizobium leguminosarum 128C53K
88/71/0.80
Rhizobium leguminosarum PB45
91/69/1.00
92/98/1.00
Rhizobium leguminosarum PB163
Rhizobium gallicum PB2
99/83/1.00
70/80/0.97
Rhizobium sullae ATCC43676
73/95/1.00
Azospirillum lipoferum 4B
100/100/1.00
Rhodococcus sp. 4N-4
Rhodococcus sp. Fp2
92/88/1.00
100/100/0.76
Pseudomonas marginalis DP3
Pseudomonas syringae GR12-2
99/99/1.00
Pseudomonas putida Bm3
70/100/0.85
Pseudomonas putida UW4
96/92/1.00
Pseudomonas brassicacearum Am3
75/58/0.99
100/100/1.00
Enterobacter aerogenes CAL3
Serratia proteamaculans SUD165
99/100/1.00
52/94/0.91
Achromobacter sp. CM1
Achromobacter xylosoxidans BM1
100/99/1.00
Achromobacter xylosoxidans A551
Burkholderia phytofirmans PsJN
Variovorax paradoxus 5C-2
Variovorax paradoxus 2C-1
Variovorax paradoxus 3P-3
Acidovorax facilis
0.1

B


100/100/1.00
Enterobacter cloacae CAL2
100/100/1.00
Pseudomonas brassicacearum Am3
72/*/1.00
Pseudomonas putida UW4
Acidovorax facilis
70/*/1.00
99/99/1.00
Variovorax paradoxus 5C-2
76/91/1.00
Variovorax paradoxus 3P-3
*/68/0.87
Variovorax paradoxus 2C-1
*/99/0.92
Burkholderia phytofirmans PsJN
Pseudomonas sp. ACP
56/58/0.66
56/99/1.00
Pseudomonas syringae GR12-2
100/100/1.00
Enterobacter serogenes CAL3
100/100/1.00
Serratia proteamaculans SUD165
Achromobacter sp. CM1
100/100/1.00
67/99/1.00
Pseudomonas marginalis DP3
Achromobacter xylosoxidans BM1
100/100/1.00
//0.57
Rhizobium leguminosarum 99A1
Rhizobium leguminosarum 128C53K
100/100/1.00
92/64/0.66
Rhizobium leguminosarum PB45
99/99/1.00
Rhizobium gallicum PB2
99/95/0.99
Rhizobium leguminosarum PB163
85/99/1.00
Agrobacterium tumefaciens d3
72/91/1.00
Azospirillum lipoferum 4B
73/54/0.53
Rhizobium sullae ATCC43676
Achromobacter xylosoxidans A551
92/100/1.00
Pseudomonas putida Bm3
Rhodococcus sp. 4N-4
Rhodococcus sp. Fp2
0.1

4 Discussion

4.1 Frequency of ACC deaminase activity in Rhizobia

Of the 233 strains obtained from Philom Bios, Inc., 27 strains had ACC deaminase activity (Table 2-1). The frequency of the presence of ACC deaminase is around 11% which is lower compared to the results of previous study on ACC deaminase in Rhizobia (Ma et al. 2003a). Ma et al. reported 5 out of 13 tested *Rhizobium* spp. shown the activity including 4 commercialized strains *Rhizobium leguminosarum* bv. *viciae* 128C53K, 128C53, 128C53G, 99A1. On the other hand, ACC deaminase containing free-living plant growth-promoting bacteria were more commonly found in polluted soil (Belimov et al. 2001; Belimov et al. 2005). Belimov et al. reported 10 out of 11 cadmium-tolerant bacterial strains isolated from the root zone of Indian mustard showed ACC deaminase activity and all 10 strains of these promoted root length significantly. However, when a collection of 597 soil bacterial strains was screened for the capability of degrading ACC, it was found only three organisms had ACC deaminase activity and two of them were *Pseudomonas* spp (Klee and Kishore 1992). The recent work of Blaha et al. (2006) indicated that one out of five *Azospirillum* strains isolated from the roots of field-grown plants in Pakistan showed ACC deaminase activity *in vitro*. Furthermore, several *acdS*$^+$ *Proteobacteria* didn't display ACC deaminase activity, which means that the phenotype-based traditional methods used for screening strain collections may not represent the real distribution of ACC deaminase gene in *Proteobacteria* (Blaha et al. 2006).

Rhizobia containing ACC deaminase had much lower activity compared to most free-living bacteria containing ACC deaminase. It has been suggested that, "High ACC deaminase-expressing organisms typically bind relatively non-specifically to a variety of plant surfaces and act as a sink for ACC" (Glick 2005) while Rhizobia only bind to host legume plants. The fact that

the *acdS* gene was positively regulated by NifA2 protein in *Mesorhizobium loti* suggested that this *acdS* gene was expressed only in mature nodules (Nukui et al. 2006). Since the amount of ethylene produced during the infection and nodulation is usually low, it may be argued that Rhizobia don't need high levels of ACC deaminase activity to degrade ACC to perform this function (Glick 2005).

4.2 16S rDNA sequence analysis of the 27 Rhizobia strains

The sequences of bacterial 16S rDNAs are generally considered to be highly conserved among bacteria (Woese 1987). Evolutionary or taxonomic relationships among bacteria, including Rhizobia, are usually estimated through 16S rDNA sequence comparisons. Based on the 16S rDNA sequences of the 27 Philom Bios Rhizobia strains which have ACC deaminase activity, 26 are considered to be *Rhizobium leguminosarum* whereas one strain, number 2, is *Rhizobium gallicum*. This is somewhat surprising since these strains were isolated from nodules from three different legume plants, *Vicia americana*, *Vicia cracca* and *Lathyrus venosus* grown in a range of geographical regions throughout Saskatchewan (350 kilometers from north to south and 320 kilometers from east to west) (Fig. 2-1).

Rhizobium leguminosarum bv. *viciae* is known to nodulate all species in the tribe Vicieae including the genera *Vicia*, *Lathyrus*, *Pisum* and *Lens* (Allen and Allen 1981; Young et al. 2003; Young and Mutch 2004). From the 26 16S rRNA gene sequences obtained from this study, it couldn't designate the subspecies for each strain. Since their host legume plants are either *Vicia* or *Lathyrus* (Table2-1), therefore the 26 Rhizobia strains are likely *Rhizobium leguminosarum* bv. *viciae*. *Rhizobium gallicum* is said to nodulate *Phaseolus vulgaris* (Amarger et al. 1997; Willems 2006). However, in this study, one strain PB2 isolated from rhizosphere of *Vicia americana* was

dertermined as *Rhizobium gallicum*. Currently, there is no published literature showing that *Rhizobium gallicum* is able to nodulate Vicieae. It will be interesting to investigate symbiotic characteristics of this strain to confirm the symbiotic relationship between *R. gallicum* and *V. americana* is the fact.

4.3 Rhizobia ACC deaminase genes and their 5' upstream regions

After assigning genus and species to the 27 isolated Rhizobia strains, the complete ACC deaminase (*acdS*) gene was cloned by PCR. Since all the 27 strains are *Rhizobium*, a set of specific primers were designed based on the sequence of the *acdS* gene from *Rhizobium leguminosarum* bv. *viciae* 128C53K. After PCR amplification, 17 strains each showed a unique band of about one kb as expected. These 17 genes were sequenced and a comparison of their DNA sequences to the *acdS* gene from *R. leguminosarum* indicated that the ACC deaminase genes in these 17 strains are highly conserved (i.e., an average of 99% identities at the gene level). This result was a little surprising as these 17 strains were isolated from 11 different locations across Saskatchewan (Fig. 2-1) and our expectation was that there would be greater variety.

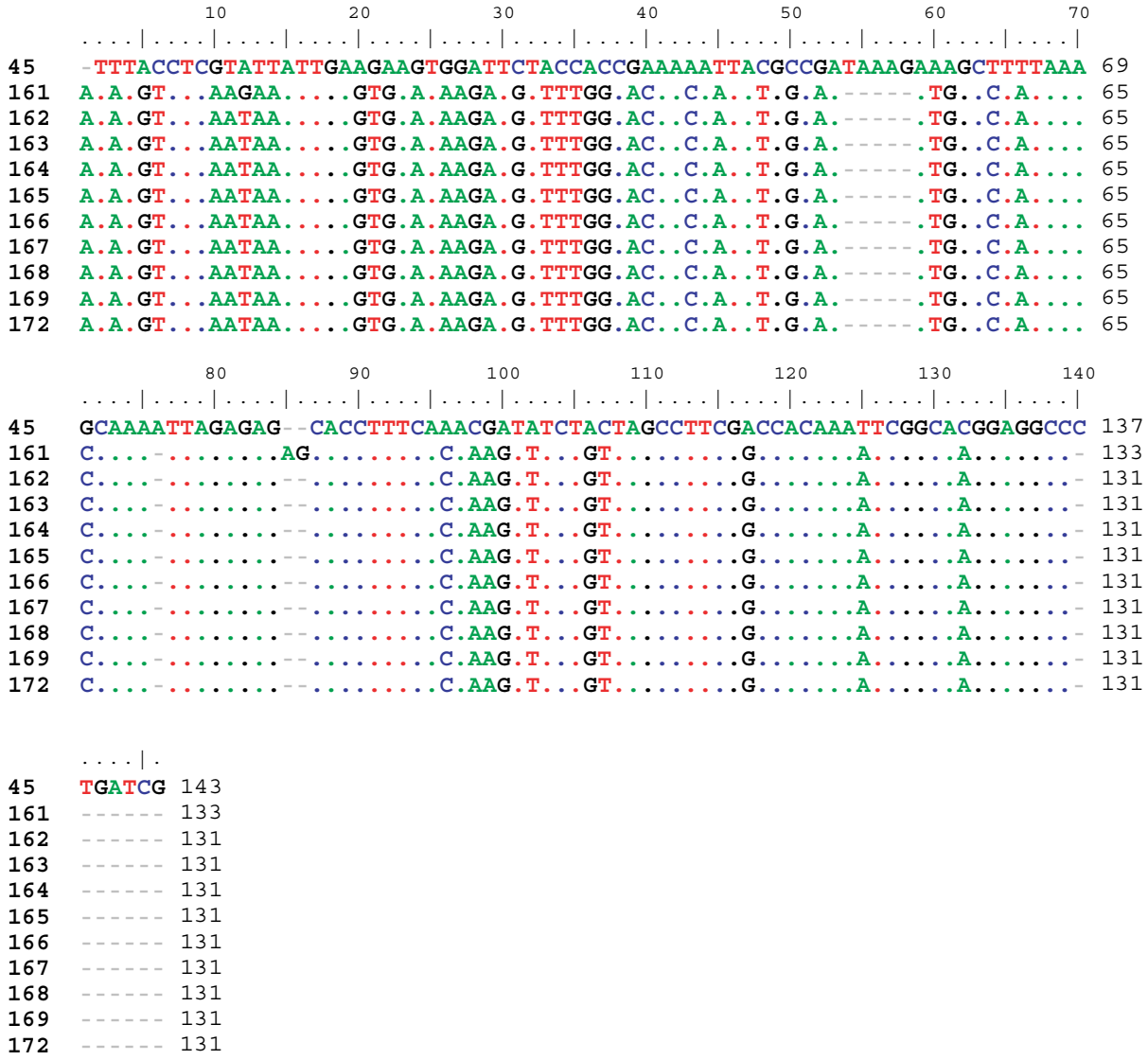
For the other 10 strains, which were isolated from Lipton, LeRoss and Mozart SK, it was not possible to PCR amplify the *acdS* gene using the primers that were effective for the other 17 strains, therefore another strategy was employed. For a start, in agreement with the activity data, Southern hybridization showed that all 27 of the Rhizobia strains had a single *acdS* gene. Thus the unsuccessful PCR was probably due to the fact that the initial set of primers were not from a region of the *acdS* gene that was conserved between the 17 strains whose *acdS* gene was amplified and the 10 strains whose *acdS* gene was not amplified. When inverse PCR was used to obtain the 5' upstream regulatory region of *acdS* gene, the 17 strains whose *acdS* gene was amplified using the initial set of primers displayed a regulatory region that was highly conserved

compared to that of the *acdS* gene of *Rhizobium leguminosarum* bv. *viciae* 128C53K. In each of these strains, there is a leucine-responsive regulatory protein (*lrp*) gene located immediately upstream of the *acdS* gene and transcribed in the opposite direction to *acdS*.

The *acdS* gene sequences obtained following inverse PCR of the 10 strains whose *acdS* gene was not amplified using the initial set of primers have lower identities when compared with the *acdS* gene of *Rhizobium leguminosarum* bv. *viciae* 128C53K. In fact, the design of the second set of PCR primers used to isolate *acdS* from the 10 recalcitrant strains was based on a partial *acdS* gene sequence obtained for one of the 10 initially recalcitrant strains by inverse PCR. The *acdS* gene sequences subsequently obtained, using the second set of primers, showed that the *acdS* gene is highly conserved among these 10 strains. However, when the *acdS* gene sequences of these 10 strains were compared to the *acdS* gene sequences from the other 17 strains, they show only 83% identities at the DNA level and 94% identities at the protein level. Examination of the amino acid sequences of all of the ACC deaminases revealed that the highly conserved lysine residue K^{51} was found in all cases. "K^{51} is the PLP-binding site in *Williopsis* (=*Hansenula*) *saturnus* (Yao et al. 2000) and is required for enzymatic activity in *Pseudomonas fluorescens* ACP (Murakami et al. 1997)" (Blaha et al. 2006).

The spacer region between *acdS* and *acdR* of the 17 strains showed that there are 143 bp nucleotides containing two LRP boxes, as was shown in previous characterized *acdS* and *acdR* in *R. leguminosarum* bv. *viciae* 128C53K. However, for the other 10 strians, the spacer region of 9 strains between *acdS* and *acdR* only contains 131 bp nucleotides whereas one strain 161 contains 133 bp nucleotides (Fig 4-1). No LRP box was found in the spacer region for these 10 strains. Sequence alignments of the spacer region among the 10 strains showed 59% identities compared to that of strain 45. The sequences close to *acdS* are more conserved than the sequences near

Figure 4-1 Sequence alignments of spacer region of *acdS* and *acdR*.


10 20 30 40 50 60 70
45 -TTTACCTCGTATTATTGAAGAAGTGGATTCTACCACCGAAAAATTACGCCGATAAAGAAAGCTTTTAAA 69
161 A.A.GT...AAGAA.....GTG.A.AAGA.G.TTTGG.AC..C.A..T.G.A.------.TG..C.A.... 65
162 A.A.GT...AATAA.....GTG.A.AAGA.G.TTTGG.AC..C.A..T.G.A.------.TG..C.A.... 65
163 A.A.GT...AATAA.....GTG.A.AAGA.G.TTTGG.AC..C.A..T.G.A.------.TG..C.A.... 65
164 A.A.GT...AATAA.....GTG.A.AAGA.G.TTTGG.AC..C.A..T.G.A.------.TG..C.A.... 65
165 A.A.GT...AATAA.....GTG.A.AAGA.G.TTTGG.AC..C.A..T.G.A.------.TG..C.A.... 65
166 A.A.GT...AATAA.....GTG.A.AAGA.G.TTTGG.AC..C.A..T.G.A.------.TG..C.A.... 65
167 A.A.GT...AATAA.....GTG.A.AAGA.G.TTTGG.AC..C.A..T.G.A.------.TG..C.A.... 65
168 A.A.GT...AATAA.....GTG.A.AAGA.G.TTTGG.AC..C.A..T.G.A.------.TG..C.A.... 65
169 A.A.GT...AATAA.....GTG.A.AAGA.G.TTTGG.AC..C.A..T.G.A.------.TG..C.A.... 65
172 A.A.GT...AATAA.....GTG.A.AAGA.G.TTTGG.AC..C.A..T.G.A.------.TG..C.A.... 65
80 90 100 110 120 130 140
45 GCAAAATTAGAGAG--CACCTTTCAAACGATATCTACTAGCCTTCGACCACAAATTCGGCACGGAGGCCC 137
161 C....-........AG.........C.AAG.T...GT.........G.......A......A.......- 133
162 C....-........--.........C.AAG.T...GT.........G.......A......A.......- 131
163 C....-........--.........C.AAG.T...GT.........G.......A......A.......- 131
164 C....-........--.........C.AAG.T...GT.........G.......A......A.......- 131
165 C....-........--.........C.AAG.T...GT.........G.......A......A.......- 131
166 C....-........--.........C.AAG.T...GT.........G.......A......A.......- 131
167 C....-........--.........C.AAG.T...GT.........G.......A......A.......- 131
168 C....-........--.........C.AAG.T...GT.........G.......A......A.......- 131
169 C....-........--.........C.AAG.T...GT.........G.......A......A.......- 131
172 C....-........--.........C.AAG.T...GT.........G.......A......A.......- 131
45 TGATCG 143
161 ------ 133
162 ------ 131
163 ------ 131
164 ------ 131
165 ------ 131
166 ------ 131
167 ------ 131
168 ------ 131
169 ------ 131
172 ------ 131

acdR. The varied spacer region and regulatory region of *acdS* in the 10 Rhizobia strains indicated there might be other regulatory mechanism of *acdS* present.

The whole genome of *Rhizobium leguminosarum* bv. *viciae* strain 3841 has been fully sequenced and annotated. The genome consists of a circular chromosome of 5,047,142 bp, and six plasmids: pRL12 (870,021 bp), pRL11 (684,202 bp), pRL10 (488,135 bp), pRL9 (352,782 bp), pRL8 (147,463 bp) and pRL7 (151,564 bp) (Yount et al. 2006). Analysis of the 5' upstream sequences (1327 bp) of the *acdS* gene from the 10 Rhizobia strains isolated using the second set of PCR primers revealed that 397 bp (from 507 bp-904 bp) are 98% identical (393/397 bp) compared to the sequences of plasmid pRL7 of *Rhizobium leguminosarum* bv. *viciae* 3841. Furthermore, a putative *acdS* gene was found on plasmid pRL10 which has 99.11% identities compared to the *acdS* gene of *Rhizobium leguminosarum* bv. *viciae* 128C53K and the 17 Rhizobia strains. However, there isn't any regulatory element at 5' upstream of this *acdS* gene, such as *acdR*. In addition, from an examination of the sequences from 170-374 bp 5' upstream of *acdS* from the 10 strains, it appears that a partial *acdR* gene is present which has 83% identities compared with the *acdR* located upstream of the *acdS* gene from *Rhizobium leguminosarum* bv. *viciae* 128C53K. However this partial *acdR* gene doesn't seem to be a functional gene, thus there might be another mode of regulation of *acdS* gene in these 10 Rhizobia strains.

Southern hybridization indicated that for all the 27 Rhizobia strains examined there is only one *acdS* gene in the genome. When *Eco*RV digested genomic DNA was probed with the corresponding complete *acdS* gene, the signals showed bands of four different sizes, 1.5 kb, 3 kb, 3.5 kb and 3.6 kb. Although based on the sequences of the 16S rDNA, 27 strains showed only two *Rhizobium* species, from the southern blot, there are 4 different patterns. Moreover, sequence

analysis of both *acdS* and its 5' upstream region indicate only tow different ACC deaminase structural and regulatory genes.

Further analysis on alleles of 16S rRNA gene and *acdS* gene showed that there are more differences among the 27 strains (Table 4-1). For example, from the results of Southern hybridization, strains 45, 61, 129, 131, 141, 142, 170, 171, 173, 180 and 194 have the same pattern. However, for 16S rRNA gene, 129 and 62 have the same alleles whereas 142, 171,173 and 180 each has unique alleles. For all the 27 strains, there are 14 different 16S rRNA alleles and 17 different *acdS* alleles. Strains 45, 61, 141 and 194 have the same Southern blot pattern, the same 16S rRNA alleles and the *acdS* alleles. From the group of 10 strains, 163 and 167 are the same for all three characters. On the whole, among the 27 Rhizobia strains, there are at least 22 different strains based on Southern hybridization, 16S rRNA alleles and *acdS* alleles.

4.4 Phylogenetic analysis of the ACC deaminase nucleotide sequences of the Rhizobia strains characterized in this study

It has been proposed that ACC deaminase genes did not evolve exclusively vertically but instead some of these genes have undergone horizontal gene transfer (Hontzeas et al. 2005; Blaha et al. 2006). Sullivan et al. (2002) reported that *acdS* is located within a symbiotic island in *Mesorhizobium loti* strain R7A. Similarly, a recent fully sequenced *Rhizobium leguminosarum* bv. *viciae* strain 3841 has a putative *acdS* gene on one of its plasmid, pRL10 **(**488,135 bp**)** (Young et al. 2006). One strain of *Sinorhizobium meliloti* has an *acdS* gene on an accessory plasmid pSmeSM11a (144,170 bp) and *acdR* is located upstream of this deduced *acdS* (Stiens et al. 2006) while another *S. meliloti* strain does not contain *acdS* at all (Ma et al. 2004). Likewise, *Rhizobium leguminosarum* bv. *trifolii* strain NZP514 has a *acdS* gene on plasmid pRtr514a (19,192 bp) with

Table 4-1 Correlations among 16S rRNA alleles, *acdS* alleles and Southern hybridization.

Strain Number	16S rRNA allele (group number)	*acdS* allele (group number)	Southern pattern
2	1	1	1
45	2	2	2
61	2	2	2
62	3	3	3
126	2	4	3
129	3	5	2
130	4	6	3
131	2	7	2
141	2	2	2
142	5	8	2
154	2	9	3
161	6	10	4
162	7	11	4
163	2	11	4
164	8	11	4
165	9	11	4
166	10	12	4
167	2	11	4
168	11	13	4
169	2	13	4
170	2	14	2
171	12	15	2
172	2	16	4
173	13	2	2
180	14	17	2
194	2	2	2
223	2	2	3

an *acdR* located upstream of *acdS* (Miller et al. 2007). Generally speaking, it is easier, both in the laboratory and in the environment, to transfer plasmid DNA than to transfer chromosomal DNA from one organism to another (Bertolla et al. 1999; Kay et al. 2003; Mercier et al. 2006). *Rhizobium leguminosarum* bv. *viciae* generally contains 1-10 plasmids which vary in size from 30 kb to more than 800 kb (Martínez-Romero and Palacios 1990; Martínez-Romero and Caballero-Mellado 1996; Mercado-Blance and Toro 1996; Young et al. 2006). And, if many *acdS* genes are plasmid encoded, it is likely that at least in some bacteria they are inherited by horizontal gene transfer.

Currently, there are four principal methods for constructing phylogenetic trees from protein and nucleic acid sequence alignments including distance methods, of which Neighbor Joining (NJ) is the favored implementation; maximum parsimony (MP); maximum likelihood (ML) and Bayesian. All four methods were used to construct phylogenetic trees of 16S rRNA gene and corresponding *acdS* nuleotide sequences. The trees shown in Fig 3-12 are ML trees with bootstrap values (1000 replicates) using NJ, bootstrap values using MP and posterior probabilities using Bayesian. For 16S rRNA gene, all methods gave the same overall topology. For *acdS* gene, ML and Bayesian trees gave the same topology, whereas NJ and MP trees showed weakly supported clades for some *acdS* genes (branches with an asterisk in Fig 3-12B). A comparison of 16S rRNA gene tree and *acdS* gene tree revealed that *acdS* gene of *Enterobacter* sp., *Pseudomonas* sp. and *Achromobacter* sp. didn't mirror the 16S rRNA tree since the *acdS* genes were distributed throughout the tree, indicating horizontal gene transfer.

Recent studies have demonstrated that in *Variovorax paradoxus* 5C2, *Achromobacter xylosoxidans* A551, *Pseudomonas putida* UW4 and *Rhizobium leguminosarum* bv. *viciae* 128C53K, *acdR* and *acdS* were inherited together and the Lrp protein is essential for the

transcription of the ACC deaminase gene (Hontzeas et al. 2005). The close juxtaposition and reverse orientation of *acdS* and *acdR* was observed here for the 17 Philom Bios Rhizobia strains whose *acdS* gene was amplified with the first set of primers. However, for the other 10 Rhizobia strains, the *acdS* and its 5' upstream region varied in this regard. Not only was the *acdS* gene sequence somewhat different in these 10 strains compared to the previously mentioned 17 strains, these 10 strains all had a truncated version of the *acdR* gene. At the present time, it isn't known whether the truncated Lrp protein is functional or not. Of these 10 strains, eight were isolated from LeRoss, SK while the other 2 strains were isolated from Lipton and Mozart, SK, respectively, towns that geographically close to LeRoss. It is possible that these 10 strains may have acquired *acdS* and its 5' upstream region from other Rhizobia strains concomitant with deletion of a portion of *acdR* and moreover, the source of these genes may have been other Rhizobia strains isolated from Lipton and Mozart, SK (strains which were part of the group of 17).

The fact that strains have ACC deaminase activity from such a wide geographic area showed relatively little diversity was somewhat surprising. There are many factors that affect microbial diversity in soil, for example, climate, soil physical and chemical properties, type and amount of plant cover (Dunfield and Germida, 2001). Among these factors, plant variety influences the biodiversity of bacteria in soils by "releasing of compounds such as amino acids, sugars and growth factors in plant root exudates, microbial activity and growth are stimulated" (Dunfield and Germida, 2001). It was considered that the isolated strains might represent the environmental remnants of the wide spread use of commercial Rhizobia inoculants. However, as far as we have been able to ascertain, this is not the case for the Rhizobia strains containing ACC deaminase activity. Alternatively, given the harsh winters and general lack of diverse vegetation

(this area has been used predominantly to grow wheat and/or canola) there may be real limits to the diversity of somewhat specialized microorganisms such as Rhizobia. In this regard, it would be important to determine how much diversity exists in the Rhizobia strains that lack ACC deaminase activity as well as in the free-living bacteria found in these locations. In addition, since the species designation based on 16S rRNA gene alone is not enough, other methods should be used as well.

4.5 Conclusions

In the symbiotic nitrogen fixation process, Rhizobia convert atmospheric dinitrogen to ammomia, which can be effectively utilized by host legume plants. However, in many legumes, ethylene has an inhibitory effect on nodulation. An enzyme, ACC deaminase, catalyzes the clevage of ACC, the immediate precursor of ethylene, to α-ketobutyrate and ammonia. Previous studies had shown that several Rhizobia strains that have ACC deaminase activity may enhance nodulation by reducing ethylene levels.

Our lab obtained 233 strains from Philom Bios Inc., in which 27 strains displayed ACC deaminase activity. ACC deaminase gene (*acdS*) was amplified and sequenced for each of the 27 strains. Southern hybridization indicated that there is only one ACC deaminase gene in the genome of each strain. Analysis based on 16S rRNA alleles, *acdS* alleles as well as Southern blot showed there are at least 22 different strains. From inverse PCR, 17 strains contain leucine-responsive regulatory gene (*acdR*), indicating that *acdS* and *acdR* were inherited together. The remaining 10 strains that isolated from 3 closely related locations all contain a partial *acdR*. Moreover, *acdS* genes in these 10 strains share less similarity with the *acdS* gene in the other 17 strains. It is possible that the *acdS* and *acdR* were introduced by horizontal gene transfer with

some mutations. This work indicates that most ACC deaminase genes and their regulatory regions from Rhizobia strains isolated over a wide geographical area are highly conserved.

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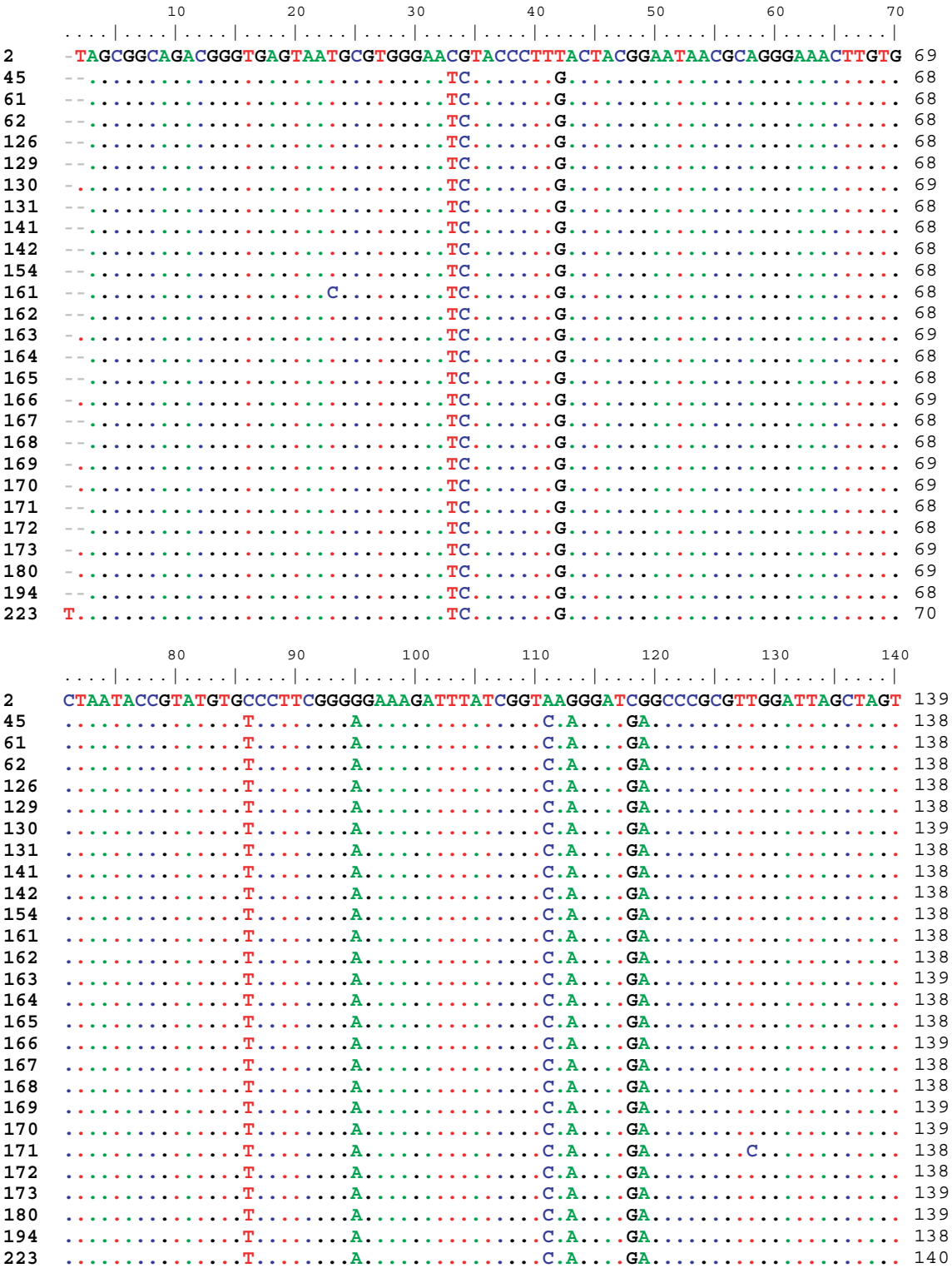
Appendix

Figure 6-1. 16S rRNA gene sequence alignments of 27 Philom Bios Rhizobia strains.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62 .....T.....A.....C.A....GA..... 138
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141 .....T.....A.....C.A....GA..... 138
142 .....T.....A.....C.A....GA..... 138
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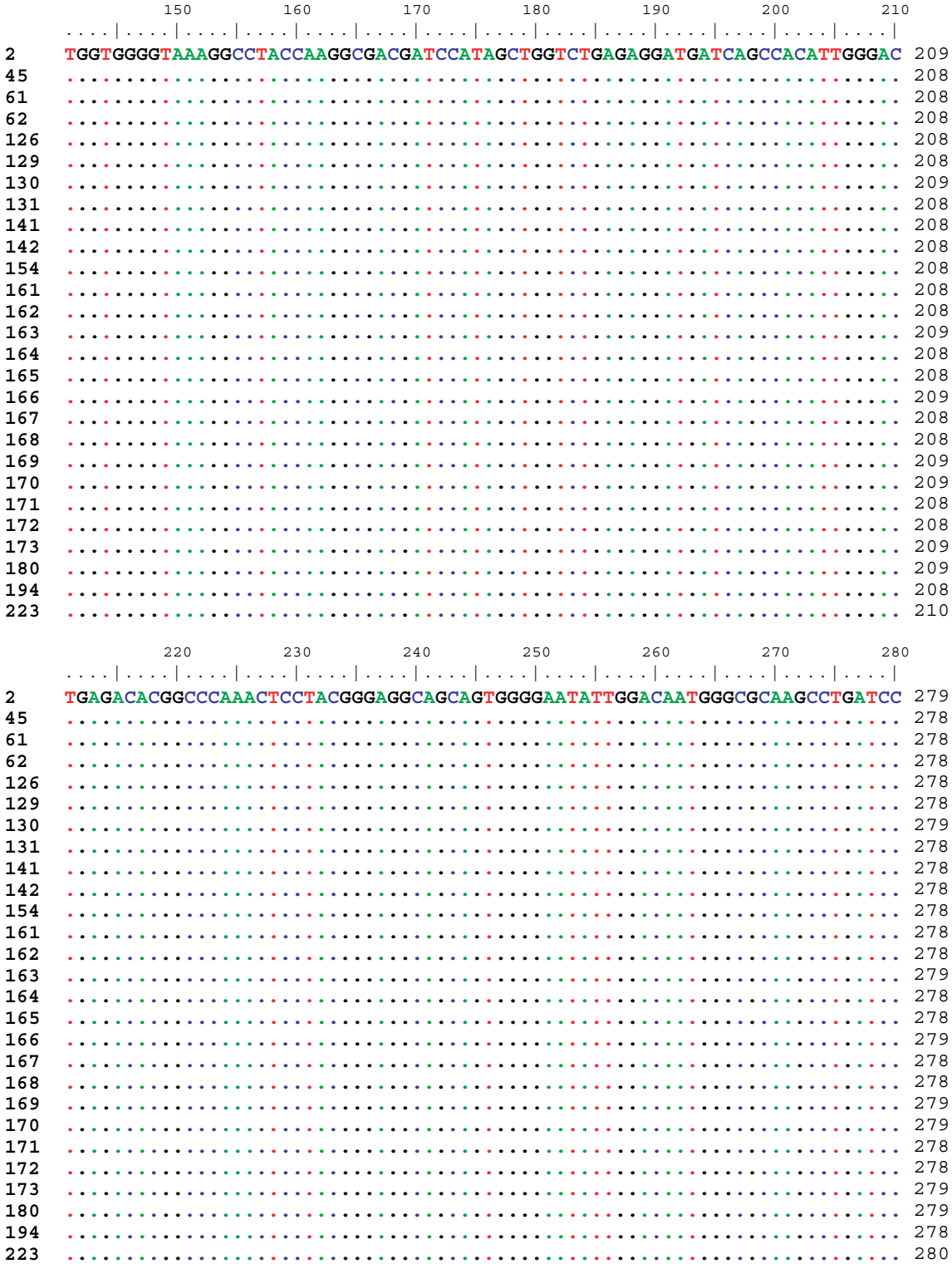
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173 209
180 209
194 208
223 210
220 230 240 250 260 270 280
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45 278
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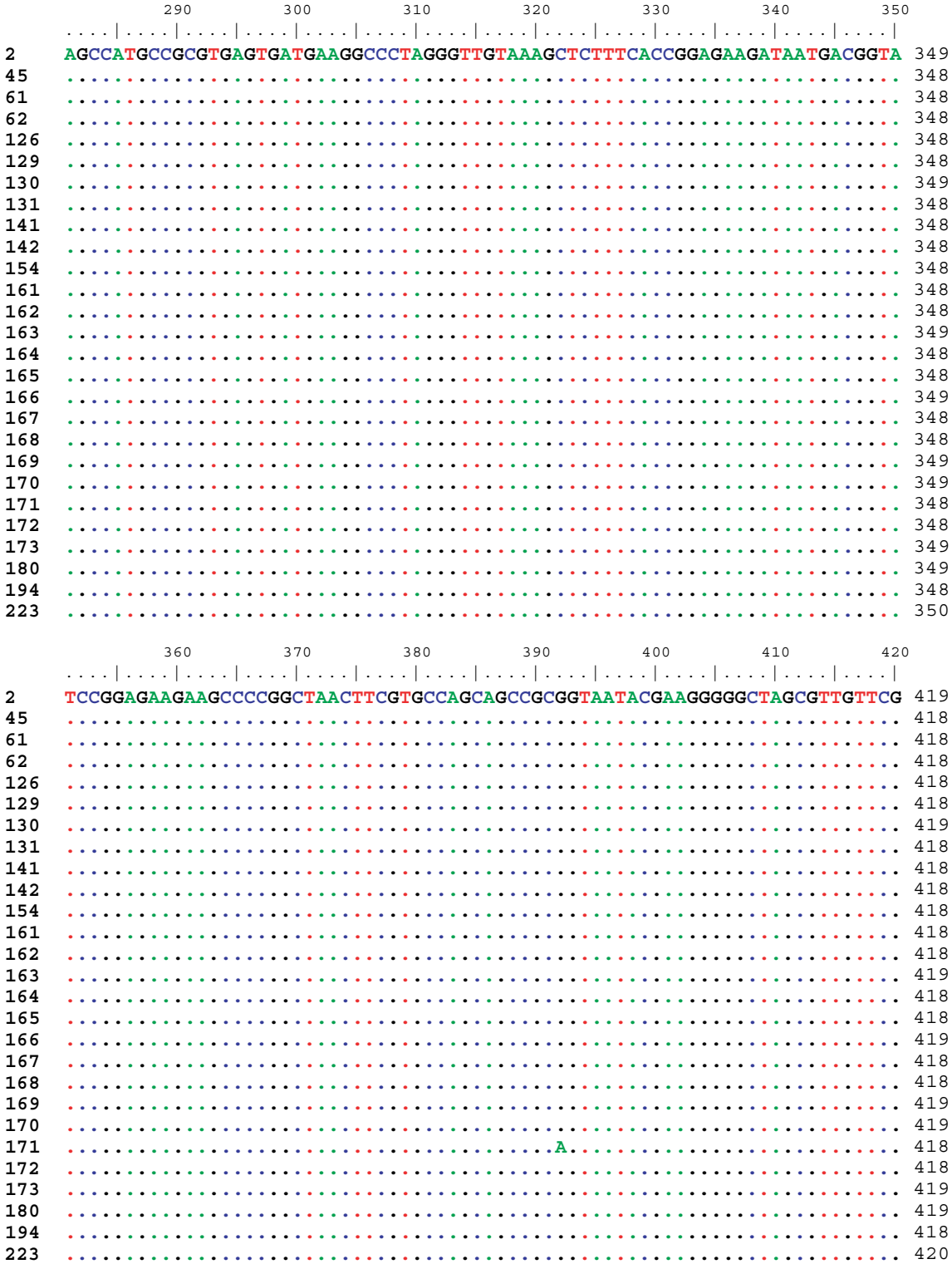
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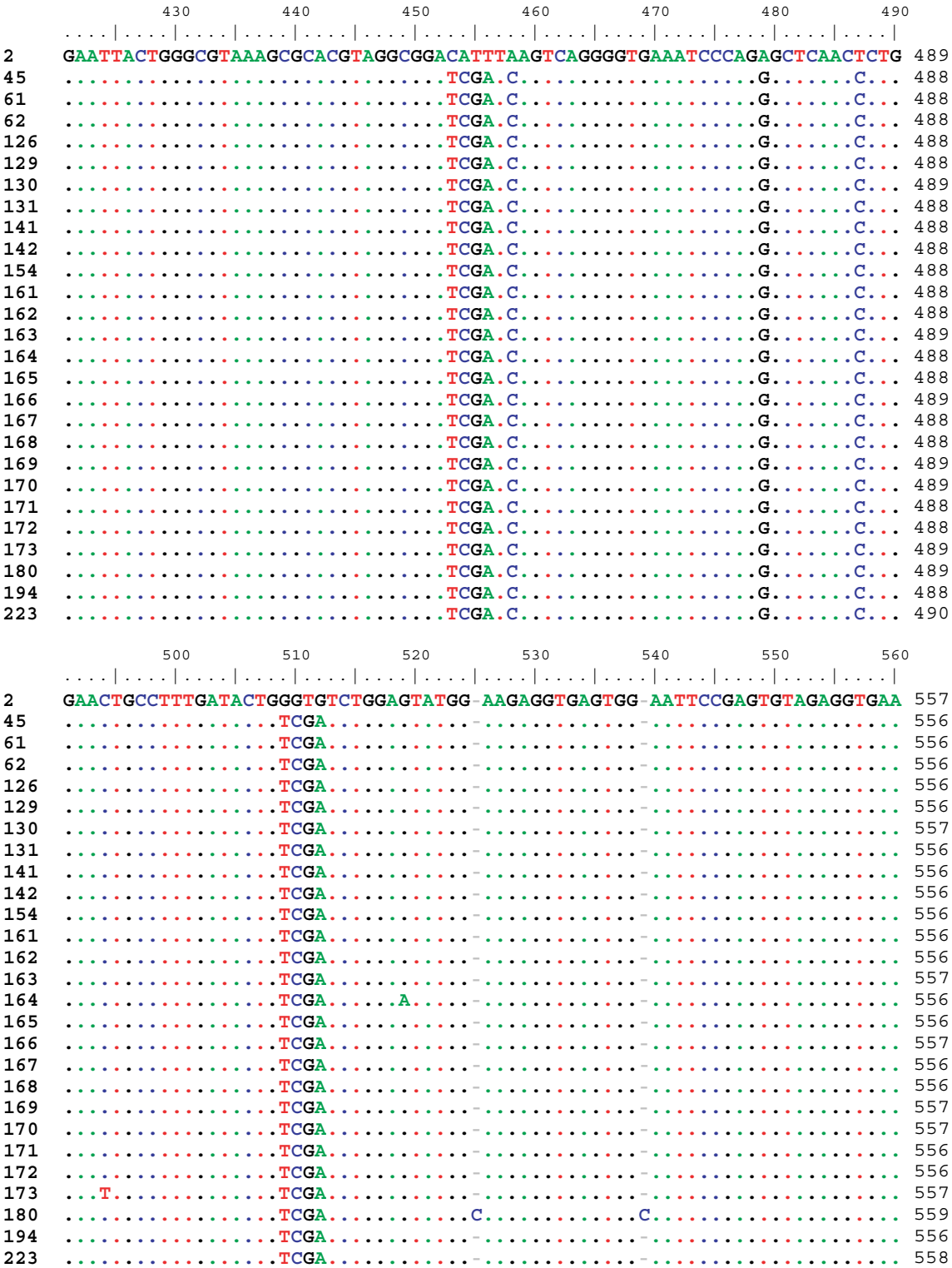
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180 TCGA.C G.C 489
194 TCGA.C G.C 488
223 TCGA.C G.C 490
500 510 520 530 540 550 560
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45 TCGA - - 556
61 TCGA - - 556
62 TCGA - - 556
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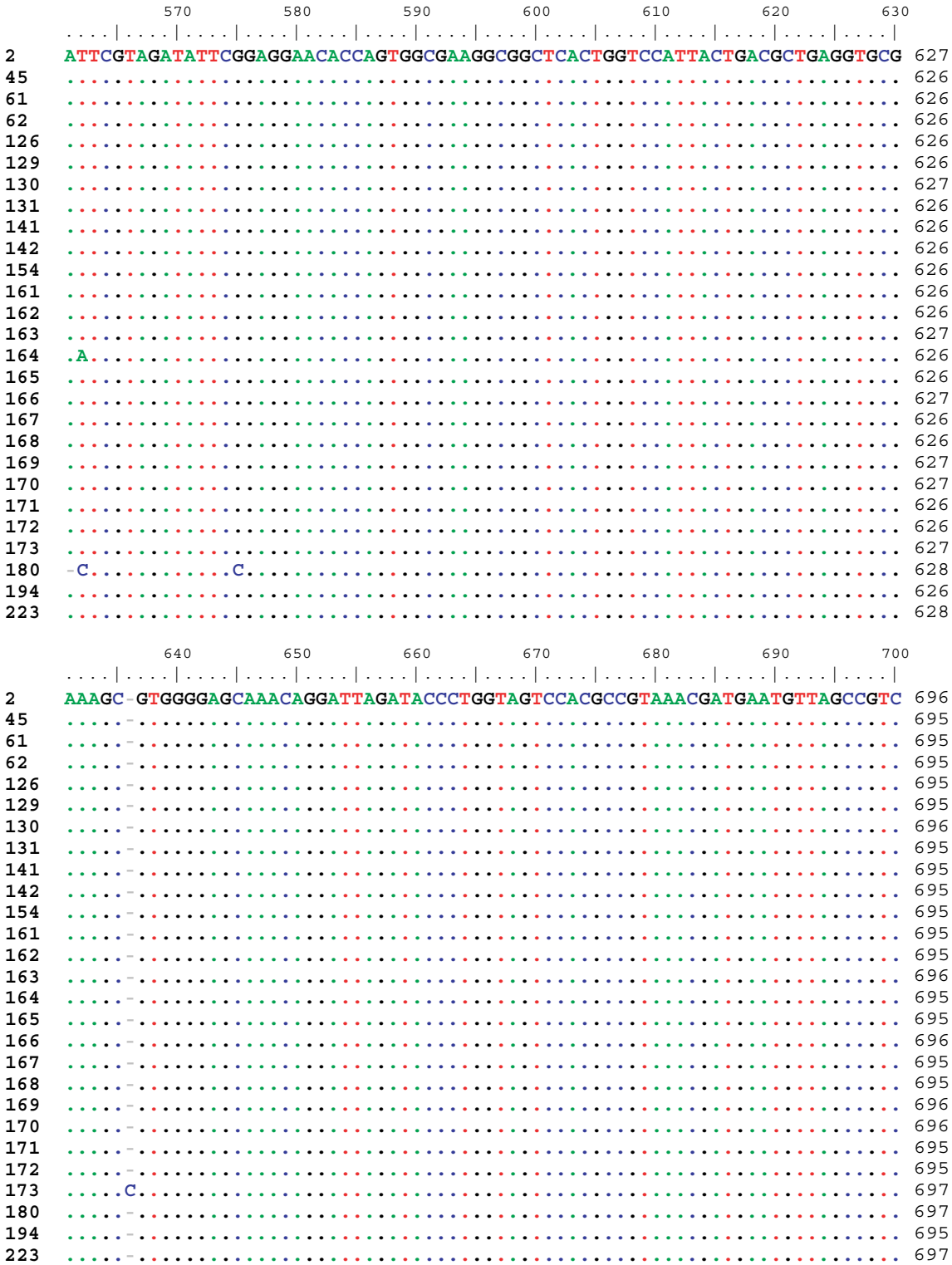
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163 627
164 .A. 626
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169 627
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173 627
180 -C.....C. 628
194 626
223 628
640 650 660 670 680 690 700
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45 .....-. 695
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173 .....C. 697
180 .....-. 697
194 .....-. 695
223 .....-. 697


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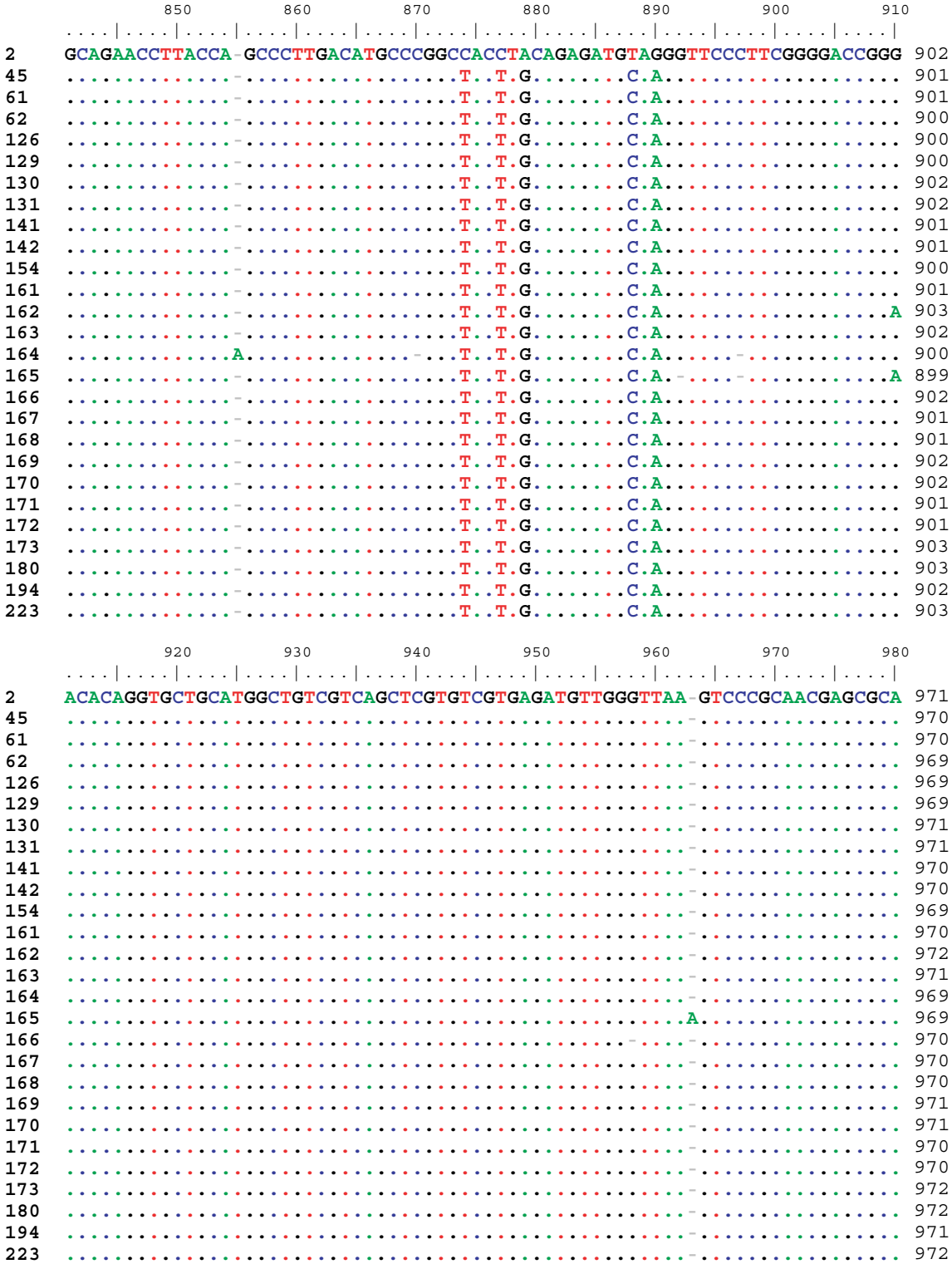
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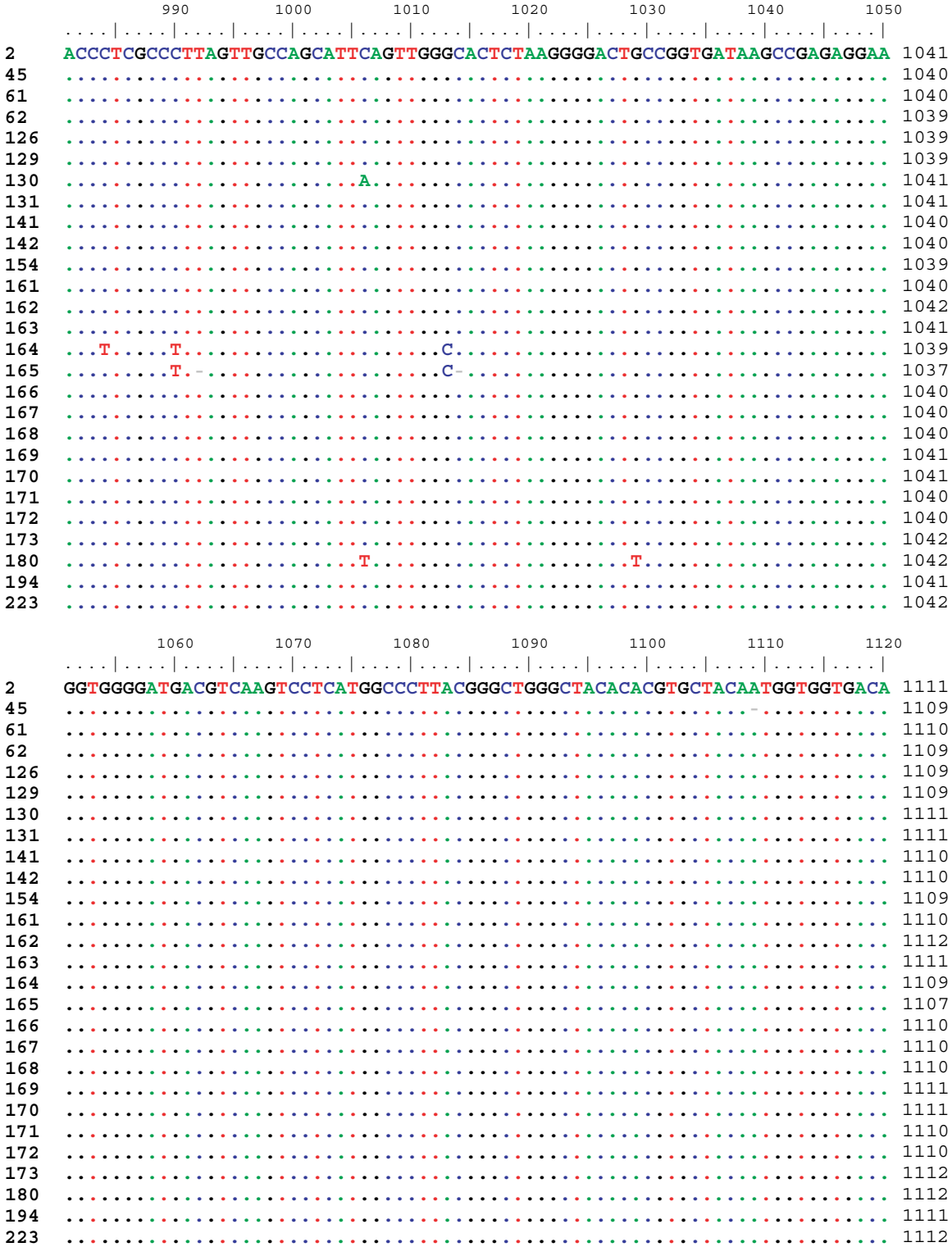
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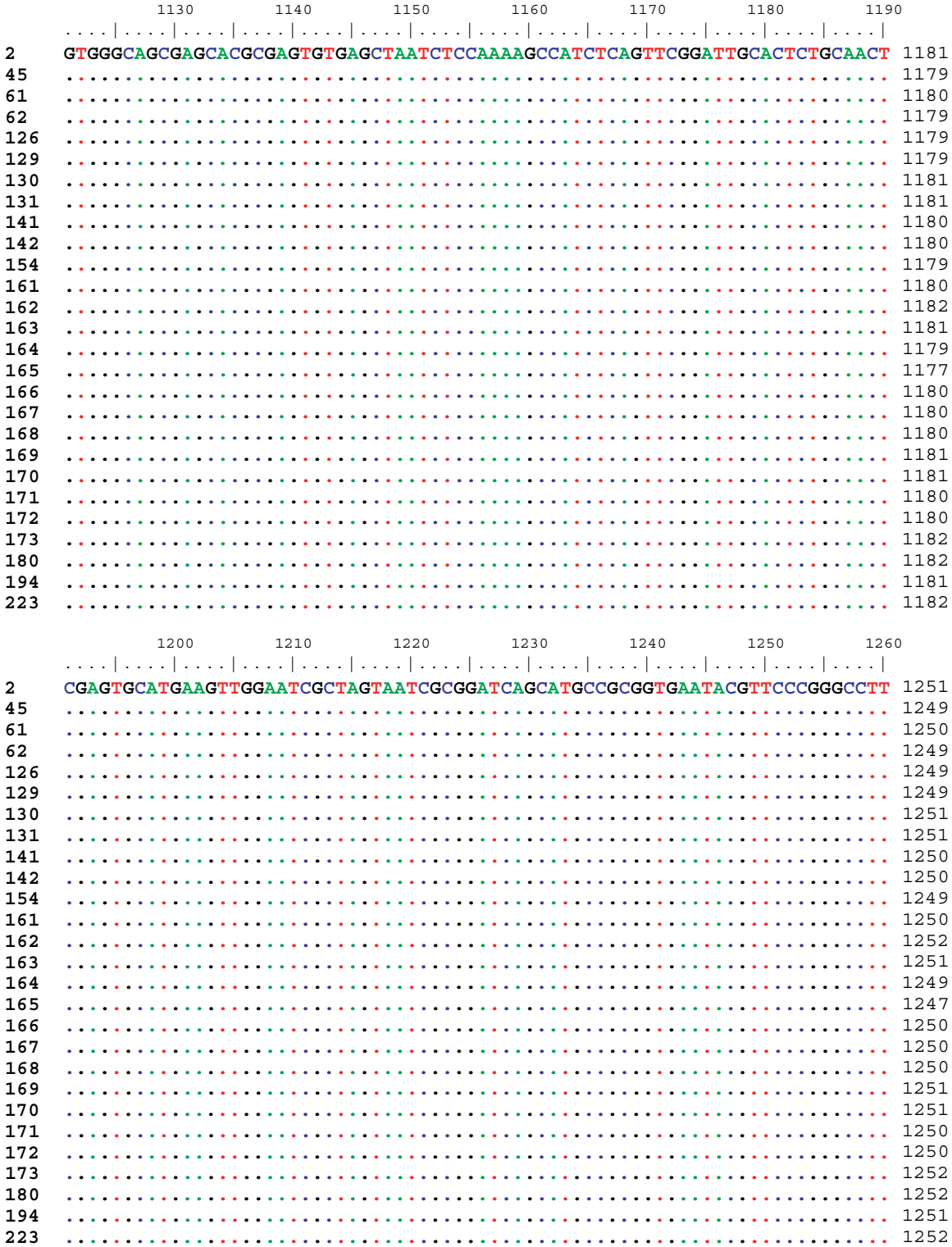
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