The role of ACC deaminase in plant growth promotion by the
endophytic bacterium *Burkholderia phytofirmans* PsJN

By

Yili Sun

A thesis
presented to the University of Waterloo
in fulfillment of the
thesis requirement for the degree of
Master of Science
in
Biology

Waterloo, Ontario, Canada, 2008

© Yili Sun 2008

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.

I understand that my thesis may be made electronically available to the public.

ABSTRACT

The endophytic bacterium *Burkholderia phytofirmans* PsJN has been previously shown to promote plant growth. This bacterium produces siderophores, indoleacetic acid (IAA) and the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, all of which have previously been implicated in the promotion of plant growth by bacteria. Following isolation of the ACC deaminase gene (*acdS*), AcdS deficient mutants of PsJN were generated. One mutant contains a tetracycline resistance gene inserted into *acdS*, and the other mutant contains a deletion in the *acdS* gene. Both of the mutants showed no detectable ACC deaminase activity, produced a decreased level of siderophores and an increased amount of IAA compared to the wild-type, and lost the ability to promote canola root elongation. In addition, the GFP-labeled *acdS* deletion mutant colonized plant interior surfaces somewhat less efficiently than the GFP-labeled wild-type strain.

ACKNOWLEDGMENTS

I would like to extend my deepest appreciation to these people who have helped me in one way or another to finish this thesis.

In the first place, I would like to acknowledge my supervisor Dr. Bernard Glick for his supervision, advice, and guidance through out the work. Over the two years, he has provided me with countless brilliant ideas helping me overcome all the difficulties in my experiments, and with the freedom to explore, which shaped me into the independent researcher I am today.

Many thanks go to my lab-mates Zhenyu Cheng, Jin Duan, Youai Hao, Jennifer Czarny, Biljana Todorovic and Merav Kamensky. It is a pleasure to work with these talented and hard-working people. I benefited a lot from the scientific discussions in our lab.

I gratefully thank Susan Lolle and Trevor Charles for their constructive comments on this thesis.

Last but not least, I would like to say thank you to my fiends Xuan Huang, Bo Yang Yu and Liangliang Hao for sharing joys and tears in the two years, and also for being supportive all the time.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

GLOSSARY

ACC   1-aminocyclopropane-1-carboxylic acid

acdS   The ACC deaminase structural gene

acdR   The ACC deaminase regulatory gene

Amp   ampicillin

DIG   digoxigenin

Km   kanamycin

Lrp   Leucine responsive regulatory protein

PGPR   Plant growth-promoting rhizobacteria

SAM   S-adenosyl-L-methionine

Tc   tetracycline

1 Introduction

1.1 Ethylene

Ethylene is one of the simplest organic molecules with biological activity. Its existence was first proposed in 1901 by Russian scientist Dimitri Neljubov, with etiolated pea seedlings (Abeles *et al.*, 1992).

In higher plants ethylene is formed from L-methionine via *S*-adenosyl-L-methionine (SAM) and the cyclic non-protein amino acid 1-aminocyclopropane-1-carboxylic acid (ACC) (Adams and Yang, 1979). The enzymes catalyzing the conversion of methionine to SAM, of SAM to ACC and of ACC to ethylene are SAM synthetase, ACC synthase and ACC oxidase, respectively (Giovanelli *et al.*, 1980; Kende, 1989; John, 1991). Besides ACC, the other ACC synthase product is 5'-methylthioadenosine (MTA), which is recycled to L-methionine through the Yang cycle (Miyazaki and Yang, 1987). By preserving the methylthio group at the cost of one molecule of ATP per cycle, this pathway ensures that a high rate of ethylene biosynthesis leaves the level of the rest of the metabolites relatively unchanged, even when the methionine pool is small (Bleecker, 2000). The genes for both ACC synthase and ACC oxidase are parts of multigene families and the transcription of different forms is induced by different environmental or physiological conditions (Theologis, 1992).

Ethylene plays important roles not only in a plant's normal development but also in its response to stress (Deikman, 1997). During normal development, ethylene is involved in seed germination, senescence, abscission and fruit ripening, as well as growth of

vegetative tissue including stems, roots, and petioles (Deikman, 1997). Under stress conditions, such as temperature extremes, water stress, salt, exposure to ultraviolet light, organic chemicals, metals, disease, insect damage and mechanical wounding, the level of ethylene increases. The term "stress ethylene" was coined by Abeles (1973), to describe the increased production of ethylene associated with environmental and biological stresses, including pathogen attack (Morgan and Drew, 1997). Many studies have shown that in stress conditions, such as pathogen infection for example, not only does the pathogen action cause damage to the plant, but also does the autocatalytic ethylene synthesis cause severe damage to the plant (Van Loon, 1984). The production of stress ethylene generally occurs in two stages. The first stage of increased plant ethylene level acts to initiate protective responses that can enhance the survival of plants under adverse conditions, while the second peak of increased ethylene is much higher than the first one, and can exacerbate some of the stress symptoms, such as senescence, chlorosis and abscission (Glick, 2005).

1.2 Plant Growth Promoting Bacteria

The interaction between bacteria and plants can be beneficial, harmful or neutral for the plant. There are two types of bacteria that are beneficial to plants, symbiotic and free-living bacteria. The former generally form specialized structures or nodules on host plants roots, while the latter live near or inside the plant roots (Glick, 1995a). Free-living beneficial soil bacteria are generally considered to be plant growth-promoting bacteria (PGPB), and they are found associated with roots of many different plants (Glick *et al.*,

1999). The most widely studied group of PGPB is plant growth-promoting rhizobacteria (PGPR) which colonize the plant rhizosphere.

Generally PGPR can stimulate plant growth directly or indirectly (Glick, 1995a). Indirect promotion happens when bacteria prevent plants from being inhibited by some phytopathogenic organisms or decrease the deleterious effects of the pathogens (Glick and Bashan, 1997). Direct promotion of plant growth occurs when these bacteria provide the plants with compounds they synthesize, such as fixed nitrogen or phytohormones; facilitate the uptake of nutrients, such as iron and phosphorus, from the environment by the plant; or synthesize the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase which can lower the ethylene level in the plant (Glick *et al.*, 1998).

1.3 ACC deaminase

ACC deaminase is a multimeric enzyme, and each of the monomeric subunits is about 35-42 kDa. It is a sulfhydryl enzyme with pyridoxal 5-phosphate as an essential co-factor. ACC deaminase catalyzes the cleavage of ACC, forming α-ketobutyrate and ammonia (reviewed by Glick, 2005; Fig. 1-1 A). It is suggested that lowering the ethylene level of plants is one of the most important mechanisms used by PGPR to stimulate plant growth (Hall *et al.*, 1996).

A model was proposed to explain the role of ACC deaminase in plant growth promotion (Glick *et al.*, 1998; Fig. 1-1 B). In this model, the plant growth-promoting bacteria bind to the surface of either the seed or root of a developing plant. Some of the indoleacetic acid (IAA) synthesized by the bacteria is taken up by the plant. However, besides stimulating plant cell proliferation, IAA can induce the transcription of ACC

synthase, the enzyme which converts SAM to ACC. Some of the ACC is exuded from seeds or roots, taken up by bacteria and hydrolysed by ACC deaminase to ammonia and α-ketobutyrate, both of which can be metabolized by most soil bacteria. This process efficiently decreases the ACC level in a plant, and may provide the bacterium with a unique source of nitrogen in the form of ACC which other soil bacteria can not use (Glick *et al.*, 1998). Thus, ACC deaminase-containing plant growth-promoting bacteria act as a sink for ACC, thereby lowering plant ethylene levels.

In most of the *Proteobacteria* that contain an ACC deaminase gene, *acdS*, there is also an *acdR* gene, which is a homolog to leucine-responsive regulator genes *lrp*, in the vicinity of *acdS* (Prigent-Combaret *et al.*, 2008). The *acdS acdR* locus is sometimes located on mobile genetic elements, especially in *Alphaproteobacteria*, while they are located within the chromosomal DNA in most *Gamma-* and *Betaproteobacteria* (Prigent-Combaret *et al.*, 2008). The regulatory role of AcdR in *acdS* transcription has been elaborated in several studies (Grichko and Glick, 2000; Li and Glick, 2001; Ma *et al.*, 2003; Prigent-Combaret *et al.*, 2008; Cheng *et al.*, 2008). Evidence for the role of AcdR in regulating expression of *acdS* include the binding sites for Lrp-like regulator AcdR in *acdS* promoter region (Grichko and Glick, 2000; Li and Glick, 2001; Ma *et al.*, 2003; Prigent-Combaret et al., 2008); AcdR inactivation abolished ACC deaminase activity in *R. leguminosarum* 128C35K (Ma *et al.*, 2003), as well as the physical interaction between the AcdR protein and the *acdS* promoter in *A. lipoferum* 4B and the abolishment of ACC deaminase activity in an *acdR* mutant in *A. lipoferum* 4B (Prigent-Combaret et al., 2008). In addition, Cheng *et al.* (2008) recently showed that AcdR acts in concert with another protein, AcdB, to regulate *acdS* expression.

To study the role of ACC deaminase in plant growth promotion, an ACC deaminase minus mutant (AcdS⁻) of *Pseudomonas putida* UW4 was constructed and characterized (Li *et al.*, 2000). Compared to the wild-type *P. putida* UW4, both ACC deaminase activity of UW4 AcdS⁻ and its ability to promote canola roots elongation under gnotobiotic conditions were greatly diminished (Li *et al.*, 2000). Furthermore, it has been shown that the wild-type UW4, not the UW4 AcdS⁻ strain, significantly improved plant growth under salt stress conditions (Cheng *et al.*, 2007).

ACC deaminase-containing plant growth-promoting bacteria have been reported to be able to protect plants against various stresses by lowering the level of stress ethylene in the plants (reviewed by Glick, 2004). For example, tomato plants inoculated with the ACC deaminase-containing bacterium *Pseudomonas putida* UW4, *Enterobacter cloacae* CAL2 or *Pseudomonas putida* ATCC17399/pRKACC showed a substantial tolerance to flooding stress (Grichko and Glick, 2001). Two biocontrol bacterial strains were transformed with the ACC deaminase gene from *Pseudomonas putida* UW4, and were found to be more effective than non-transformed biocontrol strains in suppressing phytopathogen damage of cucumbers (Wang *et al.*, 2000). Plant growth-promoting bacteria with ACC deaminase activity have also been reported to be able to accelerate plant growth in recalcitrant organic contaminated soils, diminishing the toxic effects to plants (Huang *et al.*, 2004a and b). Moreover, Mayak *et al.* (2004a and b) reported that the ACC deaminase-containing bacterium *Achromobacter piechaudii* ARV8 significantly promoted tomato growth under both drought and salt stress conditions.

Figure 1-1 Proposed role of ACC deaminase in plant growth promotion.

Panel A shows the biochemical mechanism of ACC cleavage.

A

B
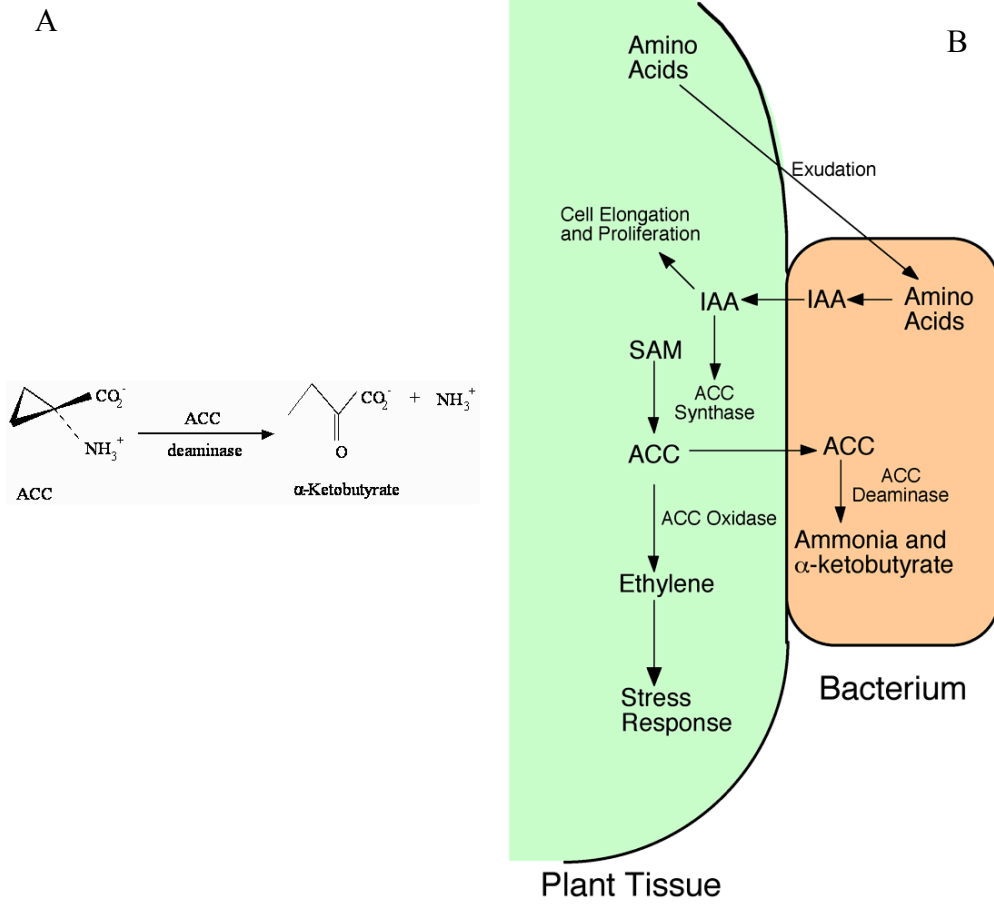
1.4 Endophytic bacteria

Endophytic bacteria can enter the root interior and establish endophytic populations. The term endophyte is derived from the Greek "endon" (within) and "phyte" (plant), and it had originally been applied exclusively to fungi (Carroll, 1988; Clay, 1988), including the mycorrhizal fungi (O'Dell and Trappe, 1992). In 1995, a new definition of endophyte was proposed, "fungi or bacteria, which for all or part of their life cycle invade the tissues of living plants and cause unapparent and asymptomatic infections entirely within plant tissues, but cause no symptoms of disease" (Wilson, 1995). In 1997, Hallmann defined endophytic bacteria from another perspective, "those bacteria that can be isolated from surface-disinfected plant tissue or extracted from within the plant, and that do not visibly harm the plant" (Hallmann *et al.*, 1997).

The rhizosphere is now considered the major source of endophytic bacteria (Sturz *et al.*, 2000; Germaine *et al.*, 2004; Compant *et al.*, 2005) although they may also originate from other sources, such as the phyllosphere, the anthosphere, or the spermosphere (Hallmann *et al.*, 1997). As early as 1973, Darbyshire and Greaves classified endophytic bacteria into the bacterial rhizosphere community. Old and Nicolson (1978) further defined the root cortex as a part of the soil-root microbial environment. Frommel (1991) found that in potatoes inoculated with an endophytic species, the endophytic population approached a higher optimal density in roots than stems, which suggested that endophytes enter plants from the rhizosphere. In addition, the similarity between bacterial species within plant organs and those found in the rhizosphere also supports the view that soil is a major source from which endophytic bacterial populations originate (reviewed by Sturz et al., 2000).

However, some evidence has shown that endophytic bacteria and rhizobacteria each have distinct features. For example, endophytic and rhizobacteria from the same genera were found to form discrete subpopulations, suitable for colonizing different locations, through an analysis of lypopolysaccharide and cell envelope protein patterns (van Peer *et al.*, 1990). Based on this observation, the authors suggested that it is likely that some selection mechanism for the entry into plants might have taken place prior to host-colonization.

1.5 Colonization of endophytic bacteria

Endophytic bacteria have been isolated from both monocotyledonous and dicotyledonous plants, ranging from woody tree species to herbaceous crop plants (reviewed by Lodewyckx et al., 2002). Many studies reported the presence of endophytic bacteria in the intercellular spaces inside plants, especially in xylem vessels, while intracellular endophytic colonization was also found in cytoplasm and vacuoles within some plants (reviewed by Sturz et al., 2000). A current view of colonization by endophytic bacteria is that they originate from the rhizosphere, penetrate and colonize root tissue, and some of them can find a route into the xylem, and then transport themselves throughout the plant (Hurek *et al.*, 1994; Shishido *et al.*, 1999; Gyaneshwar *et al.*, 2001; Lodewyckx *et al.*, 2002; James *et al.,* 2002; Compant et al., 2005, 2008a).

To initiate colonization, plants secrete root exudates which facilitate communication with the bacteria in rhizosphere and guide them to the root surfaces (Bais et al., 2004). The organic acids and amino acids in root exudates, such as ferulic acid, butanoic acid, *trans*-cinnamic acid, harmine, catechin *etc*. (reviewed by Bais et al., 2004), may initiate a

chemotactic response of bacteria which then attach to the root surface by active motility facilitated by flagella (De Weert et al., 2002; Steenhoudt and Vanderleyden, 2000; Turnbull et al., 2001).

After the initial colonization step, certain bacteria are able to enter roots through cracks at root emergence sites or by passing through root tips (reviewed by Reinhold-Hurek and Hurek, 1998) or through the middle lamella of the epidermal layer (Hurek et al., 1994). Once inside the plant tissue, endophytic bacteria either remain localized in a specific plant tissue, such as root cortex, or systematically colonize the entire plant (Lodewyckx et al., 2002). Intercellular spreading of bacteria may be mediated by pectic enzymes which can break down the middle lamella connecting plant cells, as is the case for *Azospirillum*, for example (reviewed by Reinhold-Hurek and Hurek, 1998). Intracellular colonization involves cellulolytic enzymes which are necessary to overcome the barrier of the primary or secondary cell wall. For example, the endophytic bacterium *Burkholderia phytofirmans* PsJN produces enzymes endoglucanase and endopolygalacturonase, which may help to permeate the central cylinder of *Vitis vinifera* L. Chardonnay plant by breaking the endodermis barrier (Compant et al., 2005). Once inside the plant, endophytic populations vary with different plants and different tissues, but usually achieve lower population densities than phytopathogen populations which can reach up to 10^{10} cells per gram of plant fresh weight (Grimault and Prior, 1994).

Although the root zone offers the most obvious site of entry for many endophytic bacteria, the entry may also occur at sites on the surface of seeds, leaves and flowers (Lamb et al., 1996; Sharrock et al., 1991). In plants that propagate vegetatively, such as

potatoes, parent materials can also be a source of endophytic bacteria that subsequently colonize the developing roots and shoots via vascular tissues (Sturz et al., 2000).

1.6 Burkholderia phytofirmans PsJN

Burkholderia phytofirmans PsJN, which was originally designated *Pseudomonas* sp. strain PsJN (Frommel et al., 1991) is a highly effective plant-beneficial bacterium, first isolated as a contaminant from *Glomus vesiculiferum*-infected onion roots (Nowak et al., 1998). *B. phytofirmans* PsJN has been found to establish rhizosphere and endophytic populations associated with potato, tomato, grapevine, cucumber, watermelon and chickpea (Frommel *et al*., 1991; Ait Barka *et al*., 2000; Liu *et al*., 1995; Pillay and Nowak, 1997; Sessitsch *et al*., 2005; Compant *et al*. 2005, 2008a, 2008b).

B. phytofirmans PsJN has been shown to stimulate plantlet growth, induce developmental changes, lead to better water management and enhance resistance to low levels of pathogens upon transplantation and *in vitro* infection of grapevine with *Botrytis cinerea* (reviewed by Nowak and Shulaev, 2003). Recently, additional properties of this strain as a PGPR were reported. For example, *B. phytofirmans* PsJN showed a high level of ACC deaminase activity (Sessitsch *et al*., 2005), which can lower the ethylene level in plants and stimulate plant growth. Inoculation with PsJN promoted the growth of grapevines and improved their ability to withstand cold stress (Ait Barka *et al*., 2006). Moreover, *B. phytofirmans* PsJN has been visualized colonizing the root surface of grapevine, entering into root internal tissues, translocating via stem xylem vessels to leaf tissues (Compant *et al*., 2005), inflorescence stalks and immature berries (Compant *et al*., 2008a).

1.7 Objective of this study

Endophytic bacterium *Burkholderia phytofirmans* PsJN has been previously shown to promote plant growth. ACC deaminase is known to be a very important mechanism used by PGPR to stimulate plant growth. To study how ACC deaminase affects the effectiveness of an endophyte, ACC deaminase minus mutants (AcdS⁻) of PsJN were constructed. To study the mechanisms used by PsJN to stimulate plant growth, both of the wild-type and mutant strains of PsJN were characterized for IAA secretion, siderophore production, ACC deaminase activity and the ability to promote plant growth under gnotobiotic conditions. To study colonization of canola by the PsJN strains, bacteria were labeled with a green fluorescent protein (GFP) gene marker and monitored *in situ* with a confocal microscope.

2 Materials and Methods

2.1 Bacterial strains and plasmids

The endophytic bacterium *Burkholderia phytofirmans* PsJN (Sessitsch et al., 2005) was generously provided by Dr. Jerzy Nowak, Virginia Polytechnic Institute and State University. This strain was grown on either solid or liquid tryptic soybean broth (TSB) medium (Difco Laboratories, Detroit, MI) with 20 µg/ml rifampicin at 30°C. It was also grown in DF salts minimal medium (Dworkin and Foster, 1958) supplemented with either 0.2% w/v $(NH_4)_2SO_4$ or 3 mM ACC as a nitrogen source.

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

E. coli DH5α with p519ngfp (Matthysse et al., 1996) was obtained from the American Type Culture Collection (ATCC #87453). Plasmid p519ngfp is a broad host range mob^+ plasmid containing *gfp* under the transcriptional control of an *npt2* promoter. This plasmid was used to enable *Burkholderia phytofirmans* PsJN and its ACC deaminase deficient mutants to express Green Fluorescent Protein (GFP), so that they could be visualized under confocal microscopy. *E. coli* DH5α/p519ngfp was grown at 37°C in Luria Broth medium (Difco Laboratories, Detroit, MI) with 20 µg/ml kanamycin.

E. coli strains containing plasmid pK19*mobsacB* (Schäfer et al., 1994) were grown at 37°C in Luria Broth medium (Difco Laboratories, Detroit, MI) supplemented with 20 µg/ml kanamycin. pK19*mobsacB* is derived from the pK19 plasmid, and it contains the broad-host-range transfer machinery of plasmid RP4 and a modified *sacB* gene from

Bacillus subtilis. It is a very useful tool to enhance and subsequently to detect rare double cross-over events.

Antibiotics were used at the following concentration for both *E. coli* and *B. phytofirmans* strains (μg/ml): ampicillin (Amp), 100; tetracycline (Tc), 15; kanamycin (Km), 20; rifampicin (Rif), 20.

2.2 Isolation of genomic DNA from *Burkholderia phytofirmans* PsJN

A single colony of *B. phytofirmans* PsJN was transferred into 5 ml TSB medium, and incubated at 30°C for 2 days until the cells reached stationary phase. One milliliter of the culture was transferred into a 1.5 ml microcentrifuge tube and centrifuged at 6000 ×g for 5 minutes using an Eppendorf centrifuge 5417c (Hamburg, Germany). Genomic DNA was isolated from the cell pellets using a Promega (Mississauga, ON, Canada) Wizard genomic DNA purification system according to the manufacture suggested protocol.

2.3 Isolation of the ACC deaminase gene from *Burkholderia phytofirmans* PsJN

A draft genome sequence of *Burkholderia phytofirmans* PsJN(NZ_AAUH00000000) has been determined by the US DOE Joint Genome Institute and posted on the NCBI website (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nuccore&id=118045147) on November 16th, 2006. The gene encoding the *Burkholderia phytofirmans* PsJN 1-aminocyclopropane-1-carboxylate deaminase is 1017 bp. According to the nucleotide sequence of this strain, specific PCR primers were designed as followed: 5'-TTGTTGCGTTCATAGGTTCC-3' (sense) and 5'-TCCTGAGGGAGCATTTGAG-3'

(antisense) and were synthesized by Sigma (Oakville, ON, Canada). The 25 μl PCR mixture contained 12.5 μl Go Taq® Green Master Mix from Promega (Madison, WI, USA), 2 μl of 20 pmol of each primer, 200 ng genomic DNA of *Burkholderia phytofirmans* PsJN as template DNA. PCR amplification was performed using a MJ Instrument PTC-100 thermocycler (Waltham, MA) with the following program: 4 minutes initial denaturation at 94°C, 30 cycles of 30 seconds denaturation at 94°C, 30 seconds primer annealing at 57°C, and 1 minute elongation at 72°C. A final elongation step was 10 minutes at 72°C.

2.4 Construction of ACC deaminase deficient mutants of *Burkholderia phytofirmans* PsJN

To construct an ACC deaminase deficient mutant of *B. phytofirmans* PsJN, a replacement vector which contained a disrupted ACC deaminase gene was first constructed. The replacement vector was then introduced by conjugation into wild-type *B. phytofirmans* PsJN. Subsequently, clones were selected in which a homologous double-crossover event had occurred between the endogenous *acdS* gene in the bacterial genome and the mutant *acdS* gene on the vector. Two ACC deaminase deficient mutants of *Burkholderia phytofirmans* PsJN were constructed using different methods.

2.4.1 Construction of ACC deaminase deficient replacement vectors

The construction of the first replacement vector for the construction of a PsJN ACC deaminase deficient mutant is outlined in Fig. 2-1. The PCR product of the PsJN *acdS*

gene was cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA), to make pGEMACC. The linear pGEM-T Easy vector contains a 3'-T to both ends, which result in compatible overhangs for PCR products generated by Taq DNA polymerase. To interrupt the *acdS* gene on plasmid pGEMACC, the pBR322 EcoRI-AvaI fragment containing the tetracycline resistance gene (Tc) was inserted into the EcoRV site within the *acdS* coding region to make pGEMACC⁻. Since EcoRV is a blunt end restriction enzyme, the 5' protruding ends of the EcoRI-AvaI fragment from pBR322 was filled in and made blunt ended by *E. coli* DNA Polymerase I Klenow Fragment (MBI Fermentas, Inc.) before insertion. Then, the plasmid pGEMACC⁻ was digested by EcoRI and the fragment that contains *acdS* with the tetracycline resistance gene insertion was cloned into the EcoRI site of the mobilizable cloning vector pK19*mobsacB* (Schäfer et al., 1994) to make pK19Rep. The plasmid pK19*mobsacB* was derived from *Escherichia coli* plasmid pK19, and it contains the broad-host-range transfer machinery of RP4 and a modified *sacB* gene (Selbitschka et al., 1993) from *Bacillus subtilis*. The *sacB* gene, which codes for the enzyme levansucrase and confers sucrose sensitivity to Gram-negative and some Gram-positive bacteria, was used here as a conditional lethal gene to discriminate between single and double cross-over events.

The second replacement vector was constructed as outlined in Fig. 2-2. Instead of inserting an exogenous gene to interrupt the *acdS* gene, an EcoRV-ClaI fragment was removed from the coding region of *acdS*. The mutant *acdS* gene was cloned into pK19*mobsacB* to yield pK19RepV2.

Among the molecular biological techniques used in the project, the isolation of plasmid DNA was performed using a Promega Wizard® Plus SV Minipreps DNA

Purification System (Madison, WI, USA), and DNA extraction from agarose gels were done using a QIAquick® Gel Extraction Kit from QIAGEN Sciences (Maryland, USA). The restriction enzymes EcoRV, EcoRI, AvaI and ClaI were all obtained from MBI Fermentas Incorporated, and the T4 DNA ligase was from Promega (Madison, WI, USA).

Figure 2-1 Schematic Representation of the Construction of the Replacement Vector pK19Rep.

Am: ampicillin resistance gene. Tc: tetracycline resistance gene. Km: kanamycin resistance gene. *acdS*: the ACC deaminase gene of *Burkholderia phytofirmans* PsJN. *sacB*: the gene encodes for the *Bacillus subtilis* levansucrase, which confers sucrose-sensitivity to bacteria. *rep*: the replicon responsible for the replication of plasmid (source - plasmid pMB1). *rop*: the gene codes for the Rop protein, which promotes conversion of the unstable RNA I - RNA II complex to a stable complex and serves to decrease copy number.
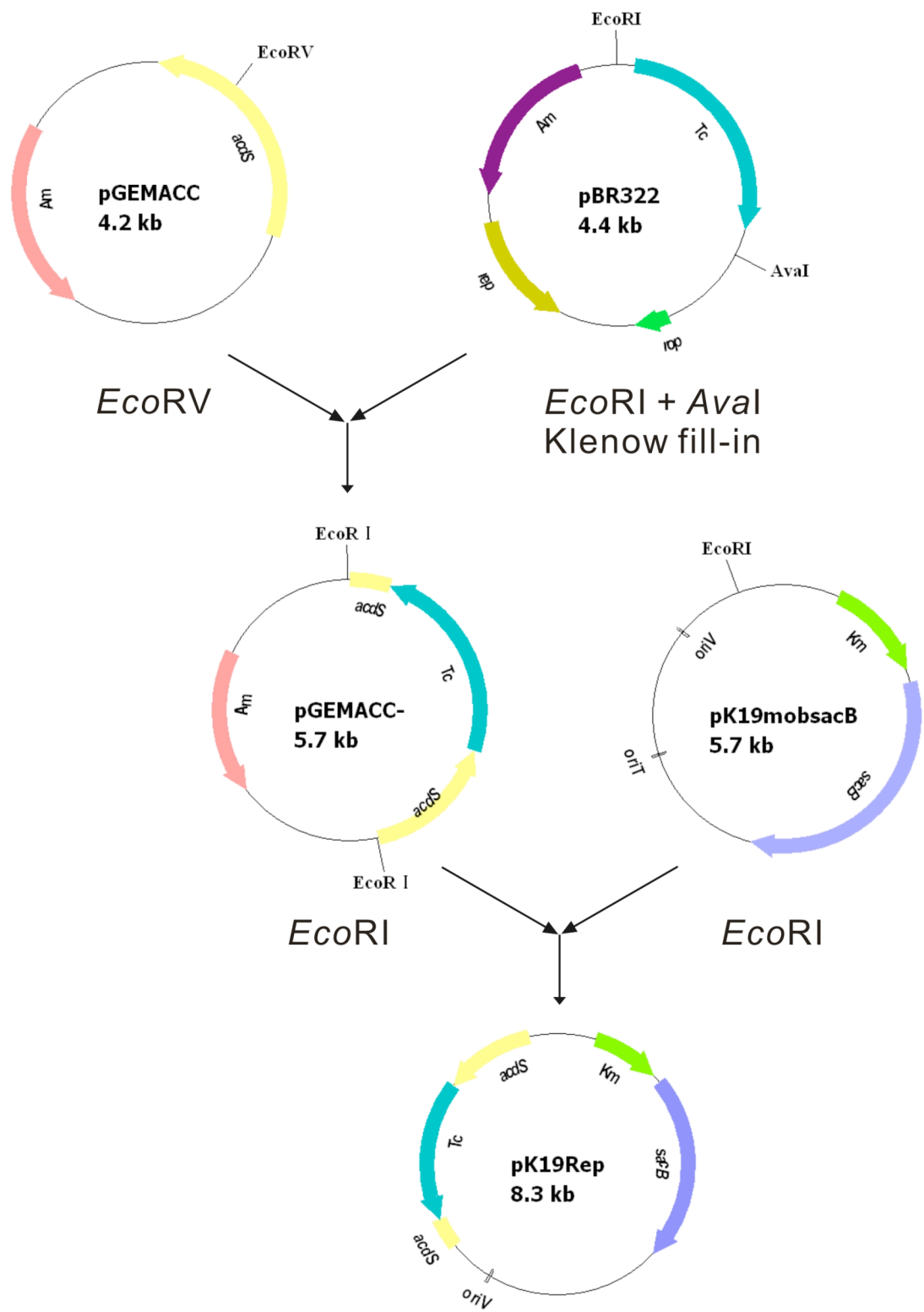
Figure 2-2 Schematic Representation of the Construction of the Replacement Vector pK19RepV2.

Am: ampicillin resistance gene. Km: kanamycin resistance gene. *acdS*: the ACC deaminase gene of *Burkholderia phytofirmans* PsJN. *sacB*: the gene encodes for the *Bacillus subtilis* levansucrase, which confers sucrose-sensitivity to bacteria.
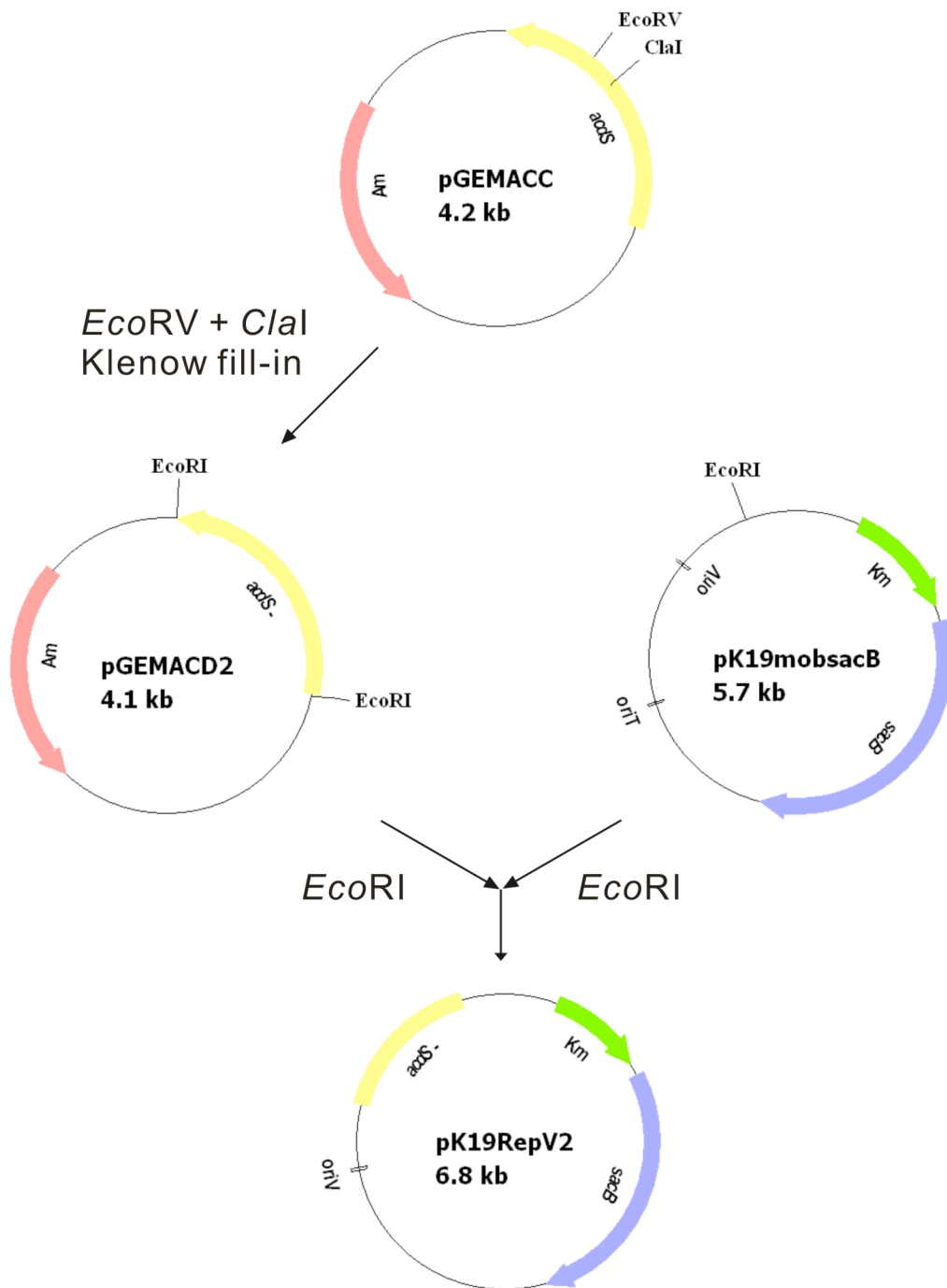
2.4.2 Conjugation and homologous recombination

After constructing the replacement vectors, the replacement vectors were transferred to *B. phytofirmans* PsJN by conjugation to obtain the mutants. In the conjugation experiments, 1.5 ml of an overnight culture of *E. coli* DH5α containing the plasmid being transferred was centrifuged at 8,000 ×g for 3 minutes, suspended in 1 ml 0.85% NaCl, and centrifuged again to collect the cells. This washing procedure aimed to remove the antibiotics in the medium. Then, 1.5 ml of an overnight culture of *B. phytofirmans* PsJN was transferred to the centrifuge tube with the *E. coli* DH5α cell pellet. After centrifugation at 8,000 ×g for 3 minutes, the liquid was discarded, and the cells were again washed with 1 ml 0.85% NaCl. The mixed cells were then suspended in 75 μl 0.85% NaCl, and 75-100 μl of the cell suspension was transferred to the center of a TSB agar plate. Following incubation at 30°C for 24 hours, the cells on the plate were suspended in 1 ml of 0.85% NaCl. The cell suspension was diluted 10^3 times, and a 100 μl aliquot was then plated onto the selective medium plates.

The medium used to select for the first ACC deaminase deficient mutant is TSB agar plus tetracycline, rifampicin and 7.5% sucrose. To select for the second mutant, the cell suspension was first plated on TSB agar with rifampicin and kanamycin to obtain the transconjugants which contain the replacement vector, and then the colonies were incubated in TSB medium with 7.5% sucrose to obtain the PsJN variants in which endogenous gene *acdS* has been replaced by the deficient *acdS*.

2.5 Southern hybridization

2.5.1 Labeling the probe with digoxigenin

Two microgram of the denatured PCR product of the tetracycline resistance gene was tagged with digoxigenin (DIG) using a DIG Oligonucleotide 3'- End Labeling Kit (2nd Generation) (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol. First, 2 μg of the denatured PCR product was diluted to a total volume of 10 μl with double distilled water. Then, 4 μl of 5× Reaction buffer, 4 μl of 5 mM $CoCl_2$-solution, 1 μl of 0.05 mM DIG-ddUTP solution and 1 μl of terminal transferase (20 U) were mixed with the diluted DNA template on ice. This mixture was incubated at 37°C for an hour and the reaction was stopped by adding 2 μl of 0.2 M EDTA (pH 8.0).

2.5.2 Preparation of the genomic DNA for Southern hybridization

Approximately 2 μg of the genomic DNA isolated from both the wild-type *B. phytofirmans* PsJN and its AcdS⁻ mutant No.1 was completely digested with restriction enzyme EcoRV (MBI Fermentas, Inc.) at 37°C for 3 hours. The digested DNA was separated by electrophoresis in a 1% agarose gel using a 1 kb DNA ladder (MBI Fermentas, Inc.) as a sizing standard. The PCR product of the tetracycline resistance gene from pBR322 was used as a positive control. The gel was stained with 0.3 μg/ml (final concentration) ethidium bromide dissolved in the gel, and DNA bands were visualized under UV light.

The agarose gel was washed in 0.25 M HCl at room temperature for 10 minutes to nick the DNA and make it easier to transfer, and then washed in denaturation solution

(0.5 M NaCl, 150 mM NaOH) twice for 15 minutes, followed by two washes in 0.5 M Tris-Cl (pH 8.0), 0.5 M NaCl neutralization solution for 15 minutes to facilitate DNA transfer. Afterward, the DNA on the gel was 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 Russell, 2001).

The nylon membrane was retrieved from the apparatus after 16 hours. The DNA on the membrane was fixed by exposing it to 150 mJoules of UV light in a Gene Linker (Bio-Rad Laboratories, Hercules, CA).

2.5.3 Southern hybridization

The membrane was sealed in plastic bag with 50 ml DIG Easy Hyb solution (Roche Diagnostics GmbH, Mannheim, Germany) and pre-hybridized for 3 hours at 60°C with gentle agitation. Then, the membrane was hybridized with the DIG labeled probe (100 pmol) in 50 ml preheated fresh DIG Easy Hyb solution at 60°C with gentle agitation for 16 hours.

After hybridization, the membrane was washed twice in 2× SSC and 0.1% SDS at room temperature for 5 minutes, followed by two additional washes in 0.5× SSC and 0.1% SDS at 60°C for 15 minutes. The membrane was then ready for the detection of hybridized oligonucleotides.

2.5.4 Visualization of DNA in Southern hybridization

After hybridization and stringency washes, the membrane was washed in 20 ml 1× Washing buffer (10× concentration buffer contains Maleic acid buffer and 3% Tween 20 v/v) for 5 minutes, incubated in 50 ml Blocking solution (Roche Diagnostics GmbH,

Mannheim, Germany) for 30 minutes and another 30 minutes in 20 ml Antibody solution (1 μl Anti-DIG-AP in 5 ml of 1× blocking solution), all with gentle agitation at room temperature. 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 1 M Tris-HCl, pH 9.5 and 1 M NaCl). The membrane was then put in a sealable bag with 20 drops (0.5 ml) of CDP-Star (Roche Diagnostics GmbH, Mannheim, Germany) solution and incubated for 5 minutes at room temperature. Finally, the excess liquid was removed, the bag was sealed and the blot was exposed to film for chemiluminescence detection.

2.6 Culture conditions for the induction of bacterial ACC deaminase activity

The induction of ACC deaminase activity is needed both in studies of bacterial ACC deaminase activity and the ability of bacteria to enhance plant growth. The bacteria *B. phytofirmans* PsJN and its mutants were grown in 15 ml TSB medium with appropriate antibiotics at 30°C for 2 days until they reached stationary phase. To induce ACC deaminase activity, the cells were collected by centrifugation, washed twice with 5 ml DF salts minimal medium (Dworkin and Foster, 1958), suspended in 7.5 ml DF salts minimal medium with 3.0 mM (final concentration) ACC as the sole nitrogen source, and incubated at 30°C with shaking for an additional 24 hours. After the incubation, the cells were harvested by centrifugation and washed twice by suspending the cell pellet in 5 ml of either 0.1 M Tris-HCl, pH 7.6 (if the cells were to be assayed for ACC deaminase activity), or 0.03 M MgSO$_4$ (if they were to be used as a bacterial treatment in the growth

pouch assay). The cell pellet was stored at -20°C overnight for the ACC deaminase activity assay.

2.7 ACC deaminase activity assays of *B. phytofirmans* PsJN and its mutants

ACC deaminase activity was assayed according to the method of Penrose and Glick (2003). In the assay, the production of α-ketobutyrate from the cleavage of ACC catalyzed by ACC deaminase was measured by comparing the absorbance at 540 nm of a sample to a standard curve of α-ketobutyrate ranging from 0.01 to 1.0 µmol. Specifically, the induced bacterial cells were resuspended in 400 µl of 0.1 M Tris-HCl (pH 8.0), labilized by adding 20 µl of toluene and then vortexed for 30 seconds. Fifty microliter of labilized cell suspension was incubated with 5 µl of 0.5 M ACC in a 1.5 ml Eppendorf tube at 30°C for 30 minutes, as well as the negative control of each sample (50 µl of labilized cell suspension without ACC) and the blank (50 µl of 0.1 M Tris-HCl, pH 8.0 with 5 µl of 0.5 M ACC). Following the addition of 500 µl of 0.56 M HCl, the mixture was vortexed and centrifuged at 16 000 ×g for 5 minutes at room temperature. A 500 µl aliquot of the supernatant or standard α-ketobutyrate solution was transferred to a 13 × 100 mm glass test tube, vortexed together with 400 µl of 0.56 N HCl and 150 µl of DNF solution (0.2% 2,4-dinitrophenylhydrazine in 2 N HCl), and incubated at 30°C for 30 minutes. One milliliter of 2 N HCl was added to the samples and the standards before the absorbance at 540 nm was measured. All samples were assayed in duplicate and standards were measured in triplicate.

2.8 Protein concentrations determination

To determine the concentration of toluenized cells，the method of Bradford (1976) was used. A 26.5 μl aliquot of the toluene-labilized bacterial cell sample from the ACC deaminase assay was diluted with 173.5 μl of 0.1 M Tris-HCl (pH 8.0). Following the addition of 200 μl of 0.1 N NaOH, the cells were boiled for 10 minutes. After the sample was cooled to room temperature, 8 μl of the sample or a standard bovine serum albumin (BSA) solution was diluted by adding 792 μl H_2O, and then 200 μl of the Bio-Rad protein dye reagent (Bio-Rad Lab., USA) was added and mixed. The mixture was incubated at room temperature for 5 minutes and the absorbance at 595 nm was measured. The protein concentration was determined by comparing the absorbance to the BSA standard curve.

2.9 Growth pouch assay

The growth pouch assay, or gnotobiotic root elongation assay, is used as a method to study the effect of bacterial strains on the growth of canola seedlings. This assay followed the protocol of Penrose and Glick (2003).

The bacteria used in the assay to treat canola seeds were prepared as described in section 2.6. On ice, the cell pellet was suspended in 0.5 ml sterile 0.03 M $MgSO_4$ and diluted about 100 times in 0.03 M $MgSO_4$ until the absorbance of the bacterial suspension at 600 nm was approximately 0.15.

Before placing the seeds in the seed-pack growth pouches (Northrup King Co., Minneapolis, MN, USA), 12 ml of distilled water was added to each pouch. The pouches

were wrapped with aluminium foil in groups of 10, placed in an upright position, and autoclaved at 121°C for 15 minutes.

Canola seeds (*Brassica napus*) used in this assay were kindly provided by Dr. Laila Yesmin of Brett-Young Seeds (Winnipeg, Manitoba, Canada). To disinfect the seeds (approximately 0.2 g per treatment) before use, the seeds were soaked in 70% ethanol for 1 minute and in 1% sodium hypochlorite for 10 minutes. They were then washed thoroughly with sterile distilled water, at least five times. The seeds were then treated with bacterial suspensions in sterile 0.3 M $MgSO_4$, or with sterile 0.3 M $MgSO_4$ as a negative control in separate dishes for 1 hour at room temperature. Following the incubation, six seeds were placed in each growth pouch with sterilized forceps; 9 pouches were used for each treatment. The pouches for each treatment were placed in a rack (Northrup King Co.) with two empty pouches at the ends of each rack so that all seeds were under similar air and light conditions. Racks with different seed treatments were placed in different clean plastic bins containing sterile distilled water (about 500 ml), and covered loosely with plastic wrap to prevent dehydration. The pouches were incubated in a growth chamber (Conviron CMP 3244; Controlled Environments Ltd, Winnipeg, MB, Canada) at 20±1°C with a cycle of 12 hours of dark followed by 12 hours of light (18μmol $m^{-2}s^{-1}$). For the first two days, the bins were covered with aluminium foil. The primary root lengths were measured on the fifth day of growth and the data were analyzed using a one-way analysis of variance (ANOVA). The roots developed from the seeds that failed to germinate two days after they were sown were not measured.

2.10 Siderophore assay

Siderophore production was detected by the CAS (Chrome azurol S) agar (Alexander and Zuberer, 1991). This medium contains the Fe-CAS-HDTMA dye complex, which gives the medium its characteristic blue color. The siderophores produced by bacteria remove Fe from the Fe-CAS-HDTMA complex and develop orange halos around the bacterial colonies.

The bacterial strains were grown in King's B medium (Proteose peptone No.3 20g/L, MgSO$_4$·7H$_2$O 1.5g/L, Glycerol 15ml/L) overnight at 30°C. An aliquot of 20 µl of the overnight growth cell suspension was inoculated on CAS agar; each strain was assessed in triplicate. After 48 hours incubation at 30°C, the orange halos form when the bacteria produce siderophores.

2.11 IAA production

To quantify the IAA production of *B. phytofirmans* PsJN and its mutants, a modified version of the procedure of Patten and Glick (2002) was used. The bacterial strains were propagated overnight in TSB medium at 30°C, and then 20-µl aliquots of each strain were transferred into two tubes each containing 5 ml of TBS medium with 200 µg/ml and 500 µg/ml L-tryptophan (Sigma) respectively, as well as 5 ml of half TSB half water medium with 200 µg/ml and 500 µg/ml L-tryptophan. After 42 hours incubation, the optical density of each culture was measured at 600 nm, and the bacteria in the culture medium were removed by centrifugation (6000 ×g, 10 minutes). A 1 ml aliquot of the supernatant or standard IAA solution (0, 0.5, 1, 5, 10, 15, 20, 25, 40 µg/ml of 3-indoleacetic acid) was mixed vigorously with 4 ml of Salkowski's reagent (150 ml

of concentrated H_2SO_4, 250 ml of distilled water, 7.5 ml of 0.5 M $FeCl_3 \cdot 6H_2O$; Gordon and Weber, 1951). The mixture was incubated at room temperature for 20 minutes and the absorbance at 535 nm was measured. The concentration of IAA in each culture medium was determined by comparison with the standard curve.

2.12 Labeling GFP

To label *B. phytofirmans* PsJN and its mutants with GFP, conjugation between *B. phytofirmans* and *E.coli* carrying p519*ngfp* were carried out as described in section 2.4.2. The transconjugants were selected for Km^R, and then for green fluorescence using an epifluorescent microscope (Axiovert 40 CFL, Carl Zeiss Inc).

2.13 Plant inoculation and growth conditions

The canola seeds were disinfected as described for the growth pouch assay, and the bacteria were also prepared as in the growth pouch assay except the bacterial suspension absorbance at 600 nm was 0.5, which corresponded to 1×10^8 cells/ml. Two disinfected canola seeds were sown in each 12.7 cm diameter green plastic pot containing wet unsterile Pro-Mix^{TM} BX (about 200 g dry mass) general-purpose growth medium (Premier Horticulture, Riviere-du-Loup, Quebec, Canada). A 2 ml aliquot of the bacterial suspension was added to the surface of each seed, and then the seeds were planted at a depth of approximately 1 cm. The pots were placed in small trays, and water was added to the trays to avoid washing the bacteria away from the growth medium. The plants were grown in a growth chamber at 20°C with a 16 h photoperiod and a light intensity of 200

μmol m^{-2}s^{-1}. After germination, one of the plantlets in each pot was removed, leaving only one plantlet per pot. Each treatment included 7 plants. The plants were harvested after two and three weeks' incubation, and the roots, stems, and leaves of the plants were observed by confocal microscopy.

2.14 Confocal microscopy of endophytic colonization by PsJN and its AcdS⁻ mutant

Different parts (roots, stems, and leaves) of the plants were cut into small pieces, put into a drop of water on a clean microscope slide, and sealed the cover slip with clear nail polish (nitrocellulose dissolved in a solvent). A Zeiss LSM 510 confocal microscope was used to detect the green fluorescence emitted by the bacteria. The excitation wavelength was 488 nm, and the emission wavelength used was 505-530 nm. Image analysis was done by Zeiss Image Examiner (Carl Zeiss Inc.).

3 Results

3.1 Isolation of the ACC deaminase gene from *Burkholderia phytofirmans* PsJN

The *acdS* gene of *Burkholderia phytofirmans* PsJN (Accession number: NC_010676) is 1017bp in length. Based on DNA sequence analysis, 165 bp upstream of the *acdS* gene, there is an ACC deaminase regulatory gene *acdR*, which belongs to the AsnC (asparagine synthase C involved in the regulation of asparagine biosynthesis genes) family (Yokoyama and Suzuki, 2005). The AsnC is a homolog of the Leucine Responsive Regulatory Protein (Lrp).

The primers used to isolate the *B. phytofirmans* PsJN *acdS* gene are shown schematically in Fig. 3-1. The PCR product should be 1225 bp according to the published sequence of the bacterium. The agarose gel electrophoresis of the PCR product (Fig. 3-2) showed the presence of a band which was approximately 1.2 kb and was thought to contain the *acdS* gene.

3.2 Construction and confirmation of the first AcdS⁻ mutant of *B. phytofirmans* PsJN

3.2.1 Construction of the first replacement vector pK19Rep

To construct the replacement vector pK19Rep, first the PCR product of the *B. phytofirmans* PsJN *acdS* gene was cloned to pGEM®-T Easy Vector (Promega, Madison, WI, USA). The recombinant plasmid pGEMACC was confirmed by *Eco*RI digestion which cleaved pGEMACC into two bands as visualized by agarose gel electrophoresis (Fig. 3-3). The larger band was approximately 3 kb which corresponds to linear pGEM-T

Easy Vector, and the smaller band was approximately 1.2 kb which corresponds to the inserted *acdS* gene.

To interrupt the *acdS* gene on plasmid pGEMACC, a tetracycline resistance gene (Tc) from pBR322 was inserted into the *Eco*RV site within the coding region of *acdS* gene to make pGEMACC⁻. Plasmid pBR322 was doubly digested by *Eco*RI and *Ava*I. The digested products were separated by agarose gel electrophoresis (Fig. 3-4). The smaller fragment (~1.5 kb) which contained the tetracycline resistance gene was extracted from the gel, the 5' protruding ends were filled-in by *E. coli* DNA Polymerase I Klenow Fragment (MBI Fermentas, Inc.), and the blunt ended fragment was ligated to *Eco*RV-digested pGEMACC. The ligation mixture was transformed into *E. coli* DH5α; the transformants were selected following growth on LB agar medium containing tetracycline and ampicillin. All four of the colonies obtained were incubated in LB medium, separately, and then the plasmid DNAs were extracted from overnight cultures. The putative pGEMACC⁻ plasmids were digested with *Sal*I (Fig. 3-5) to confirm the orientation of the insert. The gel picture in Fig. 3-5 shows that plasmids extracted from transformant Nos. 1, 3 and 4 have an insert with the same orientation while plasmids from transformant No. 2 have an insert with an orientation different from the other three. The tetracycline resistance gene fragment is about 1429 bp, and the enzyme *Sal*I cleaves this fragment into 653 bp and 774 bp fragments, while the *Eco*RV site in the *acdS* gene cleaves it into 295 bp and 930 bp fragments. Since the smaller bands in Fig. 3-5 are approximately 1600 bp (Nos .1, 3 and 4) and 1700 bp (No.2), respectively, these fragments must all contain the larger fragment (930bp) of the *acdS* gene. Thus, the *acdS* gene in the plasmid pGEMACC is oriented in counterclockwise direction; the Tc inserts

in plasmid pGEMACC⁻ from transformants Nos. 1, 3 and 4 are oriented in a counterclockwise direction, and the insert in transformant No. 2 is oriented in a clockwise direction. In all subsequent experiments, pGEMACC⁻ from transformant No. 1 was used.

To make an efficient and mobilizable carrier vector, pGEMACC⁻ was digested with *Eco*RI, and the fragment containing the interrupted *acdS* gene was separated and inserted into *Eco*RI digested pK19*mobsacB* to make the replacement vector pK19Rep. This plasmid pK19Rep (Fig. 3-6 B) incorperated the *acdS* gene with a Tc insertion, a broad-host-range transfer machinery (containing *oriT*) from RP4, a modified *sacB* gene from *Bacillus subtilis*, and a kanamycin resistance gene. After transformation of the putative replacement vector into *E. coli* DH5α, eight colonies grew on tetracycline and kanamycin containing plates. Plasmids from three of the eight colonies were extracted and digested with *Sal*I to confirm the orientation of the insertion. Plasmid pK19Rep was digested into two fragments by *Sal*I (Fig. 3-6A), and the size of the smaller fragment revealed the orientation of the inserted DNA. In lane three, the smaller fragment is approximately 1600 bp, which corresponds to the larger part of the *acdS* gene and a portion of the tetracycline resistance gene, so plasmids from colony No.1 contain a *acdS* gene in clockwise orientation containing the Tc gene. While, plasmids from colony No. 2 and 3 contain a counterclockwise interrupted *acdS* gene because the smaller fragment after digestion is approximately 1100 bp, which corresponds to the smaller part of *acdS* gene and the other portion of the tetracycline resistance gene. In the subsequent conjugation experiment, pK19Rep from colony No.2 was used.

Figure 3-1 Schematic representation of *acdS* and *acdR* gene on *Burkholderia phytofirmans* PsJN chromosome.

The red arrows represent the primers used to PCR amplify the *acdS* gene and a portion of its up- and down-stream region.
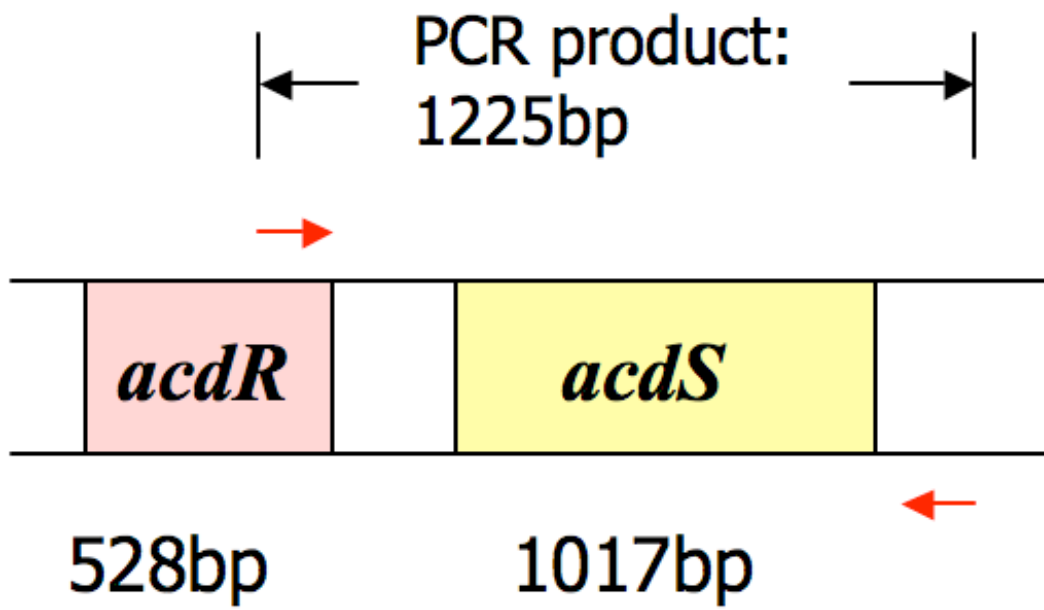
Figure 3-2 Agarose gel electrophoresis showing PCR product amplified from *Burkholderia phytofirmans* PsJN.

(-): negative control in which no DNA template was added. 1 and 2: putative *acdS* gene.

1500bp ➤
1000bp ➤
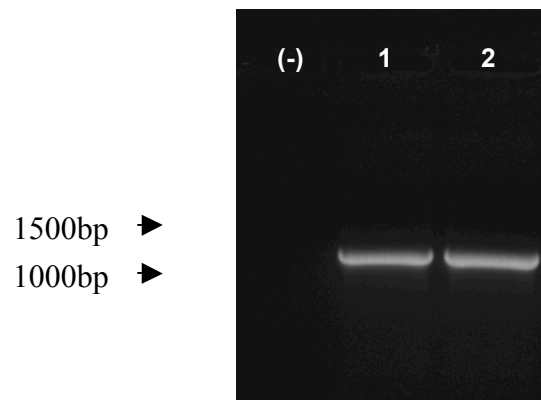
Figure 3-3 Agarose gel electrophoresis showing *Eco*RI digested products of the putative

pGEMACC. M: 1kb DNA ladder.

(-): undigested putative pGEMACC No.1. 1-5: *Eco*RI digested putative pGEMACC.
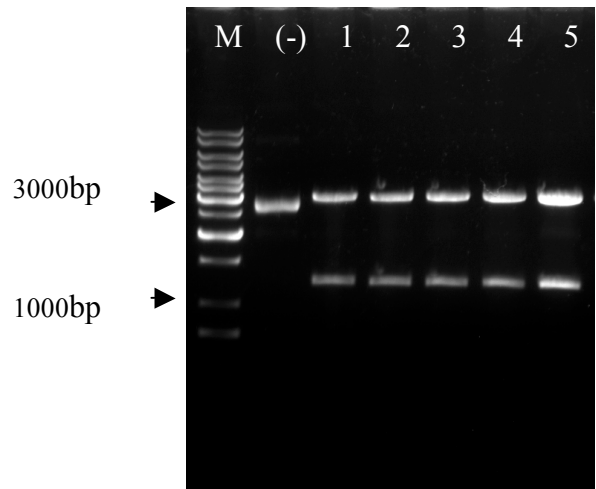
Figure 3-4 Agarose gel electrophoresis of *Eco*RI and *Ava*I double digest of pBR322.

M: 1kb DNA ladder. (-): undigested pBR322. 1 and 2: *Eco*RI and *Ava*I doubly digested pBR322
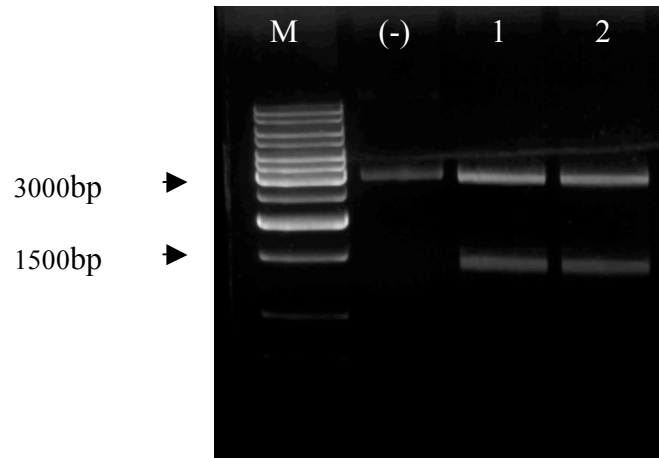
Figure 3-5 Agarose gel electrophoresis of *Sal*I-digested putative pGEMACC⁻ and schematic picture of SalI sites in pGEMACC⁻.

M: 1kb DNA ladder. (-): undigested putative pGEMACC⁻ No.1. 1-4: *Sal*I digested putative pGEMACC⁻. Am represents ampicillin resistence gene. Tc represents tetracycline resistance gene.

A

pGEMACC-
5.7 kb

B
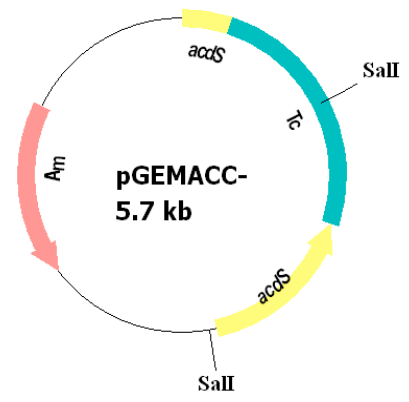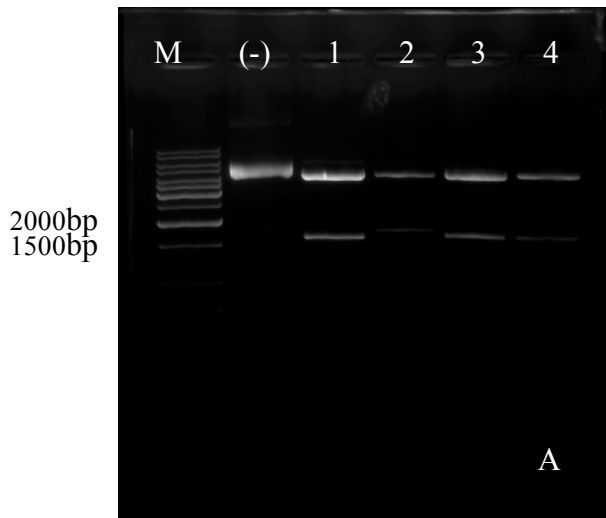
Figure 3-6 Agarose gel electrophoresis showing *Sal*I-digested pK19Rep and a schematic

representation of *Sal*I sites in pK19Rep.

M: 1kb DNA ladder. (-): undigested pK19Rep No. 1. 1-3: *Sal*I-digested putative

pK19Rep. Km represents kanamycin resistence gene. Tc represents tetracycline

resistance gene. *sacB* gene encodes the *Bacillus subtilis* levansucrase.
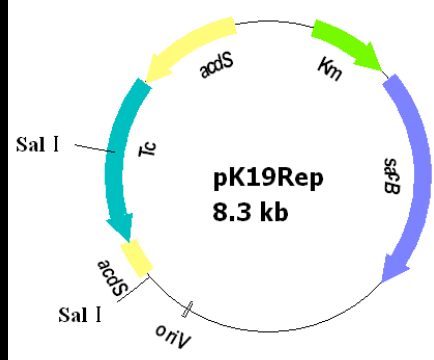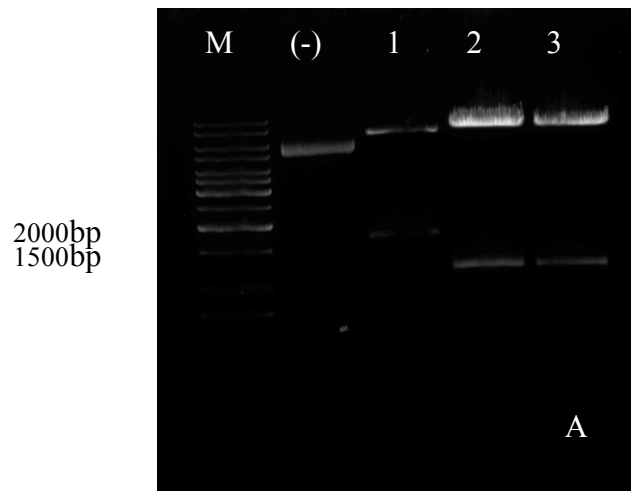
3.2.2 Conjugation and PCR confirmation

After mating *E. coli* DH5α/pK19Rep with *B. phytofirmans* PsJN and plating on TSB agar medium plus tetracycline, rifampicin and sucrose, several hundred putative transcojugants were obtained. Several transconjugants were transferred into 5 ml of TBS medium, and genomic DNA was extracted from the overnight grown cell suspension. Using genomic DNA as the template, PCR was performed to amplify the mutant *acdS* gene from these putative PsJN AcdS⁻ mutants (Fig. 3-7). The amplified fragment was approximately 2.7 kb in size and corresponds to the wild-type *acdS* gene plus the tetracycline resistance gene insertion (about 1.5 kb).

3.2.3 Identification of the *acdS* mutation in PsJN by Southern hybridization

The presence of the insertion in the PsJN AcdS⁻ strain was confirmed by Southern hybridization (Fig. 3-8). Genomic DNA from both of the wild-type and mutant *B. phytofirmans* PsJN were digested by *Eco*RI. After hybridization with the tetracycline resistance gene as a probe, one cross-hybridizing band was detected from the AcdS⁻ mutant while no band from the wild-type PsJN was detected (Fig. 3-8 right).

3.3 Construction and confirmation of the second AcdS⁻ mutant of *B. phytofirmans* PsJN

3.3.1 Construction of the second replacement vector pK19RepV2

To construct the replacement vector pK19RepV2, an *Eco*RV-*Cla*I fragment was removed from the coding region of *acdS* on pGEMACC to yield pGEMACD2. The

mutant *acdS* gene on pGEMACD2 was then cloned into the *Eco*RI site of pK19*mobsacB* to yield pK19RepV2 (Fig. 2-2).

First, pGEMACC was doubly digested by *Eco*RV and *Cla*I to yield two fragments; the larger fragment was approximately 4 kb, and the smaller one was 139 bp. The larger fragment was extracted from the agarose gel and made blunt ended by *E. coli* DNA Polymerase I Klenow Fragment (MBI Fermentas, Inc.). The blunt-end fragment was then self-ligated to make the plasmid pGEMACD2.

Plasmid pGEMACD2 was digested with *Eco*RI (Fig. 3-9) and the smaller digested fragment which contained the mutant *acdS* gene was inserted into the *Eco*RI site of pK19*mobsacB* to obtain the second replacement vector pK19RepV2.

3.3.2 Conjugation and PCR confirmation

To construct the second AcdS⁻ mutant of *B. phytofirmans* PsJN, pK19RepV2 was introduced into wild-type *B. phytofirmans* PsJN by conjugation. After 24 hours incubation, the cells were plated onto TSB agar plus 40 μg/ml rifampicin and 20 μg/ml kanamycin to select for the transconjugants containing the replacement vector. Three days later, seven colonies were obtained from the selective medium, and confirmed by colony PCR (Fig. 3-10). The primers used here were the same ones used to isolate *B. phytofirmans* PsJN *acdS* gene. PCR amplification of the seven colonies gave rise to single bands, the same size as the mutant *acdS* gene (third lane in Fig. 3-10) in vector pK19RepV2, and about 100 bp smaller than the wild-type *acdS* gene (second lane in Fig. 3-10) in *B. phytofirmans* PsJN genome. This result indicated the replacement of the wild-type *acdS* by an *acdS* with a small deletion may have happened in those transconjugants. To make sure the seven putative AcdS⁻ mutants of PsJN had lost the replacement vectors,

the seven colonies were transferred into liquid TSB medium with 7.5% sucrose. After 24 hours incubation, three tubes were randomly picked, and the genomic DNA was extracted. PCR was performed using genomic DNA as a template and the deletion mutation in the *acdS* gene of *B. phytofirmans* PsJN AcdS⁻ No.2 was confirmed (lane 5-7 in Fig. 3-11). In addition, the mutation in the *acdS* gene has been confirmed by DNA sequence analysis (Fig. 3-12).

Figure 3-7 Agarose gel electrophoresis of PCR products amplified from genomic DNA of

B. phytofirmans PsJN and putative AcdS⁻ Mutant No.1.

M: 1kb DNA ladder. (-): negative control in which no DNA template was added. 1: wild-type *acdS* gene of PsJN. 2: mutant *acdS* gene of PsJN.
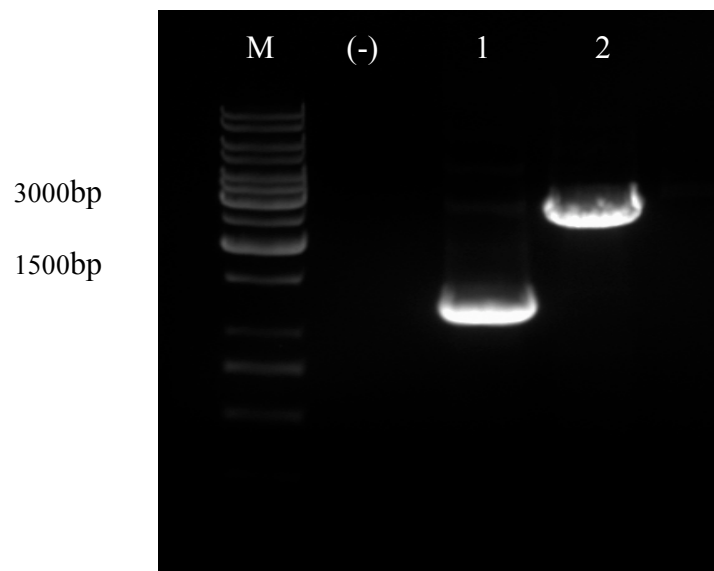
Figure 3-8 *Eco*RI-digested genomic DNA (left) and autoradiogram (right) following Southern Hybridization.

1: wild-type PsJN. 2: *B. phytofirmans* PsJN AcdS⁻ Mutant No.1. The probe used in hybridization is the tetracycline resistance gene.
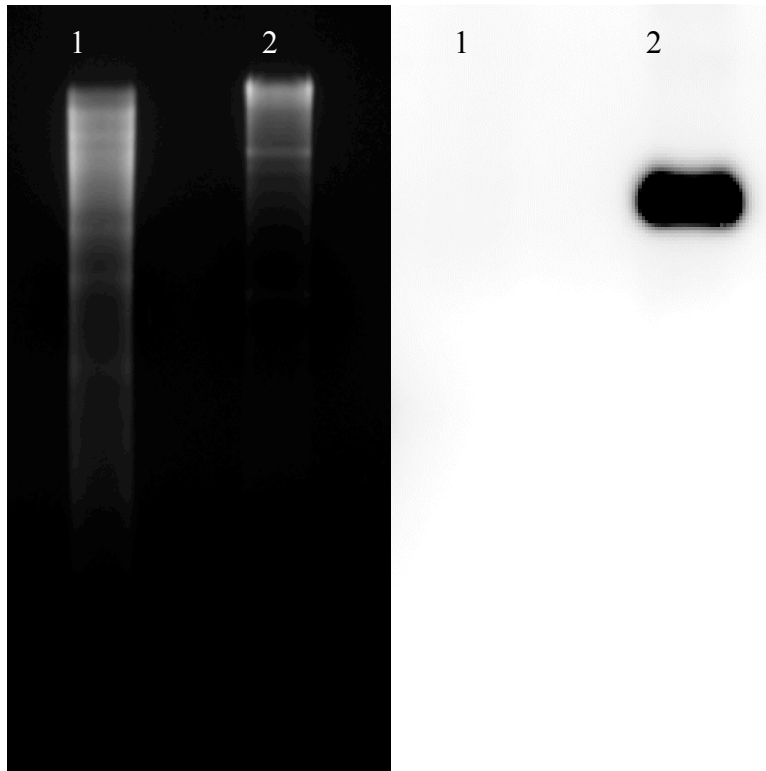
Figure 3-9 Agarose gel electrophoresis of *Eco*RI-digested pGEMACC and pGEMACD2.

M: 1 kb DNA ladder. 1: *Eco*RI digested pGEMACC. 2 and 3: *Eco*RI digested pGEMACD2.
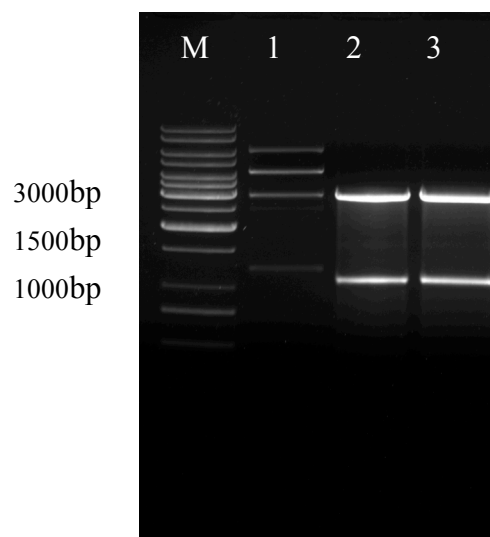
Figure 3-10 Agarose gel electrophoresis of colony PCR products amplified from wild-type *B. phytofirmans* PsJN and putative PsJN AcdS⁻ mutant No.2.

M: 1 kb DNA ladder. 1: Wild-type PsJN genomic DNA as template. 2: Plasmid pK19RepV2 as DNA template. 3-10: Transconjugant colonies as DNA templates.

Figure 3-11 Agarose gel electrophoresis of PCR products amplified from genomic DNA of wild-type PsJN and PsJN AcdS⁻ mutant No.2.

M: 1 kb DNA ladder. wt: Wild-type *B. phytofirmans* PsJN. (+): Plasmid pK19RepV2 as DNA template. (-): Negative control in which no DNA template was added. 1-3: Genomic DNA of PsJN AcdS⁻ mutant No.2 as template.
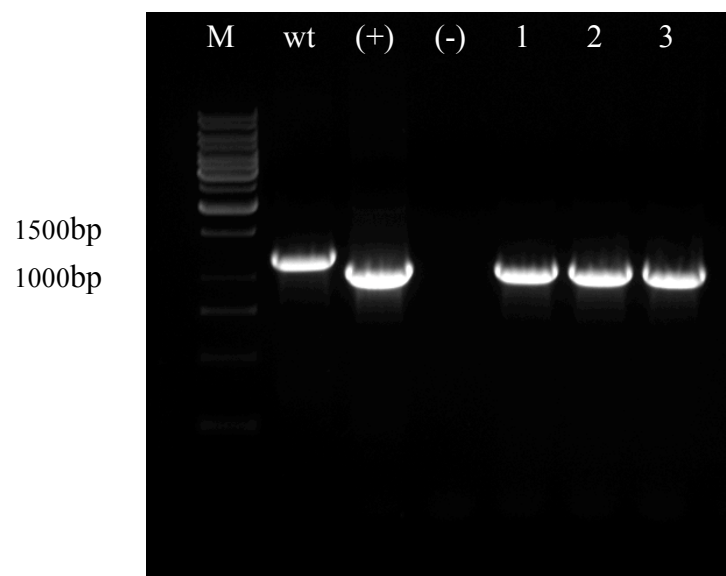
Figure 3-12 Sequence alignments of *acdS* gene of *B. phytofirmans* PsJN and PsJN AcdS⁻

mutant No.2.

```
Mutant_acdS        G G T A T C G A C G C G T C G G C C A A G C C C G C G C A G A C G C G C G A G C A G A T C A C C C G C A T C G C G A A G
Clustal Consensus  * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *


                                 730         740         750         760         770         780
                          . . . . | . . . . | . . . . | . . . . | . . . . | . . . . | . . . . | . . . . | . . . . | . . . . |
PsJN_acdS          C A G A C T G C G G A A C A G G T C G G C C T G G G A C G C G A T A T C A C C A G C A A G G A C G T G G T G C T C G A C
Mutant_acdS        C A G A C T G C G G A A C A G G T C G G C C T G G G A C G C G A T - - - - - - - - - - - - - - - - - - - - - - - - - -
Clustal Consensus  * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *


                                 790         800         810         820         830         840
                          . . . . | . . . . | . . . . | . . . . | . . . . | . . . . | . . . . | . . . . | . . . . | . . . . |
PsJN_acdS          G A G C G C T T C G G T G G C C C G G A A T A C G G T T T G C C G A A T G A C G G C A C G C T C G A A G C G A T C C G C
Mutant_acdS        - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Clustal Consensus


                                 850         860         870         880         890         900
                          . . . . | . . . . | . . . . | . . . . | . . . . | . . . . | . . . . | . . . . | . . . . | . . . . |
PsJN_acdS          T T G T G C G C G C G C C T G G A A G G C G T G T T G A C C G A T C C T G T C T A C G A G G G C A A A T C G A T G C A C
Mutant_acdS        - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - C G A T G C A C
Clustal Consensus                                                                                                  * * * * * * * *


                                 910         920         930         940         950         960
                          . . . . | . . . . | . . . . | . . . . | . . . . | . . . . | . . . . | . . . . | . . . . | . . . . |
PsJN_acdS          G G C A T G A T C G A G A T G G T G C G C A A C G G C G A G T T T C C C G A A G G T T C G C G T G T G C T G T A T G C G
Mutant_acdS        G G C A T G A T C G A G A T G G T G C G C A A C G G C G A G T T T C C C G A A G G T T C G C G T G T G C T G T A T G C G
Clustal Consensus  * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *


                                 970         980         990        1000        1010
                          . . . . | . . . . | . . . . | . . . . | . . . . | . . . . | . . . . | . . . . | . . . . | . . .
PsJN_acdS          C A C C T C G G C G G C G T G C C C G C G C T G A A C G G C T A C A G C T T C A T T T T C C G C A A C G G C T A A
Mutant_acdS        C A C C T C G G C G G C G T G C C C G C G C T G A A C G G C T A C A G C T T C A - - - - - - - - - - - - - - - - -
Clustal Consensus  * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
```

3.4 ACC deaminase activity of *B. phytofirmans* PsJN and its mutants

The ACC deaminase activity of *B. phytofirmans* PsJN, *B. phytofirmans* PsJN/gfp, *B. phytofirmans* PsJN AcdS⁻ No.1, *B. phytofirmans* PsJN AcdS⁻ No.2, and *B. phytofirmans* PsJN AcdS⁻ No.2/gfp was assayed (Table 3-1). Both the wild-type *B. phytofirmans* PsJN and GFP labeled *B. phytofirmans* PsJN (*B. phytofirmans* PsJN/gfp) showed a high level of ACC deaminase activity, while the two AcdS⁻ mutants of *B. phytofirmans* PsJN as well as the GFP labeled mutant showed no detectable activity in the assay.

3.5 Effect of *B. phytofirmans* PsJN and its AcdS⁻ mutants on root elongation of canola seedlings under gnotobiotic conditions

The ability of *B. phytofirmans* PsJN and its two AcdS⁻ mutants to promote the elongation of canola roots were compared separately. In the first test, canola seeds were inoculated with wild-type *B. phytofirmans* PsJN and *B. phytofirmans* PsJN AcdS⁻ No.1, as well as 0.3 M MgSO₄ as a negative control. The root lengths were measured on the sixth day after inoculation (Table 3-2), and the results were analyzed by NCSS Statistical and Power Analysis Software (Kaysville, Utah, USA) using the methods Kruskal-Wallis One-Way ANOVA and Newman-Keuls Multiple-Comparison Test. The results showed that the roots of canola seeds treated with wild-type *B. phytofirmans* PsJN were significantly (Alpha=0.050) longer than the roots from the negative control and *B. phytofirmans* PsJN AcdS⁻ No.1, while the latter two were not significantly different from each other. Fig. 3-13 shows representative canola roots with different treatment and a graphical representation of this growth pouch assay data.

In the second test, *B. phytofirmans* PsJN, *B. phytofirmans* PsJN/gfp, *B. phytofirmans* PsJN AcdS⁻ No.2, and *B. phytofirmans* PsJN AcdS⁻ No.2/gfp were used to inoculate canola seeds, with 0.3 M MgSO₄ treated canola seeds as a negative control. The root lengths were measured on the fifth day after inoculation (Table 3-3). The results indicated that *B. phytofirmans* PsJN- and *B. phytofirmans* PsJN/gfp- treated canola seedlings were significantly (Alpha=0.050) longer than when seeds were treated with *B. phytofirmans* PsJN AcdS⁻ No.2, *B. phytofirmans* PsJN AcdS⁻ No.2/gfp, or MgSO₄ (Fig. 3-14).

3.6 Siderophore production of *B. phytofirmans* PsJN and its AcdS⁻ mutants

The production of siderophores by *B. phytofirmans* PsJN, *B. phytofirmans* PsJN/gfp, *B. phytofirmans* PsJN AcdS⁻ No.1, *B. phytofirmans* PsJN AcdS⁻ No.2, and *B. phytofirmans* PsJN AcdS⁻ No.2/gfp was detected on CAS plates (Fig. 3-15). The results showed that both the wild-type *B. phytofirmans* PsJN and GFP labeled *B. phytofirmans* PsJN produced a high level of siderophores while both of the two AcdS⁻ mutants, as well as the GFP labeled *B. phytofirmans* PsJN AcdS⁻ No.2, produced a much lower amount of siderophores.

3.7 IAA production of *B. phytofirmans* PsJN and its AcdS⁻ mutants

The amount of IAA secreted into the growth medium by *B. phytofirmans* PsJN vs. the two *B. phytofirmans* PsJN AcdS⁻ mutants was measured. In the first test (Table 3-4), IAA secreted into TSB medium by *B. phytofirmans* PsJN AcdS⁻ No.1 was approximately

6.3-fold higher than the amount secreted by the wild-type *B. phytofirmans* PsJN; in half TSB medium, IAA produced by *B. phytofirmans* PsJN AcdS⁻ No.1 was approximately 8.7-fold higher than the wild-type *B. phytofirmans* PsJN. Similar results were obtained in the second test (Table 3-5). Wild-type *B. phytofirmans* PsJN and GFP labeled *B. phytofirmans* PsJN produced similar amount of IAA; *B. phytofirmans* PsJN AcdS⁻ No.2 and GFP labeled *B. phytofirmans* PsJN AcdS⁻ No.2 produced similar amounts of IAA; *B. phytofirmans* PsJN AcdS⁻ No.2 produced approximately 5.3-fold higher IAA than *B. phytofirmans* PsJN into TSB medium, and approximately 6.6-fold higher IAA than *B. phytofirmans* PsJN into half TSB medium.

Table 3-1 ACC deaminase activity of *B. phytofirmans* PsJN and its mutants.

This set of assays was performed twice with the same result. The result of one set of assay is shown.

Strain	ACC deaminase activity (nmol keto/mg prot/hr)
B. phytofirmans PsJN	3150.
B. phytofirmans PsJN/gfp	4030.
B. phytofirmans PsJN AcdS⁻ No.1	Not detectable
B. phytofirmans PsJN AcdS⁻ No.2	Not detectable
B. phytofirmans PsJN AcdS⁻ No.2/gfp	Not detectable

Table 3-2 Effect of *B. phytofirmans* PsJN and PsJN AcdS⁻ mutant No.1 on root elongation of canola seedlings.

The number in parentheses is the number of seedlings measured in each instance. All values are ±SEM. 1 means the root length of canola treated with wild-type PsJN was significantly different from the other treatments. The root lengths were measured on the sixth day after inoculation

Seads treated with	Root length, cm (n)
B. phytofirmans PsJN	6.02 ± 0.21^{1} (47)
B. phytofirmans PsJN AcdS⁻ No.1	4.79 ± 0.19 (52)
0.3 M MgSO$_4$	4.94 ± 0.18 (48)

Table 3-3 Effect of *B. phytofirmans* PsJN and PsJN AcdS⁻ mutant No.2 on root elongation of canola seedlings.

The number in parentheses is the number of seedlings measured in each instance. All values are ±SEM. 1 means the root length of canola treated with wild-type PsJN and GFP labeled PsJN were significantly longer than the other treatments labeled with 2. The root lengths were measured on the fifth day after inoculation.

Seeds treated with	Root length, cm (n)
B. phytofirmans PsJN	5.64 ± 0.16^{1} (56)
B. phytofirmans PsJN/gfp	5.34 ± 0.12^{1} (57)
B. phytofirmans PsJN AcdS^{-} No.2	3.76 ± 0.11^{2} (48)
B. phytofirmans PsJN AcdS^{-} No.2/gfp	3.72 ± 0.11^{2} (39)
0.3 M MgSO$_4$	4.06 ± 0.15^{2} (49)

Table 3-4 IAA secreted into the growth medium by *B. phytofirmans* PsJN and PsJN AcdS⁻ No.1.

Strain	IAA (µg/ml) secreted	
	TSB	Half TSB
B. phytofirmans PsJN	4.44	3.29
B. phytofirmans PsJN AcdS⁻ No.1	27.96	28.64

Table 3-5 IAA secreted into the growth medium by *B. phytofirmans* PsJN and PsJN AcdS⁻ No.2.

Strain	IAA (µg/ml) secreted	
	TSB	Half TSB
B. phytofirmans PsJN	4.88	4.11
B. phytofirmans PsJN/gfp	5.15	3.46
B. phytofirmans PsJN AcdS⁻ No.2	25.77	27.09
B. phytofirmans PsJN AcdS⁻ No.2/gfp	25.73	27.49

Figure 3-13 Representative canola roots treated by *B. phytofirmans* PsJN and PsJN AcdS⁻ mutant No.1.

(A), and a graphical representation of root lengths with different treatments (B). 1 means the root length of canola treated with wild-type PsJN was significantly different from the other treatments.

A

B

MgSO₄   PsJN AcdS⁻ No.1   PsJN
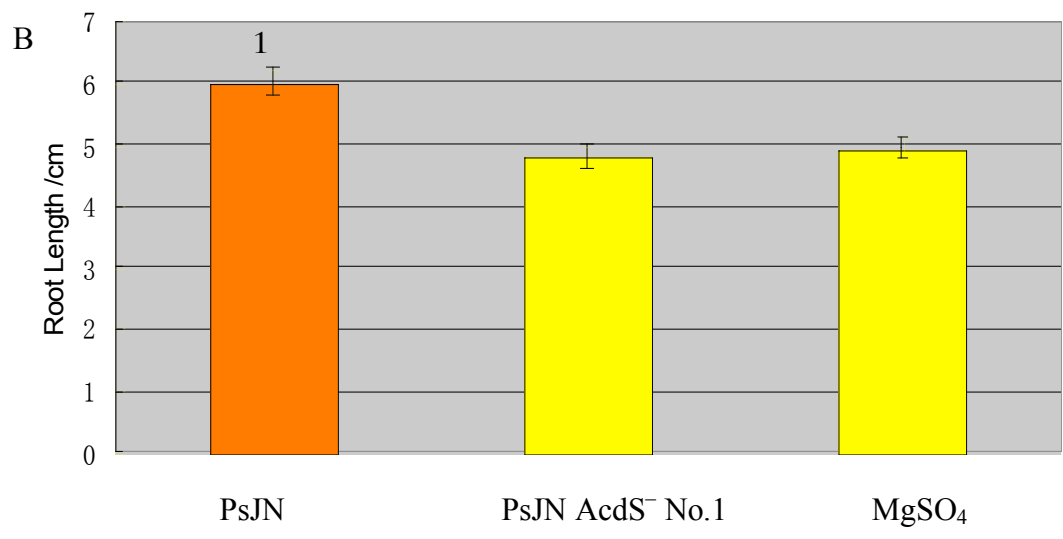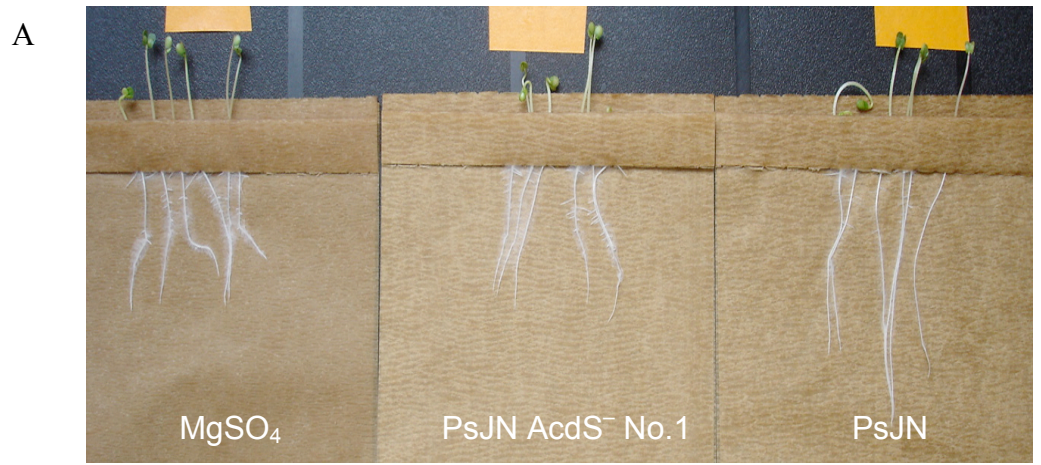
Figure 3-14 Representative canola roots treated by *B. phytofirmans* PsJN and PsJN AcdS⁻ mutant No.2.

(A), and a graphical representation of root lengths with different treatments (B). 1 means the root length of canola treated with wild-type PsJN and GFP labeled PsJN were significantly longer than the other treatments labeled with 2.
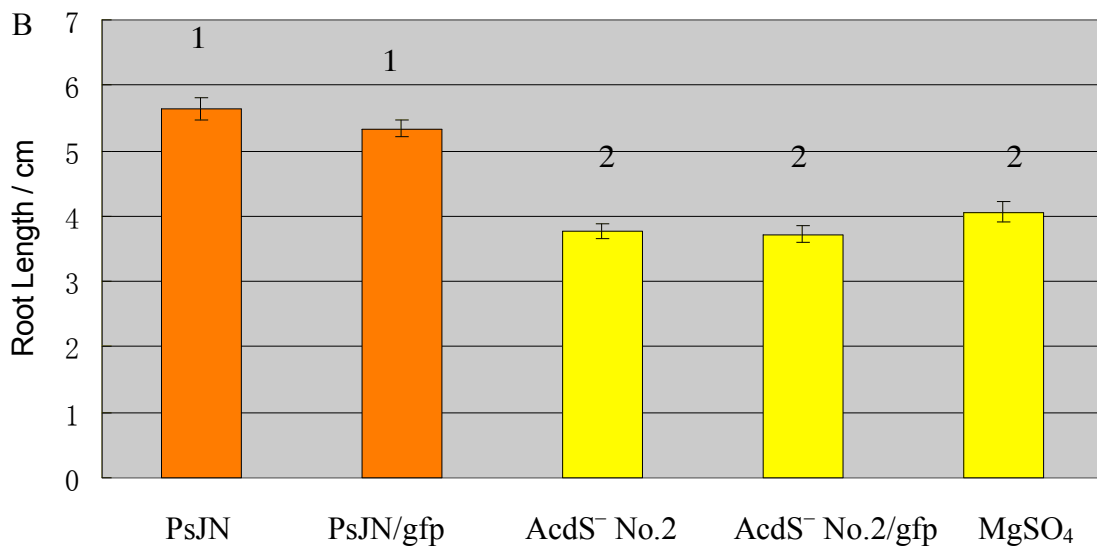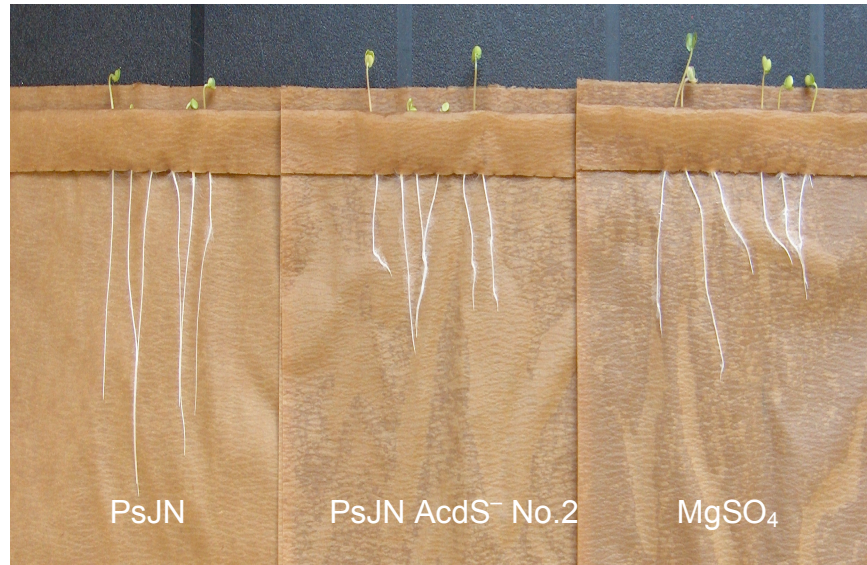
Figure 3-15 Siderophore production of *B. phytofirmans* PsJN and its AcdS⁻ mutants on a CAS plate.

The orange colour indicates siderophore production.

A

PsJN   PsJN AcdS⁻ No.1

B

PsJN   PsJN AcdS⁻ No.2

PsJN/gfp   PsJN AcdS⁻ No.2/gfp
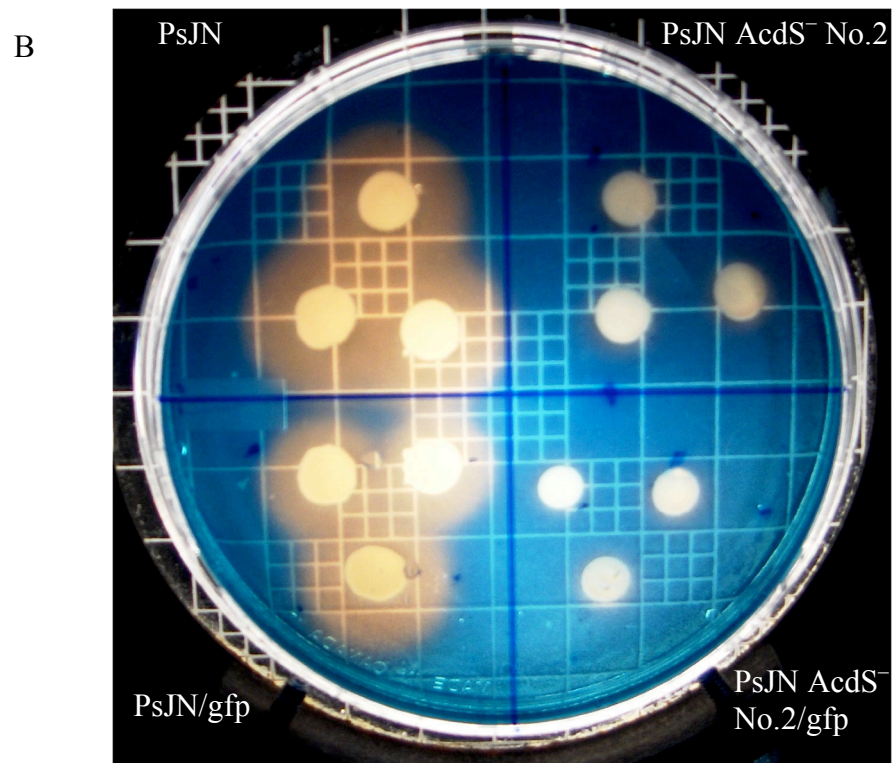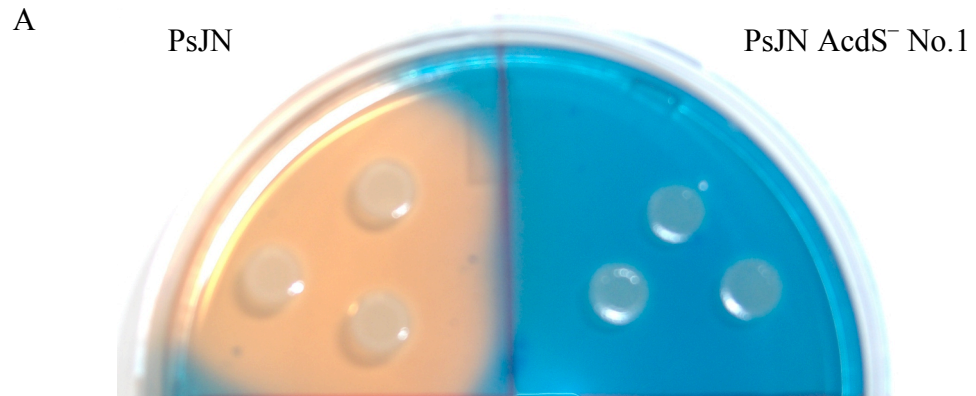
3.8 Endophytic colonization by *B. phytofirmans* PsJN and *B. phytofirmans* PsJN AcdS⁻ No.2

Plant colonization of *B. phytofirmans* PsJN and *B. phytofirmans* PsJN AcdS⁻ No.2 was monitored using a confocal microscope at various times after inoculation. To study the competition between wild-type and mutant PsJN, canola seeds were treated with several bacterial suspensions:

1) *B. phytofirmans* PsJN/gfp;

2) *B. phytofirmans* PsJN AcdS⁻ No.2/gfp;

3) 1/2 *B. phytofirmans* PsJN/gfp + 1/2 *B. phytofirmans* PsJN AcdS⁻ No.2;

4) 1/2 *B. phytofirmans* PsJN + 1/2 *B. phytofirmans* PsJN AcdS⁻ No.2/gfp;

5) 9/10 *B. phytofirmans* PsJN/gfp + 1/10 *B. phytofirmans* PsJN AcdS⁻ No.2;

6) 9/10 *B. phytofirmans* PsJN AcdS⁻ No.2/gfp + 1/10 *B. phytofirmans* PsJN;

7) 0.3 M $MgSO_4$ as a negative control.

Two weeks after inoculation, both GFP labeled *B. phytofirmans* PsJN and *B. phytofirmans* PsJN AcdS⁻ No.2 were detected as endophytes within canola roots, stems and leaves (Fig. 3-17). The xylem vessels in canola primary roots showed intense green fluorescence, which probably indicated the transport pathways of endophytes inside plant. All of these images are superimposed images (Fig. 3-16 C and F) of green fluorescence image (Fig. 3-16 A and D) and the DIC (differential interference contrast) image (Fig. 3-16 B and E).

Green fluorescence was detected at the root tip, primary root and basal leave edge of bacterial suspension sample 3 (1/2 *B. phytofirmans* PsJN/gfp + 1/2 *B. phytofirmans* PsJN AcdS⁻ No.2) treated canola, but the fluorescence was very weak. While in canola

treated with bacterial suspension sample 4 (1/2 *B. phytofirmans* PsJN + 1/2 *B. phytofirmans* PsJN AcdS⁻ No.2/gfp), green fluorescence was only detected at the root tip two weeks after soil inoculation.

Endophytic colonization of bacterial sample 5 (9/10 *B. phytofirmans* PsJN/gfp + 1/10 *B. phytofirmans* PsJN AcdS⁻ No.2) and 6 (9/10 *B. phytofirmans* PsJN AcdS⁻ No.2/gfp + 1/10 *B. phytofirmans* PsJN) was detected within canola root tips (Fig. 3-18) and basal leaves with the confocal microscope after two weeks incubation. Besides, green fluorescence was also detected within the primary root of canola treated with bacterial sample 5 (9/10 *B. phytofirmans* PsJN/gfp + 1/10 *B. phytofirmans* PsJN AcdS⁻ No.2).

Three weeks after inoculation, green fluorescence was detected at all observed parts of canola treated with all bacterial samples except the negative control. Endophytic colonization of *B. phytofirmans* PsJN and *B. phytofirmans* PsJN AcdS⁻ No.2 was detected within root tips, lateral roots, primary roots, stems, basal leaves and stem leaves (leaves that grow on the upright stems, different in size and shape from basal leaves) (Fig. 3-19, 3-20, 3-21). No significant differences were observed between different treatments except the green fluorescence from root tips of sample 1 (*B. phytofirmans* PsJN/gfp) and sample 2 (*B. phytofirmans* PsJN AcdS⁻ No.2/gfp) treated canola was more intense than canola treated with other bacterial combinations.

Canola plants above ground parts showed no significantly visible difference in growth between different treatments, including the negative control (Fig. 3-22).

Figure 3-16 Images from a confocal microscope of canola roots treated with PsJN/gfp (A, B and C) and MgSO$_4$ (D, E and F) as a negative control.

A and D are green fluorescence images. B and C are DIC (differential interference contrast) images. C and F are superimposed images of A and B, D and E respectively.
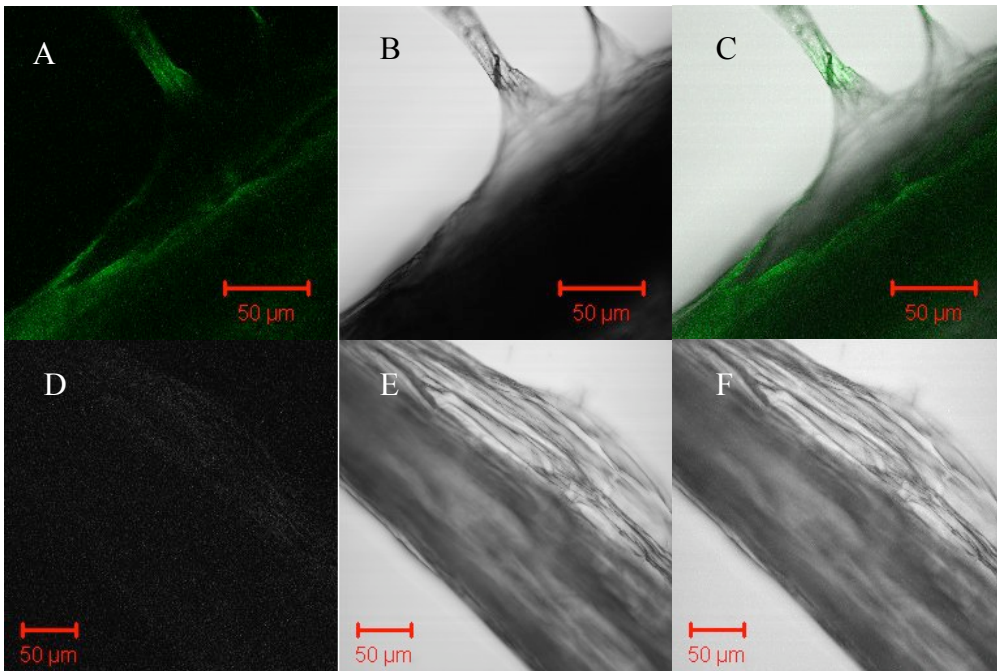
Figure 3-17 Images from a confocal microscope of canola treated with *B. phytofirmans* PsJN/gfp (A, C, E, G and I) and *B. phytofirmans* PsJN AcdS⁻ No.2/gfp (B, D, F, H and J) two weeks after inoculation.

A and B: root tip. C and D: primary root. E and F: stem. G and H: basal leaf. I and J: stem leaf.
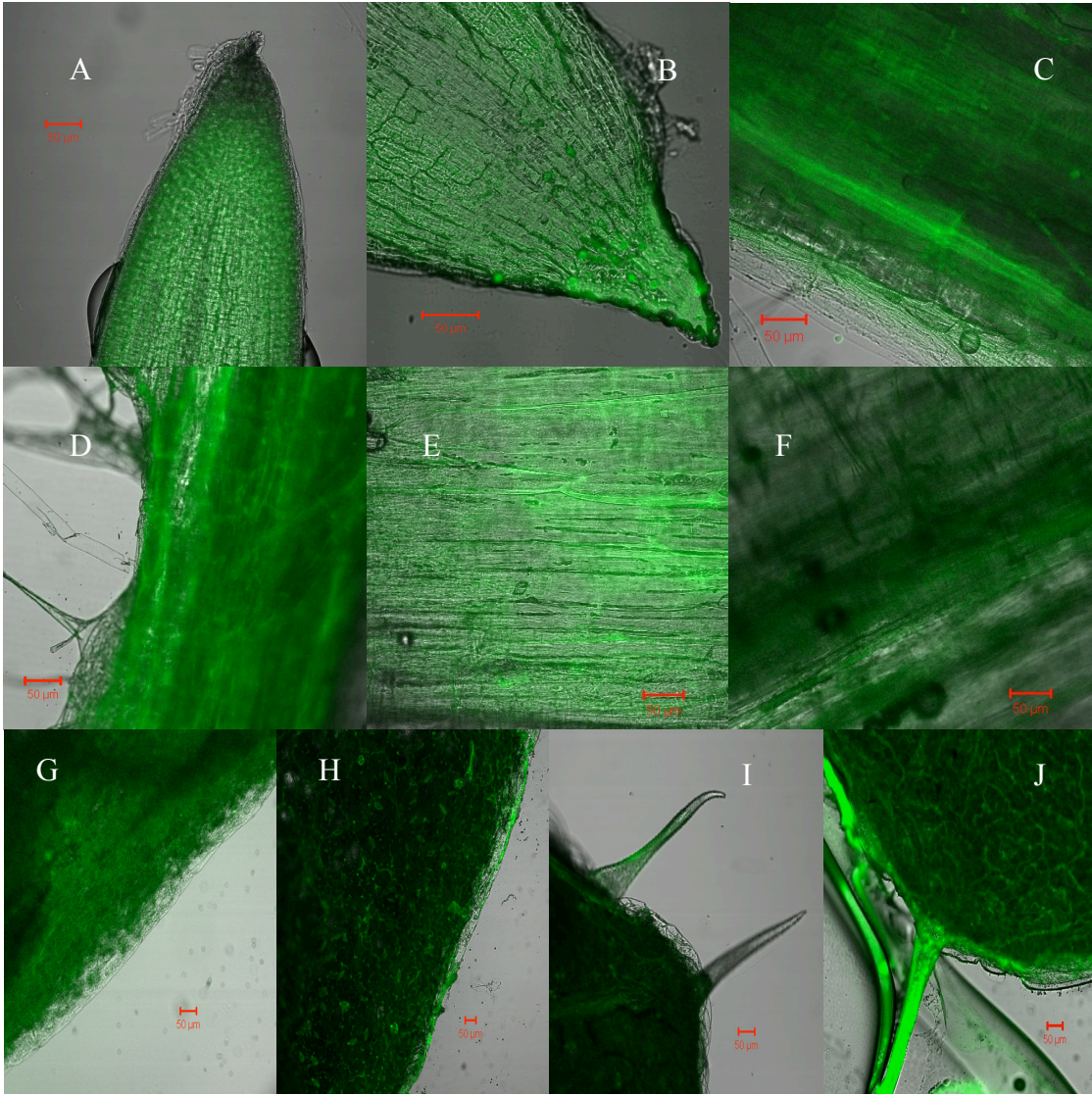
Figure 3-18 Images from a confocal microscope of canola root treated with 9/10 *B. phytofirmans* PsJN/gfp + 1/10 *B. phytofirmans* PsJN AcdS⁻ No.2 (A) and 9/10 *B. phytofirmans* PsJN AcdS⁻ No.2/gfp + 1/10 *B. phytofirmans* PsJN (B) two weeks after inoculation.
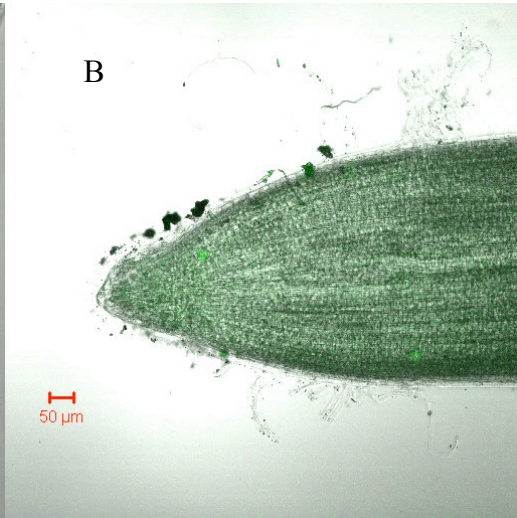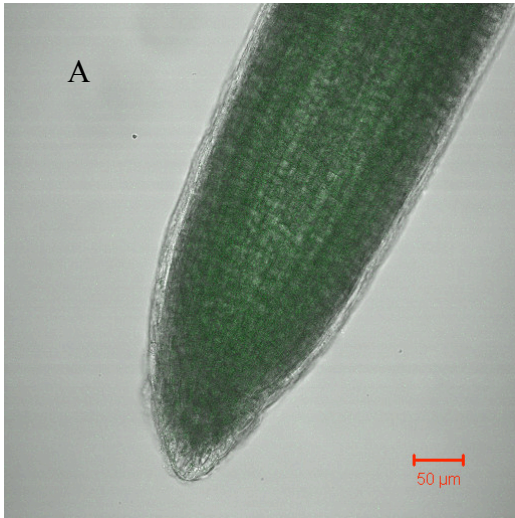
Figure 3-19 Images from a confocal microscope of canola treated with *B. phytofirmans* PsJN/gfp (A, C, E, G and I) and *B. phytofirmans* PsJN AcdS⁻ No.2/gfp (B, D, F, H and J) three weeks after inoculation.

A and B: root tip. C and D: primary root. E and F: stem. G and H: basal leaf. I and J: stem leaf.
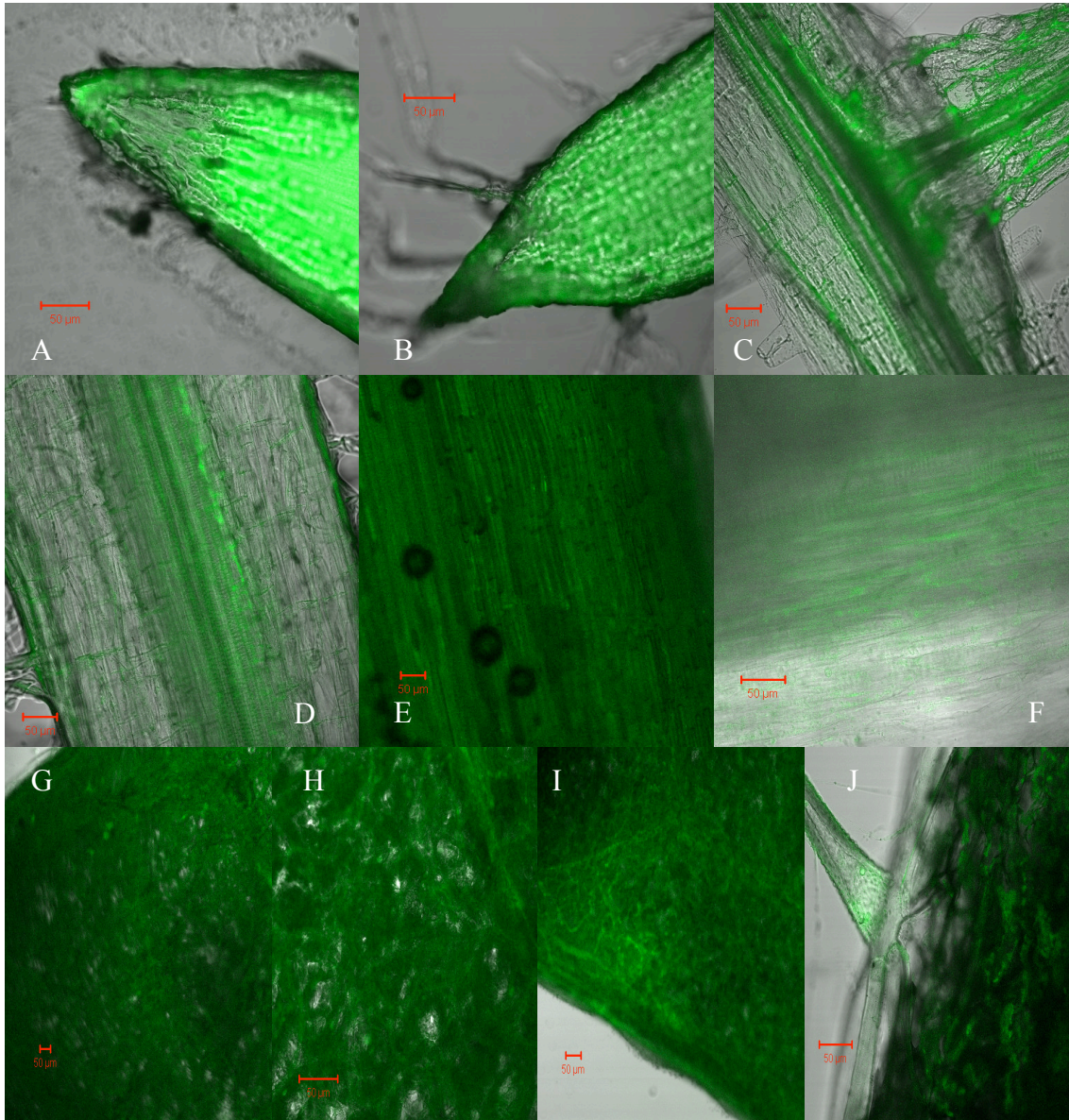
Figure 3-20 Images from a confocal microscope of canola treated with 1/2 *B. phytofirmans* PsJN/gfp + 1/2 *B. phytofirmans* PsJN AcdS⁻ No.2 (A, C, E, G and I) and 1/2 *B. phytofirmans* PsJN + 1/2 *B. phytofirmans* PsJN AcdS⁻ No.2/gfp (B, D, F, H and J) three weeks after inoculation.

A and B: root tip. C and D: lateral root. E and F: stem. G and H: basal leaf. I and J: stem leaf.
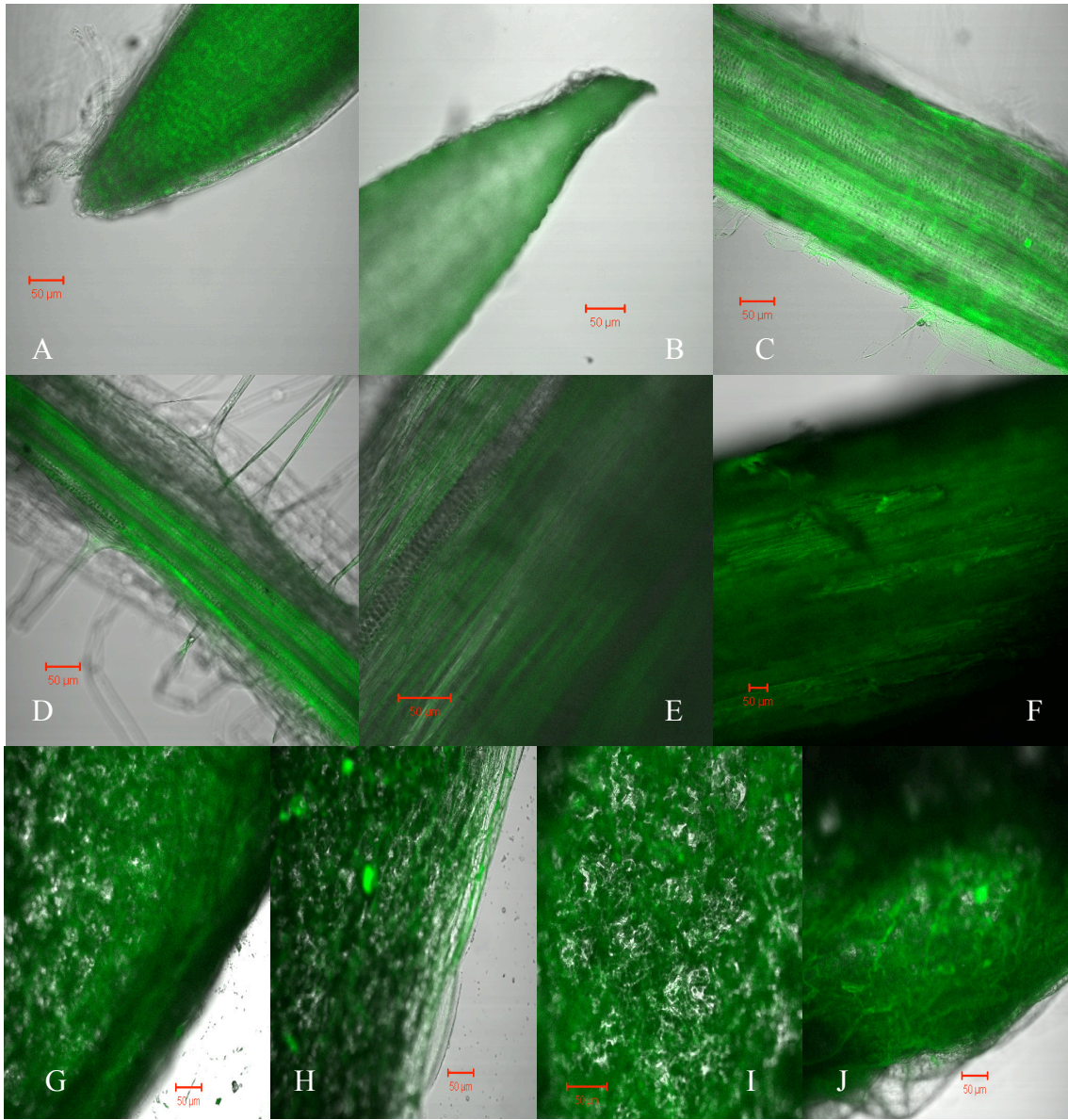
Figure 3-21 Images from a confocal microscope of canola treated with 9/10 *B. phytofirmans* PsJN/gfp + 1/10 *B. phytofirmans* PsJN AcdS⁻ No.2 (A, C, E, G, I and K) and 9/10 *B. phytofirmans* PsJN AcdS⁻ No.2/gfp + 1/10 *B. phytofirmans* PsJN (B, D, F, H, J and L) three weeks after inoculation.

A and B: root tip. C and D: primary root. E and F: lateral root. G and H: stem. I and J: basal leaf. K and L: stem leaf.
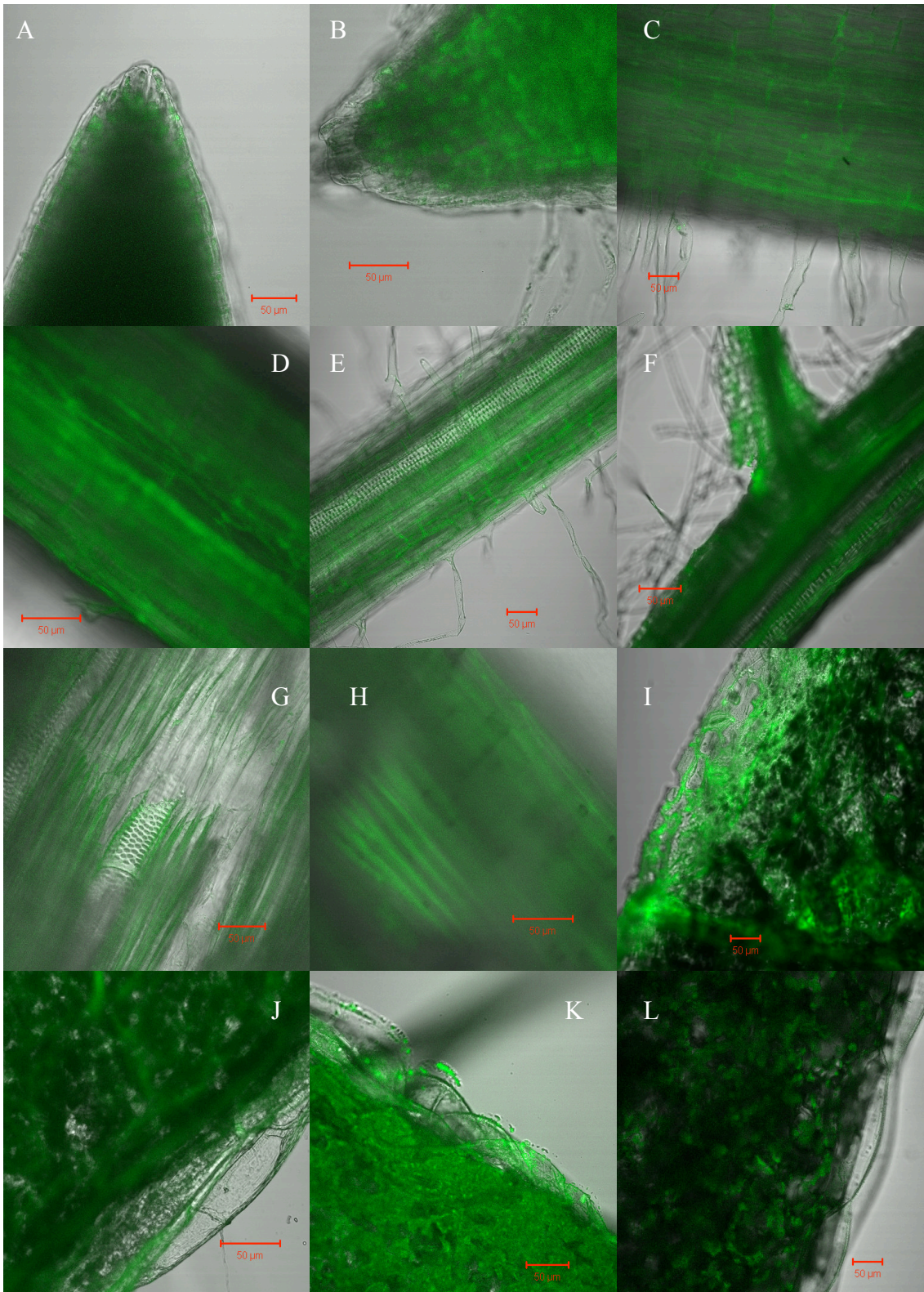
Figure 3-22 Canola plants in growth chamber three weeks after inoculation.

(-): canola seeds were treated with 0.3 M MgSO$_4$ as a negative control. 1: canola seeds were treated with *B. phytofirmans* PsJN/gfp. 2: canola seeds were treated with *B. phytofirmans* PsJN AcdS$^-$ No.2/gfp. 3: canola seeds were treated with 1/2 *B. phytofirmans* PsJN/gfp + 1/2 *B. phytofirmans* PsJN AcdS$^-$ No.2. 4: canola seeds were treated with 1/2 *B. phytofirmans* PsJN + 1/2 *B. phytofirmans* PsJN AcdS$^-$ No.2/gfp.

4 Discussion

The beneficial effects of endophytic bacteria occur through similar mechanisms as PGPR (Kloepper *et al.*, 1991; Höflich *et al.*, 1994). However, the effects of endophytic bacteria on host plants are often more pronounced than rhizobacteria because of their intimate association with plants (Conn *et al.*, 1997; Compant *et al.*, 2005). Thus, endophytic bacteria may play a very important role in agriculture. Besides promoting plant growth, and acting as natural biocontrol agents, endophytic bacteria have been manipulated genetically to explore more fully their potential in agricultural and environmental applications, such as enhancing pest control and phytoremediation (Lodewyckx *et al.*, 2002; Barac *et al.*, 2004). Some of the apparent advantages enjoyed by bacterial endophytes is based on their ability to be transported through the xylem and subsequently systematically colonize throughout the plant.

ACC deaminase-containing rhizospere bacteria can facilitate growth of a wide range of plants in the presence of various biotic and abiotic stresses, including infestation by pathogens, stresses of flooding, drought, salt and organic contaminants (Wang *et al.*, 2000; Grichko and Glick, 2001; Mayak *et al.,* 2004a and b; Cheng *et al.*, 2007; Reed and Glick, 2005). However, only very few studies have been reported that focus on the effects of ACC deaminase activity in endophytic bacteria (Idris *et al.*, 2004; Sessitsch *et al.*, 2005; Ait Barka *et al.*, 2006). In this thesis, the relationship between the ACC deaminase activity of the endophytic bacterium *B. phytofirmans* PsJN and its ability to colonize the plant interior and promote plant growth was studied.

4.1 Characterization of *B. phytofirmans* PsJN AcdS⁻ mutant No.1.

Traditionally, the generation and selection of mutant strains has been used to elaborate the role of particular proteins in the functioning of an organism. This is a particularly powerful approach in bacteria where the vast majority of genes are present in a single copy. Here, mutants of *B. phytofirmans* PsJN deficient in ACC deaminase activity were constructed and tested. The first AcdS⁻ mutant of PsJN was constructed by inserting a tetracycline resistance gene into the coding region of the *acdS* gene within the PsJN genome. This mutant showed no detectable ACC deaminase activity (Table 3-1), and lost most of its ability to promote plant growth as observed in growth pouch assays with canola seeds (Table 3-2). Interestingly, this AcdS⁻ mutant synthesized a decreased level of siderophores (Fig. 3-15A) and an increased amount of IAA (Table 3-4). The productions of siderophores and IAA are both very important mechanisms used by plant growth-promoting bacteria to promote plant growth (Glick, 1995a). Siderophore is an iron chelating compound secreted by microorganisms. Siderophores produced by plant growth-promoting bacteria can not only dissolve the insoluble ferric irons (Fe^{3+}) that are present in the rhizosphere, producing soluble iron complex which plants can use, but they also can prevent pathogens from proliferating because of their high affinity for iron which they can deplete in the vicinity of pathogens (O'Sullivan and O'Gara, 1992). IAA is a phytohormone which is known to be involved in root initiation, cell division, and cell elongation (Salisbury, 1994) and is very commonly produced by plant growth-promoting bacteria (Patten and Glick, 1996; Barazani and Friedman, 1999).

The method used to construct this PsJN AcdS⁻ mutant had been previously used by Li *et al.* (2000) to construct an ACC deaminase minus mutant of the rhizosphere

bacterium *Pseudomonas putida* UW4. As far as ACC deaminase activity and the ability to promote canola root elongation are concerned, similar results were found in the study of the *P. putida* UW4 and *B. phytofirmans* PsJN AcdS⁻ mutants. However, for the UW4 mutant, inserting a tetracycline resistance gene into the chromosomal gene encoding AcdS had no effect on IAA production compared to the wild-type UW4, while siderophore production in the UW4 wild-type and mutant strains was not tested (Li *et al*., 2000). Since the only change in *B. phytofirmans* PsJN during the construction of the mutant was an insertion in the *acdS* gene, it was thought that the difference of siderophore production and IAA secretion between wild-type *B. phytofirmans* PsJN and *B. phytofirmans* PsJN AcdS⁻ mutant might result from a metabolic load on the mutant strain caused by the expression of the introduced tetracycline resistance gene.

4.2 Metabolic load

Metabolic load has been defined as "the portion of a host cell's resources - either in the form of energy such as ATP or GTP, or raw materials such as amino acids - that is required to maintain and express foreign DNA, as either RNA or protein, in the cell" (Glick, 1995b). The extent of metabolic load caused by the addition of a foreign gene(s) depends on the location of the introduced gene(s) (on a vector or in the chromosomal DNA), the size and copy number of the vector, the metabolic state of the host cell, and the growth medium (Glick, 1995b). Metabolic load may change the physiology and functioning of the host cell greatly, with the decrease of growth rate as the most commonly observed effect (Glick, 1995b). In a study of the plant growth-promoting bacterium *Pseudomonas putida* GR12-2 (Hong *et al*., 1995), it was found that

100

introducing a plasmid carrying a tetracycline resistance gene into *P. putida* GR 12-2 decreased the rate of siderophore synthesis of the host cell to approximately 75% of the value in the nontransformed host cells, although it did not alter the growth rate of the host bacterium nor its ability to promote plant growth.

Since *B. phytofirmans* PsJN AcdS⁻ mutant has a tetracycline resistance gene inserted into the chromosomal DNA, the metabolic load of the host cell should be lower than that caused by introducing a multi-copy plasmid carrying a tetracycline resistance gene. However, it is still possible that the relatively lower level of metabolic load changed the host cell's physiology, including siderophore synthesis, IAA production, or even the ability to promote plant root elongation. If this is the case, the conclusion that endophytic strain *B. phytofirmans* PsJN AcdS⁻ mutant No.1 lost a portion of the ability to promote plant growth because of the disruption of ACC deaminase would be questionable. To eliminate the possibility that a metabolic load caused by introducing the tetracycline resistance gene is responsible for the observed decrease in the ability of *B. phytofirmans* PsJN to promote canola root elongation, a second ACC deaminase deficient mutant of *B. phytofirmans* PsJN was constructed.

4.3 Characterization of *B. phytofirmans* PsJN AcdS⁻ mutant No.2

In an effort to avoid the complication of a too-highly expressed tetracycline resistance gene inserted in the *acdS* gene of *B. phytofirmans* PsJN, a deletion mutant of the *acdS* gene without a tetracycline resistance gene was constructed. This mutant strain also showed no detectable ACC deaminase activity (Table 3-1), produced a decreased amount of siderophores compared to the wild-type (Fig. 3-15B), secreted an

approximately 5- to 7-fold higher amount of IAA than the wild-type (Table 3-5), and lost the ability to promote canola root elongation (Fig. 3-14). All of these results are quite similar to what was observed with *B. phytofirmans* PsJN AcdS⁻ mutant No.1, therefore, the changes in siderophore production and IAA secretion found in both of these mutants are unlikely to be the result of a metabolic load that was a consequence of foreign gene expression.

Another possible explanation for the observed results is that there is an increased level of RpoS, the stationary-phase sigma factor, in the AcdS⁻ mutants of *B. phytofirmans* PsJN. RpoS directs transcription of many genes expressed in the beginning of stationary phase (Loewen and Hengge-Aronis, 1994) in response to starvation and stress conditions, such as phosphate, carbon and nitrogen starvation, heat shock, acid shock and osmotic stress (Loewen and Hengge-Aronis, 1994; O'Neal *et al.*, 1994; Hengge-Aronis, 1996). In plant growth-promoting bacteria, IAA, siderophores, and ACC deaminase are primarily produced in the stationary phase of bacterial growth (Saleh and Glick, 2001).

The possible role of *rpoS* gene in regulating the transcription of some plant growth-promoting activities has previously been suggested (Saleh and Glick, 2001). The plant growth-promoting bacteria *Enterobacter cloacae* CAL2 and *Pseudomonas putida* UW4 were genetically transformed with either an *rpoS* or a *gacS* gene which encodes the global activator sensor kinase GacS from *Pseudomonas fluorescens*. The transformed strains were found to have a longer lag phase and were slower in reaching stationary phase. In addition, compared to the nontransformed strains, these transformed strains produced significantly more IAA and less siderophores in stationary phase compared to the non-transformed strains (Saleh and Glick, 2001). Since similar changes of

siderophore production and IAA secretion were found in AcdS⁻ mutants of *B. phytofirmans* PsJN, it is possible that the level of RpoS in these mutants may be elevated compared to the wild-type PsJN, thereby altering the physiology of the AcdS⁻ cells. However, this hypothesis remains to be tested. If this turns out to be the case, it suggests that in *B. phytofirmans* PsJN, unlike *P. putida* UW4, AcdS may play an important physiological role.

4.4 Endophytic colonization of canola by *B. phytofirmans* PsJN and its AcdS⁻ mutant

The bacterium *B. phytofirmans* PsJN has been shown to be able to endophytically colonize various plants. The most well studied colonization pattern of a plant by *B. phytofirmans* PsJN is of *Vitis vinifera* L. cv. Chardonnay plantlets (Compant *et al*. 2005, 2008a). The bacterium was visualized colonizing grape root surfaces, rhizodermal cells, inter- and intracellular spaces of cortical cells, endodermis, and xylem vessels (Compant *et al*. 2005). *B. phytofirmans* PsJN travels through the xylem vessels and then colonizes stems, leaves and inflorescence stalks, pedicels, and immature berries of grape (Compant *et al*. 2005, 2008a).

In the present study, canola seeds were inoculated with GFP-labeled *B. phytofirmans* PsJN strains, and the colonization was monitored two weeks after inoculation, which is sufficient time to colonize the root interior, stems and leaves. There was no visibly difference in the intensity of the green fluorescence detected from canola treated with *B. phytofirmans* PsJN or PsJN AcdS⁻ mutant No.2. For plants treated with either 1/2 PsJN/gfp + 1/2 PsJN AcdS⁻ No.2 or 1/2 PsJN + 1/2 PsJN AcdS⁻ No.2/gfp,

differences in the extent of colonization were detected two weeks after inoculation, but not in the third week. Colonization by the bacterial suspension containing half PsJN/gfp was detected at the root tip, primary root and basal leaf edge; while colonization by the bacterial suspension containing half PsJN AcdS⁻ No.2/gfp was detected only at the root tip. The results suggest that the initial colonization step may be affected by deletion of the bacterial ACC deaminase. However, this effect is diminished over time after bacteria enter the interior of plant. This difference in root colonization between the wild-type and mutant strains may reflect the somewhat modified physiological state of the mutant cells as discussed earlier. Similar results to what was observed in this work were reported by Compant *et al.* (2005), who observed significantly higher numbers of PsJN cells in leaves than in stems. This may be explained by the fact that the leaves accumulate bacteria while the stem serves only in transporting bacteria (Compant *et al.* 2005).

The existence of the mutant strain with a deletion in the *acdS* gene may be used, in comparison to the wild-type strain, to facilitate an understanding of how endophytes such as *B. phytofirmans* PsJN promote plant growth. In particular, it is expected that the mutant strain will be less capable of protecting plants against a range of different environmental stresses.

REFERENCES

Abeles FB, Morgan PW, Saltveit Jr. ME. 1992. Ethylene in Plant Biology. 2nd Ed. Academic Press, New York.

Abeles FB. 1973. Ethylene in Plant Biology. Academic Press, New York.

Adams DO, Yang SF. 1979. Ethylene biosynthesis: identification of 1-aminocyclopropane-1-carboxylic acid as an intermediate in the conversion of methionine to ethylene. *Proc Natl Acad Sci USA* 76, 170-174.

Alexander DB, Zuberer DA. 1991. Use of chrome azurol S reagents to evaluate siderophore production by rhizosphere bacteria. *Biology and Fertility of Soils* 12, 39-45.

Ait Barka E, Belarbi A, Hachet C, Nowak J, Audran JC. 2000. Enhancement of in vitro growth and resistance to gray mould of *Vitis vinifera* L. co-cultured with plant growth-promoting rhizobacteria. *FEMS Microbiol Lett* 186, 91-95.

Ait Barka E, Nowak J, Clement C. 2006. Enhancement of chilling resistance of inoculated grapevine plantlets with a plant growth-promoting rhizobacterium, *Burkholderia phytofirmans* strain PsJN. *Appl Environ Microbiol* 72, 7246-7252.

Bais HP, Park SW, Weir TL, Callaway R M, Vivanco JM. 2004. How plants communicate using the underground information superhighway. *Trends Plant Sci* 9, 26-32.

Barac T, Taghavi S, Borremans B, Provoost A, Oeyen L, Colpaert JV, Vangronsveld J, Lelie DV. 2004. Enginerred endophytic bacteria improve phytoremediation of water-soluble, volatile, organic pollutants. *Nat Biotechnol* 22, 583-588.

Barazani O, Friedman J. 1999. Is IAA the major root growth factor secreted from plant-growth-mediating bacteria? *J Chem Ecol* 25, 2397-2406.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72, 248-254.

Carroll G. 1988. Fungal endophytes in stems and leaves: from latent pathogen to mutualistic symbiont. *Ecology* 69, 2-9.

Cheng Z, Park E, Glick BR. 2007. 1-Aminocyclopropane-1-carboxylate deaminase from *Pseudomonas putida* UW4 facilitates the growth of canola in the presence of salt. *Can J Microbiol* 53, 912-918.

Cheng Z, Duncker BP, McConkey BJ, Glick BR. 2008. Transcriptional regulation of ACC deaminase gene expression in *Pseudomonas putida* UW4. *Can J Microbiol* 54, 128-136.

Clay K. 1988. Fungal endophytes of grasses: a defensive mutualism between plants and fungi. *Ecology* 69, 10-16.

Compant S, Reiter B, Sessitch A, Nowak J, Cl´ement C, Ait Barka E. 2005. Endophytic colonization of *Vitis vinifera* L. by plant growth-promoting bacterium *Burkholderia* sp. strain PsJN. *Appl Environ Microbiol* 71, 1685-1693.

Compant S, Kaplan H, Sessitsch A, Nowak J, Ait Barka E, Cl´ement C. 2008a. Endophytic colonization of *Vitis vinifera* L. by *Burkholderia phytofirmans* strain PsJN: from the rhizosphere to inflorescence tissues. *FEMS Microbiol Ecol* 63, 84-93.

Compant S, Nowak J, Coenye T, Cl ´ement C, Ait Barka E. 2008b Diversityand occurrence of *Burkholderia* spp. in the natural environment. *FEMS Microbiol Rev* 32, 607-626.

Conn KL, Nowak J, and Lazarovits G. 1997. A gnotobiotic bioassay for studying interactions between potato and plant growth-promoting rhizobacteria. *Can J Microbiol* 43, 801-808.

Deikman J. 1997. Molecular mechanisms of ethylene regulation of gene transcription. *Physiol Plant* 100, 561-566.

De Weert S, Vermeiren H, Mulders IHM, Kuiper I, Hendrickx N, Bloemberg GV, Vanderleyden J, de Mot R, Lugtenberg BJJ. 2002. Flagella-driven chemotaxis towards exudate components is an important trait for tomato root colonization by *Pseudomonas fluorescens*. *Mol Plant–Microbe Interact* 15, 1173-1180.

Dworkin M, Foster JW. 1958. Experiments with some microorganisms which utilize ethane and hydrogen. *Microbiology* 75, 502-603.

Frommel MI, Nowak J, Lazarovits G. 1991. Growth enhancement and developmental modifications of in vitro grown potato (*Solanum tuberosum* ssp. *tuberosum*) as affected by a nonfluorescent *Pseudomonas* sp. *Plant Physiol* 96, 928-936.

Germaine K, Keogh E, Borremans B, Garcia-Cabellos G, van der Lelie D, Barac T, Oeyen L, Vangronsveld J, Moore FP, Moore ERB, Campbell CD, Ryan D, Dowling DN. 2004. Colonisation of poplar trees by *gfp* expressing bacterial endophytes. *FEMS Microbiol Ecol* 48, 109-118.

Giovanelli J, Mudd SH, Datko AH. 1980. Sulfur amino acids in plants. In: Miflin BJ (ed) Amino acids and derivatives, the biochemistry of plants: a comprehensive treatise. Vol 5. Academic Press, New York, pp. 453-505.

Glick BR. 1995a. The enhancement of plant growth by free-living bacteria. *Can J Microbiol* 41, 109-117.

Glick BR. 1995b. Metabolic load and heterologous gene expression. *Biotechnol Adv* 13, 247-261.

Glick BR. 2004. Bacterial ACC deaminase and the alleviation of plant stress. *Adv Appl Microbiol* 56, 291-312.

Glick BR. 2005. Modulation of plant ethylene levels by the bacterial enzyme ACC deaminase. *FEMS Microbiol Lett* 251, 1-7

Glick BR, Bashan Y. 1997. Genetic manipulation of plant growth-promoting bacteria to enhance biocontrol of phytopathogens. *Biotech Adv* 15, 353-378.

Glick BR, Patten CL, Holguin G, Penrose DM. 1999. Biochemical and Genetic Mechanisms Used by Plant Growth Promoting Bacteria. London: Imperial College Press

Grimault V, Prior P. 1994. *Eur J Plant Pathol* 100, 259-267.

Glick BR, Penrose DM, Li J. 1998. A model for the lowering of plant ethylene concentrations by plant growth-promoting bacteria. *J Theor Biol* 190, 63-68.

Gordon SA, Weber RP. 1951. Colorimetric estimation of indole-acetic acid. *Plant Physiol* 26, 192-195.

Grichko VP, Glick BR. 2000. Identification of DNA sequences that regulate the expression of the *Enterobacter cloacae* UW4 1-aminocyclopropane-1-carboxylic acid deaminase gene. *Can J Microbiol* 46, 1159-1165.

Grichko VP, Glick BR. 2001. Amelioration of flooding stress by ACC deaminase-containing plant growth-promoting bacteria. *Plant Physiol Biochem* 39, 11-17.

Grimault V, Prior P. 1994. Invasiveness of *Pseudomonas solanacearum* in tomato, eggplant, and pepper: a comparative study. *Eur J Plant Pathol* 100, 259-267.

Gyaneshwar P, James EK, Mathan N, Teddy PM, Reinhold-Hurek B, Ladha JK. 2001. Endophytic colonization of rice by a diazotrophic strain of *Serratia marcescens*. *J Bacteriol* 183, 2634-2645.

Hall JA, Peirson D, Ghosh S, Glick BR. 1996. Root elongation in various agronomic crops by the plant growth promoting rhizobacterium *Pseudomonas putida* GR12-2. *Isr J Plant Sci* 44, 37-42.

Hallman J, Quadt-Hallman A, Mahafee WF, Kloepper JW. 1997. Bacterial endophytes in agricultural crops. *Can J Microbiol* 43, 895-914.

Hanahan D. 1983. Studies on transformation of *E. coli* with plasmids. *J Mol Biol* 166, 557-580.

Hengge-Aronis R. 1996. Back to log phase: σ^s as a global regulator in the osmotic control of gene expression in *Escherichia coli*. *Mol Microbiol* 21, 887-893.

Höflich G, Wiehe W, Kühn G. 1994. Plant growth stimulation by inoculation with symbiotic and associative rhizosphere mechanisms. *Experientia* 50, 897-905.

Hong Y, Pasternak JJ, Glick BR. 1995. Overcoming the metabolic load associated with the presence of plasmid DNA in the plant growth-promoting rhizobacterium *Pseudomonas putida* GR12-2. *Can J Microbiol* 41**,** 624-628.

Huang X-D, El-Alawai Y, Gurska J, Glick BR, Greenberg BM. 2005. A multi-process phytoremediation system for decontamination of persistent total petroleum hydrocarbons (TPHs) from soils. *Microchem J* 81, 139-147.

Huang X-D, El-Alawi Y, Penrose DM, Glick BR, Greenberg BM. 2004. Responses of plants to creosote during phytoremediation and their significance for remediation processes. *Environ Pollut* 130, 453-463.

Hurek T, Reinhold-Hurek B, Van Montagu M, Kellenbberger E. 1994. Root colonization and systemic spreading of *Azoarcus* sp. strain BH72 in grasses. *J Bacteriol* 176, 1913-1923.

Idris R, Trifonova R, Puschenreiter M, Wenzel WW, Sessitsch A. 2004. Bacterial communities associated with flowering plants of the Ni hyperaccumulator *Thlaspi goesingense*. *Appl Environ Microbiol* 70, 2667-2677.

James EK, Gyaneshwar P, Manthan N, Barraquio WL, Reddy PM, Ianetta PPM, Olivares FL, Ladha JK. 2002. Infection and colonization of rice seedlings by the plant growth-promoting bacterium *Herbaspirillum seropedicae* Z67. *Mol Plant-Microbe Interact* 15, 894-906.

John P. 1991. How plant molecular biologists revealed a surprising relationship between two enzymes, which took an enzyme out of a membrane where it was not located, and put it into the soluble phase where it could be studied. *Plant Mol Biol Rep* 9, 192-194.

Keister DL, Cregan PB. Eds., Kluwer Academic Publishers, Dordrecht, The Netherlands.

Kende H. 1989. Enzymes of ethylene biosynthesis. *Plant Physiol* 91, 1-4.

Kloepper JW, Schroth MN. 1978. Plant growth-promoting rhizobacteria on radishes. In: Station de pathologie vegetale et phyto-bacteriologie (ed.), Proceedings of the 4th International Conference on Plant Pathogenic Bacteria, vol. II. Gilbert-Clarey, Tours, France. pp. 879-882.

Kloepper JW, Tipping EM, Lifshitz R. 1991. Plant growth promotion mediated by bacterial rhizosphere colonizers. In: The Rhizosphere and Plant Growth. pp. 315-326.

Lamb TG, Tonkyn DW, Kluepfel DA. 1996. Movement of *Pseudomonas aureofaciens* from the rhizosphere to aerial plant tissue. *Can J Microbiol* 42, 1112-1120.

Li J, Glick BR. 2001. Transcriptional regulation of the *Enterobacter cloacae* UW4 1-aminocyclopropane-1-carboxylate (ACC) deaminase gene (*acdS*). *Can J Microbiol* 47, 359-367.

Liu L, Kloepper JW, Tuzun S. 1995. Induction of systemic resistance in cucumber against bacterial angular leaf spot by plant growth-promoting rhizobacteria. *Phytopathology* 85, 843-847.

Li J, Ovakim DH, Charles TC, Glick BR. 2000. An ACC deaminase minus mutant of *Enterobacter cloacae* UW4 no longer promotes root elongation. *Curr Microbiol* 41, 101-105.

Loewen PC, Hengge-Aronis R. 1994. The role of the sigma factor σ^s (KatF) in bacterial global regulation. *Annu Rev Microbiol* 48, 53-80.

Lodewyckx C, Vangronsveld J, Porteous F, Moore ERB, Taghavi S, Mezgeay M, van der Lelie D. 2002. Endophytic bacteria and their potential applications. *Crit Rev Plant Sci* 21, 583-606.

Ma W, Guinel FC, Glick BR. 2003. *Rhizobium leguminosarum* biovar viciae 1-aminocyclopropane-1-carboxylate deaminase promotes nodulation of pea plants. *Appl Environ Microbiol* 69, 4396-4402.

Matthysse AG, Stretton S, Dandie C, McClure NC, Goodman AE. 1996. Construction of GFP vectors for use in Gram-negative bacteria other than *Escherichia coli*. *FEMS Microbiol Lett* 145, 87-94.

Mayak S, Tirosh T, Glick BR. 2004a. Plant growth-promoting bacteria that confer resistance to water stress in tomato and pepper. *Plant Sci* 166, 525-530.

Mayak S, Tirosh T, Glick BR. 2004b. Plant growth-promoting bacteria that confer resistance in tomato to salt stress. *Plant Physiol Biochem* 42, 565-572.

Miyazaki JH, Yang SF. 1987. The methionine salvage pathway in relation to ethylene and polyamine biosynthesis. *Physiol Plant* 69, 366-370.

Morgan PW, Drew CD. 1997. Ethylene and plant responses to stress. *Physiol Plant* 100, 620-630.

Nowak J, Asiedu SK, Bensalim S, Richards J, Stewart A, Smith C, Stevens D, and. Sturz AV. 1998. From laboratory to applications: challenges and progress with in vitro dual cultures of potato and beneficial bacteria. *Plant Cell Tissue Organ Cult* 52, 97-103.

Nowak J, Shulaev V. 2003. Priming for transplant stress resistance in in vitro propagation. *In Vitro Cell Dev Biol-Plant* 39, 107-124.

O'Dell TE, Trappe JM. 1992. Root endophytes of lupin and some other legumes in northwestern U.S.A. *New Phytol* 122, 479-485.

Old, K. M. and Nicolson, T. H. 1978. The root cortex as part of a microbial continuum. In: Microbial Ecology. Loutit, M. V. and Miles, J. A. R., Eds., Springer-Verlag, Berlin, Germany, pp. 291–294.

O'Neal CR, Gabriel WM, Turk AK, Libby SJ, Fang FC, Spector MP. 1994. RpoS is necessary for the positive and negative regulation of starvation survival genes during phosphate, carbon, and nitrogen starvation in *Salmonella typhimurium*. *J Bacteriol* 176, 4610-4616.

O'Sullivan DJ, O'Gara F. 1992. Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens. *Microbiol Rev* 56, 662–676.

Patten CL, Glick BR. 1996. Bacterial biosynthesis of indole-3-acetic acid. *Can J Microbiol* 42, 207-220.

Patten CL, Glick BR. 2002. The role of bacterial indoleacetic acid in the development of the host plant root system. *Appl Environ Microbiol* 68, 3795-3801.

Penrose D M, Glick B R. 2003. Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria. *Physiol Plant* 118, 10-15.

Pillay V J, Nowak J. 1997. Inoculum density, temperature and genotype effects on in vitro growth promotion and epiphytic and endophytic colonization of tomato (*Lycopersicon esculentum* L.) seedlings inoculated with a *pseudomonad* bacterium. *Can J Microbiol* 43, 354–361.

Prigent-Combaret C, Blaha D, Pothier JF, Vial L, Poirier MA, Wisniewski-Dyé F, Moënne-Loccoz Y. 2008. Physical organization and phylogenetic analysis of acdR as leucine-responsive regulator of the 1-aminocyclopropane-1-carboxylate deaminase gene acdS in phytobeneficial *Azospirillum lipoferum* 4B and other Proteobacteria. *FEMS Microbiol Ecol* [Epub ahead of print]

Reed MLE, Glick BR. 2005. Growth of canola (*Brassica napus*) in the presence of plant growth-promoting bacteria and either copper or polycyclic aromatic hydrocarbons. *Can J Microbiol* 51, 1061-1069.

Reinhold-Hurek B, Hurek T. 1998. Life in grasses: diazotrophic endophytes. *Trends Microbiol* 6, 139–144.

Saleh SS, Glick BR. 2001. Involvement of *gasS* and *proS* in enhancement of the plant growth-promoting capabilities of *Enterobacter cloacae* CAL2 and UW4. *Can J Microbiol* 47, 698–705.

Salisbury FB. 1994. The role of plant hormones. In: Plant–Environment Interactions. Ed. R EWilkinson. Marcel Dekker, New York, USA. pp. 39–81.

Sambrook J, Russell RW. 2001. Molecular Cloning: A Laboratory Manual 3ed ed. Cold Spring. NY: Cold Spring Harbor Laboratory Press.

Schäfer A, Tauch A, Jäger W, Kalinowski J, Thierbach G, Pühler A. 1994. Small mobilizable multi-purpose cloning vector derived from the *Escherichia coli* plasmid pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene* 145, 69-73.

Selbitschka W, Niemann S, Ptihler A. 1993. Construction of gene replacement vectors

for Gram⁻ bacteria using a genetically modified *sacRB* gene as a positive selection marker. *Appl Microbial Biotechnol* 38, 615-618.

Sessitsch A, Coenye T, Sturz AV, Vandamme P, Ait Barka E, Salles JF, Van Elsas JD, Faure D, Reiter B, Glick BR, Wang-Pruski G, Nowak J. 2005. *Burkholderia phytofirmans* sp. nov., a novel plant-associated bacterium with plant-beneficial properties. *Int J Syst Evol Microbiol* 55, 1187–1192.

Sharrock KR, Parkes SL, Jack HK, Rees-George J, Hawthorne BT. 1991. Involvement of bacterial endophytes in storage rots of buttercup squash (*Cucurbita maxima* D. hybrid 'Delicia'). *New Zealand J Crop Horticultural Sci* 19, 157–165.

Shishido M, Breuil C, Chanway C. 1999. Endophytic colonisation of spruce by plant growth promoting rhizobacteria. *FEMS Microbiol Ecol* 29, 191–196.

Steenhoudt O, Vanderleyden J. 2000. *Azospirillum*, a free-living nitrogen- fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. *FEMS Microbiol Rev* 24, 487–506.

Sturz AV, Christie BR, Nowak J. 2000. Bacterial endophytes: potential role in developing sustainable systems of crop production. *Crit Rev Plant Sci* 19, 1–30.

Theologis A. 1992. One rotten apple spoils the whole bushel: the role of ethylene in fruit ripening. *Cell* 70, 181-184.

Turnbull GA, Morgan JAW, Whipps JM, Saunders JR. 2001. The role of motility in the in vitro attachment of *Pseudomonas putida* PaW8 to wheat roots. *FEMS Microbiol Ecol* 35, 57–65.

Van Loon LC. 1984. Regulation of pathogenesis and symptom expression in diseased plants by ethylene. In: Ethylene: Biochemical, Physiological and Applied Aspects, Ed. by Y. Fuchs and E. Chalutz. Martinus Nijhoff/Dr W. Junk Publ., The Hague. pp. 171-180.

van Peer R, Punte HLM, de Weger LA, Schippers B. 1990. Characterization of root surface and endorhizosphere *pseudomonads* in relation to their colonization of roots. *Appl Environ Microbiol* 56, 2462–2470.

Wang CX, Knill E, Glick BR, Defago G. 2000. Effect of transferring 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase genes into *Pseudomonas fluorescens* strain CHA0 and its *gacA* derivative CHA96 on their growth-promoting and disease-suppressive capacities. *Can J Microbiol* 46, 898-907.

Wilson D. 1995. Endophyte—the evolution of a term, and clarification of its use and definition. *Oikos* 73, 274–276.

Yokoyama K, Suzuki M. 2005. Orthologous and paralogous FFRPs in *E. coli* and related

proteobacteria. *Proc Japan Acad* 81, 129-139.