Plant Growth-Promoting Bacterial Endophytes that contain ACC Deaminase: Isolation, Characterization, and Use

By

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Author's Declaration

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

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Abstract

Bacteria that provide benefit to plants are considered to be plant growth-promoting bacteria (PGPB) and can facilitate plant growth by a number of different mechanisms. Plant growth-promoting bacteria that are able to utilize the plant compound 1-aminocyclopropane-1-carboxylate (ACC) as a sole source of nitrogen, as a consequence of possessing the enzyme ACC deaminase, can protect host plants from a number of environmental stresses. In addition to ACC deaminase, PGPB may utilize other mechanisms to facilitate plant growth including IAA synthesis, siderophore production, phosphate solubilization activity, ammonia production, and antibiotic production.

Plant growth-promoting bacterial endophytes employ similar plant growth promotion mechanisms to those used by rhizospheric PGPB. In fact, bacterial endophytes are PGPB that go one step further and colonize the inside of the plant tissues and provide more efficient and prompted protection to their hosts compared to those that bind exclusively to the plant's rhizosphere. Therefore, it is likely that endophytic plant growth-promoting bacteria will be superior to similar non-endophytic bacterial strains in promoting plant growth under a wide range of environmental conditions.

In the work reported here, new bacterial endophytes were isolated and characterized. Among twenty-five ACC deaminase positive strains, two best strains were selected and ACC deaminase deficient mutants were constructed. The ability of two newly isolated 1-aminocyclopropane-1-carboxylate (ACC) deaminase-containing plant growth-promoting bacterial endophytes *Pseudomonas fluorescens* YsS6, *Pseudomonas migulae* 8R6 and their ACC deaminase deficient mutants was shown to 1) delay the

senescence of mini carnation cut flowers and 2) to facilitate tomato plant growth under salinity stress.

In the mini carnation flower senescence evaluation, the only difference between wild-type and mutant bacterial endophytes was ACC deaminase activity, our results demonstrate that this enzyme is directly responsible for a significant delay in flower senescence. Despite containing ACC deaminase activity, the rhizosphere-binding PGPB *Pseudomonas putida* UW4 was not taken up by the cut flowers and therefore had no effect on prolonging flower shelf life.

In evaluating the effect of bacterial endophytes under salt stress, tomato plants treated with either of the wild-type strains of the two selected bacterial endophytes demonstrated early flowering and fruiting and had significantly greater numbers of flowers, buds, and fruits than either the corresponding ACC deaminase mutant strain-treated plants or the control plants. Although both bacterial endophytes *P. fluorescens* YsS6 and *P. migulae* 8R6 showed significant plant growth-promotion capabilities, *P. migulae* 8R6 demonstrated better plant growth facilitation under salt stress than did *P. fluorescens* YsS6. *P. migulae* 8R6 treated tomato plants demonstrated the least sodium uptake, the highest chlorophyll content, and highest fresh and dry biomass.

The results of the work presented here suggest that ACC deaminase containing selected bacterial endophytes could be employed as environmentally friendly adjuncts to agricultural and horticultural practice.

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List of Abbreviations

σ	Sigma
ACC	1-aminocyclopropane-1-carboxylate
$ACCD^+$	1-aminocyclopropane-1-carboxylate deaminase positive
acdS	1-aminocyclopropane-1-carboxylate gene
Amp	Ampicillin
ANOVA	Analysis of variance
AVG	L-α-(aminoethoxyvinyl)-glycine
BLAST	Basic local alignment search tool
CAS agar	Chrome azurol S agar
DNA	Deoxyribonucleic acid
Ery	Erythromycin
HK	Histidine kinase
IAA	Indole acetic acid
Km	Kanamycin
LB	Luria agar
MAST	Motif alignment and search tool
MEME	Multiple Em for Motif Elicitation
MFS	Major facilitator superfamily
MG-RAST	Metagenomis-RAST (Rapid Annotation using Subsystem Technology)
NADPH	Nicotinamide adenine dinucleotide phosphate

NCBI	National center for biotechnology information
PGP	Plant growth-promoting
PGPB	Plant growth-promoting bacteria
RBH	Reciprocal BLAST hit
RNA	Ribonucleic acid
rRNA	Ribosomal Ribonucleic acid
RSD	Reciprocal least distance
SEM	Scanning electron microscopy
Tc	Tetracycline
TCA	Tricarboxylic acid cycle
Trp	Tryptophan
TSB	Tryptic soy broth

1 Introduction

The world's population is currently ~7 billion people and this is predicted to increase to ~8 billion around the year 2020. Thus, in the next ten to twenty years it will be a significant challenge to feed all of the world's people, a problem that will likely increase with time. However, to do so it is necessary to greatly increase agricultural productivity in a sustainable and environmentally friendly manner. It is necessary to re-examine many of the existing approaches to agriculture that presently include the use of chemical fertilizers, herbicides, fungicides, and insecticides. Instead, sustainable agriculture will need to make much greater use of both transgenic plants (for example, see http://www.isaaa.org/inbrief/default.asp) and plant growth-promoting bacteria (PGPB) (Glick, 2012).

1.1 Plant-bacteria interactions

Of the many different microorganisms that are commonly found in soil (bacteria, fungi, protozoa, and algae), bacteria are by far the most common. In fact, it has been estimated that there are often around 10^8 to 10^9 bacterial cells per gram of soil (Dunbar *et al*., 2002). Plants are continuously in contact with bacteria throughout their lifetime. Different bacteria can affect plants differently. Based upon their mode of action and the plant's response, bacteria can be generally divided into three classes: 1) plant pathogens; those bacteria that inhibit plant growth and cause different disease symptoms in various parts of the plant, 2) plant asymptomatic bacteria; those that do not produce any observable effects on plant growth, and 3) plant growth-promoting bacteria (PGPB);

those that support plant growth to significant levels. In addition, the effect that a particular bacterium has on a plant may vary according to the environmental conditions. Thus, a bacterial strain that stimulates plant growth by providing fixed nitrogen, a compound that is typically present in limited amounts in many soils, will not provide any benefit to plants when a large amount of chemical nitrogen fertilizer is added to the soil. Moreover, a particular bacterial strain may provide just the right amount of the plant hormone indoleacetic acid (IAA) to stimulate the growth of one plant while the same level of IAA may inhibit the growth of a different plant.

There is a growing body of literature describing the potential uses of plant associated bacteria as agents that can be used to stimulate plant growth and manage soil and plant health (Glick *et al*., 1995; Hallman and Kloepper, 1997; Reed and Glick, 2004; Rovira, 1965; Sturz and Nowak, 2000; Welbaum *et al*., 2004; Reed and Glick, 2012). In addition to promoting the growth of plants used in agricultural practice, PGPB also play important roles in horticulture, silviculture and environmental remediation (Reed and Glick, 1995; Reed and Glick, 2012). PGPB can be broadly classified into three categories (Fig.1.1): those that colonize the root surface and the close neighborhood (rhizospheric bacteria), those that establish a symbiotic relationship with plants (symbiotic bacteria), and those that can enter into the root interior and colonize inside the plant (endophytic bacteria) (Bacon and White, 2000; Bacon and Hinton, 2006).

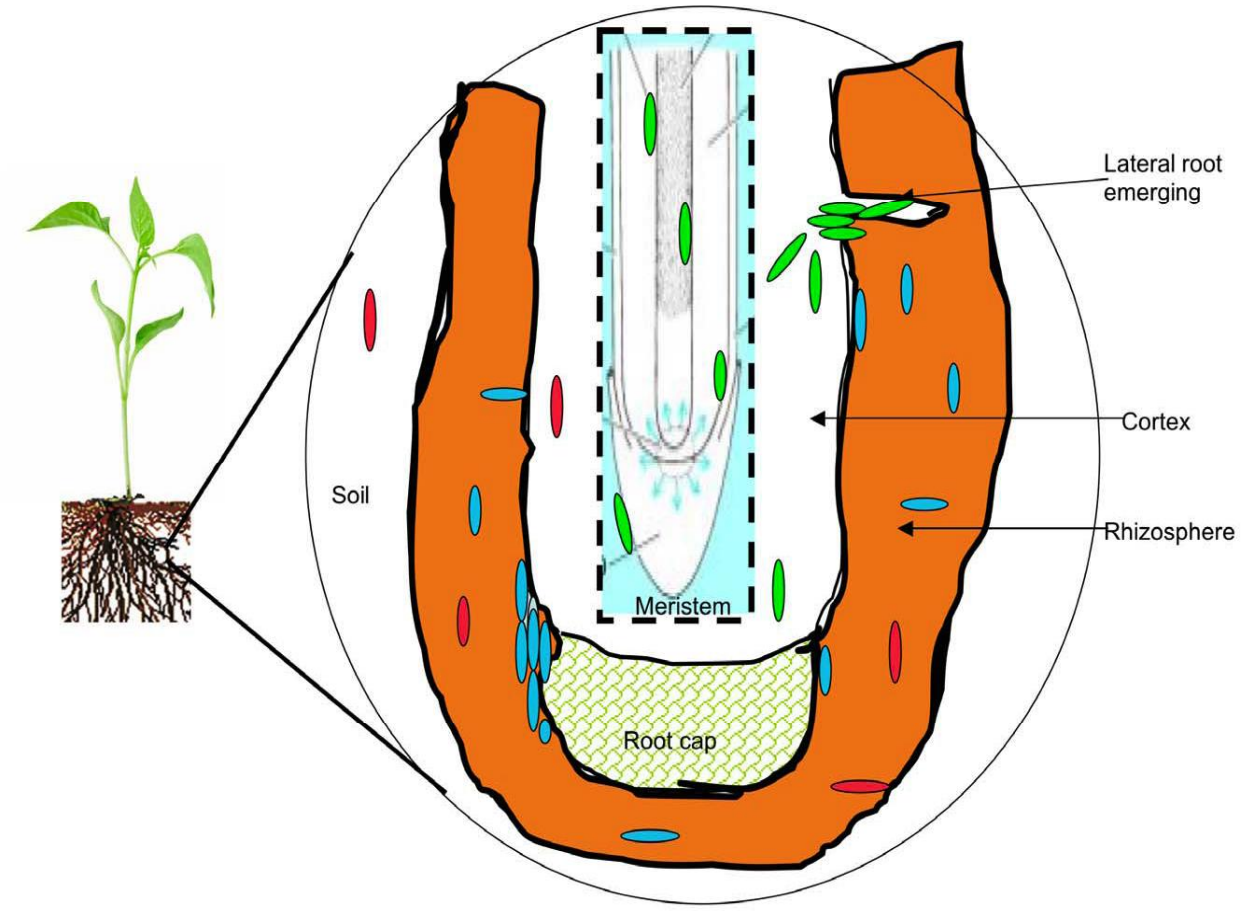


Figure 1.1 Schematic representation of plant colonization by bacteria. Root cracks are considered the main entry points for endophytes in a plant.

Color key: green rods represent bacterial endophytes, blue rods represent rhizospheric bacteria, and red rods represent plant pathogens.

1.2 Mechanisms used by PGPB

1.2.1 Direct mechanisms

Plant growth-promoting bacteria have the ability to colonize either a plant's interior or a plant's rhizosphere and to establish mutualistic relationship where both partners may derive benefits from this interaction (Hallmann *et al*., 1997; Reiter and Sessitsch, 2006). Thus, many PGPB can supply plants with substances that are essential for the proper growth of the plant. Some PGPB can fix atmospheric nitrogen and provide it to plants (Compant *et al*., 2005b and Watanabe *et al*., 1979). Although, from an agricultural perspective, the major nitrogen-fixing organisms are from the *Rhizobium* sp., many other nitrogen-fixing bacteria have been identified as members of the genera *Azospirillum, Azotobacter, Clostridium, Klebsiella* (Bashan and Holguin, 2002), *Burkholderia* (Reis *et al*., 2004) and *Azoarcus* (Krause *et al*., 2006). Several studies have demonstrated that when nitrogen fixing PGPB were used to inoculate plants, the number of nodules (Ma *et al*., 2003) and the plant overall biomass (Govindarajan *et al*., 2007) were significantly increased.

In addition to fixed nitrogen, phosphorus is an essential element for the growth of plants. Typically, phosphorus is found in an insoluble state in soil that plants cannot use directly. The insoluble forms of phosphate include apatite, phytate, phosphomonoesters, and phosphotriesters (Khan *et al*., 2007). Many soil bacteria produce low molecular weight organic acids, such as gluconic and citric acid, which convert insoluble phosphate to soluble forms in a series of reactions. Moreover, soil bacteria also possess special phosphatases to mineralize esters of phosphate (Bnayahu 1991; Rodriguez and Fraga

1999; Rodriguez *et al*., 2004). Solubilization and mineralization of phosphorus by PGPB enhances the bioavailability of soluble phosphorus for the plants and is considered an important plant growth-promotion mechanism under field conditions (Verma *et al*., 2001).

Bacterial siderophores are low molecular weight compounds with high iron (III) chelating affinity and are responsible for solubilization and transport of iron (III) into bacterial cells (Neilands 1981; Hilder and Kong 2010). The amount of bioavailable iron in the soil is very low, thus there is a competition for iron among members of the soil ecosystem. The siderophore-producing plant associated bacteria benefit plants in several ways: 1) they sequester iron from the iron-limiting environment and provide it to plants that need it for growth; 2) some PGPB and bacterial endophytes sequester iron even from heterologous siderophores produced by neighboring microorganisms, because of their high affinity towards iron, and thereby outcompete those other microorganisms (O'Sullivan and O'Gara 1992; Castignetti and Smarelli 1986; Lodewyckx *et al*., 2002; Raaijmakers *et al.*, 1995; Whipps 2001); and 3) some PGPB can act as biocontrol agents in that they outcompete plant pathogens leaving very little bioavailable iron behind so that plant pathogenic fungi, which produce siderophores that have a much lower affinity for iron, are unable to proliferate and infect plants (Kloepper *at al*., 1980; Loper and Henkels 1999; O'Sullivan and O'Gara 1992).

In addition, PGPB have been reported to promote plant growth by producing and/or modulating the level of plant hormones (Glick, 1995; Lee *et al*., 2004). Phytohormones are responsible for the proper growth and development of plants, and any deviation from the normal hormonal level is often an indication of the presence of plant

stress. Plants respond adequately to environmental changes and try to adjust their biochemistry and physiology by altering phytohormone levels (De Salamone *et al*., 2005). PGPB have been reported to produce gibberellin, cytokinin, and/or auxin (Nieto and Frankenberger, 1989; Timmusk *et al*., 1999; Garcia de Salamone *et al*., 2001; Taller and Wong, 1989; Tien *et al*., 1979; Williams and Sicardi de Mallorca, 1982; Badenochjones *et al*., 1983; Patten and Glick, 2002; Costacurta and Vanderleyden 1994; Glick, 2012). However, the total amount of phytohormones produced by the plant and the PGPB should be optimal in order to promote plant growth efficiently. Phytohormones produced by PGPB are beneficial to plants only if the combined hormone levels (the amount of each particular hormone produced by the plant plus the bacterium) is optimal; sub-optimal or super-optimal phytohormone levels do not effectively support plant growth. In fact, the production of high levels of phytohormones (IAA) is often a key characteristic of plant pathogens (Kunkel and Chen, 2006; Rezzonico *et al*., 1998; Shinshi *et al*., 1987).

A number of studies have shown plant growth promotion upon the inoculation of different plant systems with cytokinin or gibberellin-producing bacteria (Yahalom *et al*., 1990; Lorteau *et al*., 2001; Atzhorn *et al*., 1988; Joo *et al*., 2005; Kang *et al*., 2009). Moreover, IAA producing PGPB have been reported to promote plant growth in canola (Patten and Glick, 2002), in mung beans (Xie *et al*., 1996), and in tomato (Mayak *et al*., 1999; Loper and Schroth, 1986; Brown, 1974).

PGPB may also promote plant growth as a consequence of the action of bacteria expressing the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase (E.C. 4.1.99.4). ACC is the immediate precursor of ethylene in all higher plants. ACC

deaminase is a multimeric enzyme that cleaves ACC to α-ketobutyrate and ammonia and thereby decreases ethylene levels in host plants (Sessitsch *et al*., 2005; Sun *et al*., 2009; Jacobson *et al*., 1994; Glick *et al.,* 1998; Penrose *et al*., 2001; Glick, 2005). Ethylene is a plant hormone that plays a vital role in plant developmental processes as well as in stress signaling (Jackson, 1991; Abeles *et al*., 1992; Frankenberger and Arshad, 1995; Glick, 2004). Under normal conditions, ethylene helps in seed germination, root hair development, root elongation, leaf and petal abscission, fruit ripening and organ senescence (Abeles *et al*., 1992; Siddikee *et al*., 2011). However, during the stress response, plants produce high levels of ethylene that acts antagonistically for normal function and is deleterious to the plant growth. However, there is a clear distinction between ethylene responses under these two conditions (i.e. increased ethylene concentration within plant tissue and increased plant sensitivity towards increased concentrations of ethylene). The way the cells distinguish these conditions is still unknown.

ACC deaminase sequesters and cleaves ACC. As a consequence, it lowers the concentration of ethylene in plant tissues and promotes plant growth under stress conditions (Hong *et al*., 1991; Glick, 2004; Glick, 2005). A model (Fig. 1.2) describing the role of bacterial ACC deaminase in decreasing the level of ethylene in plants was posited (Glick *et al*., 1998) and, more recently, refined (Glick *et al*., 2007). In this model, it is argued that a plant growth-promoting bacterium with ACC deaminase activity can sequester and cleave the ethylene precursor, ACC, and thereby lower potentially deleterious ethylene levels in the plant host altering its physiology (Glick, 2004; Glick,

2005; Glick *et al*., 2007). In this model, bacteria that contain ACC deaminase are envisioned as acting as a sink for plant ACC.

In addition to directly facilitating plant growth and development, a number of studies have indicated that PGPB can promote plant growth by altering plant physiology including osmotic pressure regulation, changes in stomatal responses, adjustment in root size and morphology, modification of nitrogen accumulation and metabolism, and increased uptake of certain minerals (Compant *et al*., 2005a; Compant *et al*., 2005b; Rajkumar, 2009).

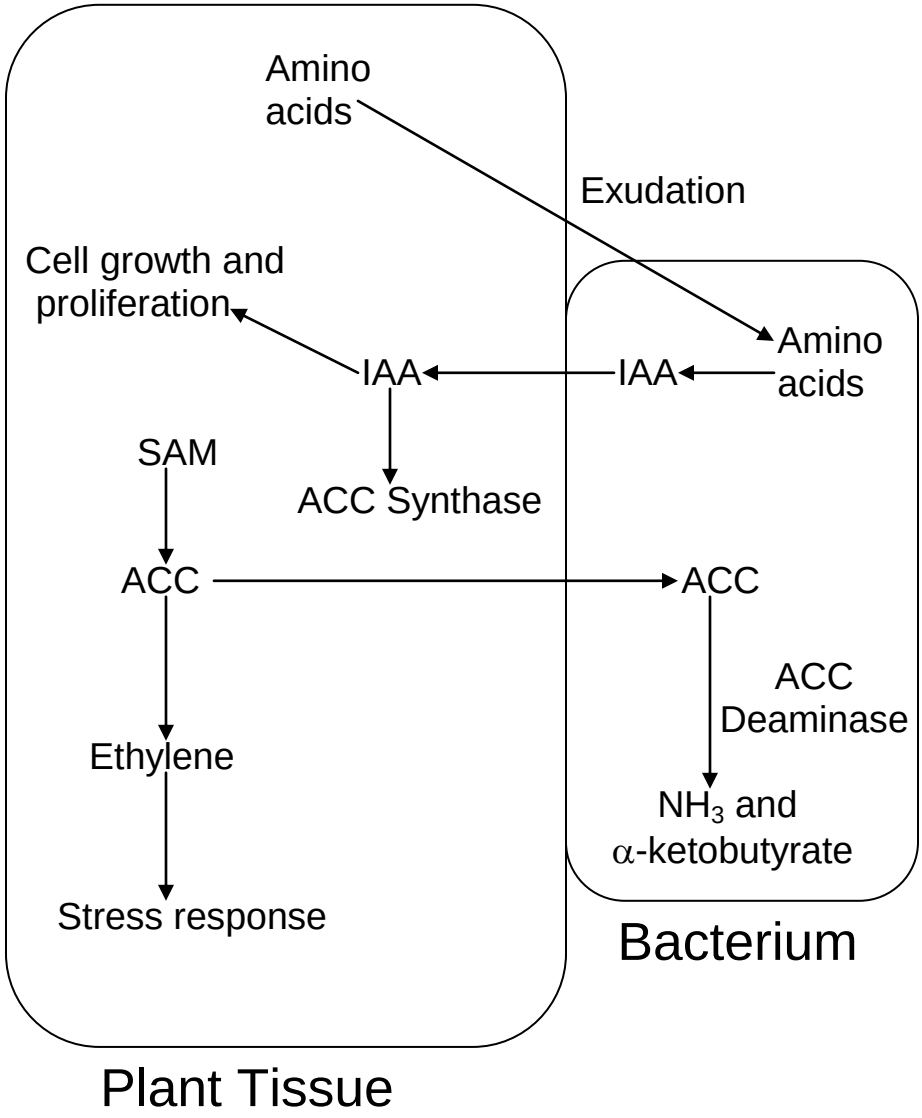


Figure 1.2 Schematic representation of the model proposed by Glick and colleagues (1998, 2007): a plant associated PGPB with ACC deaminase activity sequesters and

cleaves the ethylene precursor, ACC, and thereby lowering potentially deleterious ethylene levels in the plant.

ACC = 1-aminocyclopropane-1-carboxylate; IAA = Indoleacetic acid; SAM = S-adenosyle-methionine

1.2.2 Indirect mechanisms

PGPB can also be used as biocontrol agents. Antibiotic production (Aria, 1976; Demain, 1981; Ezra *et al*., 2004; Goodfellow, 1988; Long *et al*., 2004) and lytic enzyme production, e.g. hydrolases (Chernin and Chet, 2002), chitinases (Frankowski *et al*., 2001), laminarinases (Lim *et al*., 1991), and glucanases (Singh *et al*., 1999), are two common modes of PGPB-based biocontrol. Moreover, PGPB have also been reported to trigger induced systemic resistance (ISR)-based plant growth promotion (Ait Barka *et al*., 2000; Ait Barka *et al*., 2002; Benhamou *et al*., 1996a; Benhamou *et al*., 1996b; Brooks *et al*., 1994; M'Piga *et al*., 1997; Conn *et al*., 1997; Sharma and Nowak, 1998; Viswanathan and Samiyappan, 1999). Induced systemic resistance is generally defined as the enhanced defensive ability of a plant that is developed after appropriate stimulation.

1.3 PGPB and plant stress

Soil microorganisms continuously face environmental challenges because of the various dynamic parameters of the heterogeneous soil. Soil is not a good nutritional source for the growth of plant-associated microorganisms; soil typically contains 0.8 to 2.0% organic matter (including both carbon and nitrogen) (Timmusk *et al*., 2011), nitrogen limitation is often considered a significant factor for the plant growth. Therefore, soil bacteria constantly face nutrient scarcity during their lifetime (Timmusk *et al*., 2011). Under the environmental conditions where the nutrient concentration is insufficient for their steady growth, bacterial cells tend to enter into the stationary phase of their growth

(Llorens *et al*., 2010). Moreover, the continuous nutritional depletion drives bacteria to remain in stationary phase (Kolter *et al*., 1993). In fact, it has been estimated that quiescent microorganisms contribute 60% of the total earth's biomass (Gray *et al*., 2004; Llorens *et al*., 2010).

On the other hand, because plants continuously exude small molecules such as amino acids, sugars, and other organic acids through their roots into the surrounding area (rhizosphere), a number of bacteria (and other microorganisms) are attracted to the rhizosphere to take advantage of this nutrient-rich niche. The root exudation process boosts associated microbial growth and their relationship with the plants. In return, some bacteria (PGPB) facilitate plant growth in a number of ways (discussed earlier).

In addition to promoting plant growth, PGPB can also assuage some of the effects of different plant-growth-limiting environmental stresses (both abiotic and biotic). Plants continuously face various environmental challenges and plant hormones play a key role in signaling the stress and response, primarily to protect the plant from a number of different biotic and abiotic stresses (Fujita *et al*., 2006). A number of phytohormones are involved in stress signaling in plants, these mainly include abscisic acid, jasmonic acid, salicylic acid, and ethylene (Fujita *et al*., 2006).

In particular, ethylene is an important plant growth regulator that functions in the processes of root initiation, fruit ripening, seed germination, flower wilting, leaf abscission, biosynthesis of other phytohormones, and stress signaling (Abeles *et al*., 1992). Plants normally synthesize only small amounts of ethylene; levels that typically confer beneficial effects on plant growth and development (except during fruit ripening when ethylene concentration is much higher). However, in response to various stresses,

there is often a significant rise in endogenous ethylene biosynthesis, called "stress ethylene" (Abeles *et al*., 1992; Stearns and Glick, 2003; Glick *et al*. 2007). These stresses may include flooding, drought, salinity, wounding, temperature extremes, insect predation, pathogen infection, heavy metals, organic pollutants, mechanical stress, and nutritional stress (Hyodo, 1991; Stearns and Glick, 2003; Morgan and Drew, 1997). As a consequence of prolonged stress, ethylene induces abscission, chlorosis, and senescence in plants, ultimately leading to the death of plant tissue. Plants typically behave similarly when they are either exposed to an environmental stress stimulus or they are treated with high levels of exogenous ethylene (Morgan and Drew, 1997). The two most important ethylene-producing enzymes in plants (ACC oxidase and ACC synthase) are both induced by stress (Morgan and Drew, 1997). Moreover, it has been documented that a considerable portion of the damage that occurs in plants as a consequence of stress is due to the deleterious action of stress ethylene and not necessarily from the direct effects of the stress (Van Loon, 1984; Stearns and Glick, 2003). In addition, a number of studies have shown that there is a significantly lower level of ethylene production and, concomitantly, less plant tissue damage observed when chemical inhibitors of ethylene biosynthesis (such as L-α-(aminoethoxyvinyl)-glycine, AVG) were used to treat melon infected by the fungal phytopathogen *Fusarium oxysporum* (Cohen *et al*., 1986), cotton infected by *Alternaria* (Bashan, 1994), and roses, carnation, tomato, pepper, and cucumber infected by *Botrytis cinerea* (Elad, 1998, 1990). In contrast, when plants were treated with exogenous ethylene, the disease development and severity were increased in tomato infected by *Verticillium* (Cronshaw and Pegg, 1976) and in cucumber infected by *Colletotrichum lagenarium* (Biles *et al*., 1990).

Considering the role of ethylene in the physiology of plants under stress conditions, it is believed that agents that can lower the ethylene level in plants can be employed to facilitate plant growth under a variety of environmental stresses. For example, application of chemical inhibitors of plant ethylene production, such as silver thiosulphate (STS) (Veen and van de Geijn, 1978), cyclic olefin norbornadiene (NBD) (Reid and Wu, 1992), and L-α-(aminoethoxyvinyl)-glycine (AVG) (Nayani *et al*., 1998), have been reported to prolong the shelf life of ethylene-sensitive flowers. However the use of various chemicals has a number of drawbacks. For example, NDB has a foul odor and is carcinogenic (Reid and Wu, 1992), treatment of plants with AVG and STS increases the cost of the plant to the consumer, and above all, treating plants with high concentrations of chemicals is potentially phytotoxic and environmentally hazardous (Abeles *et al*., 1992). In this regard, PGPB that exhibit ACC deaminase activity may be the best choice to help plants to keep the stress ethylene concentration below the growth inhibitory point following a particular stress (Glick, 1995). Furthermore, a number of studies have been published describing the role of ACC deaminase containing PGPB in ameliorating plant damages caused by various stresses. For example: 1) Tomato plants treated with the ACC deaminase-containing PGPB, *Enterobacter cloacae* UW4 (currently *Pseudomonas putida* UW4), under flooding stress showed higher chlorophyll contents, increased biomass, and lower ethylene production when compared with control plants (Grichko and Glick, 2001). 2) A study revealed that an ACC deaminase-containing PGPB, *Achromobacter piechaudii* ARV8, provides resistance against water stress in tomato and pepper plants (Mayak *et al*., 2004). 3) Grapevine plantlets treated with the endophytic ACC deaminase-containing PGPB *Burkholderia phytofirmans* PsJN displayed

enhanced chilling resistance (Ait Barka *et al*., 2006). 4) Pretreating tomato plantlets with *Pseudomonas putida* UW4 significantly inhibited crown gall formation caused by *Agrobacterium tumefaciens* (Toklikishvili *et al.*, 2010; Hao *et al*., 2007). 5) Enhanced resistance against verticillium wilt was found in tomato plantlets when inoculated with the PGPB *Pseudomonas* sp. strain PsJN (currently *Burkholderia phytofirmans* PsJN) (Sharma and Nowak, 1998). 6) A variety of plants (tomato, canola, cucumber, red pepper) were found to resistant to high salinity resistant when treated with various ACC deaminase-containing plant growth-promoting bacterial strains (Mayak *et al*., 2004; Cheng *et al*., 2007; Siddikee *et al*., 2011; Gamalero *et al*., 2010; Saravanakumar and Samiyappan, 2007; Nadeem *et al*., 2007; Yue *et al*., 2007).

Moreover, several PGPB bacteria have been shown to effectively facilitate various phytoremediation strategies (Van Aken *et al*., 2004; Barac *et al*., 2004). Various studies have shown that PGPB that were isolated from soils contaminated with heavy metals, hydrocarbons, or other organic pollutants exhibited high level of phytoremediation capability (Koo *et al.*, 2010; Moore *et al*., 2006; Idris *et al*., 2004). However, in addition to resistance to high levels of the target contaminant, a PGPB that is useful for phytoremediation purposes often produces siderophores, IAA and ACC deaminase (Glick, 2010). ACC deaminase-containing plant growth-promoting bacteria, isolated from the above mentioned niches, have been demonstrated to protect plants in the presence of organic toxicants (Glick, 2003; Huang *et al*., 2004, 2005; Reed and Glick, 2005; Gurska *et al*., 2009) and a variety of different metals (Burd *et al*., 1998; Belimov *et al*., 2005; Nie *et al.*, 2002; Glick, 2003; Reed and Glick, 2005; Dell'Amico *et al*., 2005; Safronova *et al*., 2006; Farwell *et al*., 2006). In addition, heavy metals and other organic

pollutant resistance can be transferred to a PGPB by molecular cloning and genetic engineering tools. However, the genetically modified microorganisms often face political and regulatory criticism and cannot be deliberately released into the environment in most parts of the world.

Contemplating all the above-mentioned points, it is thought that an endophytic PGPB exhibiting ACC deaminase, producing IAA and siderophores can alleviate the stress level and facilitate plant growth under various environmental conditions thereby facilitating specific phytoremediation strategies (Glick, 2012).

1.4 Bacterial endophytes

Bacterial endophytes were first discovered in Germany in 1903 (Freeman, 1903; Tan and Zou, 2001) and, as the name indicates, are defined as "microorganisms that colonize healthy plant tissue without causing obvious symptoms or produce obvious injuries to the host" (Bacon and White, 2000; Bacon and Hinton, 2006). Although microorganisms other than bacteria (e.g. fungi) can also act as endophytes in plants, this thesis deals exclusively with bacterial endophytes.

Generally, bacterial endophytes are neither organ nor host specific (Rosenblueth and Martínez-Romero, 2006). A variety of endophytes have been isolated from different tissue types in numerous species of plants (hosts) (Kobayashi and Palumbo, 2000). It is also possible to isolate multiple species of endophytes from a single plant (Kobayashi and Palumbo, 2000). Bacteria that are responsible for latent infections and/or colonize senescent plant tissues and produce macroscopic signs of disease are not considered to be

endophytes. Endophytic bacteria are also distinguished from bacteria that are present within plant tissues on a transient basis and do not survive there for long periods of time.

Plant growth-promoting bacterial endophytes employ similar plant growth promotion mechanisms to those used by rhizospheric PGPB. In fact, bacterial endophytes are PGPB that go one step further to colonize the inside of the plant tissues where they can serve their host promptly and efficiently compared to those that bind exclusively to the plant's rhizosphere. Therefore, it is likely that endophytic plant growth-promoting bacteria will be more effective than similar non-endophytic bacterial strains in promoting plant growth under a wide range of environmental conditions.

1.4.1 Plant colonization

Prior to colonizing the inside of a plant, an endophyte first colonizes the rhizosphere (Sturz, 1995; Sturz and Nowak, 2000) or the phyllosphere (Pillay and Nowak, 1997), where rhizosphere refers to the area of the soil surrounding a plant's root and phyllosphere represents the aboveground surfaces of a plant that are used as microbial habitat. Microorganisms can gain entry into plants by chance as well; however, those microbes that enter a plant's interior accidentally generally do not survive for long periods of time. Thus, a microorganism that is able to enter the interior of the plant is not necessarily a true endophyte. True endophytic colonization must be confirmed with proper spread among different organs of the plant (if required) and the maintenance of endophytic state for bacterial generations within the plant environment (van Overbeek *et al*., 2006). These two most important abilities of endophytes distinguish endophytes from other transient visitors.

Like plant pathogens, endophytes employ different mechanisms to gain entry into plants. They may enter the plant through different points; these entry points include tissue wounds (Agarwhal and Shende, 1987), stomata (Roos and Hattingh, 1983), lenticels (Scot *et al*., 1996), root cracks (Sorensen and Sessitsch, 2006) and germinating radicles (Gagn *et al*., 1987). Entry through root cracks is recognized as the main portal of entry for bacterial colonization. Other mechanisms may include penetration of bacteria via root hair cells (Huang, 1986) and through the production of cell wall degradative enzymes (Quadt-Hallmann, 1997).

Upon reaching the inside of the plant, an endophyte may localize itself at the point of entry or may be spread throughout the plant (Hallmann *et al*., 1997). If endophytes are systemically spread then they can reside in the intracellular spaces (Patriquin and Dobereiner, 1978), in the vascular system (Bell *et al*., 1995), and/or within the cells (Jacob *et al*., 1985). Specialized cellular environments are selected by certain bacterial species because of their higher tendency to become established in those environments (van Overbeek, 2006). For example the plant xylem was demonstrated to be a selective environment for nitrogen-fixing endophytic bacteria such as *Acetobacter diazotrophicus* (Dong *et al*., 1994), *Bacillus pumilus* (Benhamou *et al*., 1996a; Benhamou *et al*., 1998), *Gluconacetobacter diazotrophicus* (Cocking, 2003), *Herbaspirillum seropedicae* (James *et al*., 2002), *Klebsiella pneumoniae* (Dong *et al*., 2003) and *Serratia marcescens* (Gyaneshwar *et al*., 2001). On the other hand, sieve tubes (phloem) are a habitat for certain pathogenic phytoplasma (Bove and Garnier, 2003). The effects of endophytes on their host plants vary widely; they may be beneficial to some hosts while harmful for some others (in that case they are considered as plant specific endophytes).

This mainly depends on the response of their host at various growth stages and different environmental conditions to the mechanisms employed by the bacteria (Hallmann *et al*., 1997; Sturz and Nowak, 2000; van Overbeek, 2006).

1.4.2 Isolation of bacterial endophytes

The endophytic niches likely protect the bacteria from many environmental stresses. A wide range of plants can serve as hosts for endophytic bacteria, ranging from herbaceous plants to woody plants. Bacterial endophytes have been isolated from almost all kinds of plants including monocotyledonous plants and dicotyledonous plant genera (Robert *et al*., 2008; Posada and Vega, 2005). A large number of bacterial endophytes have been isolated from almost every part or tissue of the plant (Posada and Vega, 2005). Bacterial endophytes are widely abundant in all types of plants; no known plant species are completely free of endophytes (Rosenblueth and Martínez-Romero, 2006). However, endophytic population densities are lower than those of rhizospheric bacteria or bacterial pathogens (Hallmann *et al*. 1997; Rosenblueth and Martínez-Romero, 2004, 2006). Furthermore, apparent absences of endophytes in a plant species may be either due to the procedure used for the isolation of the endophytes or the microorganism is not culturable. Operationally, the most reliable method for isolating endophytes is the tissue surface sterilization method that comprises three main steps: 1) surface sterilization of plant tissue, 2) maceration of sterilized tissue, and 3) culturing of serially diluted macerated plant tissues (Monique *et al*., 2003). Bacterial endophytes can also be extracted from the internal plant tissues (Hallmann *et al*., 1997).

1.5 Objectives of the present study

Most laboratory and field endophytic studies have not tested for the presence of ACC deaminase. However, based on extensive studies with rhizospheric plant growth-promoting bacteria, it is extremely likely that endophytic plant growth-promoting bacteria that contain this activity will also be more effective to similar strains without this activity in promoting plant growth under a wide range of environmental conditions. Therefore, the purpose of the work reported here was to isolate new ACC deaminase-containing bacterial endophytes from 18 soil samples from geographically different locations, to screen them for their plant growth-promoting capacities, and finally to test them to ascertain whether they could be employed as environmentally friendly adjuncts to agricultural and horticultural practice.

2 Materials and Methods

2.1 Isolation of bacterial endophytes

A total 18 different soil samples were collected from Canada (collected on the campus of the University of Waterloo in Waterloo; Ontario, and from Kitchener; Ontario), Lyon; France (kindly provided by Dr. Yvan Moënne-Loccoz), Haifa; Israel (kindly provided by Dr. Shimon Gepstein), Evora; Portugal (kindly provided by Dr. Solange Oliveira), and Blacksburg, Virginia; USA (kindly provided by Dr. Jerzy Nowak). Surface sterilized tomato seeds (*Solanum lycopersicum* Heinz 72) were sown in the above-mentioned soils in green plastic pots (11 cm × 9 cm), 10–15 seeds per pot. The pots were kept at room temperature on the lab bench top. The tomato plants were harvested after 6–7 weeks of growth. Bacterial endophytes were then isolated based on the method described by Sturz *et al*. (1998) and Surette *et al*. (2003). Each plant was separated into roots, stems, and leaves and then thoroughly washed with tap water to remove any adhering soil. The plant tissues were then surface disinfected by a 3 min treatment with commercial bleach (5.25% available chlorine), transferred to a 3% hydrogen peroxide solution for 3 min, and finally rinsed three times with filtered and deionized ("Milli-Q") water. A 10% solution of Tween 20 was added to the first rinse solution. Surface-sterilized tissues were used to inoculate tryptic soy agar (TSA) (BactoTM Becton, Dickinson and company Sparks, MD, USA) plates to ascertain that, subsequent to the above-mentioned washing procedure (McInroy and Kloepper, 1995a; Sturz *et al*., 1998; Surette *et al*., 2003), plant tissue surfaces did not contain any culturable microorganisms. In fact, in all cases when this was done, no bacterial growth

was found following 48 h incubation at 30°C. Tomato plant tissues (root, stem, and leaf) were then homogenized in 10 ml 3× Ringer's solution (Surette *et al*., 2003) using an ethanol-sterilized mortal and pestle, incubated at room temperature (21–23°C) in an orbital shaker for 1 h, and serially diluted to 10^{-3} with 3× Ringer's solution. Aliquots of 100 µl of each dilution were plated on tryptic soy agar (TSA), Luria agar (LA) (Fisher Scientific, New Jersey, USA), and King's B agar (KBA) (20 g proteose peptone 3, 10 ml glycerol, 1.5 g K_2HPO_4, 1.5 g $MgSO_4 \cdot 7H_2O$ and 15 g agar in 1 L of Milli-Q water) plates in duplicate, then the plates were incubated at 25°C for 72 h. Morphologically different colonies (based on size, shape, and color) were selected; not more than two colonies were selected per plate. Individual colonies were subcultured on respective growth medium plates for further screening and to make −80°C glycerol culture stocks.

2.1.1 pH determination of the soil

Five g of each soil sample was suspended in 25 ml of Milli-Q water in a separate 50 ml plastic tube. Then the tubes were vigorously shaken and left to stand for 5 h. The pH of the soils was measured with VWR Scientific pH meter model 8000 (Batavia, IL).

2.2 Characterization of bacterial endophytes

2.2.1 ACC deaminase activity measurement

ACC deaminase activity was determined for all of the newly isolated strains according to the protocol described by Penrose and Glick (2003) with a standard curve of

α-ketobutyrate between 0.05 and 0.5 μM.

2.2.2 IAA production assay

The ability of bacterial endophytes to produce IAA was measured based on the colorimetric method described by Glickmann and Dessaux (1995) and Patten and Glick (2002) with some modifications. Aliquots of 20 μl of an overnight grown bacterial culture were used to inoculate 5 ml of TSB without and with tryptophan (200 μg ml^{-1} or 500 μg ml^{-1}) and incubated at 30°C for 24 h. Overnight cultures were centrifuged and 1 ml of supernatant was mixed with 4 ml Salkowski's reagent (Gordon and Weber, 1951), incubated for 20 min at room temperature before the absorbance was measured at 535 nm. The concentration of each sample was estimated using a standard plot ranging from 0.01 to 0.4 μg ml^{-1} pure IAA (Sigma).

2.2.3 Siderophore production assay

This assay was done qualitatively and is based on competition for iron between a ferric complex of chrome azurol S (CAS), an indicator dye, and a siderophore produced by the microorganism. The iron is removed from CAS by the siderophore (which binds iron more tightly) and a positive reaction is indicated by a color change of the CAS reagent from blue to orange (Schwyn and Neilands, 1987). A 5 μl aliquot of an overnight bacterial culture in KB medium was spotted onto a CAS agar plate (Alexander and Zuberer, 1991) in triplicate and incubated at 30°C for 3–4 days.

2.2.4 Salt tolerance

A 20 μl aliquot of an overnight test culture was inoculated into 5 ml of 1% proteose peptone plus 0.0%, 0.5%, 1%, 2%, 3%, 4%, 5%, 7%, or 10% NaCl and incubated at their respective optimal temperatures in a shaking water bath. After 24–48 h, the absorbance of the culture was determined at 600 nm, with uninoculated medium serving as a blank. The entire experiment was repeated three times.

2.2.5 Antibiotic sensitivity/resistance

Five different antibiotics were selected for testing according to their modes of action. Ampicillin (50 μg ml^{-1}), erythromycin (50 μg ml^{-1}), kanamycin (50 μg ml^{-1}), novobiocin (50 μg ml^{-1}), and tetracycline (15 μg ml^{-1}) were added separately to 5 ml TSB medium, before 20 μl of an overnight test culture was added and then incubated at 30°C in a shaking water bath for 24–48 h. The absorbance at 600 nm of all samples was measured using uninoculated TSB as a blank. The entire experiment was repeated three times.

2.2.6 Optimal growth temperature

Optimal growth temperatures were investigated by growing the test strains at a range of temperatures from 15°C to 50°C in a growth curve machine (Lab Systems Thermo Electron Growth Curves Bioscreen C Microplate Reader, Diversified Equipment Company Inc. Lorton, VA) following the protocol suggested by the manufacturer. Each

growth curve experiment was done in triplicate in TSB medium and run for 3 days.

2.2.7 Production of ammonia

The ability of bacterial strains to produce ammonia was assessed as described by Marques *et al*. (2010). In this method 20 µl of an overnight grown test culture was inoculated into 5 ml of 1% proteose peptone broth and incubated at 30°C in a shaking water bath. After 24–48 h, 0.5 ml Nesseler's reagent (Krug *et al*., 1979) was added to the culture and the color change was noted, a yellow coloration indicates a positive result while the intensity of color is indicative of the amount of ammonia produced by the test strain.

2.2.8 Gnotobiotic root elongation assay

The ability of newly isolated bacterial endophytes to promote the growth of canola roots was carried out and monitored as described by Penrose and Glick (2003). Briefly, all test strains were grown under gnotobiotic conditions and then diluted with 0.03 mol $MgSO_4$ to an OD of 0.15 ± 0.02 at 600 nm. Canola seeds were surface sterilized by a 1 min treatment with 70% ethanol, 10 min with 1% commercial bleach, and several washes with sterile milli-Q water. The seeds were then treated with the test strains (1 h incubation at room temperature) and growth pouches were inoculated manually. As negative and positive controls, surface sterilized seeds were treated with 0.03 mol $MgSO_4$ and a suspension of *Burkholderia phytofirmans* PsJN, respectively. Observations were made on day five. One-way ANOVA was performed followed by a Tukey's post-test for each test strain and compared with both positive and negative controls. Values similar to

positive control are represented by “a”, values significantly greater than the negative control but lower than the positive control are represented by “b”, values similar to the negative control are represented by “c” and values significantly lower than the negative control are represented by “d”.

2.2.9 Strain identification

Strains were identified using two different methods, i.e. 96 well Biolog plates (GN2 MicroPlaTcM, Biolog Inc. Hayward, CA, USA) used as suggested by the manufacturer. For 16S rRNA gene sequencing, total genomic DNA was extracted using Wizard® Genomic DNA Purification Kit (Promega) as described by the manufacturer. With genomic DNA as the template, a portion of the bacterial 16S rRNA gene (400 bp) was amplified with the bacterial universal primers (b341 and b758) designed by Wang *et al*. (2006). However, the full 16S rRNA gene of the selected bacterial endophytes (8R6 and YsS6) was amplified with primers (fD1 and rD1) designed by Weisburg *et al*. (1991). The 25 μl of the PCR mixture (Novagen) contained 2.5 μl 10× buffer for KOD Hot Start DNA Polymerase, 1.5 mM of $MgSO_4$, 0.2 mM of each of the four dNTPs, 0.3 μM of each primer, 100 ng of genomic DNA as template, 0.02 U of KOD Hot Start Polymerase enzyme, and PCR grade water to make up the volume. A negative control (PCR mixture without DNA template) was included for each PCR reaction. Amplifications were carried out in an Eppendorf Mastercycler using the following conditions: 95°C for 2 min, 30 denaturation cycles at 95°C for 20 s, annealing temperatures suitable for primer set, primer extension at 70°C for 10 s followed by a final extension at 70°C for 5 min. In each

case, the PCR product was run on an 0.8% agarose gel containing ethidium bromide, isolated and purified using a EZ-10 Spin Column PCR purification kit (BioBasic Inc., Markham, ON, Canada), and sent to the AAC Genomic Facility at the University of Guelph, ON, Canada. The DNA sequences obtained were analyzed with basic sequence alignment BLAST program (Altschul *et al*., 1991) run against the nucleotide collection (nr/nt) database from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST).

2.3 Construction of ACC deaminase deficient mutants

2.3.1 Bacterial strains and plasmids

ACC deaminase deficient mutants were constructed for the two recently isolated bacteria endophytes *Pseudomonas fluorescens* YsS6 and *Pseudomonas migulae* 8R6. Both of the strains were grown either on solid or in liquid TSB medium with 100 μg ml^{-1} ampicillin at 30°C.

Escherichia coli DH5α (Hanahan, 1983) was used as the recipient of different recombinant plasmids and was grown in LB medium at 37°C. Plasmid pK19 *mobsacB* (Schäfer *et al*., 1994) containing *Escherichia coli* strain was also grown at 37°C in LB medium supplemented with 20 μg ml^{-1} of kanamycin. pK19 *mobsacB* contains broad host range transfer machinery of plasmid RP4 and a modified *sacB* gene from *Bacillus subtilis*. These two properties make this plasmid a very useful tool in molecular cloning and genetic engineering research. The *mob* gene enhances the rapid transfer of the recombinant gene over a wide range of hosts while the *sacB* gene makes the recipient of

the plasmid susceptible to sucrose and subsequently helps to detect rare double cross-over events.

E coli MT616 containing pRK600 (Finan *et al*., 1986) was also grown in LB containing 25 μg ml^{-1} chloromphenicol at 37°C and used as helper for the bacterial conjugation.

2.3.2 Isolation of genomic DNA from bacterial endophytes

Bacterial endophytes (*Pseudomonas fluorescens* YsS6 and *Pseudomonas migulae* 8R6) were inoculated in 5 ml of TSB supplemented with 100 μg ml^{-1} of ampicillin and incubated in a shaker water bath at 30°C for 24 h. One ml of the bacterial culture was transferred to 1.5 ml micro centrifuge tube and centrifuged at 14000 rpm for 5 min in 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 manufacturer suggested protocol.

2.3.3 Isolation of ACC deaminase gene from bacterial endophytes

The gene encoding the *Pseudomonas* species' 1-aminocyclopropane-1-carboxylic acid deaminase is ~1000 bp. Several *Pseudomonas* sp. *acdS* genes were aligned and the following two sets of strain specific primer were designed: For *Pseudomonas fluorescens* YsS6 5'- ATGAACCTGAATCGTTTTGA-3' (right) and 5'-TCAGCCGTTGCGAAACAGGA-3' (left) and for *Pseudomonas migulae* 8R6 5'-

ACAGGAAGCTGTAGGCGTTC-3’ (right) and 5’- CTGTATGCCAAGCGTGAAGA-3’ (left) were synthesized by Sigma. The 25 μl PCR mixture (Novagen) contained 2.5 μl 10× buffer for KOD Hot Start DNA Polymerase, 1.5 mM of $MgSO_4$, 0.2 mM of each of the four dNTPs, 0.3 μM of each primer, 100 ng of total genomic DNA of respective wild type bacterial endophyte as template, 0.02 U of KOD Hot Start Polymerase enzyme, and PCR grade water to make up the volume. Amplifications were carried out in an Eppendorf Mastercycler using the following conditions: 95°C for 2 min, 30 denaturation cycles at 95°C for 20 s, annealing temperatures suitable for primer set (63°C for strain 8R6 and 59°C for strain YsS6), primer extension at 70°C for 15 s, followed by a final extension at 70°C for 5 min. In each case, the PCR product was run on an 0.8% agarose gel containing ethidium bromide, isolated and purified using a EZ-10 Spin Column PCR purification kit (BioBasic Inc., Markham, ON, Canada), and sent to the AAC Genomic Facility. The DNA sequences obtained were analyzed with basic sequence alignment BLAST program (Altschul *et al*., 1991) run against the nucleotide collection (nr/nt) database from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST).

2.3.4 Construction of ACC deaminase deficient replacement vectors

The construction of ACC deaminase deficient replacement vectors for *P. fluorescence* YsS6 and *P. migulae* 8R6 is outlined in Fig. 2.1 and Fig. 2.2 respectively. The plasmid Bluescript (pBS) (Stratagene Cloning Systems, Cambridge, U.S.A.) was linearized with EcoRV, which produces blunt ends. The isolated *acdS* gene was cloned in

the plasmid to make pBSYsS6ACC and pBS8R6ACC. To disrupt the *acdS* gene on each plasmid, the tetracycline resistance gene contained within the pBR322 HindIII-AvaI fragment was inserted into the BmgBI site (strain YsS6) and TthIII1 site (strain 8R6) within the *acdS* coding region to make pBSYsS6ACC$^-$ and pBS8R6ACC$^-$. The cohesive ends produced by HindIII-AvaI on tetracycline resistance gene and by TthIII1 on *acdS* gene of strain 8R6 were filled-in and made blunt ended by *E. coli* DNA polymerase I Klenow fragment (Fermentas Inc.) before ligation, while BmgBI produces blunt ends on *acdS* gene of strain YsS6. Then, the *acdS* gene with the tetracycline fragment of each modified plasmid was cut by PstI and KpnI and cloned into mobilizable cloning vector pK19 *mobsac*B, which was already linearized by SmaI, to construct pK19 *mobsac*B YsS6ACC$^-$ and pK19 *mobsac*B 8R6ACC$^-$. All plasmid extraction and purification were done using Promega Wizard® Plus SV Minipreps DNA Purification System (Promega). All ligation reactions were incubated overnight at 4°C. The restriction enzymes AvaI, BmgBI, EcoRV, HindIII, KpnI, PstI, and TthIII1 were purchased from Fermentas Inc., and the T4 DNA ligase was purchased from Promega.

2.3.5 Conjugation and homologous recombination

In order to obtain the *acdS* mutants of bacterial endophytes, the mobilizable cloning vectors pK19 *mobsac*B YsS6ACC$^-$ and pK19 *mobsac*B 8R6ACC$^-$ were transferred to wild type strains of *P. fluorescence* YsS6 and *P. migulae* 8R6 separately in the presence of a helper plasmid (pRK600) through conjugation. For the conjugation reaction, 1 ml of each culture (recipient, strain YsS6 or 8R6; donor, *E. coli* DH5α containing pK19 *mobsac*B YsS6ACC$^-$ or pK19 *mobsac*B 8R6ACC$^-$, helper *E. coli*

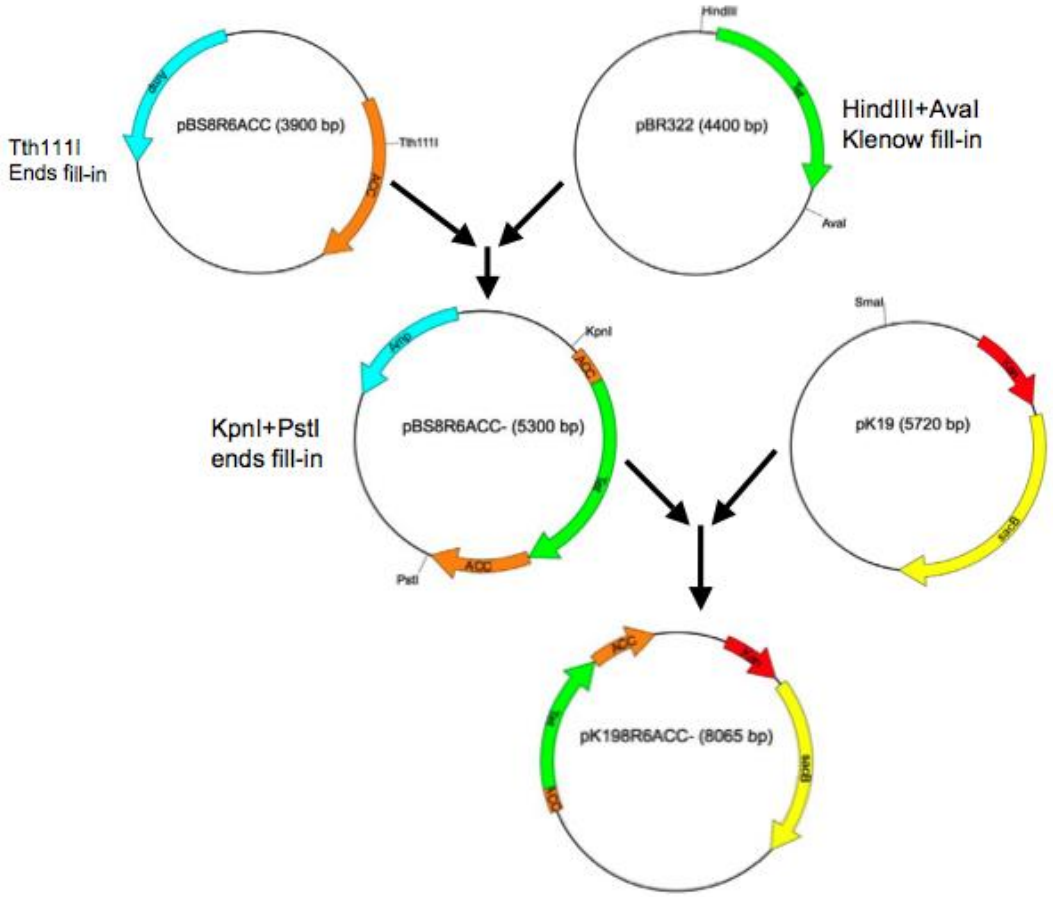


Figure 2.1 Schematic representation of the construction of the deficient *acdS* gene containing vector pK19 *mobsac*B YsS6ACC^{-}.

Amp: ampicillin resistance gene, Tc: tetracycline resistance gene, and ACC: ACC deaminase gene of *P. fluorescence* YsS6.

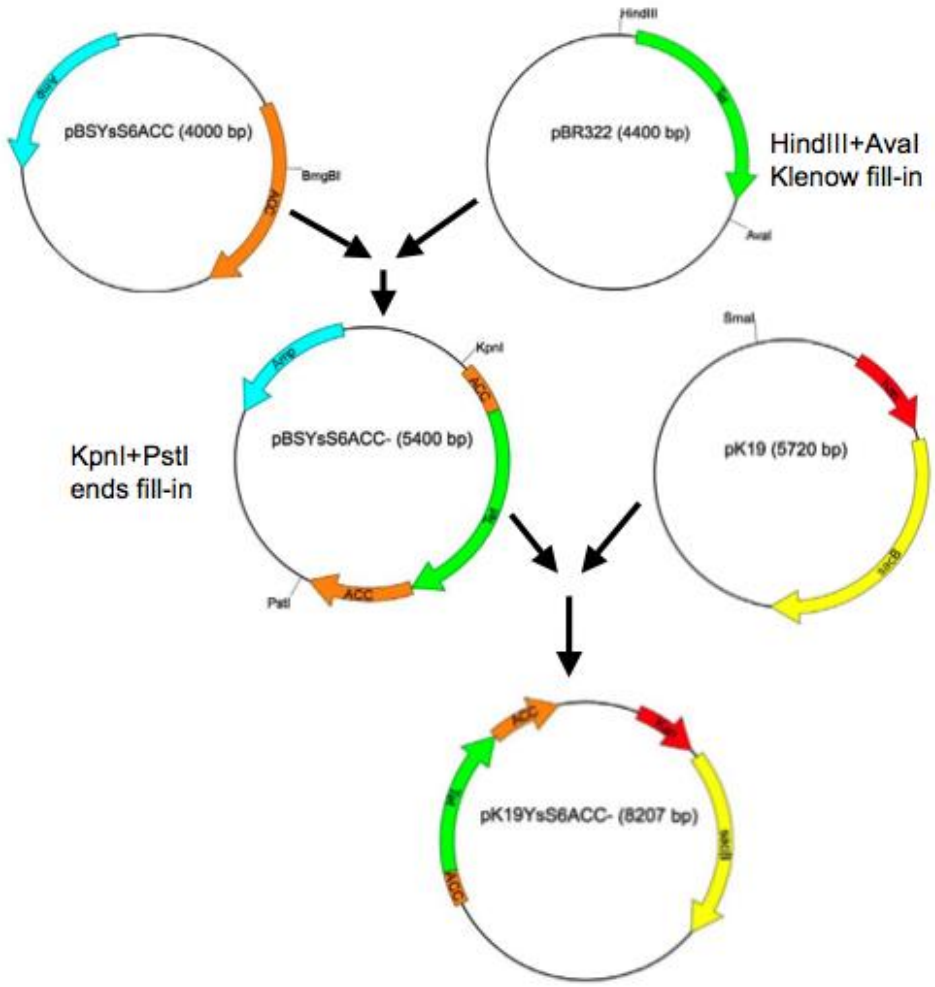


Figure 2.2 Schematic representation of the construction of the deficient *acdS* gene containing vector pK19 *mobsac*B 8R6ACC⁻.

Amp: ampicillin resistance gene, Tc: tetracycline resistance gene, and ACC: ACC deaminase gene of *P. migulae* 8R6.

MT616 containing pRK600) was taken in a separate micro centrifuge tube and centrifuged at 14000 rpm for 3 minutes. Cells were washed two times with sterile 0.85% NaCl solution to remove all of the medium components and antibiotics. Finally, cells were suspended in 100 µl of 0.85% NaCl, mixed in a 1:1:1 ratio, and transferred to the center of a plain TSB agar plate. The plate was incubated at 30°C for 24 h. After the incubation, the bacterial cells were suspended in 1 ml of 0.85% NaCl, diluted to 10^{-4}, and 100 µl of each dilution was plated onto the selective medium plates (TSB agar plate containing ampicillin 100 µg ml^{-1}, tetracycline 10 µg ml^{-1}, and 10% sucrose). The *acdS* deficient mutants were first selected by their sensitivity to kanamycin (25 µg ml^{-1}), then tested by PCR amplification of the mutant gene with the *acdS* gene primers set (Tc gene within the *acdS* gene), and finally confirmed by ACC deaminase assay of the mutants, and the results were compared with their corresponding bacterial wild-type.

Subsequently, the *acdS* deficient mutants were characterized for the ACC deaminase activity, IAA production, siderophore production, and the ability to promote plant growth under gnotobiotic conditions as described earlier.

2.4 Flower senescence experiment

2.4.1 Plant material

Carnation is a typical ethylene-sensitive flower that produces ethylene through an autocatalytic pathway and enters into rapid flower senescence (Rahemi and Jamali, 2011). Mini carnation plants (*Dianthus caryophyllus*) were grown from seed and maintained in a green house within the Department of Biology, University of Waterloo.

The flowers were cut at full bloom, the stems were trimmed to a uniform length of ~8 cm and then processed immediately.

2.4.2 Bacterial strains and growth conditions

All four bacterial strains (*P. fluorescence* YsS6, *P. migulae* 8R6 and their *acdS* deficient mutants) were grown in 15 ml of TSB with appropriate antibiotics at 30°C for 24 h. Overnight cultures were washed once with 0.85% NaCl, centrifuged at 5000 rpm for five minutes and then diluted with 0.85% NaCl to an absorbance of 0.15 ± 0.02 at 600 nm.

2.4.3 Chemicals

The compounds 1-aminocyclopropane-1-carboxylate (ACC) and sodium chloride (NaCl) were purchased from Calbiochem®,Merck KGaA, Darmstadt, Germany, and L-α-(aminoethoxyvinyl)-glycine (AVG) was purchased from Sigma-Aldrich. All three chemicals were dissolved and diluted in Milli-Q water. Stock solutions of ACC (0.5 M) and AVG (0.25 M) were prepared and then stored in small aliquots at -20°C.

2.4.4 Experimental set-up

Each flower was placed in a separate 13x100 mm glass test tube filled with 8 ml of either diluted bacterial suspension, 1 x 10^{-4} M ACC solution, or 1 x 10^{-4} M

AVG solution, then the tube was placed in a tube rack and incubated at room temperature (21-23°C for 11 days. A set of 105 flowers was used for each treatment. Flowers treated only with 0.85% NaCl were used as a negative control. Flower senescence was recorded on a scale from zero to four where zero is a freshly cut flower and four is a completely senesced flower (Fig. 2.3).

2.4.5 Scanning electron microscopy

Different portions of treated flowers were examined for the presence or absence of bacteria inside the tissue. A 1-1.5 cm piece of the flower stem or flower thalamus was removed and the interior portion of the tissue was exposed by cutting it lengthwise; it was then fixed in a 2.5% glutaraldehyde solution in 0.1 M phosphate buffer (pH 7.0) overnight at 4°C and then washed twice with phosphate buffer. After dehydration in a series of acetone solutions (20, 50, 70, 95, 100%), the specimens were dried to critical point in liquid CO_2, mounted on a stud, coated with gold, and examined under a scanning electron microscope (Hitachi s570) at 15 kV accelerating voltage (Matzk *et al*., 1996). The entire experiment was repeated three times with two samples for each treatment (i.e. control flower, flower treated with wild type strain, flower treated with mutant strain).



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Figure 2.3 Levels of senescence of different colored mini carnation flowers on a scale from 0 to 4, no senescence or fresh flower (a) to 1 (b), 2 (c), 3 (d), and 4 (e) completely senesced flower.

2.5 Effect of bacterial endophytes on the plant growth under salt stress

2.5.1 Biological material

All the bacterial endophytes and the growth conditions are the same as for the flower senescence experiment except for the culture volume, i.e. 250 ml of each culture was prepared. Tomato (*Solanum lycopersicum* H 72 Better Boy) seeds, purchased from Stokes Seeds Ltd. (Thorold, ON, Canada), were surface sterilized as mentioned earlier and treated with bacterial cell suspension having an absorbance of 0.25 ± 0.02 at 600 nm. However, the control seeds were treated with sterile Milli-Q water only (no bacteria). All of the treatments were incubated at room temperature for 1.5 h.

2.5.2 Pot experiment set-up

Four tomato seeds (per pot) that was pretreated either with wild-type bacteria, mutant bacteria, or no bacteria (control) were sowed in a green plastic pot (9.5 cm x 11 cm) filled with wet peat-based Sunshine4^{TM} mix aggregate (~180 g dry weight) general-purpose plant growth medium (Premier Horticulture, St. Catharines, Ontario, Canada). After the seed had been placed on the soil, a one ml bacterial suspension was poured on the top of each seed, and then the seeds were covered with the soil. A group of 24 pots (96 plants) was set for each treatment, and the entire experiment was repeated two times. For the first three weeks of the experiment, the pots were watered with regular tap water when needed. After the three weeks, plants were watered either with 165 mM salt, 185

mM salt, or no salt (0 mM) when required. At week 7, the plants were transferred to larger pots (25 cm x 30 cm).

2.5.3 Fresh and dry biomass measurements

After week 11, plants were collected and fresh biomass was measured and recorded. Then, the plants were dried in an oven at 85°C until there was no further drop off in the weight; the dried shoot biomass was also recorded.

2.5.4 Sodium content measurements

For the quantification of sodium content in the plant, 1 g of dried plant material was suspended in 20 ml of 1 M nitric acid (HNO_3), incubated at 70°C for 4 h, and then cooled at room temperature for 1 h (Cheng *et al*., 2007). The contents of the tubes were centrifuged at 10,050 g for 10 min. An aliquot of 100 µl of the supernatant was diluted to 1 ml with the Milli-Q water. The sodium concentration was measured using a graphite furnace atomic absorption spectroscopy (VarianSpectrAA 880 spectrophotometer; Varian, Palo Alto, California, USA). The sodium concentration in the plant tissue was calculated using a standard curve between 50 and 2000 µM of sodium.

2.5.5 Chlorophyll measurements

One g of fresh leaves was treated with 5 ml of N,N-dimethylformamide (Moran and Porath, 1980) in a small glass bottle with a tight cap. The tube was incubated at 4°C

in the dark for 48 h. The absorbance of the resulting solution was read at 663 nm and 645 nm. The amount of total chlorophyll was calculated by using the following formula (Taffouo *et al*., 2010):

$$\text{Total chlorophyll} = (20.2 \times A_{645} + 8.02 \times A_{663}) \times ½$$

2.5.6 Presence of bacterial endophytes in plant tissue

Different parts of the plant (roots, stems, leaves, fruits) were investigated for the presence of bacterial endophytes. Surface sterilized plant tissue was homogenized in an alcohol-flamed sterile mortar and pestle in 3x Ringer's solution. The success of the disinfection process was tested by the tissue impression method as discussed earlier. The plant homogenate was incubated at room temperature for one hour in an orbital shaker. A series of serial dilutions was plated onto TSB agar plates, and the plates were incubated at 30°C for 24 hours. Bacterial endophytes were confirmed with the main characterization attributes.

3 Results

3.1 Isolation of bacterial endophytes

A total of 18 different soil samples were used for growing the tomato plants for the isolation of new bacterial endophytes. The soil samples were from a range of diverse environments (for example, temperature diversity, pH diversity, water content diversity). Table 3.1 shows all of the soil sample names, country of origin, and the pH. In three soil samples (DN, DA, DH) out of eighteen, plants did not grow. Therefore, 6-7 week old tomato plants from 15 different soil samples were used as starting material. A total of 174 unique bacterial endophytes were isolated from the interior tissues of tomato plants.

3.2 Characterization of bacterial endophytes

When all of the 174 newly isolated bacterial endophytes were first tested for the presence of plant growth-promoting enzyme ACC deaminase activity, only 25 separate strains were positive with cellular levels of ACC deaminase activity (Table 3.3). Only ACC deaminase-containing strains were re-inoculated and re-tested for their ability to colonize the interior tissues of tomato plants to ensure that all of the selected strains were true endophytes (Rosenblueth and Martinez-Romero, 2006). Moreover, to avoid contaminants or false positive endophytes, the surface sterilized plant material was also tested for the possibility of carrying any adhering contaminants with them in the process of re-isolation of the true endophytes by the tissue impression method and the final-rinse water's spreading method onto the sterile media agar plates. Only ACC deaminase-

Table 3.1 Soil samples; their country of origin, name, and pH description.

No.	Country of origin	Location	Sample designation	pH
1	Canada	Kitchener	8B	8.63
2	Canada	Columbia lake	CL	8.96
3	Canada	Laurel creek lake	L	8.84
4	Canada	Biology green house lawn	G	8.81
5	France	Lyon	Yso5	8.09
6	France	Lyon	Ymo4	8.01
7	Israel	Haifa	A	10.01
8	Israel	Haifa	B	8.51
9	Israel	Haifa	C	8.89
10	Israel	Haifa	D	8.81
11	Portugal	Evora, Valverda river	V	5.79
12	Portugal	Evora, Mitra	M	7.65
13	Portugal	Evora, Guandalupe oak tree	GO	7.81
14	Portugal	Evora, Guandalupe pine tree	GP	7.04
15	U.S.A.	Virginia, Undisturbed air only	UA	7.57

Table 3.2 Identification of newly isolated ACC deaminase containing bacterial endophytes.

16S rRNA gene identification was made at the species level only when the 400 sequenced bases were at least 97% identical to the species indicated.

Strain	**16S rRNA gene identification**	**% Identity**	**Biolog plate results**	**GenBank Accession No.**
8R6	*Pseudomonas migulae*	99	*Pseudomonas* sp.	JN118619
YsS1	*Pseudomonas* sp. TF10	99	*Pseudomonas fluorescens*	JN118636
YsS2	*Pseudomonas* sp. D21	100	*Pseudomonas* sp.	JN118637
YsS3	*Pseudomonas* sp. D21	100	*Pseudomonas fluorescens*	JN118638
YsS4	*Pseudomonas* sp. TF10	100	*Pseudomonas fluorescens*	JN118639
YsS5	*Pseudomonas* sp. D21	100	*Pseudomonas fluorescens*	JN118640
YsS6	*Pseudomonas fluorescens* STAD 384	100	*Pseudomonas fluorescens*	JN118641
YsS7	*Pseudomonas* sp. strain MTQ15	99	*Pseudomonas fluorescens*	JN118642
AS1	*Microbacterium takaoensis* strain P1P4	100	Unidentifiable	JN118620
AS2	*Microbacterium* sp. 3407bBRRJ	99	Unidentifiable	JN118621
AS3	*Microbacterium* sp. 3407bBRRJ	99	Unidentifiable	JN118622
AS5	*Agrobacterium tumefaciens* strain BW62UT1570	100	*Agrobacterium tumefaciens / radiobacter*	JN118618
AS6	Uncultured *Devosia* sp.	99	*Sphingomonas macrogoltabidus*	JN118623
CR4	*Acinetobacter radioresistens* YCY7	100	Unidentifiable	JN118625
YmS1	*Bacillus horneckiae* NBB13	99	Unidentifiable	JN118635
GO-L6	*Agrobacterium tumefaciens* strain WR41	99	*Agrobacterium tumefaciens / radiobacter*	JN118628
CL-S3	*Rhodococcus equi* SB01003	100	*Chromobacterium violacium*	JN118624
LR1	*Bacillus idriensis* strain AS3-3	98	*Burkholderia glumae*	JN118632
LL4	*Agrobacterium vitis*	99	*Agrobacterium vitis*	JN118631
UA-S1	*Bacillus psychrosaccharolyticus*	100	Unidentifiable	JN118633
V2	*Bacillus simplex* strain XAS5-3	99	Unidentifiable	JN118634
DL1	Uncultured bacterium clone ncd 184bolcl	99	Unidentifiable	JN118626
DL6	Uncultured bacterium clone DP10.5.28	100	Unidentifiable	JN118627
GPR1	Bacterium 8-gw2-7	99	Unidentifiable	JN118629
GPR2	*Bacillus* sp. NIOC23	99	Unidentifiable	JN118630

Table 3.3 ACC deaminase activity of newly isolated bacterial endophytes.

Strain name	ACC deaminase activity (μmol. mg^{-1} h^{-1})	Gram reaction
8R6	10.90	−
YsS1	11.47	−
YsS2	9.88	−
YsS3	8.58	−
YsS4	10.79	−
YsS5	12.34	−
YsS6	12.50	−
YsS7	11.50	−
AS1	2.02	+
AS2	1.01	+
AS3	0.84	+
AS5	1.43	−
AS6	1.37	−
CR4	1.24	−
YmS1	1.03	−
GO-L6	0.98	−
CL-S3	3.98	+
LR1	0.84	+
LL4	3.01	−
UA-S1	0.44	+
V2	2.03	+
DL1	1.31	+
DL6	1.09	−/+[a]
GPR1	1.19	−
GPR2	1.05	+

[a] Gram staining was performed several times and each time the same ambiguous result was obtained.

containing true bacterial endophytic strains were tested for various plant growth-promotion related characteristics (i.e. production of IAA, ammonia, and siderophore, salt tolerance, antibiotic sensitivity and resistance, phosphate solubilization, and their growth temperature Table 3.3-3.6). Furthermore, the genus and species of each of these 25 strains was characterized, both through the use of the Biolog^{TM} system and the sequencing of a portion of their genes encoding 16S ribosomal RNA (Table 3.2). Gram staining results revealed that 7 individual endophytes of the ACC deaminase-containing endophytes were Gram positive, 17 individual endophytes were Gram-negative, and the Gram staining results for one ACCD^{+} endophyte (DL6) was found to be ambiguous despite the fact that the procedure was repeated several times (Table 3.3). Among all of the 25 ACC deaminase-containing strains, the *Pseudomonas* strains exhibited the highest ACC deaminase activity followed by CLS3 (*Rhodococcus equi* SB01003) and then by LL4 (*Agrobacterium vitis*) while strain UAS1 showed the least activity (Table 3.3). Furthermore, all of the ACC deaminase-containing endophytes were able to produce IAA in the absence of exogenous tryptophan, and the IAA production was significantly increased when exogenous tryptophan was added to the growth media (Table 3.4). IAA production by strains AS3 and DL1 was found to be the most inducible by the addition of tryptophan. All *Pseudomonas* strains demonstrated a relatively high level of IAA production (among 25 strains) without exogenous tryptophan and were moderately induced when the precursor was provided (Table 3.4). Moreover, the optimal growth temperature for most of these endophytes was 30°C however strains AS5, CR4, DL1, and GOL6 grow best at 25-30°C (Table 3.5). Almost all of these strains were able to produce ammonia, although the amount of the ammonia produced varied considerably

Table 3.4 Production of IAA and IAA-like molecules (µg ml^{-1}).

Trp =Tryptophan.

Strain name	No Trp	200 µg/ml Trp	500 µg/ml Trp
8R6	29.36	31.94	39.43
YsS1	30.69	32.65	39.06
YsS2	32.58	33.08	38.36
YsS3	32.02	31.69	35.91
YsS4	27.78	32.30	36.54
YsS5	29.67	40.21	34.58
YsS6	35.15	34.23	38.45
YsS7	32.35	34.97	39.91
AS1	20.26	41.71	96.50
AS2	38.40	74.03	105.50
AS3	36.76	88.34	143.34
AS5	31.53	40.51	40.50
AS6	25.37	24.56	24.56
CR4	24.50	38.90	40.17
YmS1	9.80	12.40	22.25
GO-L6	14.80	24.12	41.15
CL-S3	3.75	11.70	13.47
LR1	27.0	51.90	66.4
LL4	10.10	15.60	18.30
UA-S1	19.53	40.75	75.22
V2	14.42	37.35	76.01
DL1	11.52	82.52	104.71
DL6	22.34	41.80	48.61
GPR1	23.21	39.56	56.78
GPR2	25.35	38.80	42.71

Table 3.5 Biochemical characteristics of newly isolated bacterial endophytes.

Strain name	Siderophore production	Optimal growth temp. °C	Ammonia production	PO_4 solubilization activity
8R6	+/-	30	+	+/-
YsS1	+	30	++	+
YsS2	+	30	++	+
YsS3	++	30	+++	+
YsS4	+	30	++	+
YsS5	++	30	+++	+
YsS6	++	30	+++	+
YsS7	++	30	++	+
AS1	++	30	+	++
AS2	-	30	+++	++
AS3	-	30	++	+/-
AS5	+	25-30	++	-
AS6	+	30	++	-
CR4	-	25-30	++	+
YmS1	+	30	+	+
GO-L6	+	25-30	+	+/-
CL-S3	-	30	+/-	+
LR1	-	30	+	-
LL4	-	30	+	-
UA-S1	-	30	+	-
V2	-	30	+	+/-
DL1	-	25-30	+	+/-
DL6	+/-	30	+/-	+/-
GPR1	+	30	+	+/-
GPR2	+	30	+	+/-

among the strains (Table 3.5). Strains AS5, AS6, LR1, LL4, and UAS1 did not exhibit phosphate solubilization capability at all on the complex-phosphorus medium while strains 8R6, AS3, GOL6, V2, DL1, DL6, GPR1, and GPR2 were able to solubilize phosphate only in traces. Strains AS1 and AS2 were the best phosphate solubilizers among all of the strains, whereas all *Pseudomonas* strains exhibited moderate phosphate solubilization activity. Fig. 3.1 explains the qualitative scale of phosphate solubilization activity measurement, which was presented in the Table 3.5. In addition, only a few strains were able to produce strong siderophores (YsS3, YsS5 YsS6, YsS7, and AS1) whereas others produced siderophores either weakly or not at all (Table 3.5) on CAS agar plates. The qualitative scale for the measurement of siderophore production can be viewed in Fig. 3.2. Likewise, the antibiotic sensitivity and resistance were evaluated for all newly isolated ACC deaminase-containing endophytes. Interestingly, all of the strains were sensitive to kanamycin, and only a few were resistant to tetracycline. Resistance to erythromycin was the most common resistance in these endophytes whereas resistance to ampicillin and novobiocin showed a mixed trend (Table 3.6). Surprisingly, all of the newly isolated endophytes were found to be highly salt tolerant. In particular, all the *Pseudomonas* isolates exhibited tolerance to as high as 4% salt (Table 3.6). Moreover, the ability of each of these strains to promote plant growth was evaluated by gnotobiotic root elongation assay. In this assay, only two strains (UAS1 and YmS1) demonstrated plant growth inhibition compared to the control (no bacteria) whereas strain GOL6 was equal to the control in promoting the plant growth. Other than these three strains, all new

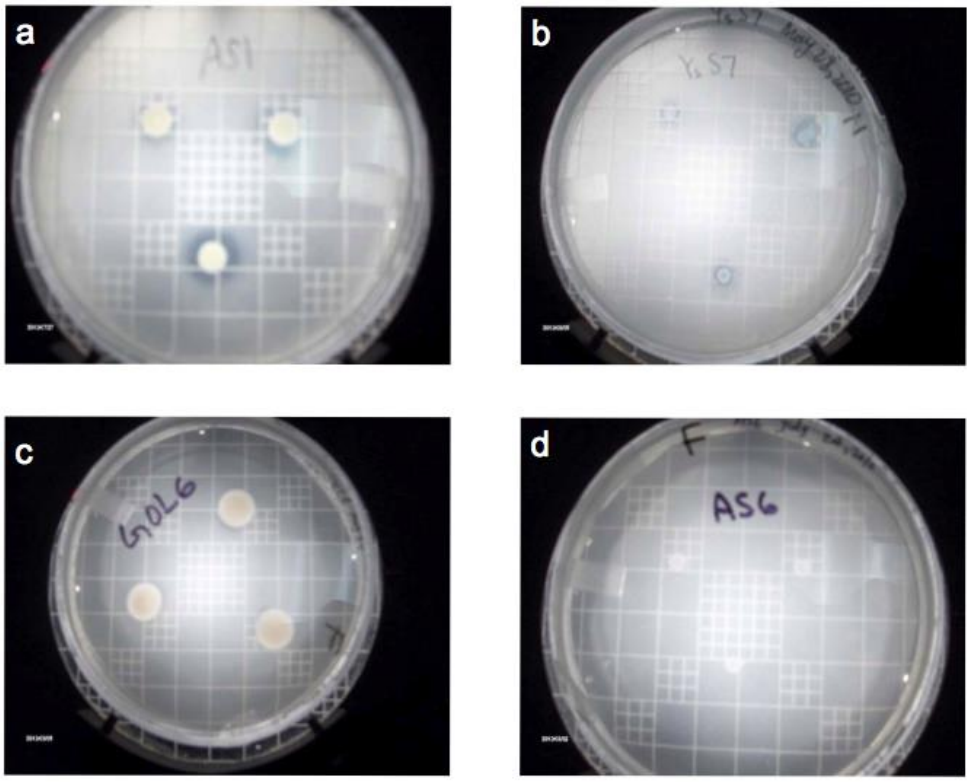


Figure 3.1 Qualitative scale for the measurement of phosphate solubilization activity by newly isolated bacterial endophytes.

Panel (a) shows ++ activity, Panel (b) shows +, Panel (c) shows +/-, and Panel (d) shows – or no activity.

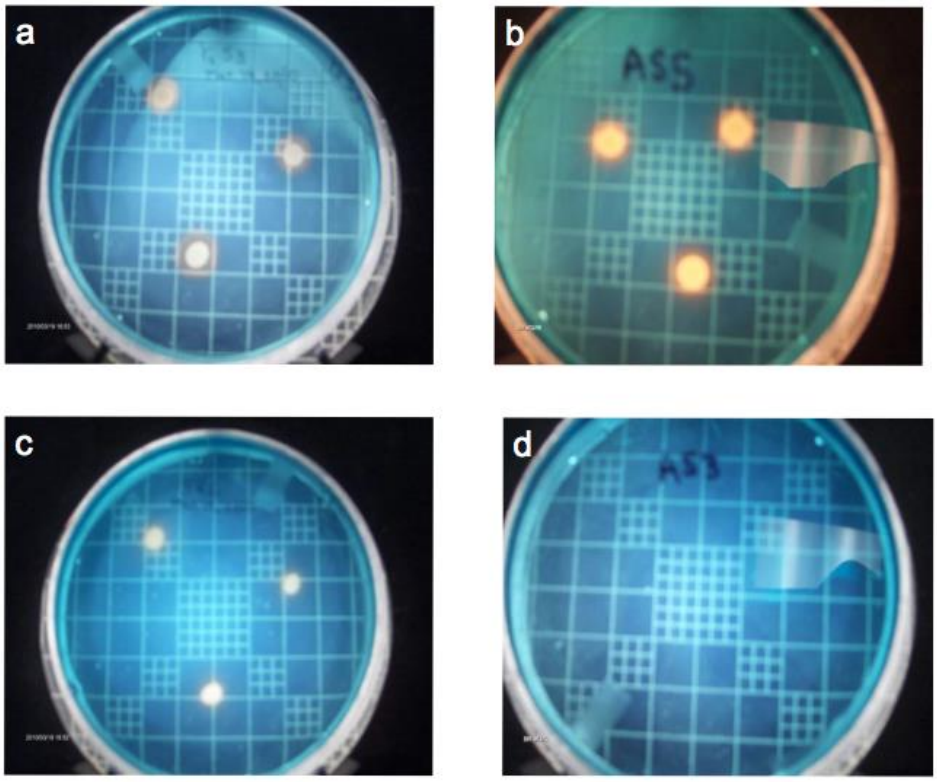


Figure 3.2 Qualitative scale for the measurement of siderophore by newly isolated bacterial endophytes.

Panel (a) shows ++ production, Panel (b) shows +, Panel (c) shows +/-, and Panel (d) shows – or no production.

Table 3.6 Antibiotic resistance and salt tolerance of newly isolated bacterial endophytes.

Strain name	Antibiotic resistance					Salt
8R6	**Amp.**	**Ery.**	**Kan.**	**Nov.**	**Tc.**	**tolerance**
YsS1	+	+	-	+	-	4%
YsS2	+	+	-	+	-	4%
YsS3	+	+	-	+	-	4%
YsS4	+	+	-	+	-	4%
YsS5	+	+	-	+	-	4%
YsS6	+	+	-	+	-	4%
YsS7	+	+	-	+	-	4%
AS1	+	+	-	+	-	4%
AS2	-	+	-	-	+	3%
AS3	-	+	-	-	+	3%
AS5	-	+	-	-	+	3%
AS6	-	+	-	-	-	0.5%
CR4	-	+	-	-	-	0.5%
YmS1	+	+	-	-	-	3%
GO-L6	+	+	-	-	+	3%
CL-S3	+	+	-	+	-	1%
LR1	+	+	-	-	+	1-1.5%
LL4	+	+	-	-	-	1%
UA-S1	-	+	-	-	-	3%
V2	-	-	-	-	-	0.5%
DL1	-	+	-	-	+	3%
DL6	-	-	-	-	-	2%
GPR1	-	-	-	-	-	1.5%
GPR2	-	-	-	-	-	2%
	-	-	-	-	-	2%

strains exhibited a high level of plant growth promotion under the assay conditions that were employed. Among all, strain YsS6 promoted the highest level of root elongation on canola seeds (Fig. 3.3).

3.3 Construction of ACC deaminase deficient mutants

3.3.1 Isolation of ACC deaminase gene from bacterial endophytes

The *acdS* gene of bacterial endophytes (*Pseudomonas fluorescens* YsS6 and *Pseudomonas migulae* 8R6) was amplified using two different primer sets. The agarose gel electrophoresis of the PCR product (Fig. 3.4) showed DNA bands that were roughly 1000 bp and were thought to contain the respective *acdS* genes. From the sequencing result, the *acdS* genes were aligned using the BLAST on NCBI website against the nucleotide collection (nr/nt) database and found to be ACC deaminase gene from *Pseudomonas* species with at least 90% identity. Moreover, from the sequencing result it was confirmed that the *acdS* gene from *Pseudomonas fluorescens* YsS6 is about 1093 bp long, and the *acdS* gene from *Pseudomonas migulae* 8R6 is approximately 902 bp in length.

3.3.2 Construction and confirmation of the replacement vector pK19 *mobsacB* ACC

To construct the deficient *acdS* replacement vector pK19 *mobsacB* ACC, first the purified PCR product of *acdS* gene of both endophytes was cloned into plasmid

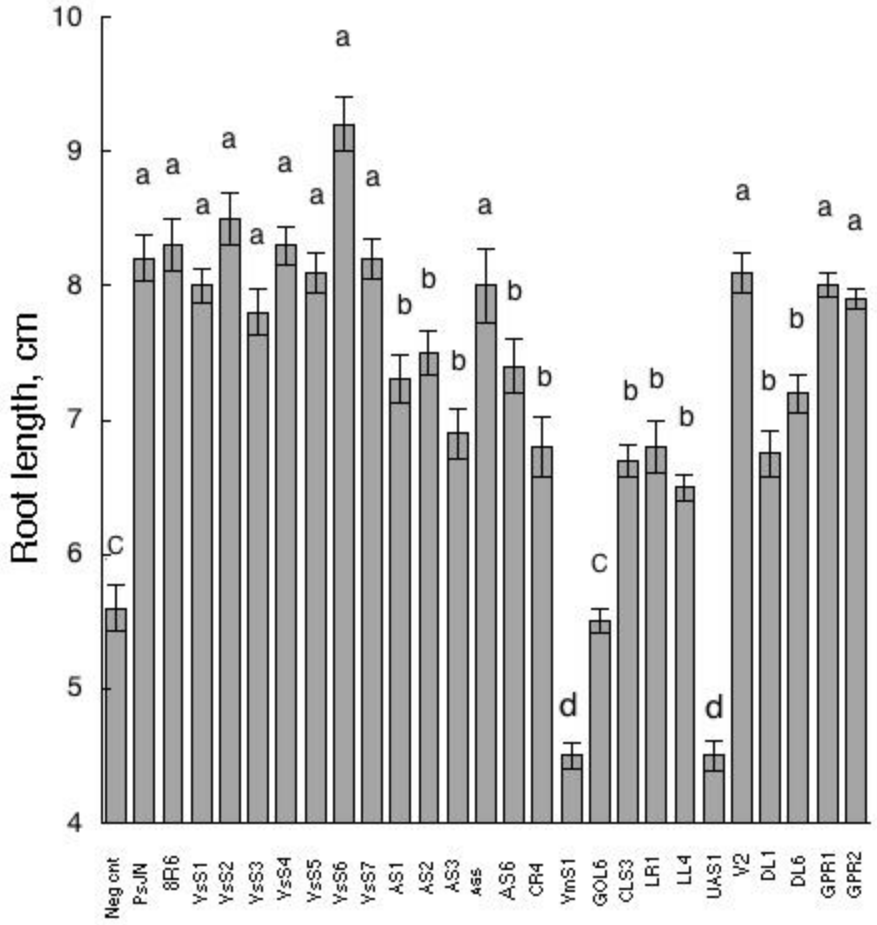


Figure 3.3 Gnotobiotic root elongation assay.

Seeds treated with 0.03 M $MgSO_4$ served as a negative control (1st bar). For the test strains, strains were grown under gnotobiotic conditions and diluted with 0.03 M $MgSO_4$ to achieve an OD of 0.15 ± 0.02 at 600 nm. Surface sterilized seeds were treated with the test strains (1 h incubation at room temperature) and growth pouches were inoculated

manually. Each bar represents a mean (± SE; n = 56) root length in cm. One-way ANOVA was performed followed by a Tukey's post-test for each test strain and compared with both positive and negative controls. Values similar to positive control are represented by "a", values significantly greater than the negative control but lower than the positive control are represented by "b", values similar to the negative control are represented by "c" and values significantly lower than the negative control are represented by "d". Observations were made on day five. *Burkholderia phytofirmans* PsJN was used as a positive control (2nd bar).

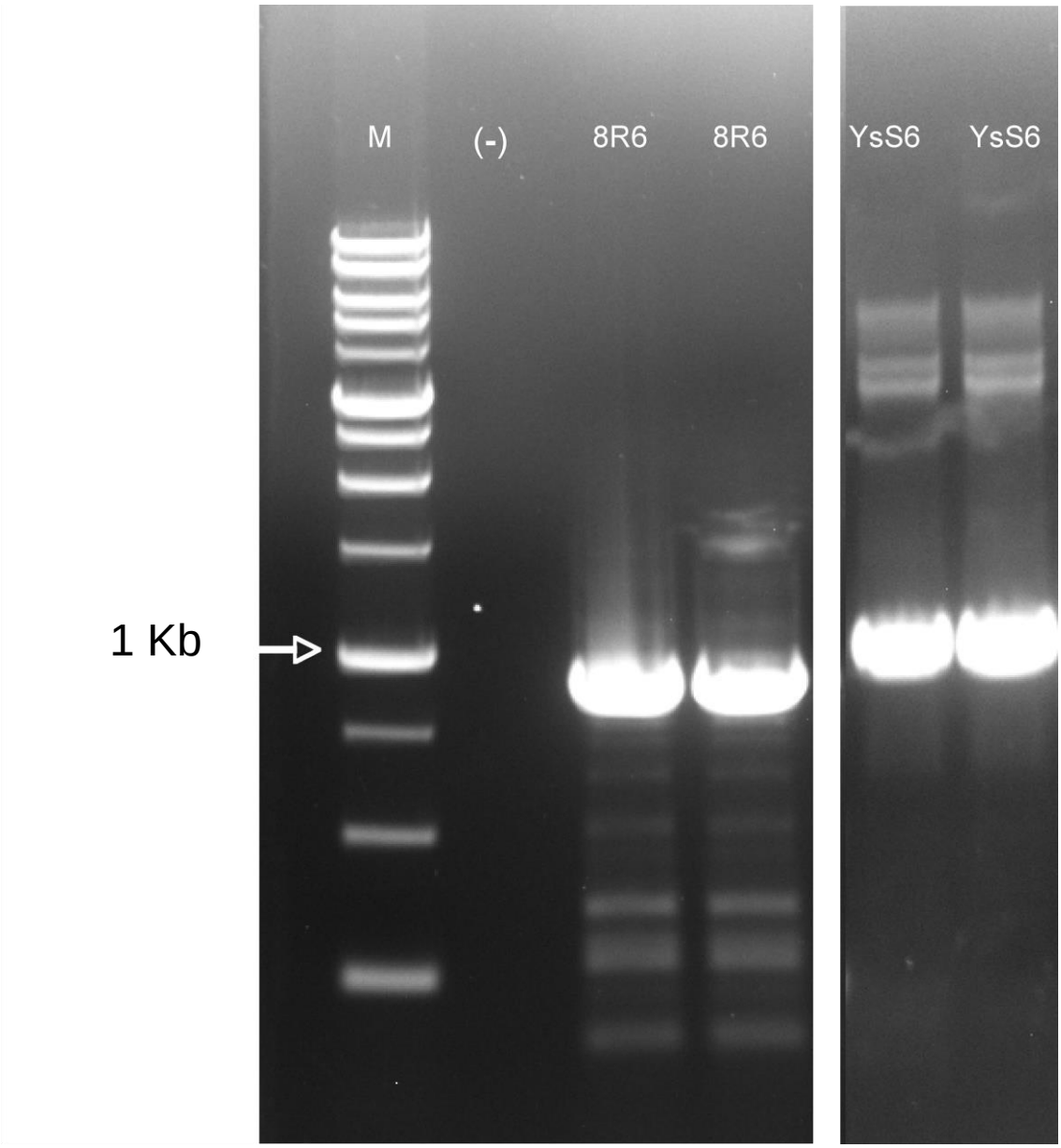


Figure 3.4 Agarose gel electrophoresis showing PCR product amplified from bacterial endophytes *P. fluorescens* YsS6 and *P. migulae* 8R6.

M: 1 Kb DNA ladder marker, (-): Negative control where no template DNA was added, 8R6: *P. migulae* 8R6 putative *acdS* gene, and YsS6: *P. fluorescens* YsS6 putative *acdS* gene.

Bluescript to make pBS8R6ACC or pBSYsS6ACC. Then the recombinant plasmids were confirmed by EcoRI and HindIII digestion, which cut the recombinant plasmids into two different size fragments. The consequent agarose gel electrophoresis (Fig. 3.5) demonstrated that the first fragment is about 3 kb (the size of native pBS) and the other fragment is about 1 kb, corresponding to the inserted *acdS* gene. To interrupt the *acdS* gene on the pBS8R6ACC and pBSYsS6ACC, a tetracycline resistance gene (Tc) from plasmid pBR322 was used. To isolate the Tc fragment, the pBR322 was double digested with HindIII and AvaI and separated on an agarose gel where the small fragment (1.4 kb) represents the Tc gene and the larger fragment (3 kb) corresponds to the remaining plasmid (Fig. 3.6). The resulting cohesive ends, from the restriction enzymes digestion, were filled in by DNA polymerase I Klenow fragment. Then the blunt-ended Tc fragment was ligated with Tth111I-digested pBS8R6ACC in case of *P. migulae* 8R6 and with BmgBI-digested pBSYsS6ACC in case of *P. fluorescens* YsS6. The ligation mixture was used to transform *E. coli* DH5α, and the transformants were selected following growth on LB agar medium plate containing tetracycline and ampicillin. Plasmids were isolated from the transformants and the *acdS* gene, interrupted by a Tc fragment, was cut by PstI and KpnI double digestion in both cases. On the agarose gel, the restriction enzyme digestion gave 2.3 kb and 3 kb bands, and 2.49 kb and 3 kb bands for the pBS8R6ACC and pBSYsS6ACC, respectively, (Fig. 3.7) where the 3 kb band represents the pBS and the 2.3 kb or 2.49 kb bands correspond to the interrupted *acdS* genes. The protruded ends from KpnI and PstI digestion were made blunt ended by DNA polymerase I Klenow fragment and then used to transform linearized pK19*mobsacB* to construct pK19 *mobsacB* 8R6ACC and pK19 *mobsacB* YsS6ACC.

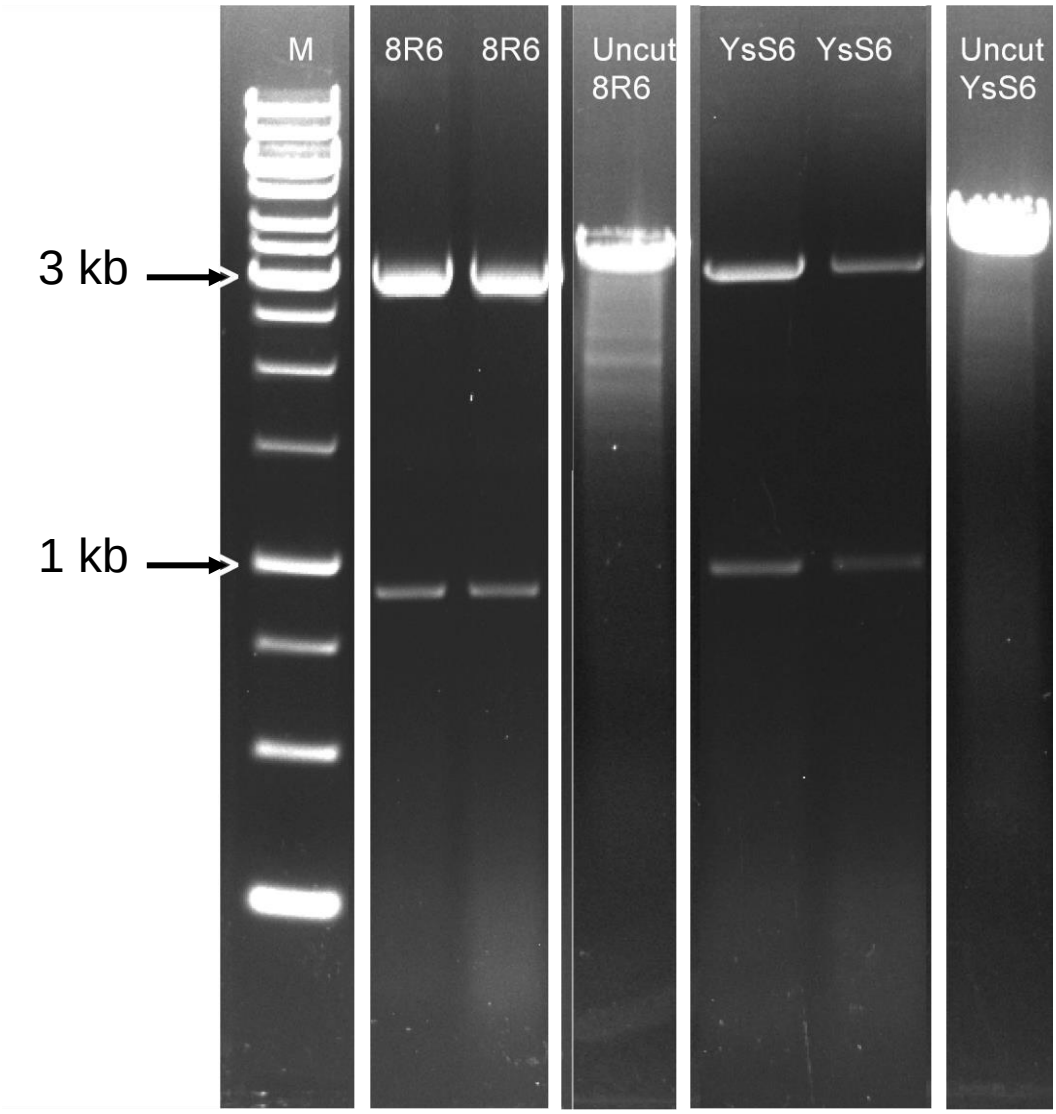


Figure 3.5 Agarose gel electrophoresis showing EcoRI and HindIII digestion of pBS containing *acdS* gene from bacterial endophytes *P. fluorescens* YsS6 and *P. migulae* 8R6.

M: 1 Kb DNA ladder marker, 8R6: Digested pBS containing *P. migulae* 8R6 *acdS* gene, uncut 8R6: Undigested pBS containing *P. migulae* 8R6 *acdS* gene, YsS6: Digested pBS

containing *P. fluorescens* YsS6 *acdS* gene, and uncut YsS6: Undigested pBS containing *P. fluorescens* YsS6 *acdS* gene.

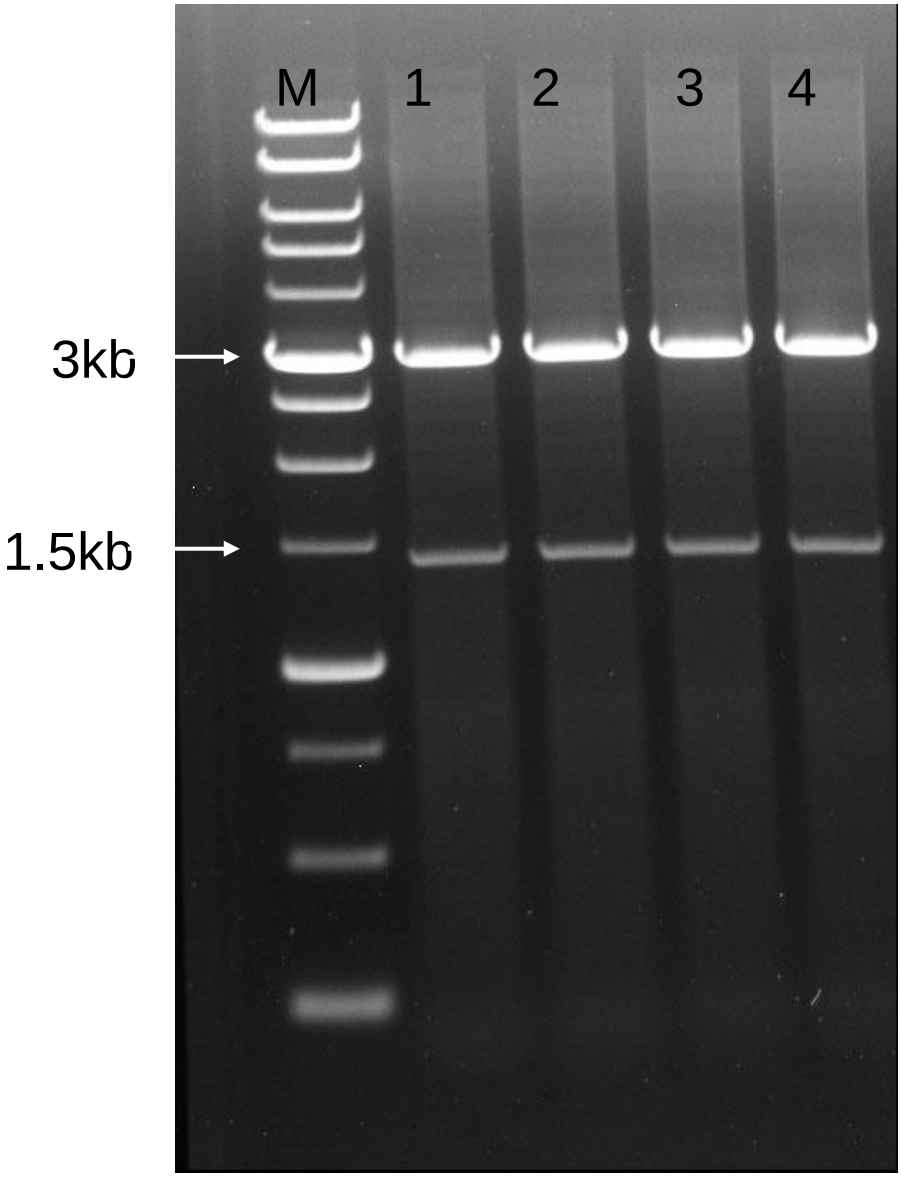


Figure 3.6 Agarose gel electrophoresis of pBR322 that was double digested with AvaI and HindIII.

M: 1 Kb DNA ladder marker, 1-4: HindIII and AvaI doubly digested pBR322.

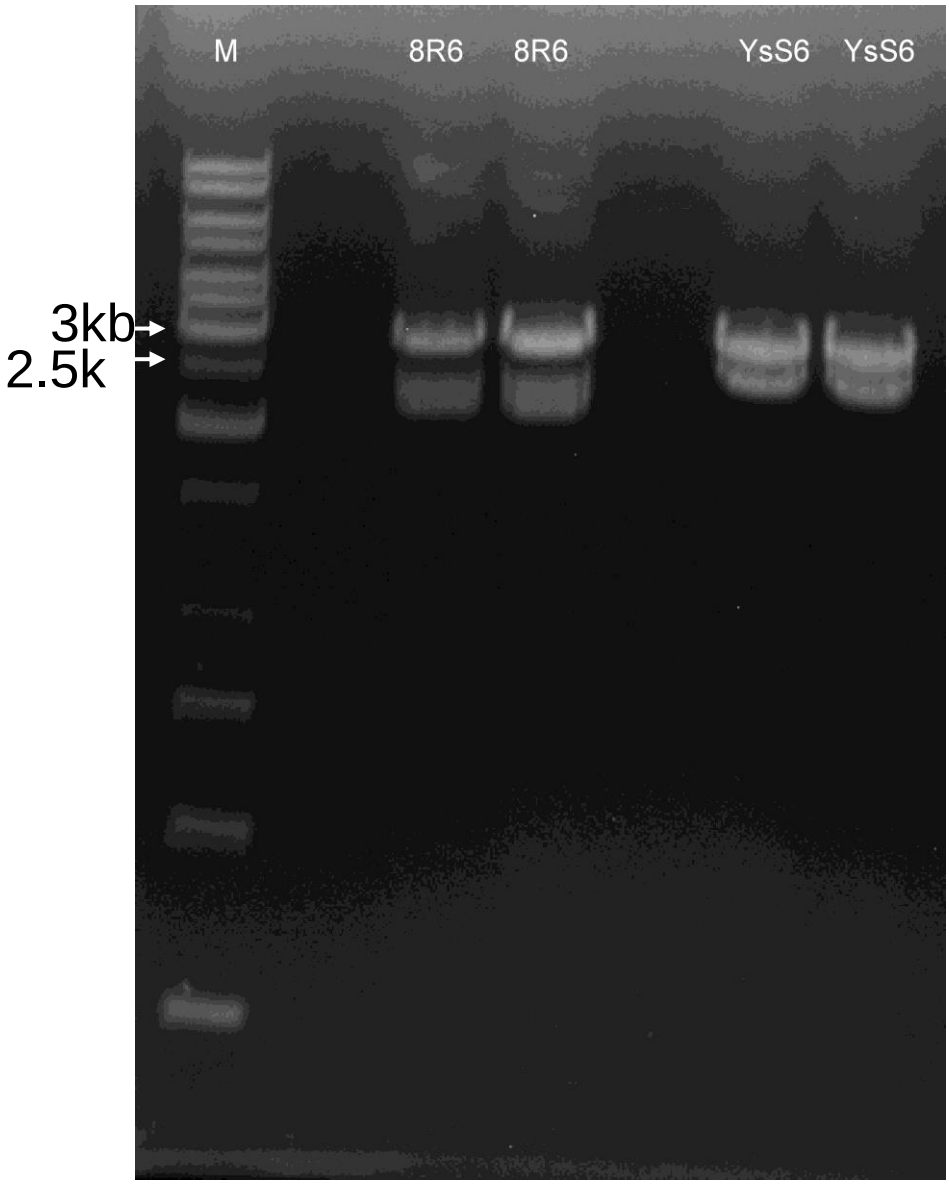


Figure 3.7 Agarose gel electrophoresis of pBS8R6ACC and pBSYsS6ACC doubly digested by PstI and KpnI.

M: 1 Kb DNA ladder marker, 8R6: Digested pBS8R6ACC8R6 by PstI and KpnI, and YsS6: Digested pBSYsS6ACC by PstI and KpnI.

E. coli DH5α (pK19 *mobsac*B 8R6ACC$^-$ and pK19 *mobsac*B YsS6ACC$^-$) was conjugated with wild-type *P. fluorescens* YsS6 and with wild-type *P. migulae* 8R6 separately in the presence of helper strain *E. coli* MT616 (tri-parental conjugation) and plated on TSB agar plate containing tetracycline, ampicillin, and 15% sucrose. Several individual colonies were selected from the plates, and checked for their sensitivity to kanamycin. Only kanamycin sensitive colonies were transferred to TSB medium, and the total genomic DNA was extracted from the overnight grown bacterial culture. Using total genomic DNA as template, the interrupted *acdS* gene was amplified from corresponding *acdS* deficient mutants (Fig. 3.8). The amplified fragment was 2.3 kb in the case of the *P. migulae* 8R6 *acdS* deficient mutant and 2.49 kb in the case of the *P. fluorescens* YsS6 *acdS* deficient mutant. The size of the bands is the sum of the wild type *acdS* gene plus the inserted tetracycline gene (1.4 kb).

3.4 Characterization of ACC deaminase deficient mutants

The a*cdS* gene mutants were characterized for ACC deaminase activity, IAA production, siderophore production, and their ability to promote plant growth under gnotobiotic conditions, and compared with the respective wild-type bacterial endophytes. The *acdS* deficient mutants' ability to produce siderophore remained unchanged (Fig 3.9), and their ability to produce IAA was not considerably changed (Table 3.7). However, it was confirmed that the *acdS* deficient mutants no longer exhibit significant ACC deaminase activity: wild type *P. fluorescens* YsS6 had an ACC deaminase activity of 12.5 μmol. mg^{-1}. h^{-1} and its ACC deaminase deficient mutant an activity of 0.11 μmol.

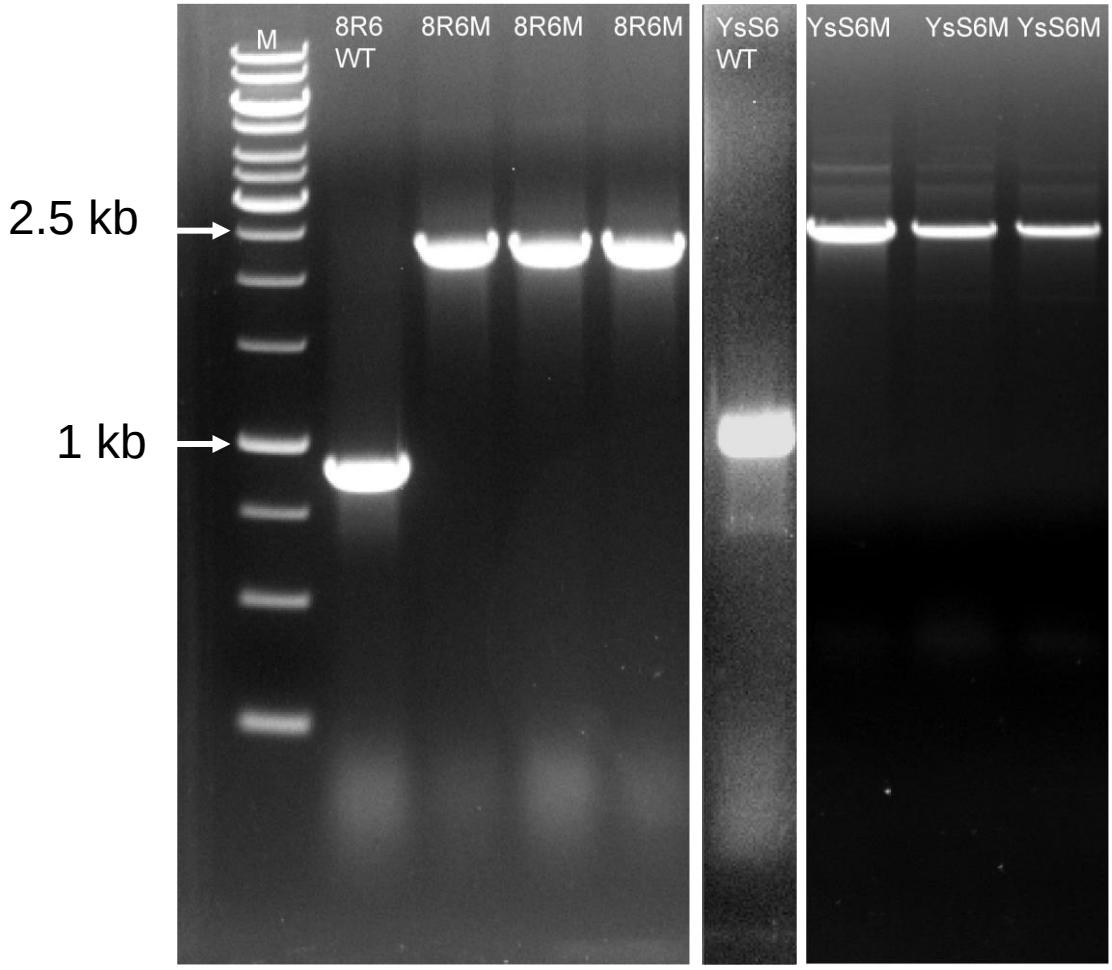


Figure 3.8 Agarose gel electrophoresis of PCR products amplified from genomic DNA of wild type bacterial endophytes *P. migulae* 8R6, *P. fluorescens* YsS6, and their *acdS* deficient mutants.

M: 1 Kb DNA ladder marker, 8R6WT: PCR product which used wild type *P. migulae* 8R6 genomic DNA as template, 8R6M: PCR product which used *P. migulae* 8R6 putative *acdS* deficient mutant as template, YsS6WT: PCR product which used wild type *P. fluorescens* YsS6 genomic DNA as template, and YsS6M: PCR product which used *P. fluorescens* YsS6 putative *acdS* deficient mutant as template.

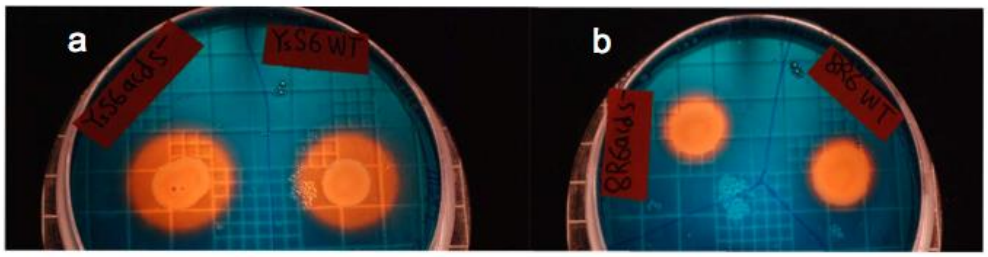


Figure 3.9 Siderophore production by *P. fluorescens* YsS6 wild type, *P. fluorescens* YsS6 ACC deaminase deficient mutant panel (a), *P. migulae* 8R6 wild type, and *P. migulae* 8R6 ACC deaminase deficient mutant panel (b).

mg^{-1}. h^{-1}. Wild- type *P. migulae* 8R6 and its ACC deaminase deficient mutant had activity levels of 10.9 and 0.03 μmol. mg^{-1}. h^{-1} respectively (Table 3.7). As a consequence of the loss of the ACC deaminase activity, the mutants no longer promote canola root growth in the growth pouch assay (Fig. 3.10).

3.5 Delay of flower senescence by bacterial endophytes

The effect of bacterial endophytes *P. fluorescens* YsS6, *P. migulae* 8R6, and their corresponding *acdS*-deficient mutants in delaying carnation flower senescence was evaluated. Carnation flowers were treated with the suspension of bacteria, ethylene inhibitor (AVG), ethylene precursor (ACC), and saline (control). Flowers treated with the ACC solution died earlier than any of the other treated flowers, i.e. by day 6 of the treatment, control flowers developed senescence symptoms and died by day 8, while the AVG treatment delayed the onset of senescence by around one day compared to the control (Fig. 3.11). *P. putida* UW4-treated flowers senesced quickly, i.e. at a rate between the control and the ACC-treated flowers (Fig. 3.11).

Both wild-type endophytes (*P. fluorescens* YsS6 and *P. migulae* 8R6) delayed flower senescence to approximately the same extent, providing an extra two days of protection as compared to AVG and about 3-4 days of protection when compared to no treatment (control) (Figs. 3.11 and 3.12). When the cut flowers were treated with ACC deaminase deficient mutants of *P. fluorescens* YsS6 and *P. migulae* 8R6 the flowers behaved in a manner similar to control flowers. That is, unlike their wild-type counterparts, the mutant bacterial strains failed to delay flower senescence compared to

Table 3.7 ACC deaminase activity and IAA production by *P. fluorescens* YsS6 wild type, *Pseudomonas fluorescens* YsS6 ACC deaminase deficient mutant, *P. migulae* 8R6 wild type, and *P. migulae* 8R6 ACC deaminase deficient mutant.

Strain	**ACC deaminase activity (μmol mg^{-1} h^{-1})**	**IAA production (μg ml^{-1}).**		
		No Trp	**200 μg/ml Trp**	**500 μg/ml Trp**
8R6WT	10.9	0.37	0.40	0.97
8R6M	0.03	0.31	0.33	0.90
YsS6WT	12.5	0.89	0.70	3.05
YsS6M	0.11	0.86	0.99	3.17

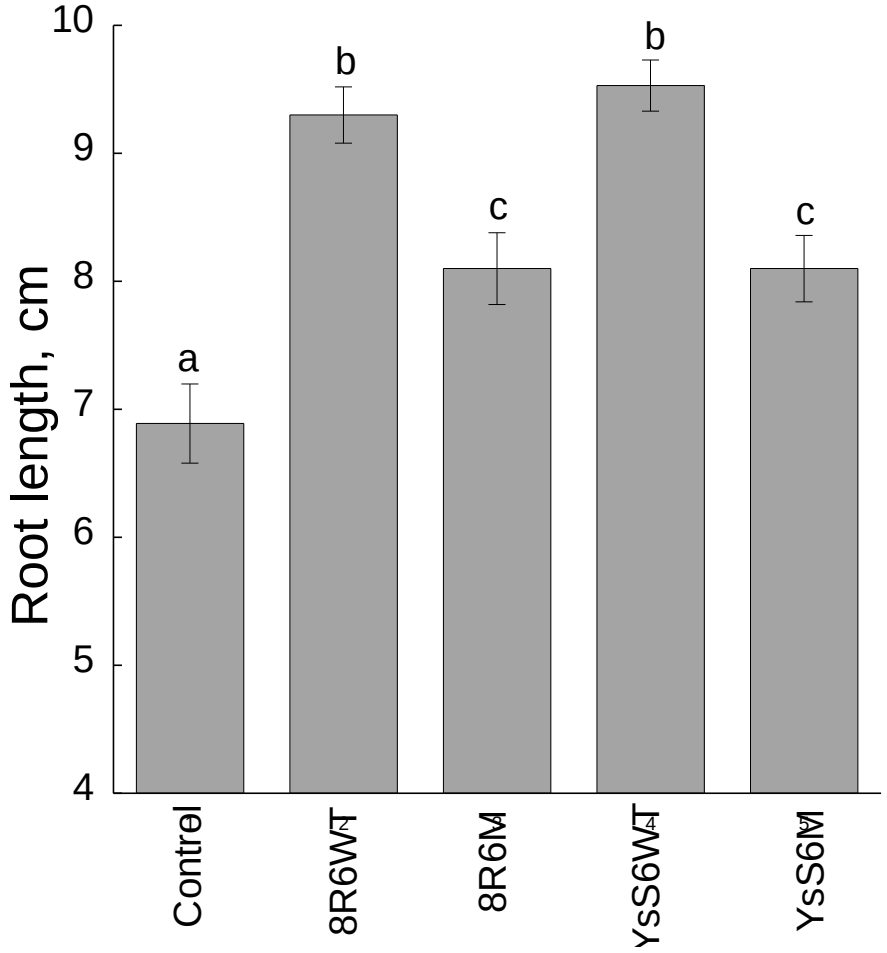


Figure 3.10 Gnotobiotic root elongation assay for *P. fluorescens* YsS6 wild type, *P. fluorescens* YsS6 ACC deaminase deficient mutant, *P. migulae* 8R6 wild type, and *P. migulae* 8R6 ACC deaminase deficient mutant.

Seeds treated with 0.03 M $MgSO_4$ served as a negative control (1st bar). For the test strains, strains were grown under gnotobiotic conditions and diluted with 0.03M $MgSO_4$ to achieve an OD of 0.15 ± 0.02 at 600 nm. Surface sterilized seeds were treated with the test strains (1 h incubation at room temperature) and growth pouches were inoculated

manually. Each bar represents a mean (± SE; n = 56) root length in cm. One-way ANOVA was performed followed by Tukey's post-test for each test strain and compared with control. For each bar, the values represented by the same lower-case letter are not significantly different at $P < 0.05$. Observations were made on day five.

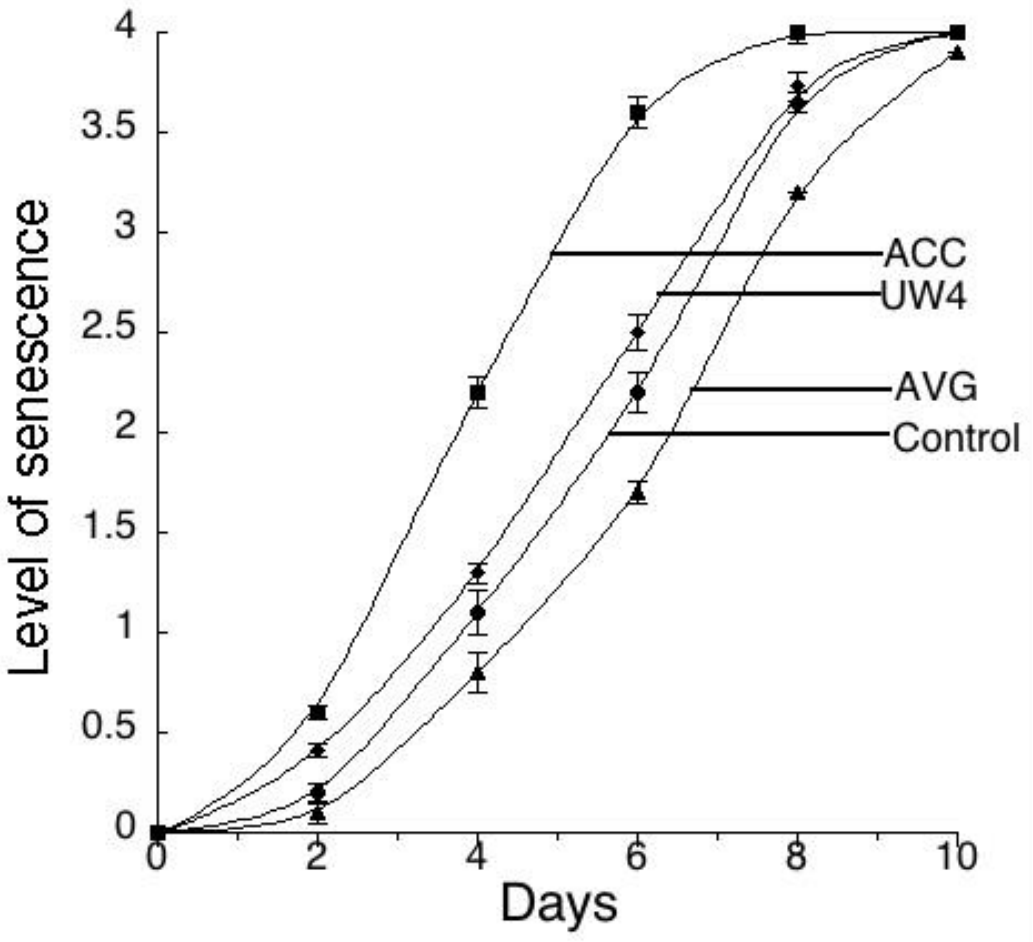


Figure 3.11 Senescence of flower from untreated carnation, served as control, and carnation treated with ACC, AVG, and *Pseudomonas putida* UW4.

Each point represents a mean (± SE; n = 105) value of senescence level.

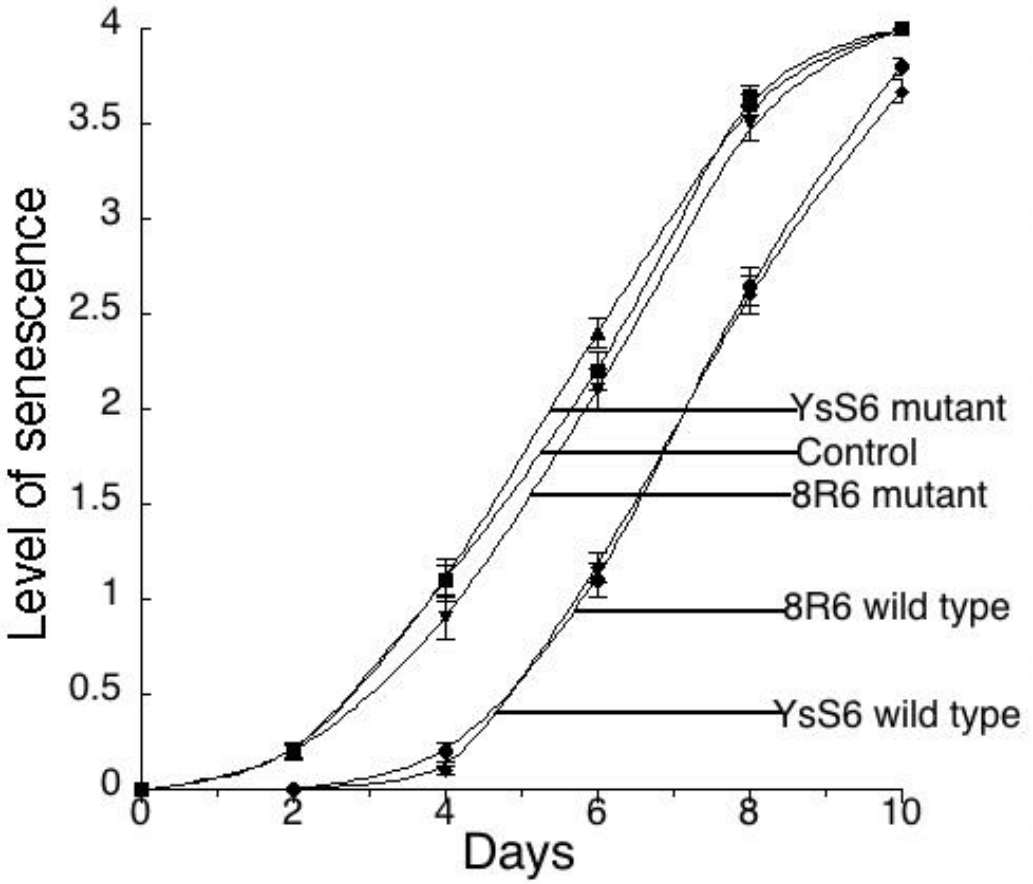


Figure 3.12 Senescence of flower from untreated carnation, served as control, and carnation treated with suspension of *P. fluorescens* YsS6 wild type, *P. fluorescens* YsS6 ACC deaminase deficient mutant, *P. migulae* 8R6 wild type, and *P. migulae* 8R6 ACC deaminase deficient mutant.

Each point represents a mean (±SE; n = 105) senescence level value.

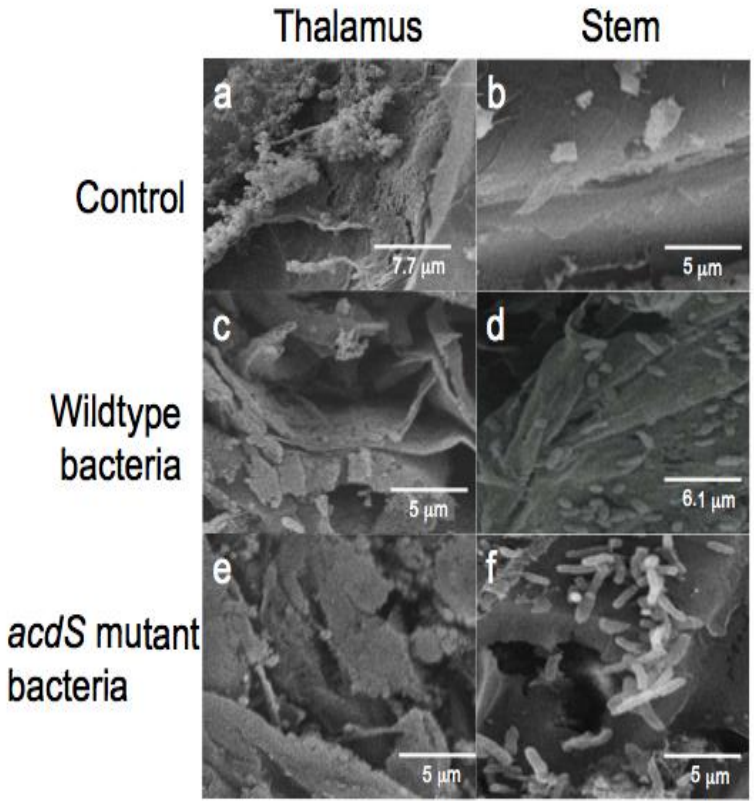


Figure 3.13 Scanning electron micrographs of treated flowers viewed on day 8 of the treatment.

No bacteria were found in the flower thalamus (Panel a) and stem (Panel b) of control flower. Thalamus of flowers treated with both wild type bacterial endophytes (Panel c) and their *acdS* mutant bacterial endophytes (Panel e) showed no bacterial presence. Rod shaped bacteria present in flower stem treated with wild type bacterial endophyte suspension (Panel d) and their *acdS* mutant bacterial endophyte suspension (Panel f).

the untreated flowers (control) (Fig. 3.12).

Moreover, scanning electron micrographs confirmed the presence of both endophytes as well as their ACC deaminase deficient mutants within the stems of the cut flowers (Fig. 3.13d and f). On the other hand, no bacteria could be seen in the flower thalamus of the endophyte-treated plants (Fig. 3.13c and e). The untreated control plants did not harbor any bacteria in either the stem or the thalamus (Fig. 3.13a and b).

3.6 Effect of bacterial endophytes on the growth of tomato plants under salt stress

The bacterial endophytes *P. fluorescens* YsS6, *P. migulae* 8R6, and their corresponding *acdS* deficient mutants were tested for their capacity to facilitate plant growth under salt stress. Bacterial treatments were given at seed level whereas seeds treated with 0.03 M $MgSO_4$ only served as control. Salt treatments were started when the plants were three weeks old. All the plants were watered, when required, with the same amount of water at the same time. Plants with no added salt provided the trends of endophytic behavior on the growth of tomato plants. Seeds treated with the wild-type bacterial endophytes exhibited the higher growth than the control whereas respective *acdS* deficient mutant-inoculated plants were significantly lower than the ones that were inoculated with the wild-type strains (Fig. 3.14a and b) and are more like the control. The sodium contents of the plants treated with no salt varied significantly; wild type strain-inoculated plants tended to prevent salt up-take from the environment, however more

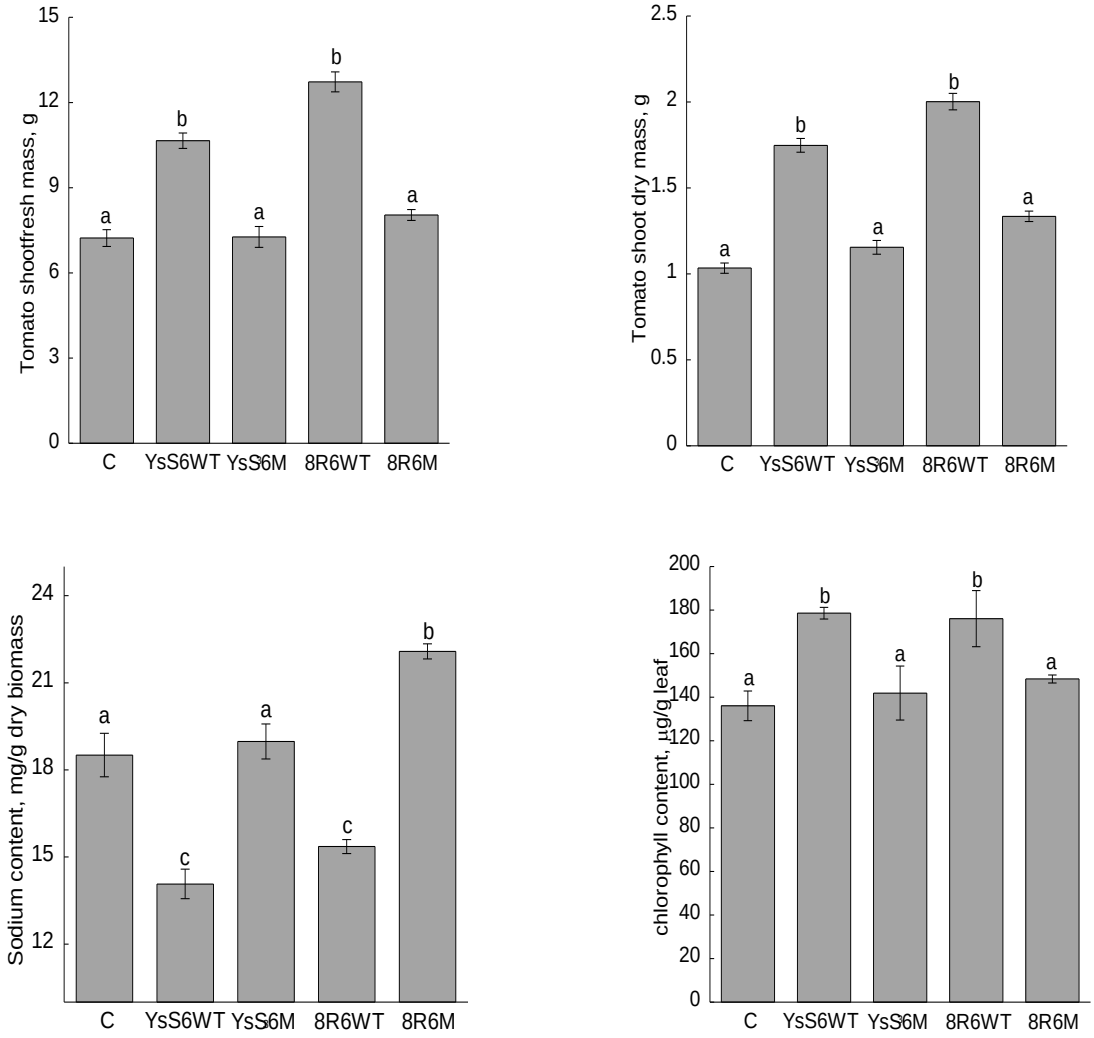


Figure 3.14 The effect of the bacterial endophytes on the tomato plants growth treated with no salt.

The effect of the bacterial endophytes *P. fluorescens* YsS6 wild type, *P. fluorescens* YsS6 ACC deaminase deficient mutant, *P. migulae* 8R6 wild type, and *P. migulae* 8R6 ACC deaminase deficient mutant *P. migulae* 8R6 on the fresh shoot mass (Panel a), dry

shoot mass (Panel b), sodium contents (Panel c), and chlorophyll contents (Panel d) of the tomato plants: Seeds treated with 0.03M $MgSO_4$, no bacteria, served as control (1st bar). For the test strains, strains were grown and diluted with 0.03M $MgSO_4$ to achieve an OD of 0.25 ± 0.02 at 600 nm. Surface sterilized seeds were inoculated with the test strains (1 h incubation at room temperature) and sown in pots manually. One-way ANOVA was performed followed by Tukeys post-test for each treatment and compared with control. For each bar, values represented by the same lower-case letters are not significantly different at $P< 0.05$. All the measurements were taken following 11 weeks of growth. Error bars indicate 1 standard error of the mean.

salt was deposited in the plants inoculated with the mutant strains (Fig. 3.14c). Because wild type strains promoted the growth of tomato plants, so as a consequence, the chlorophyll contents of the plants inoculated with wild type bacterial endophytes was higher than the plants either inoculated with the mutant strain or non-inoculated (control) plants (Fig. 3.14d). In addition, plants treated with the wild type strains of bacterial endophytes showed early flowering and fruiting, and the number of the flowers and the buds was more than the rest of the treatments (Table 3.8). On the other hand, when these plants were stressed with either 165 mM or 185 mM salt, the general effect of the bacterial endophytes on the growth of the tomato plants was similar. Wild-type strains-inoculated plants protected plants from salt stress significantly. Furthermore, when the control plants and mutant strains-inoculated plants were watered with 165 mM salt, their fresh mass and dry mass reduced and they almost died at week 11 (Fig. 13.5a and b). Wild-type strains of bacterial endophytes provided protection against salt stress by precluding the salt entry to the plants therefore plants survived longer. Fig. 13.5c shows that the wild-type strains-inoculated plants deposited the salt the least whereas the salt contents of the other plants (mutants inoculated and non-inoculated) were not significantly different. In the similar manner, the chlorophyll content was also different among the differently treated plants (Fig. 13.5d). When the salt concentration was increased to 185 mM, although the sodium content of the plants inoculated with the wild-type strains of bacterial endophytes also increased (Fig. 3.16c), but the overall fresh shoot mass for that treatment was not significantly changed (Fig. 3.16a) when compared with the 165 mM salt treatment plants (3.16a). Moreover, among the bacterial endophytes, wild type *P. migulae* 8R6 remediated the salt stress more and provided the better

Table 3.8 Number of flowers, buds, and fruits in tomato plants inoculated with bacterial endophytes in the presence of 165 mM, 185 mM, or no salt.

Plants inoculated with *P. fluorescens* YsS6 wild type, *P. fluorescens* YsS6 ACC deaminase deficient mutant, *P. migulae* 8R6 wild type, and *P. migulae* 8R6 ACC deaminase deficient mutant *P. migulae* 8R6 in the presence of no salt, 165 mM salt, and 185 mM salt. Each value is the mean value of four individual plants. Data was collected on week 13 after the seed sowing.

	No salt			**165 mM salt**			**185 mM salt**		
Treatment	**Flower**	**Bud**	**Fruit**	**Flower**	**Bud**	**Fruit**	**Flower**	**Bud**	**Fruit**
Control	5.0	3.5	0.5	0.5	0	0	0	0	0
8R6WT	7.5	2.5	1	1.5	2.5	0.25	3.5	2	0.25
8R6M	4.5	4.5	1	0.5	1	0	0.5	2	0
YsS6WT	8.5	7	2.5	3	4	0.5	2.5	4	0.25
YsS6M	3.0	4.5	0.5	1	0.5	0	0	0	0

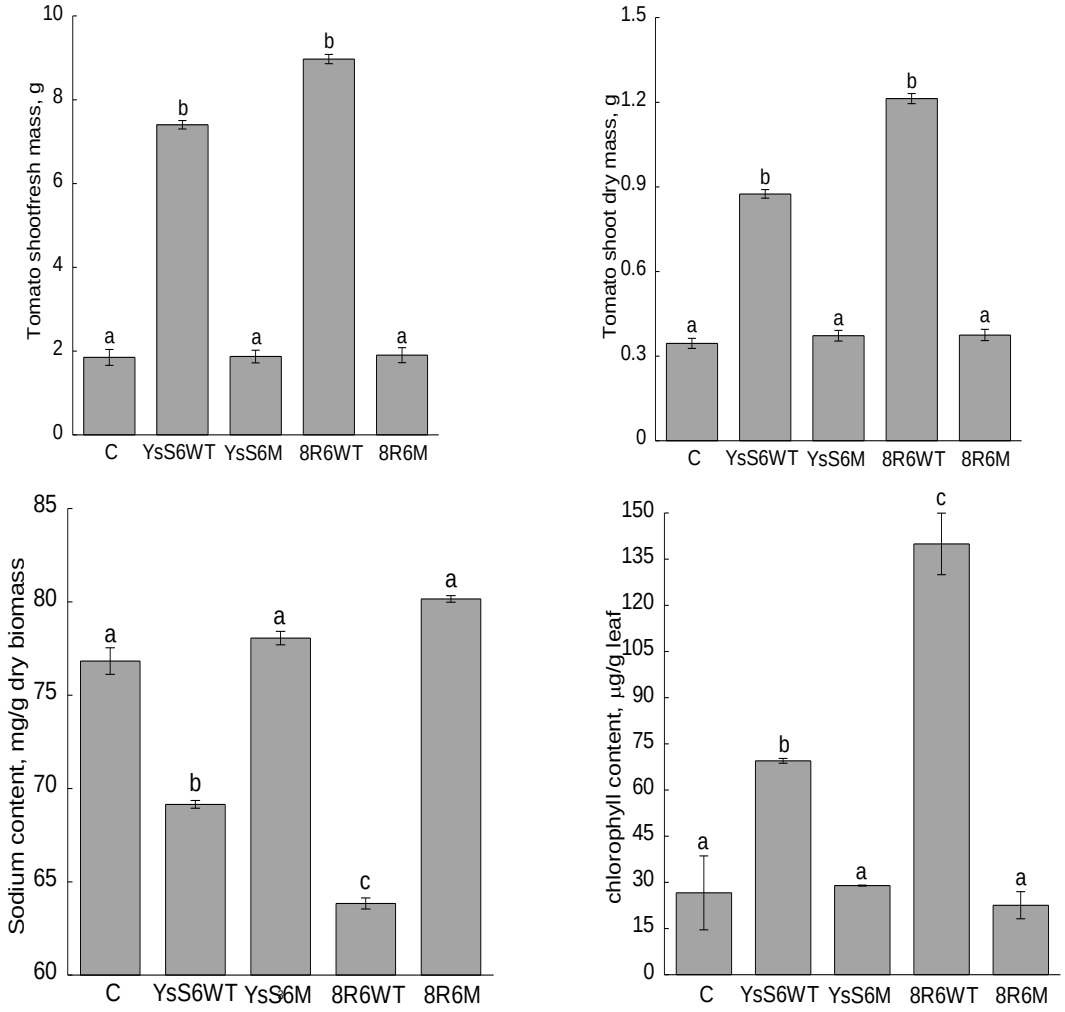


Figure 3.15 The effect of the bacterial endophytes on the growth of tomato plant in the presence of 165 mM salt.

The effect of the bacterial endophytes *P. fluorescens* YsS6 wild type, *P. fluorescens* YsS6 ACC deaminase deficient mutant, *P. migulae* 8R6 wild type, and *P. migulae* 8R6 ACC deaminase deficient mutant *P. migulae* 8R6 on the fresh shoot mass (Panel a), dry shoot mass (Panel b), sodium content (Panel c), and chlorophyll content (Panel d) of the

tomato plants in the presence of 165 mM salt: Seeds treated with 0.03 M $MgSO_4$, (no salt, no bacteria) served as control (1st bar). For the test strains, strains were grown and diluted with 0.03 M $MgSO_4$ to achieve an OD of 0.25 ± 0.02 at 600 nm. Surface sterilized seeds were inoculated with the test strains (1 h incubation at room temperature) and sown in pots manually. Salt treatment was started at 3rd week of seed sowing. One-way ANOVA was performed followed by Tukeys post-test for each treatment and compared with control. For each bar, values represented by the same lower-case letters are not significantly different at $P< 0.05$. All the measurements were taken following the 11-weeks of growth. Error bars indicate 1 standard error of the mean.

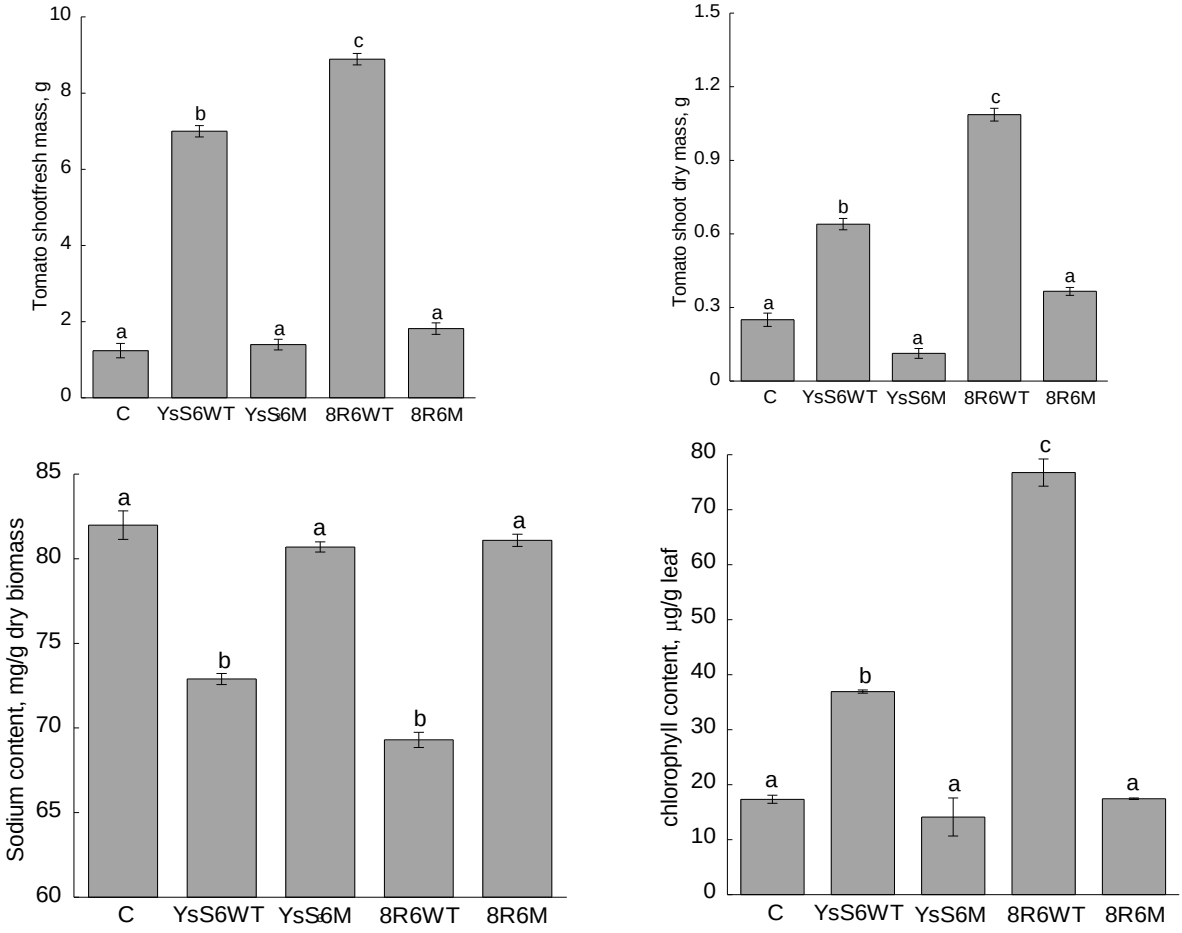


Figure 3.16 The effect of the bacterial endophytes on the growth of tomato plant in the presence of 185 mM salt.

The effect of the bacterial endophytes *P. fluorescens* YsS6 wild type, *P. fluorescens* YsS6 ACC deaminase deficient mutant, *P. migulae* 8R6 wild type, and *P. migulae* 8R6 ACC deaminase deficient mutant *P. migulae* 8R6 on the fresh shoot mass (Panel a), dry shoot mass (Panel b), sodium content (Panel c), and chlorophyll content (Panel d) of the

tomato plants in the presence of 185 mM salt: Seeds treated with 0.03 M $MgSO_4$ (no salt, no bacteria) served as control (1st bar). For the test strains, strains were grown and diluted with 0.03 M $MgSO_4$ to achieve an OD of 0.25 ± 0.02 at 600 nm. Surface sterilized seeds were treated with the test strains (1 h incubation at room temperature) and sown in pots manually. Salt treatment was started at the 3rd week of seed sowing. One-way ANOVA was performed followed by Tukeys post-test for each treatment and compared with control. For each bar, values represented by the same lower-case letters are not significantly different at $P< 0.05$. All the measurements were taken following the 11-weeks of growth. Error bars indicate 1 standard error of the mean.

protection than the wild-type strain of *P. fluorescens* YsS6.

The presence of bacterial endophytes in different plant tissue was also investigated. Plant tissue homogenization method was used to check and verify the presence of bacteria in the surface sterilized plant tissue. Plants from the seeds that were inoculated with the bacterial cell suspension (either wild-type or mutants) carry bacteria in their stem and root, however bacterial endophyte *P. migulae* 8R6 was also able to colonize in the leaves of the tomato plant. Interestingly, no evidence for the bacterial presence was found in the fruit of the plant. The bacteria were recognized and verified by their ability to synthesize ACC deaminase, IAA production, salt tolerance, and antibiotic resistance pattern.

4 Discussion

Most plants carry one or more endophytes (Strobel and Daisy, 2003). Bacterial endophytes often provide instant and enhanced protection to their hosts compared to those bacteria that bind exclusively to the plant's rhizosphere. Therefore, it is likely that many plant growth-promoting endophytic bacteria are superior to similar non-endophytic bacterial strains in promoting plant growth under a wide range of environmental conditions (Rosenblueth and Martínez-Romero, 2006). Moreover, while most scientific studies of plant growth promotion have, to date, focused on rhizospheric bacteria, bacterial endophytes have been successfully used in a number of studies to facilitate phytoremediation. This suggests that there is a need to isolate new and better plant growth-promoting bacterial endophytes, and that the work that has been reported until now may be considered to be preliminary. Therefore, the main reasons for isolating new endophytes were: 1) Many of the endophytes that have previously been isolated are not very well characterized; 2) Not all of the previously isolated bacterial endophytes possess the enzyme ACC deaminase, which is thought to be critically important in promoting plant growth; and 3) Many of the previously isolated bacterial endophytes are not environmentally friendly so that their deliberate use to promote plant growth in agriculture in the field is highly questionable and may not readily receive approval from regulatory authorities. As a consequence of the study reported here, there are several new bacterial endophytic strains that can potentially be safely used to facilitate plant growth with a variety of plant hosts and under a range of environmental conditions.

4.1 Isolation of bacterial endophytes

Based on the interactions with their host plants, bacterial endophytes may be classified as one of three types: 1) facultative endophytes, i.e. those can live inside plants and in other habitats, 2) obligate endophytes, i.e. those that can only live inside of a plant, and 3) opportunistic endophytes, i.e., those that can occasionally enter plants and live inside. The approach that was used in this work to isolate bacterial endophytes likely primarily resulted in the isolation of facultative endophytes. The simplest and most straightforward procedure for isolating facultative endophytes involves the planting of seeds in different soils, allowing the seeds to germinate and the plants to grow, and then macerating surface sterilized plant tissue and plating the cell sap on bacterial growth medium. Using this method, a total of 174 unique bacterial endophytes were isolated from the interior tissues of tomato plants grown in 15 different soil samples collected from several countries around the world. A single cultivar of tomato was sown in all soils to strengthen the argument that the emergence of different endophytes is due to the different nature of the soil samples (McInroy and Kloepper, 1995b). Soil from different climates (i.e. temperature, pH, salt contents) contain different microbial flora, so the emerging plant would have the opportunity to be occupied with the same.

4.2 Characterization of bacterial endophytes

A number of studies documented that plant growth-promoting bacteria containing ACC deaminase promote plant growth with or without stress conditions, for example Sessitsch *et al*., 2005; Sun *et al*., 2009; Jacobson *et al*., 1994; Glick *et al.*, 1998; Penrose

et al., 2001; Glick, 2005; Hong *et al*., 1991; Glick, 2004; Rashid *et al*., 2012; Ali *et al*., 2012; Cheng *et al*., 2007; Mayak *et al*., 2004 showing importance of ACC deaminase in plant growth, especially under various stress environments. The newly isolated endophytes were tested for ACC deaminase activity, and 13% of them (or 25 separate strains) were positive (i.e. $ACCD^{+}$), with cellular levels of ACC deaminase activity ranging from 0.43 to 12.50 μmol mg^{-1} h^{-1} (Table 3.3). The frequency of $ACCD^{+}$ strains in rhizosphere bacteria have been reported as 11% (Duan *et al*., 2009), and the number that was found in the present study is similar. However, the fact that ACC deaminase is an induced enzyme and its expression mainly depends on the surroundings of the bacteria and the status of the plant-microbe interactions, this percentage may underestimate the occurrence of the ACC deaminase positive bacteria in certain communities.

True endophytism can be confirmed through different techniques, these may include labeling the putative endophytic strains with green fluorescence protein (Miller *et al*., 2000) and artificial inoculation of the strain with the host plant (Reinhold- Hurek and Hurek, 1998). In the present study, each of the 25 selected strains was used to re-inoculate tomato plants grown in sterilized peat-based plant growth medium which lacked endophytic bacteria. The plants were then re-tested for the presence of the added bacteria colonizing the plant's interior tissues (Rosenblueth and Martinez-Romero, 2006). In this way, it was possible to ascertain that all of the selected strains were true facultative rather than opportunistic endophytes. Following the winnowing of the initial 174 strains to 25 ACC deaminase-containing facultative endophytes, all strains were tested for a number of traits related to their ability to facilitate plant growth and proliferate in the soil environment (Tables 3.4–3.6). In addition, the taxonomic identity of

each of these 25 strains was characterized, both through the use of the Biolog™ system and the sequencing of a portion of their genes encoding 16S ribosomal RNA (Table 3.2). The sequences were aligned using the BLAST on NCBI website against the nucleotide collection (nr/nt) database and subsequently were deposited in GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) and can be accessed by the accession numbers presented in Table 1.

When considering the ability of these bacterial strains to produce various compounds with auxin activity, that Salkowski's reagent (the method used in this work) detects almost all indole-ring compounds (including IAA but not tryptophan). Thus, although the method that was used to estimate the amount of IAA does not quantify the level of IAA per se, it does provide an estimate of the amount of auxin produced. This caveat notwithstanding, all of the compounds that react with Salkowski's reagent are used here to provide an estimate of the amount of IAA produced.

The three *Microbacterium* sp. strains all displayed a high level of IAA and IAA-like molecule synthesis (Table 3.4), following induction by exogenous L-tryptophan, in addition to a moderate level of ACC deaminase activity (Table 3.3). These three strains promoted canola root elongation to a lesser extent than the positive control, the plant growth-promoting endophytic bacteria *Burkholderia phytofirmans* PsJN (Sessitsch *et al*., 2005) (Fig. 3.3), but more than the negative control, no added bacterium, and tolerated 3% salt (Table 3.6). Although these strains were confirmed to be true endophytes by re-colonization of the disinfected seeds, under some conditions they might be opportunistic pathogens as they exhibit a very high level of IAA (and/or IAA-like molecule) production that is a characteristic of plant pathogens (Kunkel and Chen, 2006; Rezzonico

et al., 1998; Shinshi *et al*., 1987). Four strains of the $ACCD^+$ bacteria (i.e. AS6, DL1, DL6, and GPR1) were most similar to uncultured bacterial strains (Table 3.2), and were sensitive to all five tested antibiotics, except for strain AS6, which is resistant to erythromycin and sensitive to the other four antibiotics (Table 3.6). Among these strains, only DL1 showed a very high level of IAA and/or IAA-like molecule production (Table 3.4). None of the strains in this group exhibited the ability to significantly promote plant growth (Fig. 3.3).

Five out of the 25 ACC deaminase-containing strains are *Bacillus* species according to BLAST results. This group has more diverse biochemical activities (when compared to each member of this group) that a host plant may utilize during its growth cycle, and no generalization can be made for the group. Strains V2 and GPR2 are able to promote canola root length elongation in growth pouches to a significant extent while UAS1 and YmS1, surprisingly for strains with ACC deaminase activity, have no (or a negative) impact on canola root elongation (Fig. 3.3). Finally, strain LRI may be regarded as a moderate plant growth-promoter on the basis of its biochemical activities. The 16S rRNA sequence of one of the isolated strains, CR4, was more than 99% identical to *Acinetobacter radioresistens* (Table 3.2), and may be a good candidate for facilitating host plant growth under a number of stressful conditions. For example, this strain grew best at higher (3%) salt concentrations (Table 3.6), members of this species are tolerant to high levels of radioactivity (Nishimura *et al*., 1988), and this strain possesses several other biological activities consistent with plant growth promotion. Strain CLS3 showed moderate plant growth-promotion activities and was identified as *Rhodococcus equi* (Table 3.2). Members of *Rhodococcus* sp. may be important under certain environmental

conditions due to their ability to catabolize a wide range of compounds and produce bioactive steroids (McLeod *et al*., 2006). The 16S rRNA gene BLAST data suggest that three strains are *Agrobacterium* species (Table 3.2). All three showed a low level of ACC deaminase activity (Table 3.3), and IAA and IAA-like molecule production (Table 3.4). Furthermore, they did not exhibit good plant growth-promoting capabilities under gnotobiotic assay conditions (Fig. 3.3), and their level of siderophore production was relatively low (Table 3.5). On the other hand, all of the *Pseudomonas* sp. strains displayed high levels of ACC deaminase activity (Table 3.3), a medium level of IAA and IAA-like molecule production (Table 3.4), moderate to high levels of siderophore synthesis (Table 3.5), and the greatest effect of all of the 25 isolated strains on canola root elongation in gnotobiotic growth pouches (Fig. 3.3). Moreover, the eight *Pseudomonas* sp. strains had the highest level of salt tolerance (Table 3.6) of the 25 isolated strains. They also exhibit the highest ACC deaminase activity and up to 4% salt tolerance as well as several other growth-promoting characteristics, thereby making these strains the most promising candidates to use to augment the host plant's ability to withstand a range of abiotic and biotic stresses. In this study, a general trend was observed for salt tolerance among the 25 newly isolated bacterial endophytes. In fact, more than 90% of isolated strains were able to grow in media containing at least 1% salt (Table 3.6). This might reflect the fact that endophytic bacteria have to reside and multiply inside of the plant in the environment of a relatively high ionic strength compared to many soils. High salt tolerance of bacterial endophytes was also observed in previous studies on endophytes, from desert dwelling plants (Lopez *et al*., 2011; Puente *et al*., 2009a). However, the high salt tolerance observed here is somewhat unexpected given the fact that the endophytes

isolated in this study are from a wide range of locales and soils. No strain was resistant to the antibiotic kanamycin, and for the other antibiotics a mixed trend was observed (Table 3.6). Almost all newly isolated strains are able to produce ammonia (Table 3.5) when grown on a medium containing a complex nitrogen source; by producing ammonia bacteria accumulate and supply nitrogen to their host plant and promote root and shoot elongation, consequently increasing plant biomass (Marques *et al*., 2010). Moreover, it is worthwhile mentioning that excess production of ammonia can serve as a triggering factor for the virulence of opportunistic plant pathogens (Bashan *et al*., 1980). In addition, all of the 25 isolated strains may be categorized either as mesophiles or psychrotrophs as reflected in their optimal growth temperatures (Table 3.5). This may reflect the fact that endophytes do not face the soil temperature directly but rather reside in the interior of the plant where the temperature is similar to the ambient air temperature.

Similar to the argument that has been made for rhizospheric plant growth-promoting bacteria (Glick *et al*., 1998, 2007a,b), Hardoim *et al*. (2008) have suggested that the major mechanism that endophytes utilize to promote plant growth is the lowering of plant ethylene levels through the effective functioning of the enzyme ACC deaminase. They argue that, "Preferential selection by plants of bacteria with high ACC deaminase activity could confer benefits to the plant and have been favorably selected by evolution. At the same time, selected bacteria encounter a protective environment in which the supply of nutrients is approximately constant, providing a suitable niche to them. This two-sided mechanism could cause the selected bacteria to be optimally fit as endophytes, thus fitting the concept of competent endophytes, characterized by a series of traits that enable them to optimally interact with plant hosts." Following this argument, it is not

surprising that the *Pseudomonas* sp. strains described here not only displayed the highest level of ACC deaminase activity, but were also more likely than any of the other isolated bacterial strains to have a high level of other plant growth-promotion traits. Consistent with this suggestion, Belimov *et al*. (2005) previously observed, for rhizospheric plant growth-promoting bacteria, that the level of ACC deaminase activity in a series of bacterial strains was correlated with the degree of stimulation of root elongation by that bacterial strain. Moreover, when a *Sinorhizobium meliloti* strain was genetically modified to express a *Rhizobium leguminosarum* ACC deaminase gene (Ma *et al*., 2003), the transformants successfully out competed the parental *S. meliloti* in its ability to nodulate alfalfa (Ma *et al*., 2004). Only a limited number of studies have examined the role of the enzyme ACC deaminase in the functioning of endophytic plant growth-promoting bacteria (e.g. Rasche *et al*., 2006; Sun *et al*., 2009; Sziderics *et al*., 2007). A number of the strains that were isolated and characterized in this study are candidates for the purposeful use of ACC deaminase containing endophytes in agricultural practice especially in saline soils, given the high level of salt tolerance of some of the isolated strains. For this reason, two of the "best" bacterial endophytic strains (8R6 and YsS6) were selected for further characterization and to assess their potential benefits in solving agriculture-related problems. The selection criterion for the strains was based on the following: 1) these strains exhibited the highest ACC deaminase activity (10.90 and 12.50 $\mu M\ mg^{-1}\ h^{-1}$ (Table 3.3) respectively, 2) they both demonstrated promising plant growth-promotion capabilities (i.e. moderate level of IAA & ammonia production, positive for siderophore production & phosphate solubilization, and they grow optimally at moderate temperature (Table 3.4 – 3.6), 3) the fact that *Pseudomonas* sp. have been

copiously studied and is part of the normal flora of most of the natural soils therefore, permission for their deliberate release to the field (environment) by the regulatory agencies may be easier, and 4) high salt tolerance by the strains makes them potential candidates in facilitating plant grow in high salinity soils.

The full-length 16S rRNA gene of the selected bacterial endophytes was amplified to make sure that there is no ambiguity in their genus and species names. From the 16S rRNA gene BLAST result, strains 8R6 and YsS6 have 99-100% identity with *Pseudomonas migulae* and *Pseudomonas fluorescens* respectively; therefore, they were named *Pseudomonas migulae* 8R6 and *Pseudomonas fluorescens* YsS6. Furthermore, ACC deaminase deficient mutants of these strains were constructed to better understand the role of ACC deaminase in the functioning of these bacteria and to study the response of plants exposed to wild-type bacterial endophytes and their respective mutants under a number of stressful conditions.

4.3 Construction and characterization of ACC deaminase deficient mutants

Traditionally, knock out mutants of a gene have been used to determine and/or to verify the function of a particular gene by comparing the behavior of the mutant to the wild-type. As mentioned earlier, the enzyme ACC deaminase has been shown to play a crucial role in plant growth-promotion and plant stress management by rhizospheric bacteria. To validate the role of endophytic ACC deaminase in facilitating plant growth in the absence of stress and/or under a variety of different stresses, a mutant deficient in this

activity was constructed for each of the selected bacterial endophytes. A tetracycline resistance gene was inserted roughly in the middle of the coding region of the ACC deaminase gene that had been previously isolated from each of the selected endophytes and then cloned into a plasmid in *E. coli*. Transfer of the respective recombinant plasmids with the tetracycline resistance gene to the appropriate wild-type strain through tri-parental conjugation yielded the desired mutant strain where in each case, the chromosomal wild-type ACC deaminase structural gene was replaced by the corresponding inactivated gene. The desired transconjugants were selected on the basis of their sensitivity towards kanamycin and their ability to grow in the presence of tetracycline and a high concentration of sucrose. Subsequent characterization of the ACC deaminase deficient mutants has shown that they lost the ability to produce ACC deaminase activity (Table 3.7). However, their ability to produce IAA (Table 3.7) and siderophores (Fig. 3.9) did not change significantly. In addition, as expected, their ability to promote canola root growth under gnotobiotic conditions was considerably reduced (Fig. 3.10). It is thought that the apparent residual canola root elongation activity of these mutant strains is a consequence of growth promotion by IAA, consistent with what was previously demonstrated for a rhizospheric plant growth-promoting bacterium (Patten and Glick, 2002).

Generally, the introduction of a foreign gene(s) increases the metabolic load of the host cell, where metabolic load of a gene may be defined as the additional energy/resource requirement that an organism needs to function following the introduction of exogenous gene(s). Typically, in cloning experiments, the metabolic load is determined by a number of factors including: 1) the size of the plasmid carrying the

target gene, 2) the copy number of the plasmid that carries the gene, 3) the type of gene that is introduced into the cell, 4) the type of growth medium that is used, 5) the metabolic state of the host cell, and 6) the expression level of the introduced gene (Glick, 1995). Previously, it was shown that the protein encoded by the tetracycline resistance gene is expressed at a significantly lower level than are proteins encoded by other antibiotic resistance genes that are used as selectable markers (Glick, 1995; Hong *et al*., 1995; See and Glick, 1982). This means that tetracycline resistance genes are less likely to cause significant changes to the host cell's metabolism compared to other antibiotic resistance genes, therefore its use, as the marker/reporter gene in genetic engineering experiments is preferred. In the present study, it is apparent that the introduced tetracycline resistance gene did not alter the metabolic load of the wild-type strains to any measurable extent. This was evidenced by the fact that the ACC deaminase deficient mutants were able to grow at a comparable rate and reach a final cell density similar to the wild-type strains in the same period of time. Moreover, the ability of the mutant strains to produce IAA and siderophores was nearly unchanged (Table 3.7, Fig. 3.9). In characterizing the newly constructed mutant strains, the only parameter that has been altered markedly in comparison to the corresponding wild-type strains, is their decreased plant growth-promotion capabilities. This altered activity is most likely not the consequence of an altered metabolic load by the introduction of a tetracycline resistance gene, but rather is a direct consequence of the inability of the mutant strains to produce a functional ACC deaminase. These results are consistent with a large body of previous work from our laboratory documenting the role of ACC deaminase in the ability of plant growth-promoting bacteria to facilitate plant growth.

4.4 Application of bacterial endophytes

4.4.1 Flower senescence and bacterial endophytes

Carnation is a typical ethylene-sensitive flower that produces ethylene through an autocatalytic pathway and enters into rapid flower senescence (Rahemi and Jamali, 2011). Ethylene production is influenced by a number of factors inside the tissue of an ethylene sensitive flower. The presence of auxins (e.g. IAA) positively regulates ethylene production by stimulating transcription of genes encoding the enzyme ACC synthase that synthesizes ACC, immediate precursor of ethylene (Huang *et al*., 1991; Penrose and Glick, 1997). A bacterium that possesses the enzyme ACC deaminase can utilize and cleave ACC into α-ketobutyrate and ammonia and thus facilitates a reduction in the level of ethylene in the plant associated with that bacterium (Glick 1995, 2005).

Previously, it was demonstrated that ACC deaminase-containing plant growth-promoting rhizobacteria are able to delay the senescence of carnation flower petals to a significant level (Nayani *et al*., 1998). In that study, 1) only rhizospheric PGPB were utilized, and 2) senescence was delayed only when the carnation petals were removed from the flower and the individual petals were incubated in solutions of the ACC deaminase-containing PGPB. Importantly, in that study, rhizospheric bacteria did not have any effect on the senescence of cut flowers per se, only on isolated flower petals. This is presumably because the rhizospheric bacteria were not taken up by the cut flowers. These experiments demonstrated that root-colonizing bacteria are able to delay carnation flower senescence only if an exchange of plant-produced ACC from flower tissue to the bacteria can occur, in that case a wound created by excising the petal from the flower provided a

channel for this interaction. On the other hand, when root-colonizing bacteria were sprayed on whole flowers or the stems of cut flowers were incubated in a suspension of these organisms, flower senescence was not affected. This is because of the fact that rhizospheric bacteria could not establish an association with the complete flower and could not sequester and cleave ACC so that they had no influence on the senescence events.

Based on the observations that bacterial endophytes can be taken up internally by plants and establish a relationship that is generally beneficial to those "colonized" plants, and bacteria with ACC deaminase activity can act as a sink for plant ACC, it was hypothesized that ACC deaminase-containing bacterial endophytes might effectively delay flower senescence. To test this hypothesis, in the present study, cut mini carnation flowers were treated with the bacterial endophytes, *P. fluorescens* YsS6, *P. migulae* 8R6 and their respective ACC deaminase deficient mutants. In these experiments, it was first demonstrated that cut mini-carnation flowers behaved as expected when treated with either the ethylene precursor ACC or the ethylene inhibitor AVG (Fig. 3.11). That is, treatment with ACC hastened flower senescence while treatment with AVG prolonged the lifetime of the mini carnation flowers compared to the control. When flowers were incubated with a suspension of wild-type ACC deaminase-containing bacterial endophytes, flower senescence was delayed to an even greater extent (Fig. 3.12) than when AVG was added (Fig. 3.11). Flowers treated with ACC deaminase deficient mutants of the same bacterial endophytes senesced at a rate that was essentially the same as the control flowers and significantly faster than when the flowers were treated with the wild-type endophytic strains (Fig. 3.12). This observation is consistent with the suggestion that

ACC deaminase is the key factor that wild-type bacterial endophytes utilize to delay flower senescence.

Scanning electron micrographs of treated flower tissues revealed the presence of rod shaped bacteria (Fig. 3.13 d & f) in the flower stems in all instances where the flowers were treated with a bacterial endophyte regardless of whether a wild-type or mutant strain was utilized. The fact that bacteria were never found in tissues of the flower thalamus indicates that these endophytes do not colonize the reproductive part of the plant.

Interestingly, cut flowers treated with the rhizospheric ACC deaminase-containing plant growth-promoting bacterium *Pseudomonas putida* UW4 senesced slightly earlier than the control flowers (Fig. 3.11). As a consequence of the lack of a direct association of this bacterium with either the flower or the stem, *P. putida* UW4 did not decrease flower senescence. However, *P. putida* UW4 is able to synthesize IAA that may be taken up by the flower stems and thereby possibly elevate plant ethylene levels by stimulating transcription of the enzyme ACC synthase, resulting in an increase in the rate of flower senescence. Thus, a comparison of the behavior of cut flowers incubated with endophytes as compared to rhizosphere binding bacteria indicates that only endophytic bacteria (that contain ACC deaminase) were able to delay flower senescence. Bacterial endophytes (*P. fluorescens* YsS6 and *P. migulae* 8R6) were taken up by the cut flowers, colonized in the stem tissues, and established a direct interaction with the internal environment of the cut flower. Compared to flower senescence in the absence of added ACC deaminase-containing endophytes, ACC deaminase-containing bacterial endophytes were able to modulate ethylene concentrations and thereby provide protection against flower senescence. On the other hand, ACC deaminase deficient mutants of the same bacterial

endophytes, which were also taken up by the cut flowers, could not ameliorate the effects of increasing ethylene. Moreover, the rhizosphere-binding and ACC deaminase-containing bacterium *P. putida* UW4 was unable to delay ethylene-induced flower senescence. This is a consequence of strain UW4 not being taken up by the cut flower.

The use of ACC deaminase-containing plant growth-promoting endophytes to delay the senescence of cut flowers is an attractive prospect for the flower industry. The delay of cut flower senescence engendered from plants treated with ACC deaminase-containing plant growth-promoting endophytes at the seedling stage is an intriguing possibility. However, given the necessity of establishing stable endophytic colonization of each plant, it remains to be determined experimentally whether this is a viable approach. Some commercially important flowers (such as rose, carnation, zinnia and geranium) exhibit very high sensitivity to ethylene (Woltering and van Doorn, 1988) so that the treatment of these flowers with naturally occurring plant growth-promoting bacterial endophytes may extend their shelf life to a significant extent without the use of potentially problematic chemicals.

4.4.2 Salt stress and the bacterial endophytes

High salinity in agricultural land is a worldwide problem. High salt concentrations are plant growth inhibitory in many crops (Cuartero and Fernandez-Munoz, 1998, Mayak *et al*., 2004, Cheng *et al*., 2007). Tomato is a dicotyledonous, ethylene sensitive important food crop; it has been previously documented that when tomato plants are exposed to high levels of salt, their ethylene production is increased (O'Donell *et al*., 1996). ACC deaminase-containing plant growth-promoting rhizospheric bacteria have

been reported to facilitate plant growth under salt stress by reducing the ethylene level that is produced as a consequence of abiotic stress (i.e. stress ethylene) (Mayak *et al*., 2004, Cheng *at al*., 2007). In the previous studies, 1) mainly rhizospheric plant growth-promoting bacteria were employed, 2) plants were grown and observed for a shorter time periods, and 3) plants were stressed with relatively low salt concentrations. In the present study, two plant growth-promoting bacterial endophytes *P. fluorescens* YsS6, *P. migulae* 8R6 and their respective ACC deaminase deficient mutants were used. As discussed earlier, ACC deaminase is a key enzyme, produced by bacteria, which helps in releasing the plant stress under a variety of abiotic and biotic stress conditions (Glick 1995, 1998, 1999, 2005). When the tomato seeds were treated with the plant growth-promoting ACC deaminase positive bacterial endophytes (wild-type strains and their ACC deaminase deficient mutants), the emerging plant was primed to deal with a number of stresses more effectively and provided better protection because of the presence of the "pre colonized-bacterial endophytes" in various parts of the plant. Even in the absence of any stress (no salt added), tomato seeds treated with the wild-type bacterial endophytes *P. fluorescens* YsS6 and *P. migulae* 8R6 exhibited better growth when compared to the control plants (no bacterial treatment) (Fig. 3.14a and b). The plants treated with ACC deaminase deficient mutants of both endophytes were notably smaller than the plants treated with the wild-type strains and similar to the control plants (Fig. 3.14a and b). The chlorophyll content of the tomato plants treated with the wild-type bacterial endophytes was also greater compared to the control plants and the plants treated with the ACC deaminase deficient mutant strains (Fig. 3.14d). Although this set of plants was not stressed by salt, the sodium content was measured, and it was observed that the plants treated with the

wild-type bacterial endophytes somehow precluded salt entry into the plant and kept the plant tissue salt contents low. On the other hand, the sodium contents of the plants treated with the ACC deaminase mutant strains were higher, and were essentially the same or higher as the control plants (Fig. 3.14c). It was previously observed that when tomato plants were pre-treated with the ACC deaminase containing plant growth-promoting bacterium *Achromobacter piechaudii* ARV8 (which is rhizospheric and not endophytic), and grown under high salt (up to 172 mM), although the stress-mediated ethylene production by the tomato plants was significantly reduced, the overall sodium content of the treated shoots was unchanged (Mayak *et al*., 2004). Interestingly, when the salt effect (150 mM) on the growth of canola in the presence of an ACC deaminase containing plant growth-promoting rhizospheric bacterium *Pseudomonas putida* UW4 was evaluated, the results were very different (Cheng *et al*., 2007). The wild-type rhizospheric bacterium tends to accumulate much higher amounts of sodium in the shoots than the untreated plant or its ACC deaminase mutant does. However, in both reports, the biomass of the wild-type treated plants was significantly increased (Mayak *et al*., 2004; Cheng *et al*., 2007). It seems that different plant growth-promoting bacteria affect plants differently under (salt) stress conditions. Rhizospheric binding bacteria (such as *P. putida* UW4) allow the salt to accumulate but facilitate plant growth by keeping the stress-ethylene level low with its ACC deaminase activity. On the other hand, it is hypothesized that plant growth-promoting bacterial endophytes prevent sodium to get into from entering the plant (Fig. 3.14c, 3.15c, 3.16c). The mechanism by which the bacterium does this action is unknown.

When tomato plants were watered with 165 mM salt, the plants that were preinoculated with the wild-type strains of bacterial endophytes *P. fluorescens* YsS6 and *P. migulae* 8R6 survived longest and their fresh and dry biomass significantly increased (Fig. 3.15a and b). The plants that were either inoculatedwith the ACC deaminase mutant strains or no bacteria (control) were almost dead by week 11 (Fig. 3.15a and b). The chlorophyll content of the tomato plants inoculated with the wild-type strains was significantly higher than the control and the mutant treated plants (Fig. 3.15d). Moreover, the trends of the sodium content in the shoots were the same as described earlier. Wild-type strains of bacterial endophytes exhibited lower sodium levels in the plant shoots, where *P. migulae* 8R6 treated plants had the lowest sodium content and provided better protection than *P. fluorescens* YsS6 (Fig. 3.15c).

In the treatment of tomato plants with 185 mM salt, plants behaved in a similar way to what was observed at the lower level of salt, although the condition of the control plants and the ACC deaminase mutant inoculated plants are worse than the 165 mM salt treatment. The plants, either inoculated with the ACC deaminase deficient mutants or no bacterial inoculation, were completely dead before week 11. However, wild-type strains of bacterial endophytes *P. fluorescens* YsS6 and *P. migulae* 8R6 protected tomato plants against salt stress and facilitated their growth under these harsh conditions (Fig. 3.16). The chlorophyll content of the wild-type strain inoculated plants was significantly higher than the mutant inoculated plants and the control plants (Fig. 3.16d). The sodium content of the tomato plants inoculated with the ACC deaminase deficient mutant strains and the control plants was similar, and significantly higher than the plants preinculated with the

wild-type strains of bacterial endophytes *P. fluorescens* YsS6 and *P. migulae* 8R6 (Fig. 3.16c).

Tomato plants inoculated with either of the wild-type strains of the bacterial endophytes demonstrated an early flowering and fruiting and had significantly greater numbers of flowers, buds, and fruits than the corresponding ACC deaminase mutant strain inoculated plants and the control plants (Table 3.8). Although both bacterial endophytes *P. fluorescens* YsS6 and *P. migulae* 8R6 show great plant growth-promotion capabilities, the bacterial endophyte *P. migulae* 8R6 demonstrated comparatively better plant growth facilitation under the salt stress than the *P. fluorescens* YsS6. Wild-type *P. migulae* 8R6 inoculated tomato plants demonstrated the least sodium uptake, the highest chlorophyll content, and highest fresh and dry biomass (Fig. 3.14, 3.15, 3.16).

ACC deaminase containing plant growth-promoting bacteria have been documented to facilitate the growth of a variety of plants under high salinity conditions. Gamalero and colleagues (2010) reported the plant growth-promoting interaction of *Pseudomonas putida* UW4 and *Gigaspora rosea* BEG9 on the growth of cucumber under salt stress conditions. Similarly, when canola seeds were inoculated with ACC deaminase containing halotolerant bacteria under high salinity conditions, the fresh and dry mass of the treated plants was increased up to 47% (Siddikee *et al*., 2010). The effect of high salt on the growth of red pepper seedlings in the presence or absence of the plant growth-promoting ACC deaminase containing halotolerant bacteria was evaluated, and it was reported that up to 57% of the production of the stress ethylene was reduced when inoculated with PGPB, and the overall biomass of the inoculated plantlets was similar to the control plants (no salt treatment) (Siddikee *et al*., 2011). In addition, Saravanakumar

and Samiyappan (2007) also reported increased saline resistance of groundnut plant after the inoculation of ACC deaminase containing *Pseudomonas fluorescens* strain TDK1 when compared with the plants inoculated with a *Pseudomonas* strain lacking ACC deaminase activity. Another group investigated the effect of ACC deaminase containing rhizobacteria on the growth of maize plants under high salt conditions (Nadeem *et al*., 2007). In a different type of experiment, canola plants were genetically transformed to constitutively express a bacterial ACC deaminase gene. The transgenic canola was stressed with high salt, and the resultant data confirmed that, as a consequence of the expression of the bacterial ACC deaminase, the transgenic plants became resistant to the inhibitory salt concentrations and their fresh and dry weight and chlorophyll content were significantly higher than the non-transformed plants (Sergeeva *et al*., 2006). In addition to the above-mentioned examples, many more reports have been demonstrated the role of ACC deaminase containing plant growth-promoting bacteria in ameliorating plant growth in high salinity environment (Cheng *et al*., 2012; Yue *et al*., 2007; Jalili *et al*., 2008; Sadrina *et al*., 2011; Ahmed *et al*., 2011). These reports, from laboratories all over the world, clearly indicate that ACC deaminase containing plant growth-promoting bacteria can provide a high level of protection to a wide range of plants under salt stress conditions.

4.5 Conclusion

ACC deaminase-containing plant growth-promoting bacteria protect plants from a wide range of environmental changes and related stresses. Plant growth-promoting bacteria may also provide protection to plants via a number of other mechanisms including auxin production; siderophore production; resistance to high salt, heavy metal, and phyto-toxins; phosphate solubilization, and nitrogen fixation. A subset of these bacteria, i. e. bacterial endophytes is plants' natural companions and can be isolated from the interior of various plant tissues. Since bacterial endophytes are better adapted to various host plants, they may be more beneficial than their counterpart, rhizospheric-binding bacteria. Bacterial endophytes expressing ACC deaminase have some additional advantages over rhizospheric-binding bacteria and transgenic plants in the same environmental conditions in that: 1) they colonize within plants and are promptly available when needed, 2) they don't alter the plant's genetic make-up, 3) they function efficiently within a plant's tissues where rhizospheric-binding bacteria are less or not at all effective (e.g. flower senescence), and 4) being natural flora of almost all of the plants, there is likely to be less regulatory limitation to the deliberate release of them into the environment. Considering all of the above-mentioned points, ACC deaminase containing bacterial endophytes may be regarded as "ultimate agricultural adjuncts".

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Appendix I

Initial characterization of RpoS regulon in proteobacteria

In bacteria, transcription is the first step in gene expression, where an mRNA is synthesized using one strand of DNA (antisense) as template. On the chromosome, transcription is initiated when RNA polymerase binds to the DNA strand. RNA polymerase is a complex enzyme that consists of several subunits ($\alpha_2\beta\beta'\omega\sigma$); each subunit has its own specific role to play in gene transcription. RNA polymerase in the $\alpha_2\beta\beta'\omega$ configuration is called the core enzyme whereas the σ (sigma) subunit is dissociable and binds to the core enzyme only when transcription is initiated. The sigma factor is primarily responsible for the recognition of the start-point (the promoter) and DNA melting at the site of transcription. Transcription is initiated when RNA polymerase core enzyme ($\alpha_2\beta\beta'\omega$) binds to its corresponding sigma factor to make it functional holoenzyme ($\alpha_2\beta\beta'\omega\sigma$). Once gene transcription has successfully started, the sigma factor dissociates from the holoenzyme and transcription is continued until RNA polymerase is halted by a transcription terminator sequence. Therefore, the sigma factor plays a crucial role in the selection of genes to be transcribed in different conditions. In a bacterial cell undergoing exponential growth, most of the transcription events occur when RNA polymerase core enzyme binds to a housekeeping sigma factor similar to *Eschericia coli* RpoD (or sigma factor 70) (Osiriphun *et al*., 2009). However, there are several other types of sigma factors in a cell that are used under different circumstances. Different sigma factors regulate expression of different sets or subsets of genes in a number of different cellular environmental conditions. Among the alternative sigma factors, RpoS

mainly regulates the genes that are expressed when bacteria enter the stationary phase of their growth or genes that are expressed in response to a number of stress stimuli (Hengge-Aronis, 2002). In this way, sigma factor RpoS is considered to be the main stress modulator in beta and gamma proteobacteria (Osiriphun *et al*., 2009). In *E. coli,* genes that are generally induced by the sigma factor RpoS are responsible for responding to carbon starvation, oxidative stress (Subsin *et al*., 2003), cellular invasion (Osiriphun *et al*., 2009), hyperosmotic stress (Lang and Hengge-Aronis, 1994), environmental stress, and toxic chemicals stress (Wise *et al*., 1996). Despite the fact that RpoD and RpoS proteins share a common core structure in term of their amino acids sequences and bind to the same position on the promoter (i.e. core -10 and -35 elements) *in vitro,* they control different set of genes under different cellular conditions *in vivo* (Gaal *et al*., 2001; Osiriphun *et al*., 2009). The RpoS-dependent promoters have some special features that distinguish them from RpoD-dependent promoters (Typas *et al*., 2007). RpoS binding at the promoter region depends on various factors: 1) -35 region binding efficiency of the sigma factors, 2) -10 region sequence, and 3) the spacer lengths between the -10 and -35 regions (Osiriphun *et al*., 2009). First, the binding at -35 region of DNA promoter by the RpoS sigma factor is much more relaxed whereas RpoD-dependent promoters are more conserved at -35 regions (Lacour *et al*., 2003; Gaal *et al*., 2001). Next, the sequence of the -10 region is itself selective for RpoS binding, the consensus sequence for RpoD-dependent promoters is XTATAAT (-7 to -13 bp), where ‘X’ can be any nucleotide, whereas RpoS-dependent promoters recognize the sequence CTATACT (-7 to -13 bp) where the presence of cytosine at the -13 position of the -10 DNA promoter region is especially important (Osiriphun *et al*., 2009; Typas *et al*., 2007; Lee and Gralle, 2001;

Becker and Hengge-Aronis, 2001). Last, the space length between the two DNA elements (-10 and at -35) also discriminates sigma factor binding, RpoD binds most efficiently when these elements are separated by exactly 17 bp space whereas sigma factor RpoS tend to be more permissive for the spacer length and binds promoters with an optimal spacing of 17±2 bp (Typas and Hengge-Aronis, 2006; Osiriphun *et al*., 2009).

Based on the selective and differential nature of the RpoS-dependent promoters, a general model for the RpoS-dependent promoter can be designed. From this hypothetical promoter model, a complete set of potential RpoS-controlled genes of an organism under different growth conditions can be inferred. Due to the poor nutritional value of soil (Timmusk *et al*., 2011), bacteria in the field are unlikely to be in log phase growth but rather they are expected to be quiescent most of the time (Gray *et al*., 2004; Llorens *et al*., 2010). Thus, it is likely that many of the activities that are associated with plant growth-promotion may be under the control of RpoS. In fact, ACC deaminase-containing plant growth-promoting bacteria help plants in their survival under a number of biotic and abiotic stresses. Moreover, RpoS-dependent promoters control a number of bacterial genes that are supposed to be beneficial for the plant growth under stress conditions. Characterization of the complete RpoS regulon of a PGPB can help in better understanding of the role of a PGPB under field and/or stress conditions.

For this study, various proteobacteria (Table A1) were selected on the basis of their efficient plant growth-promotion abilities in a variety of ways. *Burkholderia xenovorans* LB400 is a well studied, nonpathogenic *Burkholderia* species isolate, and its genome has been sequenced (Chain *et al*., 2006). *B. xenovorans* LB400 is a plant rhizospheric bacterium that effectively degrades polychlorinated biphenyl. *Burkholderia*

Table A1 The selected bacteria for the study of RpoS-dependent promoters and their information.

Bacterium	**Genome size (Mb)**	**Accession No. (GenBank)**	**Number of genes**	**Nature**
Escherichia coli K12 substrain MG1655	4.64	NC_000913.2	4496	Control
Burkholderia xenovorans LB400	9.73	CP000270.1, CP000271.1, CP000272.1,	9043	PGP rhizospheric bacterium
Burkholderia phytofirmans PsJN	8.21	CP001052.1 CP001052.1 CP001052.1	7484	PGP endophytic bacteria
Burkholderia strain JK006	6.63	CP003514.1 CP003515.1 CP003516.1 CP003517.1	6143	PGP endophytic bacteria
Azoarcus sp. BH72	4.376	AM406670.1	3992	PGP endophytic bacteria

strain KJ006 is an endophytic bacterium of rice with antifungal activity; this bacterium also exhibits plant growth-promotion activity by degradation of aromatic compounds (Kwak *et al*., 2012). *Burkholderia phytofirmans* PsJN is an endophyte that has been well documented for its prominent and efficient plant growth-promotion (Weilharter *et al*., 2011; Compant *et al*., 2005; Pillay and Nowak, 1997; Wang *et al*., 2006). *Azoarcus* sp. BH72 is nitrogen fixing obligate endophyte of grasses (e.g. rice) that has been shown to significantly promote the growth of rice seedlings (Hurek *et al*., 1994). In addition, *Escherichia coli* K-12 substrain MG1655 was used as a control.

A training set for the RpoS promoter sequence was designed by using ninety-two known RpoS-dependent promoter sequences from the *E. coli* (Table A2). Among them, eighty-one of the promoter sequences were taken from the study done by Osiriphun and colleagues (2009), and the nucleotide sequences of eleven promoters were obtained from the study done by Wise and colleagues (1996). Using these sequences as a training set a motif model representing the RpoS promoter sequences was created by Multiple Em for Motif Elicitation (MEME) web server version 4.8.1 (http://meme.nbcr.net) (Fig. A1). The resulting RpoS model was used to search for the RpoS-dependent promoter sequences in the genome sequence database of a number of plant growth-promoting rhizospheric bacteria and bacterial endophytes (Table A1) by using Motif alignment and search tool (MAST) web server version 4.8.1 (Bailey and Gribskov, 1998) (http://meme.ebi.edu.au/meme/cgi-bin/mast.cgi).

A python script was written (courtesy of Dr. Michael Lynch) to pull out the downstream region of each hit of the putative RpoS-dependent promoter predicted by MAST. The first 1000 nucleotides of each of the down-regulated genes of RpoS regulon

Table A2 Nucleotide sequence of *rpo*S-dependent gene promoters.

Promoter sequence	σ^S-dependent promoter
CTAATGTAGCCACCAAATCATACTACAAT	*adhE*(P1)/1-36
CGTAATCAGTACCCAGAAGTGAGTAATCT	*adhE*(P2)/1-36
GCGAAGATTTCGCCAGTCACGTCTACCCT	*aldB*/1-36
GGATGAATAAACATTGTTCATGGCAACTT	*ans*(P1)/1-36
CTATCATCGCCAGGATGAATAAACATTGT	*ans*(P2)/1-36
TTATTGAATAATGCGCTTTGCTTTTAACT	*artP*(P1)/1-36
CCCCCGTTATTTTGTGCTATGTTTATTGA	*artP*(P2)/1-36
CCACCGCCCCCGTTATTTTGTGCTATGTT	*artP*(P3)/1-36
CAGAATACAGCTTATTGAATACCCATTAT	*caiT*/1-36
ATAGCGGAACACATAGCCGGTGCTATACT	*dps*/1-36
GCCAACAGAAAACGCTGAAAAAACATCCA	*fbaB*/1-77
CGGCGTAACCCGGATTTGCCGCTTATACT	*fic*
ACCCCGTACCTCTGATAATGGTCTAAAAT	*hchA*/1-36
GAAGCGGGATCTGGCTGGTGGTCTATAGT	*katE*/1-36
ACTTGCAAAAGCGGAGAATCAGCTATCCT	*msyB*/1-36
CCCCGCGGTTACGCTTTCTTATCTATTCT	*narU*/1-36
CTCGTGCTGTTTCTCACGTAGTCTATAAT	*osmC*(P2)/1-36
CGAGCGGTTTCAAAATTGTGATCTATATT	*osmY*/1-37
AAAGCTCCAATAAATCATATTGTTAATTT	*pfkB*(P2)/1-36
TATCTCTAGAAAGGCCTACCCCTTAGGCT	*pm*/1-36
CCGGAGGGTGTAAGCAAACCCGCTACGCT	*proP*(P2)/1-36
ATATAACTGTCACCTGTTTGTCCTATTTT	*pstS*/1-36
AGGCGCAGGTGGCAATAGCATGCCACTAT	*rssB*/1-36
CAGCACCCTACGCTTTAAGGTGCTATGCT	*sra*/1-36
CCAGCAATACCATGCCTGTCTGCTATGCT	*talA*(P1)/1-36
ACACTGATGTTACCTGCTTAATCCAGCAA	*talA*(P2)/1-36
AATCGGTGCGCAATATCTACGACTACTAT	t*ktB*/1-36
GGAAAAAAAACAACCTGATCTCCTACACT	*uspB*/1-36
CGGTAAGCAACGCGAAATTCTGCTACCAT	*xthA*/1-36
CACAAATATCATTTCTCAACGTCTACACT	*ybgA*(P1)/1-36
GTCAGTCCTTGCGGGGAGCAGGCTTTCGT	*ybgA*(P2)/1-36
AAAGCATTATCAGACTGATACGCTATTAT	*ybjP*/1-36
AGCTGGCGAGAGACGGTATTGCTCATGCA	*yggE*(P2)/1-36
CCGCTGTGTCTGCTTTTCCCGACTATTCT	*yiaG*/1-80
ATGAAATTTTGAGGATTACCCTACACT	*cbpA*(P2)
TGGCAACTAACATCGCAGCAGCAAGCCT	*acnA*(P1)/1-35
TTGATTTAAGATTTGTAATGGCTAGATT	*csgD*(P2)/1-35
TTCCCTTCGCCATTTCCTTGAGCAAACT	*csiE*(P2)/1-35
ATTTCAGACTTATCCATCAGACTATACT	*frdA*/1-35
TATTCAACCGTCCGGAACCTTCTATGAT	*ftsQ*(P1)/1-35
TTGACTTAAGAGGGCGGCGTGCTACATT	*gadX*/1-35
TTGTTTTGTGCAAAAGTTTCACTACGCT	*hyaA*/1-35
TGGAAAAATAAAACAGAGGCGCTAAGCT	*yggE*(P1)/1-35
TGGAAACATTGCCCGGATAGTCTATAGT	*ytfK*/1-35
TGTCATGAATCCATGGCAGTGACCATACT	*aidB*/1-36
TCTCACAAAGCCCAAAAAGCGTCTACGCT	*cfa*(P2)/1-36
TGCGCATTTTTCAGAAATGTAGATATTTT	*csiD*/1-36
TGAAGAGAGCCACGATATCAAAGAAGATT	*dnaN*(P4)/1-36
CCATCATTTTTGGCGATGTTGTCTATTAT	*ecnB*/1-36
TTGCTTCCATTGCGGATAAATCCTACTTT	*gadA*/1-36

TTGCTTACTTTATCGATAAATCCTACTTT	*gadB*/1-36
CTGATCTTATTTCCAGTAAAAGTTATATT	*gadY*/1-36
ACGCACGTTATGTTTAAAGGCACTACACT	*glgS*(P2)/1-36
CTGGCACCTATTACGTCTCGCGCTACAAT	*gor*/1-36
ATAATAATTTCTATTTTATATTATTCCCT	*htrE*(P2)/1-36
TTATCGCCACGGTTTGAGCAGGCTATGAT	*ldcC*/1-36
CCTCAGGAATCCTCACCTTAAGCTATGAT	*nhaA*(P2)/1-36
CTGTTATTAACACTCTCAAGATATAAAAT	*osmB*(P1)/1-36
ATTTCACCAGACTTATTCTTAGCTATTAT	*osmB*(P2)/1-36
TTGAAAAAGCGCCCAATGTATTCCAGGCT	*osmE*/1-36
ATGGCGACCCCCGTCACACTGTCTATACT	*otsB*/1-36
TTCACTTTCCGCTGATTCGGTGCCAGACT	*pfkB*(P1)/1-36
CCTTCCCCCTCCGTCAGATGAACTAAACT	*poxB*/1-36
AACGCAGCAGTAGCAAACTAAGCTATAAA	*pqi5*(P2)/1-36
ATGGCAATTCTCCCTTCGGCAACCATAAT	*rsd*(P1)/1-36
ATGCAGCTAGTGCGATCCTGAACTAAGGT	*treA*/1-36
AAGCTAACCCCGCCATTATCAACTATGCT	*yehZ*/1-36
TTGAATCTTAACAACAGCGTACGTATGCT	*yeiL*/1-36
TTCAGGATAAAGAGGGAGATCTACCATTAT	*appC*/1-37
TTATTAAAAATATTTCCGCAGACATACTTT	*csgBA*/1-37
TTGCAGGAAAAACTGGTCACCATCGACAAT	*dnaN*(P2)/1-37
TTCGCCGGGTGCTGCAAAACCATCTACGCT	*gabD*(P2)/1-37
TTGGCGTAAATCAGGTAGTTGGCGTAAACT	*himA*(P4)/1-37
TTTACATTGCAGGGCTATTTTTTATAAGAT	*hmp*/1-37
CTGGCAACTTTGGATTTTGCATGCTAATAA	*topA*(Px1)/1-37
TTCTCCGCTTTTCCTTGCTGTCATCTACACT	*blc*/1-38
CTGCAATGGAAACGGTAAAAGCGGCTAGTAT	*bolA*/1-38
GTGGCGTTCTTTATCGCCAAGCGTCTACGAT	*dnaN*(P1)/1-38
ATGACATATACAGAAAACCAGGTTATAACCT	*hdeAB*/1-35
ATCACGCAAATAATTTGTGGTGATCTACACT	*proU*(P1)/1-109
CCGCTCTAAGATGATTCCTGGTTGATAATTAAGA	*osmB*
CCGGCGTAACCCGATTTGCCGCTTATACTTGTGG	*fic*/1-39\/1-35
CCGGAGGGTGTAAGCAAACCCGCTACGCTTGTTACA	*proP*P2\/1-37
GCGAAGATTTCGCCAGTCACGTCTACCCTTGTTATA	*aldB*\/1-37
CTGCAATGGAAACGGTAAAAGCGGCTAGTATTTA	*bolA*\/1-35
CGGTAAGCAACGCGAAATTCTGCTACCATCCACGCA	*xthA*\/1-37
ACGCACGTTATGTTTAAAGGCACTACACTGATTGGGG	*glgS*P2\/1-38
CCTTCCCCCTCCGTCAGATGAACTAAACTTGTTACCG	*poxB*\/1-38
TCTCACAAAGCCCAAAAAGCGTCTACGCTGTTTTA	*cfa*P2\/1-36
ATAGCGGAACACATAGCCGGTGCTATACTTAATCTCG	*pexB*dps\/1-41
TCTCACAAAGCCCAAAAAGCGTCTACGCTGTTTTA	*cfa*P2\/1-36

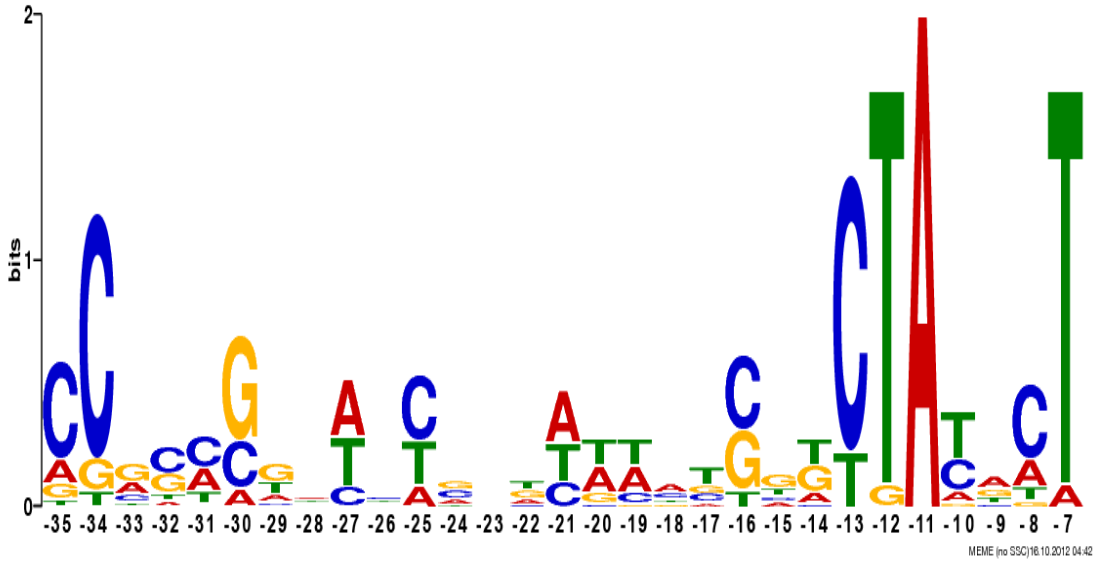


Figure A1 The putative RpoS-dependent promoters sequence of RpoS-dependent genes.

The sequence was created by the MEME analysis using a set of ninety-two RpoS-dependent promoters. The sequence logo was extensively based on the promoter core from -7 to -35 elements.

were extracted. In order to know the function of the putative RpoS-controlled genes, first, the DNA sequences obtained were searched against the GenBank non-redundant protein database using BLASTX (http://www.ncbi.nlm.nih.gov/BLAST). Sequences were subsequently uploaded to the MG-RAST (Meyer et al., 2008) server for analysis. The MG-RAST (the Metagenomics RAST) server is a web-based open-access automated analysis platform that is based on SEED framework for comparative genomics (Meyer et al., 2008). It provides quantitative insights into microbial populations based on the sequence data. The MG-RAST server was used to classify the putative proteins into various categories on the basis of their physiological role in diverse metabolic pathways, which can be used to visualize differences between metagenomics (or different input files) in a taxonomic context. Fig. A2 illustrates the distribution of functional categories of the putative genes under the control of RpoS promoter in the study bacteria. Since *E. coli* is neither a plant growth-promoting nor an endophytic bacterium, the common genes under the control of the RpoS regulon of this proteobacterium was subtracted from each of the plant growth-promoting bacteria. The putative RpoS regulon of each study bacteria was compared with *E. coli* by using the reciprocal smallest distance (RSD) algorithm. RSD (with all its basic components) is an easy access program that can be downloaded from the Internet (Wall and DeLuca, 2007). RSD utilizes the reciprocal best BLAST hits (RBH), global sequence alignment, and the highest probability estimation of evolutionary distance in the identification of orthologs between two genomes (or two input files) (Wall and DeLuca, 2007). The RSD approach was used to detect the possible orthologs present in *E. coli* and each of the study bacteria with the most stringent blast E-value ($1\ E^{-20}$) and divergence thresholds (0.2). Orthologs that are common in the RpoS regulons of *E. coli*

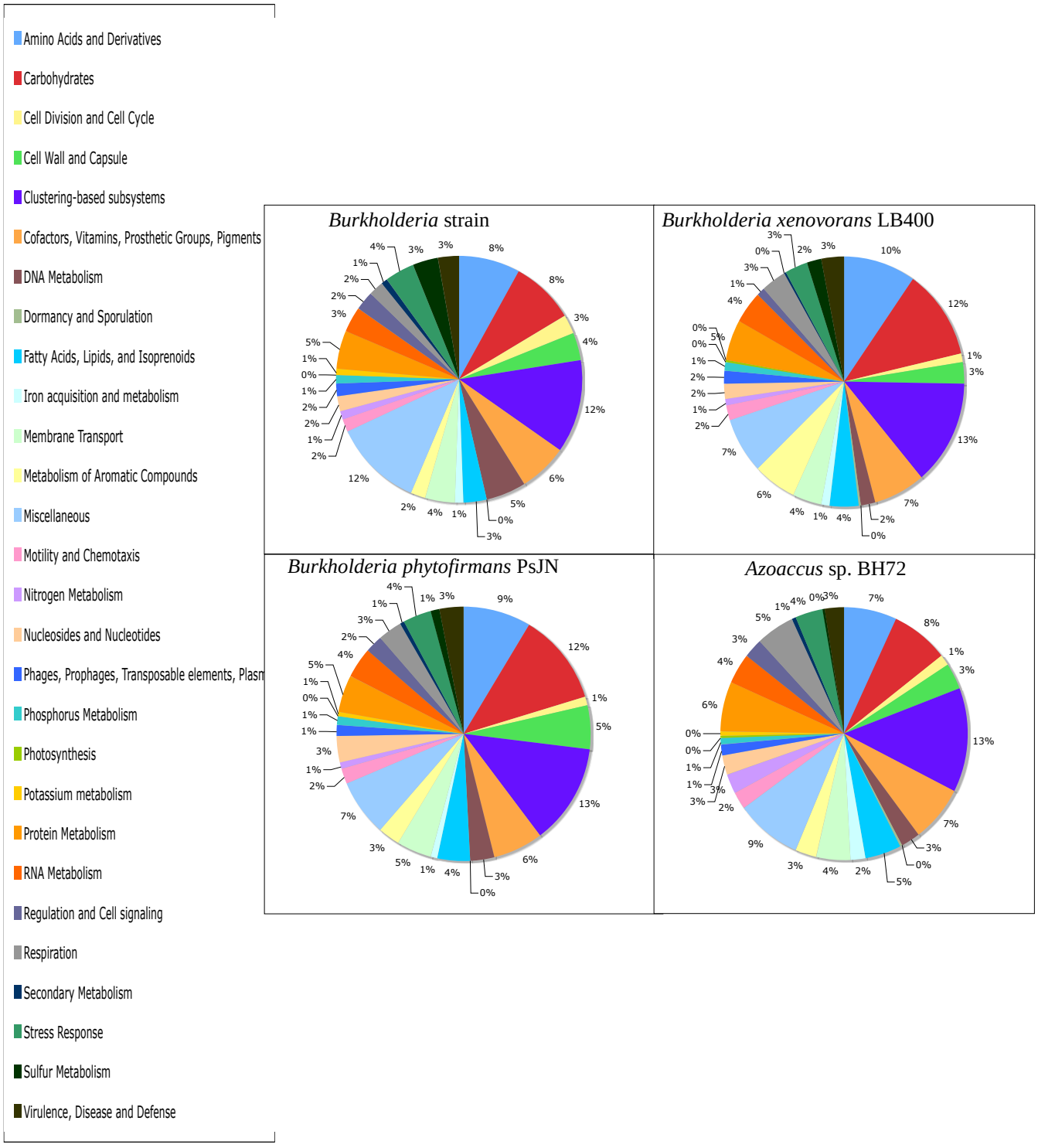


Figure A2 Functional classification of all down regulated proteins of RpoS-dependent promoters in various proteobacteria.

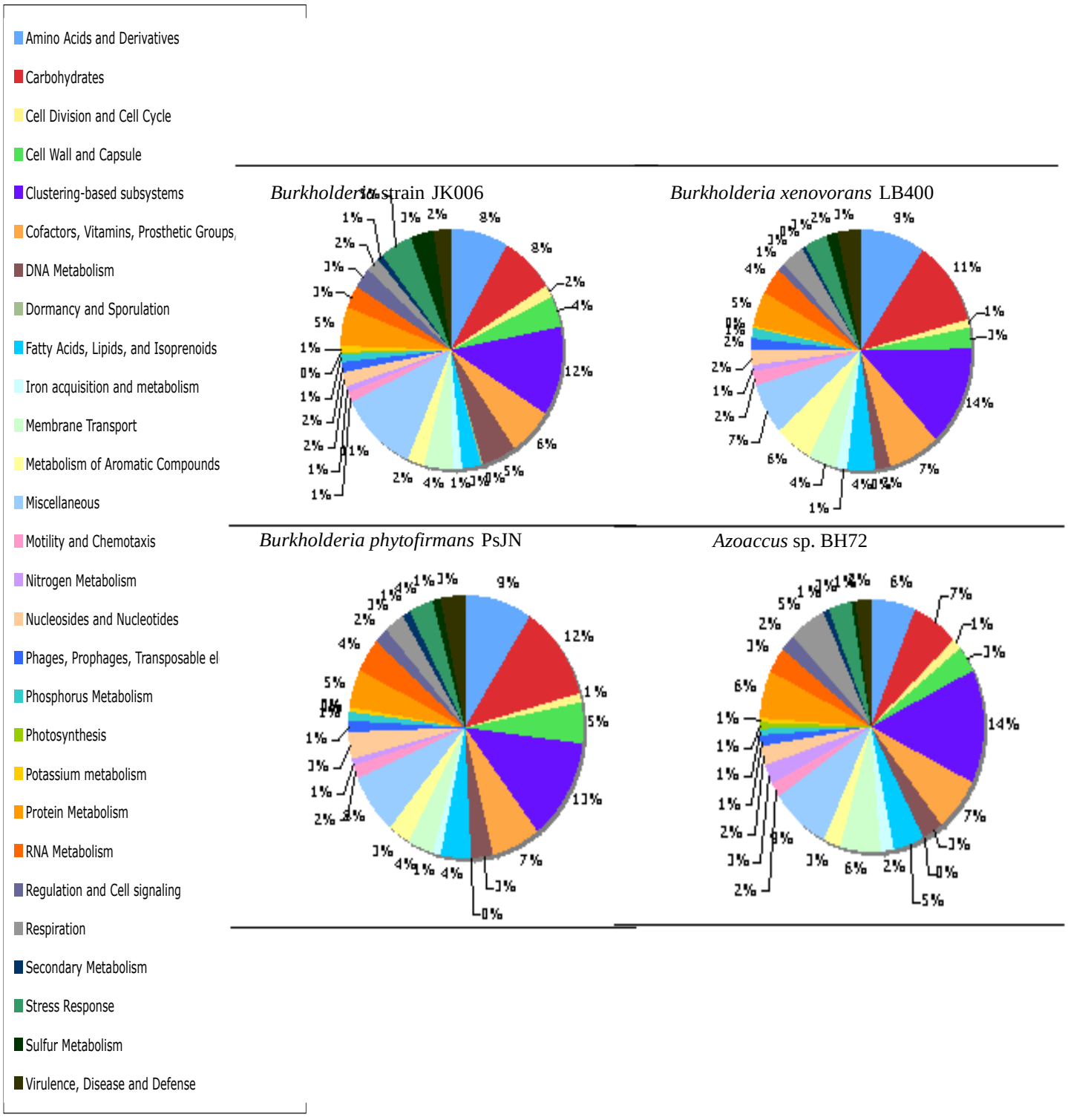


Figure A3 Functional classification of down regulated proteins of RpoS-dependent promoters in various proteobacteria.

The common RpoS-dependent genes of *E. coli* were manually subtracted from each bacterial RpoS regulon and functional classification was reconstructed.

and the study bacteria were manually deleted from the RpoS regulon of individual bacteria, and then the functional categories of each plant growth-promoting bacterial putative RpoS regulon were reconstructed using MG-RAST server (Fig. A3). Interestingly, only a few genes are common in *E. coli* and the PGP study bacteria. This strengthens the argument that although *E. coli* is a proteobacterium and has a reasonable number of genes (i.e. 568 genes) under putative RpoS regulon, these genes are different from those that are expressed in plant growth-promoting bacteria. Furthermore, the RpoS-dependent promoter training set worked equally well in a number of different proteobacteria (Table A3).

The genes in the putative RpoS regulon of all four plant growth-promoting bacteria were also compared to each other by RSD to find out the possible orthologs in various bacteria. Seventy-seven different orthologs were shared by the four bacteria. Potential functional categories for these orthologs were determined using the MG-RAST server (Fig. A4). Data from the SEED analysis indicate that of the RpoS-controlled orthologs, 14% were involved in amino acids metabolism, 14% were involved in carbohydrate metabolism, 13% were involved in clustering based subsystems, and 7% were involved in membrane transport systems. Moreover, the other dominant categories include cofactors, vitamins, and prosthetic groups, fatty acids, lipids, and isoprenoids, metabolism of aromatic compounds, cell wall and capsule, protein metabolism, RNA metabolism, and stress response. Genes involved in carbohydrate metabolism in proteobacteria have been previously shown to be under RpoS regulation (Osiriphun *et al*., 2009). These genes are associated with several pathways for energy production (glycolysis and TCA cycle), coenzyme biosynthesis, and production of NADPH to

Table A3 The number of genes in bacterial RpoS regulon before and after the subtraction of the *E.coli* RpoS regulon common genes.

Bacterium	**Number of genes in RpoS regulon**	**RpoS regulon – *E. coli***
Escherichia coli K12 substrain MG1655	568	-
Burkholderia xenovorans LB400	1029	1017
Burkholderia phytofirmans PsJN	789	776
Burkholderia strain JK006	504	500
Azoarcus sp. BH72	373	366

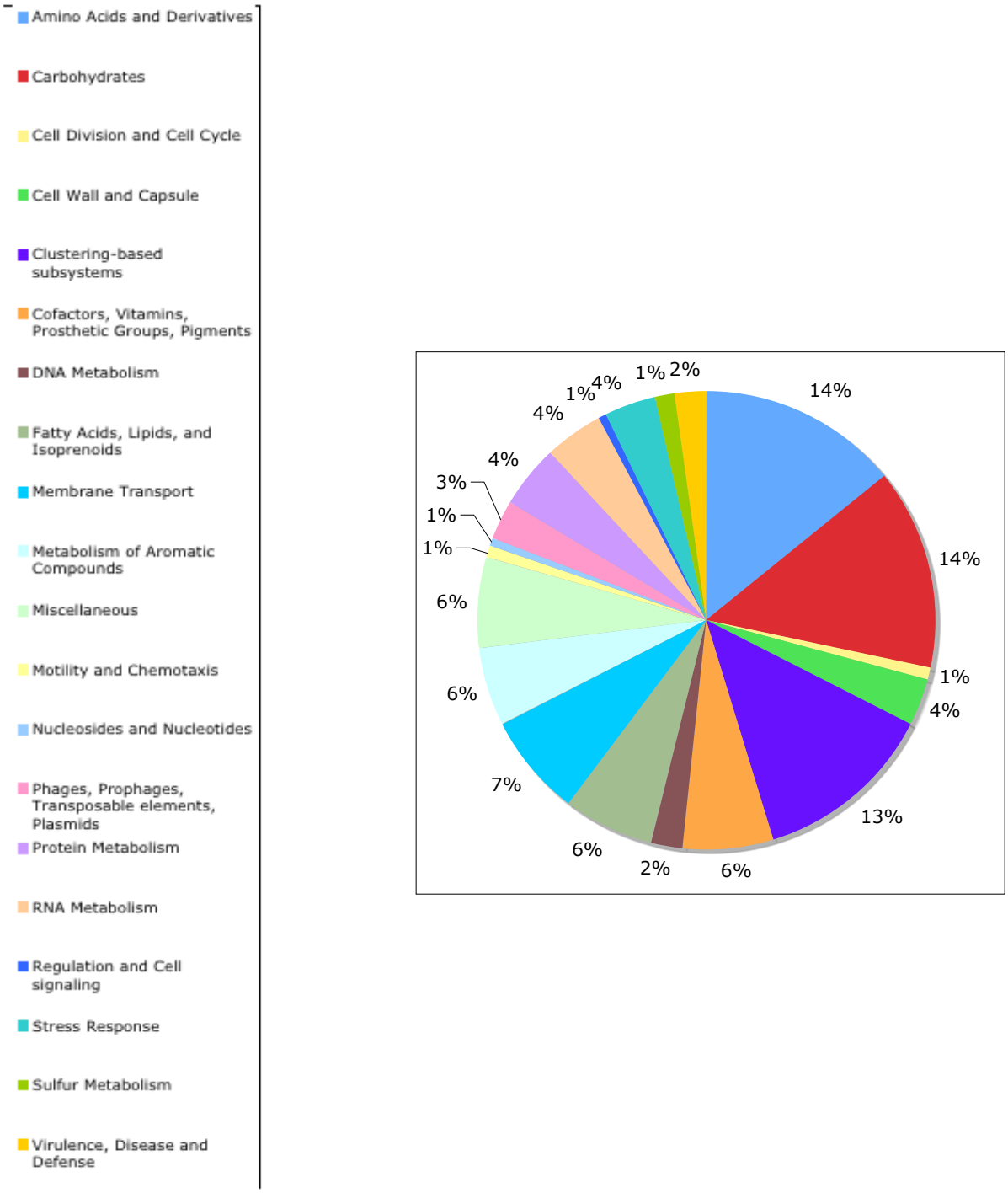


Figure A4: The functional categories of the common RpoS regulon of study proteobacteria.

Table A4 The name and the function of all putative RpoS-dependent genes in the common RpoS regulon of study proteobacteria.

Putative RpoS-dependent gene	Gene origin
uvrA1 gene product	Abh_102029_1_1_999_+
glcD1 gene product	Abh_1072429_1_1_999_+
phbB2 gene product	Abh_1100059_-1_1_999_+
etfA1 gene product	Abh_1868364_1_1_999_+
dppF gene product	Abh_2263135_-1_1_999_-
exbD1 gene product	Abh_261145_-1_1_397_+
thmS2 gene product	Abh_3320411_-1_1_999_+
livG1 gene product	Abh_3350063_1_278_999_-
Carboxyltransferase subunit of acetyl-CoA carboxylase	Abh_3382911_-1_1_402_-
rhlE1 gene product	Abh_340787_1_305_999_+
cobyric acid synthase	Abh_3859118_1_1_999_-
fadfX gene product	Abh_421442_-1_95_999_+
yeaT gene product	Abh_4231994_-1_1_999_-
Putative hybrid sensor and regulator protein	Abh_4323919_-1_1_999_-
PAS/PAC/GGDEF-domain-containing protein	Abh_482492_-1_1_573_+
ubiA gene product	Abh_516617_-1_1_999_+
lpxC gene product	Abh_954502_-1_1_360_+
hflX1 gene product	Abh_999468_-1_228_470_+
Histone deacetylase superfamily protein	Bkj_1009332_-1_69_999_+
D-methionine transport system permease protein	Bkj_1012562_-1_1_751_+
Acetyl-coa synthetase	Bkj_1027718_1_142_999_+
Glucose dehydrogenase, membrane-bound cytochrome c, class I	Bkj_1311199_-1_693_999_+
Mota/tolq/exbb proton channel	Bkj_1465624_-1_394_999_-
ABC transporter,ATP-binding protein	Bkj_1485719_1_1_518_-
Aldehyde dehydrogenase	Bkj_1682051_-1_1_780_-
Xre family transcriptional regulator	Bkj_1730246_-1_1_738_-
Clpb protein	Bkj_1764633_1_468_999_-
Outer membrane chaperone Skp (omph)	Bkj_1928767_-1_1_478_-
Amino acid ABC transporter	Bkj_2439250_1_1_349_+
NAD-dependent epimerase/dehydratase	Bkj_259639_-1_520_999_-
Excinuclease ABC subunit A	Bkj_2745777_1_603_999_+
Yihe protein	Bkj_3048874_-1_180_999_-
Methyl-accepting chemotaxis sensory transducer	Bkj_413329_1_1_999_-
Oligopeptide/dipeptide ABC transporter atpase	Bkj_448782_-1_257_999_-
Polyhydroxyalkanoic acid synthas	Bkj_716837_-1_723_999_-
Multi-sensor hybrid histidine kinase	Bkj_729256_-1_1_999_+
Lysr family transcriptional regulator	Bkj_779547_-1_496_999_-

Heavy metal RND efflux outer membrane protein	Bkj_846328_-1_1_999_+
Bifunctional glucokinase/rpir family transcriptional regulator	Bkj_905838_-1_462_999_-
Type VI secretion atpase, clpv1 family	Bphy_1045998_1_1_999_+
Rne/Rng family ribonuclease	Bphy_1453438_-1_1_999_-
Alkane 1-monooxygenase	Bphy_1592201_-1_1_363_+
Acetoacetyl-coa reductase	Bphy_1768999_1_1_999_-
General substrate transporter	Bphy_1826322_-1_639_999_+
DNA polymerase III subunits gamma and tau	Bphy_2052493_-1_524_999_+
Beta-ketoadipyl coa thiolase	Bphy_2148042_1_1_792_-
Gntr family transcriptional regulator	Bphy_2986335_1_1_784_-
Conserved hypothetical lipoprotein	Bphy_3212492_1_253_707_+
Lipoprotein, yaec family	Bphy_3370343_1_553_999_-
Glucose-methanol-choline oxidoreductase	Bphy_434506_1_316_999_+
fdx gene product	Bphy_49531_-1_556_999_-
RNA methyltransferase	Bphy_592528_1_452_999_-
NodT family RND efflux system outer membrane lipoprotein	Bphy_613821_1_1_834_+
Hypothetical protein	Bphy_690222_1_628_999_+
4-hydroxybenzoate polyprenyltransferase	Bphy_785888_1_479_999_+
Acetylornithine deacetylase	Bx_1106135_-1_1_999_+
Nitrate/sulfonate/bicarbonate ABC transporter atpase	Bx_143929_1_1_999_-
Branched chain amino acid ABC transporter atpase	Bx_1482057_-1_1_999_+
Spermidine/putrescine ABC transporter atpase	Bx_1718106_-1_1_761_+
Aldolase II superfamily protein	Bx_2020698_-1_1_467_-
Heat-shock protein, chaperone clpb	Bx_2284837_-1_1_999_+
UDP-3-O-[3-hydroxymyristoyl] glucosamine N-acyltransmembrane protein	Bx_3035465_1_1_999_-
ATP-dependent RNA helicase dbpa	Bx_33082_1_1_999_+
DNA helicase	Bx_3736282_1_1_590_-
Electron transfer flavoprotein subunit alpha	Bx_3783593_1_153_999_-
D-methionine transport system permease protein	Bx_3787394_-1_199_999_-
D-xylose ABC transporter, ATP-binding protein	Bx_465624_-1_483_999_+
Acetyl-coa acetyltransferase	Bx_475821_-1_431_999_+
Branched chain amino acid ABC transporter atpase	Bx_4817476_1_501_999_-
FAD-binding oxidase	Bx_542772_1_1_411_-
Serine hydroxymethyltransferase	Bx_613854_1_1_999_+
Glycosyltransferase	Bx_675911_1_1_999_+
Lyso-ornithine lipid acyltransferase	Bx_738188_1_434_999_+
Betaine-aldehyde dehydrogenase	Bx_812388_-1_1_999_+
Cell shape determining protein, mreb/Mrl family	Bx_81689_-1_10_999_-
Transcriptional regulator cysb-like protein	Bx_885466_-1_1_999_-
Lysine/arginine/ornithine ABC transporter periplasmic protein	Bx_958952_1_1_444_-

prevent oxidative stress-induced injuries (Osiriphun *et al*., 2009). In addition, genes that are under the regulation of RpoS are involved in morphological changes of the cell, resistance to various stress conditions , metabolic processes, and virulence and pathogenicity (Navarro Llorens *et al*., 2010; Hengge-Aronis, 1994; Martinez-Garcia *et al*., 2001; Raiger-Iustman and Ruiz, 2008). In the present study, the common RpoS regulon of proteobacteria also carries genes that are involved in the processes of cell division and cell cycle (1%), cell wall and capsule (4%), motility and chemotaxis (1%), and virulence, disease and defense (2%). It is hypothesized that the first two groups of genes (cell division and cell cycle and motility and chemotaxis) are required in determining the cell shape at the onset of stationary phase (Nystrom, 2004).

Table A4 summarizes the information about the genes comprising the common RpoS regulon of four proteobacteria. This table includes several transcriptional regulators, mainly LysR transcriptional regulator, Xre transcriptional regulator, CysB type regulator, GntR family, ClpB protein, and YihE transcriptional regulator. These transcriptional regulators regulate various events of the transition phase of the bacterial growth cycle. LysR family proteins control a diverse set of genes that are mainly involved in virulence, metabolism, quorum sensing and motility (Maddocks and Oyston, 2008). Likewise, the Clp family of proteins rescues the other cellular proteins from heat stress (Barnett *et a*l., 2000). A number of genes in Table A4 are responsible for the transport of branched chain amino acids to subcellular compartments. These branched chain amino acids (mainly leucine) may trigger the Lrp family global transcription regulators that can subsequently alter the transcription level of stationary phase proteins. In fact, more than 400 *E. coli* genes and almost three-quarters of the stationary phase

induced genes are controlled by this Lrp global regulator. The *E. coli* stationary phase genes that are under the control of Lrp global regulator work in response to nutrient scarcity, high concentrations of organic acids, and osmotic stress (Navarro Llorens *et* al., 2010; Tani *et al*., 2002). Generally, Lrp regulators function in order to syncronize the bacterial cellular metabolism to its environmental nutritional status (Navarro Llorens *et* al., 2010; Landgraf *et a*l., 1996). Moreover, it has been previously reported that ACC deaminase genes from a variety of plant growth-promoting bacteria are under the transcriptional control of Lrp regulatory protein (Grichko and Glick, 2000; Li and Glick, 2001; Ma *et al*., 2003; Prigent-Combaret *et al*., 2008). In addition, bacterial IAA (auxin) producing genes have also been reported to be under the control of an RpoS-dependent promoter (Pattan and Glick, 2002).

Knowledge of the complete set of genes under the control of RpoS-dependent promoters in plant growth-promoting bacteria may reveal information about bacterial strategies for functioning in particular environments. The knowledge of different set of genes under such a regulation can also facilitate an understanding of the diversity and versatility of bacterial genomes. In this regard, while the data obtained from the computer-based bioinformatic work can be experimentally obtained using a variety of techniques including proteomics and microarrays, the use of a bioinformatic approach provides a novel and potentially simpler approach to the same end. However, this bioinformatic approach remains to be experimentally varified.

Appendix II

A bioinformatics approach to the determination of bacterial genes involved in endophytic behavior

1 Introduction

Plant growth-promoting bacteria are a group of plant-associated bacteria that support plant growth to significant levels (Glick, 1995). Plant growth-promoting bacterial endophytes employ similar plant growth promotion mechanisms to those used by rhizospheric plant growth-promoting bacteria. Bacterial endophytes are plant growth-promoting bacteria that go one step further to colonize the inside of plant tissues where they can interact with their host more efficiently than bacteria that bind exclusively to the plant's roots. Under a wide range of environmental conditions, it is likely that endophytic plant growth-promoting bacteria will be superior to similar non-endophytic bacterial strains in promoting plant growth. It is therefore interesting to ask what makes two similar plant growth-promoting bacteria (rhizoshperic vs. endophytic) act so differently so that one can enter the plant and colonize the interior tissues without provoking the host defense system, while the other remains outside the plant (in the rhizosphere). Presumably, there are key differences at the genomic level that make a bacterium either an endophyte or a rhizosphere colonizing bacterium.

Bacterial genomes carry all the information that is required for an organism to grow under a range of both favorable conditions as well as in a number of challenging habitats. These genomes do not merely encode house keeping machinery, but they also encode genes that may be required for proliferation and survival under a range of diverse

circumstances. In this study, the complete genome sequences of a number of Proteobacterial species (Table B1) were compared to see whether it might be possible to identify some of the common genes that are present in the majority of the bacterial endophytes and, ultimately, are responsible for endophytic behavior.

2 Materials and Methods

The amino acid sequences corresponding to each of the predicted coding regions of the genomes used in the analysis were catalogued using the Artemis genome browser and annotation tool (Rutherford *et al*., 2000).

Genome coding sequences were compared using the reciprocal smallest distance (RSD) algorithm (Wall and Deluca 2007). RSD utilizes reciprocal best blast hits (RBH), global sequence alignment, and the highest probability estimation of evolutionary distance in the identification of orthologs between two genomes (or two input files) (Wall and DeLuca, 2007).

3 Results

To investigate what genes might be involved in making a bacterium an endophyte, a bioinformatics approach was used. For this study, several complete-genome sequenced endophytic and non-endophytic plant growth-promoting Proteobacteria were used, and genes putatively responsible for endophytic behavior were predicted. Table 1 lists all of the bacteria examined in this study, their GenBank accession numbers, and their behavior with respect to their interaction with plants.

Table B1 Plant growth-promoting bacteria used in this study including Genbank accession number and the nature of their interaction with plants.

Bacterium	**Accession number**	**Biological nature**
Burkholderia xenovorans LB400	NC007951 NC007952 NC007953	Rhizospheric
Burkholderia phytofirmans PsJN	CP001052 CP001053	Endophytic
Burkholderia sp. strain JK006	CP003514 CP003515 CP003516	Endophytic
Azoarcus sp. BH72	AM406670	Endophytic
Azospirillum lipoferum 4B	NC_016622	Endophytic
Enterobacter cloecae ENHKV01	CP003737	Endophytic
Enterobacter sp. 638	NC_009436	Endophytic
Pseudomonas putida W619	CP000949.1	Endophytic
Klebsiella pneumoniae 342	NC_011283	Endophytic
Serratia proteamaculans 568	NC_009832	Endophytic

First, a rhizospheric binding bacterium (i.e. *Burkholderia xenovorans* LB400) and an endophytic bacterium of the same genus (i.e. *Burkholderia phytofirmans* PsJN) were compared. Orthologs present in both genomes (rhizospheric versus endophytic) were identified. For this reason, the set of genes encoded by the rhizospheric bacterium *Burkholderia xenovorans* LB400 was subtracted from the set of genes encoded by the bacterial endophyte *Burkholderia phytofirmans* PsJN. The common orthologs set represents the genes that are present in both bacteria and are putatively responsible for house keeping functions. Because these common genes are present in both types of bacteria of the same genus, i.e. rhizospheric and endophytic, they probably do not include the genes responsible for endophytic behavior. Next, the set of the common genes was substrated from the endophytic strain's gene complement, thereby ensuring that the remaining genes from the endophytic strain have a higher probability of being involved in endophytic behavior. The set of putative endophytic genes was compared with the complete genomes of eight different (plant growth-promoting) endophytic bacterial genomes by using the RSD algorithm, thereby enriching for genes that are present in a number of different endophytic bacteria. The resulting common orthologs (Table B2) are good candidates to consider for possible involvement in endophytic behavior.

4 Discussion

Comparative genomic analysis is a powerful tool that can help to identify the set of similar and different genes between two or more organisms. In addition, it provides a deeper insight into various genomes from diverse bacteria encoding similar functions. Thus, by comparing the set of genes encoded by an endophytic strain of

Table B2 Genes putatively responsible for endophytic behavior. The RSD algorithm was used with the most stringent blast E-value (1 E^{-20}) and divergence thresholds (0.2).

Strains key: AB is *Azoarcus* sp. BH72; AL is *Azospirillum lipoferum* 4B; BK is *Burkholderia* strain JK006; BP is *Burkholderia phytofirmans* PsJN; EC is *Enterobacter clloecae* ENHKV01; E638 is *Enterobacter* sp. 638; KP is *Klebsiella pneumoniae* 342; PP is *Pseudomonas putida* W619; SP is *Serratia proteamaculans* 568. A "+" sign in columns 2 – 10 represents the presence of particular gene and a "-" indicates the absence of the gene in the genome studied.

Number	Gene Function	BP	BK	AB	EC	PP	KP	E638	SP	AL
Bphyt_0033	Lysine exporter protein lyse/ygga	+	+	-	+	+	+	+	+	+
Bphyt_0034	Major facilitator superfamily protein	+	+	-	-	+	-	-	+	-
Bphyt_0109	Regulator protein FrmR	+	+	-	+		+	+	-	-
Bphyt_0434	Asnc family transcriptional regulator	+	+	-	+	+	+	-	+	-
Bphyt_0435	Hypothetical protein	+	+	-	-	+	-	-	+	-
Bphyt_0573	2-isopropylmalate synthase	+	+	-	+	-	+	+	+	-
Bphyt_1098	Short-chain dehydrogenase/reductase SDR	+	+	-	+	-	+	+	-	-
Bphyt_1366	Glutathione S-transferase	+	+	+	+	-	+	+	+	-
Bphyt_1467	Acetaldehyde dehydrogenase	+	+	+	-	-	-	-	-	-
Bphyt_2146	Carbonate dehydratase	+	+	-	+	+	+	+	+	-
Bphyt_2287	AraCfamily transcriptional regulator	+	+	-	+	+	+	-	-	-
Bphyt_2288	Cupin	+	+	-	+	+	+	+	+	+
Bphyt_3335	Peptidase M48 Ste24p	+	+	-	+	-	+	+	+	-
Bphyt_3906	Branched-chain amino acid ABC transporter atpase	+	+	+	+	+	+	+	+	+
Bphyt_3908	Branched-chain amino acid ABC transporter inner membrane protein	+	+	+	+	+	+	+	+	+
Bphyt_4023	Aldehyde dehydrogenase	+	+	-	-	+	+	-	-	-
Bphyt_4261	NAD(P)(+) transhydrogenase (AB-specific)	+	+	-	-	+	-	-	+	-
Bphyt_4584	ABC transporter related	+	+	-	+	-	+	+	+	-
Bphyt_4604	Two component	+	+	+	+	+	+	-	-	+

	transcriptional regulator, winged helix family									
	Gluconate 2-dehydrogenase	+	+	-	-	+	+	-	+	-
Bphyt_4638	(acceptor)									
	Gluconate 2-dehydrogenase	+	+	-	-	+	+	-	+	-
Bphyt_4639	(acceptor)									
	Gluconate 2-dehydrogenase	+	+	-	-	+	+	-	+	-
Bphyt_4640	(acceptor)									
	Type VI secretion protein,	+	+	+	-	+	-	-	-	-
Bphyt_4913	VC_A0107 family									
	Type VI secretion protein,	+	+	+	-	+	-	-	-	+
Bphyt_4914	EvpB/VC_A0108 family									
	Type VI secretion ATPase,	+	+	-	-	+	-	-	-	+
Bphyt_4919	ClpV1 family									
	Transcriptional regulator,	+	+	-	+	-	+	-	+	-
Bphyt_4951	DeoR family									
	S-(hydroxymethyl)glutathione	+	+	-	+	-	-	-	-	-
	dehydrogenase/class III									
Bphyt_5114	alcohol dehydrogenase									
Bphyt_5159	2-dehydropantoate 2-reductase	+	+		+		+	+	+	-
Bphyt_5345	LrgB family protein	+	+	+	-	-	-	-	-	+
Bphyt_5350	Alpha,alpha-trehalase	+	+	-	+	-	+	+	-	-
	Malate/L-lactate	+	+	-	-	-	+	+	+	-
Bphyt_5456	dehydrogenase									
	Metabolite/H+ symporter,	+	+	-	-	-	+	-	+	-
	major facilitator superfamily									
Bphyt_5520	(MFS)									
	Extracellular solute-binding	+	+	-	+	-	+	-	+	-
Bphyt_5521	protein family 1									
	Transcriptional regulator,	+	+	-	+	+	+	-	+	-
Bphyt_5523	LysR family									
Bphyt_5655	Acetoacetyl-coa reductase	+	+	+	+	-	+	-	+	-
	3-hydroxyisobutyrate	+	+	-	-	-	+	-	-	-
Bphyt_5931	dehydrogenase									
Bphyt_6134	Alpha/beta hydrolase fold	+	+	-	+	+	+	-	-	+
Bphyt_6351	Flavoprotein WrbA	+	+	+	+	-	+	+	+	-
	Diaminopimelate	+	+	-	+	-	+	+	+	-
Bphyt_7089	decarboxylase									
	RND family efflux transporter	+	+	+	+	+	+	+	+	+
Bphyt_6992	MFP subunit									

Burkholderia with the genes encoded by a rhizospheric strain of the same genus, it should be possible to identify genes that are uniquely found within the genome of the endophytic strain. Table 2 lists forty individual gene orthologs that are putatively responsible for endophytic behavior of a number of plant growth-promoting bacterial endophytes. This gene set includes the most highly conserved genes that may play an active role in determining the endophytic behavior of a bacterium.

Generally, endophytes are not host (plant) specific so that various endophytes might be expected to utilize similar (and sometimes identical) strategies to colonize their hosts. Thus, different endophytes might be expected to contain at least a minimal set of so-called "endophytic genes". In the set of genes that are putatively associated with endophytic behavior (Table 2), a significant number of genes encode various transporter proteins. Thus, there are fourteen individual genes (Table 2) that are directly involved in transporting different molecules across membranes. In addition, there are several genes that are involved in the major facilitator superfamily (MFS) transporter system (Table 2). Members of ATP-binding cassette (ABC) transporters and MFS transport systems have previously been identified as being important to plant growth-promoting endophyte *Enterobacter* sp. 638 (Taghavi *et al*., 2010). These transport proteins allow bacterial endophytes to efficiently take up plant-synthesized nutrients that may either be present inside the plant or are exuded into the rhizosphere, primarily functioning in the exchange of a variety of carbohydrates and amino acids.

In addition to transporter protein genes, genes coding for resistance nodulation and cell division family (RND) efflux system membrane associated proteins are also found in each of the endophytic bacteria examined in Table 2. MFP subunit of the RND

family efflux systems has been reported to play an active role in the successful colonization of the host plant (apple tree) by the endophyte *Erwinia amylovora* (Taghavi *et al*., 2010; Burse *et al*., 2004). Here it should be noted that the bacterium *Erwinia amylovora* behaves as an endophyte with some plants and as a pathogen with other plants (Burse *et al*., 2004). RND efflux system membrane associated proteins have also been identified in the genome of the plant growth-promoting bacterial endophyte *Enterobacter* sp. 638 and it has been suggested that they are most likely associated with the endophytic nature of the bacterium (Taghavi *et al*., 2010). These bacterial protein secretion and delivery systems play crucial roles in infection, virulence, and pathogenicity (Reinhold-Hurek and Hurek, 2011). Generally, Type I and Type II secretion systems are present in a number of bacterial endophytes (Reinhold-Hurek and Hurek, 2011) while Type III and Type IV secretion systems are mainly present in pathogenic bacteria and are mostly absent in endophytes (Downie, 2010). Type V secretion system is regarded as an autotransporter and is found mostly in endophytes (Taghavi *et al*., 2010) while Type VI secretion systems may have a positive role in plant-microbe interactions (Mattinen *et al*., 2008) and have been identified in some bacterial endophytes (Reinhold-Hurek and Hurek, 2011). In this study, three type VI protein secretion and delivery system associated proteins were found as common orthologs in the bacterial endophytes listed in Table 2. In addition, the absence of Type III and IV protein secretion system genes and the presence of Type VI secretion system genes suggested that organisms living in an endophytic association are structured as if they were "disarmed pathogens" (Jones and Dangl, 2006). Interestingly, in almost all of the endophytes listed in Table 1, at least one gene encoding a lysine biosynthesis enzyme

(i.e., diaminopimelate decarboxylase) and one gene encoding a lysine exporter protein were found (Table 2). There is no experimental evidence for such coincidental presence of these two highly conserved orthologs in a quite diverse set of bacterial endophytes. However, it is predicted that they might be involved at some crucial step in the functioning of the bacterium as an endophyte switches from a free-living (in the soil) to an endophytic life style (in the plant).

Many plant pathogens (including bacteria and fungi) carry genes encoding plant cell wall degradation hydrolases, which mainly includes cellulases, hemicellulases, and endoglucanases. These enzymes are involved in degradation of plant cell wall components (Taghavi *et al*., 2010). In contrast, non-pathogenic plant associated bacteria do not encode proteins involved in this process. However, non-phytopathogens may possess glycoside hydrolases other than cellulase/hemicellulase (or cell wall degradation hydrolases). These glycoside hydrolases (e.g. trehalase) hydrolyze *O*- and *S*-glycosyl compounds (Kalf and Rieder, 1958). Moreover, as seen in Table 2, several genes encoding hydrolases were found in almost all of the endophytes listed in Table 1; and in fact the presence of non-plant-cell-wall-breaking hydrolases has previously been noted to be part of the genome of the plant growth-promoting bacterial endophytes *Enterobacter* sp. 638 and *Serratia proteamaculans* 568 (Taghavi *et al*., 2010). The presence of this enzyme in numerous endophytes is consistent with its possible role in the diversity of sugar utilization that might be a useful component of a competent endophyte. Interestingly, almost all of the endophytic bacteria contain genes that encode the protein cupin. It has been suggested that members of the cupin superfamily may function in the modification of plant cell wall carbohydrates, and that many proteins from this family

utilize substrates that are involved in plant growth and development (Dunwell *et al*., 2004).

Another class of genes that was found to be conserved and is putatively responsible for endophytic behavior includes various transcriptional regulators. A number of different transcriptional regulators are present in the list including AraC, FrmR, AsnC, LrgB, LysR, DeoR, WrbA, and two components of winged helix transcriptional regulator proteins (Table 2). These transcriptional regulators work differently under different conditions. The majority of them are global regulators while some, for example AsnC, function in a more specific manner (Thaw *et al*., 2006). All of these regulators affect cellular metabolism, however, the effector molecules appear to be different for different regulators. The AsnC regulator functions in response to the presence of branched-chain amino acids (there are two orthologs present in the list involved in the specific transport of branched-chain amino acids, Table 2), and LysR recognizes different co-inducers (some members respond to the cellular concentration of the amino acid lysine). LysR family proteins control a diverse set of genes that are mainly involved in bacterial virulence, metabolism, quorum sensing and motility (Maddocks and Oyston, 2008). In addition, AraC transcriptional regulators function in carbon metabolism, stress responses, and virulence management (Gallegos *et al*., 1997; Martin and Rosner, 2001; Cristiane *et al*., 2004). FrmR is a regulatory protein that is not very well characterized; this regulator is also a global transcriptional regulator and negatively controls cellular carbohydrate metabolism (Hyeon *et al*., 2012). Similarly, the DeoR family of transcriptional regulators contains proteins that negatively control genes involved in carbohydrate metabolism (Elgrably-Weiss *et al*., 2005). LrgB family proteins are mainly involved in controlling

hydrolase activity (Groicher *et al*., 2000). The presence of this regulatory protein indicates its most likely function (non-invasive breakdown of plant cell wall), occurs when endophytes come into contact with plant hosts at the time of plant infection. WrbA flavoproteins have been documented to act as RpoS-dependent stationary phase proteins (Yang *et al*., 93; Lacour and Landini, 2004). However, the possible role of this class of transcriptional regulators in determining the endophytic interaction of a bacterium with its host is still unclear. Nevertheless, these regulators may function in response to the nutrients and small molecules that an endophyte finds in its surroundings when it comes in contact with cells inside of its host. Many of these regulators work in modulating carbohydrate metabolism, which is very important when cells enter the stationary phase of their growth. Moreover, some of the transcriptional regulators function in communication pathways that are critical for altering the behavior of an organism that switches its physiology when adapting from the soil to the plant environment. Generally speaking, these transcriptional regulators may work in specialized signaling cascades by triggering each other in a way that does not trigger the activation of any host defense system and therefore allows the bacterium to effectively colonize the plant tissues.

Four glutathione synthesis and reductase related genes were also identified (Table 2). The presence of these genes is consistent with a possible protective response of the bacteria under oxidative stress created as a consequence of the endophytic bacteria infecting a plant host. Glutathione is a secondary messenger and is a part of a complex regulatory network. The enzymes responsible for the transport of this molecule play an important role in the expression of the oxidative stress response defense systems (Storz and Imlay, 1999; Taghavi *et al*., 2010). The fact that this set of genes is present in all of

the bacterial endophytes studied as well as in *Enterobacter* sp. 638 is a strong indication of a possible relationship between endophytic behavior and the glutathione regulatory network. Additionally, when bacterial endophytes enter and start colonizing plants, they must circumvent a variety of defense mechanisms. One of these mechanisms is the production of reactive oxygen species. As a consequence of the production of reactive oxygen species by plants as well as the oxygen rich environment (photosynthetic) inside of plants cells, endophytes likely encounter an oxidative stress environment. Therefore, in order to effectively colonize their plant hosts, endophytes must be metabolically capable of surviving the reactive-oxygen rich environment. The presence of such a system in the bacterium could contribute to the ability of endophytes to detoxify some reactive oxygen species and may function as part of a bacterium's defense and survival strategy (Taghavi *et al*., 2010).

In addition to all of the above-mentioned genes, there are some general-purpose enzymes that are present in the list (Tables 2). They include dehydrogenases, synthases and hydratases (Table 2). Some of these gene products are likely involved in central metabolic pathways to generate energy, both at substrate level and at electron transfer chain level. Moreover, the presence of a large number (eight) of hydrogenases/dehydrogenases suggests their possible function in the transfer of protons (H^+) to and from various substrates during the transport of diverse substances across the membranes and in keeping the membrane redox potential constant during metabolism.

4 Conclusion

Genome comparison analysis is a powerful tool that may be used to predict the possible gene-related functions that are involved in switching lifestyles adapted by bacteria at different growth stages or/and in different environmental conditions. The predicted functions of genes and their role in determining the bacteria's endophytic behavior reveal one possible dimension of the multi-faceted study of plant-endophyte interactions. By definition, the list of putative endophyte genes only includes the common orthologs found in Proteobacterial endophytes. In addition, it is expected that each individual endophyte utilizes several more species-specific gene-functions in establishing an endophytic association with various host plants. Importantly, while the conclusions based on the genome comparison analysis that was conducted provide a number of intriguing possibilities in terms of identifying endophytic genes, the conclusions drawn from this approach need to be tested experimentally. This might be done by constructing and testing both over-producing and under-producing mutants of some of the genes identified as being involved in the endophytic functioning of a particular bacterial strain.