Two of the Mechanims Used by Bacteria to Modify the Environment: Quorum Sensing and ACC Deaminase

by

Youai Hao

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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 revisions, as accepted by my examiners.

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

Abstract

Quorum sensing (QS) cell-cell communication systems are utilized by bacteria to coordinate their behaviour according to cell density. Several different types of QS signal molecules have been identified, among which acyl-homoserine lactones (AHLs) produced by *Proteobacteria* have been studied to the greatest extent. QS has been shown to be involved in many aspects of bacterial life, including virulence, bioluminescence, symbiosis, antibiotic production, swarming and swimming motility, biofilm formation, conjugation and growth inhibition. Although QS has been studied extensively in cultured microorganisms, little is known about the QS systems of uncultured microorganisms and the roles of these systems in microbial communities. To extend our knowledge of QS systems and to better understand the signalling that takes place in the natural environment, in the first part of this thesis, isolation and characterization of new QS systems from metagenomic libraries constructed using DNA from activated sludge and soil were described. Using an *Agrobacterium* biosensor strain, three cosmids (QS6-1, QS10-1 and QS10-2) that encode the production of QS signals were identified and DNA sequence analysis revealed that all three clones encode a novel *luxI* family AHL synthase and a *luxR* family transcriptional regulator. Thin layer chromatography revealed that these LuxI homolog proteins are able to synthesize multiple AHL signals. Tandem mass spectrometry analysis revealed that $LuxI_{QS6-1}$ directs the synthesis of at least three AHLs, 3-O-C14:1 HSL, 3-O-C16:1 HSL and 3-O-C14 HSL; $LuxI_{QS10-1}$ directs the synthesis of at least 3-O-C12 HSL and 3-O-C14 HSL; while $LuxI_{QS10-2}$ directs the synthesis of at least C8 HSL and C10 HSL. Two

possible new AHLs, C14:3 HSL and (?)-hydroxymethyl-3-O-C14 HSL, were also found to be synthesized by $LuxI_{QS6-1}$.

Agrobacterium tumefaciens is a plant pathogen that causes crown gall disease. Its ability to transfer and integrate foreign DNA into plant genome also makes it a useful tool for plant genetic engineering. Ethylene, the gaseous plant hormone, has been reported to be important for both crown gall development and *A. tumefaciens* mediated transformation efficiency to plants. ACC deaminase, an enzyme that can break down ACC, the direct precursor of ethylene biosynthesis in plants, is a mechanism used by some plant growth promoting bacteria (PGPB) to promote plant growth by reducing stress ethylene levels. In the second part of this thesis, the effect of ACC deaminase on *A. tumefaciens* induced crown gall development and on *A. tumefaciens* mediated transformation efficiency was studied. By either introduction of an ACC deaminase encoding gene into the virulent strain *A. tumefaciens* C58 or co-inoculation of *A. tumefaciens* C58 with an ACC deaminase containing PGPB *P. putida* UW4, using different plant systems including tomato plants and castor bean plants, it was found that the presence of an ACC deaminase significantly inhibited crown gall development. It was also found that introduction of an *acdS* gene into the disarmed *A. tumefaciens* strain GV3101::pMP90 reduced the ethylene levels evolved by plants during infection and cocultivation process and increased the transformation efficiency of commercialized canola cultivars. The *A. tumefaciens* D3 strain was reported to contain an ACC deaminase encoding gene (*acdS*). In this study it was determined that this strain is an avirulent strain and shows plant growth promoting activity. When co-inoculated with *A. tumefaciens* C58 on castor bean stems, both the wild type and the

acdS knockout mutant showed biocontrol activity and were able to significantly inhibit crown gall formation, with the wild type strain showing slightly better tumor inhibition effects. The mutation of *acdS* and its regulatory gene *lrpL* in *A. tumefaciens* D3 was also found to affect QS signal production of this strain, which indicates a cross talk between the two sets of genes.

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List of Abbreviations

ACC, 1-aminocyclopropane-1-carboxylic acid

ACP, acyl carrier protein

AHL, acyl homoserine lactone

AI-2, autoinducer 2

Ap, ampicillin

AVG, aminoethoxyvinylglycine

CFU, colony forming units

Cm, chloromphenical

CTAB, cetyl trimethylammonium bromide

EDTA, ethylenediaminetetraacetic acid

ESI MS, electrospray ionization mass spectrometry

GC, gas chromatography

Gm, gentamycin

HTH, helix turn helix

Km, kanamycin

MCP, 1-methylcyclopropane

ML, maximum likelihood

MS/MS, tandem mass spectrometry

MTA, 5-methylthioadenosine

OA, organogenesis medium A

OB, organogenesis medium B

PGPB, plant growth promoting bacteria

QS, quorum sensing

PPT, phoshinotricin

Rf, rifampicin

SAM, S-adenosylmethionine

Sp, spectinomycin

Sm, streptomycin

Tc, tetracycline

TLC, thin layer chromatography

Claims of contributions to knowledge

- Using the *Agrobacterium tumefaciens* HC103(pJZ381) quorum sensing (QS) biosensor strain, three new QS systems, QS6-1, QS10-1 and QS10-2 were isolated from metagenomic libraries containing total community DNA from either activated sludge or from soil.
- With the help of an *in vitro* transposition insertion mutagenesis system, about 7 kb of DNA sequences from each of the above mentioned clones were obtained, characterized and analyzed.
- Sequence analysis revealed that each clone contains a *luxI* type acyl homoserine lactone (AHL) synthesis gene and a *luxR* type transcriptional regulator gene located adjacent to one another.
- Transposon insertion mutagenesis revealed that mutation of the *luxR* homologs as well as mutation of the *luxI* homologs changes the behaviour of the metagenomic clones QS10-1 and QS10-2 so that they are no longer able to induce the biosensor strain to form blue colonies. This implies that similar to what has been observed in many LuxR/LuxI type QS systems, the two *luxI* homolog genes are regulated by their cognate LuxR homolog proteins.
- Possible *lux*-box like elements were identified upstream of $luxI_{QS6\text{-}1}$, $luxI_{QS10\text{-}1}$ and $luxR_{QS10\text{-}2}$. It is very likely that $luxR_{QS10\text{-}2}$ and $luxI_{QS10\text{-}2}$ and the two downstream genes encoding phytanoyl-CoA dioxygenase and a GntR family transcriptional regulator form an operon which is QS regulated.

- Phylogenetic analysis of the sequenced DNA fragments revealed that the genes encoded within QS6-1 and QS10-1 are closely related and are most probably from *β* or *γ proteobacteria,* while QS10-2 is most probably from *α proteobacteria*.
- The three *luxI* homologs were subcloned and expressed in *E. coli*. The *luxI* homolog from QS6-1 was also subcloned and then expressed in *A. tumefaciens* HC103(pJZ381) under the transcriptional control of the *lac* promoter. The active signal compounds were extracted from the culture supernatants of different *A. tumefaciens* or *E. coli* strains expressing corresponding *luxI* homologs. Analytical thin layer chromatography (TLC) assay was performed to separate the signals. It was found that each LuxI homolog was able to direct synthesis of multiple AHLs.
- The active signals were partially purified from TLC plates and then electrospray ionization mass spectrometry (ESI MS) and tandem MS were performed to elucidate the structures of the signal molecules synthesized by each LuxI homolog.
- It was found that $LuxI_{QS6-1}$ was able to direct the synthesis of 3-O-C14 HSL, 3-O-C14:1 HSL, 3-O-C16:1 HSL and two new AHLs, C14:3 HSL and (?)-hydroxymethyl-3-O-C14 HSL; $LuxI_{QS10-1}$ was able to direct the synthesis of 3-O-C12 HSL and 3-O-C14 HSL; and $LuxI_{QS10-2}$ was able to direct synthesis of C8 HSL and C10 HSL.
- When the three *luxR* homologs were expressed in *E. coli,* it was found that the presence of the cognate AHLs could significantly increase the solubility of $LuxR_{QS10-1}$, suggesting that the $LuxR_{QS10-1}$ requires the binding of the cognate AHLs for proper folding. $LuxR_{QS6-1}$ and $LuxR_{QS10-2}$ were mainly expressed in an insoluble form regardless of the presence or absence of the cognate AHLs.

- This is only the second report of the successful isolation of new QS systems from environmental samples using functional metagenomics. Compared to the first report (Williamson *et al.*, 2005) in which only one QS clone (QS1) has been isolated, here three clones not closely related to any known species producing several unique AHL structures were identified.
- It has been reported that *A. tumefaciens* infection induces ethylene levels in the crown gall tumor and that this increased level of ethylene plays an important role in crown gall development. Some plant growth promoting bacteria contain an enzyme, ACC deaminase, which can reduce the level of ACC, the immediate precursor in ethylene biosynthesis and therefore reduce the ethylene level in plants. When an ACC deaminase encoding gene from *Pseudomonas putida* UW4 under the transcriptional control of the *E. coli lac* promoter was introduced into the plant pathogen *A. tumefaciens* C58, it was found that compared to wild type *A. tumefaciens* C58, crown gall tumor development on castor bean plants induced by the strain expressing ACC deaminase was significantly inhibited. This indicates that the presence of ACC deaminase reduces the pathogenicity of *A. tumefaciens* C58.
- Co-inoculation of *A. tumefaciens* C58 with wild-type *P. putida* UW4 (which produces ACC deaminase) showed a much greater amount of crown gall inhibition than when *A. tumefaciens* C58 was co-inoculated with an *acdS* knockout mutant of *P. putida* UW4. This again emphasizes the role that ACC deaminase plays in the inhibition of crown gall development.
- Compared to inoculation with *A. tumefaciens* C58 alone, co-inoculation with *A. tumefaciens* C58 and the *acdS* knockout mutant strain of *P. putida* UW4 also

- An *A. tumefaciens* D3 strain was previously reported to contain a putative *acdS* gene. In this study, the ACC deaminase activity of this strain was characterized and was found to have an activity that is about 1/5th that of *P. putida* UW4 and about 4 times that of *Rhizobium leguminosarum* bv. viciae 128Sm.
- Using primers designed according to the conserved regions of the Ti or Ri plasmid, it was found that *A. tumefaciens* D3 strain does not contain a Ti or Ri plasmid. Virulence assays using carrot discs, tomato stems, castor bean stems, *Arabidopsis* stems, tobacco stems and canola stems all showed that *A. tumefaciens* D3 does not induce either tumors or hairy roots on these plants. These results indicate that *A. tumefaciens* D3 is an avirulent strain.
- An *acdS* and *lrpL* double mutant strain of *A. tumefaciens* D3 was constructed by replacement of the C-terminal fragments of both genes with a tetracycline resistance gene. The mutant strain showed no ACC deaminase activity. Complementation of either *lrpL* or *acdS* or both *lrpL* and *acdS* revealed that consistent with what has been observed with numerous other species, the *lrpL* gene is the regulator of the *acdS* gene.
- IAA and siderophore production assays performed on wild type and mutant strains of *A. tumefaciens* D3 revealed that the two strains showed similar IAA and siderophore production activity.

- *A. tumefaciens* D3 was found to produce an AHL QS signal that behaved similarly to C6 HSL, however, the AHL production of the *A. tumefaciens* D3 *acdS lrpL* mutant strain was severely reduced. Complementation of *lrpL* or *acdS* or both *lrpL* and *acdS* results in strains that produce an AHL that is different from that produced by the wild-type *A. tumefaciens* D3.
- Gnotobiotic root elongation assay using canola revealed that *A. tumefaciens* D3 promotes root elongation while the mutant strain does not.
- Co-inoculation of *A. tumefaciens* D3 with pathogenic *A. tumefaciens* C58 indicated that although *A. tumefaciens* D3 has a lower level of ACC deaminase activity than *P. putida* UW4, it showed better biocontrol activity. With a 1:1 cell ratio of *A. tumefaciens* D3 and *A. tumefaciens* C58, crown gall development was almost totally inhibited.
- Co-inoculation of the *acdS* knockout mutant strain of *A. tumefaciens* D3 with *A. tumefaciens* C58 also showed some crown gall tumor inhibition effects, indicating that besides the role of ACC deaminase other factors in *A. tumefaciens* D3 can affect *A. tumefaciens* C58 growth or pathogenicity.
- The effect of ACC deaminase on *A. tumefaciens* mediated transformation efficiency was evaluated using three commercial canola cultivars. Of the three infection dilutions used (OD_{600}=1; OD_{600}=0.1, OD_{600}=00.1), the optimal infection concentration of *A. tumefaciens* that yielded the highest transformation frequency for the cultivars *Brassica napus* cv. Westar and *B. napus* cv. 4414RR was found to be the OD_{600}=1 dilution, and optimal infection concentration of *A. tumefaciens* for the cultivars *B. napus* cv. Hyola 401 was found to be the OD_{600}=0.1 dilution. Under

- This is also the first transformation efficiency assay of the two important commercial canola cultivars *B. napus* cv. 4414RR and *B. napus* cv. Hyola 401 that are widely grown in North America, providing an important reference for future genetic modification of these cultivars.

Chapter 1 Overview of Projects

Bacteria are ubiquitous on earth and can be found in almost any environment, ranging from soil to water, from hot springs to radioactive waste, from organic matter to live bodies of plants and animals, and even deep in the earth's crust. It has been estimated that in one gram of a typical soil sample, there are about 40 million bacterial cells (Whitman et al, 1998); within an average healthy adult human body, the bacteria cell numbers are ten times the number of human cells (Sears, 2005). To date, the vast majority of bacteria have not been characterized, and less than 1% of the bacteria in most environments, which are from only half of the bacterial phyla, can be cultured in current laboratory conditions (Rappé et al., 2003).

Bacteria play fundamental roles in the biogeochemical cycles of earth, including the nitrogen cycle, the carbon cycle, the sulphur cycle, the phosphorus cycle and the oxygen cycle, with many steps of these cycles depending on the functioning of bacteria. For example, bacteria provide essential functions in the nitrogen fixation, nitrification, denitrification and ammonification steps of the nitrogen cycle. Bacteria are also important for human beings. Some bacteria are plant or animal pathogens, while others are beneficial for plants or animals. Moreover, bacterial interactions with other members of the same species, with other species of bacteria, and with organisms from other kingdoms, including plants and animals, are important for their survival and function.

Within or between bacterial species, bacteria can communicate with each other by producing, releasing and detecting small diffusible chemical molecules into the environment. By detecting the concentration of the signal molecules in the environment,

bacteria can keep track of the population density of their own members and other species and thereby coordinate their behaviour accordingly. This cell-cell communication system of bacteria is called quorum sensing (QS). QS has been found in many of the cultured bacterial species, including both gram positive and negative species. Different types of signal molecules have been identified. Gram negative bacteria can produce and utilize acyl homoserine lactones (AHL), 2-alkyl-4-quinolones, long-chain fatty acids and fatty acid methyl esters as signal molecules; Gram positive bacteria can use linear, modified or cyclic peptides as signal molecules; while *Streptomycetes* synthesize c-butyrolactones such as A-factor as QS signals (Williams, 2007). Another type of QS signal is autoinducer 2 (AI-2), which is a group of interconvertible furanones derived from dihydroxypentanedione (DPD) (the product of the LuxS protein) and has been found to be synthesized by both Gram positive and negative bacteria and may be used for interspecies communication. As a mechanism of bacteria-bacteria interaction, QS has been reported to regulate a wide range of functions, including bioluminescence, biofilm formation, swimming and swarming motility, antibiotic production, exopolysaccharide (EPS) production, symbiosis, virulence of pathogenic organisms, pigmentation and sporulation (Diggle *et al*., 2007).

At the present time, scientists do not know how to culture the majority of bacteria, thus it is possible that novel QS systems and signals are present among these uncultured bacteria. In this study, using an *Agrobacterium tumefaciens* QS biosensor strain HC103(pJZ381), metagenomic libraries previously constructed using total DNA from activated sludge or from soil (Wang *et al*., 2006) were screened for novel QS systems.

Isolation and characterization of several new LuxR/I type QS systems are presented in the first part of the thesis.

Bacteria can also form complex association with other organisms, such as plants and animals. These relationships can be characterized as parasitism, mutualism or commensalism. Bacteria are correspondingly called pathogens, mutualists or commensals. Commensal bacteria have no effect on other organisms; pathogenic bacteria have an adverse effect on the organisms they associate with; and mutualists form a relationship with other organisms that is beneficial for both partners. Plant associated mutualist bacteria are called plant growth promoting bacteria (PGPB) which can promote plant growth using either direct or indirect mechanisms. Indirect mechanisms include the production of antibiotics to inhibit growth of plant pathogens, competition with plant pathogens and induction of plant systemic acquired resistance (Haas and Defago, 2005; van Loon *et al.*, 2006). Direct mechanisms include production of plant growth hormones; nitrogen fixation; production of growth modulating enzymes; and enhancement of the acquisition of nutrients such as phosphorus, nitrogen and iron (Glick, 1995; Glick *et al.*, 1999). One direct mechanism that is utilized by many PGPB is the lowering of ethylene levels in plants by the production of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase.

Biotic and abiotic stresses such as pathogen attack, insect damage, mechanical wounding, drought, flooding, high salt and the presence of organic contaminants and heavy metals, induces the production of ethylene. This induced ethylene may exacerbate some of the effects of various stresses by initiating a senescence response and is called stress ethylene. ACC deaminase-containing PGPB can attach to plant tissues and break

down ACC, the direct precursor of ethylene biosynthesis in plants, into ammonia and α-ketobutyrate which it can use as a nitrogen and carbon source. As a result, less ACC is available for conversion to ethylene in plants and the stress symptoms of plants are relieved.

Agrobacterium tumefaciens is a plant pathogen that causes crown gall disease. It can transfer and integrate a strand of DNA (T-DNA) containing oncogenic genes (indole-3-acitic acid (IAA) and cytokinin biosynthesis genes) and opine synthesis genes. Expression of the oncogenic genes in transformed plant cells results in the production of elevated levels of the plant hormones IAA and cytokinin, and as a consequence, induces unlimited cell division and forms tumor like tissue on the infection site of the plants called crown galls. The transformed plant cells also synthesize opine which can be used by *A. tumefaciens* as a carbon and energy source. The level of another plant hormone, ethylene is also enhanced in infected plant tissues. This increased level of ethylene plays an important role in crown gall development and it has been reported that application of ethylene inhibitors can inhibit crown gall development (Aloni *et al.*, 1998; Wächter *et al.*, 1999). In the second part of this thesis, the possibility of using ACC deaminase or ACC deaminase-containing PGPB to control *A. tumefaciens* induced crown gall disease was investigated. An ACC deaminase encoding gene, *acdS*, from *Pseudomonas putida* UW4 was introduced into *A. tumefaciens* C58, and the pathogenicity of the resulting strain was studied using carrot discs, tomato plants and castor bean plants. ACC deaminase-containing *P. putida* UW4 and *A. tumefaciens* D3, and their corresponding *acdS* knockout mutants were also co-inoculated with *A. tumefaciens* C58 in tomato plants

and/or castor bean plants, and the tumor inhibition effects of the different strains were evaluated by comparison of the tumor size and tumor fresh weight.

The ability of *A. tumefaciens* to transfer foreign DNA into the plant genome also makes it a very useful tool for plant genetic engineering. The oncogenic genes that are responsible for the pathogenicity of *A. tumefaciens* are not necessary for the transfer of T-DNA. By modification of the Ti plasmid, including removing the oncogenic genes and opine synthesis genes, adding plant and bacteria selectable markers and multiple cloning sites, different disarmed *A. tumefaciens* strains (*A. tumefaciens* strains that are no longer virulent) and Ti plasmids have been developed for the introduction of foreign genes into plant genomes. *A. tumefaciens* mediated transformation has many advantages and has been widely used. However, for most commercial cultivars of crops, the transformation efficiency is very low thereby limiting its use. Among different factors that affect the transformation efficiency, ethylene inhibits gene delivery efficiency as well as plant regeneration frequency (Chakrabarty *et al.*, 2002; Burgos and Alburquerque, 2003; Han *et al.*, 2005; Petri *et al.*, 2005; Seong *et al.*, 2005). In this thesis, the effect of ACC deaminase on *A. tumefaciens* mediated transformation efficiency of commercial canola cultivars has also been studied.

Chapter 2 Isolation and Characterization of Novel Quorum Sensing Systems from Metagenomic Libraries

2.1 Introduction

2.1.1 Quorum sensing

Quorum sensing (QS) is a term used to describe the cell-cell communication of bacteria. QS bacteria can produce and secrete into the environment small hormone-like chemical molecules. As the population density increases, the signal molecules accumulate, and when a certain threshold concentration is reached, bacteria can respond to them and alter gene expression and thus cell behaviour. QS was first described in the marine bacterium *Vibrio fischeri* (Nealson and Hastings, 1979) in 1979. *Vibrio fischeri* is a proteobacterial organism that can live freely in seawater as well as in symbiosis with certain fish or squid. In the symbiotic state, this organism colonizes the specialized light organs of the host and is responsible for the production of luminescent light. While the host provides nutrition for the bacteria, the luminescent light produced by the bacteria may help hide the shadows of the hosts on moonlit nights and protects them from predators. Studies of the molecular mechanism of bioluminescence of *Vibrio fischeri* revealed the presence of a LuxI-LuxR two component regulatory system. The LuxI protein can synthesize the small signal molecule N-acyl-homoserine lactone (AHL) 3-oxo-C6 HSL, which can diffuse outside of the cell membrane. At low cell density, the AHL synthesis is limited. As the population density increases, the AHL molecules in the environment accumulate, and when a certain threshold concentration is reached, the AHLs can bind to the LuxR protein and the LuxR/AHL complex can activate the transcription of the *lux* operon thereby producing light. Since this system can detect the population density in the environment, it is called quorum sensing (QS) (Figure 2.1).

To date, QS has been found in many bacteria, including both *Proteobacteria* and

Firmicutes. QS systems that are homologous to the *Vibrio fischeri* LuxI-LuxR system have been experimentally identified and studied in more than 40 different *Proteobacteria* species (Table 2.1). Moreover, genome sequencing projects revealed that many more *Proteobacteria* may contain this type of QS system. In this system, the LuxI homolog is responsible for the synthesis of AHL signal molecules, while the LuxR homolog is an AHL dependent transcriptional regulator. Although all AHLs contain a core of the homoserine lactone ring, the acyl chain length, the degree of saturation of the acyl chain and the substitution position C3 determine the specificity of the AHL molecules (Figure 2.2). Usually, each LuxR homolog can detect the AHLs produced by the cognate LuxI homolog, making the AHL dependent cell-cell communication system resonably specific. In many LuxI-LuxR type QS systems, the AHLs can drive their own production via an amplification loop leading to the AHL synthesis gene, the *luxI* homolog gene. Thus, the AHLs are sometime called autoinducers (Figure 2.3). QS has also been found in many *Firmicutes* including *Staphylococcus* species and *Enterococcus* species. However, rather than using AHLs, Gram-positive bacteria use modified oligopeptides as signal molecules (Cangelosi *et al.*, 1991; Booth *et al.*, 1996; Mayville *et al.*, 1999; Nakayama *et al.*, 2001). Another type of QS signal molecule, autoinducer 2 (AI-2), which was first identified in *Vibrio harveyi*, includes a family of interconvertible furanones derived from the same precursor molecule, the LuxS product, 4,5-dihydroxy-2,3-pentanedione (DPD). It has been reported to be produced by both Gram-positive and Gram-negative bacteria and is believed to be used for interspecies communication (Chen *et al.*, 2002; Xavier and Bassler, 2003).

QS allows bacteria to monitor the changes in the population number and/or community composition and respond to it. It enables the bacteria to communicate with each other and (to an extent) mimic multi-cellular organisms. It is involved in many aspects of bacterial life, including bioluminescence, virulence, symbiosis, antibiotic production, swarming and swimming motility, biofilm formation, conjugation and growth inhibition (Table 2.1). Quorum sensing influences a significant portion of bacterial transcriptome (4%-10%) and proteome (more than 20%) (Dunman *et al.*, 2001; Arevalo-Ferro *et al.*, 2003; Schuster *et al.*, 2003; Wagner *et al.*, 2003) and is now considered to be fundamental to the functioning of bacterial strains.

2.1.2 Quorum sensing in soil bacteria

Quorum sensing has been found in many soil bacteria and has been shown to play important roles in plant-microbe interactions. QS systems have been found in many phytopathogenic bacteria including *Agrobacterium tumefaciens, Agrobacterium vitis, Agrobacterium rhizogenes, Pectobacterium carotovorum*, *Pantoea stewartii, Xanthomonas campestris and Ralstonia solanacearum,* and regulate the virulence of those phytopathogenic bacteria, albeit using different mechanisms. QS systems have been implicated in controlling genes involved in major pathogenicity factors, such as exopolysaccharides (EPS), type III secretion system, and exoenzyme production, as well as in regulating genes involved in epiphytic fitness of the plant pathogen by the production of antibiotics or an increase of UV light resistance (Sharma *et al.* 2003; von Bodman *et al.* 2003). QS systems have also been characterized in many plant growth promoting bacteria (PGPB) including *Burkholderia cepacia*, *Pseudomonas aureofaciens*,

and *Pseudomonas fluorescens* (Lewenza *et al.* 2003; Pierson *et al.*, 1994; Wood *et al.*, 1997). For example, QS has been shown to control protease and siderophore production in *B. cepacia* (Lewenza *et al.* 2003), and the production of phenazine antibiotics which inhibit *Gaeumannomyces graminis var. tritici*, the causative agent of wheat take-all disease, in *P. aureofaciens* 30-84 and *P. fluorescens*2-79 (Pierson *et al.*, 1994; Wood *et al.*, 1997). QS systems have also been found in rhizobial species including *Rhizobium etli*, *Rhizobium leguminosarum* and *Sinorhizobium meliloti*. Furthermore, mutant analysis showed that QS in rhizobia plays an important role in the symbiotic process (Gray *et al.*, 1996; Rosemeyer *et al.*, 1998; Lithgow *et al.*, 2000; Gurich and Gonzalez, 2009).

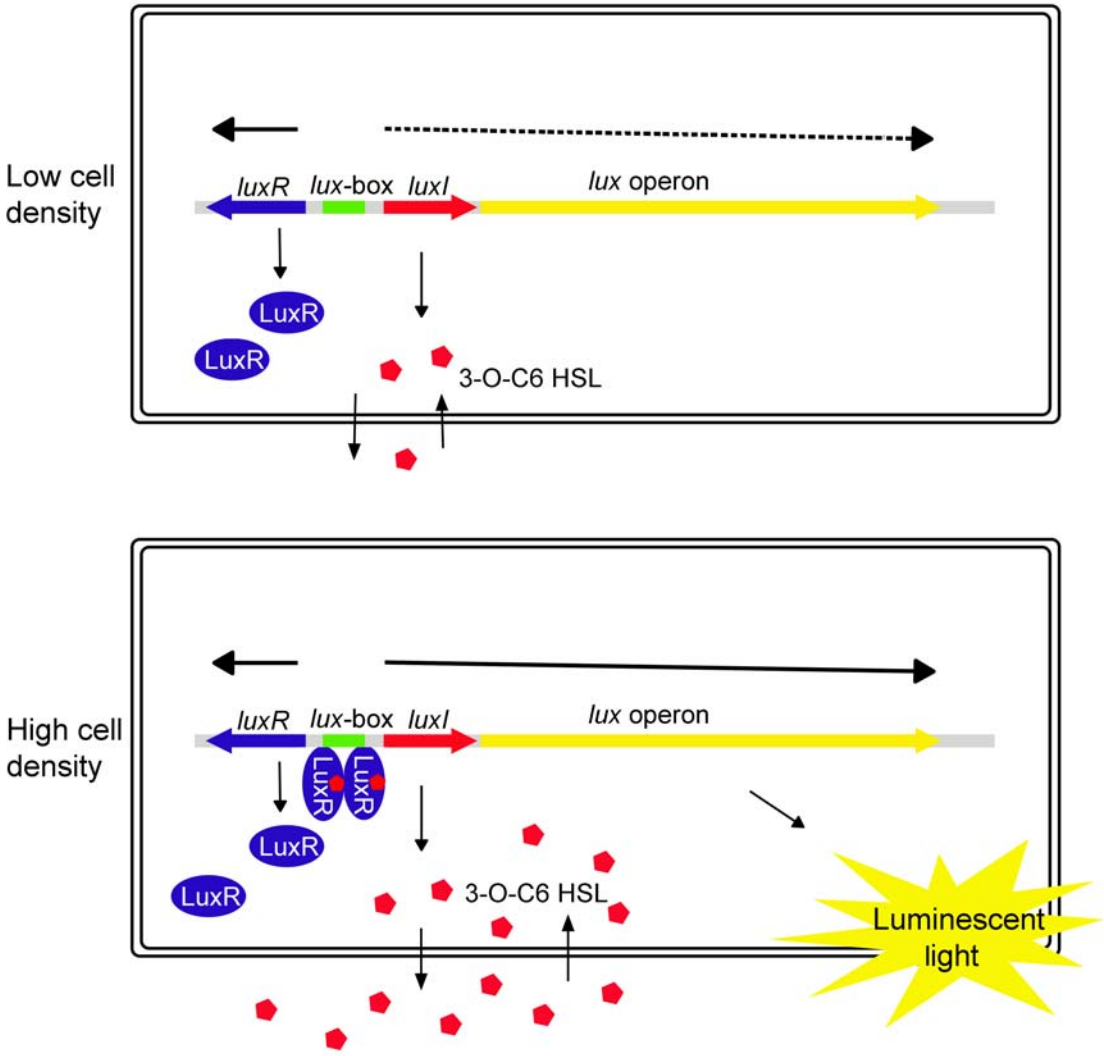


Figure 2.1. LuxI and LuxR QS system in *Vibrio fischeri.* At low cell density, the LuxI protein is expressed at low level and is able to direct synthesis of only small amount of 3-O-C6 HSL. The concentration of the 3-O-C6 HSL in the environment is low and is not able to activate LuxR protein. The *lux* operon is not expressed, so there is no light production. At high cell density, the concentration of the 3-O-C6 HSL in the environment and inside the cell is high and can bind to LuxR protein and activate it. The activated LuxR protein binds to the *lux* box region and activates the transcription of *lux* operon. As a result luminescent light is produced.

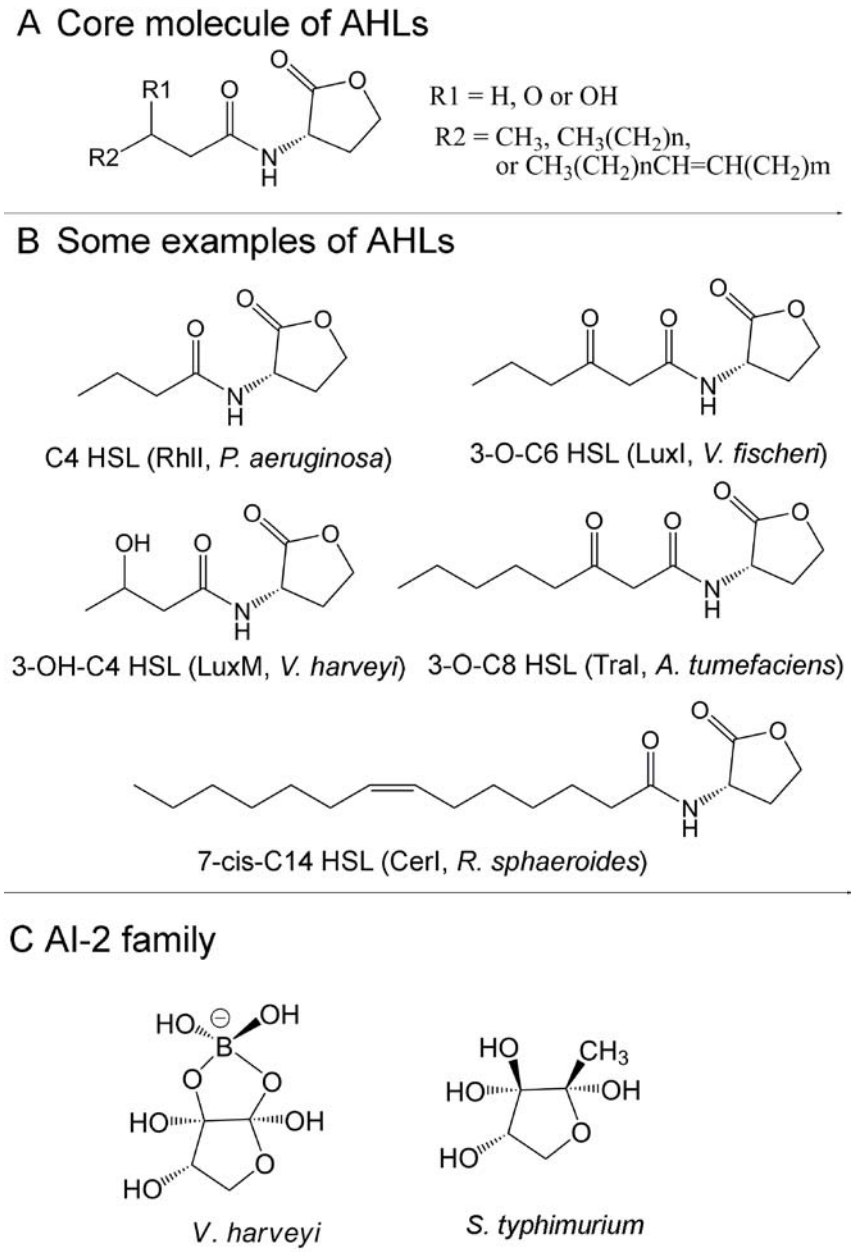


Figure 2.2. Structure of QS signal molecules. A, the core molecule of AHLs. B, some examples of AHLs synthesized by different bacteria. C, two examples of AI-2 family signals. Structures were drawn using ChemBioDraw Ultra 11.0 software (http://www.cambridgesoft.com/software/ChemBioDraw/).

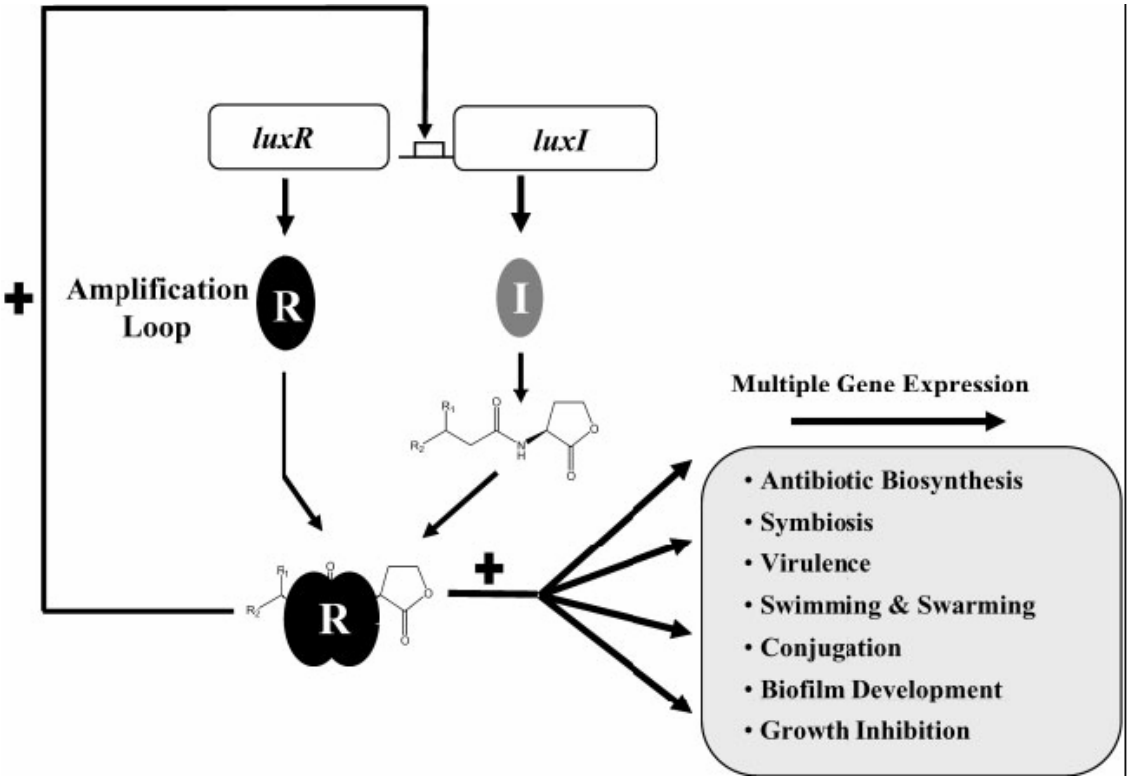


Figure 2.3. A model of AHL-mediated QS systems. The *luxI* and *luxR* genes are homologs of the *V. fischeri* AHL synthase and AHL receptor genes, respectively. The *luxI* homolog gene encodes the I protein which is an AHL synthase, while the *luxR* homolog gene encodes the R protein which is an AHL dependent transcriptional regulator. After binding to an AHL, the R protein is activated and regulates different target genes (including those involved in antibiotic production, symbiosis and virulence) in different bacterial species. In many (but not all) AHL-dependent QS systems, the AHLs can drive their own production via an amplification loop leading to the AHL synthesis gene, the *luxI* homolog gene. (Picture is from Williams, 2007)

Table 2.1. Some QS systems that have been studied experimentally.

Organisms	Name	Protein ID	AHL Products	Function of the QS system	Major reference
Aeromonas hydrophila	AhyI/ AhyR	ABD59318/ ABD59317	C4	Extracellular protease, biofilm formation	Swift *et al.*, 1997; Swift *et al.*, 1999b
Aeromonas salmonicida	AsaI/ AsaR	P70774/ ABO91724	C4	Extracellular protease	Swift *et al.*, 1997
Agrobacterium. tumefaciens C58	TraI/ TraR	P33907/ P54294	3-O-C8	Conjugation	Piper *et al.*, 1993; Hwang *et al.*, 1994; Li *et al.*, 1998; Piper *et al.*, 1999
Agrobacterium. tumefaciens R10	TraI/ TraR	AAB95104/ AAC28121	3-O-C8	Conjugation	Fuqua and Winans, 1994; Fuqua *et al.*, 1994
Agrobacterium vitis F2/5	AvsI/ AvsR	AAY97862.1/ AAY97861.1	C14:1, 3-O-C16:1	Virulence	Hao and Burr, 2006
Acidithiobacillus ferrooxidans	AfeI/ AfeR	AAZ20805.1/ AAV53702.2	3-OH-C8, 3-OH-C10, 3-OH-C12, C12, C14, 3O-C14, 3-OH-C14, 3-OH-C16	Biofilm formation	Rivas *et al.*, 2005
Azospirillum lipoferum TVV3	AlpI/ AlpR	ABD97989/ ABD97988	3-O-C8, C8, 3-O-C10, 3-OH-C10, C10	unknown	Vial *et al.*, 2006
Burkholderia cepacia	CepI/ CepR	Q9ZIU1/ Q9ZIU0	C8	Protease, siderophore	Lewenza *et al.*, 1999
Burkholderia. glumae	TofI/ TofR	CAM12357/ CAM12358	C6, C8	Production of lipid A and phytotoxin (toxoflavin)	Kim *et al.*, 2004; Devescovi *et al.*, 2007
Burkholderia mallei ATCC 23344	Bmal/ BmaR1	YP_106161/ YP_102421.1	C8	Virulence	Duerkop *et al.*, 2007
Burkholderia. vietnamiensis	BviI/ BviR	ABK32015.1/ ABK32016.1	C10	Unknown	Conway and Greenberg, 2002; Malott and Sokol, 2007

Burkholderia pseudomallei pp844	BpsI/ BpsR	AAM21707/ AAS90557	C8, 3-O-C8, C10, 3-OH-C10, 3-OH-C12	*dpsA* and the oxidative stress response	Lumjiaktase *et al.*, 2006
Chromobacterium violaceum	CviI/ CviR	AAP32920.1/ AAP32919.1	C6	Exoenzymes, antibiotics, cyanide, violacein	McClean *et al.*, 1997
Enterobacter agglomerans	EagI/ EagR	P33881/	3-O-C6	Unkown	Swift *et al.*, 1993
Erwinia. chrysanthemi	EchI/ EchR	Q46968/ Q46967	3-O-C6	Pectinases	Nasser *et al.*, 1998
Escherichia coli	/SdiA	/P07026		Cell division, attaching and effacing lesion formation	Sitnikov *et al.*, 1996
Mesorhizobium tianshanense	MrtI/ MrtR	AAZ32755/ AAZ32754	Different strains produce different AHLs	Symbiotic process(root hair adherence and root nodule formation)	Zheng *et al.*, 2006 and *Cao et al*. 2009
Methylobacterim extorquens	MsaI	ABI17430.1	C14:1, C14:2	Unknown	Nieto Penalver *et al.*, 2006
Pectobacterium carotovorum ssp. carotovora	CarI/ CarR	P33880/ AAC45995	3-O-C6	Carbapenem antibiotic, exoenzymes	Swift *et al.*, 1993; McGowan *et al.*, 1997; McGowan *et al.*, 1998
P. carotovorum ssp. carotovora	ExpI/ ExpR	P33882/ CAA56646	3-O-C6	Exoenzymes	Pirhonen *et al.*, 1993; Andersson *et al.*, 2000
Pantoea stewartii	EsaI/ EsaR	P54656/ P54293	3-O-C6	Exopolysaccharide	Beck von Bodman and Farrand, 1995
Pseudomonas aeruginosa PAO1	LasI/ LasR	P33883/ AAG04819	3-O-C12	Exoenzymes, Xcp, biofilm formation, RhlR, cell-cell spacing.	Gambello and Iglewski, 1991
Pseudomonas. aeruginosa PAO1	RhlI/ RhiR	P54291/ P54292	C4	Exoenzymes, cyanide, lectins, pyocyanin, rhamnolipid, type 4 pili.	Winson *et al.*, 1995; Latifi *et al.*, 1996
Pseudomonas aureofaciens	PhzI/ PhzR	AAC41535/ AAA21841	C6	Phenazine antibiotic	Pierson *et al.*, 1994; Wood *et al.*, 1997

Pseudomonas fluorescens 2-79	PhzI/ PhzR	Q51785/ Q51786	Unknown	Phenazine antibiotic	Shaw *et al.*, 1997
P. fluorescens 2P24	PcoI/ PcoR	AAT42217.1/ AAT42219.1	3-O-C6, 3-O-C8	Root colonization and suppress of plant disease	Wei and Zhang, 2006; Yan *et al.*, 2009a; Yan *et al.*, 2009b
P. fluorescens NCIMB 10586	MupI/ MupR	AAK28505.1/ AAK28504.1	C6, C10, C14:1	Mupirocin production	El-Sayed *et al.*, 2001; Anastasakis and Antsaklis, 2008
Pseudomonas. putida IsoF	PpuI/ PpuR	AAM75411.1/ AAM75413.1	3-O-C6, 3-O-C8, 3-O-C10	Influences biofilm structural development	Elasri *et al.*, 2001; Steidle *et al.*, 2002; Dubern *et al.*, 2006
Pseudomonas. syringae pv. tabaci	PsyI/ PsyR	P52990/ Q52408	C6, 3-O-C6	Unknown	Swift *et al.*, 1999a
Ralstonia solanacearum	SolI/ SolR	O30920/ O30919	C8	Unknown	Flavier *et al.*, 1997
Rhizobium etli	RaiI/ RaiR	O54451/ O54452	Unknown	Restriction of nodule number	Rosemeyer *et al.*, 1998
Rhizobium leguminosarum bv. viciae.	CinI/ CinR	AAF89990/ Q03316	3-OH-7-*cis*-C14	Nodulation, bacteriocin, stationary phase survival	Gray *et al.*, 1996; Lithgow *et al.*, 2000
Rhodobacter capsulatus	GtaI/	RRC03805/	C14 HSL, C16	Regulate gene transfer agent production	Schaefer *et al.*, 2002
Rhodobacter sphaeroides	CerI/ CerR	O30761/ O30760	7-*cis*-C14	Community escape (Dispersal from aggregates)	Puskas *et al.*, 1997
Serratia liquefaciens	SwrI/ SwrR	P52989/	C4 and C6	Swarming, protease, T1SS	Eberl *et al.*, 1996; Givskov *et al.*, 1997; Lindum *et al.*, 1998; Riedel *et al.*, 2001
Serratia plymuthica	SphI/ SphR	AAW27921/ AAW27922	3-O-C6 (major), C6, C4	Production of antibiotic pyrrolnitrin	Liu *et al.*, 2007

Sinorhizobium meliloti 1021	TraI/ TraR	AAR19278.1/ AAR19282.1	different strains produce different AHLs	Plasmid transfer	Marketon and Gonzalez, 2002
S.meliloti 1021	SinI/ SinR	Smc00168/ Smc00170	3-O-C14, C16, 3-O-C16, C16:1, 3-O-C16:1, C18	Exopolysaccharide II synthesis, swarming	Marketon *et al.*, 2003; Gao *et al.*, 2005
Vibrio anguillarum	VanI/ VanR	P74945/ P74946	3-O-C10	Unknown	Milton *et al.*, 1997
Vibrio. fischeri	LuxI/ LuxR	CAA68562/ CAA68561	3-O-C6	Bioluminescence	Engebrecht and Silverman, 1987
Yersinia enterocolitica	YenI/ YenR	P52988/ P54295	3-O-C6, C6	Unknown	Throup *et al.*, 1995
Yersinia pestis	YpeI/ YpeR	NP_404601/ NP_404602	3-O-C8, 3-O-C6	Unknown	Kirwan *et al.*, 2006
Yersinia pseudotuberculosis	YpsI/ YpsR	Q9XDD0/ Q9XDD1	3-O-C6	Motility, clumping	Atkinson *et al.*, 1999
Yersinia pseudotuberculosis	YtbI/ YtbR	AAC28704.2/ AAC28703.2	C8, 3-O-C6, C6	Unknown	Atkinson *et al.*, 1999
Yersinia ruckeri	YukI/ YukR	O87970/ O87971	Unknown	Unknown	Atkinson *et al.*, 1999
Uncultured proteobacterium QS1	QS1	AAT90822.1/ AAT90827.1	3-O-C6	Unknown	Williamson *et al.*, 2005

2.1.3 Metagenomics

Using current laboratory approaches, the vast majority of the world's microorganisms can not be cultured (more than 99% in most soils) (Amann *et al.*, 1995; Daniel, 2005). Those uncultured microorganisms may contain a large number of interesting genes and encoded products, such as novel enzymes, antibiotics and signal molecules. In order to characterize the metabolic capacity of the as-yet-uncultured organisms, culture-independent methods were introduced. Metagenomics, also referrered to as "community genomics" or "environmental genomics", is the sequencing and analysis of DNA of microorganisms from a particular environment without culturing them (Handelsman *et al.*, 1998; Rondon *et al.*, 2000; Handelsman, 2004). Metagenomic libraries have been constructed by directly extracting DNA from environmental samples and cloning the extracted DNA into vectors that are maintained in a bacterial host. By directly sequencing or functionally screening metagenomic libraries, the genetic properties of those uncultured microrganisms can be analyzed. The vectors used include plasmids for small inserts (up to several kb of DNA), cosmids or fosmids for medium sized inserts (up to tens of kb of DNA), and bacterial artificial chromosomes (BAC) for large fragments (up to 150 kb of DNA) (Sjöling *et al.* 2007). While *E. coli* is the most common host cell used, other bacterial hosts including *Bacillus* and *Streptomyces sp.* and some eukaryal hosts such as *Saccharomyces*, *Pichia* and *Aspergillus* have also been used (Sjöling *et al.* 2007).

Metagenomics studies can help to develop an understanding of the phylogenetic diversity of a certain habitat using 16S rRNA or other phylogenetically conserved genes. In this case, it is possible to monitor the effects of environmental conditions and changes on the diversity of microorganisms. Screening the metagenomic libraries may also enable

researchers to recover novel enzymes and antibiotics that can be exploited for agricultural, industrial or medical applications. By examining genes/operons for nutrient acquisition, QS, central intermediary metabolism and other microbial processes, it is possible to gain an understanding of the microbial community responses and interactions, and reveal the basis for the success of particular organisms in their environment. Metagenomic studies have been applied in many different environments, including oceans (Venter *et al.*, 2004; Hallam *et al.*, 2004; Delong, 2005; Worden *et al.*, 2006), soils (Handelsman *et al.*, 1998; Rondon *et al.*, 2000; Williamson *et al.*, 2005; Wang *et al.*, 2006), hot springs (Rhee *et al.*, 2005), acid mines (Tyson *et al.*, 2004), and the human mouth (Diaz-Torres *et al.*, 2003) and gastrointestinal tract (Breibart *et al.*, 2003; Gill *et al*., 2006; Gloux *et al.*, 2007). It is considered of particular value in the areas of medicine, alternative energy, environmental remediation, biotechnology, agriculture, biodefense and forensics (National Research Council, 2007).

2.1.4 Previous studies of QS of uncultured microorganims

In regard to QS, although it has been extensively studied in cultured microorganisms, only a few attempts have been made to study QS systems of uncultured organisms. In 2005, Williamson *et al.* (Williamson *et al.*, 2005) first reported the isolation of new QS inducers from metagenomic libraries constructed using DNA from soil on the floodplain of the Tanana River in Alaska. They isolated a clone that encoded a LuxI family protein, which synthesizes the AHL 3-O-C6 HSL. A metagenomic analysis of the gypsy moth gut microbiota led to the identification of a gene that encodes a monooxygenase homologue. The gene product mediates an indole oxidation pathway and leads to production of signal mimics that induce QS (Guan *et al.*, 2007). Most recently, the

screening of the metagenomic libraries generated using DNA from a pasture soil from France failed to find any AHL synthase, although one clone containing a quorum quenching lactonase was identified (Riaz *et al.*, 2008).

2.1.5 Objectives of this study.

Metagenomic libraries were previously constructed using DNA from activated sludge and from soil in our lab into cosmid vector pRK7813 and maintained in *E. coli* HB101 (Wang *et al.*, 2006). Four of the libraries, CX4 (activated sludge), CX6 (municipal waste), CX9 (soil) and CX10 (soil) (Table 2.2), which previous studies have shown to be of good quality (Wang *et al*. 2006), were screened in this study using an *A. tumefaciens* QS biosensor strain HC103(pJZ381) for novel QS inducers. Isolation, sequencing and characterization of three novel QS systems are described below.

2.2 Materials and Methods

2.2.1 Metagenomic libraries, bacterial strains, plasmids and culture conditions.

Metagenomic libraries used in this study are listed in Table 2.2. The bacterial strains, plasmids use in this study are listed in Table 2.3. *Agrobacterium* strains were cultured in Luria broth (LB) (Bertani, 1951) medium (10 g tryptone, 5 g yeast extract, 10 g NaCl) or ABM (Chilton *et al.*, 1974) minimal medium at 28°C. When required, antibiotics were supplied as follows: gentamicin (Gm), 50 μg/ml; kanamycin (Km), 50 μg/ml; chloramphenicol (Cm), 17 μg/ml; tetracycline (Tc), 2 μg/ml. *Escherichia coli* strains were cultured in LB medium at 37°C. When necessary, antibiotics were supplied as follows: ampicillin (Ap), 100 μg/ml; Km, 100 μg/ml; Cm, 34 μg/ml; Tc, 20 μg/ml.

2.2.2 Screening of metagenomic libraries.

The *Agrobacterium tumefaciens* biosensor strain HC103(pJZ381) was used to screen the above mentioned libraries for novel QS inducers. Figure 2.4 shows the screening strategy that was used. The biosensor strain HC103(pJZ381) contains a *traI* nonpolar deletion and a *traC-lacZ* translational fusion on the Ti plasmid. In pJZ381, *traR* is overexpressed under the transcriptional control of the *lac* promoter of pBBR1MCS5. Unless it binds to AHL, the TraR protein is not stable and degrades quickly after it is synthesized (Zhu and Winans, 2001). When metagenomic clones were introduced into the biosensor strain, if QS inducer synthases were present, and the produced active signals could bind to the TraR protein and activate transcription of the *traC-lacZ* fusion, the bacterium would then form blue colonies in the presence of the indicator substrate X-Gal. However, if the metagenomic clone contains a *lacZ* gene, it would also enable the

biosensor strain to form blue colonies. Metagenomic clones were transferred to the biosensor strain *A. tumefaciens* HC103(pJZ381) by triparental mating using the helper strain *E. coli* DH5α(pRK600) (Finan *et al.*, 1986). The transconjugants were selected and screened on LB agar containing 50 μg/ml of Gm, 2 μg/ml of Tc and 20 μg/ml of X-Gal. After 48-72 hours incubation at 28°C, plates were checked for blue colonies. Blue colonies were picked and then streak purified.

The metagenomic plasmids from the identified blue colonies were transferred into *E.coli* DH5α by triparental mating using *E. coli* DH5α(pRK600) as the helper strain. The conjugants were selected on LB agar with Tc (20 μg/ml) at 37°C overnight (14h to16h), under which conditions the *Agrobacterium* strains can not form visible colonies in 14 to 16 hours, and only *E.coli* DH5α that has obtained a metagenomic cosmid clone which confers resistance to tetracycline was able to form visible colonies. The colonies were streak purified once on LB agar with Tc (20 μg/ml), and then one single colony was inoculated into liquid LB medium with Tc and plasmids were extracted (Sambrook *et al*., 1989). The plasmids were digested using *Bam*HI, and the digestion patterns were analyzed and those with the same digestion pattern were deemed to be the same clone.

All unique plasmids were transferred back into *A. tumefaciens* HC103(pJZ381) by electroporation (Cangelosi *et al.* 1991) to confirm that they form blue colonies in presence of X-gal. In order to determine whether the metagenomic clones contain QS inducers or *lacZ* genes, plasmids from each of the unique clones were also electroporated into wild-type *A. tumefaciens* C58 which does not contain a *lacZ* gene, and incubated at 28°C for 48 to 72 hours. If the wild-type colonies turn blue on LB agar containing X-Gal, then the plasmid contains a *lacZ* gene, otherwise it contains QS genes.

Table 2.2. Metagenomic libraries used in this study. All four libraries were described in Wang *et al.* (2006)

Libraries	**DNA source**	**Vector**	**No. of clones**
CX4	Activated sludge DNA from Domtar Cornwall mill (pulp)	Cosmid pRK7813	3879
CX6	Activated sludge DNA from Vaudreuil (municipal waste)	Cosmid pRK7813	3322
CX9	DNA from soil samples collected from the banks of Laurel Creek, University of Waterloo Campus	Cosmid pRK7813	22180
CX10	DNA from soil samples collected from the banks of Laurel Creek, University of Waterloo Campus	Cosmid pRK7813	8696

Table 2.3. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
Agrobacterium tumefaciens		
HC103(pJZ381)	HC103 is R10 based strain, the Ti plasmid contains a *traC-lacZ* translational fusion and a *traI* nonpolar deletion. In pJZ381, *traR* is under *lac* promoter of pBBR1MCS5. Km^r and Gm^r	This study
C58	Wild type strain	(Goodner *et al.*, 2001), (Wood *et al.*, 2001)
Escherichia coli		
DH5α	*recA1* and *endA1* cloning strain	Lab stock
DH5α(pRK600)	DH5α strain containing helper plasmid pRK600, Cm^r	(Finan *et al.*, 1986)
BL21(DE3)	(λDE3) F^- *ompT hsdS*$_B$($r_B^-m_B^-$) *dcm gal*	Novagen
Plasmid		
pBluescript II SK(+)	Cloning vector, Amp^r	(Alting-Mees and Short, 1989), (Alting-Mees *et al.*, 1992)
pRK415	Broad host cloning vector, Tc^r	(Keen *et al.*, 1988)
pET30(a+)	IPTG-inducible expression vector; Km^r	Novagen
pET30(b+)	IPTG-inducible expression vector; Km^r	Novagen
pRK6-1LuxI	pRK415 derivative, carrying full-length PCR amplified *luxI*$_{QS6-1}$ under the control of the *lac* promoter of pRK415, Tc^r	This study

pET6-1LuxI	pET30(a+) derivative, carrying full-length *luxI*$_{QS6\text{-}1}$ under the T7 promoter, Km^r	This study
pET10-1LuxI	pET30(a+) derivative, carrying full-length *luxI*$_{QS10\text{-}1}$ under the T7 promoter, Km^r	This study
pET10-2LuxI	pET30(b+) derivative, carrying full-length *luxI*$_{QS10\text{-}2}$ under the T7 promoter, Km^r	This study
pET6-1LuxR	pET30(a+) derivative, carrying full-length *luxR*$_{QS6\text{-}1}$ under the T7 promoter, Km^r	This study
pET10-1LuxR	pET30(a+) derivative, carrying full-length *luxR*$_{QS10\text{-}1}$ under the T7 promoter, Km^r	This study
pET10-2LuxR	pET30(a+) derivative, carrying full-length *luxR*$_{QS10\text{-}2}$ under the T7 promoter, Km^r	This study

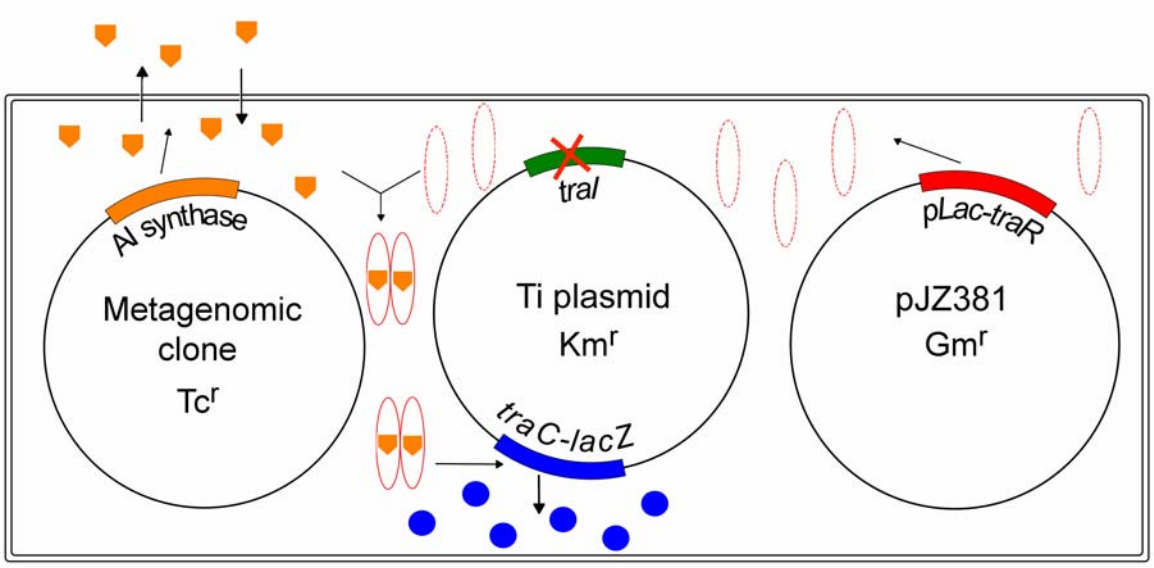


Figure 2.4. Screening of metagenomic libraries for QS inducer synthase using biosensor strain *A. tumefaciens* HC103(pJZ381). The Ti plamid contains a *traI* nonpolar deletion and a *traC-lacZ* translational fusion. In pJZ381, *traR* is overexpressed under control of the *lac* promoter of pBBR1MCS5. Without binding to AHL, the TraR protein is not stable and degrades quickly after synthesis. When metagenomic clones are introduced into the biosensor strain, if QS inducer synthases are present, the produced active signals bind to TraR protein and activate transcription of the *traC-lacZ* fusion, enabling the bacteria to form blue colonies in the presence of X-Gal.

2.2.3 Transposon insertiontional mutagenesis and sequencing of clones

EZ-Tn5™ <KAN-2> and HyperMu™ <CHL-1> in vitro insertion kits were purchased from EPICENTRE Biotechnologies (Madison). In vitro transposon insertion of the metagenomic clones was performed according to the manual provided by the supplier. The mutagenized clones were transferred to the biosensor stain *A. tumefaciens* HC103(pJZ381) by electroporation. Transformants were incubated at 28°C on LB agar with appropriate antibiotics and 20 μg/ml of X-Gal. White colonies, indicating disruption of the QS genes, were picked and streak purified. The metagenomic clones from the white colonies were transferred to *E. coli* DH5α by triparental mating. Plasmids were extracted and sequenced using primers from the transposon: MUCHL-1 FP-1, MUCHL-1 RP-1, KAN-2 FP-1 and KAN-2 RP-1 (Table 2.4). Up and down stream regions were further sequenced using new primers designed from the sequenced area using a chromosome walking method. All primers used for sequencing are listed in Table 2.4. All sequencing was performed at MOBIX (McMaster University, Hamilton, ON, Canada) using an ABI 3730 DNA analyzer (Applied Biosystems) or at York University's Core Facility for Molecular Biology (Department of Biology, York University, 4700 Keele Street, Toronto, ON, Canada).using ABI 373A Sequencer (Applied Biosystems)

Table 2.4. Primers used for DNA sequencing.

Name of primer	Sequence (from 5' to 3')
MUCHL-1 FP-1	CACAGGTATTTATTCGGTCGA
MUCHL-1 RP-1	TGGAGGTAATAATTGACGATA
KAN-2 FP-1	ACCTACAACAAAGCTCTCATCAACC
KAN-2 RP-1	GCAATGTAACATCAGAGATTTTGAG
6-1FP1	GGCACATTTCACTGGCAT
6-1RP1	TGGCGATTCTGCGATGTT
6-1FP2	GCCTCTTGGACTGACTAAGG
6-1FP3	TCCAACACATTACAAGCGG
6-1 FP4	CAGCGATTGCAGGACCGAGCCA
6-1 RP4	AAGCTGGAGACCACCTCGATGTACG
6-1 RP5	CTTCCTGTCGGTGCGTGT
10-1FP1	CTTCGTGAAAAGGTTGCG
10-1RP1	GGCACTTCCACATGCAGAT
10-1FP2	ATCCAAGACGCTGTGGCAT
10-1FP3	GCTATTGGCAGGAAAGTCG
10-1 FP4	TGGAGCACTATCCGCAACTCACGC
10-1 RP4	TGGCCGCCAGGATGCCCAGT
10-1 FP5	ACGCCGCCCTTCACTCACCTCTTG
10-1RP5	GTGTACAACGCCAGGCTCAT
10-2FP1	CGATACAGACCCGACCAAA
10-2RP1	TGGTGCCTTTCTTCTGGGA
10-2FP2	TACGAGCAATGTTTCGCC
10-2 FP3	AGTCATCTTGTCGGCAACC
10-2 FP4	CCACGGATCGGCACATCG
10-2 RP4	TCCGCATAACCGCCTCCCT
10-2 FP5	GGTGTATCTCGGCTCGGTTA
10-2 RP5	CGGCGGATAATGACCTTTAC
10-2 FP6	ATCGGGTCACTGGCAGAGGAGC
10-2 RP6	TCGCCGACCACGACGGAATA

2.2.4 Phylogenetic analysis

All sequences used were obtained from Genbank or SwissProt. For LuxI and LuxR homologs, sequences with experimental evidence and sequences of top BlastP hits to NCBI non-redundant protein sequences were used. For LuxI homologs, 44 sequences with experimental evidence were used. When obtaining the top BlastP hits, for $LuxI_{QS6\text{-}1}$ and $LuxI_{QS10\text{-}1}$, sequences with a score of more than 120 (inclusive) were selected (12 and 18 sequences were obtained respectively), while for $LuxI_{QS10\text{-}2}$, sequences with a score of more than 88 (inclusive) were selected (15 sequences were obtained). Those LuxI homologue sequences were combined and redundant sequences were deleted. Finally, in total 70 sequences were used for multiple sequence alignment and phylogenetic analysis. For LuxR homologues, 42 sequences with experimental evidence were used. When obtaining top Blast hits, for $LuxR_{QS6\text{-}1}$, sequences with a score of more than 100 (inclusive) were selected (16 sequences); for $LuxR_{QS10\text{-}1}$, sequences with a score of more than 115 (inclusive) were selected (13 sequences); while for $LuxR_{QS10\text{-}2}$, sequences with a score of more than 95 (inclusive) were selected (14 sequences). After getting rid of the redundant sequences, 71 LuxR homologue protein sequences in total were used for the phylogenetic analysis. For other genes, sequences of top BlastP hits were obtained from Genbank.

Multiple sequence alignments were performed using "Muscle" (version 3.6) (Edgar, 2004) and were refined by eye. Some gap containing areas were removed using BioEdit (Version 7.00) (Hall, 1999). The best fitting models were tested using Protest (Abascal *et al.*, 2005). Phylogenetic tree topologies were determined using Phyml version 2.4.4 (Guindon and Gascuel, 2003). Bootstrap values were obtained from 1000 replicates

employing Neighbour Joining (NJ) and Maximum Parsimony (MP) algorithms using PAUP 4.0b program (Swofford, 2000).

2.2.5 β-Galactosidase activity assay

Agrobacterium strains were grown to stationary phase in ABM minimal medium at 28°C, then 50 µl of the stationary phase cultures were transferred to 5 ml ABM minimal medium and returned to a 28°C waterbath shaker. For the positive control, 3-O-C8 HSL was added to a final concentration of 10 nM to the HC103(pJZ381) culture at this point. Approximately 20 hours after subculture, the cultures were at exponential growth phase, β-galactosidase activity assay was performed as described (Miller, 1972).

2.2.6 Subcloning of *luxI* and *luxR*

To subclone the *luxI* and *luxR* homologs, primers were designed to include the start and stop codons of the *luxI* or the *luxR* homolog genes (Table 2.5). KOD hot start DNA polymerase (Novagen) was used to amplify the fragment. The PCR fragments were purified (using QIAGEN PCR Purification Kit) and were ligated into the EcoRV site of pBluescriptII SK(+) (Alting-Mees and Short, 1989; Alting-Mees *et al.*, 1992). Diagnostic PCR was performed using T7 primer and the forward or reverse primer of the corresponding gene to screen for insertions with appropriated orientations. Clones were sequenced and after they were confirmed that no mutation has been introduced, the DNA was digested with BamHI and HindIII and the excised *luxI* or the *luxR* gene fragment was gel purified (using QIAGEN Gel Purification Kit). The *luxI* or *luxR* gene was then ligated into BamHI and HindIII digested pET30a+ or pET30b+ to construct the corresponding

vectors, pET6-1LuxI, pET10-1LuxI, pET10-2LuxI, pET6-1LuxR, pET10-1LuxR and pET10-2LuxR (Table 2.3). Plasmid pRK6-1luxI was also constructed by inserting $luxI_{QS6\text{-}1}$ into BamHI and HindIII digested pRK415.

Table 2.5. Primers used to amplify *luxI* and *luxR* homologs.

Name of primers	Sequences
$luxI_{QS6\text{-}1}$ F	ATGCATCACCAGATTTTTACG
$luxI_{QS6\text{-}1}$ R	GGCTAGGCCGTTGCGTCC
$luxI_{QS10\text{-}1}$ F	AAAGTTGTTACACATTCGCTCAGG
$luxI_{QS10\text{-}1}$ R	GCAAATCCGGCTGTACTCCCT
$luxI_{QS10\text{-}2}$ F	GCCGATGATTCTGATCATCAACGC
$luxI_{QS10\text{-}2}$ R	TGTTTCTTTACGCGGCGATCTTT
$luxR_{QS6\text{-}1}$ F	CGAAGACGTCGATCGGGTGC
$luxR_{QS6\text{-}1}$ R	GCGCAGCTAGGTGATCAGTCC
$luxR_{QS10\text{-}1}$ F	GAGGAAGGATTGACAATGACGAG
$luxR_{QS10\text{-}1}$ R	GCCTCTAGACAATCAGTCCCATC
$luxR_{QS10\text{-}2}$ F	AATCAGTTCGAAGTGGCGC
$luxR_{QS10\text{-}2}$ R	GAGGGTCCGGAATCATGTTAG

2.2.7 Extraction of AHL and analytical TLC assay

AHL standards (C6, C8, C10, C12, 3-O-C6, 3-O-C8) were purchased from Sigma-Aldrich (St. Louis, MO). *Agrobacterium* strains were grown in 5 ml ABM medium or LB medium at 28°C with shaking for 48 to 72 hours until stationary phase was attained. *E. coli* strains BL21(DE3)(pET6-1LuxI), BL21(DE3)(pET10-1LuxI) and BL21(DE3)(pET10-2LuxI) were grown overnight at 37°C in LB medium with aeration and then subcultured (using a 1:50) to 20 ml LB and incubated for one hour at 37°C until the OD_{600} reached 0.4-0.5, then IPTG was added to a final concentration of 0.5 mM and incubated for 6 hours at 28°C. The AHLs produced were extracted and the TLC plates were developed mainly as described by Shaw *et al.* (1997). Briefly, 5 ml of the culture was centrifuged at 5000 x g for 10 min and the supernatant of the culture was extracted twice using an equal volume of ethyl acetate, and water was removed from the combined extracts using anhydrous $MgSO_4$. The extractions were evaporated to dryness. Residues were then dissolved in 20 µl HPLC grade ethyl acetate. Two µl of AHL standards or samples were applied to C18 reverse phase TLC plates (200 µm layer, Whatman) and chromatographs were developed with methanol/water (70/30, vol/vol). Plates were dried and overlayed with a layer of 0.7% ABM agar containing the biosensor strain *A. tumefaciens* HC103(pJZ381) and X-Gal at a concentration of 40 µg/ml. The plates were incubated overnight at 28°C or until sufficient blue colour had developed, before the plates were dried and scanned.

2.2.8 Preparative TLC and mass spectrometry

For *A. tumefaciens* strains HC103(pJZ381)(pQS6-1), HC103(pJZ381)(pQS10-1), HC103(pJZ381)(pQS10-2) and HC103(pJZ381)(pRK6-1LuxI), a single colony was inoculated into 5ml of LB medium and incubated at 28°C until the culture reached stationary phase, it was then transferred to 500 ml of LB medium in a 2 L flask and incubated for about 24 h until the culture reached early stationary phase. The supernatant from the 500 ml culture was extracted twice with equal volumes of ethyl acetate, and then the extracts were combined in a glass beaker and dried over anhydrous $MgSO_4$. When about 10 ml of ethyl acetate was left, it was then filtered using Whatman filter paper to remove the magnesium sulphate, and then dried using a Savant Speed Vac. The residues were then dissolved in 50 µl of ethyl acetate.

For *E. coli* strains BL21(DE3)(pET6-1LuxI), BL21(DE3)(pET10-1LuxI), and BL21(DE3)(pET10-2LuxI), 5 ml of overnight culture was transferred to 500 ml of LB medium and incubated at 37 °C for about one and a half hour until OD_{600} reached about 0.4-0.5, then IPTG was added to a final concentration of 0.5 mM and the cells were incubated at 28 °C for 6 hours. Then the culture supernatant was extracted and dried as described above.

For a given sample, 50 µl of the extracts were applied in a series of spots to the bottom of a 20 x 20 cm^2 C18 reverse phase TLC plate (Whatman) and developed using 70:30 methanol:water. About 3 cm of the plate was removed from one side using a glass cutter and overlayed with biosensor strain *A. tumefaciens* HC103(pJZ381) and incubated overnight until sufficient blue colour developed. By comparison to the overlayed area of the TLC plate, the sillica matrix from the rest of the TLC plate at the corresponding positions was removed, transferred to a 1.5 ml Enpendorf tube, and extracted 3 times with

1 ml of 1:1 chloroform:dichloromethane. The extracts were centrifuged and then filtered through a 0.7 µm glass filter (Whatman) to remove any solid particles, and then dried using a Savant Speed Vac. The residues were dissolved in 20 µl of 1:1 acetonitrile:water with 0.2% formic acid. Electrospray ionization mass spectrometry (ESI MS) and ESI MS/MS were performed using a micromass Q-TOF ultima global mass spectrometer**.** Argon gas was used as the collision gas, and the collision energy was kept at 15 eV for all experiments.

2.2.9 Expression of LuxR homologs in *E. coli* and determination of solubility.

To determine whether the LuxR homologs were expressed in *E. coli*, single colonies of *E. coli* BL21(DE3)(pET6-1luxR), *E. coli* BL21(DE3)(pET10-1luxR) and *E. coli* BL21(DE3)(pET10-2luxR) strains were inoculated into 5 ml LB medium with appropriate antibiotics and incubated overnight at 37°C and then subcultured using a 1:50 ratio in 20 ml LB in 100 ml flasks and returned to the waterbath shaker for about 90 min until the OD_{600} of the cultures reached 0.4-0.5. Before adding IPTG, 1 ml of each culture was transferred to a 1.5 ml centrifuge tube and the cells were pelleted by centrifugation at 8000 x g for 2 min (uninduced control). IPTG was added to the rest of the culture to a final concentration of 1 mM and protein expression was induced by incubation at 37°C for 3 h. Then 0.5 ml of the culture was pelleted as described above (induced total protein). An aliquot of 50µl of 1 x SDS loading buffer (0.045 M Tris·Cl, pH 6.8; 10% glycerol; 1% SDS; 0.01% bromophenol blue; 0.05 M DTT) was added to the pellets and vortexed to resuspend the cells. The cells were then boiled for 5 mins, cooled on ice, centrifuged at 12000 x g for 1 min to get rid of the cell debris, and 20 µl of the supernatants were applied

to a SDS-PAGE (4% condensing gel, 12% separating gel) to separate the proteins. The gels were stained with Coomassie blue to help visualize the proteins bands.

To determine whether the expressed LuxR homologs were in the soluble portion or formed inclusion bodies, the above mentioned *E. coli* strains were incubated in 5 ml LB medium at 37°C overnight, subcultured using a 1:50 ratio in 50 ml LB medium in 200 ml flasks, and incubated at 37°C for about 90 minutes until the OD_{600} reached 0.4-0.5. An aliquot of one ml was removed and pelleted (uninduced control, 50 µl of 1 x SDS loading buffer was added to resuspend the cells) before IPTG was added to a final concentration of 0.5 mM, and the flasks were returned to a 28°C shaker water bath and incubated for 2 to 5 h. Another ml aliquot was removed and pelleted and 100 µl of 1 x SDS loading buffer was added to resuspend the cells (induced total proteins). The rest of the cells were pelleted by centrifugation at 5000 x g for 10 min using a Sorval centrifuge, and the supernatants were discarded. The cell pellets were resuspended in 5 ml cell lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, the pH was adjusted to 8.0 using NaOH), and lysozyme was added to a final concentration of 1mg/ml and incubated on ice for 30 min before sonication at 10 x 10 s with 10 s pauses at 200 W on ice. The lysate was centrifuged at 10,000 g for 25 min at 4°C, the supernatant (the soluble part) was transferred to a new tube, and the pellet was resuspended in 5 ml lysis buffer (insoluble part). An equal volume of 2 x SDS loading buffer (0.09 M Tris·Cl, pH 6.8; 20% glycerol; 2% SDS; 0.02% bromophenol blue; 0.1 M DTT) was added to the soluble and insoluble extracts. All of the samples including uninduced control and induced total protein control were boiled for 5 min, cooled on ice, centrifuged at 12,000 g for 1 min, and 20 µl of each sample was loaded onto a 12% SDS-PAGE. Alternatively, the *E. coli* cells were lysed using BugBuster®

master mix (Novagen) according to manufacturer's instructions to obtain the soluble and insoluble extracts.

In order to determine if the cognate AHLs are required for the LuxR protein to be expressed in the soluble portion of the cell extract, 30 mins before induction with 0.5 mM IPTG, AHLs extracted from an equal volume of culture supernatant of *E.coli* strains expressing corresponding *luxI* genes were added. Then the protein expression was induced at 28°C for 2.5 h with shaking. The cells were broken down as described above to determine the solubility of the proteins.

2.3 Results

2.3.1 Screening of the metagenomic libraries for QS inducers

In this study, four metagenomic libraries, CX4, CX6, CX9 and CX10 (Table 2.2), constructed using DNA from activated sludge or soil (Wang *et al.*, 2006) were screened for QS inducer synthase as described. In the initial screening, not only QS inducers, which induces expression of *traC-lacZ* in *A. tumefaciens* HC103(pJZ381), but also *lacZ* genes, could be detected. After the initial screening, a number of colonies that were blue on LB agar containing X-gal were isolated from each library (Table 2.6). Since the signals were weak, the five weak blue colonies obtained from library CX4 were not further analyzed in this study. The plasmids from each of the isolated blue colonies were digested with BamHI to check whether they are unique clones or siblings of each other (Figure 2.5). It was found that the plasmids from the six blue colonies isolated from library CX6 showed exactly the same digestion pattern, thus were deemed to be from one unique clone (QS6-1), the plasmids from the six blue colonies isolated from library CX9 showed two different digestion patterns, 9-1 showed one digestion pattern and all of the others showed another digestion pattern, thus, there are two unique clones (QS9-1 and QS9-2). The six blue colonies from library CX10 also showed two digestion patterns, the isolates 10-1 and 10-6 were from the same clone (QS10-1), and the others were from another clone (QS10-2). The plasmids from the five unique clones were then transferred back to the biosensor strain HC103(pJZ381) to confirm that they are positive. While all the other four plasmids were, as expected, able to make the biosensor strain to form blue colonies, the cosmid from the isolate QS9-1 was not, which means that it was a false positive result. The unique clones were then transferred to *A. tumefaciens* C58, which does not contain a *lacZ* gene, to

determine whether these isolates contain a *lacZ* gene. It was found that only the clone from QS9-2 encodes an environmental *lacZ* gene. Finally, three clones containing QS inducers were identified and named QS6-1, QS10-1 and QS10-2.

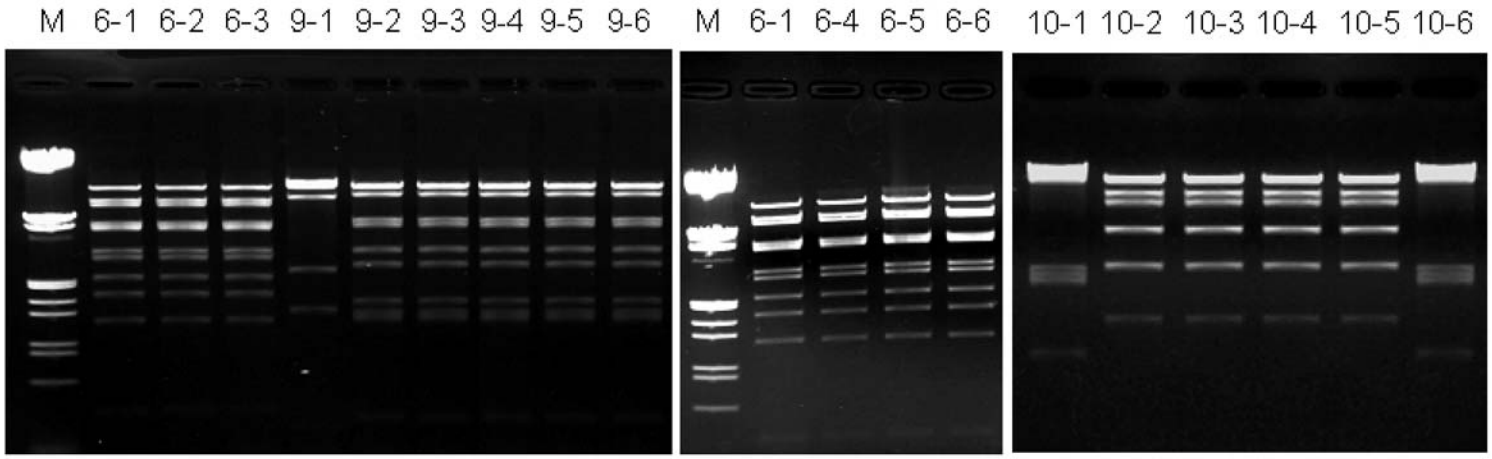


Figure 2.5. BamHI digestion pattern of isolated clones. M, λ DNA/EcoRI and HindIII marker. 6-1, 6-2, 6-3, 6-4, 6-5 and 6-6; the six blue colonies isolated from library CX6. 9-1, 9-2, 9-3, 9-4 and 9-5; the five blue colonies isolated from library CX9. 10-1,10-2, 10-3, 10-4, 10-5, 10-6; the 5 colonies isolated from library CX10.

Table 2.6. Screening of metagenomic libraries for QS autoinducers

Library	**DNA source**	**No. of clones**	**No. colonies screened**	**No. of blue colonies**	**No. unique clones**
CX4	Pulp waste activated sludge	3879	≈14000	5 (weak blue)	ND (not further analyzed)
CX6	Municipal waste activated sludge	3322	≈5600	6	1 (QS6-1)
CX9	Soil collected from UW campus	22180	≈20000	6	2 (one false positive, one with a *lacZ* gene)
CX10	Soil collected from UW campus	8696	≈16300	6	2 (QS10-1, QS10-2)

2.3.2 In vitro transposon insertional mutagenesis and sequencing results of the isolated QS systems

Since the cosmid clones contain inserts with an average size of more than 30 kb, to sequence the entire insert would be time consuming and costly. To help localize and sequence the QS signal synthase genes in the cosmid clones, *in vitro* transposition systems HyperMu™<CHL-1> insertion kit and the EZ-Tn5™<KAN-2> insertion kit (Epicenter) were used. After random mutagenesis and screening, one white clone for QS6-1, three for QS10-1 and four for QS10-2 were identified. DNA sequences were obtained from the transposon insertion sites using the transposon specific primers and adjacent sequences were then obtained utilizing a primer walking method. Finally, 7.6 kb, 7.3 kb and 7.1 kb of sequences for QS6-1, QS10-1 and QS10-2 respectively were obtained. The sequences were deposited in the Genbank database under the following accession numbers: QS6-1, FJ041295; QS10-1, FJ041296; QS10-2, FJ041297. Figure 2.6 shows the gene arrangements in each of the clones and the transposon insertion sites. All three clones contain a *luxI* family AHL synthase gene and a *luxR* family transcriptional regulator gene adjacent to one another.

Transposon insertional mutagenesis indicated that not only mutation of the *luxI* homologs, but also mutation of the *luxR* homologs in QS10-1 and QS10-2 (Figure 2.6) caused those two clones to lose the ability to induce the biosensor strain to turn blue. A similar phenomenon has been reported for other *luxI-luxR* family QS systems (Zheng *et al.*, 2006). This may be explained by the fact that in many *luxI-luxR* type systems the *luxI* type genes are regulated by their cognate R proteins (Waters and Bassler, 2005; Williams, 2007). Mutation of the *luxR* homologs in QS10-1 and QS10-2 disrupted the

positive regulation loop leading to their cognate AHL synthesis genes, so that only very low levels of AHLs were synthesized by the mutants even at high population density that could not be detected by the biosensor strain. Since the R protein usually binds to *lux* box-like sequences which are typically 20-bp inverted repeats close to or partially overlapping with the -35 box of the σ^{70} promoter (Devine *et al.*, 1988), the DNA sequences upstream of the *luxI* homologs were examined. Using promoter prediction software BPROM (Softberry, Inc., Mt. Kisco, NY) or SAK (Gordon *et al.*, 2003), a possible σ^{70} promoter was identified upstream of both the $luxI_{QS6\text{-}1}$ and $luxI_{QS10\text{-}1}$ regions. Possible *lux*-box-like sequences with imperfect dyad symmetry were also identified near the -35 box of both promoters (Figure 2.7 A). No promoter was predicted upstream of the $luxI_{QS10\text{-}2}$ region. However, a possible σ^{70} promoter was found upstream of the $luxR_{QS10\text{-}2}$ gene and a putative *lux*-box like sequence was also found close to the -35 box region (Figure 2.7 A). Considering that the $luxR_{QS10\text{-}2}$ and the $luxI_{QS10\text{-}2}$ are oriented in the same direction, and there is only 102 bp of intergenic region between them, it is possible that they form an operon. Comparison of the three possible *lux*-box like elements with known *lux*-box like sequences indicated conservation at certain residues as well as specificity (Figure 2.7 B). It was found that interruption of the acyl-CoA carboxylase biotin carboxylase subunit of QS10-2 also affects the AHL synthesis of that clone.

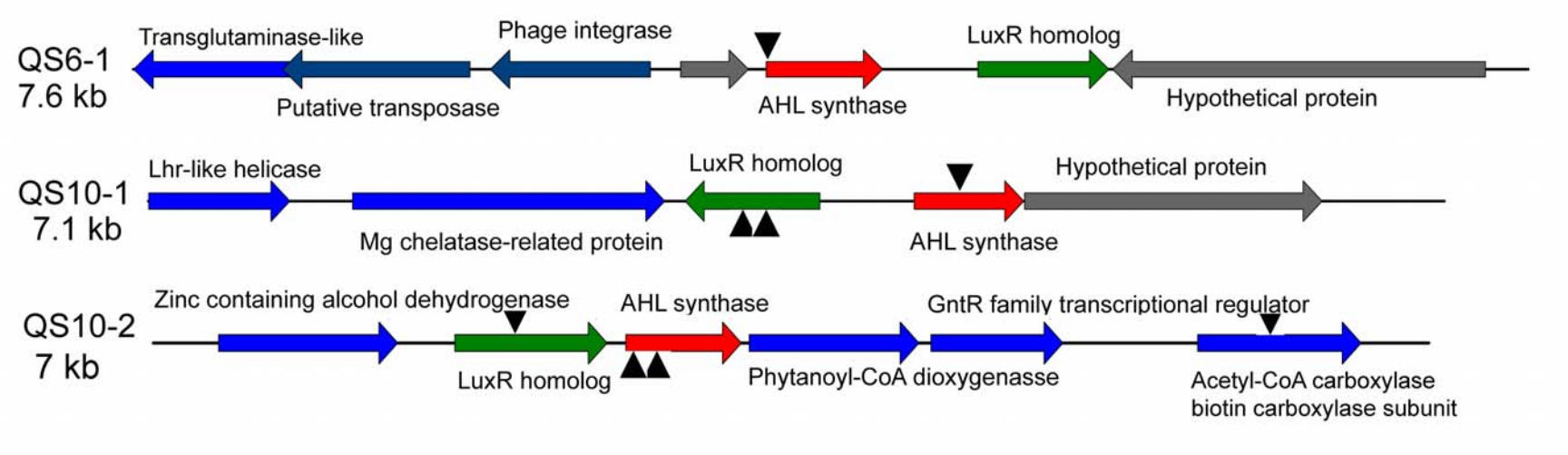


Figure 2.6. Gene arrangements on clones QS6-1, QS10-1 and QS10-2. The *luxI* homolog AHL synthase genes are highlighted in red, and the *luxR* homolog genes are highlighted in green. Black triangles indicate the position of transposon insertion sites.

Figure 2.7. Identification of *lux*-box-like elements. A, *lux*-box-like elements upstream of $luxI_{QS6\text{-}1}$, $luxI_{QS10\text{-}1}$ and $luxR_{QS10\text{-}2}$. B, Comparison of elements with known *lux*-box-like elements from *Ralstonia solanacearum SolI* (Flavier *et al.*, 1997), *Pseudomonas aeruginosa RhlI* and *LasI* (Latifi *et al.*, 1995), *Vibrio fischeri LuxI* (Devine, 1988), *Acidithiobacillus ferrooxidans AfeI* (Rivas *et al.*, 2005), *Burkholderia cepacia CepI* (Lewenza *et al.*, 1999). Sequences with more than 60% identity were shaded.

A

LuxI$_{QS6\text{-}1}$

TAACGCAAAGACTGTGGGGGTGCCCAGTGGCCTCGCGTGGGGGC

-35

GTTCCGTTAAATCGACTTCGTCGATTTACCAGCGGAAACACTCAC

-10 RBS

ATGCAT……

LuxI$_{QS10\text{-}1}$

ATTCTACCTATGGACCTTCGTAGGTTACGAAAAAAAGGCTGCTCCA

-35

GTCAAATCCGCGCTACGGATAACTGGACACGCCCATGCCA……

-10 RBS

LuxR$_{QS10\text{-}2}$

GATGCATAACTACCCGGTCTGTTAGTTTTTGGTCGGGTCTGTATC

-35

GCTGCGTATCAGTTGGGATGCCCGAAAGCACATTGCGGGTATGA

-10

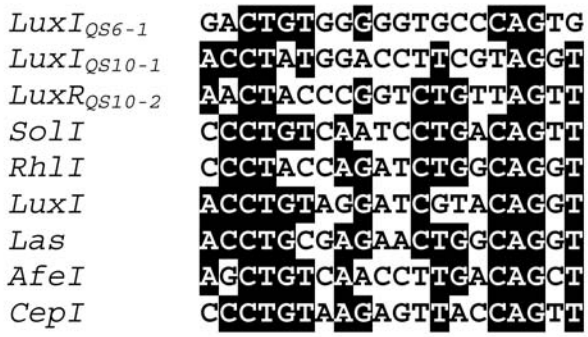
CGCGGTGGATGCAGCAATACAGGCGACGTCGGTGGGCCGGCTCTC

TCGCTAGGAAGCAAGGAGCGTCGGTCAACAGGGATGCGCGACG

TCCATAAGCGGGTAGTGGTTGAGGGAATCGGCCATGAAT……

RBS

B



2.3.3 Bioinformatic analysis of the isolated clones

2.3.3.1 BLAST search results of the LuxI and LuxR homologs BLASTP searches against NCBI Non-redundant Protein Sequences database and TBLASTN searches against NCBI Environmental Samples database were performed, and it was found that the identified LuxI and LuxR homologs showed from 32% to 54% identity to their closest matches (Table 2.7). BLAST searches were also performed against the CAMERA All Metagenomic ORF Peptides database, the CAMERA All Metagenomic 454 Reads, the CAMERA Non-Identical Peptide Sequences database, and the JGI all IMG genes database; in all cases, no sequence with a higher identity was found.

2.3.3.2 Phylogenetic analysis. In order to obtain information of the closest known relatives to the identified clones, phylogenetic analyses were performed. For the LuxI and LuxR homologs, sequences that have been experimentally determined together with those putative LuxI and LuxR homologs obtained from the top BLASTP hits against NCBI Non-redundant Protein Sequences database were used. For LuxI homologs, 44 sequences with experimental evidence of functionality were used. The top 12 to 18 sequences of the BLASTP hits to each of the three LuxI homologs were obtained from NCBI. After deleting redundant sequences, in total 70 sequences were used for multiple sequence alignment and phylogenetic analysis. For LuxR homologs, 42 sequences with experimental evidence of functionality were combined with 13 to 16 top BLASTP hits of each of the three LuxR homologs. After deleting redundant sequences, 71 LuxR homologue protein sequences in total were used for the phylogenetic analysis.

The midpoint rooted maximum likelihood (ML) trees of LuxI and LuxR homologs showed similar topologies (Figure 2.8 A and B). Most sequences from *α proteobacteria* grouped together; while sequences from *β proteobacteria* and *γ proteobacteria* are intermingled which is different from previously published phylogenetic analysis of LuxI and LuxR homologs (Gray and Garey, 2001; Lerat and Moran, 2004). This may be because more sequences from *β proteobacteria* and *γ proteobacteria* were used in this analysis. This reflects the relative close relationship of *β* and *γ proteobacteria* and suggests the relative frequent lateral gene transfer of the *luxI* and *luxR* genes between them. In both LuxI and LuxR homologs trees, QS6-1 and QS10-1 are grouped together. In fact, $LuxI_{QS6\text{-}1}$ and $LuxI_{QS10\text{-}1}$ showed 61% identity in amino acid sequence, and $LuxR_{QS6\text{-}1}$ and $LuxR_{QS10\text{-}1}$ showed 53% identity in amino acid sequence, both of which are higher than the sequence identities they showed to their closest matches from NCBI BLASTP search. They tend to group with *β* or *γ proteobacteria*. From the LuxI homolog tree, the most closely related species to QS6-1 and QS10-1 is *Nitrosospira multiformis*. Both the LuxI and LuxR homolog of QS10-2 are grouped with sequences from *α proteobacteria*. However, they are not closely related to any known species.

In agreement with the LuxI and LuxR homolog protein, phylogenetic analysis of all other sequences from QS10-1 showed that it grouped with *β proteobacteria* (Figure 2.10), and phylogenetic analysis of all other sequences from QS10-2 showed that it grouped with *α proteobacteria* (Figure 2.11 and Figure 2.12). However, for QS6-1, although most of its sequences group with *β* and *γ proteobacteria*, some sequences group with sequences from *Flavobacteria* (Figure 2.8 and Figure 2.9).

Table 2.7. Closest matches to the identified LuxI and LuxR homologs obtained from BLAST searches.

Protein	**Closest match from NCBI nonredundant protein sequence database (BLASTP search)**	**Closest match from NCBI Environmental Samples database (TBLASTN search)**
LuxIQS6-1	AHL synthase of *Nitrosospira multiformis* ATCC 25196 (47% identity)	Freshwater sediment metagenome lwFormaldehyde_BCIB15374_yl (ABSN01034438.1) (53% identity)
LuxIQS10-1	Same as above (51% identity)	Same as above (54% identity)
LuxIQS10-2	AHL synthase of *Sphingomonas Sp*. SKA58 (37% identity)	Marine metagenome ctg_1101668694332 (AACY023886981.1) (37% identity)
LuxRQS6-1	LuxR family protein of *Nitrococcus mobilis* Nb-231 (34% identity)	Marine metagenome 1096626424210 (AACYo20346372.1) (26% identity)
LuxRQS10-1	LuxR family protein of *N. multiformis* ATCC 25196 (32% identity)	Same as above (31% identity)
LuxRQS10-2	LuxR family protein *Sphingomonas Sp*. SKA58 (37% identity)	metagenome sequence XZS25054.x1 (AAFX01052353.1) (41% identity)

Figure 2.8. Midpoint rooted maximum likelihood tree of LuxI (A) and LuxR (B) homologs. Abbreviations for bacterial genus names: *Ac, Acidthiobacillus; Ae, Aeromonas; Ag, Agrobacterium; Az, Azospirillium; B, Burkholderia; Br, Bradyrhizobium; C, Chromobacterium; E, Erwinia; En, Enterobacter; Es, Escherichia; M, Mesorhizobium; Mb, Methylobacterium; Ms, Magnetospirillum; Nb, Nitrobacter; Nc, Nitrococcus; Ns, Nitrosospira; P, Pseudomonas; Pe, Pectobacterium; R, Rhizobium; Ra, Ralstoina; Rb, Rhodobacter; Rp, Rhodopseudomonas; S, Sinorhizobium; Sa, Sacharophagus; Se, Sericia; Sph, Spingomonas; V, Vibrio; Y, Yersinia.* Tree topology was determined using phyml version 2.4.4 under WAG model. The scale bar indicates the mean number of substitutions per site. Bootstrap values were obtained from 1000 replicates under NJ (upper) and MP (lower) algorithms using PAUP 4.0b. Bootstrap values less than 50% are not shown. The red highlighted branches are sequences with experimental evidence. Purple highlighted sequences are from *α proteobacteria*, green highlighted sequences are from *β proteobacteria*, and blue highlighted ones are from *γ proteobacteria.*

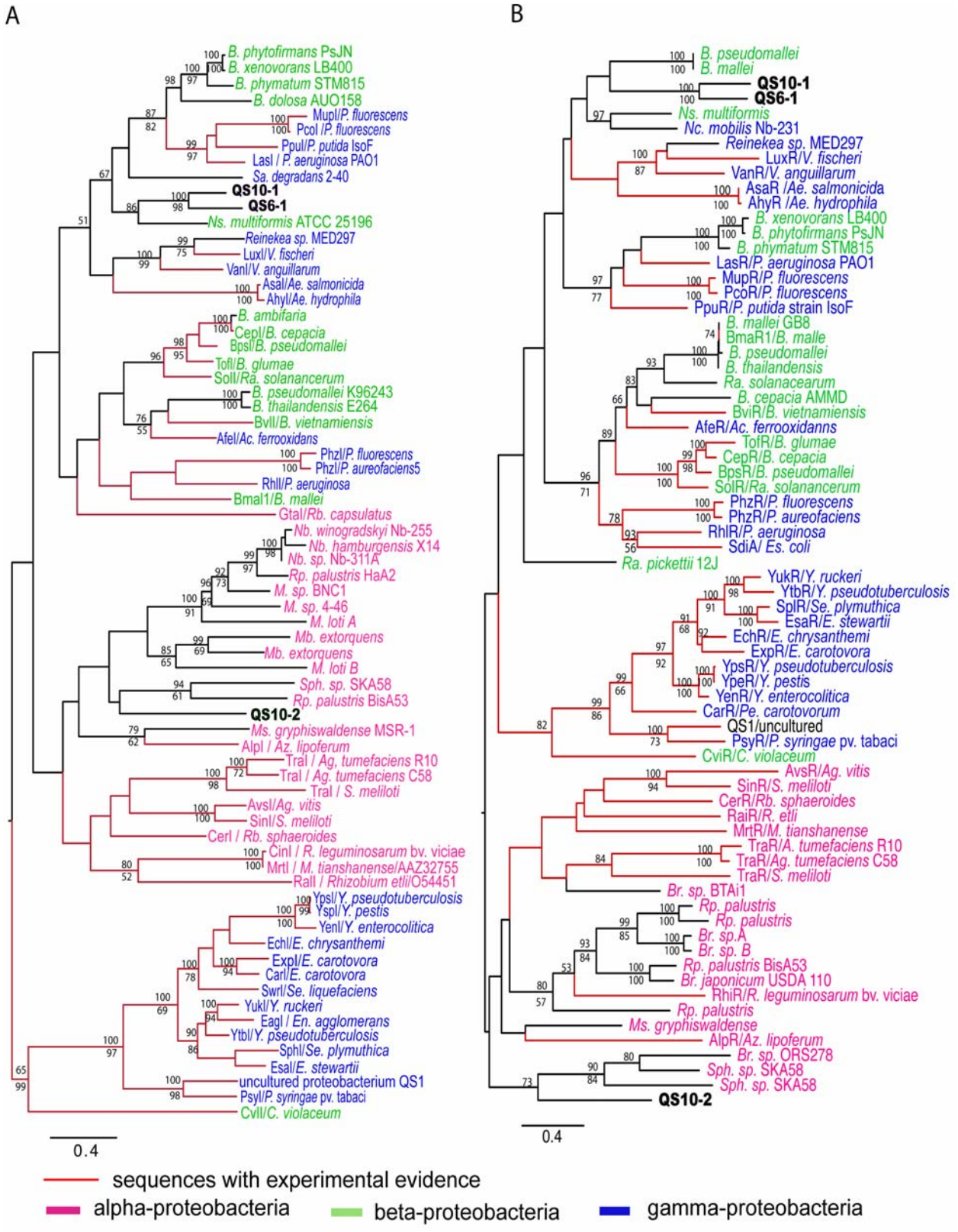

A
B. phytofirmans PsJN
B. xenovorans LB400
B. phymatum STM815
B. dolosa AUO158
MupI/P. fluorescens
PcoI /P. fluorescens
PpuI/P. putida IsoF
LasI / P. aeruginosa PAO1
Sa. degradans 2-40
QS10-1
QS6-1
Ns. multiformis ATCC 25196
Reinekea sp. MED297
LuxI/V. fischeri
VanI/V. anguillarum
AsaI/Ae. salmonicida
AhyI/Ae. hydrophila
B. ambifaria
CepI/B. cepacia
BpsI/B. pseudomallei
TofI/B. glumae
SolI/Ra. solanancerum
B. pseudomallei K96243
B. thailandensis E264
BviI/B. vietnamiensis
AfeI/Ac. ferrooxidans
PhzI/P. fluorescens
PhzI/P. aureofaciens5
RhlI/P. aeruginosa
BmaI1/B. mallei
GtaI/Rb. capsulatus
Nb. winogradskyi Nb-255
Nb. hamburgensis X14
Nb. sp. Nb-311A
Rp. palustris HaA2
M. sp. BNC1
M. sp. 4-46
M. loti A
Mb. extorquens
Mb. extorquens
M. loti B
Sph. sp. SKA58
Rp. palustris BisA53
QS10-2
Ms. gryphiswaldense MSR-1
AlpI / Az. lipoferum
TraI / Ag. tumefaciens R10
TraI / Ag. tumefaciens C58
TraI / S. meliloti
AvsI/Ag. vitis
SinI/S. meliloti
CerI / Rb. sphaeroides
CinI / R. leguminosarum bv. viciae
MrtI / M. tianshanense/AAZ32755
RalI / Rhizobium etli/O54451
YpsI/Y. pseudotuberculosis
YspI/Y. pestis
YenI/Y. enterocolitica
EchI/E. chrysanthemi
ExpI/E. carotovora
CarI/E. carotovora
SwrI/Se. liquefaciens
YukI/Y. ruckeri
EagI / En. agglomerans
YtbI/Y. pseudotuberculosis
SphI/Se. plymuthica
EsaI/E. stewartii
uncultured proteobacterium QS1
PsyI/P. syringae pv. tabaci
CviI/C. violaceum
0.4
B. pseudomallei
B. mallei
QS10-1
QS6-1
Ns. multiformis
Nc. mobilis Nb-231
Reinekea sp. MED297
LuxR/V. fischeri
VanR/V. anguillarum
AsaR /Ae. salmonicida
AhyR /Ae. hydrophila
B. xenovorans LB400
B. phytofirmans PsJN
B. phymatum STM815
LasR/P. aeruginosa PAO1
MupR/P. fluorescens
PcoR/P. fluorescens
PpuR/P. putida strain IsoF
B. mallei GB8
BmaR1/B. malle
B. pseudomallei
B. thailandensis
Ra. solanacearum
B. cepacia AMMD
BviR/B. vietnamiensis
AfeR/Ac. ferrooxidanns
TofR/B. glumae
CepR/B. cepacia
BpsR/B. pseudomallei
SolR/Ra. solanancerum
PhzR/P. fluorescens
PhzR/P. aureofaciens
RhlR/P. aeruginosa
SdiA/ Es. coli
Ra. pickettii 12J
YukR/Y. ruckeri
YtbR/Y. pseudotuberculosis
SplR/Se. plymuthica
EsaR/E. stewartii
EchR/E. chrysanthemi
ExpR/E. carotovora
YpsR/Y. pseudotuberculosis
YpeR/Y. pestis
YenR/Y. enterocolitica
CarR/Pe. carotovorum
QS1/uncultured
PsyR/P. syringae pv. tabaci
CviR/C. violaceum
AvsR/Ag. vitis
SinR/S. meliloti
CerR/Rb. sphaeroides
RaiR/R. etli
MrtR/M. tianshanense
TraR/A. tumefaciens R10
TraR/Ag. tumefaciens C58
TraR/S. meliloti
Br. sp. BTAi1
Rp. palustris
Rp. palustris
Br. sp.A
Br. sp. B
Rp. palustris BisA53
Br. japonicum USDA 110
RhiR/R. leguminosarum bv. viciae
Rp. palustris
Ms. gryphiswaldense
AlpR/Az. lipoferum
Br. sp. ORS278
Sph. sp. SKA58
Sph. sp. SKA58
QS10-2
0.4
sequences with experimental evidence
alpha-proteobacteria
beta-proteobacteria
gamma-proteobacteria

Figure 2.9. Phylogenetic analysis of sequences of QS6-1.

A, Midpoint rooted ML tree of homolog sequences of QS6-1-1, transglutaminase-like enzyme; B, Midpoint rooted ML tree of homologs of QS6-1-7, hypothetical protein; C, Midpoint rooted ML tree of homologs of QS6-1-4, hypothetical protein. Tree topologies were determined using phyml version 2.4.4. Numbers at the nodes are bootstrap values obtained from 1000 replicates using PAUP (Version 4.0 b) under neighbour joining algorithm (heuristic search). Abbreviations used: *A., Alteromonadales; B., Burkholderia; Br., Bradyrhizobium; Ca., Campylobacter; Ce., Cellulophaga; F., Flavobacterium; G., Gramella; H., Hahella; Hs., Halorhodospira; M., Mesorhizobium; Mc., Methylococcus; Ms., Magnetospirillum; P., Polaribacter ; Pc., Prosthecochloris; Pf., Psychroflexus; S., Shewanella; X., Xanthomonas; V., Vibrio*.

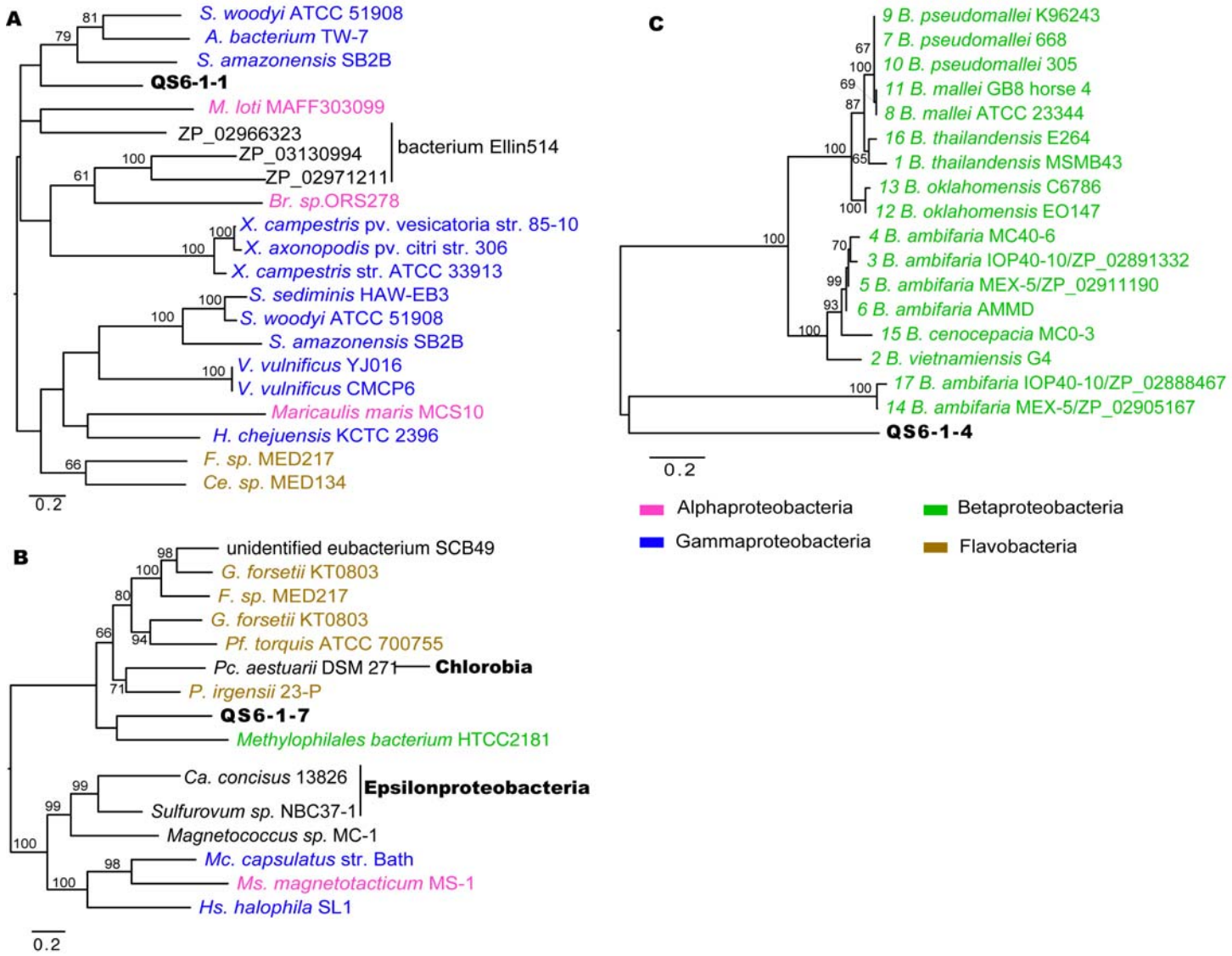

A
S. woodyi ATCC 51908
A. bacterium TW-7
S. amazonensis SB2B
QS6-1-1
M. loti MAFF303099
ZP_02966323
ZP_03130994
ZP_02971211
bacterium Ellin514
Br. sp.ORS278
X. campestris pv. vesicatoria str. 85-10
X. axonopodis pv. citri str. 306
X. campestris str. ATCC 33913
S. sediminis HAW-EB3
S. woodyi ATCC 51908
S. amazonensis SB2B
V. vulnificus YJ016
V. vulnificus CMCP6
Maricaulis maris MCS10
H. chejuensis KCTC 2396
F. sp. MED217
Ce. sp. MED134
0.2
B
unidentified eubacterium SCB49
G. forsetii KT0803
F. sp. MED217
G. forsetii KT0803
Pf. torquis ATCC 700755
Pc. aestuarii DSM 271
Chlorobia
P. irgensii 23-P
QS6-1-7
Methylophilales bacterium HTCC2181
Ca. concisus 13826
Epsilonproteobacteria
Sulfurovum sp. NBC37-1
Magnetococcus sp. MC-1
Mc. capsulatus str. Bath
Ms. magnetotacticum MS-1
Hs. halophila SL1
0.2
C
9 B. pseudomallei K96243
7 B. pseudomallei 668
10 B. pseudomallei 305
11 B. mallei GB8 horse 4
8 B. mallei ATCC 23344
16 B. thailandensis E264
1 B. thailandensis MSMB43
13 B. oklahomensis C6786
12 B. oklahomensis EO147
4 B. ambifaria MC40-6
3 B. ambifaria IOP40-10/ZP_02891332
5 B. ambifaria MEX-5/ZP_02911190
6 B. ambifaria AMMD
15 B. cenocepacia MC0-3
2 B. vietnamiensis G4
17 B. ambifaria IOP40-10/ZP_02888467
14 B. ambifaria MEX-5/ZP_02905167
QS6-1-4
0.2
Alphaproteobacteria
Betaproteobacteria
Gammaproteobacteria
Flavobacteria

Figure 2.10. Midpoint rooted ML tree of homologs of QS10-1-2, Mg-chelatase related protein. Tree topology was determined using phyml version 2.4.4. Bootstrap values were obtained from using PAUP (Version 4.0 b) under neighbour joining algorithm ("Fast" stepwise-addition). Abbreviations: *Ac., Acidovorax; Ae, Aeromonas; B ., Burkholderia; C., Comamonas; Cb., Chromobacterium; Ch., Chromohalobacter; Co., Coxiella; D., Delftia; Dc, Dechloromonas; F., Francisella; H., Herminiimonas; J., Janthinobacterium; L., Leptothrix; Mb., Methylobacillus; Mc., Methylococcus; Nc., Nitrococus; Ne., Neisseria; Ns., Nitrosomonas; Nsc. Nitrosococcus; P., Polaromonas; R., Ralstonia; Rf., Rhodofera; T., Thiobacillus; V., Verminephrobacter; X, Xanthomonas.*

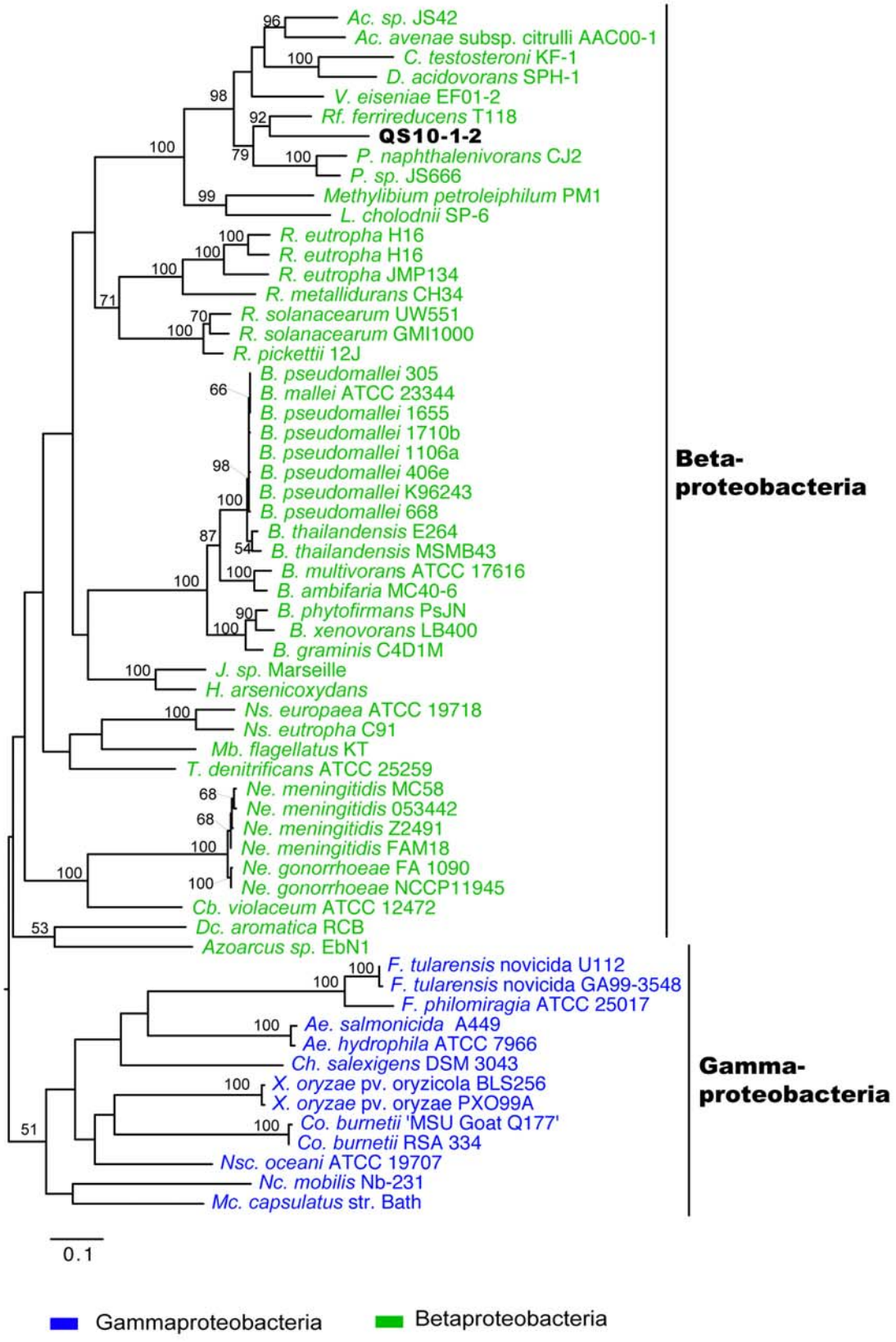

Ac. sp. JS42
Ac. avenae subsp. citrulli AAC00-1
C. testosteroni KF-1
D. acidovorans SPH-1
V. eiseniae EF01-2
Rf. ferrireducens T118
QS10-1-2
P. naphthalenivorans CJ2
P. sp. JS666
Methylibium petroleiphilum PM1
L. cholodnii SP-6
R. eutropha H16
R. eutropha H16
R. eutropha JMP134
R. metallidurans CH34
R. solanacearum UW551
R. solanacearum GMI1000
R. pickettii 12J
B. pseudomallei 305
B. mallei ATCC 23344
B. pseudomallei 1655
B. pseudomallei 1710b
B. pseudomallei 1106a
B. pseudomallei 406e
B. pseudomallei K96243
B. pseudomallei 668
B. thailandensis E264
B. thailandensis MSMB43
B. multivorans ATCC 17616
B. ambifaria MC40-6
B. phytofirmans PsJN
B. xenovorans LB400
B. graminis C4D1M
J. sp. Marseille
H. arsenicoxydans
Ns. europaea ATCC 19718
Ns. eutropha C91
Mb. flagellatus KT
T. denitrificans ATCC 25259
Ne. meningitidis MC58
Ne. meningitidis 053442
Ne. meningitidis Z2491
Ne. meningitidis FAM18
Ne. gonorrhoeae FA 1090
Ne. gonorrhoeae NCCP11945
Cb. violaceum ATCC 12472
Dc. aromatica RCB
Azoarcus sp. EbN1
F. tularensis novicida U112
F. tularensis novicida GA99-3548
F. philomiragia ATCC 25017
Ae. salmonicida A449
Ae. hydrophila ATCC 7966
Ch. salexigens DSM 3043
X. oryzae pv. oryzicola BLS256
X. oryzae pv. oryzae PXO99A
Co. burnetii 'MSU Goat Q177'
Co. burnetii RSA 334
Nsc. oceani ATCC 19707
Nc. mobilis Nb-231
Mc. capsulatus str. Bath
Beta-proteobacteria
Gamma-proteobacteria
0.1
Gammaproteobacteria
Betaproteobacteria

Figure 2.11. ML tree of homologs of QS10-2-1, Zinc containing alcohol dehydrogenase protein. Tree topology was determined using phyml version 2.4.4. Bootstrap values were obtained from using PAUP (Version 4.0 b) under neighbour joining algorithm (full heuristic). Abbreviations: *A., Acidovorax; Ag., Agrobacterium; Ar., Azorhizobium; B., Burkholderia; Bt. Bordetella; Co., Comamonas; Cu., Cupriavidus; D., Delftia; G., Granulibacter; H., Herminiimonas; Ho., Hoeflea; J., Janthinobacterium; Jb., Janibacter; Mb., Methylibium; Mr., Mesorhizobium; Ns., Nitrosospira; O., Oceanicola; Pa., Parvibaculum; Pn., Polynucleobacter; Po., Polaromonas; R., Rhizobium; Rb., Roseobacter; Rf., Rhodoferax; Rhb., Rhodobacterales; Rhs., Rhodospirillum; Rt., Ralstonia; S., Serratia; Sb., Solibacter; T., Thermobifida; V., Verminephrobacter; X., Xanthobacter; Y., Yersinia.*

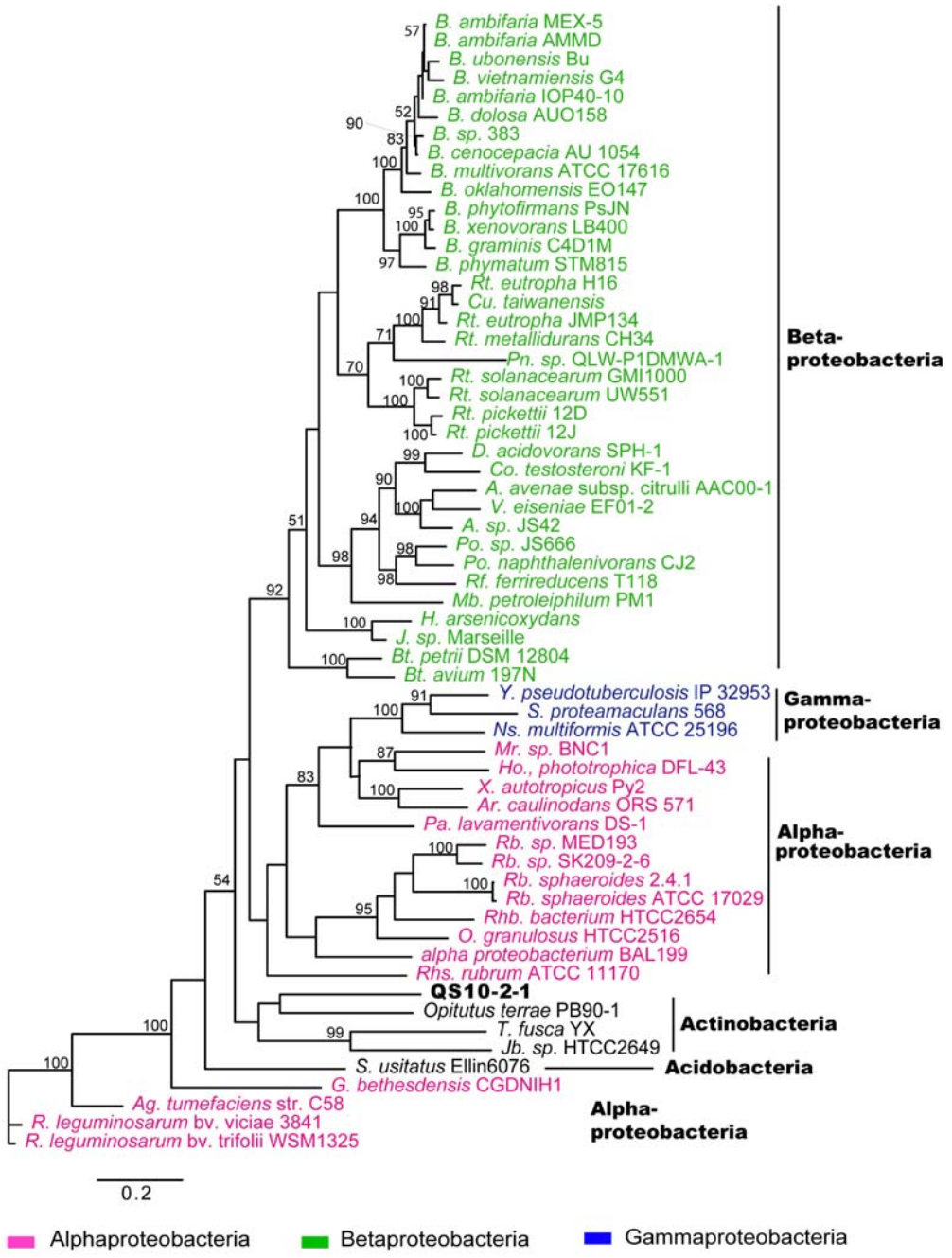
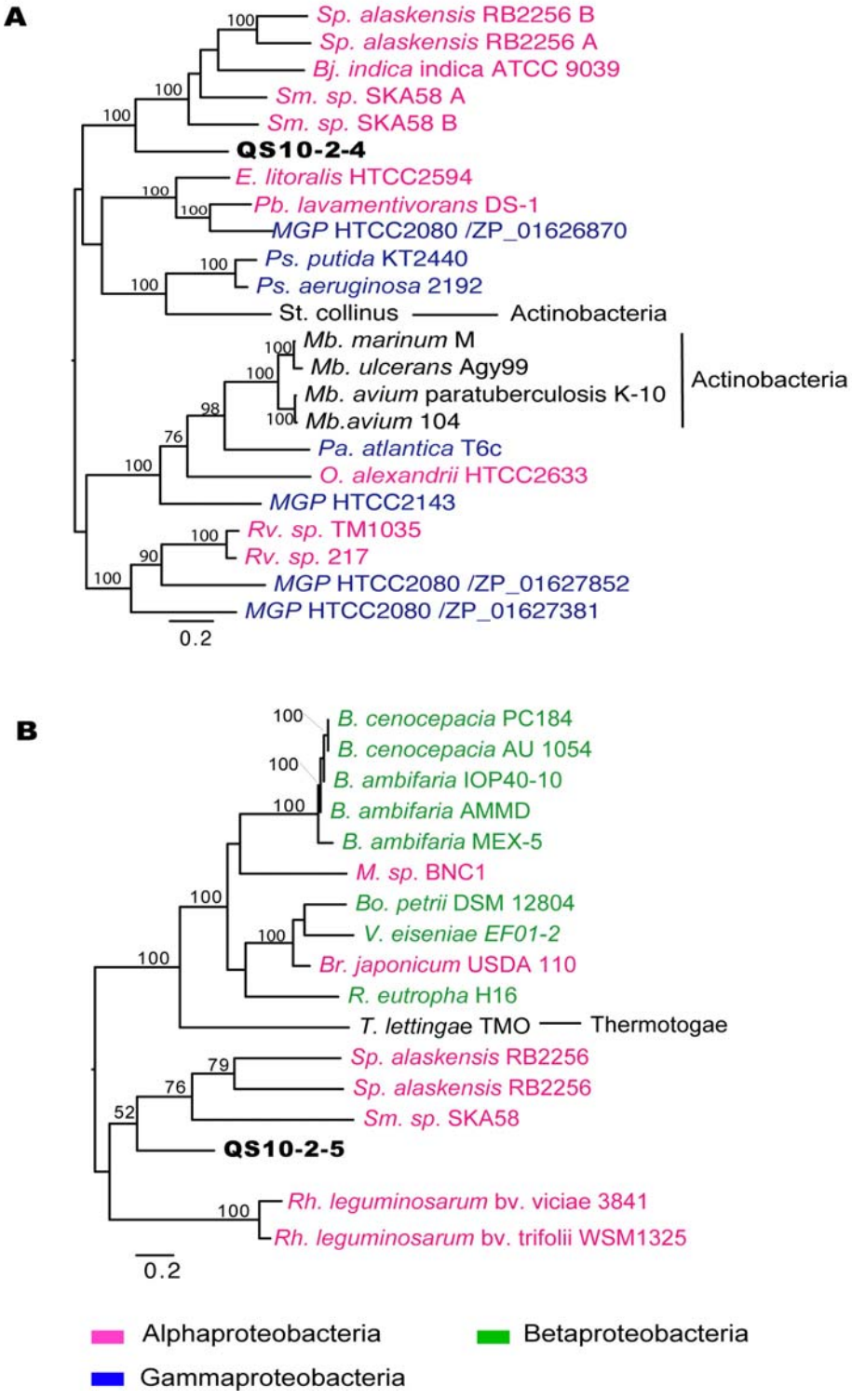

B. ambifaria MEX-5
B. ambifaria AMMD
B. ubonensis Bu
B. vietnamiensis G4
B. ambifaria IOP40-10
B. dolosa AUO158
B. sp. 383
B. cenocepacia AU 1054
B. multivorans ATCC 17616
B. oklahomensis EO147
B. phytofirmans PsJN
B. xenovorans LB400
B. graminis C4D1M
B. phymatum STM815
Rt. eutropha H16
Cu. taiwanensis
Rt. eutropha JMP134
Rt. metallidurans CH34
Pn. sp. QLW-P1DMWA-1
Rt. solanacearum GMI1000
Rt. solanacearum UW551
Rt. pickettii 12D
Rt. pickettii 12J
D. acidovorans SPH-1
Co. testosteroni KF-1
A. avenae subsp. citrulli AAC00-1
V. eiseniae EF01-2
A. sp. JS42
Po. sp. JS666
Po. naphthalenivorans CJ2
Rf. ferrireducens T118
Mb. petroleiphilum PM1
H. arsenicoxydans
J. sp. Marseille
Bt. petrii DSM 12804
Bt. avium 197N
Beta-proteobacteria
Y. pseudotuberculosis IP 32953
S. proteamaculans 568
Ns. multiformis ATCC 25196
Gamma-proteobacteria
Mr. sp. BNC1
Ho., phototrophica DFL-43
X. autotropicus Py2
Ar. caulinodans ORS 571
Pa. lavamentivorans DS-1
Rb. sp. MED193
Rb. sp. SK209-2-6
Rb. sphaeroides 2.4.1
Rb. sphaeroides ATCC 17029
Rhb. bacterium HTCC2654
O. granulosus HTCC2516
alpha proteobacterium BAL199
Rhs. rubrum ATCC 11170
Alpha-proteobacteria
QS10-2-1
Opitutus terrae PB90-1
T. fusca YX
Jb. sp. HTCC2649
Actinobacteria
S. usitatus Ellin6076
Acidobacteria
G. bethesdensis CGDNIH1
Ag. tumefaciens str. C58
R. leguminosarum bv. viciae 3841
R. leguminosarum bv. trifolii WSM1325
Alpha-proteobacteria
0.2
Alphaproteobacteria
Betaproteobacteria
Gammaproteobacteria

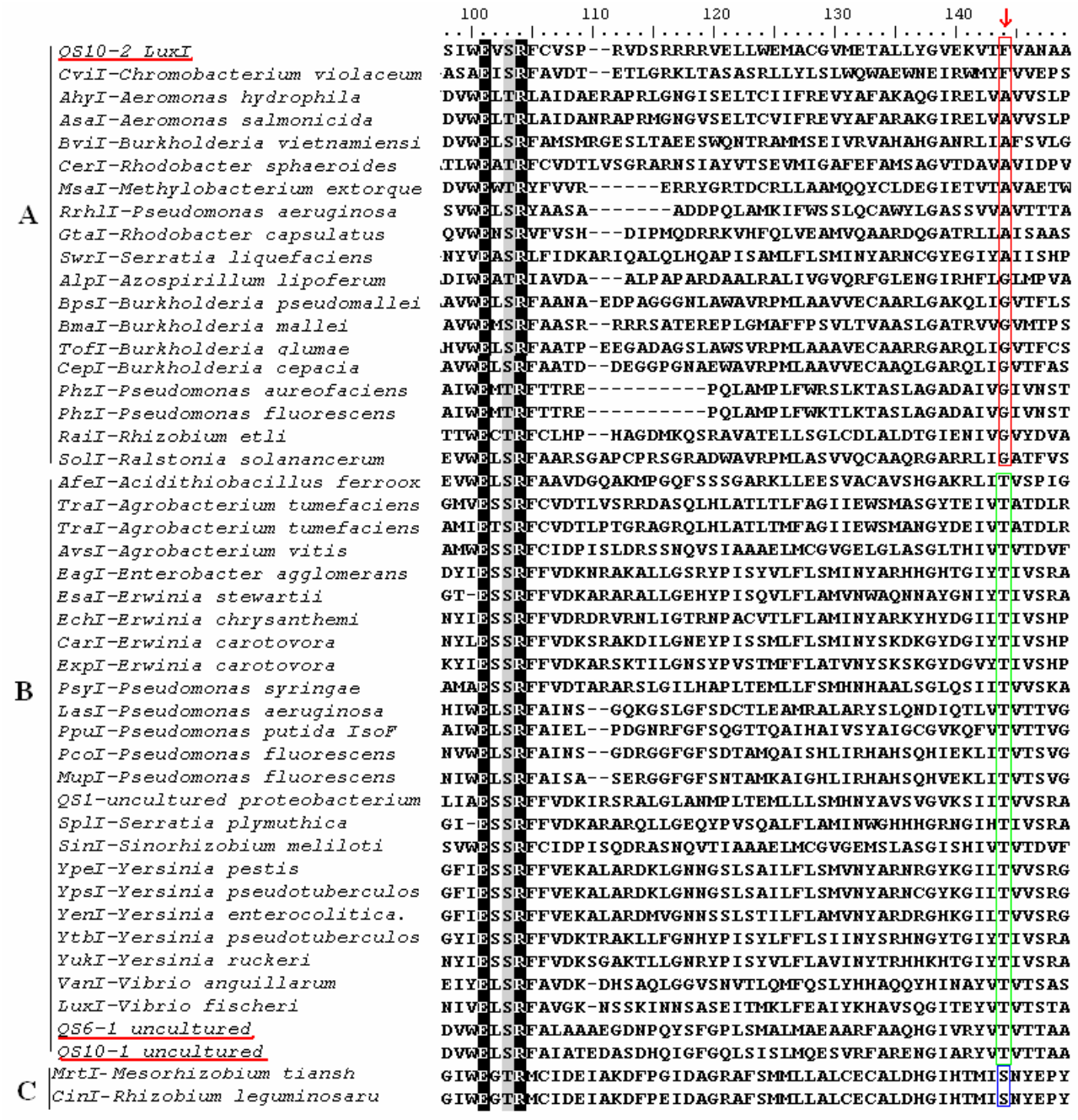
Figure 2.12. Phylogentic analysis of QS10-2-4 and QS10-2-5. A, ML tree of homologs of QS10-2-4, a phytonyl-CoA dioxygenase related protein. B, ML tree of homologs of QS10-2-5, a putative GntR family transcriptional regulator. Tree topologies were determined using phyml version 2.4.4. Bootstrap values were obtained from using PAUP (Version 4.0 b) under neighbour joining algorithm (full heuristic). Abbreviations: *B., Burkholderia; Bj., Beijerinckia; Bo, Bordetella; Br. Bradyrhizobium; E., Erythrobacter; M., Mesorhizobium; Mb., Mycobacterium; MGP, marine gamma proteobacterium; O., Oceanicaulis; Pb., Parvibaculum; Ps., Pseudomonas; Pa.,Pseudoalteromonas; R., Ralstonia; Rh. Rhizobium; Rv., Roseovarius; Sm., Sphingomonas ; Sp., Sphingopyxis; St., Streptomyces; T., Thermotoga; V., Verminephrobacter*.


A
Sp. alaskensis RB2256 B
Sp. alaskensis RB2256 A
Bj. indica indica ATCC 9039
Sm. sp. SKA58 A
Sm. sp. SKA58 B
QS10-2-4
E. litoralis HTCC2594
Pb. lavamentivorans DS-1
MGP HTCC2080 /ZP_01626870
Ps. putida KT2440
Ps. aeruginosa 2192
St. collinus
Actinobacteria
Mb. marinum M
Mb. ulcerans Agy99
Mb. avium paratuberculosis K-10
Mb.avium 104
Actinobacteria
Pa. atlantica T6c
O. alexandrii HTCC2633
MGP HTCC2143
Rv. sp. TM1035
Rv. sp. 217
MGP HTCC2080 /ZP_01627852
MGP HTCC2080 /ZP_01627381
0.2
B
B. cenocepacia PC184
B. cenocepacia AU 1054
B. ambifaria IOP40-10
B. ambifaria AMMD
B. ambifaria MEX-5
M. sp. BNC1
Bo. petrii DSM 12804
V. eiseniae EF01-2
Br. japonicum USDA 110
R. eutropha H16
T. lettingae TMO
Thermotogae
Sp. alaskensis RB2256
Sp. alaskensis RB2256
Sm. sp. SKA58
QS10-2-5
Rh. leguminosarum bv. viciae 3841
Rh. leguminosarum bv. trifolii WSM1325
0.2
Alphaproteobacteria
Betaproteobacteria
Gammaproteobacteria

2.3.3.3 Sequence analysis of the key position of LuxI homologs.

It has been reported that the 140 position of the EsaI protein (the LuxI homolog in *Pantoea stewartii*), which is part of the active site of the acyl chain binding region, is critical for the specificity of the C3-substitution of AHLs (Watson *et al.*, 2002). By comparing several other LuxI homologs, it was found that those that synthesize 3-O-substituted AHLs usually contain a threonine (T) at the corresponding position. On the other hand, LuxI homologs that synthesize 3-OH-substituted AHLs contain a serine (S), and LuxI homologs that synthesize C3-unsubstituted AHLs could contain an alanine (A) or glycine (G) at this position (Watson *et al.*, 2002). By aligning 43 LuxI homolog sequences that have been experimentally studied with the 3 LuxI homologs identified in this study, it was found that $LuxI_{QS6\text{-}1}$ and $LuxI_{QS10\text{-}1}$, together with LuxI homolog sequences that mainly synthesize 3-O-substituted AHLs, contain a T at the position corresponding to the 140 position of EsaI (Figure 2.13 group B). It was also found that, in addition to A and G, a phenylalanine (F) at that position is also conserved for synthesis of C3-unsubstitued AHLs. The $LuxI_{QS10\text{-}2}$, which has later been shown to synthesize C8 HSL and C10 HSL, together with the CviI of *Chromobacterium violaceum*, which synthesizes C6 HSL, contain an F at the corresponding position of T140 of EsaI (Figure 2.13 group A).

Figure 2.13. Multiple sequence alignment of LuxI homologs with experimental evidence. Group A are sequences that mainly synthesize C3-unsubstituted AHLs. Group B are sequences that mainly synthesize 3-O-substituted AHLs. Group C are sequences that synthesize 3-OH-substituted AHLs. The red arrow indicates the corresponding position of the EsaI T140.


100
110
120
130
140
A
B
C
QS10-2 LuxI
CviI-Chromobacterium violaceum
AhyI-Aeromonas hydrophila
AsaI-Aeromonas salmonicida
BviI-Burkholderia vietnamiensi
CerI-Rhodobacter sphaeroides
MsaI-Methylobacterium extorque
RrhlI-Pseudomonas aeruginosa
GtaI-Rhodobacter capsulatus
SwrI-Serratia liquefaciens
AlpI-Azospirillum lipoferum
BpsI-Burkholderia pseudomallei
BmaI-Burkholderia mallei
TofI-Burkholderia glumae
CepI-Burkholderia cepacia
PhzI-Pseudomonas aureofaciens
PhzI-Pseudomonas fluorescens
RaiI-Rhizobium etli
SolI-Ralstonia solanancerum
AfeI-Acidithiobacillus ferroox
TraI-Agrobacterium tumefaciens
TraI-Agrobacterium tumefaciens
AvsI-Agrobacterium vitis
EagI-Enterobacter agglomerans
EsaI-Erwinia stewartii
EchI-Erwinia chrysanthemi
CarI-Erwinia carotovora
ExpI-Erwinia carotovora
PsyI-Pseudomonas syringae
LasI-Pseudomonas aeruginosa
PpuI-Pseudomonas putida IsoF
PcoI-Pseudomonas fluorescens
MupI-Pseudomonas fluorescens
QS1-uncultured proteobacterium
SplI-Serratia plymuthica
SinI-Sinorhizobium meliloti
YpeI-Yersinia pestis
YpsI-Yersinia pseudotuberculos
YenI-Yersinia enterocolitica.
YtbI-Yersinia pseudotuberculos
YukI-Yersinia ruckeri
VanI-Vibrio anguillarum
LuxI-Vibrio fischeri
QS6-1 uncultured
QS10-1 uncultured
MrtI-Mesorhizobium tiansh
CinI-Rhizobium leguminosaru

2.3.4 Expression of LuxI in *E. coli.* $LuxI_{QS6-1}$, $LuxI_{QS10-1}$ and $LuxI_{QS10-1}$ were subcloned into pET30a+ and pET30b+ to get pET6-1LuxI, pET10-1LuxI and pET10-2LuxI. When induced with 0.5 mM IPTG for 5 h at 28ºC, all three proteins were able to be expressed at high levels (Figure 2.14). $LuxI_{QS6-1}$ was also subcloned into the broad-host-range vector pRK415 under the control of the *lac* promoter. When it was introduced into the biosensor strain HC103(pJZ381), it was expressed constitutively and enabled the biosensor strain to form blue colonies (Figure 2.15).

2.3.5 β-galactosidase activity of the isolated clones. When streaked on agar plates, HC103(pJZ381)(pQS10-1) and HC103(pJZ381)(pQS10-2) produced signals that diffused in agar and induce the biosensor strain adjacent to them to turn blue (Figure 2.16 shows this phenomenon for HC103(pJZ381)(pQS10-1)), while diffusion of signals was not observed for HC103(pJZ381)(pQS6-1). When the $luxI_{QS6-1}$ homolog gene was subcloned into the broad host vector pRK415 and expressed under the the control of the *lac* promoter, the diffusion of QS inducing signals was observed for the resulting strain *A. tumefaciens* HC103(pJZ381)(pRK6-1LuxI).

The isolated clones showed different levels of β-galactosidase activity (Figure 2.17). Of the three isolated clones, QS6-1 showed the lowest activity, QS10-1 showed the highest activity, and QS10-2 showed an intermediate level of activity. When $LuxI_{QS6-1}$ was expressed under the control of the *lac* promoter in pRK6-1LuxI, the resulting strain *A. tumefaciens* HC103(pJZ381)(pRK6-1LuxI) showed a high level of activity.

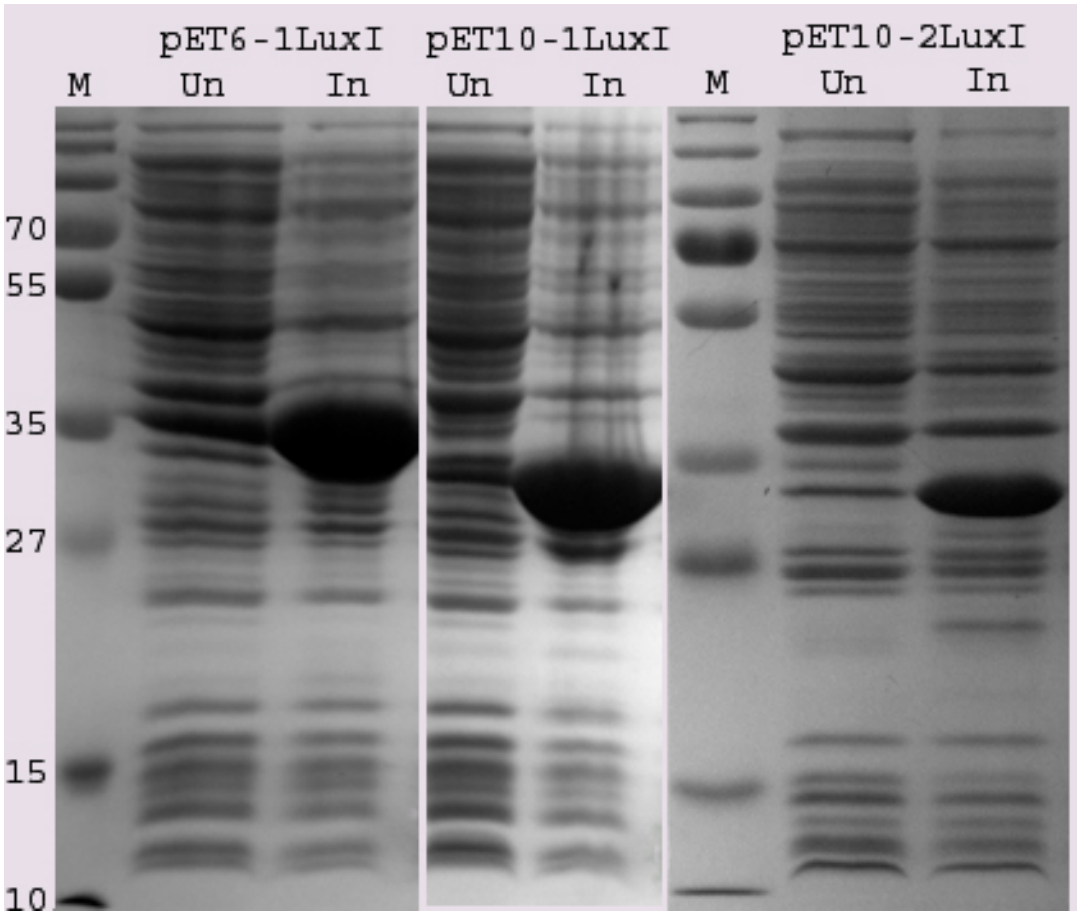


Figure 2.14. LuxI expression in *E. coli* BL21(DE3). M, molecular weight marker, Un, uninduced total protein. In, induced total protein.

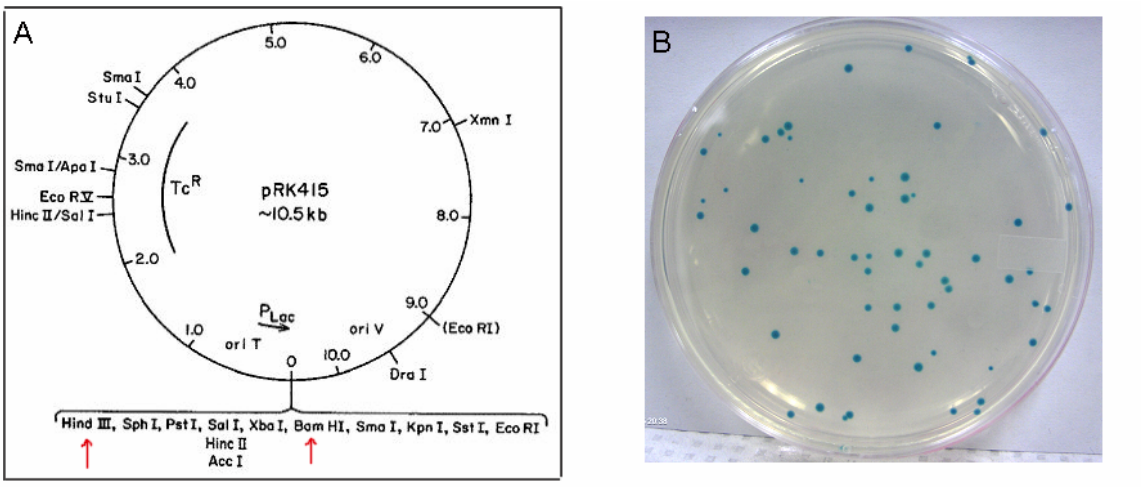


Figure 2.15. Construction of pRK6-1LuxI and expression of *luxI*$_{QS6\text{-}1}$ in *A. tumefaciens* HC103(pJZ381). A. The map of the vector pRK415. *luxI*$_{QS6\text{-}1}$ was inserted into the HindIII and BamHI sites of the vector and was expressed under the control of the *lac* promter. B. The plasmid pRK6-1LuxI was electroporated into the biosensor strain *A. tumefaciens* HC103(pJZ381), and the expression of *luxI*$_{QS6\text{-}1}$ enables the biosensor strain to form blue colonies in presence of X-Gal.

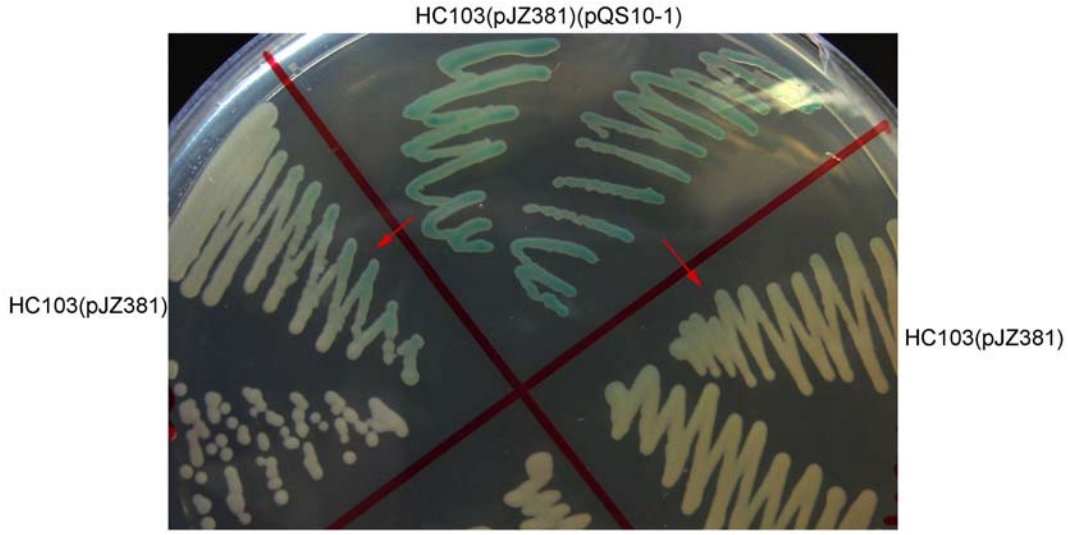


Figure 2.16. Diffusion assay for HC103(pJZ381)(pQS10-1). *A. tumefaciens* HC103(pJZ381)(pQS10-1) (the top quarter of the plate) and the biosensor strain *A. tumefaciens* HC103(pJZ381) (the left and right quarters of the plate) were streaked on LB agar with Km (50 μg/ml), Gm (50 μg/ml) and X-Gal (20 μg/ml). After 2 days incubation at 28°C, *A. tumefaciens* HC103(pJZ381)(pQS10-1) was able to secrete diffusible signals to induce the colonies of the biosensor strain adjacent to it to turn blue (indicated by red arrows).

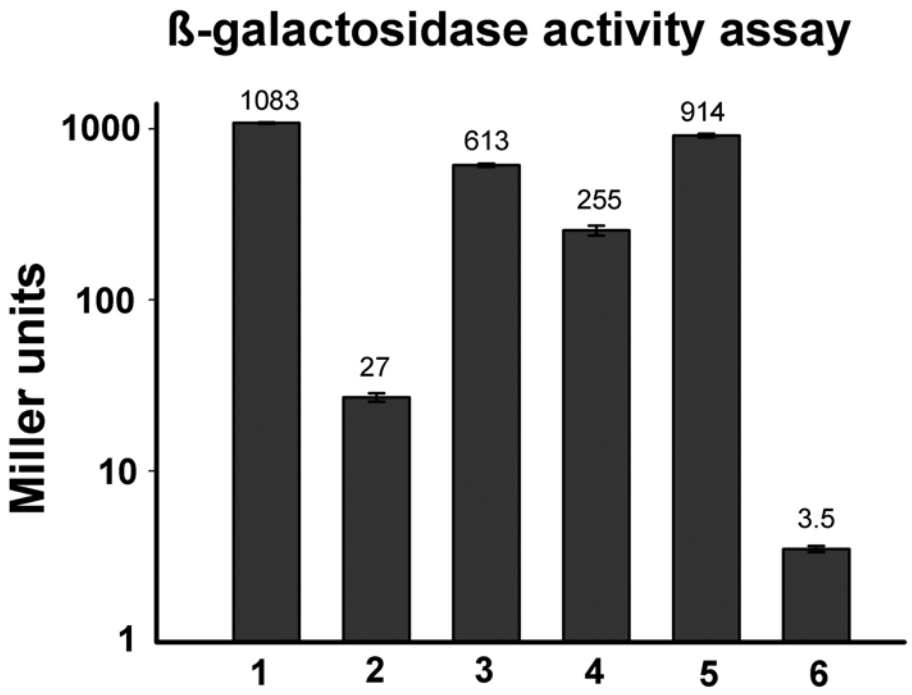


Figure 2.17. Activity of the isolated clones. 1, *A. tumefaciens* HC103(pJZ381)(pRK6-1LuxI); 2, *A. tumefaciens* HC103(pJZ381)(pQS6-1); 3, *A. tumefaciens* HC103(pJZ381)(pQS10-1); 4, *A. tumefaciens* HC103(pJZ381)(pQS10-2); 5, *A. tumefaciens* HC103(pJZ381) + 10nM OOHL; 6, *A. tumefaciens* HC103(pJZ381). Error bars are standard error from 3 replicates of independent cultures.

2.3.6 Analytical TLC assay

In order to characterize the AHLs produced by each clone, an analytical TLC assay was performed as described in the Materials and Methods. Figure 2.18 shows the results of the TLC assay. On the TLC plate, 3-O-AHLs formed tailed spots while C3-unsubstituted AHLs formed round spots (Figure 2.18 S1 and S2). When expressed in *A. tumefaciens* under its own promoter, no signal (lane 1) or only a very weak signal (lane 1') was detected for $luxI_{QS6-1}$. When the $luxI_{QS6-1}$ was subcloned into pRK415 under the *lac* promoter and expressed in *A. tumefaciens*, or into pET30a+ under the T7 promoter and expressed in *E. coli*, multiple signals were detected, including both short and long chain AHLs (lanes 2 and 3). $LuxI_{QS10-1}$ directed synthesis of multiple AHLs when expressed in either *A. tumefaciens* or *E. coli* (lanes 4 and 5). Similar to the 3-O-AHL standards, the AHLs synthesized by both $LuxI_{QS6-1}$ and $LuxI_{QS10-1}$ tend to form tailed spots on the TLC plate. Extractions from *A. tumefaciens* HC103(pJZ381)(pQS10-2) showed two round spots on the TLC plate with similar shape and migration rate to C6-HSL and C8-HSL (lane 6). When expressed in *E. coli*, some additional signals were detected with one of them showing similarity to C10-HSL (lane 8).

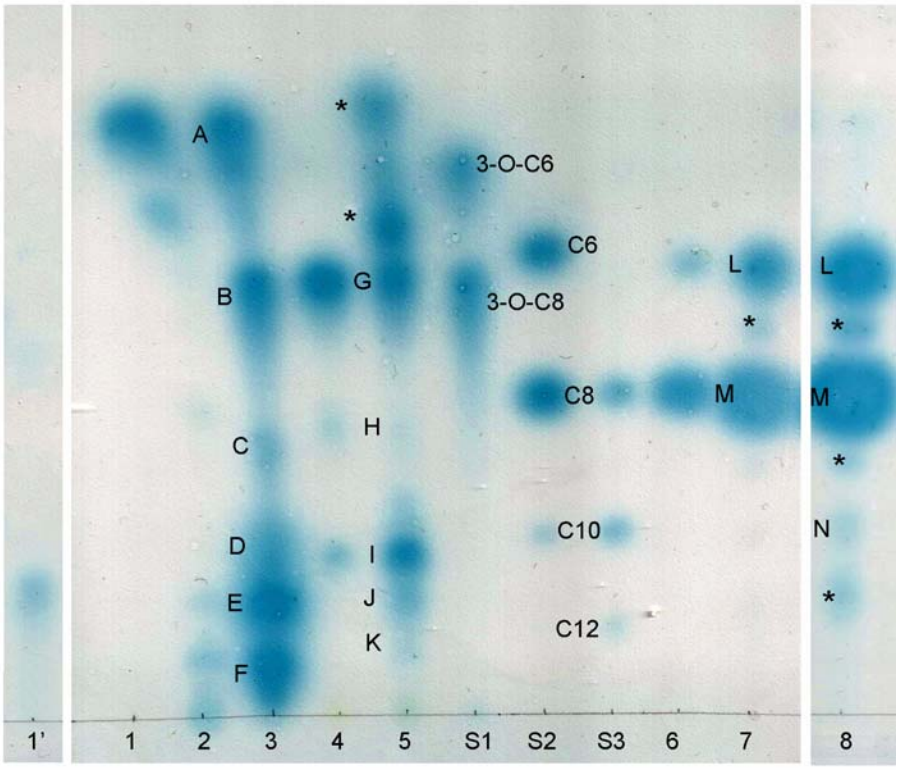


Figure 2.18. Analytical TLC assay. S1 and S2, standards: S1, 3-O-C6 HSL (2 pmol) and 3-O-C8 HSL(0.1 pmol); S2, C6 HSL(4000 pmol), C8 HSL(100 pmol), C10 HSL(1000 pmol) and C12 HSL(9000 pmol). 1-8, condensed extractions of different strain cultures: 1, HC103(pJZ381)(pQS6-1) (10 ml); 2, HC103(pJZ381)(pRK6-1LuxI) (5 ml); 3, BL21(DE3)(pET6-1LuxI) (2 ml); 4, HC103(pJZ381)(pQS10-1) (5 ml); 5, BL21(DE3)(pET10-1LuxI) (5 ml); 6, HC103(pJZ381)(pQS10-2) (5 ml); 7, BL21(DE3)(pET10-2LuxI)(3 ml) ; 8, BL21(DE3)(pET10-2LuxI) (5 ml). The numbers in parenthesis are the corresponding volumes of culture supernatant extracted. The spots labeled from A to N were purified from preparative TLC plates and further analyzed using ESI MS and MS/MS. The spots labeled with "*" are unknown compounds and were not analyzed using ESI MS and MS/MS.

2.3.7 Preparative TLC and Mass spectrometry analysis

The active compounds at the corresponding spots (from A to M) on Figure 2.18 were partially purified by removing the silica matrix from a preparative TLC plate and extracting and drying as described in the the Materials and Methods section. The residues were dissolved in 20 to 50 µl of 1:1 acetonitrile:water with 0.2 % formic acid and subjected to ESI mass spectrometry. Suspected peaks were then chosen to perform MS/MS to elucidate the structures. Aliquots of 20 pmol of standards (3-O-C6, 3-O-C8, C6, C8, C12, 3-O-C16:1) were also subjected to ESI MS and MS/MS. By comparison with the standards and data published in the literature, we were able to identify most of the active compounds synthesized by each LuxI homolog (Table 2.8).

Figure 2.19 shows the MS and MS/MS results for compounds synthesized by $LuxI_{QS6\text{-}1}$ at the corresponding spots D, E and F on analytical TLC plate (Figure 2.18). There are two AHLs identified from spot F (Figure 2.19 d, e and f). One showed exactly the same MS/MS pattern as a commercial sample of the compound N-3-oxo-hexadec-11Z-enoyl-L-homoserine lactone (3-O-C16:1-Δ11cis-(L)-HSL) (Cayman Chemical) (Figure 2.19 b and e) which indicates it is 3-O-C16:1 HSL. The other is 3-oxo-tetradecanoyl-homoserine lactone (3-O-C14 HSL) as it shows a similar MS/MS pattern to synthesized N-3-oxo-dodecanoyl-L-homoserine lactone (3-O-C12 HSL) (Cayman chemical), except that the molecular weight (MW) and all of the acyl chain derived fragments are 28 units bigger (corresponding to one C_2H_4 unit) (Figure 2.19 c and f). Another major AHL was identified from both spot D and E (Figure 2.19 g and i), and the MS/MS pattern (Figure 2.19 h) confirms that it is 3-O-C14:1 HSL, as it shows a pattern similar to 3-O-C16:1-Δ11cis-(L)-HSL except that the mass is 28 units smaller (Figure

2.19 b and h). Two minor AHLs were also identified from spot E, one with m/z 306 and another with m/z 356 during MS (Figure 2.19 i). The MS/MS pattern for the ion at m/z 306 (Figure. 2.19 k) showed a similar pattern to that of the previously published C14:1 HSL and C14:2 HSL (Nieto Penalver *et al.*, 2006) except that the mass is 4 and 2 mass units smaller than C14:1 HSL and C14:2 HSL, respectively. This suggests that the AHL at m/z 306 contains 3 unsaturated bonds on the acyl chain, and is therefore likely to be a novel AHL, C14:3 HSL. When collisionally activated, the ion at m/z 356 gave a major peak at m/z 338 (Figure 2.19 j), which most likely results from losing a water molecule. According to the relative abundance of the ions at m/z 338 and 356, the molecule most probably contains an OH group. The peak at m/z 324 is 32 mass units smaller than the ion 356, which most probably results from losing a methanol moiety. All of the other peaks of the MS/MS spectrum are very similar to that of 3-O-C14:1 HSL. So the most probable structure of the compound at m/z 356.24 is (?)-hydroxymethyl-3-oxo-C14 HSL, which contains a -CH_2OH.group substitution on the acyl chain of 3-O-C14 HSL. The exact position of the substitution needs further experiments to be determined. After losing a methanol, the compound will turn into 3-O-C14:1 HSL. Although the synthesized standard 3-O-C16:1 HSL does not give the adduct ion [M+MeOH+H] during MS, there is still possibility that the compound at m/z 356 is the adduct ion [M+MeOH+H] of 3-O-C14:1 HSL, which is formed during TLC or MS assay. Further experiments are required to determine whether it is a new AHL synthesized by bacteria or just an adduct ion of 3-O-C14:1 HSL and to determine the exact structure.

The compound at spot I synthesized by $LuxI_{QS10\text{-}2}$ showed a major peak at m/z 298 during MS analysis and the MS/MS pattern is the same as the synthesized 3-O-C12

except there is an extra peak at m/z 175 (Figure 2.20 a and b). This is most probably from contamination by another compound produced by the *E. coli* host that showed the same m/z as 3-O-C12 HSL in MS. In fact, a control sample which is extracted from the same position of a TLC plate loaded with extracts from *E. coli* BL21(DE3) culture showed a peak at m/z 299 in the mass spectrum, and during MS/MS, it does give a major peak at m/z 175 (Figure 2.20 c). For the substance at spot K, the mass spectrum showed an ion with m/z of 326 and the fragmentation pattern confirmed that the active compound at spot K is 3-O-C14 HSL (Figure 2.20 d).

The MS and MS/MS results for compounds at spot M synthesized by $LuxI_{QS10\text{-}2}$ exhibit exactly the same spectrum as the synthesized standard C8 HSL (Figure 2.21 a and b), confirming that the active compound at spot M is C8 HSL. The MS for the compounds at spot N showed a major ion at m/z 256 and the MS/MS analysis confirms that it is C10 HSL (Figure 2.21 c).

According to spot shapes and Rf values, the active compounds at spots B and G are similar to 3-O-C8, spot L is similar to C6-HSL. The compounds at spot A showed a similar shape to 3-O-AHLs, but migrated faster than 3-O-C6 AHL, which could be the product of the recyclization of the open ringed 3-O-C6 AHL (Yates *et al.*, 2002) (*Agrobacterium* can not detect C4 derivatives). ESI MS and MS/MS were tried twice for those samples, but no suspect AHLs was identified. The attempts to identify the active compounds at spots C and H were also not successful. This may be due to the low abundance of the active compounds, or due to the technical limitations of preparative TLC for purifying AHLs. There is also a possibility that these active compounds could be

some signal mimics other than AHLs. The spots marked with an asterisk (*) were not analyzed in this study.

Figure 2.19. ESI MS and MS/MS results of the active compounds produced by $LuxI_{QS6\text{-}1}$ (at spot D, E and F on Figure 2.18). a, MS results for synthesized 3-O-C16:1-Δ11cis-(L)-HSL; b, MS/MS for synthesized 3-O-C16:1-Δ11cis-(L)-HSL; c, MS/MS for synthesized 3-O-C12 HSL; d, MS for compounds purified from spot F in Figure 2.18; e, MS/MS for the ion at m/z 352.2 in panel d; f, MS/MS for the ion at m/z 326.2 in panel d; g, MS for compounds purified from spot D in Figure 2.18; h, MS/MS for the ion at m/z 324.2 in panel g and i; i, MS for the compound purified at spot E on TLC plate. j, MS/MS for the ion at m/z 356.2 in panel i; k, MS/MS for the ion at m/z 306.2 in panel i.

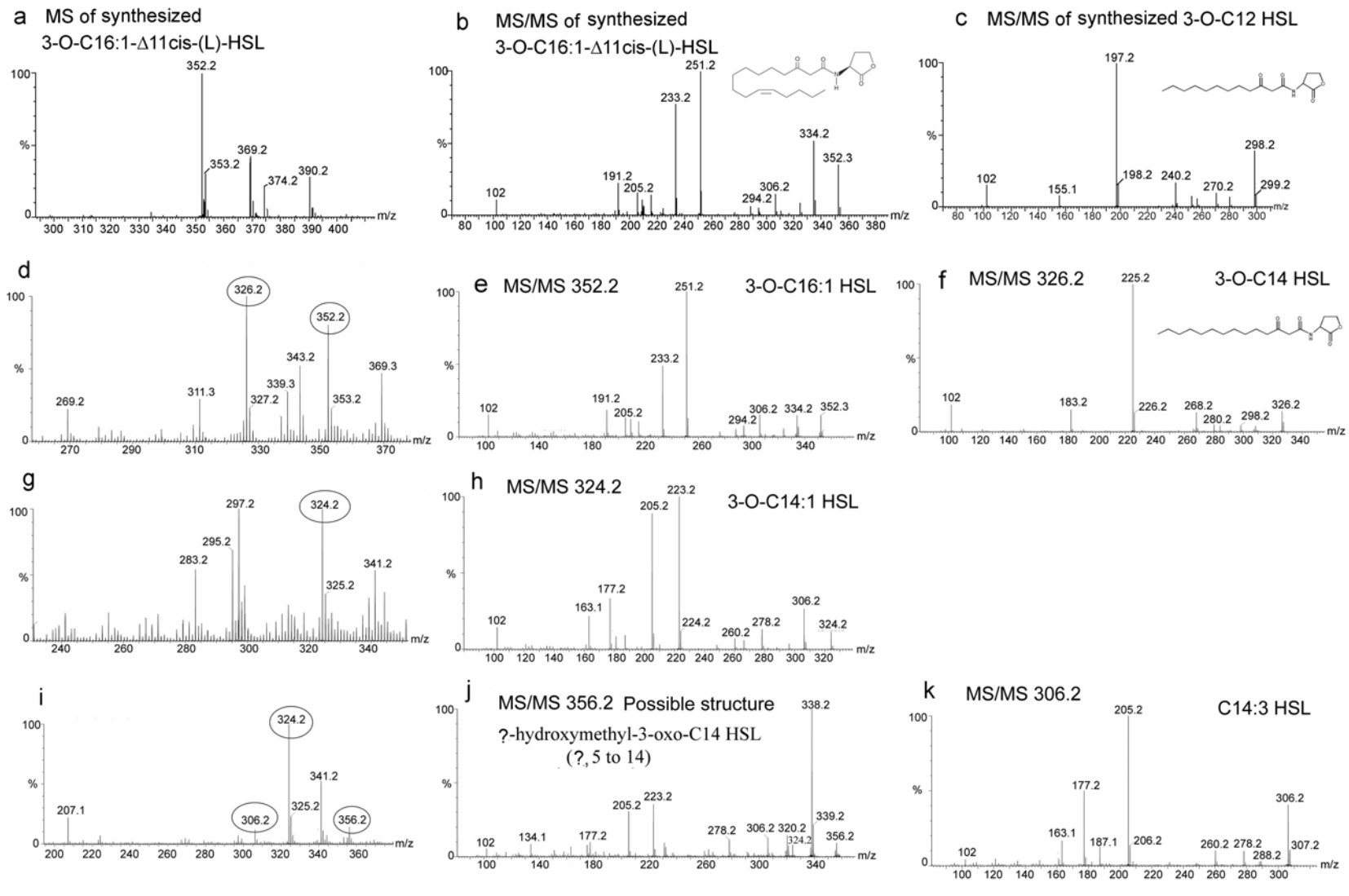

a MS of synthesized
3-O-C16:1-Δ11cis-(L)-HSL
b MS/MS of synthesized
3-O-C16:1-Δ11cis-(L)-HSL
c MS/MS of synthesized 3-O-C12 HSL
d
e MS/MS 352.2 3-O-C16:1 HSL
f MS/MS 326.2 3-O-C14 HSL
g
h MS/MS 324.2 3-O-C14:1 HSL
i
j MS/MS 356.2 Possible structure
?-hydroxymethyl-3-oxo-C14 HSL
(?, 5 to 14)
k MS/MS 306.2 C14:3 HSL
m/z

Figure 2.20. ESI MS and MS/MS results of the standard 3-O-C12 HSL and the active compounds produced by $LuxI_{QS10\text{-}1}$ (at spot I and K on Figure 2.18). A, MS (left) and MS/MS (right) of synthetic 3-O-C12 HSL; B, MS (left) and MS/MS (right) of the active compounds at spot I. C, MS (left) and MS/MS (right) of control for spot I; D, MS (left) and MS/MS (right) of the active compounds at spot K.

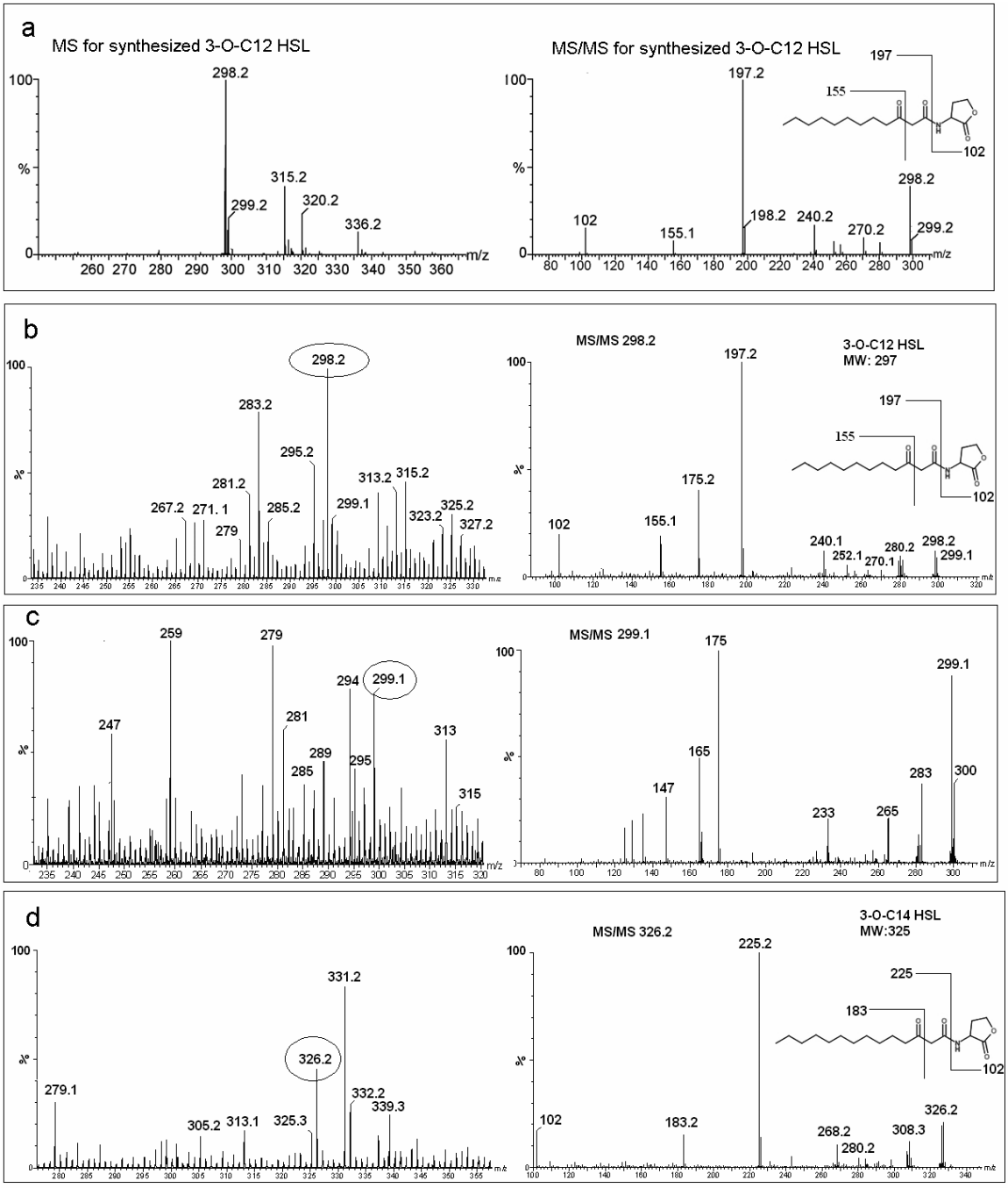

a
MS for synthesized 3-O-C12 HSL
298.2
315.2
299.2
320.2
336.2
MS/MS for synthesized 3-O-C12 HSL
197
197.2
155
102
298.2
102
155.1
198.2
240.2
270.2
299.2
b
298.2
283.2
295.2
281.2
313.2
315.2
267.2
271.1
285.2
299.1
325.2
323.2
279
327.2
MS/MS 298.2
197.2
3-O-C12 HSL
MW: 297
197
155
175.2
102
102
155.1
240.1
252.1
280.2
298.2
270.1
299.1
c
259
279
294
299.1
247
281
313
289
285
295
315
MS/MS 299.1
175
299.1
165
283
300
147
233
265
d
331.2
326.2
279.1
332.2
339.3
305.2
313.1
325.3
MS/MS 326.2
225.2
3-O-C14 HSL
MW:325
225
183
102
102
183.2
326.2
268.2
308.3
280.2

Figure 2.21. ESI MS and MS/MS results of the standard C8-HSL and the AHLs produced by $LuxI_{QS10\text{-}2}$ (at spot M and N on Figure 2.18). A, MS (left) and MS/MS (right) of synthetic C8 HSL; B, MS (left) and MS/MS (right) of the compounds at spot M; B, MS (left) and MS/MS (right) of the compounds at spot N.

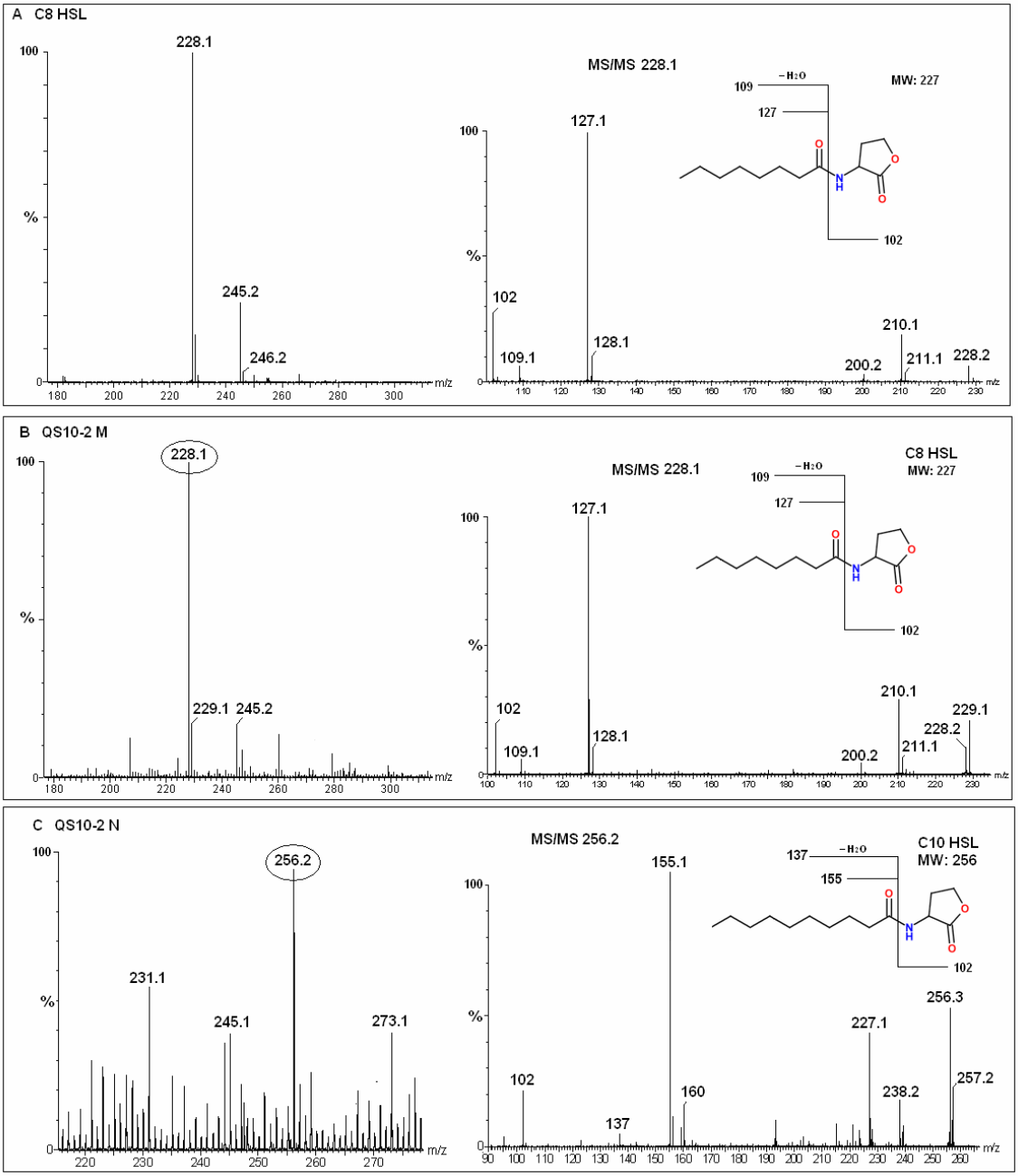

A C8 HSL
228.1
245.2
246.2
MS/MS 228.1
MW: 227
127.1
102
109.1
128.1
210.1
200.2
211.1
228.2
B QS10-2 M
C8 HSL
MW: 227
229.1
245.2
C QS10-2 N
256.2
231.1
245.1
273.1
MS/MS 256.2
C10 HSL
MW: 256
155.1
137
155
160
227.1
238.2
256.3
257.2
m/z

Table 2.8. AHLs synthesized by each LuxI homolog.

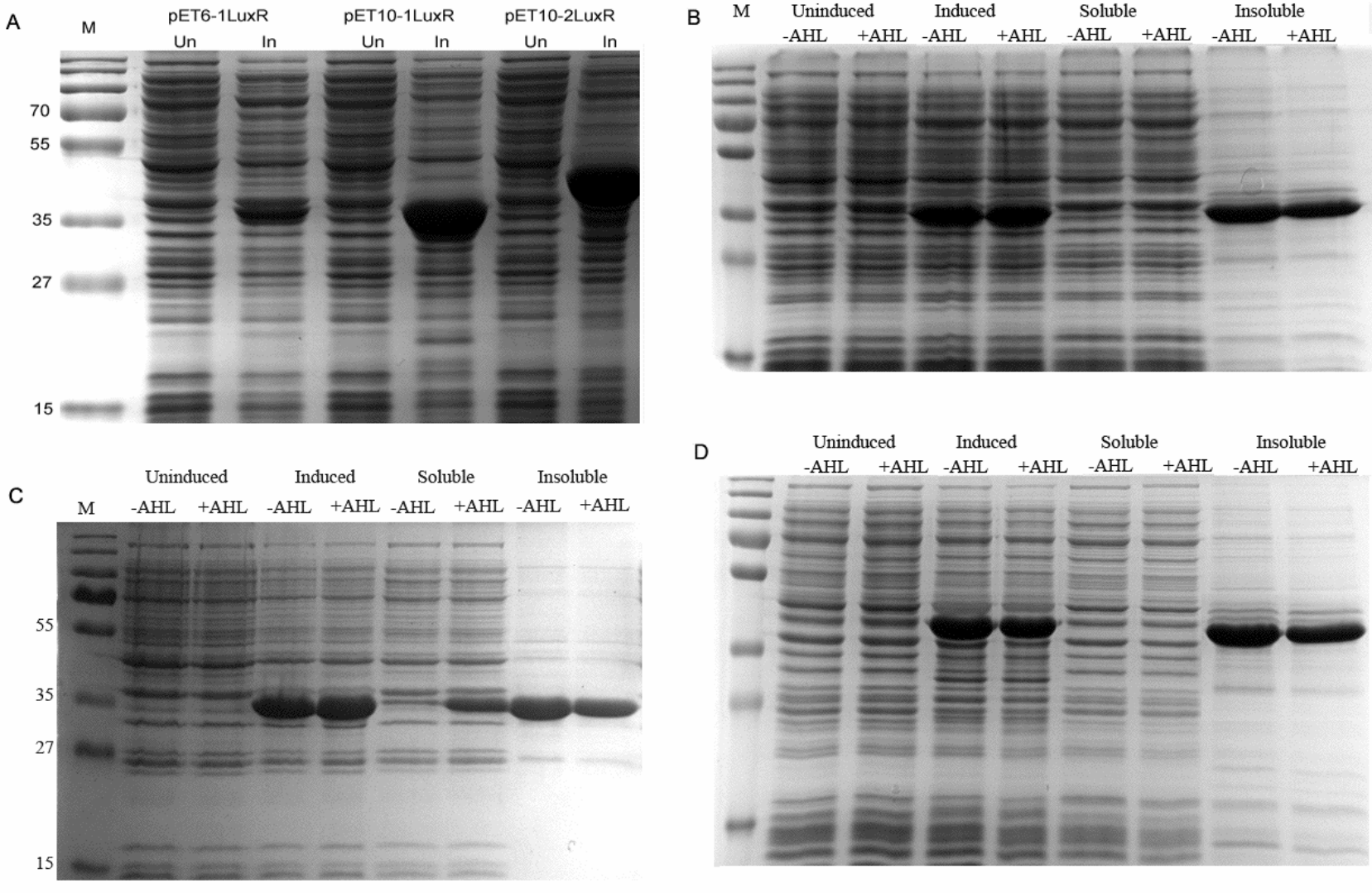
AHL synthase	Identified AHLs (corresponding position on TLC)
$LuxI_{QS6-1}$	3-O-C14:1 HSL (spot D and E), 3-O-C16:1 HSL (Spot F), 3-O-C14 HSL (Spot F), C14:3 HSL (Spot E), (?)-hydroxymethyl-3-oxo-C14 HSL (?, 5 to 14) (Spot E)
$LuxI_{QS10-1}$	3-O-C12 HSL (spot I), 3-O-C14 HSL(spot K)
$LuxI_{QS10-2}$	C8 HSL (Spot M), C10 HSL (Spot N)

2.3.8 Expression of *luxR* homologs in *E. coli*

The three *luxR* homologs were PCR amplified, spliced into the pET30a+ expression vector and then expressed in *E. coli* BL21(DE3). All three LuxR homologs were able to be expressed at a high level when induced with 1mM IPTG at 37 °C for 3 h (Figure 2.22, A). Different induction conditions have been tried, including using different IPTG concentrations (0.1 mM, 0.2 mM, 0.5 mM and 1mM), use of autoinduction medium (6 g/l Na_2HPO_4, 3 g/l KH_2PO_4, 20 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, 0.5 g/l glucose, 2 g/l lactose, and 6 g/l glycerol, pH was adjusted to 7.2), and different induction temperatures (37°C, 30°C, 28°C and 25°C). With all of these conditions, following expression in *E. coli* the LuxR protein was found mainly in the insoluble part of the cell extract (data not shown).

To determine if the cognate AHLs are required for the LuxR protein to be expressed in the soluble portion of the cell extract, 30 mins before induction with 0.5 mM IPTG, AHLs extracted from equal volumes of culture supernatant of an *E.coli* strain expressing the corresponding *luxI* gene were added. Then the protein was induced at 28°C for 2.5 h with shaking. It was found that the presence of cognate AHLs significantly increased the solubility of the $LuxR_{QS10\text{-}1}$ homolog protein, and the amount of over-expressed protein formed in the insoluble portion of the cell extract decreased correspondingly (Figure 2.22 B). However, the majority of $LuxR_{QS6\text{-}1}$ and $LuxR_{QS10\text{-}2}$ were localized in the insoluble part regardless of the presence or absence of cognate AHLs (Figure 2.22 C and D).

Figure 2.22. Expression of *luxR* homologs in *E.coli* BL21(DE3). A: Expression of $LuxR_{QS6-1}$, $LuxR_{QS10-1}$ and $luxR_{QS10-2}$. Un, uninduced total protein. In, total protein induced with 1mM IPTG for 3 h at 37°C. B-C: Expression of $LuxR_{QS6-1}$ (B), $LuxR_{QS10-1}$ (C) and $luxR_{QS10-2}$ (D) with and without the application of the cognate AHLs. Expression was induced with 0.5 mM IPTG for 2.5 h at 28°C


A
M
pET6-1LuxR
pET10-1LuxR
pET10-2LuxR
Un
In
Un
In
Un
In
70
55
35
27
15
B
M
Uninduced
Induced
Soluble
Insoluble
-AHL
+AHL
-AHL
+AHL
-AHL
+AHL
-AHL
+AHL
C
M
Uninduced
Induced
Soluble
Insoluble
-AHL
+AHL
-AHL
+AHL
-AHL
+AHL
-AHL
+AHL
55
35
27
15
D
Uninduced
Induced
Soluble
Insoluble
-AHL
+AHL
-AHL
+AHL
-AHL
+AHL
-AHL
+AHL

2.4 Discussion

In this study, three clones containing novel LuxI-LuxR type QS systems were obtained from environmental samples. Phylogenetic analysis of the protein sequences showed that, like all other known LuxI-LuxR systems, they most probably come from *Proteobacteria*, however, they are not closely related to LuxI-LuxR from any known and sequenced species (Figure 2.8). Phylogenetic analysis of the sequences of known LuxI-LuxR systems also revealed that α *Proteobacteria* tend to group together, while β and γ *Proteobacteria* are intermingled, which likely reflects the relatively frequent horizontal gene transfer (HGT) events between the later two. The CviI/R of *Chromobacterium violacearum*, as previously reported (Gray and Garey, 2001), groups with γ *Proteobacteria* instead of β *Proteobacteria,* which indicates that this may reflect HGT.

Possible *lux* box elements were identified in the promoter region of $luxI_{QS6\text{-}1}$, $luxI_{QS10\text{-}1}$ and $luxR_{QS10\text{-}2}$. No other putative *lux* box elements were found in the characterized sequences. The gene $luxI_{QS10\text{-}1}$ seems to form an operon with its downstream gene, encoding a hypothetical protein (QS10-1-5). The N-terminus of this protein contains a CheY-like signal receiver domain (REC), while the C-terminus contains the domain of an uncharacterized conserved protein. Based on the results of transposon insertional mutagenesis experiments and the short intergenic distance between them, the four genes in the clone QS10-2, $luxR_{QS10\text{-}2}$, $luxI_{QS10\text{-}2}$, phytanoyl-CoA dioxygenase (*phyH*) and *gntR* family transcriptional regulator, seem to form an operon which is QS regulated. The four gene homologs were in the same order and also contain very short intergenic regions in the genome of *Sphingopyxis alaskensis* RB2256 (NCBI genome projects, gene tags: Sala_2588, Sala_2589, Sala_2590 and Sala_2591). The gene *phyH* belongs to the sub-family of Fe(II)/α-

ketoglutarate-dependent hydroxylases, it catalyzes the α-hydroxylation of phytanoyl-CoA. Its role in bacteria is not very well understood, while the deficiency of the *phyH* homolog in humans causes Refsum's disease (Jansen et al. 2000). The GntR family of bacterial regulators is named after the GntR protein of *Bacillus subtilis*, a repressor of the gluconate operon (Haydon and Guest, 1991). This family of transcriptional regulators contains a conserved N-terminal helix-turn-helix (HTH) DNA binding domain, a more heterologous C-teriminal effector binding and oligomerization domain, and is involved in different regulatory pathways (Rigali, et al. 2001). Transposon insertional mutagensis revealed that mutation of one of the downstream genes, the biotin carboxylase subunit of acetyl-CoA carboxylase, also affects the production of AHLs. This could be explained by the fact that acetyl-CoA carboxylase catalyzes the carboxylation of acetyl-CoA to produce malonyl-CoA, which is the first committed step in the biosynthesis of acyl-acyl carrier proteins (acyl-ACPs) used for fatty acid biosynthesis, while the AHL synthesis pathway shares the same pool of acyl-ACPs as fatty acid biosynthesis (Miller and Bassler, 2001).

Each of the LuxI homologs is able to direct the synthesis of multiple AHLs. One of the major AHLs produced by LuxIQS6-1 is 3-O-C16:1 HSL, which has been reported to be the AHL produced by the plant pathogen *A. vitis* F2/5 strain (Hao and Burr, 2006). Some possible AHLs with new structures have been shown to be synthesized by $LuxI_{QS6-1}$, including an AHL with three unsaturated carbon bonds, C14:3 AHL, and a possible -CH2-OH substituted 3-O-C14 HSL. It was also found that AHLs synthesized by the same LuxI homolog in *A. tumefaciens* and in *E. coli* were slightly different (Figure 2.18). Not only is the relative abundance of each signal different, but also more AHLs were detected in the TLC assay when the genes were expressed in *E. coli*. A similar phenomenon was reported

for the LasI protein of *P. aeruginosa* (Gould *et al.*, 2006). This could be due to the fact that the expression level is much higher in *E. coli* than in *Agrobacterium*, so that acyl-ACPs with lower affinities for the AHL synthase proteins could also be used to synthesize AHLs in *E. coli*. However, there is also a possibility that the expression host could affect the species of AHLs synthesized. Their different growth temperatures and growth rates, the difference in their protein synthesis and matrix transportation mechanisms, and different acyl-ACP pools in the cells could affect the synthesized species of AHLs.

It has been noted that for some LuxR homologs it is very difficult to get soluble protein expression, probably because of the membrane association properties of the proteins (Kaplan and Greenberg, 1987; Smith *et al.*, 2003). In this study, when the three R proteins were overexpressed in *E. coli*, $LuxR_{QS10\text{-}1}$ could be expressed in the soluble portion of the cell only in the presence of its cognate signalling AHLs. The dependence on cognate AHL for solubility was also reported for the TraR protein of *A. tumefacien*s (Zhu and Winans, 2001), CepR protein of *Burkholderia cenocepacia* (Weingart *et al.*, 2005), the LuxR protein of *V. fischeri* (Urbanowski *et al.*, 2004) and the LasR protein of *P. aeruginosa* (Schuster *et al.*, 2004; Bottomley *et al.*, 2007). This means that $LuxR_{QS10\text{-}1}$, like the above mentioned other R proteins, needs the binding of its cognate signalling ligand for proper protein folding. However the majority of the $LuxR_{QS6\text{-}1}$ and $LuxR_{QS10\text{-}1}$ remained insoluble even in the presence of cognate AHLs.

The LuxI and LuxR homologs of the three clones were able to synthesize and detect multiple signals with different structures and a wide range of chain lengths. This should make it possible to construct novel biosensor strains that can detect a broad range of AHLs.

These biosensor strains could be very useful in the screening and study of *luxI-luxR* type QS systems for both cultured and uncultured organisms.

Earlier attempts to isolate QS systems using functional metagenomics approaches were less successful than the present study in isolating novel AHL-based systems (Williamson *et al.*, 2005; Riaz *et al.*, 2008). The specificity of the biosensor systems often requires a cognate *luxR* to be cloned along with the *luxI*, as we demonstrated, and this would only occur if the genes are clustered on the same metagenomic library clone. Functional screening also requires that the genes are expressed in the surrogate host. Continuing the use of large-insert libraries, and screening in a number of biosensor strains of diverse genomic backgrounds, should result in increased numbers of novel QS systems isolated.

Chapter 3. The Effect of ACC Deaminase or ACC Deaminase Containing PGPB on *Agrobacterium tumefaciens* C58 Induced Crown Gall Development and on *A. tumefaciens* Mediated Transformation Efficiency

3.1 Introduction

3.1.1 Ethylene, an important plant hormone. Although it was used in practice by ancient Egyptians, Hebrews and Chinese to stimulate the ripening of fruit, and even though, in 1864, gas leaks from street lights were found to change plant morphology, it was not until 1901, that a Russian scientist, Dimitry Neljubow, found that the active component was ethylene, the smallest unsaturated hydrocarbon (Neljubow, 1901). It took another 30 more years for scientists to discover that plants can synthesize ethylene (Gane, 1934). In 1935, ethylene was proposed as the plant hormone responsible for fruit ripening (Crocker *et al.*, 1935). To date, ethylene has been shown to be produced by essentially all parts of higher plants and is considered to be an essential plant hormone. It is involved in a variety of plant developmental processes, including seed germination, root elongation, tissue differentiation, flowering and fruit ripening, senescence and abscission (Jackson and Osborne, 1970; Bleecker and Kende, 2000; Glick, 2004). It also plays an important role in biotic and abiotic stress responses, nodulation, and systemic resistance to pathogens (Bleecker and Kende, 2000). In higher plants, ethylene is produced from L-methionine. Methionine is activated by ATP to form S-adenosylmethionine (SAM) through the catalytic activity of SAM synthase. Then, SAM is converted by the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) synthase to the non-protein amino acid ACC and 5-methylthioadenosine (MTA). ACC is converted to ethylene by ACC oxidase, while the MTA is diverted back into the Yang cycle and recycled to synthesize L-methionine (Figure 3.1) (Yang and Hoffman, 1984) so that the level of L-methinoine available to synthesize SAM remains the same even during higher rates of ethylene production (Abeles, 1992). It has been argued that formation of ACC is the rate-limiting step in ethylene biosynthesis (Bleecker and Kende, 2000) despite the fact that

the enzyme ACC oxidase, like ACC synthase, is also an induced enzyme.

The production of ethylene is regulated by various developmental and environmental factors. For example, during seed germination, senescence of flowers and ripening of fruits, ethylene production is induced (Yang and Hoffman, 1984). Ethylene production can also be induced by environmental stresses (Hyodo, 1991), such as water stress, high salt, the presence of metals and organic contaminants, insect damage, disease and mechanical wounding, and the induced production of ethylene is called stressed ethylene. This stress ethylene may both alleviate and exacerbate some of the effects of various stresses, especially phytopathogens (Abeles, 1992). These contradictory results may be explained by the presence of two peaks of stress ethylene, a small peak that occurs close in time to the stress and a much larger peak that occurs some time later (Stearns and Glick, 2003; Pierik *et al.*, 2006; Glick *et al*., 2007). The first peak initiates a protective response by the plant, while the second peak initiates a senescence response. Ethylene can also interact with other plant hormones. For example, it is known that auxin and ethylene can interact with each other at many points. They promote each other's biosynthesis by up-regulation of the biosynthesis genes (Swarup *et al.*, 2002; Stepanova *et al.*, 2005; Arteca and Arteca, 2008). There is also evidence that ethylene inhibits the expression of auxin response regulators and auxin transport (Lehman *et al.*, 1996; Li *et al.*, 2004; Prayitno *et al.*, 2006). Cytokinin has also been reported to enhance ethylene biosynthesis (Babiker *et al.*, 1993; Arteca and Arteca, 2008).

3.1.2 ACC deaminase, a strategy used by PGPB to lower ethylene levels in plant.

Plant growth promoting bacteria (PGPB) are a group of bacteria that can colonize root surfaces or plant tissues and benefit plant growth directly or indirectly. Indirect methods

include the production of antibiotics to inhibit growth of plant pathogens, competition with plant pathogens and induction of plant systemic acquired resistance (Haas and Defago, 2005; van Loon *et al.*, 2006). Direct mechanisms include production of plant growth hormones; nitrogen fixation; production of growth modulating enzymes; and enhancement of nutritients acquisition, such as phosphorus, nitrogen and iron (Glick, 1995; Glick *et al.*, 1999). One mechanism that is utilized by many PGPB is the lowering of ethylene levels in plants by the production of the enzyme ACC deaminase. After binding to the plant surface or interior, the PGPB can break down the ACC that is exuded by the plant cells into ammonia and α-ketobutyrate, and use as nitrogen and carbon sources, respectively (Figure 3.2 a). PGPB that contain ACC deaminase act as a sink for ACC, the direct precursor of ethylene synthesis, reducing its overall concentration. Thus, less ACC is available for conversion to ethylene by ACC oxidase (Figure 3.2 b). Many ACC deaminase containing PGPB can also produce and secrete the plant hormone indole-3-acetic acid (IAA), part of which can be taken by plant cells and subsequently activate the transcription of ACC synthase, resulting in the production of increasing amounts of ACC (Glick *et al.*, 1998).

To date, many aspects of the plant growth promotion and plant stress reduction by ACC deaminase-containing PGPB have been reported, including promotion of nodulation (Ma *et al.*, 2003a; Ma *et al.*, 2004; Tittabutr *et al.*, 2008), decreasing the severity of a range of different stresses including flooding (Grichko and Glick, 2000), heavy metals (Reed and Glick, 2005; Zhang *et al*., 2008), polycyclic aromatic hydrocarbons (Reed and Glick, 2005), pathogens (Wang *et al.*, 2000; Belimov *et al.*, 2007), drought (Mayak *et al.*, 2004b; Arshad *et al*., 2008) and salt *(Mayak et al., 2004a; Cheng et al., 2007; Nadeem et al.*, 2007; Zahir *et al.*, 2009).

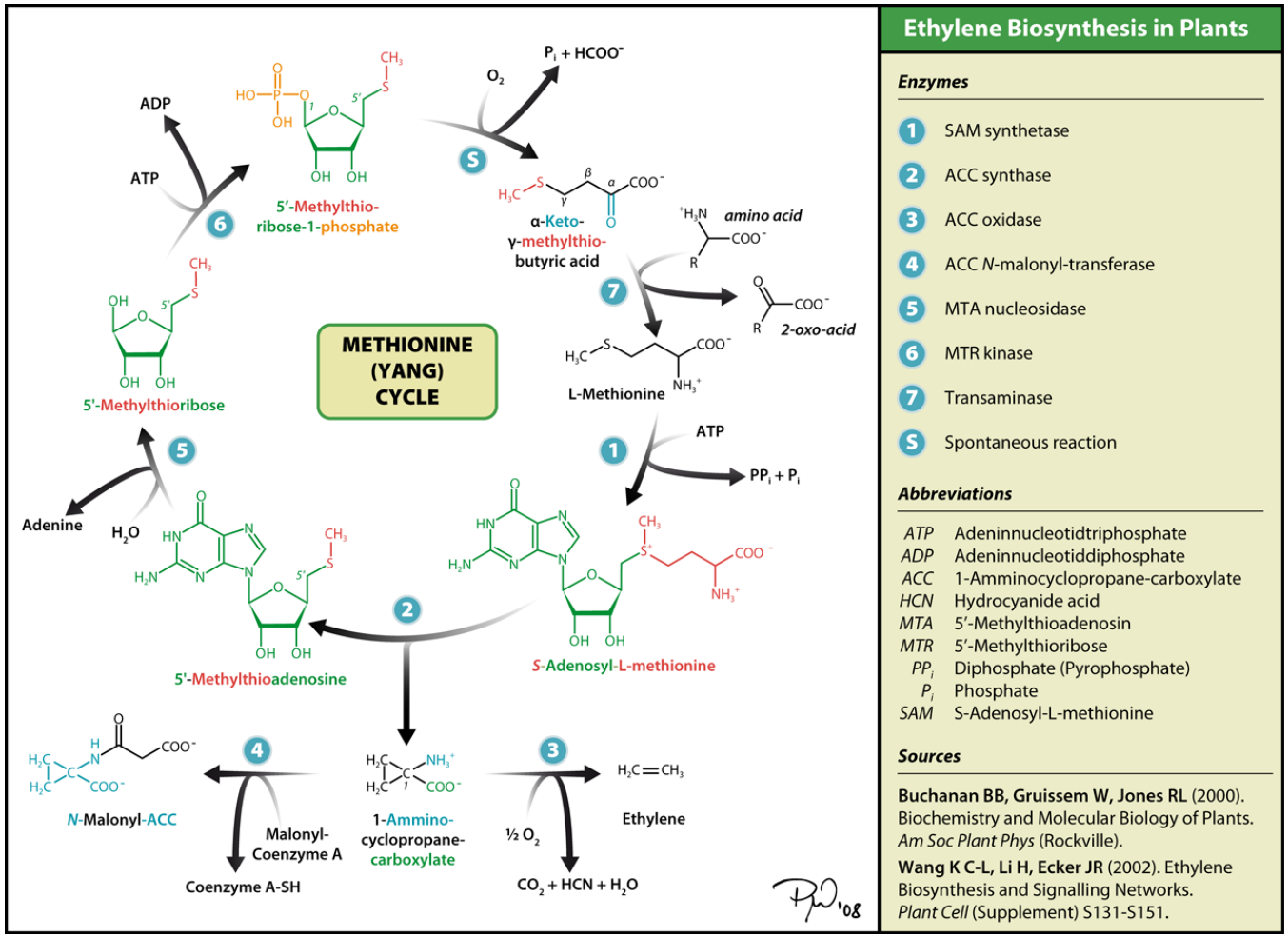
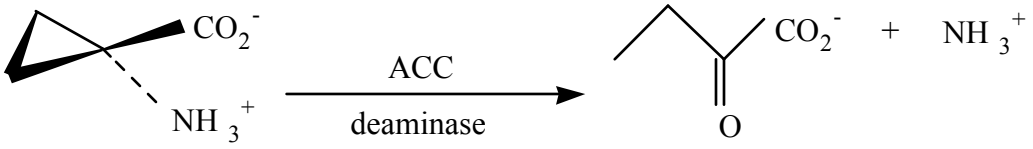


Figure 3.1. TheYang cycle and the ethylene biosynthesis pathway. Picture is from http://commons.wikimedia.org/wiki/File:Yang-cycle.png

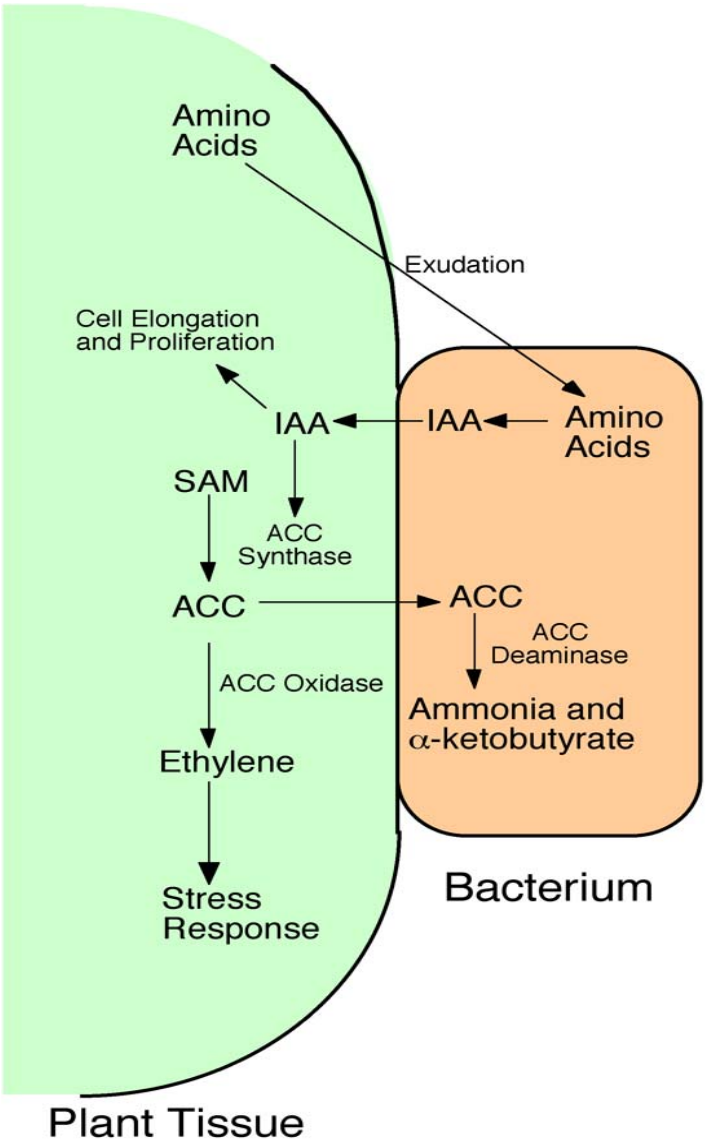
Figure 3.2. A model of plant growth promotion by ACC deaminase-containing PGPB. A. Catalytic activity of ACC deaminase. B. PGPB model. PGPB can attach to the surface of a plant cell. It can degrade the ACC exuded from the plant cell to ammonia and α-ketobutyrate. To maintain the equilibrium between the interior and external ACC levels, more ACC is exuded and drawn away from the ethylene synthesis pathway. Thus, PGPB that express ACC deaminase effectively reduces ethylene synthesis in plants. (Glick *et al.*, 1998)

A



ACC α-Ketobutyrate

B



3.1.3 *Agrobacterium sp.*

Agrobacterium sp. is a Gram negative soil bacterium that can cause crown gall or hairy root disease of plants. Accordingly, the two major species have been named *A. tumefaciens* (Smith and Townsend, 1907) and *A. rhizogenes* (Riker *et al.*, 1930), reflecting their distinctive pathogenic features. The avirulent strains were named *A. radiobacter* (Beijerinck and van Delden, 1902). The infectious ability relies on the presence of the extrachromosomal replicons, the Ti (tumor inducing) plasmid for *A. tumefaciens* and the Ri (root inducing) plasmid for *A. rhizogenes*. Since the plasmid can transfer between strains and can also be cured, the classification and nomenclature based on pathogenicity may not be consistent with the natural classification of *Agrobacterium* species (Young *et al.*, 2001). More recently, based on numerical analysis of phenotypic characters (White, 1972; Kersters *et al.*, 1973; Holmes and Roberts, 1981), biochemical and physiological analysis (Keane *et al.*, 1970; Kersters *et al.*, 1973; Kerr and Panagopoulos, 1977; Holmes and Roberts, 1981), fatty acid methyl ester profiles (Sawada *et al.*, 1992; Jarvis *et al.*, 1996), and electrophoregrams of soluble proteins (Kersters and De Ley, 1975), the *Agrobacterium sp*. were classified into three biovars: biovar I includes most *A. tumefaciens* and *A. radiobacter* strains; biovar II corresponds to *A. rhizogenes*; and biovar III are a group of *Agrobacteria* that have a narrow host range, and are mainly found in grapevines and is now named *A.vitis* (Young *et al.*, 2001). However, due to its very widespread use and historical reasons, the former nomenclature is still widely used.

3.1.3.1 *A. tumefaciens* is a plant pathogen that causes crown gall disease

A. tumefaciens causes crown gall disease of a wide range of dicotyledonous plants, especially members of the rose family such as apples, pears, peaches, cherries, almonds, raspberries and roses. Crown galls usually are callus shaped, and are not protected by an epidermis, thus increases evaporation. The vascular tissue near and upside the gall also changes dramatically; the vessel diameters decrease and rays remain unlignified and become multiseriate (Wächter *et al.*, 1999; Veselov *et al.*, 2003). The host stem downside of the tumor remains mainly unchanged. These features ensure water and nutrition supply priority to the tumors. Plants carrying tumors usually grow poorly and their fruit production is greatly reduced. Biovar III of *Agrobacteria*, *A. vitus*, causes the growth of galls on grapevines and is considered to be one of the major threats to the grape industry.

A. tumefaciens contains a large plasmid called the Ti (tumor inducing) plasmid that is mainly responsible for crown gall disease. Wounded plants produce some phenolic compounds, which can be sensed by *Agrobacterium* and induce the production of virulence protein factors encoded by the *vir* region of the Ti plasmid. With the help of these virulence protein factors, *Agrobacterium* can transfer a strand of DNA (T-DNA) from the Ti plasmid into the host plant cell and integrate it into the plant genome. The T-DNA encodes some oncogenic genes, including IAA and cytokinin synthesis genes and also opine synthesis genes. The expression of the oncogenic genes in a plant promotes production of the plant hormones auxin and cytokinin, leading to a breakdown in the natural balance of plant hormone levels within a plant. The final result is unlimited cell division and the growth of tumors (Sigee, 1993; Tinland, 1996), while the expression of the opine synthesis genes provides an energy source for *Agrobacterium*. Since the oncogenic genes are integrated into the plant genome, even after the removal of the *Agrobacterium*, the infected plant cannot be

cured. Prevention is the only method to control the disease. *A. radiobacter* K84, which produces agrocin 84 which kills noplaine producing strains of *A. tumefaciens*, is one of the most successfully used biocontrol agents and has been commercialized and used world wide (Farrand, 1990).

3.1.3.2 *A. tumefaciens* is a powerful tool for plant genetic engineering *Agrobacterium*'s ability to transfer and integrate a strand of DNA into plants makes it a very useful tool for plant genetic engineering. Since the transfer of T-DNA does not require the oncogenic or opine synthesis genes between the T-DNA borders, engineering the Ti plasmid (by removing all the oncogenic genes between the T-borders, and adding a plant selectable marker gene and multicloning sites) allows *Agrobacterium* to be used to transfer foreign DNA to the plant genome. Ti plasmids and their host *Agrobacterium* strains that are no longer oncogenic are called "disarmed."

Two types of Ti-plamid derived cloning vectors, the cointegrate cloning vector and the binary cloing vector have been developed to deliver foreign DNA into plants (Hille *et al*, 1983). The cointegrate vector contains a replication origin for *E. coli*, a selectable marker, a T-DNA right border, a target gene and a sequence that is homologous to Ti plasmid. By homologous recombination, the cointegrate vector will be integrated into the disarmed Ti plasmid which cotains T-DNA left border and *vir* gene cluster, thus the recombinant plasmid contains the target gene between the T-DNA left and right boarders (Hille *et al*, 1983). After scientists discovered that the T-DNA and the virulence (*vir*) genes required for T-DNA processing and transfer could be separated into different vectors and that the transfer would still be effective, binary systems were developed (Fraley *et al.*, 1983; Hoekema *et al.*, 1983).

The T-DNA and the *vir* region were separated into two replicons, and the vector with the T-DNA is usually called a binary vector, which is much smaller than Ti plasmid and can be maintained in both *E. coli* and *Agrobacterium*. Since then, many different *A. tumefaciens* strains and binary vector systems have been developed with different features, including some supervirulent strains used for transformation of recalcitrant plants (Hellens *et al.*, 2000; Lee and Gelvin, 2008). To date, most dicot species can be transformed routinely by *Agrobacterium*-mediated transformation. In recent years, some monocot species such as rice, wheat, barley and maize, some gymnosperms (Norway spruce), and even fungi have been transformed via *Agrobacterium*-mediated transformation.

Compared with other plant transformation methods, such as transposon insertion, protoplast transformation, microprojectile bombardment or particle bombardment and infiltration, *Agrobacterium*-mediated transformation has several advantages. It results in stable transformation, it introduces single or low copy numbers of transgenes, which reduces transgene silencing, and it does not need any special equipment so that the transformation procedure is convenient to perform. However, for some recalcitrant dicot species and most monocot species, especially for trees, the transformation efficiency is still very low. Improving the transformation efficiency will further extend the range of plants that can be transformed via *Agrobacterium* (Cheng M. *et al.*, 2004).

3.1.3.3 Genome sequencing progress of *Agrobacterium sp.*

As an important plant pathogen, a unique organism to be able to perform trans-kingdom DNA transfer, and a widely used tool for plant genetic engineering, several *Agrobacterium* strains have been fully sequenced or sequencing projects are in progress. *A.*

tumefaciens strain C58, which is a representative of the biovar I of agrobacteria has been fully sequenced in 2001(Goodner *et al.*, 2001; Wood *et al.*, 2001). Most recently, two other *A. tumefaciens* strains, the biocontrol agent *A. radiobacter* K84, a representative of biovar II, and *A. vitis* S4 which belongs to biovar III and mainly infects grapevines, are also been fully sequenced (Slater *et al.*, 2009). The genome sequencing project for *A. rhizogenes* A4 is currently under progress (http://depts.washington.edu/agro/).

3.1.4 Ethylene affects *A. tumefaciens* induced crown gall development

3.1.4.1 *A. tumefaciens* infection induces sustained ethylene production

It has been reported that *Agrobacterium* infection induces sustained ethylene production. As early as the 1980s, scientists noticed that carrot discs inoculated with *A. rhizogenes* or *A. tumefaciens* produced more ethylene than non-inoculated control plants (Canfield, 1983; Goodman *et al.*, 1986). More recently, it has been reported that the ethylene level from *A. tumefaciens* induced crown galls could be many times higher than that from non-tumorized stems. For example, the ethylene level from *A. tumefaciens* C58 induced stem tumors of *Ricinus communis* (castor bean) is 140 times greater than that emitted by non-tumorized control stems (Wächter *et al.*, 1999). In tomatoes, ethylene evolution from isolated internodes carrying galls was up to 50-fold greater than that from isolated internodes of control plants (Aloni *et al.*, 1998).

There are several sources of elevated ethylene levels. The expression of the oncogenic genes in transformed plant cells results in overproduction of auxin and cytokinin. It is known that high levels of auxin and cytokinin can stimulate the transcription of ACC synthase (Nakagawa *et al.*, 1991; Olson *et al.*, 1991; Yoon *et al.*, 1997), one of the critical

enzymes for ethylene biosynthesis, so it has been proposed that the elevated production of ethylene is a secondary result of auxin and cytokinin synthesis in the transformed tissue. Recently scientists found that the *Agrobacterium*-plant interaction alone can induce elevated ethylene production. Using disarmed *Agrobacterium* (*Agrobacterium* without the *tms* genes) to transform plant or plant tissues, scientists still observed elevated ethylene production from the plant or explant, although compared to the ethylene levels in crown galls, it is much lower. For example, Ezura *et al.* reported that ethylene production from cotyledonary explants excised from the melon cotyledon was significantly increased by the disarmed *Agrobacterium* inoculation and reached 2.5 times that of the control after 90 minutes (Ezura H, 2000).

3.1.4.2 Induced level of ethylene affects crown gall morphogenesis

In addition to auxin and cytokinin, the induced production of ethylene also plays an important role in crown gall morphogenesis and affects the severity of the crown gall disease. Aloni *et al's* research on the anatomical structures of *Agrobacterium* induced crown galls revealed that the galls are not unorganized, but have a sophisticated vascular network connected to the host plant and that vascularization is a general requirement for the growth of the tumors (Aloni, 1995; Aloni *et al.*, 1997; Ullrich and Aloni, 2000). Ethylene plays an essential role in the differentiation and development of the tumors (Ullrich and Aloni, 2000). In the ethylene insensitive *Never ripe* mutant of tomato infected by *A. tumefaciens*, the growth of the gall was severely suppressed (Aloni *et al.*, 1998). In *Ricinus communis* and tomatoes, by exogenous application of ethrel (a chemical which is converted to ethylene in plants) to the wounded stem, anatomical changes developed similar to those previously

observed on the stem near the gall (Yamamoto *et al.*, 1987; Yamamoto and Kozlowski, 1987; Aloni *et al.*, 1998; Wächter *et al.*, 1999).

3.1.4.3 Study of the effect of ACC deaminase on crown gall disease

As reviewed above, *A. tumefaciens* infection induces ethylene production in plants, and this increased level of ethylene is critical for crown gall tumor development and the severity of crown gall disease. Searching of *A. tumefaciens* C58 genome sequence revealed that it contains no putative ACC deaminase gene. One of our hypotheses is that the presence of an ACC deaminase enzyme, which can help reduce ethylene levels, will help to inhibit crown gall development and may be useful in controlling crown gall disease. To test this hypothesis, an ACC deaminase gene from the PGPB *P. putida* UW4, which showed a high level of ACC deaminase activity, was introduced into *A. tumefaciens* C58, and the virulence of the resulting strain was analyzed using carrot discs, tomato stems and castor bean stems. Wild type or an *acdS*$^-$ mutant strain of *P. putida* UW4 was also co-inoculated with *A. tumefaciens* C58 to study their effects on crown gall disease.

3.1.5 *A. tumefaciens* D3, an *Agrobacterium* strain contains ACC deaminase gene

Although ACC deaminase encoding genes have not been found in the sequenced *Agrobacterium* genomes, there is an *A. tumefaciens* strain D3 which was reported to contain a putative ACC deaminase gene (Trott *et al.*, 2001). This strain was first isolated from soil (Stuttgart, Germany) using an enrichment method for nitrile-hydrolysing bacteria and was found to contain nitrile hydratase and amidase activity (Layh *et al.*, 1997). Using API and BIOLOG tests, strain D3 was taxonomically classified and named as *A. tumefaciens* (Layh *et*

al., 1997). Later, when cloning the amidase gene, a putative *acdS* gene and *lrpL* gene were found upstream of the amidase encoding gene (Genbank accession number: AF3155580, ORF3 and ORF2) (Trott *et al.*, 2001). As in *P. putida* UW4 (Grichko and Glick, 2000; Li and Glick, 2001) and *Rhizobium leguminosarum* bv. viciae (Ma *et al.*, 2003a) and many other species (Duan *et al*. 2009; Sun *et al*, 2009), the *acdS* and *lrpL* are located immediately next to each other and are oriented in the opposite direction. However, unlike in those strains, the *lrpL* gene is located downstream of the *acdS* structural gene in strain D3. It has been shown that in *P. putida* UW4 and *R. leguminosarum* bv. viciae, mutation of the *lrpL* gene affects the ACC deaminase activity of these strains (Grichko and Glick, 2000; Li and Glick, 2001; Ma et al., 2003 a), and it was proposed that the LrpL protein can bind ACC and regulate the transcription of the *acdS* gene (Ma et al. 2003 a).

The production of ACC deaminase is mainly considered as a strategy used by PGPB to promote plant growth. It is interesting to study why a plant pathogen might contain such a gene. In this study, a series of experiments were performed to characterize *A. tumefaciens* D3. First, an ACC deaminase activity assay was performed to test whether the putative *acdS* gene encodes an active ACC deaminase, and if so to characterize its activity. One *lrpL* and *acdS* double mutant strain D3-1 was constructed and characterized. To study whether the LrpL protein is the regulator of the *acdS* gene, the mutations were complemented using *lrpL* or *acdS* alone or both genes and ACC deaminase activity of the resulting strains were characterized. Then the *A. tumefaciens* D3 strain was studied for its virulence using both molecular techniques and virulence assays on various plant species. The wild type and *lrpL* and *acdS* double mutant strain of *A. tumefaciens* D3 were also studied for their biocontrol activity towards *A. tumefaciens* C58 induced crown gall disease using castor bean stems.

3.1.6 Ethylene affects *A. tumefaciens* mediated transformation efficiency.

As a useful tool for plant genetic engineering, *A. tumefaciens* mediated transformation has many advantages compared with other methods. However, the low transformation efficiency with many commercially important crops is the major factor limiting its use. A lot of effort has been made to improve *A. tumefaciens* mediated transformation efficiency with various plant species, including construction of supervirulent *A. tumefaciens* strains and Ti plasmids, use of different plant material, and optimization of transformation and regeneration procedures (Cheng M. *et al.*, 2004). Among the different factors affecting *A. tumefaciens* mediated transformation efficiency, ethylene was found to be one thing that affected both the gene delivery efficiency and plant regeneration efficiency.

3.1.6.1 Ethylene inhibits *A. tumefaciens* mediated gene delivery efficiency and plant regeneration efficiency

Various plant materials have been used for *Agrobacterium* mediated transformation. For example, some plants (such as *Arabidopsis*) can be transformed using a floral dip method: the flowers are dipped into an *Agrobacterium* cell suspension and the germline cells of the plants are transformed so that the transgenic seeds can then be screened. For most other plants, explants cut from hypocotyls, cotyledons, stems, leaves, roots or embryos are used, and the transformed explants or tissues are then regenerated using plant tissue culture methods. The ethylene level is increased during the transformation and regeneration process for the following reasons: the cutting of the plant material causes mechanical wounding, which induces the production of stress ethylene; *Agrobacterium* infection increases ethylene biosynthesis (Ezura, 2000); and during tissue culture, addition of plant hormones such as 2,4-

Dichlorophenoxyacetic acid (2,4-D) also induces ethylene levels (Garcia and Einset, 1983). The elevated level of ethylene has been reported to inhibit both gene delivery efficiency and plant regeneration efficiency.

Aminoethoxyvinylglycine (AVG), an inhibitor of the enzyme ACC synthase, and silver ions and 1-methylcyclopropane (MCP), which inhibit ethylene binding to its receptor, are the most widely used ethylene inhibitors. It has been reported that application of AVG or Ag^{+} can improve transformation efficiency of many plant species, such as bottle gourd, cauliflower, apricot, and apple (Chakrabarty *et al.*, 2002; Burgos and Alburquerque, 2003; Han *et al.*, 2005; Petri *et al.*, 2005; Seong *et al.*, 2005), while application of ACC or ethylene to the cocultivation medium reduces *Agrobacterium*-mediated gene transfer efficiency (Ezura, 2000). Most recently, it has been reported that introduction of ACC deaminase into *A. tumefaciens* increases the efficiency of the transient gene transfer to melon cotyledon explants (Nonaka *et al.*, 2008a). One possible mechanism underlining this is that ethylene suppresses *vir* gene expression of *A. tumefaciens* (Nonaka *et al.*, 2008b). In some cases, the lack of efficient regeneration systems is the major limiting factor preventing the development of gene transfer technologies for many plants (Burgos and Alburquerque, 2003). The enhanced production of ethylene causes necrosis of the explants during histoculture, and many studies indicate that ethylene inhibitors can improve organogenesis and thereby improve transformation efficiency (Chi *et al.*, 1990; Chraibi *et al.*, 1991; Burgos and Alburquerque, 2003; Petri *et al.*, 2005; Seong *et al.*, 2005).

3.1.6.2 Canola, an important crop with increasing demand for genetic modification

Canola (*Brassica napus L.*) is an oilseed crop developed from the rapeseed (*Brassica rapa*) through conventional breeding. Natural rapeseed oil contains a high concentration of erucic acid and is not suitable for human consumption. In the 1970s, plant breeders in Saskatchewan, Canada developed a variety of rapeseed with low erucic acid and named it Canola, from Canadian oil, low acid. Since then, canola has been improved in many aspects, including both nutritional contents and disease and pathogen resistance. Today, canola oil is considered to be one of the healthiest cooking oils due to its low saturated fat content, high monounsaturated fat content, and the presence of omega-3 fatty acids. Canola is now one of the most important sources of vegetable oil, ranked second worldwide only to soybeans (Raymer, 2002). It is produced extensively in Canada, Europe, Asia, Australia, and the United States. The high protein content also makes canola an excellent source for livestock feed. In recent years, canola oil has also been used as a source for manufacturing biodiesel, which is a renewable fuel and helps reduce greenhouse gas.

As it is such an important crop, there is increasing interest in improving canola breeding. Compared with conventional breeding, which is time consuming, genetic engineering of canola provides an alternative to produce new cultivars in a relatively short time. As a close relative to *Arabidopsis thaliana*, a model plant whose genome has been fully sequenced and studied extensively, canola can benefit from the information obtained from *Arabidopsis* study (Ostergaard *et al.*, 2006). Various transformation methods have been used to genetically modify canola, including microinjection, microprojectile bombardment, electroportion and *A. tumefaciens*-mediated transformation. Among these methods, *A. tumefaciens*-mediated transformation is usually the method of choice because of its ease and cost effectiveness.

Many properties of canola have been modified by genetic engineering, including improving oil quality (Facciotti *et al.*, 1999; Knutzon *et al.*, 1999; Stoutjesdijk *et al.*, 2000; Katavic *et al.*, 2001; Liu *et al.*, 2001), introducing herbicide resistance features (Oelck *et al.*, 1991), and making it insect (Stewart *et al.*, 1996) or fungal resistant (Grison *et al.*, 1996). Some of the genetically modified cultivars are commercially available. For example, in 2007, 80% of the canola growing in western Canada included genetically modified herbicide resistance cultivars (Canola Council of Canada, 2009). In recent years, researchers have moved into new directions in genetically modifying canola, including making it tolerant to heavy metals and other toxic compounds so that it can be used as a component of phytoremediation protocols (Basu *et al.*, 2001), using it to produce pharmaceutically active proteins and edible vaccines (Giddings *et al.*, 2000), and also improving it as a source for producing biofuel (http://www.canolacouncil.org/biodiesel/).

Many studies have been performed to improve canola's transformation efficiency. Among the plant tissues used, such as cotyledons, hypocotyl segments, mature plant stem segments, leaves, seedling root segments, and protoplasts, the first two have been proved to be the best materials for transformation and regeneration (Cardoza and Stewart, 2003; Zhang and Bhalla, 2004; Zhang *et al.*, 2005; Bhalla and Singh, 2008). By optimizing the pre-conditioning and cocultivation time, Cardoza and Stewart have reported increasing the transformation efficiency of *B. napus cv*. Westar from 4% to 25% using hypocotyl explants (Cardoza and Stewart, 2003). Using cotyledon explants, a 33% of transformation efficiency was reported for *B. napus cv*. Westar (Zhang *et al.*, 2005). While most of the studies used the model cultivar *B. napus cv.*Westar, which is an old spring cultivar and is no longer grown in the fields due to some agronomic deficiencies, the transformation and regeneration of canola

is genotype dependent, and the commercialized cultivars are often recalcitrant to transformation. It is, therefore, important to evaluate and optimize the transformation protocols for commercialized cultivars.

3.1.6.3 Study of the effect of ACC deaminase on *A. tumefaciens* mediated transformation efficiency to Canola hypocotyls.

As stated above, ethylene inhibits *A. tumefaciens* mediated gene delivery efficiency and plant regeneration efficiency. Most transformation protocols require the addition of $AgNO_3$ to the tissue culture medium to inhibit ethylene production and obtain higher transformation rates (De Block *et al.*, 1989; Cardoza and Stewart, 2003; Bhalla and Singh, 2008). Here it is hypothesized that the introduction of an ACC deaminase gene into *A. tumefaciens* will help its ability to genetically transform plants cells. By introducing an ACC deaminase gene into *A. tumefaciens* GV3101::pMP90, a disarmed strain that contains an *A. tumefaciens* C58 chromosome background and a Ti plasmid derived from pTiC58(Koncz and Schell, 1986), the transformation efficiency of the resulting strain on the model canola cultivar Westar as well as two cultivars that have been commercialized in Canada, the hybrids Hyola 401 and 4414RR, was evaluated using hypocotyl explants.

3.1.7 Conclusion

A. tumefaciens as a plant pathogen causes crown gall disease by transferring and integrating oncogenic genes into plant genomes. The overproduction of the plant hormones auxin and cytokinin induces the biosynthesis of another plant hormone, ethylene; together, these plant hormones define the morphogenesis of crown galls and trigger the corresponding

responses of the plants carrying crown galls. An ACC deaminase gene, which can help to reduce ethylene level in plants by breaking down ACC, was introduced from the PGPB *P. putida* UW4 into wild type *A. tumefaciens* C58 and the effects of ACC deaminase on crown gall development was studied using tomato and castor bean plants. As an alternative means of studying the effect of ACC deaminase on crown gall development, the wild type *P. putida* UW4 and the *acdS* knockout mutant strain of *P. putida* UW4 were co-inoculated with *A. tumefaciens* C58, and their effects on crown gall development were also studied.

A. tumefaciens strain D3 has been reported to contain a putative *acdS* gene. To elucidate why a plant pathogen would contain this activity, the ACC deaminase activity, the root elongation promotion ability and the virulence of that strain was also studied. The wild type and the mutant strains were co-inoculated with *A. tumefaciens* C58 on wounded castor bean stems and their biocontrol activity was studied.

Disarmed *A. tumefaciens* is used by scientists to transfer foreign DNA into plant genomes and serves as a useful tool for plant genetic engineering. Although the oncogenic genes have been removed from the Ti plasmid, *Agrobacterium* infection still induces ethylene synthesis in plants (Ezura *et al.*, 2000). Cutting of plant tissue before agrobacterium infection and histoculture processes also induce stress ethylene production. The enhanced levels of ethylene affect both DNA transfer and plant regeneration efficiency. The ACC deaminase gene was also introduced into a disarmed *A. tumefaciens* strain GV3101::pMP90(pPZP-eGFP), and the transformation efficiency of the resulting strain was evaluated using canola hypocotyls.

3.2 Materials and Methods

3.2.1 Bacterial strains and plasmids used in this study and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 3.1. *A. tumefaciens* strains were cultured in MG/L (Garfinkel and Nester, 1980) or LB medium (10 g tryptone, 5 g yeast extract, 10 g NaCl) at 28°C. M9 minimal medium (Atlas, 1993) supplemented with 0.3 mg of biotin ml^{-1} was used to grow *A. tumefaciens* cells prior to being assayed for ACC deaminase activity. When required, antibiotics were supplied at the following concentration: rifampicin (Rf), 50 μg/ml; spectinomycin (Sp), 50 μg/ml; streptomycin (Sm), 20 μg/ml; kanamycin (Km), 50 μg/ml; and tetracycline (Tc), 2 μg/ml. *P. putida* strains were grown at 30°C in TSB medium (Difco Laboratories, Detroit, MI) or LB medium. DF minimal salts medium (Dworkin and Foster, 1958) was used for the assay of ACC deaminase activity. When required, 20 μg/ml of tetracycline was used. *E. coli* strains were grown at 37°C in LB medium. When required, antibiotics were supplied at the following concentrations: ampicillin (Ap) 100 μg/ml; Tc, 20 μg/ml; Km, 50 μg/ml; Sm, 50 μg/ml; Sp, 50 μg/ml; chloromphenical (Cm) 17 μg/ml.

3.2.2 Construction of strains

To construct *A. tumefaciens* C58(pRK415), *A. tumefaciens* C58(pRKACC), *A. tumefaciens* C58(pRKLACC), or *A. tumefaciens* C58(pWM2), plasmid pRK415, pRKACC, pRKLACC or pWM2 was electroporated into electrocompetent cells of *A. tumefaciens* C58, and was selected on LB agar with 2 μg/ml tetracycline. Since *A. tumefaciens* C58 gives rise to spontaneous mutants that are resistant to tetracycline at a high frequency, colony PCR or

plasmid minipreps was performed to confirm that the corresponding plasmid was transferred to the bacterium. Primers used for pRKLACC were: UW4acdS-F, acgttatccgttgaccttcg; and UW4acdS-R, cgaaacaggaagctgtaggc. Primers used for pWM2 were: RLacdS-F, ggcaaggtcgacatctatgc; and RLacdS-R, ggcttgccattcagctatg. To confirm the transfer of pRK415, Tc^r *Agrobacterium* colonies were picked and plasmids were extracted and used to transform *E. coli* DH5α. *Agrobacterium* colonies that contain plasmids able to transform *E. coli* DH5α to tetracycline resistance were selected.

The binary vectors pPZP-eGFP, which contains a CAMV 35S promoter driven eGFP gene (reporter) and a plant selectable marker *bar* gene driven by an *ocs* promoter, (kindly provided by Dr. Barbara Moffatt in Department of Biology, University of Waterloo) was electroporated into *A. tumefaciens* GV3101::pMP90 to obtain the strain GV3101::pMP90(pPZPeGFP). The plasmid pRKLACC or the empty vector pRK415, was then electroporated into *A. tumefaciens* GV3101::pMP90(pPZP-EGFP) to obtain the strain YH-1, which is GV3101::pMP90(pPZP-eGFP)(pRK415), and the strain YH-2, which is GV3101::pMP90 (pPZP-EGFP)(pRKLACC). DNA minipreps and agarose gel electrophoresis were performed to confirm the transfer of the plasmids. Since the plasmid pRK415/pRKLACC and pPZP-eGFP belong to different incompatibility groups, they can simultaneously be maintained stably in *A. tumefaciens*.

A. tumefaciens D3 was obtained from Deutsche Sammlung von Mikroorganismen (DSM) (Braunschweig, Germany) (*Agrobacterium tumefaciens* D3 DSM 9674). Figure 3.3 shows a schematic representation of the construction of the *A. tumefaciens* D3-1, the *acdS* and *lrpL* double mutant strain. The *lrpL* and *acdS* gene were PCR amplified using KOD hot start DNA polymerase with the following primers: D3F, cattcaaagggctaccgaaa; D3R,

ggggtcatggtaccaatctg. The 1.5 kb fragment was ligated into the HincII site of pBluescriptII SK(+) to get pYH1. Then the resulting plasmid was digested with HincII to get rid of the 536 bp covering the C termini of the *lrp* regulator gene and *acdS* gene, and a tetracycline resistance encoding gene (from pBR322) was inserted into the HincII sites to obtain pYH2. The 2.2 kb fragment containing the N-termini of both *lrpL* and *acdS* and the tetracycline resistance gene was amplified using KOD hot start DNA polymerase using the primers D3F and D3R, and ligated into the SmaI site of pK19mobSacB plasmid to obtain pYH3. The plasmid pK19mobSacB is a broad host range vector containing a modified *sacB* gene from *Bacillus subtilis* which encodes the enzyme levansucrase and confers sucrose sensitivity to the bacteria expressing that gene. Plasmid pYH3 was conjugated into *A. tumefaciens* D3 using triparental mating. Since the *A. tumefaciens* D3 strain is resistant to ampicillin (150 μg/ml), the transconjugants were selected on LB with Ap (150 μg/ml, which counter selects against the donor and helper) and Tc (5 μg/ml, to select for the presence of the plasmid pYH3). The resulting strain *A. tumefaciens* D3(pYH3) was then grown in liquid LB medium with 5 μg/ml of tetracycline for 24 h to allow homologous recombination to occur between the wild type *lrpL* and *acdS* genes and the disrupted ones on the plasmid. Then, 100 μl of serial dilutions of the culture were spread on LB agar with 5 μg/ml of tetracycline and 5% sucrose to select for the mutants. Since the *sacB* gene confers sensitivity to sucrose, only the mutant resulting from a double crossover that contains a tetracycline resistance gene on the chromosome will be able to grow on this medium. The replacement of the wild type *acdS* gene in the mutant was then confirmed by PCR using genomic DNA as template.

To complement the *lrpL* and *acdS* mutations, the following primers were used to amplify the *lrpL* gene and its promter (lrpL-F, lrpL-R), the *acdS* gene and its promoter

(acdS-F, acdS-R), and both the *lrpL* and *acdS* gene and their promoters (lrpL-F, acdS-R) as shown in Figure 3.4: LrpL-F, tggcaggtcccgatttgtgg; LrpL-R, tttgctgatgttcggcactttt; acdS-F, tcaagattcgcgtcagcagc; acdS-R, gaaggttctttacgcccacc. KOD hot start DNA polymerase was used to amplify those fragments, and PCR products were gel purified and ligated into EcoRV digested broad host vector pBBR1MCS-2 (Km^r) (Kovach et al. 1995) to obtain pYH4 (containing *lrpL* and it's promoter), pYH5 (containing *acdS* and it's promoter) and pYH6 (containing both *lrpL* and *acdS* and their promoters). The three plasmids were then transferred to *A. tumefaciens* D3-1 by tri-parental conjugation using *E. coli* DH5α(pRK600) as the helper strain, and the conjugants were seletected on LB with ampicillin (150 μg/ml, counter selecting against the donor and helper *E. coli* strains) and kanamycin (80 μg/ml, to select for the presence of the plasmid pYH4, pYH5, or pYH6). The resulting strains were named *A. tumefaciens* D3-2 (D3-1 containing pYH4), D3-3 (D3-1 containing pYH5), and D3-4 (D3-1 containing pYH6).

Table 3.1. Strains and plasmids used in the study

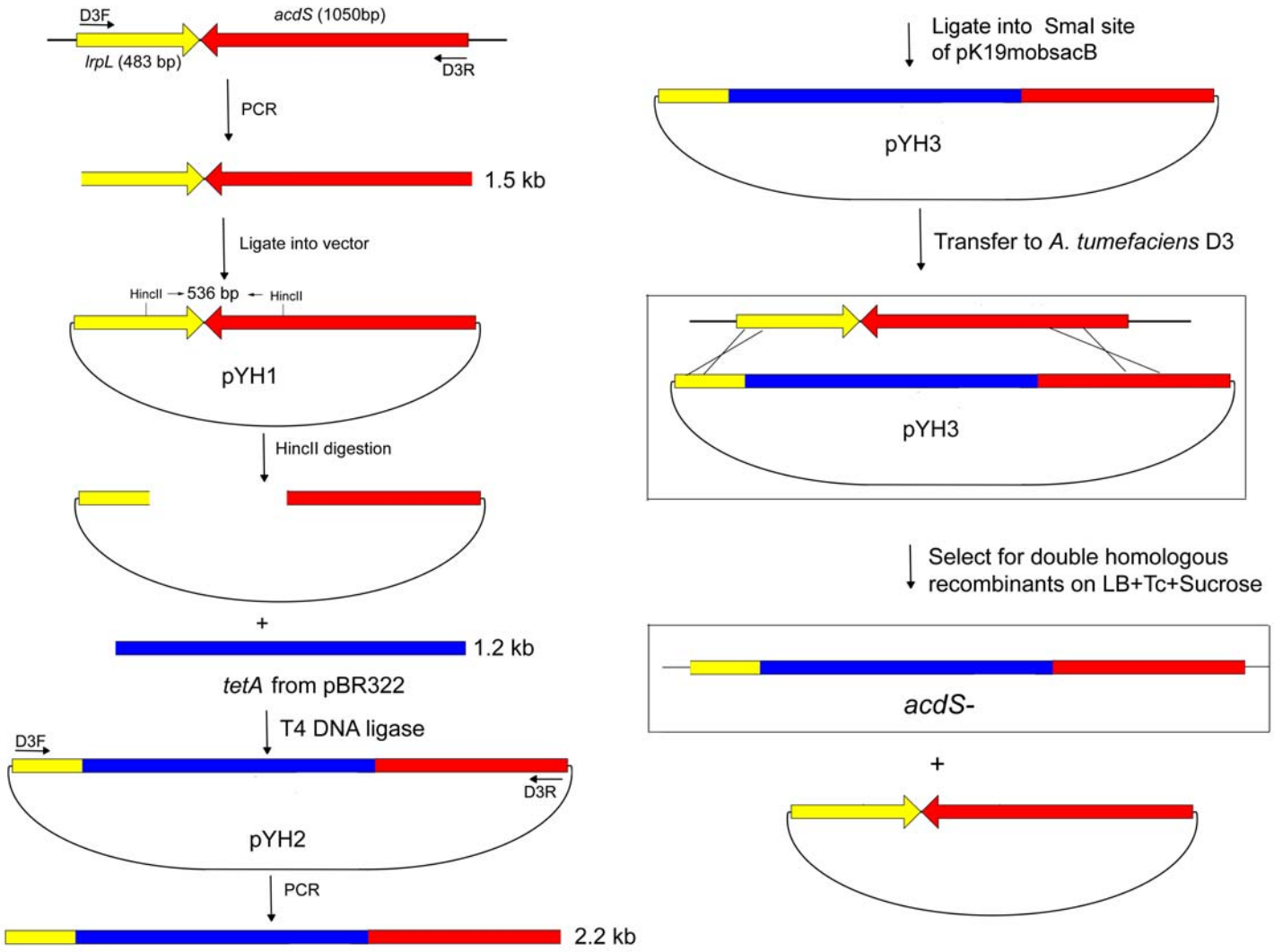
Plasmid or Bacterium	Relevant features	Reference or source
Plasmids		
pSP329	RP4 replicon, Broad-host-range vector, Tc^r	Cangelosi *et al.*, 1991
pWM2	pSP329 containing the *acdS* and *lrpL* genes of *Rhizobium leguminosarum* bv. viciae 128Sm, Tc^r	Ma *et al.*, 2003b; Ma *et al.*, 2004
pRK415	Broad-host-range vector, Pα incompatibility group, Tc^r	Keen *et al.*, 1988
pRKLACC	pRK415 containing the *acdS* gene from *Pseudomonas putida* UW4 under control of the *lac* promoter, Tc^r	Shah *et al.*, 1998
pPZP-eGFP	pPZP-RCS2 base T-DNA binary vector containing 2x35S promoter driven *eGFP* gene and *osc* promoter driven *bar* gene in the T-DNA region. pVS1 incompatibility group plasmid. Sp^r and Sm^r in bacteria, phoshinotricin (PPT) resistance in plants.	Provided by Dr. Barbara Moffatt
pBluescript II SK(+)	Cloning vector, Amp^r	Stratagene
pYH1	pBluescriptII containing 1.5 kb fragment of *acdS* and *lrpL* of *A. tumefaciens* D3 at HincII site.	This study
pYH2	pYH1 with a tetracycline resistance gene replaced the 536 bp of the acdS and lrpL genes between the HincII sites.	This study

pYH3	pK19mobSacB with the 2.2 kb fragment containing a tetracycline resistance gene disrupted *acdS* and *lrpL* from pYH2 inserted into the SmaI restriction site.	This study
pBBR1MCS-2	Broad host cloning vector. Km^r.	Kovach *et al.* 1995
pYH4	pBBR1MCS-2 with 862 bp sequence containing *lrpL* gene and 302 bp upstream promoter sequence of *A. tumefaciens* D3 inserted into the EcoRV site.	This study
pYH5	pBBR1MCS-2 with 1505 bp sequence containing *acdS* gene and 314 bp upstream promoter sequence of *A. tumefaciens* D3 inserted into the EcoRV site.	This study
pYH6	pBBR1MCS-2 with 2152 bp sequence containing both the *acdS* and the *lrpL* gene and their promoters of *A. tumefaciens* D3 inserted into the EcoRV site.	This study
Bacteria		
A. tumefaciens strains		
C58	Initially isolated from cherry tree crown gall. Genome has been fully sequenced	Goodner *et a.*, 2001; Wood *et al.*, 2001
C58(pRK415)	*A. tumefaciens* C58 containing plasmid pRK415, Tc^r	This study
C58(pRKLACC)	*A. tumefaciens* C58 containing plasmid pRKLACC, Tc^r	This study

C58(pWM2)	*A. tumefaciens* C58 containing plasmid pWM2, Tc^r	This study
GV3101::pMP90	A disarmed *A. tumefaciens* strain with C58 chromosome background and Ti plasmid derived from pTiC58. Rif^r	Koncz and Schell, 1986
GV3101::pMP90(pPZPeGFP)	GV3101::pMP90 containing pPZPeGFP.	This study
YH-1	GV3101::pMP90(pPZPeGFP)(pRK415), GV3101::pMP90 containing pPZPeGFP and pRK415.	This study
YH-2	GV3101::pMP90(pPZPeGFP)(pRKLACC), GV3101::pMP90 containing pPZPeGFP and pRKLACC	This study
D3	Isolated from soil in area of Stuttgart, Germany	Deutsche Sammlung von Mikroorganismen (DSM 9674), Trott *et al.*, 2001.
D3-1	*acdS* and *lrpL*double mutant of strain D3. The C terminal region of both *acdS* and *lrpL* between the HincII restriction sites were replaced with the *tetA* gene from pBR322. Tc^r	This study
D3-2	D3-1 containing pYH4	This study
D3-3	D3-1 containing pYH5	This study
D3-4	D3-1 containing pYH6	This study
Pseudomonas putida strains		
UW4	Plant growth-promoting bacterium with high ACC deaminase activity	Glick *et al.* 1995

UW4*(acdS⁻)*	*P. putida* UW4 with a tetracycline resistance gene inserted into the *acdS* gene. It has no ACC deaminase activity. Tc^r	Giddings *et al*, 2000
Escherichia coli strains		
DH5α	*recA1* and *endA1* cloning strain	Hanahan, 1983
DH5α(pRK600)	DH5α strain containing helper plasmid pRK600, Cm^r	Finan *et al.*, 1986

Figure 3.3. Schematic representation of the construction of the *lrpL* and *acdS* double mutant. Wild type *acdS* and *lrpL* were PCR amplified using primers D3F and D3R, and the 1.5 kb fragment was ligated into the HincII site of pBluescript II SK(+) to yield pYH1. A 536 bp fragment between the two HincII sites covering both the C terminal region of *lrpL* and *acdS* was excised and a *tetA* gene from pBR322 encoding tetracycline resistance was ligated into the HincII digested pYH1 vector to yield pYH2. The 2.2 kb fragment containg the *tetA* disrupted *acdS* and *lrpL* was PCR amplified using D3F and D3R primers and was ligated into the SmaI site of the pK19mobsacB to obtain the replacement vector pYH3, which was then transferred into *A. tumefaciens* D3 by triparental conjugation. After 24 h of incubation in LB medium with Tc (5 μg/ml) to allow the double homologous recombination to occur, the mutant was selected on LB medium containig Tc (5 μg/ml) and 5% sucrose.


D3F
acdS (1050bp)
lrpL (483 bp)
D3R
PCR
1.5 kb
Ligate into vector
HincII
536 bp
HincII
pYH1
HincII digestion
+
1.2 kb
tetA from pBR322
T4 DNA ligase
D3F
D3R
pYH2
PCR
2.2 kb
Ligate into SmaI site
of pK19mobsacB
pYH3
Transfer to A. tumefaciens D3
pYH3
Select for double homologous
recombinants on LB+Tc+Sucrose
acdS-
+

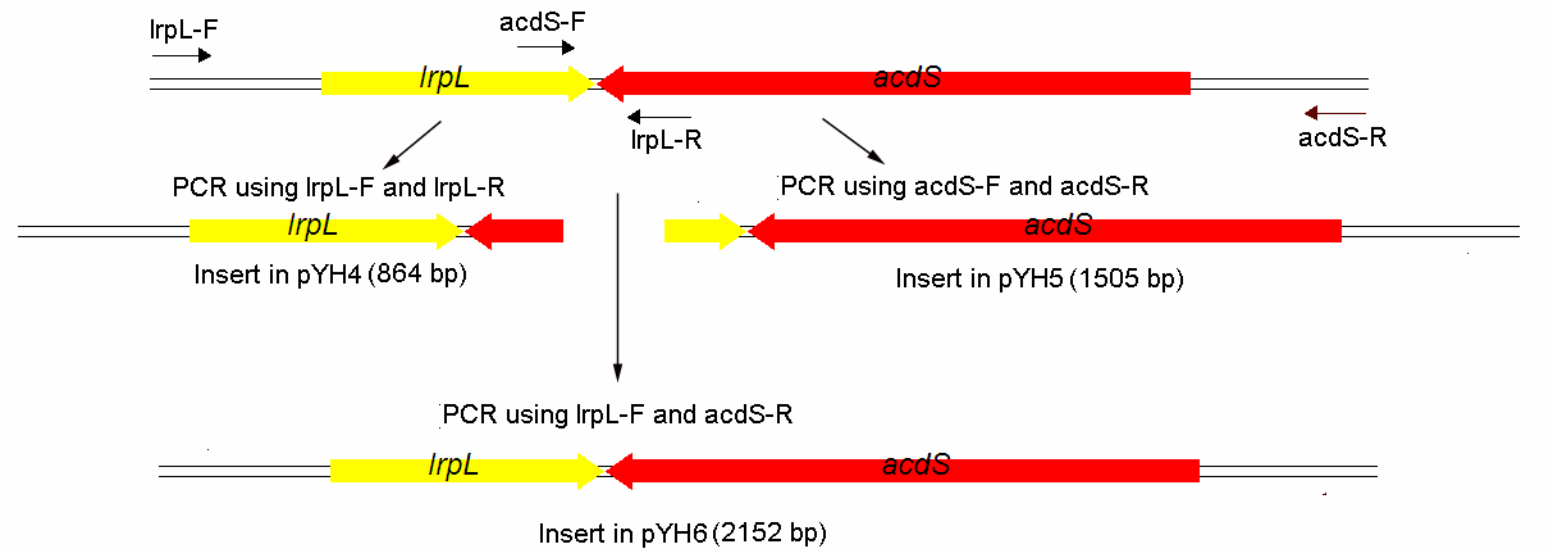


Figure 3.4. Schematic reprentation of the complementation of *lrpL* and *acdS* mutations. The gene *lrpL* and its promoter were amplified using primer lrpL-F and lrpL-R, and the PCR fragement was inserted into the EcoRV site of pBBR1-MCS2 to obtain pYH4. The gene *acdS* and its promoter were amplified using primers acdS-F and acdS-R and the amplified fragment was ligated into the EcoRV site of pBBR1-MCS2 to obtain pYH5. The *lrpL* and *acdS* gene and their promoters were amplified using primers lrpL-F and acdS-R and the PCR fragment was inserted into into the EcoRV site of pBBR1-MCS2 to obtain pYH6.

3.2.3 Plant varieties

Castor bean (*Ricinus comunis Zanzibariensis*) seeds were from Floribunda Seed Company (Indian River, ON, Canada). Tomato (*Lycopersicon esculentum,* cultivar Beefsteak) seeds were obtained from Ontario Seed Company (Waterloo, ON, Canada). Tobacco (*Nicotiana tabacum* cv. Xanthi) seeds were kindly provided by Dr. Saleh Shah (Alberta Research Council), *Arabidopsis* (*Arabidopsis thaliana*, cv. Columbia) seeds were provided by Dr. Barbara Moffat (Dept. of Biology, University of Waterloo). Canola seeds (*Brassica napus* cv. Westar, cv. Thunder, cv. Hyola401, and cv. 4414RR) were kindly provided by BrettYoung Seeds, Inc. (Winnipeg, MB, Canada).

3.2.4 Plant growth conditions

The soil used was Pro-Mix BX general purpose growth medium (Premier Horticulture, Rivière-du-Loup, Quebec, Canada), a peat-based soil used for the cultivation of a variety of plants. It contains sphagnum peat moss (75%–85%, by volume), perlite, vermiculite macronutrients (calcium, magnesium, nitrogen, phosphorus, potassium, sulphur), micronutrients (boron, copper, iron, manganese, molybdenum, zinc), dolomitic limestone, calcite limestone, and a wetting agent. The soil pH was approximately 6.0.

Similar sized castor bean seeds were selected and soaked in water for 2-3 days and then were sowed in Pro-Mix BX soil in 6 inch plastic pots about 5 cm deep. Tomato seeds were first sowed in an eight inch plastic pot, and after germination, seedlings were first transplanted to 2.5 inch pots and then to 6 inch plastic pots containing Pro-Mix BX soil. Plants were grown in a growth chamber with 16 h of light at 25°C, with a light

intensity of 250 μmol m^{-2} s^{-1} from cool-white fluorescent lamps (Sylvania), 8 h of dark at 22°C, or in green house with temperatures that ranged from 18°C to 26°C and 10 to 14 h of daylight.

3.2.5 ACC deaminase activity assay

To measure the ACC deaminase activity, bacterial strains were first grown in 10 ml of LB medium with appropriate antibiotics at 28°C for approximately 24-40 h, i.e. until stationary phase. Cells were centrifuged at 5000 x g for 10 min and washed twice with M9 minimal salts (without a nitrogen source), then resuspended in 10 ml M9 minimal medium with 5 mM ACC as the sole nitrogen source and incubated at room temperature for 40 h with shaking. Cells were then centrifuged for 10 min at 5000 x g and washed twice with 0.1 M Tris-HCl (pH 7.6) before ACC deaminase activity was determined by measuring the production of α-ketobutyrate as described previously (Honma and Shimomura, 1978; Penrose and Glick, 2003).

3.2.6 Virulence assay of *A. tumefaciens*

A. tumefaciens strains were grown at 28°C with aeration in LB broth containing the appropriate antibiotics until late log phase (OD_{600} reached about 2). Cells were pelleted by centrifugation, resuspended in fresh LB medium and normalized to OD_{600}=2.0. When serial dilutions were performed, fresh LB medium was used to dilute the culture.

Carrots purchased from a local supermarket were peeled and the ends were cut off. To surface sterilized them, the peeled carrots were first washed with soap and water,

rinsed with sterile distilled water, soaked in 10% commercial bleach for 20 min, rinsed with 70% ethanol, and finally rinsed 3 times with sterile distilled water. The carrots were cut into slices about 0.5 cm thick with a sterilized knife, and put onto 1.5% water agar in 100 x 20 mm Petri plates, with the basal surface facing upwards. Approximately 4-5 carrot discs can be placed on each plate. Aliquots of 15µl of the various *A. tumefaciens* suspensions were applied to the basal surface of each carrot disc and incubated at room temperature (~22°C) for 3 weeks.

Inoculation of tomato stems followed the procedure described by Aloni *et al* (1998). A V-shaped wound was made, with a single edged razor blade, in the middle of the main stem of three to four week old tomato plants. The wound reached about half of the stem width. Aliquots of 10 µl of the bacterial suspensions were applied to the wounded site with a pipette.

Inoculation of castor bean stems was as described by Aloni *et al* (1998). After the first pair of true leaves had appeared, castor bean hypocotyls were V-shape wounded, with a single edged razor blade, about 2 cm below the cotyledons, 10 µl of the bacterial suspensions (about 5 x 10^7 cells for the non-diluted culture) were then inoculated.

To test the virulence of *A. tumefaciens* D3, it was also inoculated on tobacco stems, canola stems and *Arabidopsis* stems using a toothpick to poke a small hole in the stems of these plants and apply the cell pellet.

The inoculated plants were grown in a growth chamber (16 h of light at 25°C, with a light intensity of 250 µmol m^{-2} s^{-1}, 8 h of dark at 22°C) for 3 to 6 weeks before the tumor size and the extent of plant growth were examined.

3.2.7 Biocontrol assay of *P. putida* UW4 and *A. tumefaciens* D3

To study the effects of *P. putida* UW4 or *A. tumefaciens* D3 on the crown gall disease caused by *A. tumefaciens* C58, the bacterial cells were grown in LB medium until they reached early stationary phase, the culture was then diluted with LB medium to OD_{600}=2.0. Tomato plants or castor bean plants were grown and cut as described in the virulence assay (sec 2.2.6), 10 µl of the *A. tumefaciens* C58 suspensions were mixed with 10 or 20 µl of the culture suspension of UW4 or D3 and were applied onto the plant to get 1:1 or 1:2 ratio of co-inoculation (C58:UW4 or C58:D3). Thus, for example, when co-inoculating *A. tumefaciens* C58 and *P. putida* UW4 using a 1:1 ratio, 10 µl of each was applied to the same plant stem. Inoculated plants were grown in a growth chamber (16 h of light at 25°C, with a light intensity of 250 µmol m^{-2} s^{-1}, 8 h of dark at 22°C) for 3 to 6 weeks before examined for the presence and size of the crown gall tumors.

3.2.8 Recovery of bacteria from the crown gall tumor

Approximately 0.1 g of fresh tumor tissue induced by *A. tumefaciens* C58, or *A. tumefaciens* C58(pRKLACC) was removed, weighed and macerated in a sterile saline solution, and following serial dilution, 100 µl of each dilution was spread on LB plates and incubated at 28°C overnight. Colonies were counted and colony forming units per gram of tumor tissue were calculated.

3.2.9 Siderophore production assay

Siderophore produced by the wild type *A. tumefaciens* D3 and the mutant D3-1 strain was analyzed using CAS (Chrome azurol S) agar (Alexander and Zuberer, 1991).

The Fe-CAS-HDTMA dye complex gives the medium a chracteristic blue color, and the siderophores produced by bacteria can remove Fe from the Fe-CAS-HDTMA dye complex so that orange halos are developed around siderophore producing bacterial colonies.

One single colony of wild type or *lrpL* and *acdS* double mutant *A. tumefaciens* D3 strain was inoculated in 5 ml LB medium and incubated for 24 h at 28°C, then 2 µl of each culture suspension were spotted on the center of each quarter of the CAS agar plate, and incubated for 48 h at 28°C. The diameters of the orange halos were measured to determine the relative amount of siderophre production by the strains.

3.2.10 IAA production assay

IAA produced by the wild type and *acdS*⁻ mutant strains was measured according to Patten and Glick (2002) with some modifications. Single colonies of the wild type and *acdS*⁻ mutant strains of *A. tumefaciens* D3 were inoculated in 5 ml M9 minimal medium and incubated at 28°C for 24 h, and then 20 µl aliquots were transferred to 5 ml M9 minimal medium supplemented with the following concentrations of L-tryptophan (Sigma-Aldrich, St. Louis, MO): 50, 100, 200 and 500 µg/ml. After another 42 h incubation at 28°C, the optical density of each culture was measured at 600nm. The cultures were centrifuged at 6000 x g for 10 mins to remove the cells and a 50 µl aliquot of each culture supernatant was mixed with 200 µl of Salkowski's reagent (150 ml concentrated H_2SO_4, 250 ml distilled H_2O, and 7.5 ml of 0.5 M $FeCl_3 \cdot 6H_20$ (Gordon and Weber, 1951)) in a 96 well microplate and incubated at room temperature for 20 min

before the absorbance at 535 nm was measured. Three replicates were performed for each sample.

3.2.11 Extraction of AHLs and analytical TLC assay.

A. tumefaciens strains D3, D3-1, D3-2, D3-3 and D3-4 were inoculated in 5 ml LB medium and incubated at 28°C for about 24 h until the cultures reached early stationary phase, then 200 µl of each culture were transferred to 20 ml LB in a 100 ml flask and incubated for about 14 h until the cultures reached stationary phase. The QS signal AHLs were then extracted using ethyl acetate and analytical TLC assay was peformed as described in sec 2.2.7.

3.2.12 Gnotobiotic root elongation assay

To study the plant growth promotion ability of different strains, a gnotobiotic root elongation assay was performed as described (Penrose and Glick, 2003). *P. putida* UW4, *A. tumefaciens* D3 and *A. tumefaciens* D3-1 were first grown in 10 ml LB medium for about 24 hour at 28°C until the cultures reached early stationary phase. The cells were pelleted (5000 x g, 10 min) and washed twice with M9 minimal medium (without a nitrogen source) and then resuspended in 10 ml M9 minimal medium supplemented with 3 mM ACC as the sole nitrogen source and returned to a waterbath shaker at 28°C for 24 h to induce ACC deaminase activity. The bacterial cells were pelleted again and then resuspended in sterile 0.03 M $MgSO_4$ and was adjusted to OD_{600} = 0.15.

Seed-pack growth pouches (Northrup King Co., Minneapolis, MN, USA) were wrapped in aluminium foil in groups of 10 and autoclaved at 121°C for 15 min. Just

before use, 12 ml of sterile distilled water were added to each growth pouch. Canola seeds (*Brassica napus* cv. Thuder) were disinfected as following: seeds were soaked in 70% ethanol for 1 min, then in 20% commercial bleach for 15 min at room temperature with shaking at intervals, and finally the seeds were rinsed five times with sterile distilled water for five times.

The seeds were placed in petri plates and incubated at room temperature for 1 h with one of the following treatments: *P. putida* UW4 suspension, *A. tumefaciens* D3 suspension, D3-1 suspension and sterile 0.03 M MgSO4 (used as a negative control). Following the incubation period, eight seeds were placed in each growth pouch with sterilized forceps and 18 pouches were used for each treatment. The pouches were incubated upright in a growth chamber (Conviron CMP 3244; Controlled Environments Ltd, Winnipeg, MB, Canada) under the following conditions: 20 ± 1°C, 12 h of light (18 μmol m^{-2} s^{-1}) and 12 h of dark. The root lengths were measured one week later.

3.2.13 PCR detection of the presence of a Ti or Ri plasmid in *A. tumefaciens* D3

Total DNA of *A. tumefaciens* C58 (positive control) and *A. tumefaciens* D3 were extracted using a commercial genomic DNA purification kit (Promega, Madison, WI). Then, PCR was performed using two pairs of primers that are designed for detection of tumorigenic or rhizogenic *Agrobacterium* and have been proven to be both sensitive and specific to detect whether *A. tumefaciens* D3 contains a Ti or Ri plasmid. Primer pair 1: designed from *tms2* gene (Pulawska and Sobiczewski, 2005): tms2F1, tttcagctgctagggccacatcag; and tms2R2, tcgccatggaaacgccggagtagg. Primer pair 2

designed from the *virC* operon (Sawada *et al.*, 1995): VCF, atcatttgtagcgact; and VCR, agctcaaagctgcttc.

3.2.14 *A. tumefaciens* mediated transformation and regeneration process of canola

3.2.14.1 Media used for tranformation and regeneration

Seed germination medium: 0.5 x MS basal salt medium (Sigma-Aldrich, St. Louis, MO) with 10 g/l of sucrose, adjusted to pH5.8 using 2N NaOH and solidified with 4 g/l of phytagel (Sigma-Aldrich, St. Louis, MO).

Cocultivation medium: MS medium with 1mg/l 2,4-D (Sigma-Aldrich, St. Louis, MO), and 30 g/l sucrose and solidified with 4 g/l of phytagel (Sigma-Aldrich, St. Louis, MO). The pH is adjusted to 5.8.

Agrobacterium resuspension and infection medium: MS medium with 50 μM of acetosyringone (Sigma-Aldrich, St. Louis, MO). The pH is adjusted to 5.8.

Callus induction medium: Cocultivation medium with 500 mg/l carbenicillin (Fisher scientific, Ottawa, ON) and 20 mg/l phophinotricin (PPT) (Sigma-Aldrich, St. Louis, MO).

Organogenesis medium A (OA): MS medium (pH5.8) with 4 mg/l BAP (Sigma), 2 mg/l zeatin (Fisher scientific, Ottawa, ON), 5 mg/l silver nitrate (Sigma-Aldrich, St. Louis, MO), 500 mg/l carbenicillin and 20 mg/l PPT, 30 g/l sucrose and solidified with 4 g/l phytagel.

Organogenesis medium B (OB): Same as organogenesis medium A but no silver nitrate was added.

Shoot regeneration medium: MS medium (pH5.8) with 3 mg/l BAP, 2 mg/l zeatin, antibiotics as above and 30 g/l sucrose and 4 g/l phytagel.

Shoot elongation medium: MS medium (pH5.8) with 0.05 mg/l BAP and 30 g/l sucrose antibiotics as above and 5 g/l phytagel.

Rooting medium: 0.5 x MS salts, 10 mg/l sucrose, 5 g/l phytagel and 0.5 mg/l indole-3-butyric acid (IBA) (Sigma-Aldrich, St. Louis, MO). Antibiotics added as above. The pH is adjusted to 5.8.

3.2.14.2 Transformation protocol

Seeds of *B. napus* cv. Westar, *B. napus* cv. Hyola 401 and *B. napus* 4414 RR were kindly provided by the BrettYoung Seed Company. Seeds were surface sterilized by soaking in 70% ethanol for 1 min, followed by 20% commercial bleach for 20 min. The seeds were then rinsed 4 times with sterilized distilled water and planted at a density of 10-12 seeds per Petri dish (100 x 25 mm) (Fisher scientific, Ottawa, ON) on the seed germination medium. Seeds were germinated at 22-25°C in darkness for 5 days for cv. Westar, 6 days for cv. 4414 RR, and 7 days for cv.Hyola 401.

The transformation and regeneration protocol was modified from Cardoza and Stewart (2003).

1. Hypocotyls from 5-6 day old seedlings were cut into about 1 cm pieces and placed on the cocultivation medium in 100 x 15 mm Petri dishes and preconditioned for 3 days. Approximately 20 explants were placed on each Petri dish.

2. Single colonies of *A. tumefaciens* strains YH-1 and YH-2 were grown at 28°C in 5ml LB medium with 50 μg/ml of Rf, 50 μg/ml Sp, 20 μg/ml Sm and 2 μg/ml Tc for about 2 days until reached early stationary phase. Culture aliquots of 100 μl were transferred to 50 ml LB medium with the same antibiotics and subcultured overnight until the culture reached $OD_{600} \approx 1$. The cells were pelleted, resuspended in the infection medium and normalized to $OD_{600} = 1$ to get the 1x dilution. Serial dilutions were then performed using the infection medium to obtain 10^{-1} x and 10^{-2} x.

3. Approximately 100-150 of the preconditioned explants were transferred to an empty Petri dish and 15 ml of an *Agrobacterium* suspension was added to the Petri dish to infect for 30 minutes, with gentle shaking.

4. The explants were then transferred to the cocultivation medium and co-cultured for 48 h.

5. The explants were then transferred to the callus induction medium supplemented with 500 mg/l carbenicillin (to kill *Agrobacterium*) and 20 mg/l phosphinothricin (PPT) (to select for transformed explant).

6. Two weeks later, the explants were transferred to the organogenesis medium with (OA) or without $AgNO_3$ (OB).

7. After another two weeks, the calli were transferred to shoot induction medium.

8. The calli were transferred to new shoot induction medium every 2 weeks until shoots emerged (usually 3-6 weeks). The shoots were then transferred to shoot elongation medium in 100 x 25 mm Petri dishes. With 4-5 shoots in each plate.

9. After another 2 weeks, the elongated shoots were transferred to the rooting medium.

10. The transformed plants were then transferred to Pro-Mix soil in 6-inch pots and grown in a greenhouse.

Plant tissue cultures were maintained in a growth chamber with 25°C, 16 h of light and 8 h of dark, with a light intensity of 40 μmol m^{-2} s^{-1} from cool-white fluorescent lamps.

3.2.14.3 Calculation of stable transformation efficiency

The stable transformation efficiency was caculated using the following formula:

Transformation efficiency (TE): $TE = \frac{\text{number of transgenic plants obtained}}{\text{number of explants used for transformation}}$

3.2.15 Estimation of bacterial population in plant tissue

Two days after infection with various dilutions of *A. tumefaciens* strain YH-1 or YH-2 and cocultivation on the cocultivation medium (MS with 1mg/L 2,4-D), the *Agrobacterium* population per gram of canola hypocotyl explants was estimated. Firstly, 20 explants from each treatment were aseptically transferred to a sterile ependorf tube, weighed, and macerated using a flame sterilized motar and pestle. Then, sterile saline solution was used to prepare serial dilutions (10^{-1} to 10^{-7}). Aliquots of 100 μl of each dilution were spread on LB agar with antibiotics. After 48 h of incubation at 28°C, colonies were counted, and the colony-forming units (CFU) per gram of plant tissue were calculated. Three repeats with a total of about 60 hypocotyl segments from two independent experiments were performed for each treatment.

3.2.16 Detection of ethylene level using gas chromatography (GC)

One week old canola hypocotyls were cut into 1 cm fragments and infected immediately with *Agrobacterium*. After infection, 40 to 50 explants were transferred to a 25 ml glass vial, carefully weighed, sealed with a rubber stopper, and incubated for 24 h at 25°C. For each treatment, 5 replicates were used. To analyze the ethylene level, 1 ml of the gas from each glass vial was removed using a plastic syringe and analyzed using a GC-17A equipped with an aluminum oxide column (Agilent technologies, HP-AL/M, 30m x 0.537mm x 15 μm) and a hydrogen flame ionization detector under the following conditions: injector temperature, 90°C; column temperature, 50°C; detector temperature, 110°C; carrier gas, Helium; and a flow rate of 5.8 ml/min. Ethylene standard was purchased from Alltech associates Inc (1000 ppm in Helium), and was diluted using Helium. The ethylene concentration in gas samples was estimated by comparing the area below the peaks to areas given by 1 ml of diluted ethylene standards. Ethylene production rates (pmol ethylene/gram fresh weight/h) were then calculated as following:

Since $N_{C2H4} = V_{C2H4}/V_{mol}$, ($N_{C2H4}$, moles of C_2H_4; V_{C2H4}, volume of C_2H_4; V_{mol}, molar volume of an ideal gas),

and $V_{C2H4} = V_{Total} \times [C_2H_4]$ in ppm,

so, $N_{C2H4} = V_{Total} \times [C_2H_4]$ ppm $/V_{mol}$.

V_{Total} =25 ml,

Under standard laboratory conditions (25^{o}C (298K) and 101.3kPa (1atm)), 1 mole of an ideal gas (V_{mol}) has a volume of 24.47 liters.

1ppm = $1/10^6$ volume of air=10^{-6} volume of air

So $N_{C2H4} = (25ml \times [C_2H_4] \times 10^{-6})/24.47\ L = (25 \times [C_2H_4] \times 10^{-9})/24.47 = 1.022 \times [C_2H_4] \times 10^{-9}$ mole=$1.022 \times [C_2H_4] \times 10^3$ pmol

Ethylene evolution rate= N_{C2H4}(pmol)/fresh weight (g)/time (h)= 1.022 x [C_2H_4] x10^3 pmol/ fresh weight (g)/time (h).

3.2.17 Other general protocols

3.2.17.1 Bacterial genomic DNA extraction and plasmid extraction

Bacterial genomic DNA was extracted using Wizard Genomic DNA purification kit (Promega, Madison WI). Plasmid DNA was extracted using Wizard plus SV Miniprep DNA purification kit (Promega, Madison WI) or using alkaline lysis method (Sambrook *et al*., 1989).

3.2.17.2 Plant genomic DNA extraction

Plant genomic DNA was isolated using the CTAB method (Doyle and Doyle, 1990). About 1 g of fresh leaf tissue was frozen in liquid nitrogen and then was grounded to fine powder with a liquid nitrogen-cooled mortar and pestle. Then 0.2 g of the ground powder was transferred to a 1.5 ml Eppendorf tube and 650 µl of 1.5 x CTAB buffer (1.5% cetyl trimethylammonium bromide (CTAB), 75mM Tris-HCl (pH8.0), 15mM ethylenediaminetetraacetic acid (EDTA) (PH8.0), 1.05 M NaCl) was added to it and mixted by votex. The mixture was incubated at 65°C for 20 min. After incubation, the cell debris was spun down by centrifugation at 12000 x g for 5 min, and the supernatant was transferred to a new 1.5 ml Eppendorf tube. The supernatant was extracted once with an eaqual volume of 25:24:1 (phenol:chloroform:isoamyl alcohol) and then with an equal volume of 24:1 (chloroform:iso amyl alcohol). The supernatant was then transferred to a new tube and the genomic DNA was

precipitated by adding 1/10 volume of 3 M sodium acetate (pH 5.2) and 2 volume of absolute ethanol, incubated at -20°C for 2 h and centrifuged at 12000 x g for 10 min. The supernatant was discarded and the pellet was washed with 70% ethanol once and air dried for 10 min at room temperature. The DNA was resuspend in 100 µl sterile DNase free water, and RNaseA was added to a final concentration of 10 ng/ml and incubated for 30 min at 37°C to remove RNA.

3.2.17.3 Gel purification of DNA fragments

Gel purification of DNA fragments were performed using Wizard SV. Gel and PCR cleanup kit (Promega, Madison, WI) according to the manufacturer's manual.

3.2.17.4 Klenow fill in of sticky ends

DNA sticky ends were made blunt ended using Klenow fragment (Fermentas, Burlington, ON) under the following conditions (20 µl system): 500 ng of DNA to be blunt ended, 2 µl of 10 x reaction buffer, 1 µl of 0.5 mM mixture of dNTPs, Klenow fragment (10 u), and sufficient double distilled H_2O to adjust the total volume to 20 µl. The mixture was incubated at room temperature for 30 min, and then incubated at 75°C for 10 min to inactivate the enzyme.

3.2.17.5 CIAP dephosphorylation of linearized plasmid DNA

Calf intestinal alkaline phosphatase (CIAP) was purchased from Fermentas (Burlington, ON). The CIAP treatment of linearized plasmid was carried out as following:

- Linearized DNA sample (90 µl) (~1 µg)
- 10x CIAP buffer (15 µl)
- CIAP diluted in 1x CIAP buffer (0.01 unit/pmol ends)
- Add double distilled H_2O to a final volume of 150 µl.

The mixture was incubated at 37°C for 1 h and then 2 µl of 0.5 M EDTA buffer (pH8.0) was added to stop the reaction. The mixture was extracted with one volume of phenol:chloroform (25:24) and then with one volume of 24:1 chloroform:isoamyl alcohol. The supernatant was transferred to a new tube and DNA was precipitated by adding 0.1 volume of 3 M sodium acetate buffer (pH5.2), 2 volumes of chilled ethanol, incubated at -20°C for 2 h, and then centrifuged at 15000 x g for 10 min. The pellet was then rinsed with 70 % ethanol and dried before and the DNA was redissolved in 30 µl of double distilled H_2O.

3.2.17.6 Electrocompetent *Agrobacterium* preparation and Electroporation process

To prepare electrocompetent *A. tumefaciens* cells, a single colony of an *A. tumefaciens* strain was grown in 5ml of liquid LB medium with appropriate antitiotics at 28ºC with shaking until the cells reached stationary phase, then 2 ml of the culture was transferred to 200 ml of fresh medium and returned to the 28ºC waterbath shaker until the OD_{600} reached 0.8 to 1. The culture was centrifuged at 4ºC using Sorvall rotor SLA1500 for 5 min at 5000 rpm, the cell pellet was resuspended and washed once with 100 ml of ice cold 0.1 mM HEPES buffer in double distilled H_2O, centrifuged as above, and then the cell pellet was washed twice with 100 ml of ice-

cold 0.1 mM HEPES buffer in 10% glycerol. Finally, the cell pellet was resuspended in 1 ml of 0.1 mM HEPES in 10% glycerol and aliquoted into 100 µl volumes. To perform electroporation, 2 µl of the plasmids pWM2, pRK415, pRKACC or pRKLACC (about 300 ng) was added to 100 µl of the competent cells and transferred to the bottom of a pre-chilled 2 mm eletroporation cuvette (Fisherscitific, Ottwa, ON), and transfer of the plasmid to *Agrobacterium* cells was achieved using a Gene Pulser (Bio-rad, Hercules, CA) at 2.5 kV, 400 ohms, and 25 µF. Following electroporation, 1 ml of LB was added immediately and transferred to a test tube and allowed to recover for 2 h at 28ºC before spreading onto LB agar with antibiotics to select for transformants. The plates were incubated at 28ºC for about 2 days until colonies appeared.

3.2.17.7 Tri-parental conjugation

E. coli DH5α(pRK600) was used as the helper strain for all the tri-parental conjugation experiments performed in this study. The donor strain, the helper strain and the recipient strain were grown to stationary phase in LB medium with appropriate antibiotics, then 1 ml of each culture was transferred to a 1.5 ml microcentrifuge tube and then centrifuged at 5000 x g for 5 min, the supernatant was discarded and the cell pellet was resuspended in 1 ml LB medium and then centrifuged at 5000 x g for 5 min to wash the cells. Each of the three cell pellets was then resuspended in 1 ml LB and mixed together. The cells mixture was filtered onto a sterile nitrocellulose membrane (0.45 µm, Whatman) held in a filter unit using a sterile plastic syringe. The membrane was incubated on top of LB agar at appropriate

temperature for 8 h, the cells were washed off from the membrane, serial dilutions were performed, and 100 µl of each dilution was spread onto LB agar with appropriate antibiotics to select for the conjugants.

3.2.17.8 Primer design

All primers were designed using the commercial software Primer Premier 5.0 (http://www.premierbiosoft.com/primerdesign/).

3.3 Results

3.3.1 ACC deaminase and ACC deaminase containing PGPB inhibit *A. tumefaciens* C58 induced crown gall development.

3.3.1.1 Construction and characterization of *A. tumefaciens* strains

Several plasmids containing an *acdS* gene from *P. putida* UW4 (high activity) or from *Rhizobium leguminosarum* bv. viciae 128Sm (low activity) were introduced into *A. tumefaciens* C58. Plasmid pWM2 contains the *acdS* and its regulatory sequence from *R. leguminosarum* bv. viciae 128Sm in the broad-host-range vector pSP329 (Ma *et al.*, 2003b; Ma *et al.*, 2004). Plasmid pRKACC contains the *acdS* gene with its promoter and regulatory sequence from *P. putida* UW4 in the broad-host-range vector pRK415 (Shah *et al.*, 1998), while pRKLACC contains the *acdS* gene from *P. putida* UW4 under the control of the *E. coli lac* promoter in pRK415 (Holguin and Glick, 2001). Since there is no *lac* repressor in *A. tumefaciens* C58, the *acdS* gene from pRKLACC is expressed constitutively. When the ACC deaminase activity of the *Agrobacterium* strains was determined (Figure 3.5), the results showed that, as expected, wild-type *A. tumefaciens* C58 and the strain containing an empty vector C58(pRK415) have no ACC deaminase activity, the strain with the plasmid pRKLACC or pRKACC exhibits a high level of ACC deaminase activity, which is similar to that of wild type *P. putida* UW4, while the strain with pWM2 shows a low level of activity. It was also confirmed that *P. putida* UW4-*acdS*¯ has no ACC deaminase activity.

The growth rates of different strains in MG/L or LB medium were also examined. For strains with plasmids, 2 μg/ml of tetracycline was added. It was found that the growth rates in complete medium of the different strains were similar regardless of the presence of any plasmid.

3.3.1.2. Virulence assay using carrot discs

Carrots were surface sterilized and sliced into ~4 mm thick discs. *A. tumefaciens* strains were grown up to stationary phase and the cultures were adjusted to $OD_{600} = 2$ using fresh LB medium. Serial dilutions of the *Agrobacteria* cultures were prepared and an aliquot of 15 μl of each dilution was applied on the basal surface of each carrot disc. The inoculated carrot discs were then incubated on top of 1.5% water agar at room temperature (~22°C) for 3 weeks before the tumor-inducing ability of the strains was determined by counting the number of carrot discs carrying tumors as well as the number of tumors on each disc. The results showed that there was no significant difference in the tumor-inducing ability among the wild type strain C58, the strain with the empty vector C58(pRK415) and the strain with an ACC deaminase encoding gene C58(pRKLACC) (Figure 3.6).

3.3.1.3 Virulence assay using tomato stems.

Since it has previously been observed that ethylene levels are low before and shortly after *Agrobacterium* infection, and only after the tumor has been initiated does the ethylene level begin to increase dramatically (Goodman *et al.*, 1986; Aloni, 1995; Wächter *et al.*, 1999), it is likely that ethylene plays a more important role in tumor

development than in tumor initiation. Therefore, tomato plants and castor bean plants were used to evaluate the effect of ACC deaminase on the later phases of crown gall development.

Tomato plants are known to be very sensitive to ethylene, and it has been reported that crown gall development was severely diminished in ethylene insensitive *Never ripe* mutant tomato plants (Aloni *et al.*, 1998). To study the effects of the presence of an *acdS* encoding gene in the pathogenicity of *A. tumefaciens*, wild-type tomato stems were inoculated with different *A. tumefaciens* strains with or without an *acdS* gene, and the size and fresh weight of the induced tumors were quantified four weeks after inoculation. The experiments were repeated three times with three to five replicates for each treatment. All experiments were performed under controlled conditions in a growth chamber; similar results were obtained with each replicate (Figure 3.7).

Unexpectedly, in terms of tumor size and fresh weight, there was no significant difference between the wild-type *A. tumefaciens* C58, the strain with the empty vector C58(pRK415), and the strain with ACC deaminase activity C58(pRKLACC). One possible explanation is that the level of ACC deaminase in the *Agrobacterium* cells was not sufficient to lower the ethylene in the infected plants to a level that could significantly modulate the tumor development. Another possibility was that since the *Agrobacterium* with an *acdS* gene can use ACC as nitrogen source, it may survive/proliferate better in the tumor than the wild type. Although we inoculated the same number of cells on the plant stem, after the first several days of inoculation, *A. tumefaciens* C58(pRKLACC) might survive better and proliferate faster on the plant stem, and infect more plant cells. Thus, although the ACC and ethylene levels may have been reduced to a greater extent in

the tumor induced by *A. tumefaciens* C58(pRKLACC) than in tumors induced by *A. tumefaciens* C58 or *A. tumefaciens* C58(pRK415), the increased number of *Agrobacterium* cells could counteract the effect of the lowered ethylene on the total tumor fresh weight. To test this possibility, *Agrobacterium* cells were recovered from four-week old tumors and the colony forming units (CFU) per gram of tumor tissue were calculated. In fact, it was found that there were approximately twenty times more bacterial cells in the tumor induced by *A. tumefaciens* C58(pRKLACC) than bacterial cells in the tumor induced by *A. tumefaciens* C58 (i.e., $8.2 \pm 1.4 \times 10^6$ CFU per gram vs. $4.2 \pm 1.1 \times 10^5$ CFU per gram. Data was obtained from 3 independent tumors for each treatment).

Since expressing *acdS* in *Agrobacterium* makes the bacteria grow better on the wounded plant stem, it becomes difficult to evaluate any inhibitory effects of ACC deaminase on crown gall development. To obviate this problem, wild-type *A. tumefaciens* C58 was co-inoculated with either wild-type ACC deaminase-containing plant growth promoting bacteria *P. putida* UW4 or *P. putida* UW4-*acdS*$^-$ (the ACC deaminase minus mutant of this bacterium), using a 1:1 ratio of cell numbers. The only difference between *P. putida* UW4 and *P. putida* UW4-*acdS*$^-$ is that the latter has no ACC deaminase activity. The results showed that in both cases the tumor fresh weight was significantly reduced (Figure 3.7). A comparison of the tumor fresh weight for the co-inoculation of *A. tumefaciens* C58 with wild-type and mutant *P. putida* UW4 indicates that the tumor fresh weight is about 20-30 percent lower when *A. tumefaciens* C58 is co-inoculated with wild-type *P. putida* UW4 than with *P. putida* UW4-*acdS*$^-$. This means that ACC deaminase contributes to the inhibition of crown gall tumor development. However, even the mutant

strain of *P. putida* UW4 has a significant effect on reducing tumor growth. The fresh weight of the tumor is only about half of that inoculated with *A. tumefaciens* C58 alone (Figure 3.7 B). This suggests that mechanisms other than ACC deaminase are operative in this case. To investigate whether these effects are unique to tomato plants, additional experiments were performed with castor beans.

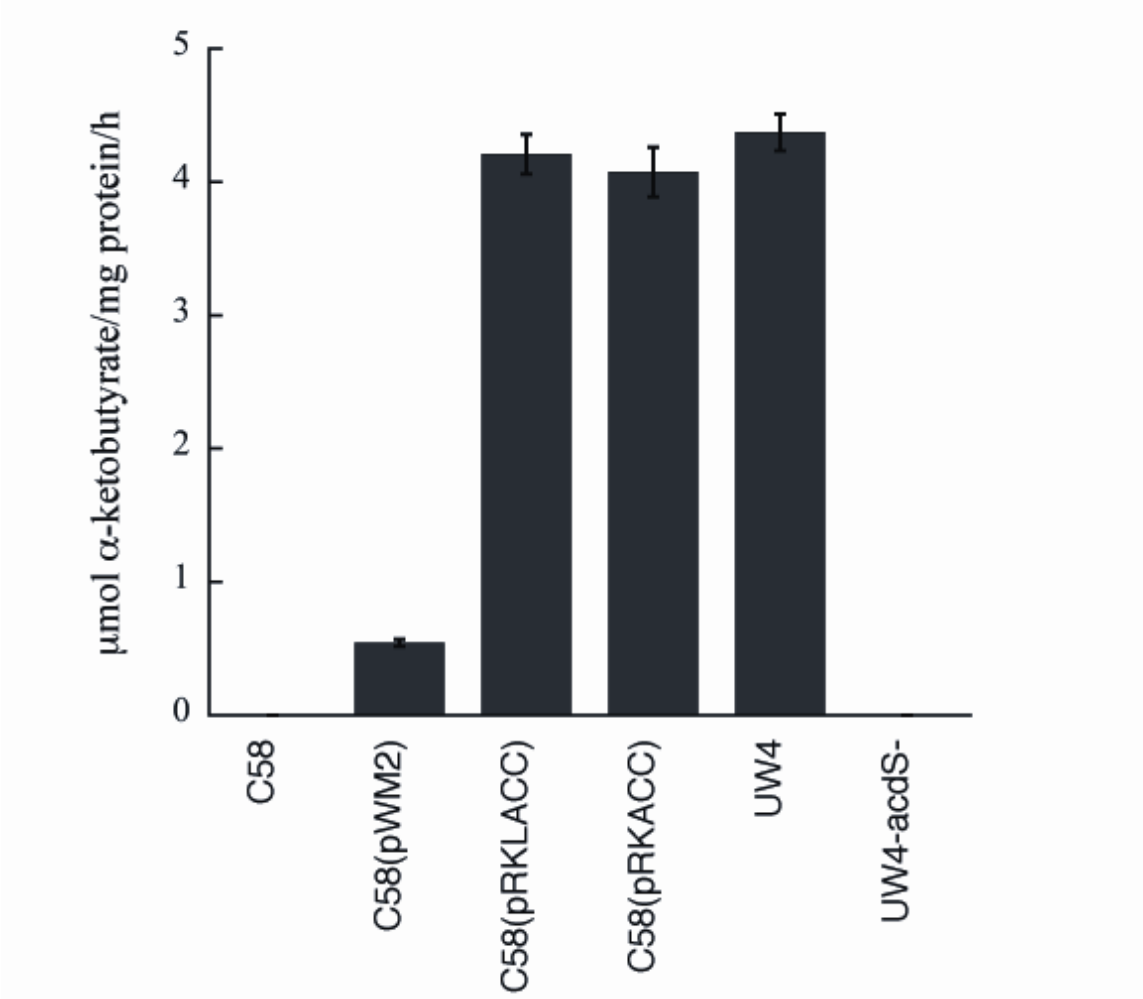


Figure 3.5. ACC deaminase activity assay of different strains. Abbreviations: C58, *A. tumefaciens* C58; C58(pRK415), *A. tumefaciens* C58(pRK415); C58(pRKACC), *A. tumefaciens* C58(pRKACC); C58(pRKLACC), *A. tumefaciens* C58(pRKLACC); UW4: *P. putida* UW4; UW4-*acdS*¯, *P. putida* UW4-*acdS*¯. The bars are standard errors from 3 independent test.

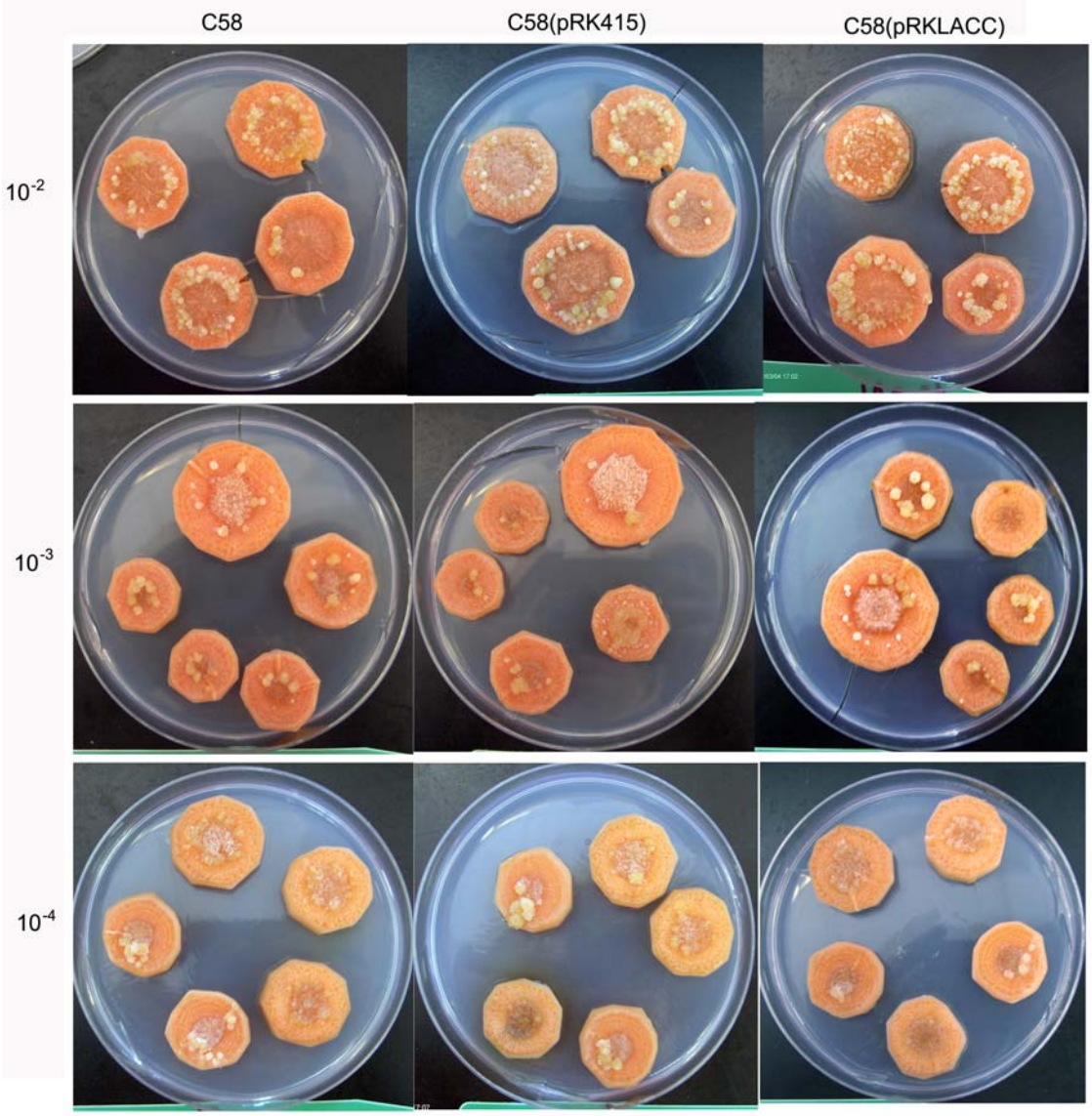
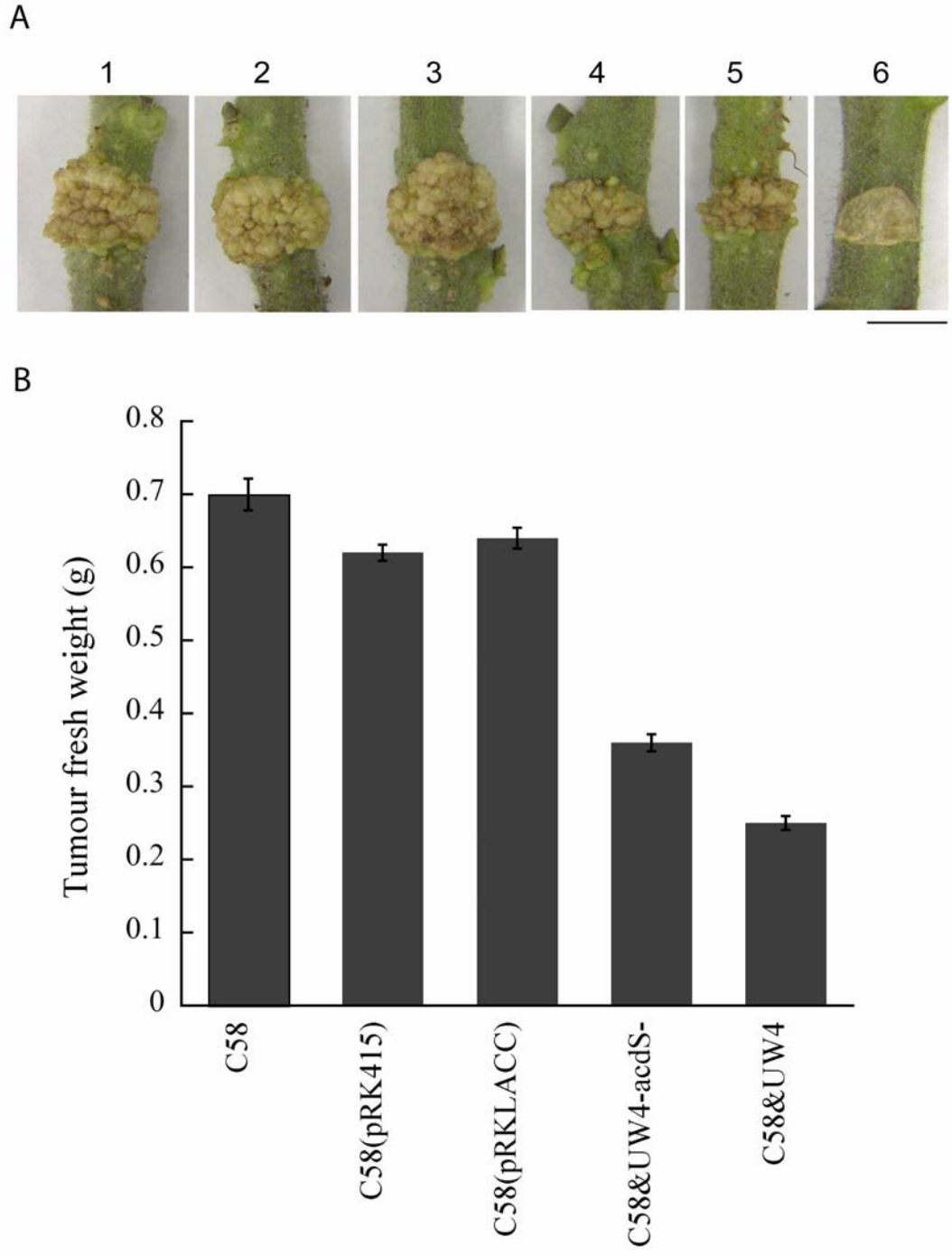


Figure 3.6. Carrot discs inoculated with different dilutions of *A. tumefaciens* strains. Pictures were taken three weeks after inoculation. Row 1 to Row 3, carrot discs inoculated with 10^{-2} (row 1), 10^{-3} (row 2) or 10^{-4} (row 3) dilution of $OD_{600} = 2$ cultures of *A. tumefaciens* C58 (left), *A. tumefaciens* C58(pRK415) (middle) and *A. tumefaciens* C58(pRKLACC) (right)..

Figure 3.7. Virulence assay using tomato stems. A: Example of four week old crown gall tumors on wild type tomato stems induced by different treatments: 1, *A. tumefaciens* C58; 2, *A. tumefaciens* C58(pRK415); 3, *A. tumefaciens* C58(pRKLACC); 4, *A. tumefaciens* C58 and *P. putida* UW4-*acdS*$^{-}$; 5, *A. tumefaciens* C58 and *P. putida* UW4; 6, saline water control. The bar at the bottom represents 1 cm. B: Fresh weight of tumors on tomato stems induced by different treatments as indicated. The results were obtained from 6 plants for each treatment. The bars are standard errors.


A
1
2
3
4
5
6
B
Tumour fresh weight (g)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
C58
C58(pRK415)
C58(pRKLACC)
C58&UW4-acdS-
C58&UW4

3.3.1.4 Virulence assay using castor bean stems

Infection of castor bean plants by *A. tumefaciens* C58 results in the formation of large tumors on the stem as well as the inhibition of plant growth (Figure 3.8). This makes it an attractive system to examine the effects of various treatments on tumor growth. Castor bean plants were inoculated with different *Agrobacterium* strains with or without *acdS*, and after four to six weeks, tumor size as well as plant growth were measured. The experiment was repeated five times, each time with three to five replicates for each treatment. Since seed size can affect growth of the castor bean plants, seeds of similar size were used for each replicate. The size of the tumors induced by wild-type *A. tumefaciens* C58 at different times can vary substantially. Thus, the data for each time point was analyzed separately and it was found that although the absolute values were different, the trends were the same. The tumors induced by strain *A. tumefaciens* C58(pRKLACC) were always smaller than the tumors induced by wild-type strain *A. tumefaciens* C58 or strain *A. tumefaciens* C58(pRK415), and the castor bean plants inoculated with *A. tumefaciens* C58(pRKLACC) always grew better than plants inoculated with *A. tumefaciens* C58 or *A. tumefaciens* C58(pRK415). Figure 3.9 and Figure 3.10 showed the results of one experiment. Figure 3.11 showed results from another experiment. The variation notwithstanding, in all cases, the results showed that the size of the tumor is significantly reduced when the ACC deaminase gene is expressed in *A. tumefaciens*. For the plants inoculated with *A. tumefaciens* C58(pRKLACC), the shoot length is 20-30% greater than plants inoculated with *A. tumefaciens* C58 or *A. tumefaciens* C58(pRK415) (Figure 3.10).

Similar to what has been found using tomato plants, when *Agrobacterium* cells were recovered from the tumor, there were about twenty times more *Agrobacterium* cells for every gram of tumor induced by strain *A. tumefaciens* C58(pRKLACC) than was found when wild-type *A. tumefaciens* C58 was used (i.e., $7.9 \pm 1.2 \times 10^6$ vs. $2.6 \pm 0.9 \times 10^5$. Data was obtained from 3 independent tumors for each treatment).

In order to obviate the complication that *A. tumefaciens* C58 expressing an *acdS* gene proliferated to a greater extent within the tumor than the wild-type, *A. tumefaciens* C58 was co-inoculated with either *P. putida* UW4 (wild-type) or UW4-*acdS*$^-$ (*acdS* knockout mutant). The experiments were performed with three or five replicates for each treatment and repeated twice. The data in Figure 3.12 is from one experiment. Consistent with the results observed using tomato stems, tumor size was significantly reduced when the plants were co-inoculated with *A. tumefaciens* and *P. putida* UW4 using a 1:1 ratio (Figure 3.12 A). The wild-type *P. putida* UW4 with ACC deaminase activity was more effective than *P. putida* UW4-*acdS*$^-$ in inhibiting tumor development. The tumor fresh weight was reduced about 40% when *P. putida* UW4-*acdS*$^-$ was applied and by approximately 55% reduced when wild-type *P. putida* UW4 was applied (Figure 3.12 B).

Figure 3.8. Growth of castor bean plants with and without tumor. Left, six week old castor bean plants with a four week old tumor caused by *A. tumefaciens* C58. Right, six week old castor bean plants that are not infected with *A. tumefaciens* C58.

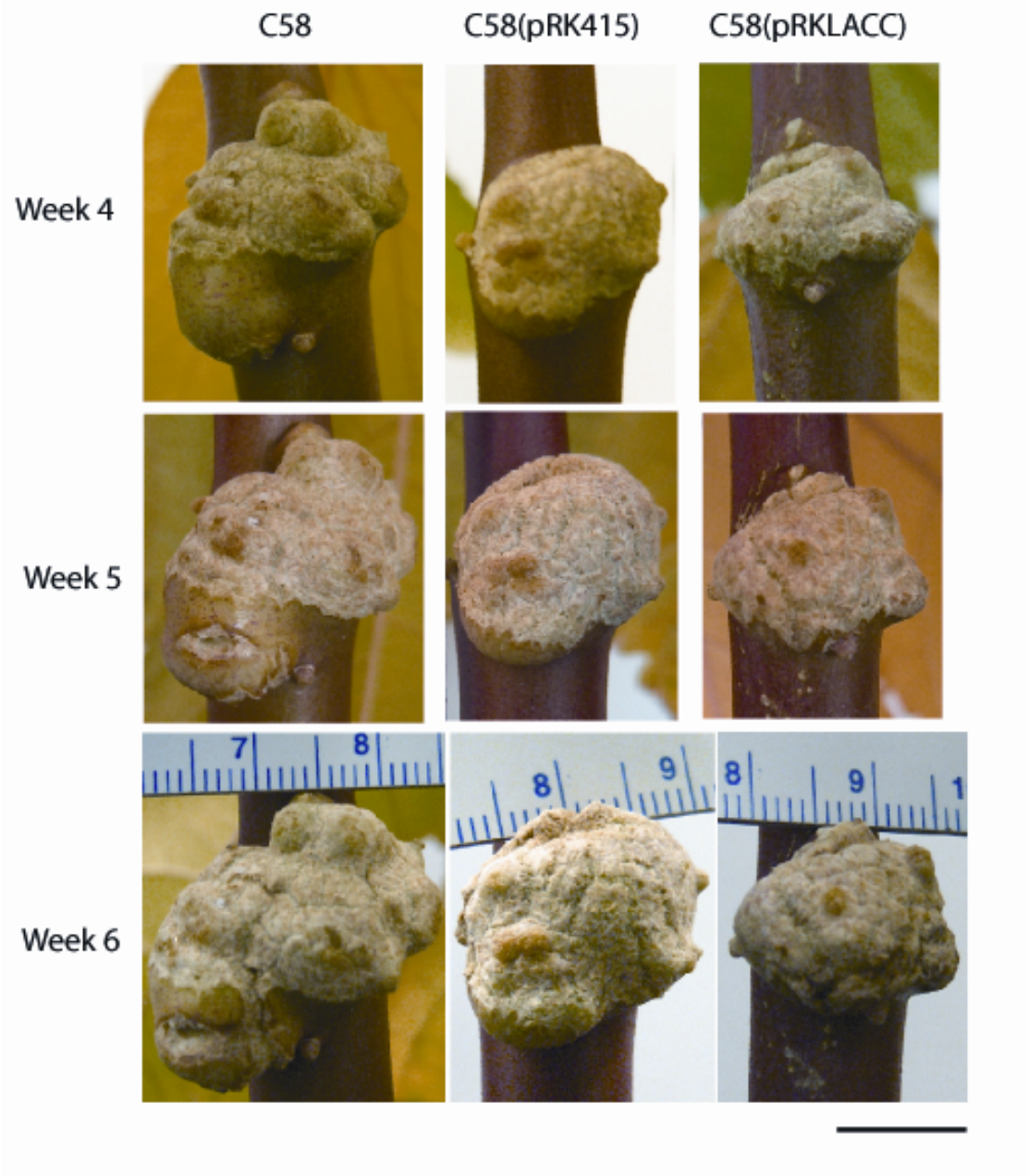


Figure 3.9. Crown gall tumors induced by *A. tumefaciens* C58, *A. tumefaciens* C58(pRK415) and *A. tumefaciens* C58(pRKLACC). Four week (first row), Five week (second row) and six week (third row) old tumors on castor bean stems induced *by A. tumefaciens* C58 (left column), *A. tumefaciens* C58(pRK415) (middle column) or *A. tumefaciens* C58(pRKLACC) (right column). The bar at the bottom represents 1 cm.

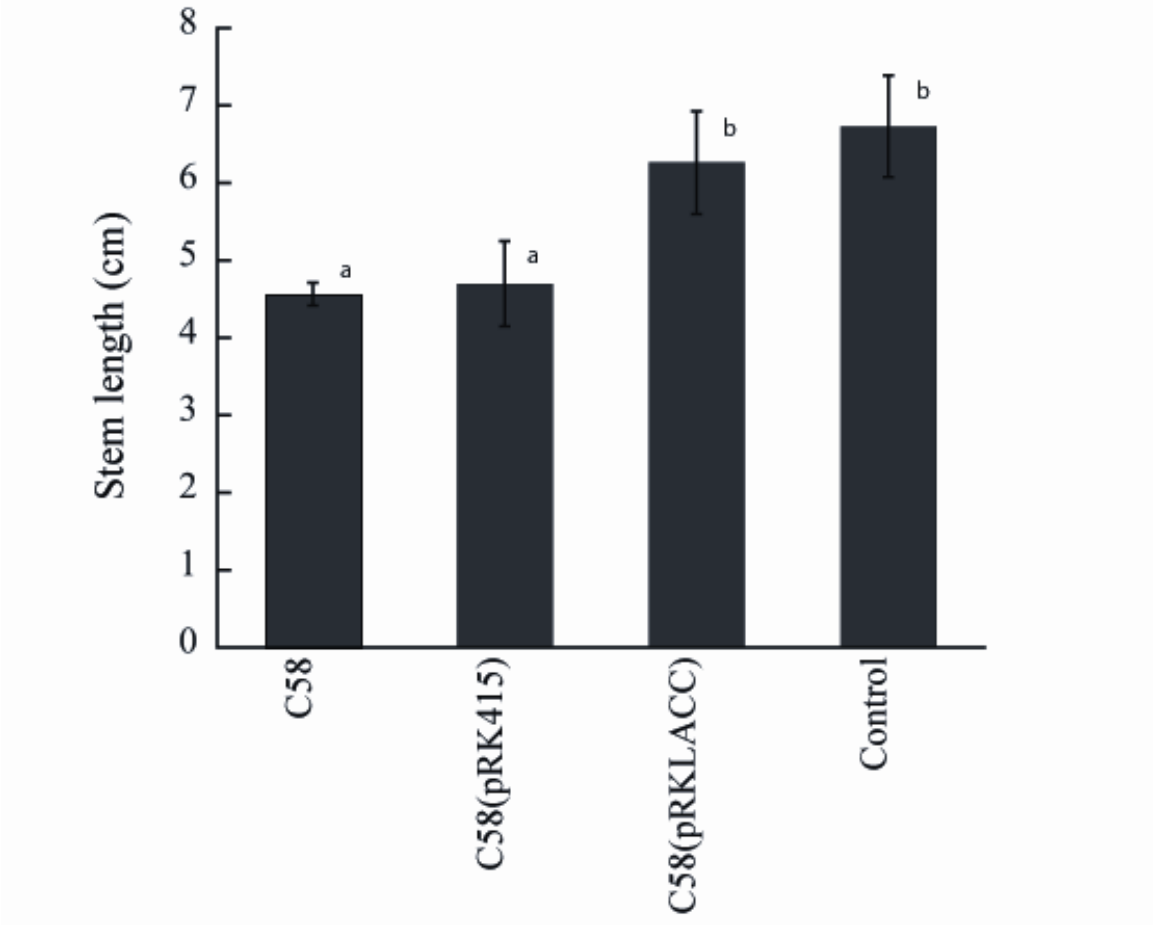


Figure 3.10. Length of castor bean stems from the tumor induced by different treatments (or the wound site of control plants) to the shoot apex (cm). Treatments: C58, *A. tumefaciens* C58; C58(pRK415), *A. tumefaciens* C58(pRK415); C58(pRKLACC), *A. tumefaciens* C58(pRKLACC); Control, uninoculated control. The results were obtained from 3 replicates for each treatment. The vertical bars that do not possess the same letter (a or b) are statistically different (Student's t test with $\alpha < 0.05$). The error bars are standard errors. The plants were from the same experiment as those shown in Figure 3.9.

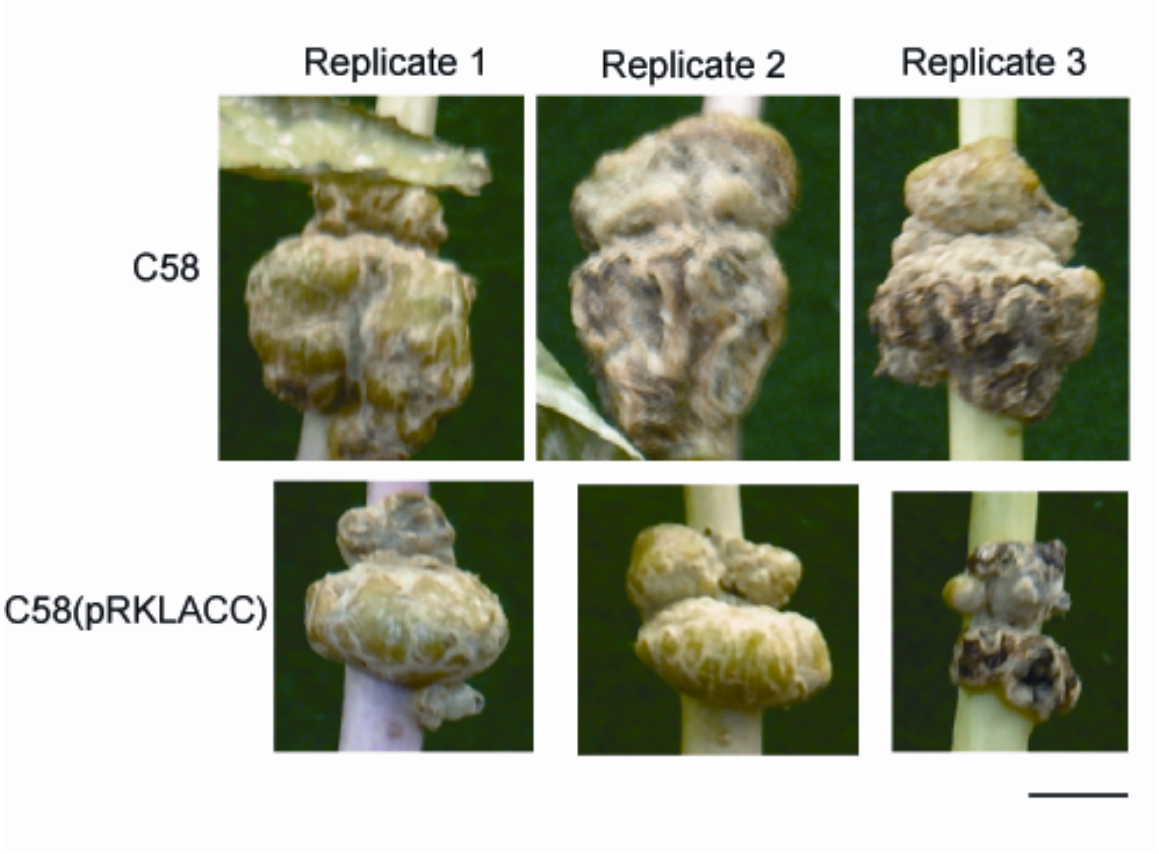
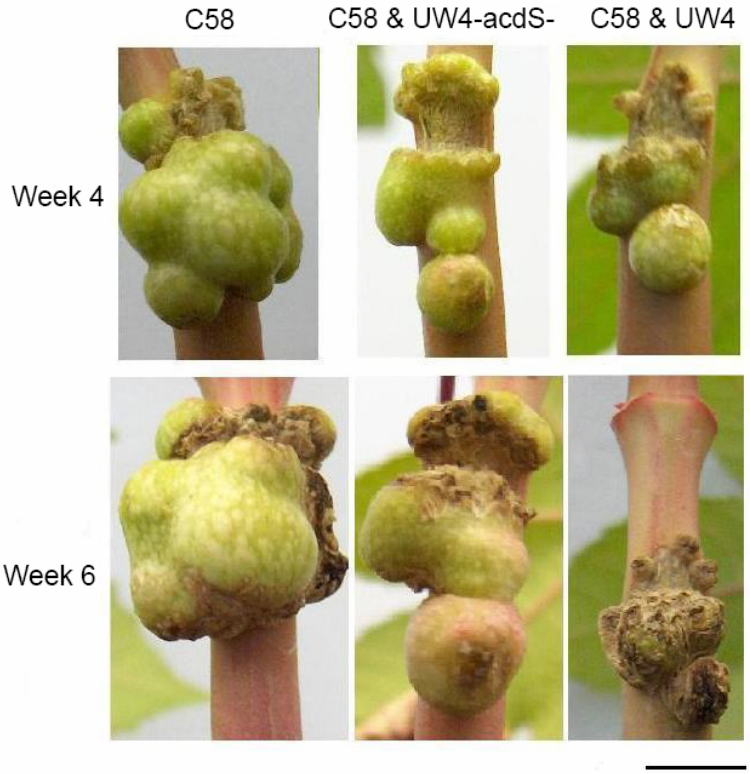


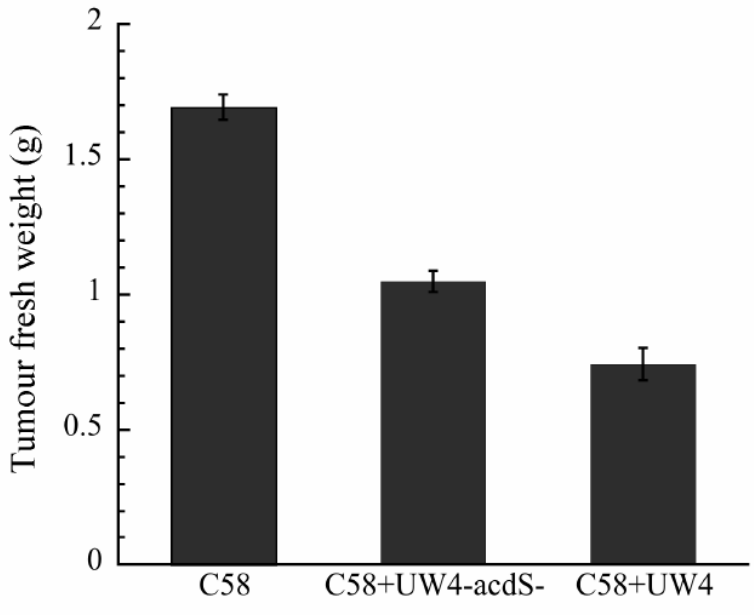
Figure 3.11. Six week old castor bean tumors induced by *A. tumefaciens* C58 (first row) or *A. tumefaciens* C58(pRKLACC) (second row). Each treatment includes three repeats (from left to right). The bar at the bottom represents 1 cm.

Figure 3.12. Co-inoculation of *A. tumefaciens* C58 with *P. putida* UW4 or UW4-*acdS*⁻. A, typical four week (upper row) and six week (lower row) old castor bean tumors resulting from inoculation with *A. tumefaciens* C58 alone (left column), or co-inoculation of *A. tumefaciens* C58 with a 1:1 ratio of either *P. putida* UW4-*acdS*⁻ (middle column) or *P. putida* UW4 (right column). The bar at the bottom represents 1 cm. B, fresh weight of six week old castor bean plant stem tumors resulting from the corresponding treatment indicated. The results were obtained from 3 replicates for each treatment.

A


C58
C58 & UW4-acdS-
C58 & UW4
Week 4
Week 6

B


Tumour fresh weight (g)
2
1.5
1
0.5
0
C58
C58+UW4-acdS-
C58+UW4

3.3.2 An ACC deaminase containing *A. tumefaciens* strain D3 shows plant growth promoting activity and biocontrol activity to crown gall disease

3.3.2.1 Characterization of the ACC deaminase activity of the wild type *A. tumefaciens* D3 strain.

Although Trott *et.al*. (2001) reported that *A. tumefaciens* D3 strain contains a putative ACC deaminase gene and the DNA sequence analysis revealed that it shows 66.9% amino acid sequence identity to the ACC deaminase protein of *Pseudomonas. sp.* strain ACP (Q00740) (Trott *et. al.* 2001), no ACC deaminase activity assay was performed. To test whether the putative *acdS* gene encodes an active ACC deaminase enzyme, and to characterize the activity, an in vitro ACC deaminase activity assay was performed as described in the Materials and Methods. It was found that *A. tumefaciens* D3 displays ACC deaminase activity that is about four times that of *R. leguminosarum* bv. viciae 128Sm, but only about $1/5^{th}$ of the activity found in strain *P. putida* UW4 (Figure 3.13).

3.3.2.2 Construction and characterization of *lrpL* and *acdS* knockout mutant.

Similar to what has been observed in *P. putida* UW4 (Grichko and Glick, 2000; Li and Glick, 2001) and *R. leguminosarum* bv. viciae 128Sm (Ma *et al.*, 2003a) and many other species (Duan *et al*, 2009; Prigent-Combaret *et al.*, 2008), the *lrpL* and *acdS* genes are located adjacent to each other and oriented in the opposite direction. However, unlike what has been found in many other species, the *lrpL* gene is located downstream of the *acdS* gene. The C-terminal fragments of both of the *lrpL* and *acdS* genes were replaced

with a tetracycline resistance gene to construct the *acdS* and *lrpL* double mutant strain *A. tumefaciens* D3-1 as described in the Materials and Methods. Replacement of the wild type *lrpL* and *acdS* genes in the mutant was confirmed by PCR using primers D3-F and D3-R (Figure 3.14). As expected, when using the wild type D3 genomic DNA as the template, a 1.5 kb fragment containing the wild type *acdS* and *lrpL* genes was amplified, while when using the genomic DNA of the mutant strain D3-1 as the template, a PCR fragment of 2.2 kb which encodes the N terminal fragments of the *acdS* and *lrpL* genes and the inserted *tetA* gene was obtained. An ACC deaminase activity assay confirmed that the mutant strain lost the ability to degrade ACC (Figure 3.13). Since it had been previously found that mutation of the *acdS* gene in *Burkholderia phytofirmans* PsJN affects the siderophore and IAA production of the bacterium (Sun *et al*, 2009), the wild type strain *A. tumefaciens* D3 and the mutant strain *A. tumefaciens* D3-1 were analyzed for their ability to produce siderophores and IAA. Unlike in *B. phytofirmans* PsJN, it was found that there is no difference between the wild type and mutant strains of *A. tumefaciens* D3 in terms of both siderophore and IAA production (Figure 3.15 and Figure 3.16). However, when the QS signal acyl homoserine lactones (AHLs) were extracted from the culture supernatants and analyzed using thin layer chromatography (TLC), it was found that the wild type D3 strain produced and secreted a major AHL that shows a migration rate and shape that is similar to the compound C6 HSL, while the mutant strain D3-1 is severely reduced in its ability to produce this compound (Figure 3.17 lane D3 and D3-1).

3.3.2.3 Complementation of the D3-1 mutant.

To complement the *lrpL* and/or *acdS* mutations of *A. tumefaciens* D3-1, pYH4, pYH5 and pYH6 were constructed by inserting the *lrpL* gene from *A. tumefaciens* D3 and its upstream promoter (pYH4), the *acdS* gene from *A. tumefaciens* D3 and its upstream promoter (pYH5), or both the *lrpL* and *acdS* genes and their upstream promoters (pYH6) into the EcoRV site of the broad host vector pBBR1MCS-2. Insertions of these fragments were confirmed by restriction digestion using restricition enzymes EcoRI and HindIII (Figure 3.18 A). As expected, digestion of pYH4 gave a fragment of about 860 bp, an insert of about 1.5 kb was excised from pYH5, and digestion of pYH6 showed an insert of about 2.1 kb. The clones were sequenced to confirm that no mutation was introduced. The plasmids were introduced into to *A. tumefaciens* D3-1 by conjugation to obtain *A. tumefaciens* D3-2 (containing *lrpL* in pYH4), *A. tumefaciens* D3-3 (containing *acdS* in pYH5) and *A. tumefaciens* D3-4 (containing *lrpL* and *acdS* in pYH6). By performing minipreps and agarose gel electrophoresis, the presence of the corresponding plasmids in those strains was confirmed (Figure 3.18 B). When inoculated in M9 minimal medium with ACC as the sole nitrogen source, it was found that only the wild type strain *A. tumefaciens* D3 and strain *A. tumefaciens* D3-4 in which both the *lrpL* and *acdS* genes were complemented were able to show obvious growth (Figure 3.19 A). An ACC deaminase activity assay revealed that *A. tumefaciens* D3-4 has a level of ACC deaminase activity that is about twice that of the wild type *A. tumefaciens* D3 strain; strain *A. tumefaciens* D3-3 in which the *acdS* and its promoter were provided in plasmid pYH5 showed a level of ACC deaminase activity that is about $1/5^{th}$ of that found in the wild type *A. tumefaciens* D3 strain; while *A. tumefaciens* D3-1 and *A. tumefaciens* D3-2 showed no detectable activity (Figure 3.19 B). This result is consistent with the notion

that similar to what has been demonstrated in *P. putida* UW4 (Grichko and Glick, 2000; Li and Glick, 2001) and *R. leguminosarum* (Ma *et al*., 2003 a), the *lrpL* gene is the regulator of the *acdS* gene in *A. tumefaciens* D3.

In order to study whether the complementation of the *lrpL* and/or *acdS* genes would be able to restore the QS signal production ability of *A. tumefaciens* D3-1, the culture supernatants of *A. tumefaciens* D3-2, D3-3 and D3-4 were extracted twice with equal volumes of ethyl acetate, and the extractions were analyzed using TLC. It was found that all three strains were able to produce an active signal that formed a tailed spot that migrated on a TLC plate in a manner that was similar to 3-O-C8 HSL (Figure 3.17).

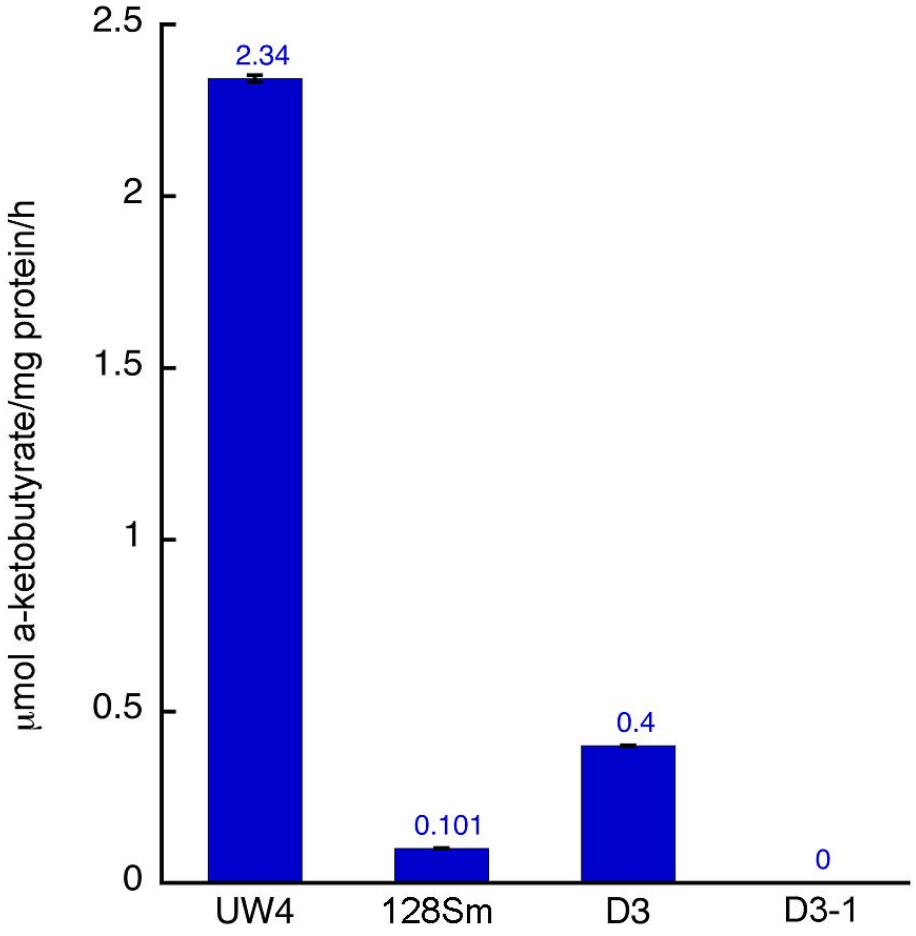


Figure 3.13. ACC deaminase activity assay: UW4, *Pseudomonas putida* UW4; 128Sm, *Rhizobium leguminosarum* bv. viciae 128Sm; D3, *A. tumefaciens* D3; D3-1, *lrpL* and *acdS* double mutant strain *A. tumefaciens* D3-1. Bars were standard errors from 3 replicates.

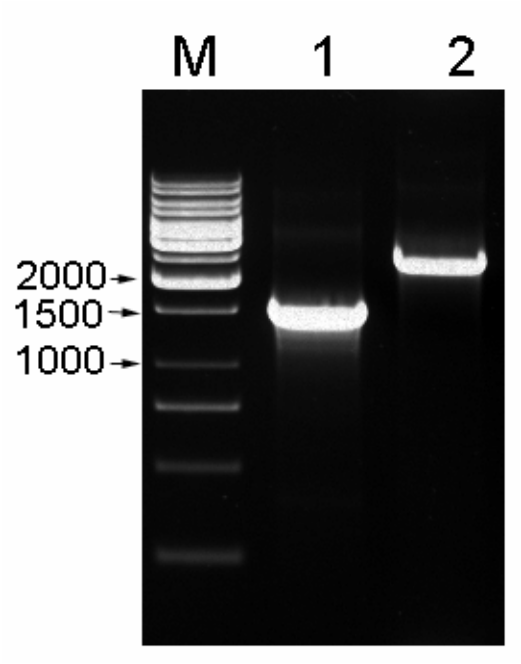


Figure 3.14. PCR confirmation of the construction of the *A. tumefaciens* D3-1 mutant strain. M, molecular weight marker (GeneRuler 1kb DNA Ladder, Fermentas, Burlington, ON). Lane 1, PCR product using the total DNA of wild type *A. tumefaciens* D3 as template. Lane 2, PCR product using the total DNA of *A. tumefaciens* D3-1 as template.

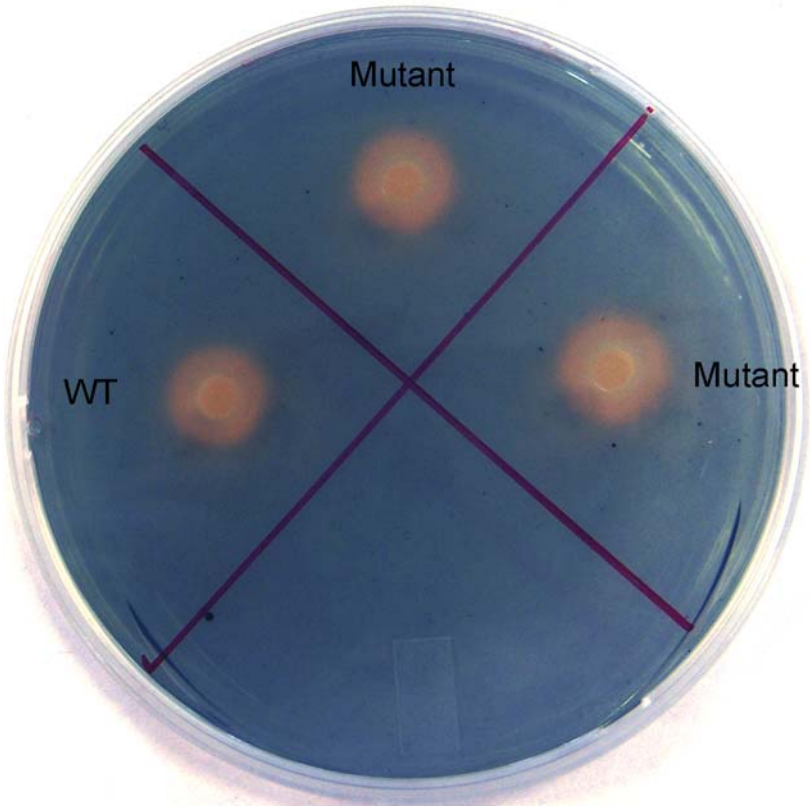
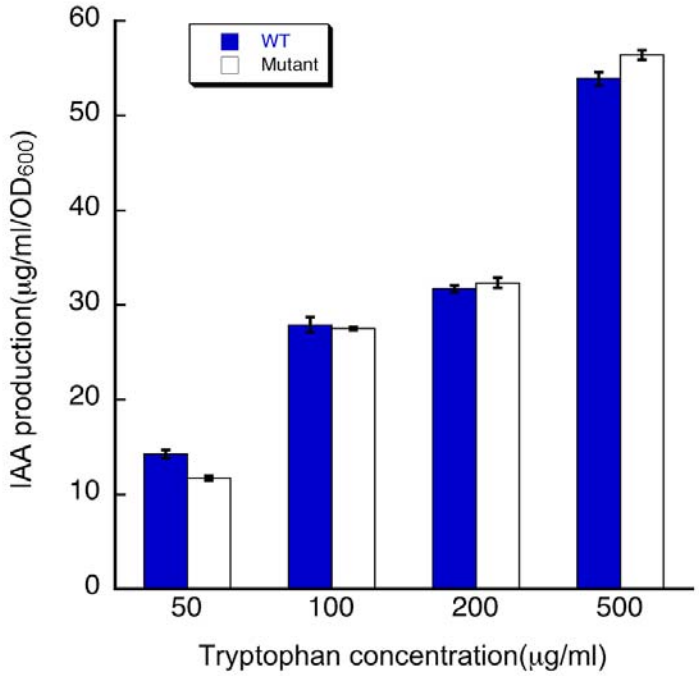


Figure 3.15. Siderophore production assay of the wild type *A. tumefaciens* D3 strain and the mutant D3-1 strain. All the orange halo diameters were estimated to be ~1.3-1.4 cm, and the colony diameters were ~0.5 cm.



Tryptophan concentration (μg/ml)	IAA production ((μg/ml/OD600 unit)	
	Wild type (D3)	Mutant (D3-1)
50	14.23 ± 0.44	11.72 ± 0.24
100	27.85 ± 0.8	27.5 ± 0.16
200	31.69 ± 0.36	32.34 ± 0.53
500	53.89 ± 0.7	56.39 ± 0.51

Figure 3.16. IAA production of the wild type *A. tumefaciens* D3 (solid bars) and *acdS* and *lrpL* mutant D3-1 strain (empty bars) when supplied with different concentrations of tryptophan. Bars represent standard errors obtained from 3 replicates.

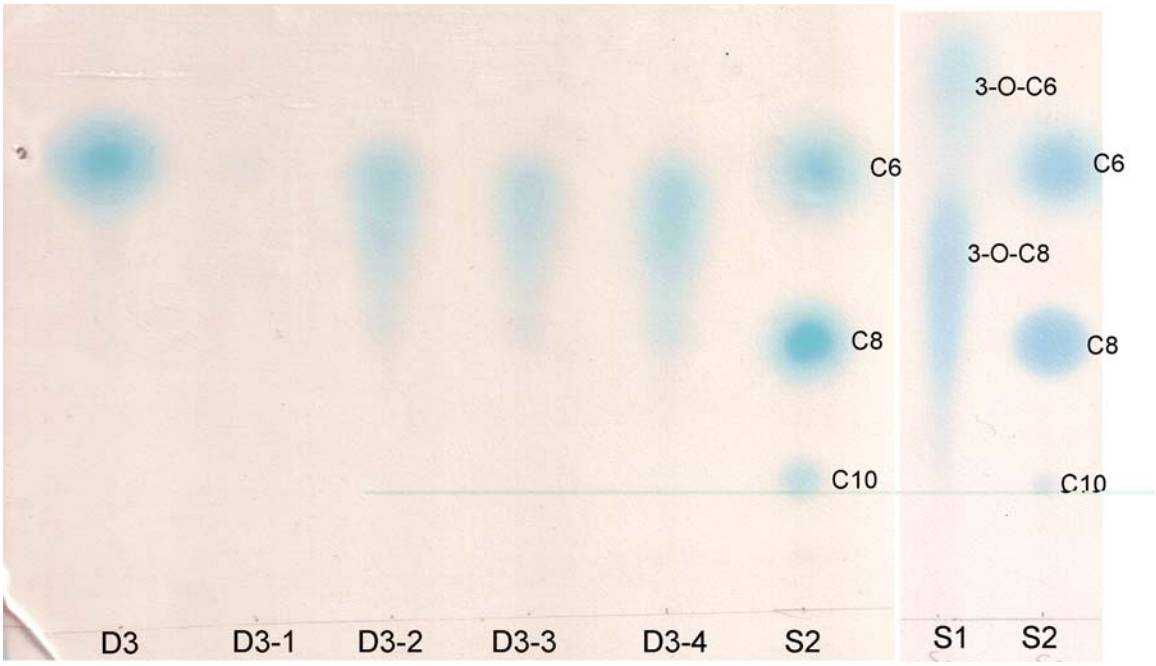


Figure 3.17. Analytical TLC assay of QS signals produced by *A. tumefaciens* strain D3 and D3-1. S1, synthetic standards 3-O-C6 HSL and 3-O-C8 HSL. S2, synthetic standards C6 HSL, C8 HSL, and C10 HSL. D3, D3-1, D3-2, D3-3 and D3-4: AHLs extracted from 5 ml of *A. tumefaciens* D3, D3-1, D3-2, D3-3 or D3-4 stationary phase culture supernatant

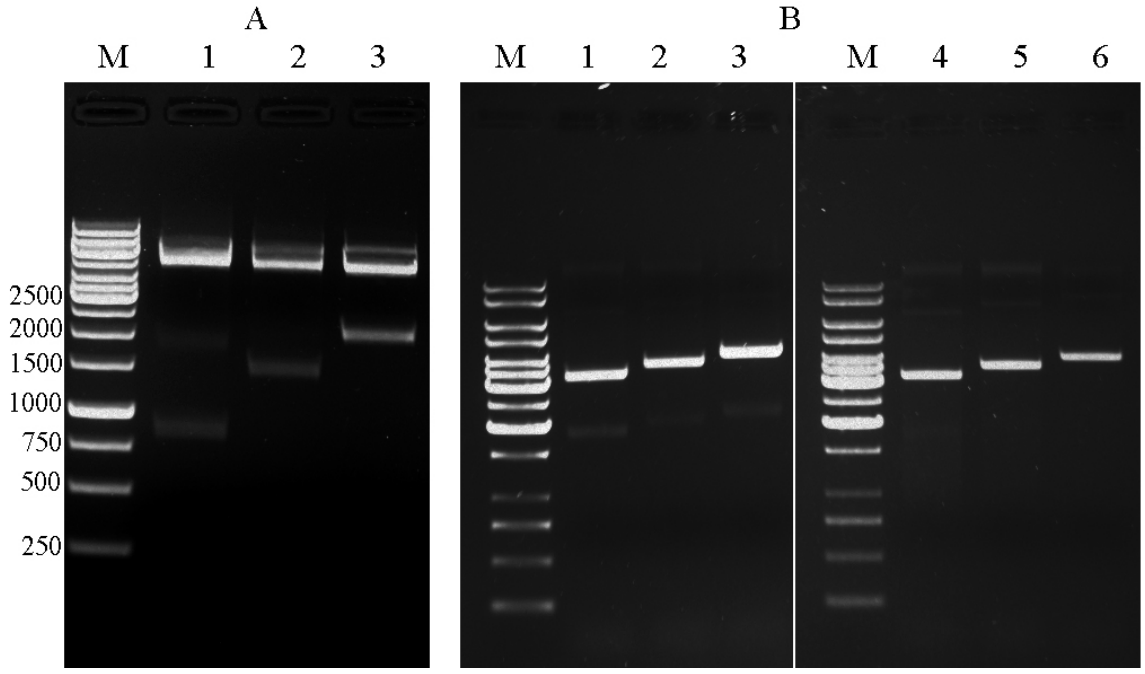


Figure 3.18. Construction of *A. tumefaciens* strains D3-2, D3-3 and D3-4.

A. EcoRI and HindIII digestions of pYH4, pYH5 and pYH6. Lane M, molecular weight marker (GeneRuler 1kp DNA Ladder, Fermentas, Burlington, ON); Lane 1, pYH4; land 2, pYH5; and lane 3, pYH6.

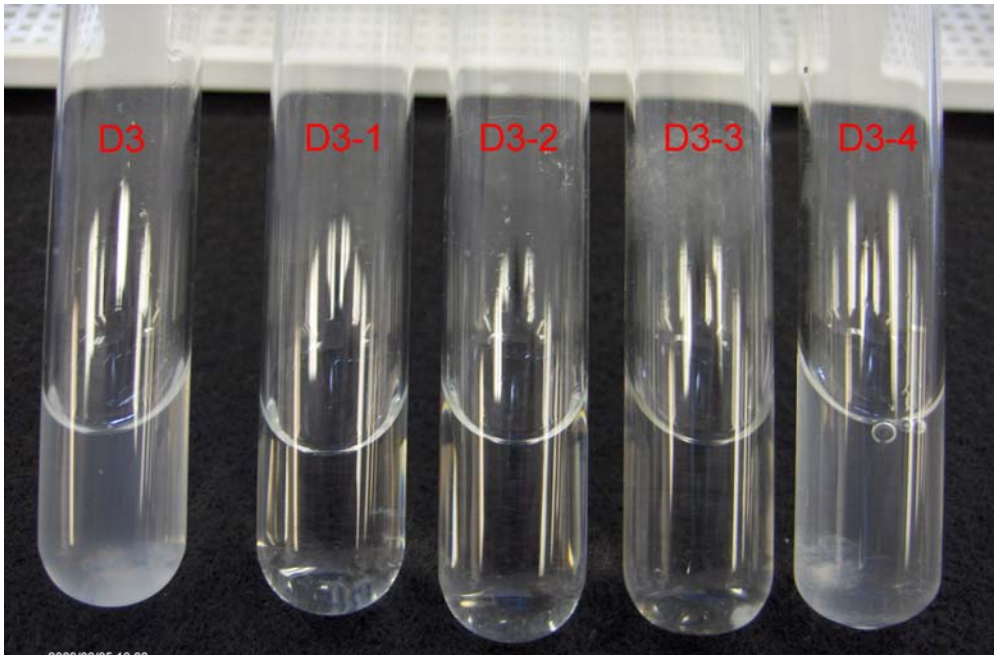
B, Minipreps from *A. tumefaciens* D3-2, D3-3 and D3-4. Lane M, molecular weight marker (GeneRuler 1kp DNA Ladder, Fermentas, Burlington, ON); lane 1-3, control for plasmids pYH4 (lane 1), pYH5 (lane 2) and pYH6; lane 4 (lane 3); lane 4-6, plasmids exacted from *A. tumefaciens* D3-2 (lane 4), D3-3 (lane 5) and D3-4 (lane 6).

Figure 3.19. ACC deaminase activity assay of strains *A. tumefaciens* D3, D3-1, D3-2, D3-3 and D3-4.

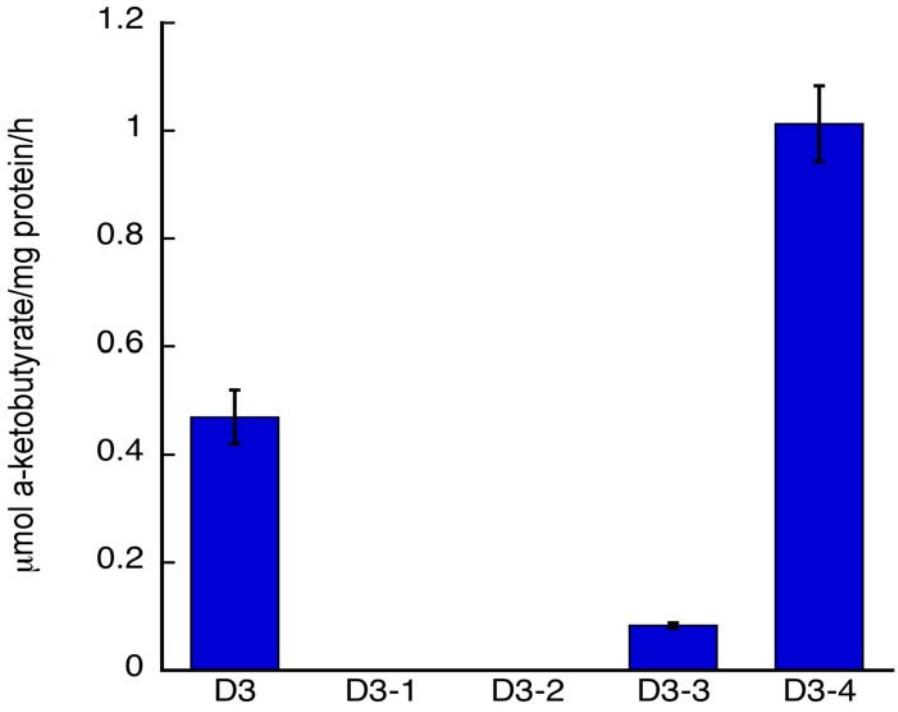
A. Growth of *A. tumefaciens* D3, D3-1, D3-2, D3-3 and D3-4 in M9 minimal medium with ACC as the sole nitrogen source.

B. ACC deaminase activity of *A. tumefaciens* D3, D3-1, D3-2, D3-3 and D3-4. Bars are standard errors from 3 replicates.

A. D3 D3-1 D3-2 D3-3 D3-4



B.



3.3.2.4 Gnotobiotic root elongation assay

A gnotobiotic root elongation assay was performed for the wild type *A. tumefaciens* D3 strain and the *lrpL* and *acdS* double mutant strain *A. tumefaciens* D3-1. *P. putida* UW4 was used as a positive control. Canola seeds were sterilized and treated with suspensions of *P. putida* UW4, *A. tumefaciens* D3, *A. tumefaciens* D3-1 or 0.03 M $MgSO_4$ buffer (as negative control) before transfer to sterile growth pouches and incubation in a growth chamber at 20 ±1°C as described in Sec 3.2.12. The root lengths of the canola seedlings were measured one week later. It was found that the average root length treated with *A. tumefaciens* D3-1 is similar to that treated with $MgSO_4$ buffer (average length is 6.09 and 6.12 cm respectively), while the average root length treated with wild type *A. tumefaciens* D3 strain is 7.07 cm, and the average root length treated with *P. putida* UW4 is 8.13 cm, both of which are significantly longer than the previous two treatments (student t test, $p<0.001$) (Figure 3.21). This result indicates that wild type *A. tumefaciens* D3 promotes canola (*Brasica napus* cv. Thunder) root elongation to a lesser degree than *P. putida* UW4, while the *acdS* mutant strain *A. tumefaciens* D3-1 shows no root elongation promotion activity.

Figure 3.20. Gnotobiotic root elongation assay.

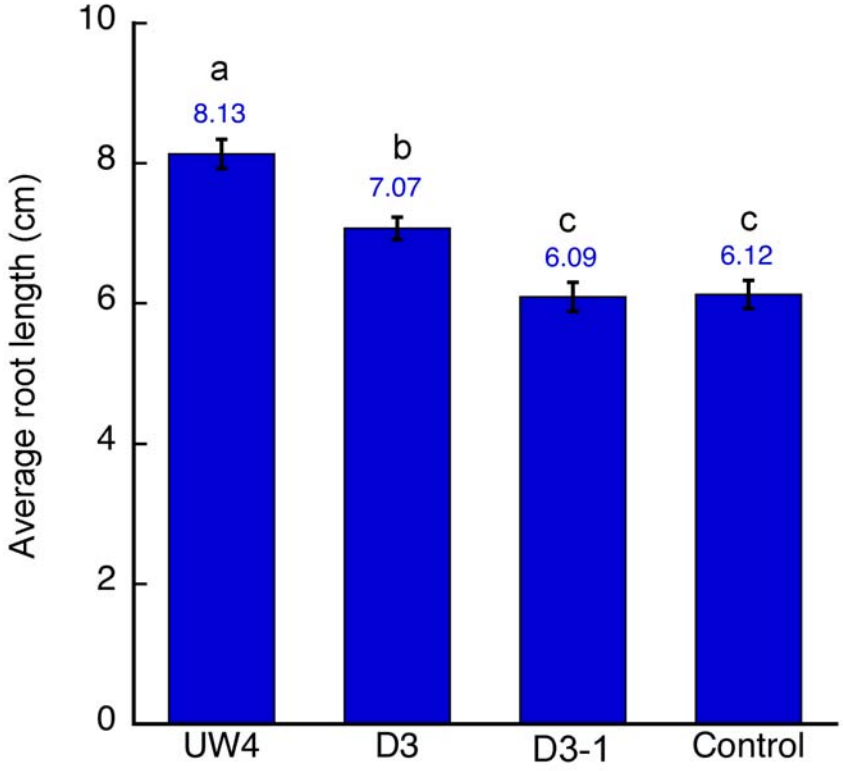
A. One-week-old canola seedlings germinated from seeds treated with *P. putida* UW4, *A. tumefaciens* D3, *A. tumefaciens* D3-1 or MgSO4 buffer.

B. Average root length of one-week-old canola after treatment with *P. putida* UW4, *A. tumefaciens* D3, *A. tumefaciens* D3-1 or MgSO4 buffer. Error bars represent standard errors obtained from about 100 samples. The bars labelled with different letters were significantly different from one another (student t-test, $p<0.001$).

A


UW4
D3
D3-1
MgSO4

B


Average root length (cm)
10
8
6
4
2
0
a
8.13
b
7.07
c
6.09
c
6.12
UW4
D3
D3-1
Control

3.3.2.5 PCR detection of the presence of a Ti or Ri plasmid and virulence assay

The *A. tumefaciens* strain D3 was taxonomically classified and named according to the API and BIOLOG tests (Layh *et al.*, 1997); however, there are no reports regarding the virulence of this strain. To test its virulence, firstly, two pairs of primers that were designed for detection of tumorigenic or rhizogenic *Agrobacterium* and have been proven to be both sensitive and specific were used to detect whether *A. tumefaciens* D3 contains a Ti or Ri plasmid. Primer pair 1 was designed from the conserved region of *tms2* gene (Pulawska and Sobiczewski, 2005): tms2F1, tttcagctgctagggccacat ag; and tms2R2, tcg ccatggaaacgccggagtagg. Primer pair 2 was designed according to the conserved region of the *virC* operon (Sawada *et al.*, 1995):VCF, atcatttgtagcgact; and VCR, agctcaaagctgcttc. The PCR results using primer pair 1 showed that there was no product that was amplified from the genomic DNA of *A. tumefaciens* D3, while for the virulent strain C58, there was a specific band at a size of about 800 bp. Using primer pair 2, *A. tumefaciens* C58 showed a specific PCR product at the size of about 750 bp, while for strain D3, no specific PCR product was detected (Figure 3.21).

Secondly, a virulence assay was performed using carrot discs, tomato (*Lycopersicon esculentum,* cv. Beefsteak) stems, and castor bean (*Ricinus comunis Zanzibariensis*) stems. It was found that *A. tumefaciens* D3 does not induce tumors or hairy roots on any of these plants (Figure 3.22). A virulence assay was also performed using tobacco (*Nicotiana tabacum* cv. Xanthi) stems, canola (*Brassica napus* cv. Westar) stems and *Arabidopsis* (*Arabidposis thaliana*, cv. Columbia) stems; similarly none of these plants showed any tumors or hairy roots (data not shown).

3.3.2.6 Biocontrol assay

To study whether *A. tumefaciens* D3 can inhibit the crown gall disease caused by *A. tumefaciens* C58, a biocontrol assay was performed using castor bean plants as described in the Materials and Methods (Sec 2.2.7). For comparison, *P. putida* UW4 was also co-inoculated with *A. tumefaciens* C58. Four weeks after inoculation, the tumor sizes were examined (Figure 3.23). Consistent with the results presented earlier (Sec 3.3.1.4), co-inoculation of *P. putida* UW4 with *A. tumefaciens* C58 significantly inhibited the tumor development (Figure 3.23 row 2 and row 3). Inoculation using a 2:1 (UW4:C58) cell number ratio provides better inhibition of the crown gall disease than using a 1:1 (UW4:C58) cell number ratio. The results also showed that, in comparison to *P. putdia* UW4, although *A. tumefaciens* D3 has a lower level of ACC deaminase activity, it exhibits a better biocontrol activity towards *A. tumefaciens* C58 induced crown gall disease. The tumor was almost completely inhibited when co-inoculated using either a 1:1 (D3:C58) or a 2:1 (D3:C58) cell number ratio (Figure 3.23 row 4 and 5).

To investigate if ACC deaminase is critical for the biocontrol activity, in a second experiment, the *lrpL* and *acdS* mutant strain *A. tumefaciens* D3-1 was also co-inoculated with *A. tumefaciens* C58 using a 1:1 cell number ratio on wounded castor bean plant stems. It was found that although the wild type D3 strain shows a slightly better biocontrol activity than the mutant strain D3-1, in these experiments the difference is not significant (Figure 3.24).

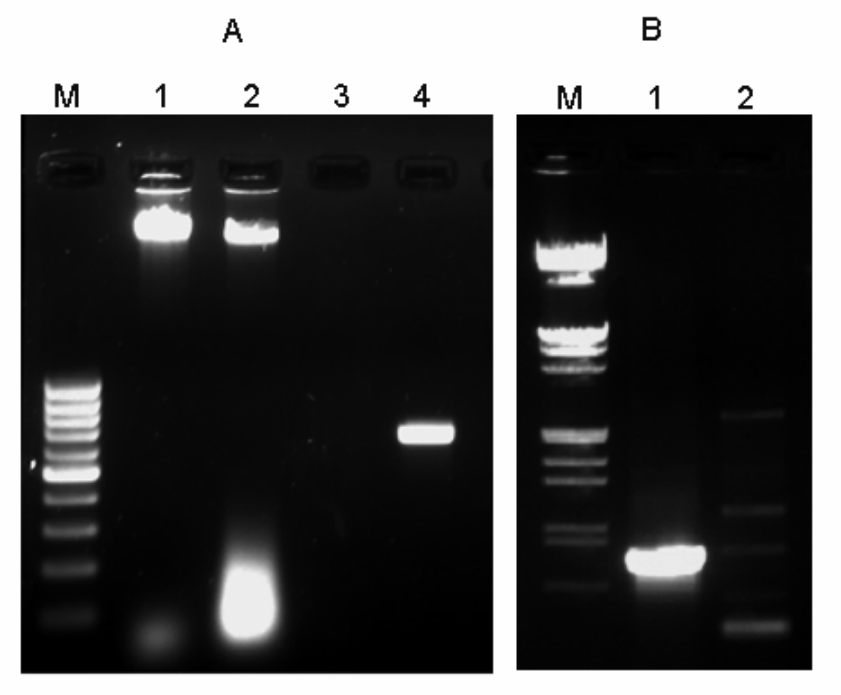


Figure 3.21. PCR detection of the presence of a Ti or Ri plasmid in *A. tumefaciens* D3.

A. PCR results using primer pair 1: Lane M, molecular weight markers (GeneRuler 100 bp DNA Ladder, Fermentas, Burlington, ON); lane 1, genomic DNA of *A. tumefaciens* D3; lane 2, genomic DNA of *A. tumefaciens* C58; lane 3, PCR product for *A. tumefaciens* D3; lane 4, PCR result for *A. tumefaciens* C58.

B. PCR results using primer pair 2: Lane M, molecular weight markers (λ DNA/EcoRI+HindIII, Fermentas, Burlington, ON); lane 1, PCR product for *A. tumefaciens* C58; lane 2. PCR result for *A. tumefaciens* D3.

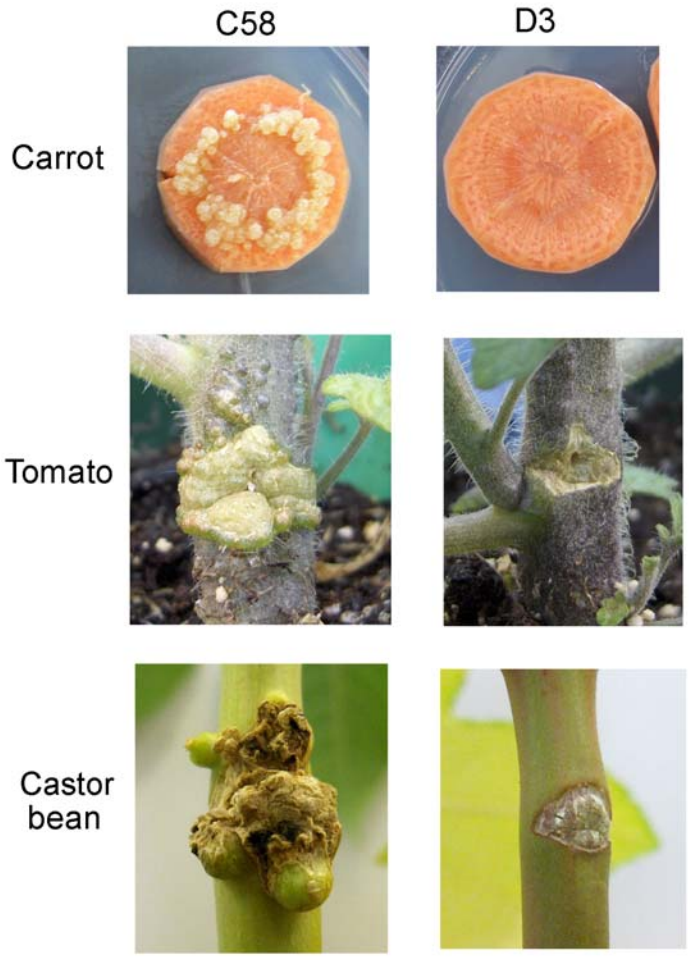
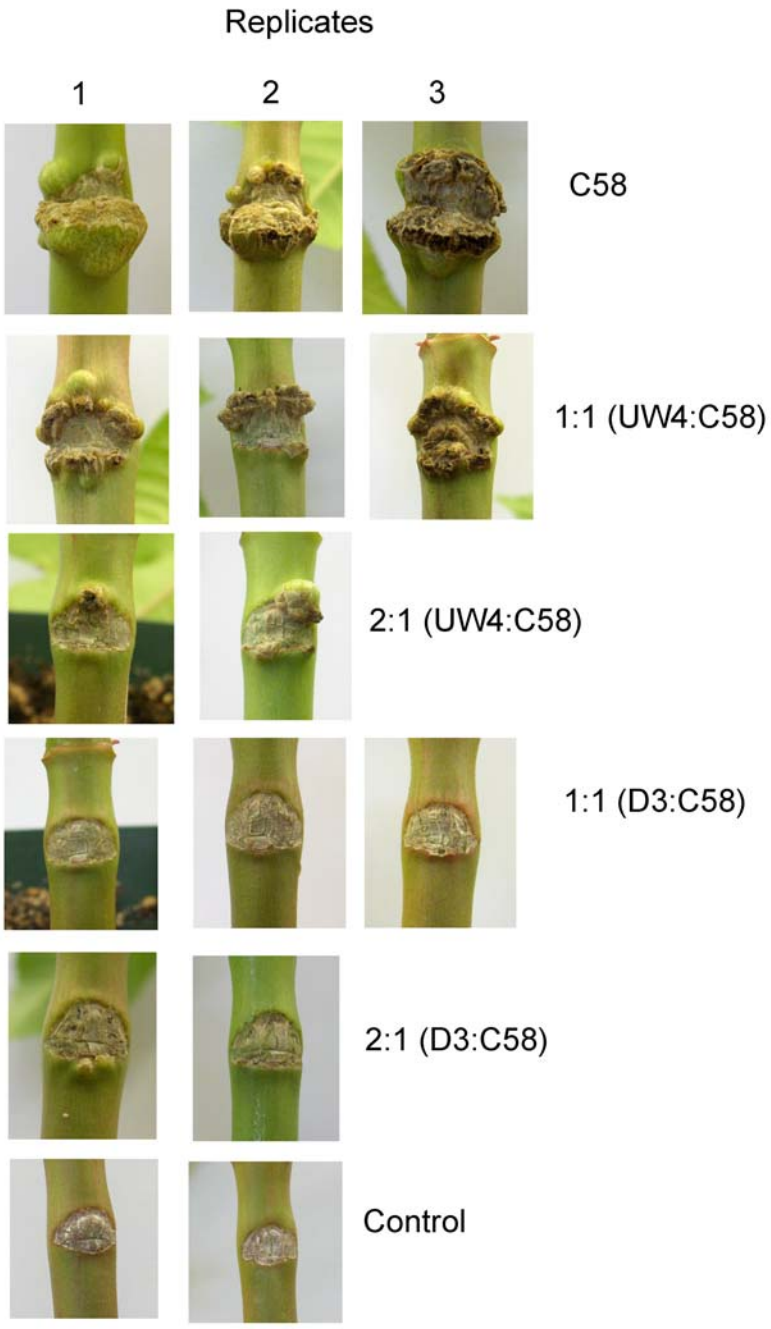


Figure 3.22. Virulence assay of *A. tumefaciens* D3. Row 1, carrot discs three weeks after inoculation with *A. tumefaciens* C58 (left) or *A. tumefaciens* D3 (right). Row 2, tomato stems three weeks after inoculation with *A. tumefaciens* C58 (left) or *A. tumefaciens* D3 (right). Row 3, castor bean stems four weeks after inoculation with *A. tumefaciens* C58 (left) or *A. tumefaciens* D3 (right).

Figure 3.23. Biocontrol activity assay of *A. tumefaciens* D3 using castor bean plant stems. The castor bean plant stems were V-shape wounded and were inoculated with 10 µl of *A. tumefaciens* C58 culture suspension alone (row1), or with a mixture of *A. tumefaciens* C58 and *P. putida* UW4 (row 2 and row 3), or a mixture of *A. tumefaciens* C58 and *A. tumefaciens* D3 (row 4 and row 5), using a 1:1 ratio (10 µl of each culture suspension) or 2:1 ratio (20 µl of UW4/D3 and 10 µl of C58 culture suspension). The control plants were inoculated with LB medium. The pictures were taken four weeks after inoculation.


Replicates
1
2
3
C58
1:1 (UW4:C58)
2:1 (UW4:C58)
1:1 (D3:C58)
2:1 (D3:C58)
Control

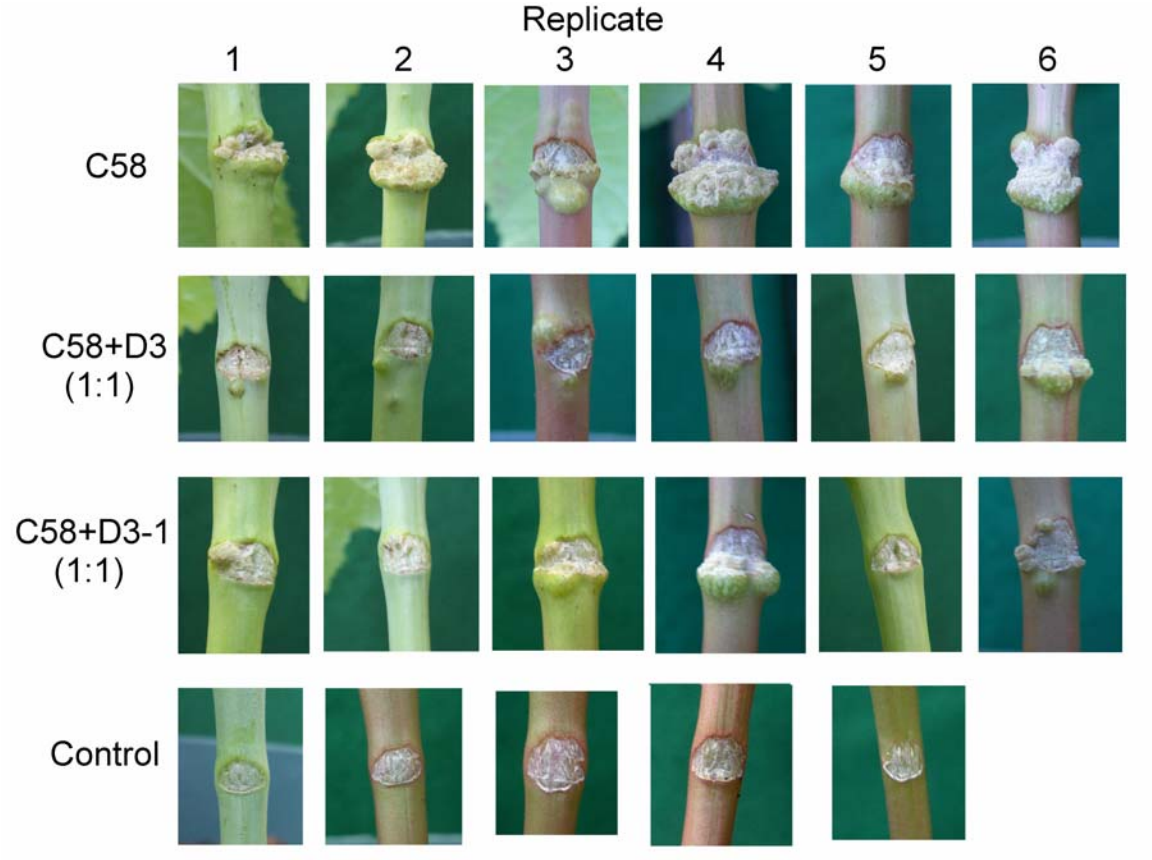


Figure 3.24. Biocontrol activity assay of *A. tumefaciens* D3 and D3-1 strains.

Row 1, inoculated with 10 µl of *A. tumefaciens* C58 culture suspension alone.

Row 2, co-inoculation of *A. tumefaciens* C58 and *A. tumefaciens* D3 using a 1:1 cell number ratio (10 µl of each culture suspension).

Row 3, co-inoculation of *A. tumefaciens* C58 and *A. tumefaciens* D3-1 using a 1:1 cell number ratio (10 µl of each culture suspension).

Row 4, control that is inoculated with fresh LB medium.

Plants were grown in a greenhouse. The pictures were taken four weeks after inoculation.

3.3.3 ACC deaminase improves *A. tumefaciens* mediated transformation efficiency to canola

3.3.3.1 Construction and characterization of *A. tumefaciens* strains.

A. tumefaciens strains GV3101::pMP90(pPZP-eGFP), YH-1 and YH-2 were constructed as described in the Materials and Methods. The presence of the plasmids was confirmed by performing plasmid minipreps and agarose gel electrophoresis (Figure 3.25 A). When inoculated in M9 minimal medium with ACC as the sole nitrogen source, strain *A. tumefaciens* YH-2, which contains an ACC deaminase gene, was able to grow, while the *Agrobacterium* strain with the empty plasmid pRK415 (YH-1) could not grow (Figure 3.25, B), which indicated that the ACC deaminase gene was expressed and active in *A. tumefaciens* YH-2. During an in vitro ACC deaminase activity assay, the *A. tumefaciens* strain YH-2 showed an activity of about 2.5 μmol α-ketobutyrate/mg protein/hour, while the strains GV3101::pMP90(pPZP-eGFP) and YH-1 showed no detectable activity (Figure 3.25, C).

3.3.3.2 Transformation efficiency assay

The canola hypocotyl segments were transformed and regenerated as described in the Materials and Methods. Figure 3.26 shows the different stages of the transformation and regeneration process. When observed using a fluorescent microscope, the transgenic calli and shoots were able to produce faint green fluorescence (data not shown). Since the untransformed control calli and shoots were also able to produce some background fluorescence, the genomic DNA was extracted from the regenerated plants and PCR was

performed using primers specific for the *eGFP* gene to confirm its presence in the transgenic plants. It was found that all of the 11 randomly selected regenerated plants contain the *eGFP* gene (Figure 3.27). Transformation frequencies were determined using the following formula: Transformation frequency = the number of transgenic plants obtained / the number of hypocotyl segments used for transformation.

Table 3.2 shows the transformation frequencies obtained for the three canola cultivars using different dilutions of *A. tumefaciens* YH-1 orYH-2. Of the three dilutions used, when using organogenesis medium A (OA), for both strains YH-1 and YH-2, the cultivars Westar and 4414 RR showed the highest transformation frequency when using 1x dilution (OD_{600} = 1 culture suspension), while for Hyola 401, the optimal condition was 0.1x dilution (OD_{600} = 0.1 culture suspension). The presence of the ACC deaminase gene in strain YH-2 significantly increased the transformation frequency of all three cultivars when optimal dilutions were used. For the Westar cultivar, using *A. tumefaciens* YH-1, the highest transformation frequency obtained was 8.66%, while the *A. tumefaciens* strain YH-2 gave a peak transformation frequency of 13.16%. For the canola cultivar 4414RR, the presence of ACC deaminase increased the transformation frequency from 8.64% to 12.35%. For the cultivar Holya 401, when using a 0.1x dilution, the *A. tumefaciens* YH-1 gave a transformation frequency of 2.92%, while *A. tumefaciens* YH-2 gave a transformation frequency of 6.5%.

It is well known that omitting the ethylene inhibitor $AgNO_3$ from the organogenesis medium severely inhibits plant regeneration and thus the transformation efficiency (Eapen and George, 1997). To study whether the introduction of an *acdS* gene can replace the role of $AgNO_3$, the transformation efficiency was also compared for the

two *A. tumefaciens* strains YH-1 and YH-2 using organogenesis medium B (OB) which does not contain $AgNO_3$. Similar to the results obtained using OA medium (organogenesis medium with $AgNO_3$), the presence of ACC deaminase in *A. tumefaciens* YH-2 increases the transformation efficiency. For example, for the cultivar 4414RR, when transformed with a 1x dilution of *A. tumefaciens* YH-1, when OB organogenesis medium was used, of the 111 explants used for transformation, no transgenic plants were obtained, while for *A. tumefaciens* YH-2, which contains an ACC deaminase gene, a transformation frequency of 2.94% was obtained. However, compared to using OA medium, the transformation frequency obtained with both *A. tumefaciens* YH-1 and YH-2 strains were significantly lower, which indicates that the presence of ACC deaminase can only partially replace $AgNO_3$ in inhibiting ethylene levels and promoting transformation frequency.

3.3.3.3 ACC deaminase reduces the ethylene level during the infection and cocultivation process.

To determine whether the presence of an *acdS* gene in *A. tumefaciens* can reduce the ethylene levels produced by the infected plant tissues, the amounts of ethylene evolved from the plant tissues treated with *A. tumefaciens* YH-1, YH-2 or infection medium alone were measured by gas chromatography. One week old canola (cv. 4414RR) seedling hypocotyls were cut into about 1 cm fragments and were treated with OD_{600} = 1 suspension of *A. tumefaciens* YH-1 or YH-2 in infection medium or infection medium alone (uninfected control) for 30 minutes at room temperature (~22°C), and then 50 hypocotyl segments (about 0.4-0.5g) from each treatment were transferred to a 25 ml

sterile glass vial and sealed tightly with a rubber stopper. After 24 hours incubation at 25°C in a growth chamber with dim light, the amounts of ethylene evolved were determined using gas chromotography. It was found that *A. tumefaciens* infection induces ethylene evolution from plant tissues to a level that is more than twice that of the uninfected control (Figure 3.28). Comparing the two strains, *A. tumefaciens* YH-1 and YH-2, it was found that the presence of an *acdS* gene in *A. tumefaciens* YH-2 significantly reduced the amount of ethylene evolved from infected plant tissues (student t test, $p<0.05$) (Figure 3.28).

3.3.3.4 Estimation of *A. tumefaciens* populations

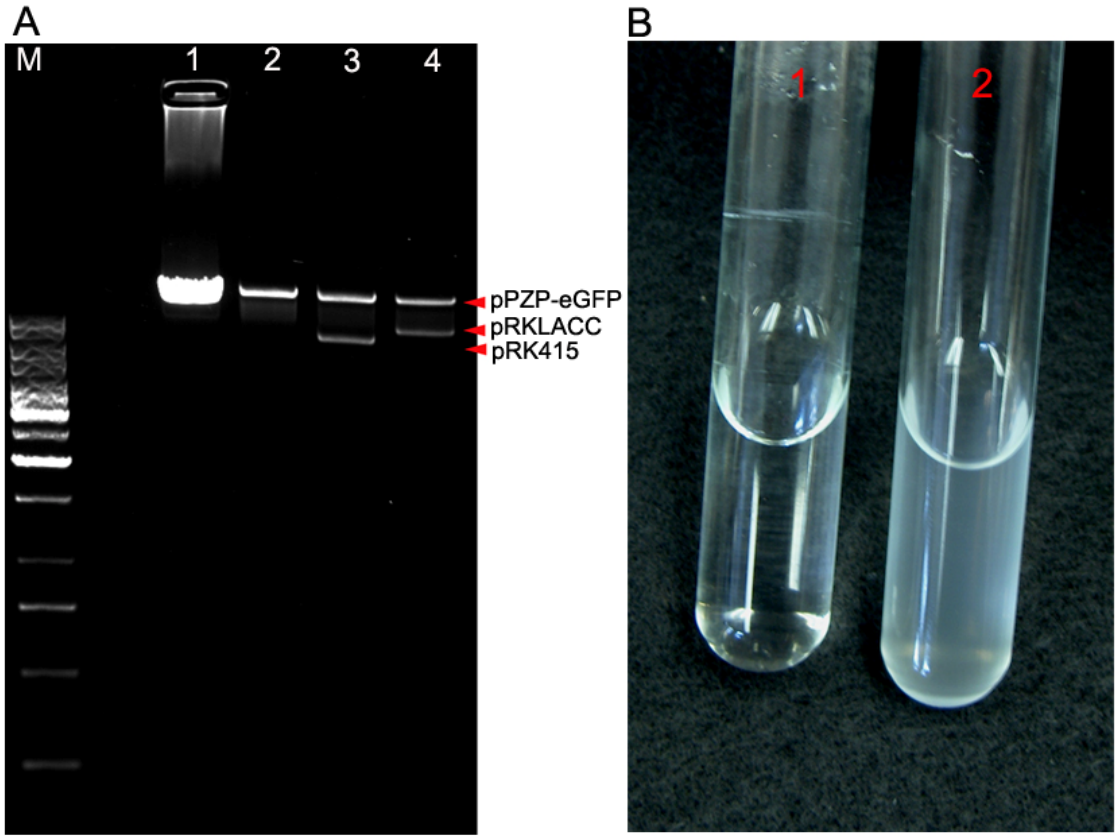
To study whether the presence of an *acdS* gene affected *Agrobacterium* proliferation during the transformation process, bacterial populations in the infected plant tissues were estimated two days after infection. Both of the canola cultivars 4414RR and Hyola 401 tested gave similar results (Table 3.3). It was found that when plants were infected with either an $OD_{600} = 1$ or $OD_{600} = 0.1$ culture suspension (about 5×10^8 or 5×10^7 cell per ml, respectively), after two days of cocultivation on MS with 2,4-D (1 mg/l) medium, both *A. tumefaciens* YH-1 and *A. tumefaciens* YH-2 were able to propagate to a population of about 10^9 CFU/gram fresh weight of plant tissue. When plants were infected with an $OD_{600} = 0.01$ culture suspension, after two days of cocultivation, both *A. tumefaciens* strains were able to propagate to a population of about 10^8 CFU/gram fresh weight of plant tissue. This result indicates, unlike what is observed with crown gall tumors (Sec 3.3.1), that ACC deaminase does not have a significant effect on the growth rate of *A. tumefaciens* during the cocultivation process.

Figure 3.25. Construction and characterization of strains GV3101::pMP90(eGFP), YH-1 and YH-2.

A. Minipreps of plasmids. M, 1kb DNA ladder (Fermentas, Burlington, ON. ; 1, plasmid pPZP-eGFP; 2, plasmid extracted from GV3101::pMP90(pPZP-eGFP); 3, plasmids extracted from *A. tumefaciens* YH-1; 4, plasmids extracted from *A. tumefaciens* YH-2.

B. Growth of *A. tumefaciens* YH-1 (tube 1, no growth, indicated by clearance of the medium) and YH-2 (tube 2, growth indicated by turbidity of the medium) in M9 minimal medium with ACC as the sole nitrogen source.

C. ACC deaminase activity assay of the *A. tumefaciens* strains: 1, *A. tumefaciens* GV3101::pMP90(pPZP-eGFP); 2, *A. tumefaciens* YH-1; 3, *A. tumefaciens* YH-2. Bars indicate standard deviation of two independent assays.


A
M
1
2
3
4
pPZP-eGFP
pRKLACC
pRK415
B
1
2

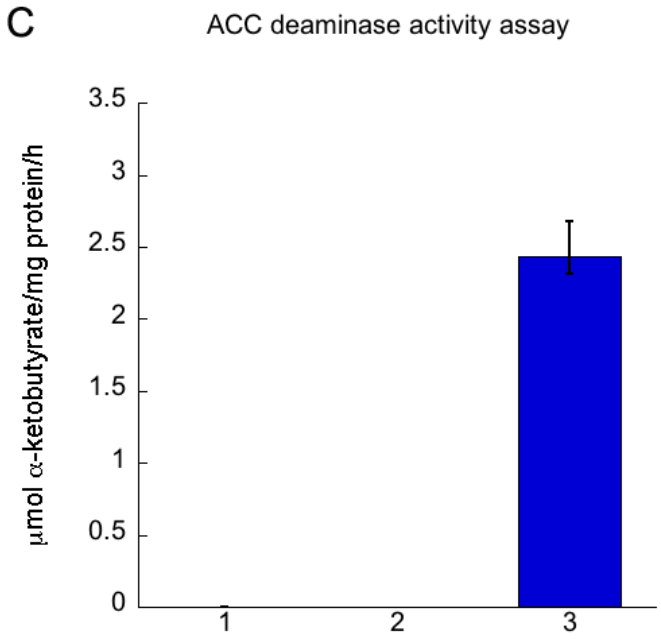

C
ACC deaminase activity assay
μmol α-ketobutyrate/mg protein/h
3.5
3
2.5
2
1.5
1
0.5
0
1
2
3

Figure 3.26. Transformation and regeneration process.

A. Canola seedling hypocotyls were cut into 1 cm fragments and then preconditioned by growth on cocultivation medium.

B. After two weeks, the infected hypocotyl segments were incubated on callus induction medium.

C. After an additional two weeks, hypocotyls were incubated on organogenesis medium.

D. After two weeks further incubation, hypocotyls were transferred to shoot induction medium.

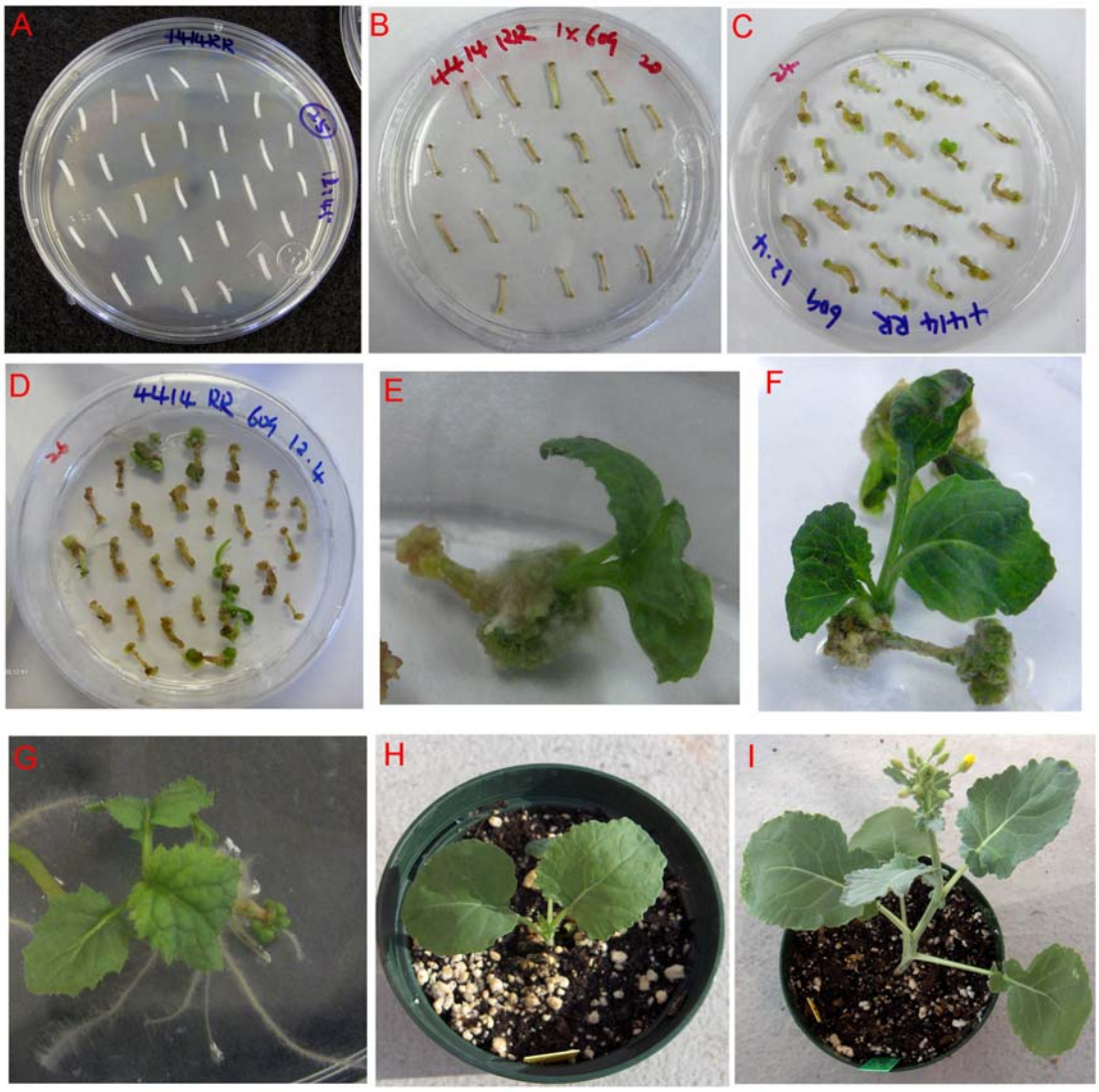
E. One induced shoot.

F. One of the transgenic shoots after two weeks incubation on shoot elongation medium.

G. One rooted transgenic plant (two weeks after transferring to rooting medium).

H. One transgenic plant after transferring to soil for two weeks.

I. One transgenic canola plant after transferring to soil for six weeks.


A
B
C
D
E
F
G
H
I

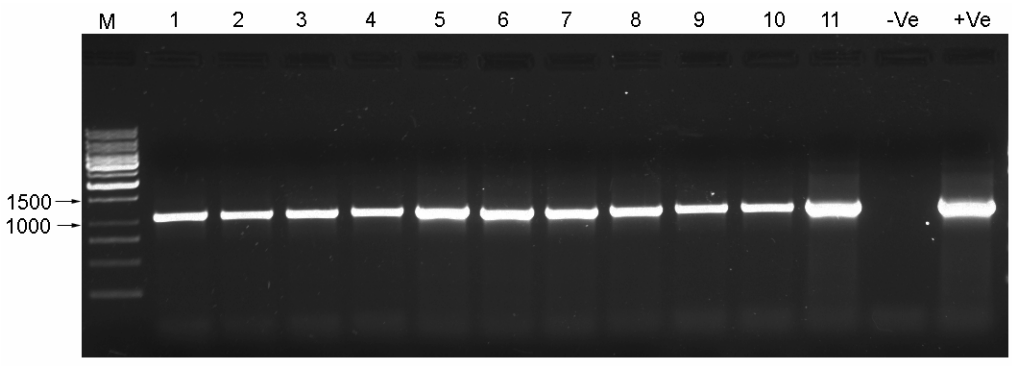
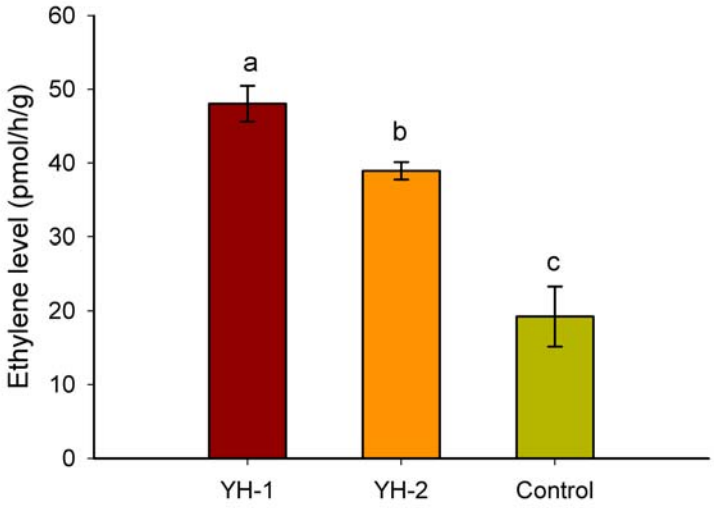


Figure 3.27. PCR confirmation of transgenesis using specific primers to amplify the *eGFP* gene.

M, 1 kb DNA ladder. 1-11, PCR products using the genomic DNA of 11 randomly selected regenerated plants as template DNA. –Ve, PCR product using the genomic DNA of wild type Canola (cv. Westar) as template, serves as a negative control. +Ve, PCR product using plasmid pPZP-eGFP as template DNA, serves as positive control.

Table 3.2. Transformation frequency assay.

Canola	*Agrobacterium* strain and concentration	Organogenesis medium	# of explants tested	# of transgenic plants obtained	Transformation frequency
cv. Westar	1x YH-1	OA	358	31	8.66%
	1x YH-2	OA	342	45	13.16%
	0.1x YH-1	OA	239	6	2.51%
	0.1x YH-2	OA	282	13	4.61%
	0.01x YH-1	OA	209	9	4.31%
	0.01x YH-2	OA	242	9	3.72%
cv. 4414RR	1x YH-1	OA	220	19	8.64%
	1x YH-2	OA	243	30	12.35%
	1x YH-1	OB	111	0	0.00%
	1x YH-2	OB	136	4	2.94%
	0.1x YH-1	OA	109	5	4.59%
	0.1 x YH-2	OA	116	9	7.76%
	0.1x YH-1	OB	128	2	1.56%
	0.1x YH-2	OB	117	4	3.42%
cv. Hyola 401	1x YH-1	OA	184	6	3.26%
	1x YH-2	OA	180	4	2.22%
	0.1x YH-1	OA	171	5	2.92%
	0.1x YH-2	OA	200	13	6.50%
	0.01x YH-1	OA	109	2	1.83%
	0.01x YH-2	OA	122	3	2.46%
	1 xYH-1	OB	104	0	0.00%
	1 xYH-2	OB	109	0	0.00%



A. tumefaciens strain	C_2H_4 (pmol/h/g)
YH-1	48.0480 ± 2.43
YH-2	38.9400 ± 1.18
Control	19.2500 ± 4.08

Figure 3.28. Ethylene evolution levels from canola (cv. 4414RR) hypocotyls following different treatments. YH-1, infection with *A. tumefaciens* strain YH-1 culture suspension (OD_{600} = 1). YH-2, infection with *A. tumefaciens* strain YH-2 culture suspension (OD_{600} = 1). Control, treated with infection medium alone. Error bars represent standard error of the mean from 5 replicates.

Table 3.3. Estimation of *A. tumefaciens* populations.

Canola	*Agrobacterium* strains and concentration	CFU/g fresh weight
cv. 4414RR	1x YH-1	1.21×10^9
	1x YH-2	1.54×10^9
	0.1x YH-1	6.10×10^8
	0.1x YH-2	1.12×10^9
	0.01x YH-1	8.00×10^7
	0.01x YH-2	3.20×10^8
cv. Hyola 401	1x YH-1	1.08×10^9
	1x YH-2	2.02×10^9
	0.1x YH-1	2.11×10^9
	0.1x YH-2	1.41×10^9
	0.01x YH-1	3.00×10^8
	0.01x YH-2	4.90×10^8

3.4 Discussion

3.4.1 Ethylene and *Agrobacterium* growth

It has been reported that the low levels of ethylene initially synthesized by plants when challenged with pathogens may inhibit bacterial growth by triggering the expression of genes involved in the plant defence system such as chitinase, β-1,3-glucanase and pathogen related gene 1 (PR1) (Deikman, 1997; Glick et al., 2007). For example, it has been reported that bacterial growth in the ethylene insensitive *Arabidopsis* mutants *ein2* and *coi1* was increased about 7 to 10 times more than in wild type *Arabidopsis* (Norman-Setterblad *et al*., 2000). Similarly, the growth of the plant pathogen *Xanthomonas campestris* in the highly ethylene sensitive tomato plant mutant LeETR4AS, was inhibited about 10 fold more than in the wild type tomato plants (Ciardi, 2001). However, in conflict with these findings, using the melon cotyledon segments, Nonaka *et al.* reported that inclusion of ACC in the germination and cocultivation medium, increased ethylene evolution by the plant tissue, but did not inhibit *A. tumefaciens* growth (Nonaka *et al.* 2008 b).

In the present study, an ACC deaminase encoding gene from *P. putida* UW4 was introduced into *A. tumefaciens* C58 to construct the strain *A. tumefaciens* C58(pRKLACC) which can help reduce ethylene levels evolved by plants during the infection process. Using both tomato plants and castor bean plants, it was found that there were approximately twenty times more bacterial cells in the tumor induced by *A. tumefaciens* C58(pRKLACC) than there were bacterial cells in the tumor induced by *A. tumefaciens* C58 five weeks after infection. One reason for this could be, as discussed above, that when the presence of ACC deaminase in *A. tumefaciens* C58(pRKLACC)

reduced ethylene levels, the expression of plant defence genes was also reduced, so that the modified *A. tumefaciens* that expressed ACC deaminase is less inhibited than the wild type *A. tumefaciens* C58. Alternatively, *A. tumefaciens* C58(pRKLACC) could use ACC as a nitrogen and carbon source and thereby survive better and proliferate faster in the tumor than the wild type strain.

To study whether the presence of ACC deaminase in disarmed *A. tumefaciens* could increase the bacterial population size in the infected plant tissue, two days after infection with different dilutions of strains *A. tumefaciens* YH-1 and YH-2, the bacterial population sizes per gram of canola hypocotyl segments were estimated. The results showed that there was no significant difference between the two strains, which means that the presence of ACC deaminase in *A. tumefaciens* YH-2 does not make it proliferate better during the cocultivation process. This result agrees with the results obtained by Nonaka *et al.* (2008 b) that inclusion of ACC in the cocultivation medium does not inhibit the growth of *A. tumefaciens*.

The controversy in the literature and of the results obtained in crown galls and in plant tissue culture may be explained by the fact that the tissue culture environment is very different from intact plants. The cocultivation medium used in tissue culture contains sufficient nutrients to support the growth of the bacteria, while in the plant tumor, the conditions are very different. Compared to intact plants, the plant segments may react differently to ethylene, and may not induce the expression of plant defence genes that can inhibit bacterial growth.

3.4.2 Ethylene and crown gall development

As reviewed in the introduction, *A. tumefaciens* infection induces sustained ethylene production in plants, and this increased level of ethylene is critical for crown gall tumor development as well as the severity of the crown gall disease (Aloni, 1995; Aloni *et al.*, 1997; Ullrich and Aloni, 2000). For example, it was reported that the growth of crown galls in the ethylene insensitive *Never ripe* mutant of tomato plants was severely suppressed (Aloni *et al.*, 1998). Thus, reducing the level of ethylene helps to inhibit crown gall development. However, reducing the ethylene level also promotes the gene delivery efficiency of *Agrobacterium*, and the application of ethylene inhibitors in the transformation and regeneration process help increase *A. tumefaciens* mediated transformation efficiency (Chakrabarty *et al.*, 2002; Ezura H, 2000; Burgos and Alburquerque, 2003; Han *et al.*, 2005; Petri *et al.*, 2005; Seong *et al.*, 2005; (Nonaka *et al.*, 2008a). Recently it was reported that ethylene may inhibit the expression of the *A. tumefaciens* virulence genes, and the frequency of tumor formation in ethylene insensitive *Arabidopsis* is higher than in wild type *Arabidopsis* plants (Nonoka *et al.* 2008 b). The apparently contradictory results may be explained by the possibility of different levels of ethylene affecting these processes and the different roles ethylene plays at different stages of *A. tumefaciens* infection and crown gall development. For example, following the first week after infection, the ethylene level evolved from the tumors on castor bean stems is very low. As the infection proceeds, the level of ethylene continues to increase and reaches a peak around week five before it begins to drop (Wächer *et al.* 1999). A similar pattern was found for tomato stem tumors and tumors on carrot discs (Aloni *et al.* 1995; Goodman *et al.* 1986). The data are consistent with the possibility that at the initial infection stage, the relatively low level of ethylene acts as a signal to trigger

the expression of plant defence genes. Thus, in the ethylene insensitive mutant plants, the frequency of tumor occurrence is higher (Nonaka. *et al*. 2008 b); while after the tumor has been initiated and during the later stages of crown gall development, the high level of ethylene promotes the differentiation and development of the crown gall tumor and increases the severity of the crown gall disease, so that tumor development in the ethylene insensitive plants is greatly inhibited (Aloni *et al.*, 1998).

In this study, the *acdS* gene from *P. putida* UW4 was introduced into the virulent strain *A. tumefaciens* C58. In order to evaluate the effect of ACC deaminase on plant tumor induction and development following *A. tumefaciens* infection, carrot discs, tomato stems and castor bean stems were used as experimental systems. The results using carrot discs indicated that there is no significant difference in the number of tumors induced between the wild type *A. tumefaciens* C58 strain and the ACC deaminase-producing strain *A. tumefaciens* C58(pRKLACC). The results using tomato stems also indicated that the tumor fresh weight was not significantly affected when *acdS* was expressed in *A. tumefaciens* C58, while the results using castor bean stems showed that the tumor size was significantly reduced and the plants grew better when ACC deaminase activity was present in *Agrobacterium*. The difference between the results observed with different plants could be a consequence of the different sensitivity and response of these plants to ethylene (Pierik *et al.* 2006). In the cases of both tomato stems and castor bean stems, *A. tumefaciens* C58(pRKLACC) proliferated to a much greater extent in the tumor than did the strains without ACC deaminase. This may partly explain why tumor development was not dramatically diminished when *acdS* was present in *Agrobacterium* while tumor development was severely inhibited in ethylene insensitive *Never ripe* tomato mutant

plants (Aloni *et al.* 1998) and application of the ethylene inhibitor AVG almost totally inhibited tumor growth in castor bean plants (Wächner *et al.* 1999). Another possible reason for the smaller than expected effect of introducing the *acdS* gene into *A. tumefaciens* could be that the level of ACC deaminase was insufficient to reduce the ethylene to a level that would severely decrease tumor development.

As an alternative to directly introducing an *acdS* gene into *A. tumefaciens*, the ACC deaminase-containing PGPB *P. putida* UW4 or the *acdS* mutant strain *P. putida* UW4-*acdS*$^-$ was co-inoculated with *A. tumefaciens* C58 on tomato and castor bean stems. In both cases, tumor development was significantly decreased by the co-inoculation with the wild-type *P. putida* UW4 being more effective than the *acdS* knockout mutant in inhibiting tumor development in terms of reducing tumor fresh weight. Castor bean plants also grew better when wild type *P. putida* UW4 was used. These results confirm that ethylene does promote tumor development, and indicate that ACC deaminase from an exogenous PGPB can be used to inhibit crown gall development and to relieve some of the stress symptoms of plants carrying crown galls.

The *acdS* gene from *P. putida* UW4 was also introduced into the disarmed *A. tumefaciens* strain GV3101::pMP90(pPZP-eGFP) and the effect of ACC deaminase on *A. tumefaciens* mediated transformation efficiency was analyzed using canola hypocotyl segments. The presence of the *acdS* gene was found to be able to significantly reduce the ethylene levels evolved by the plant tissues during the infection and cocultivation process. In addition, a much higher transformation frequency was obtained with the *A. tumefaciens* strain containing ACC deaminase for all three canola cultivars used. This implies, as suggested in the literature, that at the initial stage of *Agrobacterium* infection,

ethylene inhibits the gene delivery efficiency. Thus, ACC deaminase which acts to reduce plant ethylene levels, can be used to promote the transformation frequency of recalcitrant plants by promoting the gene delivery efficiency and maybe also the plant regeneration efficiency.

3.4.3 PGPB and crown gall disease

When co-inoculated with *A. tumefaciens* C58, both the wild type and the *acdS*⁻ mutant strains of *P. putida* UW4 inhibited crown gall tumor development. Co-inoculation of *A. tumefaciens* C58 with wild-type *P. putida* UW4 inhibited tumor development more than 50% on both tomato and castor bean stems, while co-inoculation of *A. tumefaciens* C58 with *P. putida* UW4(*acdS*-) inhibited the tumor development by about 40% on both tomato and castor bean stems. Although the wild type *P. putida* UW4 demonstrates superior tumor inhibition effects, the mutant strain also significantly inhibits tumor formation.

The *A. tumefaciens* strain D3, which contains an *acdS* gene in its genome, was proven to be an avirulent strain and was shown to be able to promote root elongation under gnotobiotic conditions. An *acdS*⁻ mutant strain *A. tumefaciens* D3-1 was constructed and characterized. When co-inoculated with *A. tumefaciens* C58 on castor bean stems, both wild type *A. tumefaciens* D3 and the mutant *A. tumefaciens* D3-1 were able to significantly inhibit tumor development, with wild type *A. tumefaciens* D3 showing slightly better biocontrol activity than the mutant *A. tumefaciens* D3-1. Compared to *P. putida* UW4, which can reduce the tumor size about 50%, *A. tumefaciens* D3 showed a better biocontrol activity towards *A. tumefaciens* C58 in that it almost

totally inhibited tumor formation despite having a lower level of ACC deaminase activity.

These results indicate that besides ACC deaminase, there are other factors in these two potential biocontrol strains that can affect *A. tumefaciens* C58 growth or pathogenicity. One possibility is that *P. putida* UW4 or *A. tumefaciens* D3 might produce some chemical compounds that inhibit the growth or kill the *A. tumefaciens* C58 cells. However, preliminary experiments make this possibility unlikely since neither *P. putida* UW4 or *A. tumefaciens* D3 culture filtrates have any effect on *A. tumefaciens* C58 growth or survival. It is also possible that the two PGPB might produce proteins that are capable of degrading QS signals produced by *A. tumefaciens* that are related to *A. tumefaciens* pathogenicity. Another, more likely, possibility is that the two potential biocontrol strains grow faster, or attach with a greater affinity to surfaces of the plant stems, therefore outcompeting *A. tumefaciens* C58 for binding to the stem. In this regard, it was recently reported (An *et al.* 2006) that during a study of the coculture of *A. tumefaciens* C58 and *P. aeruginosa*, *P. aeruginosa* can outgrow *A. tumefaciens* C58. In fact, after co-inoculation with a 1:1 ratio (*A. tumefaciens:P. aeruginosa*), in the mature biofilm, the biomass of *A. tumefaciens* was found to represent only about 1% of the total biomass (*An et al.* 2006).

3.4.4 ACC deaminase and QS in *A. tumefaciens* D3.

A. tumefaciens D3 has been reported to contain a putative *acdS* gene and an *lrpL* gene located adjacent to each and oriented in opposite directions on a megaplasmid (Trott *et al*. 2001). In this study, it was found that this strain displays a higher level of ACC

deaminase activity than *R. leguminosarum* bv. viciae 128Sm, although lower than *P. putida* UW4. An *lrpL* and *acdS* double mutant strain D3-1 was constructed by replacing the C-termini of both genes with a *tetA* gene. When complemented with the *acdS* and its promoter alone, the resulting strain D3-3 showed only very low level of ACC deaminase activity, while when complement with both the *lrpL* and *acdS* genes, the ACC deaminase activity was fully restored. This result indicates that like in *P. putida* UW4 and *R. leguminosarum* bv. viciae and other species, the *lrpL* gene is also the regulator of *acdS* gene, although unlike in those strains, the *lrpL* is located downstream of the *acdS* gene in *A. tumefaciens* D3. In strain D3-3, when only *acdS* gene and its promoter is provided, with the absence of its regulator (the *lrpL* gene), only very basic level of AcdS is expressed and thus the strain shows very low level of ACC deaminase activity, while in D3-4, when both the *acdS* and its regulator (the *lrpL* gene) are provided, the ACC deaminase activity is fully restored. Due to the multicopy nature of the plasmid pYH6, the D3-4 strain shows higher activity than the wild type strain (Figure 3.19).

It was found that the wild *A. tumefaciens* D3 synthesized a QS signal that shows a similar shape and migration rate to C6 HSL during TLC assay. Interestingly, in the *acdS* and *lrpL* double mutant *A. tumefaciens* D3-1, the QS signal production or secretion was significantly reduced. When the mutant was complemented with *lrpL* or *acdS* or both of these genes, the resulting strains produced an AHL that on TLC plates appeared to be similar to 3-O-C8 HSL. It is unclear at this point why deletion of *lrpL* or *acdS* affects the synthesis or secretion of QS signal in *A. tumefaciens* D3, and why complementation of *lrpL* and/or *acdS* would make it produce a different AHL. Futher experiments need to be

done in the future to determine the exact mechanism of the cross talk of the two regulatory systems.

Chapter 4 Conclusions and Future Directions

In the first part of this study, four metagenomic libraries constructed using total DNA from activated sludge or soil were screened for novel QS signal synthesis genes using an *A. tumefaciens* biosensor strain. One clone (QS6-1) from municipal waste activated sludge metagenomic library CX6, and two clones (QS10-1 and QS10-2) from soil metagenomic library CX10 were isolated. Sequencing results revealed that all three clones contain new LuxR/LuxI type QS systems. The majority of AHLs produced by each LuxI homolog were characterized by TLC assay, ESI MS and MS/MS. Two new AHL signals, C14:3 HSL and (?)-hydroxymethyl-3-oxo-C14 HSL were identified to be synthesized by $LuxI_{QS6-1}$. These findings broaden the range of bacterial species that contain LuxR/LuxI type QS systems, add to the knowledge of the structures of AHLs that are synthesized and used by bacteria as QS signals, indicate the prevalence of QS containing-bacteria in the natural environment and demonstrate the usefulness of functional screening in the isolation of new QS systems from uncultured bacterial species. However, the following aspects need to be further investigated in the future.

First, the exact structures of the newly discovered AHL signals need to be determined. The positions and geometries (*cis* or *trans*) of the unsaturated carbon bonds in C14:3 HSL, and the position of the hydroxymethyl (CH_2OH) group substitution in (?)-hydroxymethyl-3-oxo-C14 HSL remain to be elaborated. This might be done by performing HPLC purification followed by nuclear magnetic resonance (NMR) spectroscopy.

Second, although all three LuxR homologs were expressed in *E. coil* using the pET30 system, only $LuxR_{QS10\text{-}1}$ was able to be expressed in the soluble portion of the cell lysate. In the future, different AHL and IPTG concentrations, lower expression temperatures, and different expression vectors and host strains need to be tried in an effort to obtain the soluble expression of $LuxR_{QS6\text{-}1}$ and $LuxR_{QS10\text{-}2}$.

In this study, putative *lux*-box like elements that are LuxR homolog binding sites were identified in the promoter regions of $luxI_{QS6\text{-}1}$, $luxI_{QS10\text{-}1}$ and $luxR_{QS10\text{-}2}$ by sequence analysis. In the future, using the purified active LuxR homologs, by performing a protein-DNA binding assay, the putative function of predicated *lux*-box elements may be confirmed. Using this method, other new LuxR homolog binding DNA sequences in these clones could also be identified and therefore the QS regulated target genes could be determined in these clones.

Third, transposon insertion mutagenesis of the gene encoding the biotin carboxylase subunit of acetyl-CoA carboxylase of the clone QS10-2 was found to affect the production of AHLs. This could be explained by the fact that acetyl-CoA carboxylase is an enzyme that catalyzes the carboxylation of acetyl-CoA to produce malonyl-CoA, which is the first committed step in the biosynthesis of long chain fatty acid, while AHL synthesis requires the intermediate of fatty acid biosynthesis pathway, the acyl-acyl carrier proteins (Miller and Bassler, 2001). In the future, it would be interesting to obtain the sequence of the other two subunits of the acetyl-CoA carboxylase enzyme in QS10-2, the carboxyl carrier protein and the carboxyl transferase, and study the activity of this enzyme. Since mutation of the acetyl-CoA carboxylase of the clone QS10-2 affects AHL production even in the presence of the homolog enzyme of the expression host *A*.

tumefaciens HC103(pJZ381), it indicates that $LuxI_{QS10\text{-}2}$ requires the presence of its own acetyl-CoA carboxylase for the synthesis of AHLs. It would also be interesting to study the mechanism of this specificity.

Fourth, the biosensor strain used in this study are most sensitive for the detection of medium to long chain AHLs, and can not detect AHLs with C4 chain length and other types of QS signals. Future studies might benefit from the use of other QS biosensor strains that have different sensitivities to AHLs and strains that can detect other types of QS signals. In this way, more novel QS systems might be identified.

Finally, the three QS systems were able to synthesize and detect multiple AHLs, including some new AHL structures, and the three *luxI* homologs seem to be regulated by their cognate LuxR homolog proteins. By construction of fusions of the LuxR homolog regulated promoters with a reporter gene (such as *lacZ*), and overexpression of the *luxR* homolog gene in the same host, new biosensor strains could be constructed. These new biosensor strains may be useful in the screening and study of *luxI-luxR* type QS systems from both cultured and uncultured bacteria.

In the second part of this thesis, the effect of ACC deaminase on the pathogenicity of virulent *A. tumefaciens* strain C58 and on *A. tumefaciens* mediated transformation efficiency were studied. It was found that introduction of ACC deaminase into disarmed *A. tumefaciens* strain GV3101::pMP90 significantly increased the *A. tumefaciens*-mediated transformation frequency of the commercial canola cultivars *B. napus* cv. 4414RR and *B. napus* cv. Hyola 401. This finding confirms and extends the results reported by Nonaka *et al.* (2008a) that ACC deaminase increases *A. tumefaciens* facilitated gene delivery efficiency to melon cotyledons, and suggests that ACC

deaminase could be used as a general mechanism to improve *A. tumefaciens*-mediated transformation efficiency of various recalcitrant plants. This is also the first transformation efficiency assay of the two widely grown spring canola cultivars, and the data obtained should provide a very useful reference in the future genetic modification of these canola cultivars.

It was also found that introduction of ACC deaminase into the virulent strain *A. tumefaciens* C58 or co-inoculation of *A. tumefaciens* C58 with the ACC deaminase-containing PGPB *P. putida* UW4 significantly inhibits crown gall development in both tomato and castor bean plants. These results confirm the findings that ethylene plays an important role in crown gall development (Aloni, 1995; Aloni *et al.*, 1997; Ullrich and Aloni, 2000) and suggests the possibility of using ACC deaminase as a mechanism to control crown gall disease.

A. tumefaciens D3 was shown to be an avirulent strain with ACC deaminase activity and was able to promote canola root elongation under gnotobiotic conditions. When co-inoculated with *A. tumefaciens* C58, it shows better biocontrol activity than the PGPB *P. putida* UW4. Crown gall development was almost totally inhibited when *A. tumefaciens* C58 was co-inoculated with a 1:1 cell ratio of *A. tumefaciens* D3. However, it was found that the *acdS* mutant strain of *A. tumefaciens* D3 was also able to significantly inhibit crown gall development. This means that in addition to the role of ACC deaminase, other mechanisms present in *A. tumefaciens* D3 can act to inhibit the growth or reduce the pathogenicity of *A. tumefaciens* C58. Additional experiments need to be done to determine the precise nature of these other mechanisms. This notwithstanding, *A. tumefaciens* D3 has the potential to be developed as a biocontrol

agent towards crown gall disease. To date, the only commercialized biocontrol agent for crown gall disease is *A. radiobacter* K84, which produces agrocin 84 that kills nopaline producing *A. tumefaciens* strains but is not effective for other types of pathogenic *A. tumefaciens* strains. The ability of *A. tumefaciens* D3 to inhibit crown gall development as well as to promote plant growth might make it a better biocontrol agent than *A. tumefaciens* K84. In addition, unlike *A. tumefaciens* K84, it is unnecessary to genetically engineer *A. tumefaciens* D3.

Construction and complementation of the *acdS* and *lrpL* double mutant strain of *A. tumefaciens* D3 revealed that like many other bacteria that express ACC deaminase (Duan *et al*, 2009; Grichko and Glick, 2000; Li and Glick, 2001; Ma *et al.*, 2003a; Prigent-Combaret *et al.*, 2008; Sun *et al*, 2009), the *lrpL* gene is involved in the regulation of the *acdS* gene. It was also found that in the double mutant strain D3-1, the production of the AHL QS signal was inhibited which indicates that there is cross talk between these two regulatory systems. Additional experiments are required to determine the exact mechanism of this cross talk. As a first step, proteomic analysis using two-dimensional differential gel electrophoresis could be performed to identify differentially expressed proteins in the *lrpL* and *acdS* double mutant strain *A. tumefaciens* D3-1. Then mutagenesis, complementation and overexpression of the identified differentially expressed target proteins could be performed in an effort to elucidate the regulatory network. In this regard, it is also interesting to study whether the cross talk between ACC deaminase and QS is unique to *A. tumefaciens* D3 or whether this mechanism also exists in other organisms.

References

Abeles, F.B. (1992) *Ethylene in Plant Biology*. New York: Academic Press.

Abascal, F., Zardoya, R., and Posada, D. (2005) ProtTest: selection of best-fit models of protein evolution. *Bioinformatics* **21**: 2104-2105.

Alexander, D.B., and Zuberer, D.A. (1991) Use of chrome azurol S reagents to evaluate siderophore production by rhizosphere bacteria. *Biol Fert Soils* **12**: 39-54.

Aloni, R., Wolf, A., Feigenbaum, P., and Klee, H.J. (1997) Evidence that tumor-induced ethylene controls the morphogenesis of *Agrobacterium tumefaciens*-induced crown galls on tomato stems. *Isr J Plant Sci* **45**: 255.

Aloni, R., Wolf, A., Feigenbaum, P., Avni, A., and Klee, H.J. (1998) The *Never ripe* mutant provides evidence that tumor-induced ethylene controls the morphogenesis of *Agrobacterium tumefaciens*-induced crown galls on tomato stems. *Plant Physiol* **117**: 841-849.

Aloni, R., Pradel, K.S., and Ullrich, C. I. (1995) The three-dimensional structure of vascular tissues in *Agrobacterium tumefaciens*-induced crown galls and in the host stem of *Ricinus communis* L. *Planta* **196**: 597-605.

Alting-Mees, M.A., and Short, J.M. (1989) pBluescript II: gene mapping vectors. *Nucleic Acids Res* **17**: 9494.

Alting-Mees, M.A., Sorge, J.A., and Short, J.M. (1992) pBluescriptII: multifunctional cloning and mapping vectors. *Methods Enzymol* **216**: 483-495.

Amann, R.I., Ludwig, W., and Schleifer, K.H. (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol Rev* **59**:143-169.

An, D., Danhorn, T., Fuqua, C., and Parsek, M.R. (2006) Quorum sensing and motility mediate interactions between *Pseudomonas aeruginosa* and *Agrobacterium tumefaciens* in biofilm cocultures. *Proc Natl Acad Sci U S A* **103**: 3828-3833.

Anastasakis, E., and Antsaklis, A. (2008) Maternity unit performance index. A novel approach for evaluation of the changing obstetric practice in a single maternity unit. *Arch Gynecol Obstet* **277**: 121-126.

Andersson, R.A., Eriksson, A.R., Heikinheimo, R., Mae, A., Pirhonen, M., Koiv, V. *et al.* (2000) Quorum sensing in the plant pathogen *Erwinia carotovora* subsp. carotovora: the role of expR(Ecc). *Mol Plant Microbe Interact* **13**: 384-393.

Arevalo-Ferro, C., Hentzer, M., Reil, G., Gorg, A., Kjelleberg, S., Givskov, M. *et al.* (2003) Identification of quorum-sensing regulated proteins in the opportunistic pathogen *Pseudomonas aeruginosa* by proteomics. *Environ Microbiol* **5**: 1350-1369.

Arshad, M., Shaharoona, B. and Mahmood, T. (2008) Inoculation with *Pseudomonas* spp. containing ACC-deaminase partially eliminates the effects of drought stress on growth, yield, and ripening of pea (*Pisum sativum* L.). *Pedosphere*. 18(5): 611–620.

Arteca, R.N., and Arteca, J.M. (2008) Effects of brassinosteroid, auxin, and cytokinin on ethylene production in *Arabidopsis thaliana* plants. *J Exp Bot* **59**: 3019-3026.

Atkinson, S., Throup, J.P., Stewart, G.S., and Williams, P. (1999) A hierarchical quorum-sensing system in *Yersinia pseudotuberculosis* is involved in the regulation of motility and clumping. *Mol Microbiol* **33**: 1267-1277.

Atlas, R.M. (1993) *Handbook of microbiological media*. Boca Raton, Fla: CRC Press Inc.

Babiker, A.G.T., Butler, L.G., Ejeta, G., and Woodson, W.R. (1993) Enhancement of Ethylene Biosynthesis and Germination by Cytokinins and 1-Aminocyclopropane-l-Carboxylic Acid in *Striga Asiatica* Seeds. *Physiologia Plantarum* **89**: 21-26.

Basu, U., Good, A.G., and Taylor, G.J. (2001) Transgenic *Brassica napus* plants overexpressing aluminium-induced mitochondrial manganese superoxide dismutase cDNA are resistant to aluminium. *Plant, Cell & Environment* **24**: 1278-1269.

Beck von Bodman, S., and Farrand, S.K. (1995) Capsular polysaccharide biosynthesis and pathogenicity in *Erwinia stewartii* require induction by an N-acyl homoserine lactone autoinducer. *J Bacteriol* **177**: 5000-5008.

Belimov, A.A., Dodd, I.C., Safronova, V.I., Hontzeas, N., and Davies, W.J. (2007) *Pseudomonas brassicacearum* strain Am3 containing 1-aminocyclopropane-1-carboxylate deaminase can show both pathogenic and growth-promoting properties in its interaction with tomato. *J Exp Bot* **58**: 1485-1495

Bertani, I., and Venturi, V. (2004) Regulation of the N-acyl homoserine lactone-dependent quorum-sensing system in rhizosphere *Pseudomonas putida* WCS358 and cross-talk with the stationary-phase RpoS sigma factor and the global regulator GacA. *Appl Environ Microbiol* **70**: 5493-5502.

Beijerinck, M.W., and van Delden, A. (1902) Ueber die Assimilation des freien Stickstoffs durch Bakterien. *Zentbl Bakt Parasitenk Infekt Abt II* **9**: 3-44.

Bertani, G. (1951). Studies on lysogenesis. I. The mode of phage liberation by lysogenic Escherichia coli. J. Bacteriol. 62:293-300.

Breitbart, M., Hewson, I., Felts, B., Mahaffy, J.M., Nulton, J., Salamon, P., and Rohwer, F. (2003) Metagenomic analyses of an uncultured viral community from human feces. *J Bacteriol* **185**: 6220-6223.

Bhalla, P.L., and Singh, M.B. (2008) *Agrobacterium*-mediated transformation of Brassica napus and *Brassica oleracea. Nat. Protoc.* **3**: 181-189.

Bleecker, A.B., and Kende, H. (2000) Ethylene: a gaseous signal molecule in plants. *Annu Rev Cell Dev Biol* **16**: 1-18.

Booth, M.C., Bogie, C.P., Sahl, H.G., Siezen, R.J., Hatter, K.L., and Gilmore, M.S. (1996) Structural analysis and proteolytic activation of *Enterococcus faecalis* cytolysin, a novel lantibiotic. *Mol Microbiol* **21**: 1175-1184.

Bottomley, M.J., Muraglia, E., Bazzo, R., and Carfi, A. (2007) Molecular insights into quorum sensing in the human pathogen *Pseudomonas aeruginosa* from the structure of the virulence regulator LasR bound to its autoinducer. *J Biol Chem* **282**: 13592-13600.

Bouvier, J., Gordia, S., Kampmann, G., Lange, R., Hengge-Aronis, R., and Gutierrez, C. (1998) Interplay between global regulators of *Escherichia coli*: effect of RpoS, Lrp and H-NS on transcription of the gene *osmC*. *Mol Microbiol* **28**: 971-980.

Burgos, L., and Alburquerque, N. (2003) Ethylene inhibitors and low kanamycin concentrations improve adventitious regeneration from apricot leaves. *Plant Cell Rep* **21**: 1167-1174.

Canfield, M.L., and Moore, L.W. (1983) Production of ethylene by *Daucus carota* inoculated with *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*. *Z. Pflanzenphysiol* **112**: 471-474.

Cangelosi, G.A., Best, E.A., Martinetti, G., and Nester, E.W. (1991) Genetic analysis of *Agrobacterium. Methods Enzymol* **204**: 384-397.

Cao, H., Yang, M., Zheng, H., Zhang, J., Zhong, Z., and Zhu, J. (2009) Complex quorum-sensing regulatory systems regulate bacterial growth and symbiotic nodulation in *Mesorhizobium tianshanense*. *Arch Microbiol* **191**: 283-289.

Cardoza, V., and Stewart, C.N. (2003) Increased *Agrobacterium*-mediated transformation and rooting efficiencies in canola *(Brassica napus* L.) from hypocotyl segment explants. *Plant Cell Rep* **21**: 599-604.

Chakrabarty, R., Viswakarma, N., Bhat, S.R., Kirti, P.B., Singh, B.D., and Chopra, V.L. (2002) *Agrobacterium*-mediated transformation of cauliflower: optimization of protocol and development of Bt-transgenic cauliflower. *J Biosci* **27**: 495-502.

Chen, K., and Pachter, L. (2005) Bioinformatics for whole-genome shotgun sequencing of microbial communities. *PLoS Comput Biol* **1**: 106-112.

Chen, X., Schauder, S., Potier, N., Van Dorsselaer, A., Pelczer, I., Bassler, B.L., and Hughson, F.M. (2002) Structural identification of a bacterial quorum-sensing signal containing boron. *Nature* **415**: 545-549.

Cheng M., Lowe B.A., Spencer T. M., X.D., Y., and C.L., A. (2004) Invited Review: Factors Influencing *Agrobacterium*-Mediated Transformation Of Monocotyledonous Species. *In Vitro Cell. Dev. Biol.-Plant ,* **40**: 31-45.

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

Chi, G.L., Barfield, D.G., Sim, G.E., and Pua, E.C. (1990) Effect of AgNO3 and aminoethoxyvinylglycine on in vitro shoot and root organogenesis from seedling explants of recalcitrant *Brassica* genotypes. *Plant Cell Rep* **9**: 195-198.

Chilton, M.D., Currier, T.C., Farrand, S.K., Bendich, A.J., Gordon, M.P., and Nester, E.W. (1974) *Agrobacterium tumefaciens* DNA and PS8 bacteriophage DNA not detected in crown gall tumors. *Proc Natl Acad Sci U S A* **71**: 3672-3676.

Chraibi, K.M., Latche, A., Roustan, J.P., and Fallot, J. (1991) Stimulation of shoot regeneration from cotyledons of *Helianthus annuus* by the ethylene inhibitors, silver and cobalt. *Plant Cell Rep* **10**: 204-207.

Ciardi, J.A., Tieman, D.M., Jones, J.B., and Klee, H.J. (2001) Reduced expression of the tomato ethylene receptor gene LeETR4 enhances the hypersensitive response to *Xanthomonas campestris* pv. vesicatoria. *Mol Plant Microbe Interact* **14**: 487-495.

Conway, B.A., and Greenberg, E.P. (2002) Quorum-sensing signals and quorum-sensing genes in *Burkholderia vietnamiensis*. *J Bacteriol* **184**: 1187-1191.

Crocker, W., Hitchcock, A.E., and Zimmerman, P.W. (1935) Similarities in the effects of ethylene and the plant auxins. *Contrib. Boyce Thompson Inst.* **7**: 231-248.

Czarny, J.C. (2008) Effects of a bacterial ACC deaminase on plant growth-promotion. In *Department of Biology*. Waterloo: University of Waterloo.

Daniel, R. (2005) The metagenomics of soil. *Nat Rev Microbiol* **3**: 470-478.

De Block, M., De Brouwer, D., and Tenning, P. (1989) Transformation of *Brassica napus* and Brassica oleracea Using Agrobacterium tumefaciens and the Expression of the bar and neo Genes in the Transgenic Plants. *Plant Physiol.* **91**: 694-701.

DeLong, E.F. (2005) Microbial community genomics in the ocean. *Nat Rev Microbiol* **3**: 459-469.

Devescovi, G., Bigirimana, J., Degrassi, G., Cabrio, L., LiPuma, J.J., Kim, J. *et al.* (2007) Involvement of a quorum-sensing-regulated lipase secreted by a clinical isolate of *Burkholderia glumae* in severe disease symptoms in rice. *Appl Environ Microbiol* **73**: 4950-4958.

Devine, J.H., Shadel, G.S., and Baldwin, T.O. (1989) Identification of the operator of the *lux* regulon from the *Vibrio fischeri* strain ATCC7744. *Proc Natl Acad Sci U S A* **86**: 5688-5692.

Devine, J.H.C., C; Baldwin, T O. (1988) Nucleotide sequence of the *luxR* and *luxI* genes and the structure of the primary regulatory region of the lux regulon of *Vibrio fischeri* ATCC 7744. *Biochemistry* **27**: 837–842.

Diaz-Torres, M.L., McNab, R., Spratt, D.A., Villedieu, A., Hunt, N., Wilson, M., and Mullany, P. (2003) Novel tetracycline resistance determinant from the oral metagenome. *Antimicrob Agents Chemother* **47**: 1430-1432.

Diggle, S. P., Crusz, S. A. and Camara, M. (2007) Quorum sensing. Current Biology. **17:** R907-R910

Doyle, J.J., and Doyle, J.L. (1990) Isolation of plant DNA from fresh tissue. *Focus* **12**: 13-15.

Duan, J., Muller, K.M., Charles, T.C., Vesely, S., and Glick, B.R. (2009) 1-aminocyclopropane-1-carboxylate (ACC) deaminase genes in rhizobia from southern Saskatchewan. *Microb Ecol* **57**: 423-436.

Dubern, J.F., Lugtenberg, B.J., and Bloemberg, G.V. (2006) The ppuI-rsaL-ppuR quorum-sensing system regulates biofilm formation of *Pseudomonas putida* PCL1445 by controlling biosynthesis of the cyclic lipopeptides putisolvins I and II. *J Bacteriol* **188**: 2898-2906.

Duerkop, B.A., Ulrich, R.L., and Greenberg, E.P. (2007) Octanoyl-homoserine lactone is the cognate signal for *Burkholderia mallei* BmaR1-BmaI1 quorum sensing. *J Bacteriol* **189**: 5034-5040.

Dunman, P.M., Murphy, E., Haney, S., Palacios, D., Tucker-Kellogg, G., Wu, S. *et al.* (2001) Transcription profiling-based identification of *Staphylococcus aureus* genes regulated by the *agr* and/or *sarA* loci. *J Bacteriol* **183**: 7341-7353.

Dworkin, M., and Foster, J. W. **(**1958). Experiments with some microorganisms which utilize ethane and hydrogen. *J. Bacteriol.* 75**:**592-601.

Eberl, L., Winson, M.K., Sternberg, C., Stewart, G.S., Christiansen, G., Chhabra, S.R. *et al.* (1996) Involvement of N-acyl-L-homoserine lactone autoinducers in controlling the multicellular behaviour of *Serratia liquefaciens*. *Mol Microbiol* **20**: 127-136.

Edgar, R.C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* **32**: 1792-1797.

El-Sayed, A.K., Hothersall, J., and Thomas, C.M. (2001) Quorum-sensing-dependent regulation of biosynthesis of the polyketide antibiotic mupirocin in *Pseudomonas fluorescens* NCIMB 10586. *Microbiology* **147**: 2127-2139.

Elasri, M., Delorme, S., Lemanceau, P., Stewart, G., Laue, B., Glickmann, E. *et al.* (2001) Acyl-homoserine lactone production is more common among plant-associated *Pseudomonas spp*. than among soilborne *Pseudomonas spp*. *Appl Environ Microbiol* **67**: 1198-1209.

Engebrecht, J., and Silverman, M. (1987) Nucleotide sequence of the regulatory locus controlling expression of bacterial genes for bioluminescence. *Nucleic Acids Res* **15**: 10455-10467.

Eapen, S., and George, L. (1997). Plant regeneration from peduncle segments of oil seed *Brassica* species: influence of silver nitrate and silver thiosulfate. *Plant Cell Tissue Organ Cult.* 51: 229-232.

Ezura, H., Yuhashi, K.I., Yasuta, T., and Minamisawa, K. (2000) Effect of ethylene on *Agrobacterium tumefaciens*-mediated gene transfer to melon. *Plant Breed* **119**: 75-79.

Facciotti, M.T., Bertain, P.B., and Yuan, L. (1999) Improved stearate phenotype in transgenic canola expressing a modified acyl-acyl carrier protein thioesterase. *Nat Biotechnol* **17**: 593-597.

Farrand, S.K. (1990) *Agrobacterium radiobacter* K84: a model biocontrol system. In *New Directions in Biological Control: Alternatives for Suppressing Agricultural Pests and Diseases.* Baker, R.R., and Dunn, P.E. (eds): Publ. Alan R Liss Inc., pp. 679-691.

Finan, T.M., Kunkel, B., De Vos, G.F., and Signer, E.R. (1986) Second symbiotic megaplasmid in *Rhizobium meliloti* carrying exopolysaccharide and thiamine synthesis genes. *J Bacteriol* **167**: 66-72.

Flavier, A.B., Ganova-Raeva, L.M., Schell, M.A., and Denny, T.P. (1997) Hierarchical autoinduction in *Ralstonia solanacearum*: control of acyl-homoserine lactone production by a novel autoregulatory system responsive to 3-hydroxypalmitic acid methyl ester. *J Bacteriol* **179**: 7089-7097.

Flavier, A.B., Schell, M.A., and Denny, T.P. (1998) An RpoS (sigmaS) homologue regulates acylhomoserine lactone-dependent autoinduction in *Ralstonia solanacearum*. *Mol Microbiol* **28**: 475-486.

Fraley, R.T., Rogers, S.G., Horsch, R.B., Sanders, P.R., Flick, J.S., Adams, S.P. *et al.* (1983) Expression of bacterial genes in plant cells. *Proc Natl Acad Sci U S A* **80**: 4803-4807.

Fuqua, W.C., and Winans, S.C. (1994) A LuxR-LuxI type regulatory system activates *Agrobacterium* Ti plasmid conjugal transfer in the presence of a plant tumor metabolite. *J Bacteriol* **176**: 2796-2806.

Fuqua, W.C., Winans, S.C., and Greenberg, E.P. (1994) Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *J Bacteriol* **176**: 269-275.

Gambello, M.J., and Iglewski, B.H. (1991) Cloning and characterization of the *Pseudomonas aeruginosa* lasR gene, a transcriptional activator of elastase expression. *J Bacteriol* **173**: 3000-3009.

Gane, R. (1934) Production of ethylene by some ripening fruits. *Nature* **134**: 1008.

Gao, M., Chen, H., Eberhard, A., Gronquist, M.R., Robinson, J.B., Rolfe, B.G., and Bauer, W.D. (2005) *sinI*- and *expR*-dependent quorum sensing in *Sinorhizobium meliloti*. *J Bacteriol* **187**: 7931-7944.

Garcia, F.G., and Einset, J.W. (1983) Ethylene and ethane production in 2,4-D treated and salt treated tobacco tissue cultures. *Ann Botany* **51**: 287-295.

Garfinkel, D.J., and Nester, E.W. (1980) *Agrobacterium tumefaciens* mutants affected in crown gall tumorigenesis and octopine catabolism. *J Bacteriol* **144**: 732-743.

Giddings, G., Allison, G., Brooks, D., and Carter, A. (2000) Transgenic plants as factories for biopharmaceuticals. *Nat Biotechnol* **18**: 1151-1155.

Gill, S.R., Pop, M., Deboy, R.T., Eckburg, P.B., Turnbaugh, P.J., Samuel, B.S. et al. (2006) Metagenomic analysis of the human distal gut microbiome. *Science* **312**: 1355-1359.

Givskov, M., Eberl, L., and Molin, S. (1997) Control of exoenzyme production, motility and cell differentiation in *Serratia liquefaciens*. *FEMS Microbiol Lett* **148**: 115-122.

Glick, B.R., Karaturovíc, D.M., and Newell, P.C. (1995) A novel procedure for rapid isolation of plant growth-promoting pseudomonads. *Can. J. Microbiol*. **41**: 533–536.

Glick B.R., C., Z., Czarny J. and Duan J. (2007) Promotion of plant growth by ACC deaminase-producing soil bacteria. *Eur. J. Plant Pathol.* **119**: 329-339.

Glick, B.R. (2004) Bacterial ACC deaminase and the alleviation of plant stress. *Adv Appl Microbiol* **56**: 291-312.

Glick, B.R., Penrose, D.M., and Li, J. (1998) A model for the lowering of plant ethylene concentrations by plant growth-promoting bacteria. *J. Theor. Biol.* **190**: 63-68.

Glick, B.R., Patten, C.L., Holguin, G., and Penrose, D.M. (1999) *Biochemical and genetic mechanisms used by plant growth promoting bacteria*. London.

Glick, B.R., and Pasternak, J.J. (2003) *Molecular biotechnology: Principles and applications of recombinant DNA*. Washington, D.C.: ASM Press. 517-521.

Gloux, K., Leclerc, M., Iliozer, H., L'Haridon, R., Manichanh, C., Corthier, G., Nalin, R., Blottiere, H. M., Dore, J. (2007). Development of high-throughput phenotyping of metagenomic clones from the human gut microbiome for modulation of eukaryotic cell growth. *Appl. Environ. Microbiol.* 73: 3734-3737.

Guindon, S., and Gascuel, O. (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol* **52**: 696-704.

Gurich, N., and Gonzalez, J.E. (2009) Role of quorum sensing in *Sinorhizobium meliloti-Alfalfa* symbiosis. *J Bacteriol* **191**: 4372-4382.

Goodman, T.C., Montoya, A.L., Williams, S., and Chilton, M.D. (1986) Sustained ethylene production in Agrobacterium-transformed carrot disks caused by expression of the T-DNA tms gene products. *J Bacteriol* **167**: 387-388.

Goodner, B., Hinkle, G., Gattung, S., Miller, N., Blanchard, M., Qurollo, B. *et al.* (2001) Genome sequence of the plant pathogen and biotechnology agent *Agrobacterium tumefaciens* C58. *Science* **294**: 2323-2328.

Gordon, L., Chervonenkis, A.Y., Gammerman, A.J., Shahmuradov, I.A., and Solovyev, V.V. (2003) Sequence alignment kernel for recognition of promoter regions. *Bioinformatics* **19**: 1964-1971.

Gordon, S.A., and Weber, R.P. (1951) Colorimetric Estimation of Indoleacetic Acid. *Plant Physiol* **26**: 192-195.

Gray, K.M., and Garey, J.R. (2001) The evolution of bacterial LuxI and LuxR quorum sensing regulators. *Microbiology* **147**: 2379-2387.

Gray, K.M., Pearson, J.P., Downie, J.A., Boboye, B.E., and Greenberg, E.P. (1996) Cell-to-cell signaling in the symbiotic nitrogen-fixing bacterium *Rhizobium leguminosarum*: autoinduction of a stationary phase and rhizosphere-expressed genes. *J Bacteriol* **178**: 372-376.

Grichko, V.P., and Glick, B.R. (2000) Identification of DNA sequences that regulate the expression of the *Enterobacter cloacae* UW4 1-aminocyclopropane-1-carboxylate deaminase gene. *Can. J. Microbiol.* **46**: 1159-1165.

Grison, R., Grezes-Besset, B., Schneider, M., Lucante, N., Olsen, L., Leguay, J.-J., and Toppan, A. (1996) Field tolerance to fungal pathogens of *Brassica napus* constitutively expressing a chimeric chitinase gene. *Nat. Biotechnol.* **14**: 643-646.

Guan, C., Ju, J., Borlee, B.R., Williamson, L.L., Shen, B., Raffa, K.F., and Handelsman, J. (2007) Signal mimics derived from a metagenomic analysis of the gypsy moth gut microbiota. *Appl Environ Microbiol* **73**: 3669-3676.

Haas, D., and Defago, G. (2005) Biological control of soil-borne pathogens by fluorescent *Pseudomonads*. *Nat Rev Microbiol* **3**: 307-319.

Hall, T. (1999) BioEdit: a user friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**: 95-98.

Han, J.S., Kim, C.K., Park, S.H., Hirschi, K.D., and Mok, I. (2005) *Agrobacterium*-mediated transformation of bottle gourd (*Lagenaria siceraria* Standl.). *Plant Cell Rep* **23**: 692-698.

Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. 166:557–580.

Handelsman, J., Rondon, M.R., Brady, S.F., Clardy, J., and Goodman, R.M. (1998) Molecular biological access to the chemistry of unknown soil microbes: a new frontier for natural products. *Chem Biol* **5**: R245-249.

Handelsman, J. (2004) Metagenomics: application of genomics to uncultured microorganisms. *Microbiol Mol Biol Rev* **68**: 669-685.

Hao, G., and Burr, T.J. (2006) Regulation of long-chain N-acyl-homoserine lactones in *Agrobacterium vitis*. *J Bacteriol* **188**: 2173-2183.

Hellens, R., Mullineaux, P., and Klee, H. (2000) Technical Focus:a guide to *Agrobacterium* binary Ti vectors. *Trends Plant Sci* **5**: 446-451.

Hille J, Van Kan J, Klasen I, Schilperoort R. 1983. Site-directed mutagenesis in *Escherichia coli* of a stable R772::Ti cointegrate plasmid from *Agrobacterium tumefaciens*. *J* Bact 154:693-701.

Hoekema, A., Hirsch, P.R., Hooykaas, P.J.J., and Schilperoort, R.A. (1983) A binary plant vector strategy based on separation of vir- and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. *Nature* **303**: 179-180.

Holguin G. and Glick, B.R. (2001) Expression of the ACC deaminase gene from *Enterobacter cloacae* UW4 in *Azospirillum brasilense*. Microb. Ecol. 41: 281-288. *Microb. Ecol.* **41**: 281-288.

Holmes, B., and Roberts, P. (1981) The classification, identification and nomenclature of agrobacteria. *J Appl Bacteriol* **50.**: 443-467.

Honma, M., and Shimomura, T. (1978) Metabolism of 1-aminocyclopropane-1-carboxylic acid. *Agric. Biol. Chem.* **42**: 1825-1831.

Hwang, I., Li, P.L., Zhang, L., Piper, K.R., Cook, D.M., Tate, M.E., and Farrand, S.K. (1994) TraI, a LuxI homologue, is responsible for production of conjugation factor, the Ti plasmid N-acylhomoserine lactone autoinducer. *Proc Natl Acad Sci U S A* **91**: 4639-4643.

Hyodo, H. (1991) Stress/wound ethylene. In *The Plant Hormone Ethylene*. Mattoo, A.K., and sUTTLE, J.C. (eds). Boca Raton: CRC press.

Jackson, M.B., and Osborne, D.J. (1970) Ethylene, the natural regulator of leaf abscission. *Nature* **225**: 1019-1022.

Jarvis, B.D.W., Sivakumaran, S., Tighe, S.W., and Gillis, M. (1996) Identification of *Agrobacterium* and *Rhizobium* species based on cellular fatty acid composition. *Plant Soil* **184**: 143-158.

Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W. (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *Embo J* **6**: 3901-3907.

Kaplan, H.B., and Greenberg, E.P. (1987) Overproduction and purification of the luxR gene product: Transcriptional activator of the *Vibrio fischeri* luminescence system. *Proc Natl Acad Sci U S A* **84**: 6639-6643.

Katavic, V., Friesen, W., Barton, D.L., Gossen, K.K., Giblin, E.M., Luciw, T. *et al.* (2001) Improving erucic acid content in rapeseed through biotechnology: What

can the *Arabidopsis fae1* and the yeast *slc1-1* genes contribute? *Crop Sci* **41**: 739-747.

Keane, P.J., Kerr, A., and New, P.B. (1970) Crown gall of stone fruits. II. Identification and nomenclature of *Agrobacterium* isolates. *Aust. J. Biol. Sci.* **23**: 585–595.

Keen, N.T., Tamaki, S., Kobayashi, D., and Trollinger, D. (1988) Improved broad-host-range plasmids for DNA cloning in gram-negative bacteria. *Gene* **70**: 191-197.

Kerr, A., and Panagopoulos, C.G. (1977) Biotypes of *Agrobacterium radiobacter var. tumefaciens* and their biological control. *Phytopathol Z* **90**: 172-179.

Kersters, K., and De Ley, J. (1975) Identification and grouping of bacteria by numerical analysis of their electrophoretic protein patterns. *J Gen Microbiol* **87**: 333-342.

Kersters, K., De Ley, J., Sneath, P.H.A., and Sackin, M. (1973) Numerical taxonomic analysis of *Agrobacterium*. *J Gen Microbiol* **78**: 227-239.

Kim, J., Kim, J.G., Kang, Y., Jang, J.Y., Jog, G.J., Lim, J.Y. *et al.* (2004) Quorum sensing and the LysR-type transcriptional activator ToxR regulate toxoflavin biosynthesis and transport in *Burkholderia glumae*. *Mol Microbiol* **54**: 921-934.

Kirwan, J.P., Gould, T.A., Schweizer, H.P., Bearden, S.W., Murphy, R.C., and Churchill, M.E. (2006) Quorum-sensing signal synthesis by the *Yersinia pestis* acyl-homoserine lactone synthase YspI. *J Bacteriol* **188**: 784-788.

Knutzon, D.S., Hayes, T.R., Wyrick, A., Xiong, H., Maelor Davies, H., and Voelker, T.A. (1999) Lysophosphatidic acid acyltransferase from coconut endosperm mediates the insertion of laurate at the sn-2 position of triacylglycerols in lauric rapeseed oil and can increase total laurate levels. *Plant Physiol* **120**: 739-746.

Koncz, C., and Schell, J. (1986) The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vector. *Mol Gen Genet* **204**: 383–396.

Kovach, M.E., Elzer, P.H., Hill, D.S., Robertson, G.T., Farris, M.A., Roop, R.M., 2nd, and Peterson, K.M. (1995) Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* **166**: 175-176.

Lanahan, M.B., Yen, H.C., Giovannoni, J.J., and Klee, H.J. (1994) The never ripe mutation blocks ethylene perception in tomato. *Plant Cell* **6**: 521-530.

Latifi, A., Foglino, M., Tanaka, K., Williams, P., and Lazdunski, A. (1996) A hierarchical quorum-sensing cascade in *Pseudomonas aeruginosa* links the transcriptional activators LasR and RhIR (VsmR) to expression of the stationary-phase sigma factor RpoS. *Mol Microbiol* **21**: 1137-1146.

Latifi, A., Winson, M.K., Foglino, M., Bycroft, B.W., Stewart, G.S., Lazdunski, A., and Williams, P. (1995) Multiple homologues of LuxR and LuxI control expression of virulence determinants and secondary metabolites through quorum sensing in *Pseudomonas aeruginosa* PAO1. *Mol Microbiol* **17**: 333-343.

Layh, N., Hirrlinger, B., Stolz, A., and Knackmuss, H.J. (1997) Enrichment strategies for nitrile-hydrolysing bacteria. *Appl Microbiol Biotechnol* **47**: 668-674.

Lee, L.Y., and Gelvin, S.B. (2008) T-DNA binary vectors and systems. *Plant Physiol* **146**: 325-332.

Lehman, A., Black, R., and Ecker, J.R. (1996) HOOKLESS1, an ethylene response gene, is required for differential cell elongation in the *Arabidopsis* hypocotyl. *Cell* **85**: 183-194.

Lerat, E., and Moran, N.A. (2004) The evolutionary history of quorum-sensing systems in bacteria. *Mol Biol Evol* **21**: 903-913.

Lewenza, S., Conway, B., Greenberg, E.P., and Sokol, P.A. (1999) Quorum sensing in *Burkholderia cepacia*: identification of the LuxRI homologs CepRI. *J Bacteriol* **181**: 748-756.

Li, H., Johnson, P., Stepanova, A., Alonso, J.M., and Ecker, J.R. (2004) Convergence of signaling pathways in the control of differential cell growth in Arabidopsis. *Dev Cell* **7**: 193-204.

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

Li, P.L., Everhart, D.M., and Farrand, S.K. (1998) Genetic and sequence analysis of the pTiC58 trb locus, encoding a mating-pair formation system related to members of the type IV secretion family. *J Bacteriol* **180**: 6164-6172.

Lindum, P.W., Anthoni, U., Christophersen, C., Eberl, L., Molin, S., and Givskov, M. (1998) N-Acyl-L-homoserine lactone autoinducers control production of an extracellular lipopeptide biosurfactant required for swarming motility of *Serratia liquefaciens* MG1. *J Bacteriol* **180**: 6384-6388.

Lithgow, J.K., Wilkinson, A., Hardman, A., Rodelas, B., Wisniewski-Dye, F., Williams, P., and Downie, J.A. (2000) The regulatory locus cinRI in *Rhizobium*

leguminosarum controls a network of quorum-sensing loci. *Mol Microbiol* **37**: 81-97.

Liu, J.W., DeMichele, S., Bergana, M., Bobik, E.J., Hastilow, C., Chuang, L.T. *et al.* (2001) Characterization of oil exhibiting high gamma-linolenic acid from a genetically transformed canola strain. *J. Am. Oil Chem. Soc.* **78**: 489-493.

Liu, X., Bimerew, M., Ma, Y., Muller, H., Ovadis, M., Eberl, L. *et al.* (2007) Quorum-sensing signaling is required for production of the antibiotic pyrrolnitrin in a rhizospheric biocontrol strain of *Serratia plymuthica*. *FEMS Microbiol Lett* **270**: 299-305.

Lumjiaktase, P., Diggle, S.P., Loprasert, S., Tungpradabkul, S., Daykin, M., Camara, M. *et al.* (2006) Quorum sensing regulates *dpsA* and the oxidative stress response in *Burkholderia pseudomallei*. *Microbiology* **152**: 3651-3659.

Ma, W., Guinel, F.C., and Glick, B.R. (2003a) *Rhizobium leguminosarum* biovar viciae 1-aminocyclopropane-1-carboxylate deaminase promotes nodulation of pea plants. *Appl Environ Microbiol* **69**: 4396-4402.

Ma, W., Charles, T.C., and Glick, B.R. (2004) Expression of an exogenous 1-aminocyclopropane-1-carboxylate deaminase gene in *Sinorhizobium meliloti* increases its ability to nodulate alfalfa. *Appl Environ Microbiol* **70**: 5891-5897.

Ma, W., Sebestianova, S.B., Sebestian, J., Burd, G.I., Guinel, F.C., and Glick, B.R. (2003b) Prevalence of 1-aminocyclopropane-1-carboxylate deaminase in *Rhizobium* spp. *Antonie Van Leeuwenhoek* **83**: 285-291.

Malott, R.J., and Sokol, P.A. (2007) Expression of the *bviIR* and *cepIR* quorum-sensing systems of *Burkholderia vietnamiensis*. *J Bacteriol* **189**: 3006-3016.

Marketon, M.M., and Gonzalez, J.E. (2002) Identification of two quorum-sensing systems in *Sinorhizobium meliloti*. *J Bacteriol* **184**: 3466-3475.

Marketon, M.M., Gronquist, M.R., Eberhard, A., and Gonzalez, J.E. (2002) Characterization of the *Sinorhizobium meliloti sinR/sinI* locus and the production of novel N-acyl homoserine lactones. *J Bacteriol* **184**: 5686-5695.

Marketon, M.M., Glenn, S.A., Eberhard, A., and Gonzalez, J.E. (2003) Quorum sensing controls exopolysaccharide production in *Sinorhizobium meliloti*. *J Bacteriol* **185**: 325-331.

Mayak, S., Tirosh, T., and Glick, B.R. (2004a) Plant growth-promoting bacteria confer resistance in tomato plants to salt stress. *Plant Physiol Biochem* **42**: 565-572.

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

Mayville, P., Ji, G., Beavis, R., Yang, H., Goger, M., Novick, R.P., and Muir, T.W. (1999) Structure-activity analysis of synthetic autoinducing thiolactone peptides from *Staphylococcus aureus* responsible for virulence. *Proc Natl Acad Sci U S A* **96**: 1218-1223.

McClean, K.H., Winson, M.K., Fish, L., Taylor, A., Chhabra, S.R., Camara, M. *et al.* (1997) Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones. *Microbiology* **143 (Pt 12)**: 3703-3711.

McGowan, S.J., Bycroft, B.W., and Salmond, G.P. (1998) Bacterial production of carbapenems and clavams: evolution of beta-lactam antibiotic pathways. *Trends Microbiol* **6**: 203-208.

McGowan, S.J., Sebaihia, M., O'Leary, S., Hardie, K.R., Williams, P., Stewart, G.S. *et al.* (1997) Analysis of the carbapenem gene cluster of *Erwinia carotovora*: definition of the antibiotic biosynthetic genes and evidence for a novel beta-lactam resistance mechanism. *Mol Microbiol* **26**: 545-556.

Miller, J. (1972) *Experiments in molecular genetics*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Milton, D.L., Hardman, A., Camara, M., Chhabra, S.R., Bycroft, B.W., Stewart, G.S., and Williams, P. (1997) Quorum sensing in *Vibrio anguillarum*: characterization of the *vanI/vanR* locus and identification of the autoinducer N-(3-oxodecanoyl)-L-homoserine lactone. *J Bacteriol* **179**: 3004-3012.

Nadeem SM, Zahir ZA, Naveed M, Arshad M (2007) Preliminary investigations on inducing salt tolerance in maize through inoculation with rhizobacteria containing ACC deaminase activity. Can J Microbiol 53:1141–1149.

Nakagawa, N., Mori, H., Yamazaki, K., and Imaseki, H. (1991) Cloning of a complementary DNA for auxin-induced 1-aminocyclopropane-1-carboxylate synthase and differential expression of the gene by auxin and wounding. *Plant Cell Physiol* **32**: 1153-1163.

Nakayama, J., Cao, Y., Horii, T., Sakuda, S., Akkermans, A.D., de Vos, W.M., and Nagasawa, H. (2001) Gelatinase biosynthesis-activating pheromone: a peptide lactone that mediates a quorum sensing in *Enterococcus faecalis*. *Mol Microbiol* **41**: 145-154.

Nasser, W., Bouillant, M.L., Salmond, G., and Reverchon, S. (1998) Characterization of the *Erwinia chrysanthemi expI-expR* locus directing the synthesis of two N-acyl-homoserine lactone signal molecules. *Mol Microbiol* **29**: 1391-1405.

National Research Council (U.S.). 2007. *New science of metagenomics: revealing the secrets of our microbial planet*. Washington, DC: National Academies Press

Nealson, K.H., and Hastings, J.W. (1979) Bacterial bioluminescence: its control and ecological significance. *Microbiol Rev* **43**: 496-518.

Nieto Penalver, C.G., Morin, D., Cantet, F., Saurel, O., Milon, A., and Vorholt, J.A. (2006) *Methylobacterium extorquens* AM1 produces a novel type of acyl-homoserine lactone with a double unsaturated side chain under methylotrophic growth conditions. *FEBS Lett* **580**: 561-567.

Nonaka, S., Sugawara, M., Minamisawa, K., Yuhashi, K., and Ezura, H. (2008a) 1-Aminocyclopropane-1-carboxylate deaminase enhances *Agrobacterium tumefaciens*-mediated gene transfer into plant cells. *Appl Environ Microbiol* **74**: 2526-2528.

Nonaka, S., Yuhashi, K., Takada, K., Sugaware, M., Minamisawa, K., and Ezura, H. (2008b) Ethylene production in plants during transformation suppresses *vir* gene expression in *Agrobacterium tumefaciens*. *New Phytol* **178**: 647-656.

Norman-Setterblad, C., Vidal, S., and Palva, E.T. (2000) Interacting signal pathways control defense gene expression in *Arabidopsis* in response to cell wall-degrading enzymes from *Erwinia carotovora. Mol Plant Microbe Interact* **13**: 430-438.

Oelck, M.M., Phan, C.V., Eckes, P., Donn, G., Rakow, G., and Keller, W.A. (1991) Field resistance of canola transformants (*Brassica napus* L.) to Ignite (phosphinothricin).

In *Proceedings of the GCIRC Eighth International Rapeseed Congress*. McGregor, D.I. (ed). Saskatoon, Saskatchewan Canadap,, pp. 292-297.

Olson, D.C., White, J.A., Edelman, L., Harkins, R.N., and Kende, H. (1991) Differential expression of two genes for 1-aminocyclopropane-1-carboxylate synthase in tomato fruits. *Proc Natl Acad Sci U S A* **88**: 5340-5344.

Ostergaard, L., Kempin, S.A., Bies, D., Klee, H.J., and Yanofsky, M.F. (2006) Pod shatter-resistant Brassica fruit produced by ectopic expression of the FRUITFULL gene. *Plant Biotechnol J* **4**: 45-51.

Patten, C.L., and Glick, B.R. (2002) Role of *Pseudomonas putida* indoleacetic acid in development of the host plant root system. *Appl Environ Microbiol* **68**: 3795-3801.

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

Petri, C., Alburquerque, N., Perez-Tornero, O., and Burgos, L. (2005) Auxin pulses and a synergistic interaction between polyamines and ethylene inhibitors improve adventitious regeneration from apricot leaves and *Agrobacterium*-mediated transformation of leaf tissues. *Plant Cell Tiss Org* **82**: 105-111.

Pierik, R., Tholen, D., Poorter, H., Visser, E.J., and Voesenek, L.A. (2006) The Janus face of ethylene: growth inhibition and stimulation. *Trends Plant Sci* **11**: 176-183.

Pierson, L.S., 3rd, Keppenne, V.D., and Wood, D.W. (1994) Phenazine antibiotic biosynthesis in *Pseudomonas aureofaciens* 30-84 is regulated by PhzR in response to cell density. *J Bacteriol* **176**: 3966-3974.

Piper, K.R., Beck von Bodman, S., and Farrand, S.K. (1993) Conjugation factor of *Agrobacterium tumefaciens* regulates Ti plasmid transfer by autoinduction. *Nature* **362**: 448-450.

Piper, K.R., Beck Von Bodman, S., Hwang, I., and Farrand, S.K. (1999) Hierarchical gene regulatory systems arising from fortuitous gene associations: controlling quorum sensing by the opine regulon in *Agrobacterium*. *Mol Microbiol* **32**: 1077-1089.

Pirhonen, M., Flego, D., Heikinheimo, R., and Palva, E.T. (1993) A small diffusible signal molecule is responsible for the global control of virulence and exoenzyme production in the plant pathogen *Erwinia carotovora*. *Embo J* **12**: 2467-2476.

Prayitno, J., Rolfe, B.G., and Mathesius, U. (2006) The Ethylene-insensitive sickle mutant of *Medicago truncatula* shows altered auxin transport regulation during nodulation. *Plant Physiol* **142**: 168-180.

Prigent-Combaret, C., Blaha, D., Pothier, J.F., Vial, L., Poirier, M.A., Wisniewski-Dye, F., and Moenne-Loccoz, Y. (2008) Physical organization and phylogenetic analysis of *acdR* as leucine-responsive regulator of the 1-aminocyclopropane-1-carboxylate deaminase gene *acdS* in phytobeneficial *Azospirillum lipoferum* 4B and other Proteobacteria. *FEMS Microbiol Ecol* **65**: 202-219.

Pulawska, J., and Sobiczewski, P. (2005) Development of a semi-nested PCR based method for sensitive detection of tumorigenic *Agrobacterium* in soil. *J Appl Microbiol* **98**: 710-721.

Puskas, A., Greenberg, E.P., Kaplan, S., and Schaefer, A.L. (1997) A quorum-sensing system in the free-living photosynthetic bacterium *Rhodobacter sphaeroides*. *J Bacteriol* **179**: 7530-7537.

Rappe, M.S., and Giovannoni, S.J. (2003) The uncultured microbial majority. *Annu Rev Microbiol* **57**: 369-394.

Raymer, P.L. (2002) Canola: An emerging oilseed crop. . In *Trends in new crops and new uses.* Janick , J., and Whipkey, A. (eds). Alexandria, VA: ASHS Press, pp. 122–126.

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

Riaz, K., Elmerich, C., Moreira, D., Raffoux, A., Dessaux, Y., and Faure, D. (2008) A metagenomic analysis of soil bacteria extends the diversity of quorum-quenching lactonases. *Environ Microbiol* **10**: 560-570.

Riedel, K., Ohnesorg, T., Krogfelt, K.A., Hansen, T.S., Omori, K., Givskov, M., and Eberl, L. (2001) N-acyl-L-homoserine lactone-mediated regulation of the *lip* secretion system in *Serratia liquefaciens* MG1. *J Bacteriol* **183**: 1805-1809.

Riker, A.J., Banfield, W.M., Wright, W.H., Keitt, G.W., and Sagen, H.E. (1930) Studies on infectious hairy root of nursery trees of apples. *J Agric Res* **41**: 507-540.

Rivas, M., Seeger, M., Holmes, D.S., and Jedlicki, E. (2005) A Lux-like quorum sensing system in the extreme acidophile *Acidithiobacillus ferrooxidans*. *Biol Res* **38**: 283-297.

Rhee, J.K., Ahn, D.G., Kim, Y.G., and Oh, J.W. (2005) New thermophilic and thermostable esterase with sequence similarity to the hormone-sensitive lipase family, cloned from a metagenomic library. *Appl Environ Microbiol* **71**: 817-825.

Rondon, M.R., August, P.R., Bettermann, A.D., Brady, S.F., Grossman, T.H., Liles, M.R. *et al.* (2000) Cloning the soil metagenome: a strategy for accessing the genetic and functional diversity of uncultured microorganisms. *Appl Environ Microbiol* **66**: 2541-2547.

Rosemeyer, V., Michiels, J., Verreth, C., and Vanderleyden, J. (1998) *luxI*- and *luxR*-homologous genes of *Rhizobium etli* CNPAF512 contribute to synthesis of autoinducer molecules and nodulation of *Phaseolus vulgaris*. *J Bacteriol* **180**: 815-821.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular cloning: a laboratory manual*: Cold Spring Harbor Laboratory Press **1**: 25-27.

Sawada, H., Takikawa, Y., and Ieki, H. (1992) Fatty acid methyl ester profiles of the genus *Agrobacterium*. *Ann Phytopathol Soc Japan* **58**: 46-51.

Sawada, H., Ieki, H., and Matsuda, I. (1995) PCR detection of Ti and Ri plasmids from phytopathogenic *Agrobacterium* strains. *Appl Environ Microbiol* **61**: 828-831.

Schaefer, A.L., Taylor, T.A., Beatty, J.T., and Greenberg, E.P. (2002) Long-chain acyl-homoserine lactone quorum-sensing regulation of *Rhodobacter capsulatus* gene transfer agent production. *J Bacteriol* **184**: 6515-6521.

Schuster, M., Lostroh, C.P., Ogi, T., and Greenberg, E.P. (2003) Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis. *J Bacteriol* **185**: 2066-2079.

Sears, C.L. (2005) A dynamic partnership: celebrating our gut flora. *Anaerobe* **11**: 24

Seong, E.S., Song, K.J., Jegal, S., Yu, C., and Chung, I.M. (2005) Silver nitrate and aminoethoxyvingylglycine affect *Agrobacterium* mediated apple transformation. *Plant Growth Regul* **45**: 75-82.

Shah, S., Li, J., Moffatt, B.A., and Glick, B.R. (1998) Isolation and characterization of ACC deaminase genes from two different plant growth-promoting rhizobacteria. *Can J Microbiol* **44**: 833-843.

Shaw, P.D., Ping, G., Daly, S.L., Cha, C., Cronan, J.E., Jr., Rinehart, K.L., and Farrand, S.K. (1997) Detecting and characterizing N-acyl-homoserine lactone signal molecules by thin-layer chromatography. *Proc Natl Acad Sci U S A* **94**: 6036-6041.

Sigee, D.C. (1993) *Bacterial Plant Pathology: Cell and Molecular Aspects.*: Cambridge University Press.

Sitnikov, D.M., Schineller, J.B., and Baldwin, T.O. (1996) Control of cell division in *Escherichia coli*: regulation of transcription of *ftsQA* involves both *rpoS* and *SdiA*-mediated autoinduction. *Proc Natl Acad Sci U S A* **93**: 336-341.

Slater, S.C., Goldman, B.S., Goodner, B., Setubal, J.C., Farrand, S.K., Nester, E.W. et al. (2009) Genome sequences of three agrobacterium biovars help elucidate the evolution of multichromosome genomes in bacteria. *J Bacteriol* **191**: 2501-2511.

Smith, E.F., and Townsend, C.O. (1907) A plant-tumor of bacterial origin. *Science* **25**: 671-673.

Smith, K.M., Bu, Y., and Suga, H. (2003) Library screening for synthetic agonists and antagonists of a *Pseudomonas aeruginosa* autoinducer. *Chem Biol* **10**: 563-571.

Sjöling, S., Stafford, W., and Cowan, D.A. (2007) Soil metagenomics: Exploring and expoiting the soil microbial gene pool. In *Modern Soil Microbiology*. Elsas, J.D. van, Jansson, J.K., and Trevors, J.T. (eds): CRC press/Taylor and Francis Group.

Stearns, J.C., and Glick, B.R. (2003) Transgenic plants with altered ethylene biosynthesis or perception. *Biotechnol Adv* **21**: 193-210.

Steidle, A., Allesen-Holm, M., Riedel, K., Berg, G., Givskov, M., Molin, S., and Eberl, L. (2002) Identification and characterization of an N-acylhomoserine lactone-dependent quorum-sensing system in *Pseudomonas putida* strain IsoF. *Appl Environ Microbiol* **68**: 6371-6382.

Stepanova, A.N., Hoyt, J.M., Hamilton, A.A., and Alonso, J.M. (2005) A link between ethylene and auxin uncovered by the characterization of two root-specific ethylene-insensitive mutants in *Arabidopsis*. *Plant Cell* **17**: 2230-2242.

Stewart Jr, C.N., Adang, M.J., All, J.N., Raymer, P.L., Ramachandran, S., and Parrott, W.A. (1996) Insect control and dosage effects in transgenic canola containing a synthetic *Bacillus thuringiensis cryIAc* gene. *Plant Physiol* **112**: 115-120.

Stoutjesdijk, P.A., Hurlestone, C., Singh, S.P., and Green, A.G. (2000) High-oleic acid Australian *Brassica napus* and *B. juncea* varieties produced by co-suppression of endogenous Delta12-desaturases. *Biochem Soc Trans* **28**: 938-940.

Sun, Y., Cheng, Z., and Glick, B.R. (2009) The presence of a 1-aminocyclopropane-1-carboxylate (ACC) deaminase deletion mutation alters the physiology of the endophytic plant growth-promoting bacterium *Burkholderia phytofirmans* PsJN. *FEMS Microbiol Lett* **1:** 131-136

Swarup, R., Parry, G., Graham, N., Allen, T., and Bennett, M. (2002) Auxin cross-talk: integration of signalling pathways to control plant development. *Plant Mol Biol* **49**: 411-426.

Swift, S., Williams, P., and Stewart, G.S.A.B. (1999a) N-acylhomoserine lactones and quorum sensing in proteobacteria. In *Cell-cell signalling in bacteria*. Dunny, G.M., and Winans, S.C. (eds). Washington, D.C: ASM press, pp. 291-313.

Swift, S., Lynch, M.J., Fish, L., Kirke, D.F., Tomas, J.M., Stewart, G.S., and Williams, P. (1999b) Quorum sensing-dependent regulation and blockade of exoprotease production in *Aeromonas hydrophila*. *Infect Immun* **67**: 5192-5199.

Swift, S., Karlyshev, A.V., Fish, L., Durant, E.L., Winson, M.K., Chhabra, S.R. *et al.* (1997) Quorum sensing in *Aeromonas hydrophi*la and *Aeromonas salmonicida*: identification of the LuxRI homologs AhyRI and AsaRI and their cognate N-acylhomoserine lactone signal molecules. *J Bacteriol* **179**: 5271-5281.

Swift, S., Winson, M.K., Chan, P.F., Bainton, N.J., Birdsall, M., Reeves, P.J. *et al.* (1993) A novel strategy for the isolation of luxI homologues: evidence for the widespread distribution of a LuxR:LuxI superfamily in enteric bacteria. *Mol Microbiol* **10**: 511-520.

Swofford, D. (2000) PAUP*. Phylogenetic analysis using Parsimony (*and Other Methods). In. Sunderland, MA: Sinauer Associates.

Throup, J.P., Camara, M., Briggs, G.S., Winson, M.K., Chhabra, S.R., Bycroft, B.W. *et al.* (1995) Characterisation of the *yenI*/*yenR* locus from *Yersinia enterocolitica* mediating the synthesis of two N-acylhomoserine lactone signal molecules. *Mol Microbiol* **17**: 345-356.

Tinland, B. (1996) The integration of T-DNA into plant genomes. *Trends in Plant Science* **1**: 178-184.

Tittabutr, P., Awaya, J.D., Li, Q.X., and Borthakur, D. (2008) The cloned 1-aminocyclopropane-1-carboxylate (ACC) deaminase gene from *Sinorhizobium sp*. strain BL3 in *Rhizobium sp*. strain TAL1145 promotes nodulation and growth of *Leucaena leucocephala*. *Syst Appl Microbiol* **31**: 141-150.

Tringe, S.G., von Mering, C., Kobayashi, A., Salamov, A.A., Chen, K., Chang, H.W. et al. (2005) Comparative metagenomics of microbial communities. *Science* **308**: 554-557.

Trott, S., Bauer, R., Knackmuss, H.J., and Stolz, A. (2001) Genetic and biochemical characterization of an enantioselective amidase from *Agrobacterium tumefaciens* strain D3. *Microbiology* **147**: 1815-1824.

Tyson, G.W., Chapman, J., Hugenholtz, P., Allen, E.E., Ram, R.J., Richardson, P.M. et al. (2004) Community structure and metabolism through reconstruction of microbial genomes from the environment. *Nature* **428**: 37-43.

Ullrich, C.I., and Aloni, R. (2000) Vascularization is a general requirement for growth of plant and animal tumors. *J. Exp. Bot.* **51**: 1951-1960.

van Loon, L.C., Geraats, B.P., and Linthorst, H.J. (2006) Ethylene as a modulator of disease resistance in plants. *Trends Plant Sci* **11**: 184-191.

Venter, J.C., Remington, K., Heidelberg, J.F., Halpern, A.L., Rusch, D., Eisen, J.A. et al. (2004) Environmental genome shotgun sequencing of the Sargasso Sea. *Science* **304**: 66-74.

Veselov, D., Langhans, M., Hartung, W., Aloni, R., Feussner, I., Gotz, C. *et al.* (2003) Development of *Agrobacterium tumefaciens* C58-induced plant tumors and impact on host shoots are controlled by a cascade of jasmonic acid, auxin, cytokinin, ethylene and abscisic acid. *Planta* **216**: 512-522.

Vial, L., Cuny, C., Gluchoff-Fiasson, K., Comte, G., Oger, P.M., Faure, D. *et al.* (2006) N-acyl-homoserine lactone-mediated quorum-sensing in *Azospirillum*: an exception rather than a rule. *FEMS Microbiol Ecol* **58**: 155-168.

Von Bodman, S.B., Bauer, W.D., and Coplin, D.L. (2003) Quorum sensing in plant-pathogenic bacteria. *Annu Rev Phytopathol* **41**: 455-482.

Wächter, R., Fischer, K., Gäbler, R., Kühnemann, F., Urban, W., Bögemann, G.M. *et al.* (1999) Ethylene production and ACC-accumulation in *Agrobacterium tumefaciens*-induced plant tumors and their impact on tumor and host stem structure and function. *Plant Cell Environ.* **22**: 1263-1273.

Wagner, V.E., Bushnell, D., Passador, L., Brooks, A.I., and Iglewski, B.H. (2003) Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment. *J Bacteriol* **185**: 2080-2095.

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

Wang, C., Meek, D.J., Panchal, P., Boruvka, N., Archibald, F.S., Driscoll, B.T., and Charles, T.C. (2006) Isolation of poly-3-hydroxybutyrate metabolism genes from

complex microbial communities by phenotypic complementation of bacterial mutants. *Appl Environ Microbiol* **72**: 384-391.

Waters, C.M., and Bassler, B.L. (2005) Quorum sensing: cell-to-cell communication in bacteria. *Annu Rev Cell Dev Biol* **21**: 319-346.

Watson, W.T., Minogue, T.D., Val, D.L., von Bodman, S.B., and Churchill, M.E. (2002) Structural basis and specificity of acyl-homoserine lactone signal production in bacterial quorum sensing. *Mol Cell* **9**: 685-694.

Wei, H.L., and Zhang, L.Q. (2006) Quorum-sensing system influences root colonization and biological control ability in Pseudomonas fluorescens 2P24. *Antonie Van Leeuwenhoek* **89**: 267-280.

White, L.O. (1972) The taxonomy of the crown-gall organism *Agrobacterium tumefaciens* and its relationship to rhizobia and other agrobacteria. *J Gen Microbiol* **72**: 565-574.

Whitman, W.B., Coleman, D.C., and Wiebe, W.J. (1998) Prokaryotes: the unseen majority. *Proc Natl Acad Sci U S A* **95**: 6578-6583.

Whiteley, M., Parsek, M.R., and Greenberg, E.P. (2000) Regulation of quorum sensing by RpoS in *Pseudomonas aeruginosa*. *J Bacteriol* **182**: 4356-4360.

Williams, P. (2007) Quorum sensing, communication and cross-kingdom signalling in the bacterial world. *Microbiology* **153**: 3923-3938.

Williamson, L.L., Borlee, B.R., Schloss, P.D., Guan, C., Allen, H.K., and Handelsman, J. (2005) Intracellular screen to identify metagenomic clones that induce or inhibit a quorum-sensing biosensor. *Appl Environ Microbiol* **71**: 6335-6344.

Winson, M.K., Camara, M., Latifi, A., Foglino, M., Chhabra, S.R., Daykin, M. *et al.* (1995) Multiple N-acyl-L-homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **92**: 9427-9431.

Wood, D.W., Gong, F., Daykin, M.M., Williams, P., and Pierson, L.S., 3rd (1997) N-acyl-homoserine lactone-mediated regulation of phenazine gene expression by *Pseudomonas aureofaciens* 30-84 in the wheat rhizosphere. *J Bacteriol* **179**: 7663-7670.

Wood, D.W., Setubal, J.C., Kaul, R., Monks, D.E., Kitajima, J.P., Okura, V.K. *et al.* (2001) The genome of the natural genetic engineer *Agrobacterium tumefaciens* C58. *Science* **294**: 2317-2323.

Worden, A.Z., Cuvelier, M.L., and Bartlett, D.H. (2006) In-depth analyses of marine microbial community genomics. *Trends Microbiol* **14**: 331-336.

Xavier, K.B., and Bassler, B.L. (2003) LuxS quorum sensing: more than just a numbers game. *Curr Opin Microbiol* **6**: 191-197.

Yamamoto, F., and Kozlowski, T.T. (1987) Effects of flooding, tilting of stems, and ethrel application on growth, stem anatomy and ethylene production of *Pinus densiflora* seedlings. *J Exp Bot* **38**: 293-310.

Yamamoto, F., Angeles, G., and Kozlowski, T.T. (1987) Effect of ethrel on stem anatomy of *Ulmus americana* seedlings. *IAWA Bulletin,* **n.s.**: 3-9.

Yan, Q., Gao, W., Wu, X.G., and Zhang, L.Q. (2009a) Regulation of the PcoI/PcoR quorum-sensing system in *Pseudomonas fluorescens* 2P24 by the PhoP/PhoQ two-component system. *Microbiology* **155**: 124-133.

Yan, Q., Wu, X.G., Wei, H.L., Wang, H.M., and Zhang, L.Q. (2009b) Differential control of the PcoI/PcoR quorum-sensing system in *Pseudomonas fluorescens* 2P24 by sigma factor RpoS and the GacS/GacA two-component regulatory system. *Microbiol Res* **164**: 18-26.

Yang, S.F., and Hoffman, N.E. (1984) Ethylene biosynthesis and its regulation in higher plants. *Ann Rev Plant Physiol* **35**: 155-189.

Yoon, I.S., Mori, H., Kim, J.H., Kang, B.G., and Imaseki, H. (1997) VR-ACS6 is an auxin-inducible 1-aminocyclopropane-1-carboxylate synthase gene in mungbean (*Vigna radiata*). *Plant Cell Physiol* **38**: 217-224.

Young, J.M., Kuykendall, L.D., Martinez-Romero, E., Kerr, A., and Sawada, H. (2001) A revision of Rhizobium Frank 1889, with an emended description of the genus, and the inclusion of all species of *Agrobacterium* Conn 1942 and *Allorhizobium* undicola de Lajudie et al. 1998 as new combinations: *Rhizobium radiobacter*, *R. rhizogenes*, *R. rubi*, *R. undicola* and *R. vitis*. *Int J Syst Evol Microbiol* **51**: 89-103.

Zahir, Z.A., Ghani, U., Naveed, M., Nadeem, S.M., and Asghar, H.N. (2009) Comparative effectiveness of *Pseudomonas* and *Serratia sp*. containing ACC-deaminase for improving growth and yield of wheat (*Triticum aestivum* L.) under salt-stressed conditions. *Arch Microbiol* **191**: 415-424.

Zhang, Y., and Bhalla, P.L. (2004) In vitro shoot regeneration from commercial cultivars of Australian canola (*Brassica napus* L.). *Austr. J. Agric. Res.* **55**: 753-756.

Zhang, Y., Singh, M.B., Swoboda, I., and Bhalla, P.L. (2005) Agrobacterium-mediated transformation and generation of male sterile lines of Australian canola. *Austr. J. Agric. Res.* **56**: 353-361.

Zhang, Y., Zhao, L., Wang, Y., Yang, B., and Chen, S. (2008) Enhancement of heavy metal accumulation by tissue specific co-expression of *iaaM* and ACC deaminase genes in plants. *Chemosphere* **72**: 564-571

Zheng, H., Zhong, Z., Lai, X., Chen, W.X., Li, S., and Zhu, J. (2006) A LuxR/LuxI-type quorum-sensing system in a plant bacterium, *Mesorhizobium tianshanense,* controls symbiotic nodulation. *J Bacteriol* **188**: 1943-1949.

Zhu, J., and Winans, S.C. (2001) The quorum-sensing transcriptional regulator TraR requires its cognate signaling ligand for protein folding, protease resistance, and dimerization. *Proc Natl Acad Sci U S A* **98**: 1507-1512.