Functional Study of a Protein (UnkG) in *Pseudomonas putida* UW4

by

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AUTHOR'S DECLARATION

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

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

Abstract

The role played by the protein UnkG from the plant growth-promoting bacterium *Pseudomonas putida* UW4 in the ability of the bacterium to facilitate plant growth was studied. Previous work showed that over-expressing UnkG decreased the ability of *P. putida* UW4 to facilitate plant growth. In contrast, an *unkG* knock-out mutant of *P. putida* UW4 displayed an increased ability to promote plant growth. Various biological activities of *P. putida* UW4, *P. putida* UW4/pETP and *P. putida* UW4/pETP-*unkG* have been compared. Thus, the growth curves were measured; the Biolog™ system was used to test the ability of these strains to utilize various carbon sources; the strains were observed by scanning electron microscopy to assess their relative cell sizes; biochemical assays were conducted to quantify 3-indoleacetic acid production and to measure the enzymatic activity of 1-aminocyclopropane-1-carboxylate (ACC) deaminase; proteome-level changes of *P. putida* UW4/pETP and *P. putida* UW4/pETP-*unkG* were profiled using two-dimensional difference in-gel electrophoresis (DIGE), followed by mass spectrometry identification of the altered proteins. After running DIGE, sixteen altered proteins were identified and their possible roles in the interactions between the bacterium and plants were discussed. Based on the preliminary results, we hypothesize that 1) UnkG may be detrimental to plant growth; 2) UnkG may negatively regulate a number of key cellular functions in a general way related to the energy balance of the bacterium.

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List of Abbreviations

2,4-D	2,4-dinitrophenylhydrazine
2-DE	two-dimensional electrophoresis
ACC	1-aminocyclopropane-1-carboxylate
ACN	acetonitrile
BSA	bovine serum albumin
cICAT	cleavable isotope-coded affinity tags
DHDPS	dihydrodipicolinate synthase
DIGE	difference in-gel electrophoresis
ETF	electron transfer flavoprotein
FAD	flavin adenine dinucleotide
Fpr	ferredoxin-NADP reductase
GlnRS	glutaminyl-tRNA synthetase
IAA	indole-3-acetic acid
IEF	isoelectric focusing
IPG	immobilized pH gradients
IPTG	isopropyl-ß-D-thiogalactoside
ISR	induced systemic resistance
iTRAQ	isobaric tags for relative and absolute quantification
LB	Luria-Bertani
LPS	lipopolysaccharides
MS	mass spectrometry
NCBI	National Center for Biotechnology Information
NHS	N-hydroxysuccinimide

OA	A–inner core–O-antigenic side chain
OD	optical density
P	phosphorus
PCMH	p-cresol methylhydroxylase
PGPB	plant growth-promoting bacterium
PR	pathogenesis-related
PTM	posttranslational modifications
SA	salicylic acid
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	scanning electron microscopy
TSB	Tryptic Soy broth
TSI	triple sugar iron

Chapter 1: Introduction

1.1 Plant growth-promoting bacterium (PGPB)

The term plant growth-promoting bacterium (PGPB) refers to the group of bacteria that exhibit beneficial effects on the growth of plants (Bashan and Holguin, 1998). Many bacteria of different genera have been studied and are considered to be PGPB, including *Azotobacter, Azospirillum, Pseudomonas, Acetobacter, Burkholderia* and *Bacillus* (Bashan and Holguin, 1997; Barraquio et al., 2000; Bilal and Malik, 1987; Boddey et al., 1995; James and Olivares, 1997; James et al., 2000; Malik et al., 1997; Reinhold-Hurek et al., 1993). It has been suggested that PGPB may be an ideal replacement for traditional fertilizers and pesticides because they are more environmentally friendly and sustainable (Glick et al., 1999).

1.2 Mechanisms of promoting by PGPB

Generally speaking, the mechanisms used by PGPB to promote the growth of plants can be classified into two groups: direct and indirect promotion (Glick, 1995a). Direct promotion includes facilitating the uptake of nutrients, providing some phytohormones, regulating the growth of plants with low molecular mass compounds or enzymes, and other mechanisms that have not been well characterized. Indirect promotion is mainly achieved through antibiosis, i.e. suppressing the growth of plant pathogens. The forms of antibiosis include secreting inhibitory molecules (mainly antibiotics), producing lytic enzymes that can degrade fungal cell walls, competing for nutrients in the vicinity of plant pathogens, and induced systemic resistance (ISR). One specific PGPB strain may use one or several mechanisms together to promote plant growth (Glick, 1995a).

1.2.1 Direct promotion

This kind of promotion has a direct effect on the growth of plants but not on plant pathogens. Direct promotion can be further divided into several classes based on different mechanisms used.

Facilitating nutrient uptake

Some PGPB strains can help plants take up nutrients, such as nitrogen, iron and phosphorus. Nitrogen fixing bacteria have been extensively studied for many years. Some of them can form a symbiotic relationship with plants while the others are free living. They convert free nitrogen in the atmosphere into ammonia. Plants can utilize the latter form, but not the former form of nitrogen. Plants, primarily those of the Fabaceae family, having a symbiotic relationship with nitrogen fixing bacteria, usually develop nodules on roots to provide these bacteria with nutrients (Glick, 1995a). However, it is worth noting that some PGPB strains can promote the growth of plant roots, thus increasing the uptake of nutrients. For example, in addition to nitrogen fixation, *Azospirillum* bacteria can promote the development and function of grass and legume roots, thereby improving mineral as well as water uptake (Okon et al., 1998).

Phosphorus (P) plays an important role in the nutrition of plants partly because it is a necessary component for cell membranes and nucleic acids. However, most of the P in soil is insoluble, in either organic or inorganic form. Every year, tons of inorganic P fertilizers are used to support the growth of plants in agriculture, but a large portion of the soluble P in these fertilizers quickly becomes insoluble after applying the P to the soil (Dey, 1988). To be utilized by plants, the insoluble P must first be converted into soluble ionic phosphate (Pi, HPO_4^{2-}, $H_2PO_4^-$), or low molecular-weight organic phosphate (Goldstein, 1994). A large number of phosphate-solubilizing bacteria exist in soil, especially in the rhizosphere of plants, including *Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Micrococcus, Aereobacter,*

Flavobacterium and *Erwinia* (McGrath et al., 1998; Sperberg, 1958; Katznelson et al., 1962; Raghu and MacRae, 1966; Louw and Webley, 1959; Das, 1963; Ostwal and Bhide, 1972; Bardiya and Gaur, 1974; Darmwall et al., 1989; Illmer and Schinner, 1992).

Mineral phosphate solubilization is mainly achieved through the action of organic acids synthesized by soil microorganisms (Halder et al., 1990; Duff and Webley, 1959; Sundara and Sinha, 1963; Banik and Dey, 1982; Craven and Hayasaka, 1982; Leyval and Berthelin, 1989; Salih et al., 1989). Gluconic acid acts as a major phosphate-solubilizing agent in the species of *Pseudomonas sp.* (Illmer and Schinner, 1992), *Erwinia herbicola* (Liu et al., 1992), *Pseudomonas cepacia* (Goldstein et al., 1993) and *Burkholderia cepacia* (Lin et al., 2006). Other organic acids that can solubilize mineral phosphate include 2-ketogluconic, lactic, isovaleric, isobutyric, glycolic, oxalic, malonic and succinic acid (Illmer and Schinner, 1992; Banik and Dey, 1982). In addition to the action of organic acids, other mechanisms, such as chelating substances and inorganic acids have also been considered though their effectiveness is not as apparent as organic acids (Rodríguez and Fraga, 1999).

Organic phosphate solubilization relies on the action of several phosphatases (i.e. phosphohydrolases), which, under acidic or alkaline conditions, catalyze the hydrolysis of phosphoester or phosphoanhydride bonds within the organic phosphorous compounds. To date, there has not been a comprehensive understanding of the properties, regulation and roles of these phosphatases, in part due to their complexity (Rodríguez and Fraga, 1999). Only a few organic phosphate solubilization genes have been cloned, sequenced and studied.

Providing phytohormones

Some studies show that various soil microorganisms can produce phytohormones, such as indole-3- acetic acid (IAA). IAA is one of the most important phytohormones. It has various effects on

plant growth and development, such as cell enlargement and division, tissue differentiation, responses to light and gravity (Taiz and Zeiger, 1998; Woodward and Bartel, 2005; Teale et al., 2006). It is estimated that about 80% of rhizosphere bacteria can synthesize IAA (Patten and Glick, 1996; Khalid et al., 2004). To date, the studies on IAA synthesis have identified five tryptophan-dependent pathways, i.e. tryptophan as a precursor, and one tryptophan-independent pathway. These pathways have been reviewed by Spaepen et al. (2007). It should be noted that some bacteria possess more than one pathway for IAA synthesis (Spaepen et al., 2007). For example, in 1998, Manulis et al. identified the genes for both the IAM and the IPyA pathways in *Pantoea agglomerans.*

Since many plant pathogens can also produce IAA, IAA has been suspected to be involved in plant disease development (Yamada, 1993; Spaepen et al., 2007). However, Sequeira's study (1965) indicates that during the initial critical interactions between tobacco and *Pseudomonas solanaceraum* (here as a pathogen), most of the IAA in the host is contributed by the host itself, supported by the fact that extracts of the host plant rather than that of the pathogen, can convert tryptamine (the precursor) to IAA. Subsequent studies suggest that IAA can repress the expression of pathogenesis-related (PR) genes (Kazan and Manners, 2009; Shinshi et al., 1987; Jouanneau et al., 1991). Spaepen et al. (2007) proposed two factors that may determine the positive or negative effect of bacterial IAA on the host plant: (1) the amount of IAA produced that is available to the plant and (2) the sensitivity of the plant tissue to changes in IAA concentration.

The study of Dobbelaere et al. (1999) on *Azospirillum* mutants (minus in IAA production) shows that IAA, produced by *Azospirillum*, is involved in increased rooting of wheat. In addition, Patten and Glick (2002) proved that the bacterial IAA played a major role in the development of the host plant root system. The uptake of minerals, water and nutrients by the host plant can be then

enhanced. In addition, the dose-response curve of roots to cultures with increasing concentrations of *Azospirillum* fits well with the dose-response curve of roots to increasing concentrations of IAA (Fallik et al., 1994). But in other cases, the production of bacterial IAA alone cannot account for the bacterial promoting effects on plants (Bashan and Holguin, 1997). So some bacteria may use other mechanisms, along with IAA production, to promote plant growth.

Regulating plant growth with low molecular mass compounds or enzymes

Some PGPB strains possess the ability to regulate the levels of low molecular mass compounds that can inhibit or promote plant growth, within plants. One such well-established example is the relationship between plant ethylene and bacterial 1-aminocyclopropane-1-carboxylate (ACC) deaminase. Ethylene plays a dual role in plant growth: beneficial and deleterious. It is involved in seed germination, root elongation, tissue differentiation, formation of root and shoot primordia, lateral bud development, flowing initiation, anthocyanin synthesis, flower opening and senescence, fruit ripening and de-greening, production of volatile organic compounds responsible for aroma formation in fruits, storage product hydrolysis, leaf and fruit abscission, and the response of plants to biotic and abiotic stress (Glick, 2004). The optimal concentration of ethylene on plant growth may vary at different stages of plant development. Higher concentration of ethylene may lead to an inhibitory effect on plant growth (Abeles et al., 1992). The high concentration of ethylene is associated with biological or environmental stress, such as pathogen attack (Wang et al., 2000), flooding (Grichko and Glick, 2001), salt (Mayak et al., 2004a), drought (Mayak et al., 2004b), heavy metals (Belimov et al., 2001, 2005; Burd et al., 1998) and so on.

The enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase (EC: 4.1.99.4), having been identified in many bacterial strains and fungi (Klee et al., 1991; Sheehy et al., 1991; Honma, 1993; Jacobson et al., 1994; Glick et al., 1995; Campbell and Thomson, 1996; Burd et al., 1998;

Minami et al., 1998; Belimov et al., 2001; Mayak et al., 2004b; Babalola et al., 2003; Ghosh et al., 2003; Ma et al., 2003b, c; Uchiumi et al., 2004; Belimov et al., 2005; Hontzeas et al., 2005; Blaha et al., 2006; Madhaiyan et al., 2006), can cleave ACC, the immediate precursor of ethylene, into α-ketobutyrate and ammonia. Glick et al. (1998) have proposed a model for this process (Figure 1-1). Briefly speaking, bacteria containing ACC deaminase first bind to the surface of either the seed or root of a plant. With the induction of tryptophan and other small molecules in seed and/or root exudates (Bayliss et al., 1997; Penrose and Glick, 2001; Whipps, 1990), the bacteria synthesize and secrete indole-3-acetic acid (IAA) (Fallik et al., 1994; Frankenberger and Arshad, 1995; Patten and Glick, 1996; Xie et al., 1996). A portion of these IAA can be taken by plants and then work together with plant endogenous IAA to stimulate plant cell proliferation or plant cell elongation and/or can induce the synthesis of the plant enzyme ACC synthase that converts S-adenosylmethionine to ACC (Kende, 1993). Along with other small molecules, some of these ACC will be exuded from seeds or plant roots. Bacteria containing ACC deaminase can then cleave the ACC in the exudates into ammonia and α-ketobutyrate, both of which are readily metabolized by soil microorganisms. As a result, the concentration of ethylene within the plant will decrease (Glick et al., 1998; Glick, 2004).

1.2.2 Indirect promotion

Secreting inhibitory molecules

Over the past several decades, there have been many studies demonstrating that some PGPB metabolites are hazardous to various plant pathogens. A great part of those are antibiotics. Many bacterial strains of different genera have been identified as antibiotic producers as summarized by Raaijmakers et al. (2002). Among these bacteria, *Pseudomonas fluorescens* is the most widely studied group (Raaijmakers et al., 2002). Studies show that PGPB strains vary in the type and number of antibiotics produced (Raaijmakers et al., 2002). For example, *Bacillus cereus* UW85 (Handelsman and Stabb, 1996) and *Pseudomonas fluorescens* CHA0 and Pf5 (Keel et al., 1996)

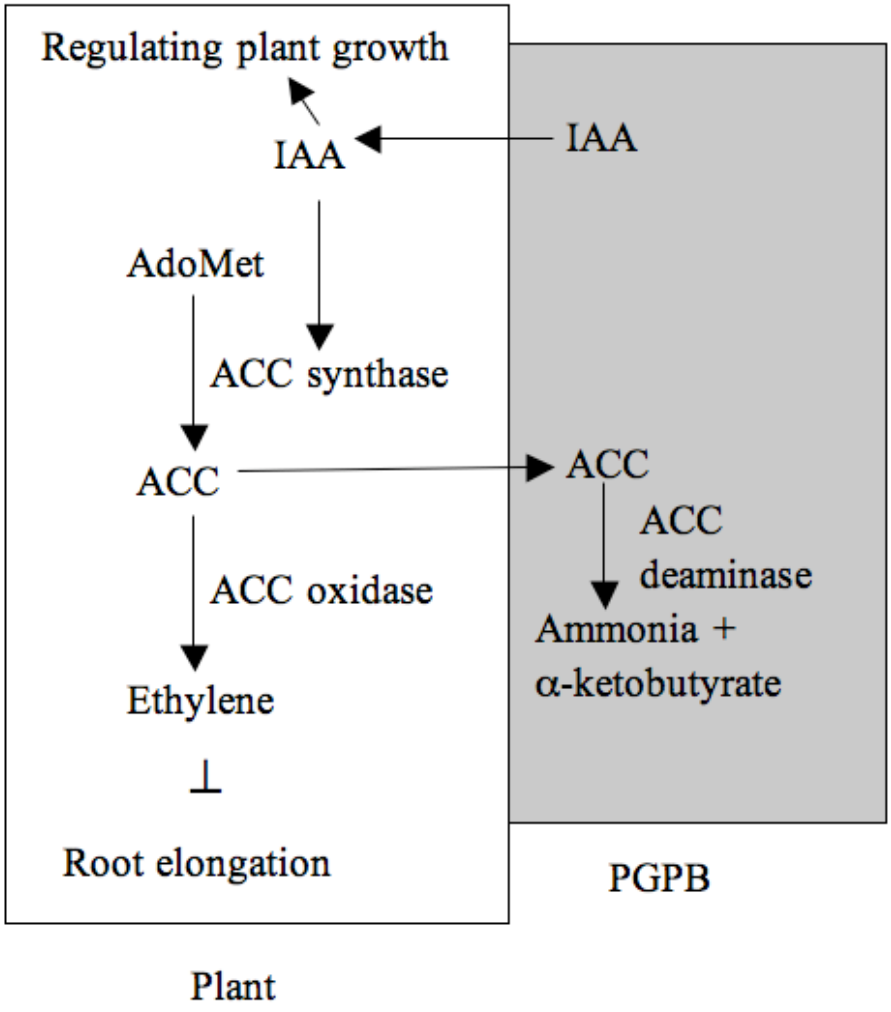


Figure 1- 1 Schematic representation of how a PGPB regulates plant ethylene levels. Adapted from Glick et al., 1998. The arrows indicate a chemical of physical step in the mechanism and the symbol ⊥ indicates the inhibition of root elongation by ethylene. Key: IAA, indole-3-acetic acid; ACC, 1-aminocyclopropane-1-carboxylate; AdoMet, S-adenosyl-methionine.

produce several types of antibiotics with overlapping or different degrees of activity against specific pathogenic fungi. These observations suggest that in some PGPB strains, different kinds of antibiotics may work together to suppress the growth of plant pathogens. Some antibiotics are broad-spectrum, i.e. able to inhibit more than one type of pathogen. One example is pyrrolnitrin. It has been shown in many studies to have activity against human pathogenic bacteria and fungi, such as *Basidiomycetes, Deuteromycetes, Ascomycetes*, as well as several Gram-positive bacteria, in particular *Streptomyces* species (Ligon et al., 2000; Ei-Banna and Winkelmann, 1998). As summarized by Raaijmakers et al. (2002), the role of antibiotics in biological control can be supported by the evidence that: (1) culture filtrates or purified antibiotics can achieve almost the same biological control level as achieved by the antibiotic-producing wild-type strain (Howell and Stipanovic, 1979; Kang et al., 1998; Nakayama et al., 1999); (2) in many cases, inactivation of antibiotic production by mutagenesis leads to a reduced ability of the bacteria to control the pathogen (Thomashow and Weller, 1988; Vincent et al., 1991; Cronin et al., 1997; Anjaiah et al., 1998; Chin-A-Woeng et al., 1998; Hokeberg et al., 1998); (3) over-production of antibiotics via introduction or modification of antibiotic biosynthetic or regulatory genes in the same strain results in enhanced biological control ability (Maurhofer et al., 1994; Ligon et al., 2000).

Producing lytic enzymes

Lytic enzymes such as chitinase, glucanase and protease, produced and released by some PGPB strains, can hydrolyze various molecules within plant pathogens, including chitin, proteins, cellulose, hemicellulose and DNA (Pal and Gardener, 2006). In some cases the activities of these enzymes may contribute to the suppression of plant pathogens. For example, chitinase seems to mediate the inhibition of *Sclerotium rolfsii* by *Serratia marcescens* (Ordentlich et al., 1988). Similarly, α β-1, 3-glucanse contributes significantly to biological control activities of *Lysobacter enzymogenes* strain C3 (Pal and Gardener, 2006; Palumbo et al., 2005). Lytic enzymes are needed to digest host cell walls when some biocontrol agents parasitize pathogens (Di Pietro et al., 1993).

Furthermore, some products of lytic enzyme activity may be involved in indirect disease suppression. For example, oligosaccharides derived from fungal cell walls can sometimes induce systemic resistance of plant hosts (Howell et al., 1988). However, due to the lack of sufficient studies on the interactions between lytic enzymes and plant pathogens, the exact role played by lytic enzymes in plant pathogen suppression is still unclear.

Competing for nutrients in the vicinity of plant pathogens

Within a given vicinity, nutrients, water and minerals will be finite and hence can only support the growth of a limited number of microorganisms. Thus, some bacteria have the potential to inhibit the growth of a plant pathogen if they have a greater ability to assimilate one or more kinds of nutrients and/or minerals and/or water when competing with the pathogen. Iron is a good example of this phenomenon. On the earth, most of iron is in the form of Fe^{3+}. However, Fe^{3+} is sparingly soluble in soil, about 10^{-18} M at pH 7.4 (Neilands et al., 1987). Some soil bacteria can secrete low molecular mass (~400-1000 Da) siderophores that have a high affinity (K_d=10^{-20} to 10^{-50}) for iron (Glick, 1995a; Castignetti and Smarrelli, 1986; Neilands and Leong, 1986; Briat, 1992). An iron-siderophore complex, which can be taken up by the bacteria but not the pathogen, will be formed after the siderophore binds to iron. Because of the high affinity of siderophores for iron, the bacteria will assimilate most of the soluble iron and thus the growth of pathogens will be inhibited. Since most plants can grow at much lower (about 1000-fold) iron concentrations than soil microorganisms (Glick, 1995a; O'Sullivan and O'Gara, 1992), and some plants can also utilize the iron-siderophore complex (Crowley et al., 1988; Bar-Ness et al., 1991, 1992; Wang et al., 1993), the growth of plants will not be harmed by the low concentration of soluble iron caused by siderophore-producing bacteria. In addition to their role in iron assimilation, siderophores may act as growth factors, and some are potent antibiotics (Leong, 1986; Neilands, 1981).

Induced systemic resistance (ISR)

PGPB can induce latent resistance mechanisms, i.e. enhanced defensive capacity, possessed by plants against some pathogens. ISR may be triggered by some chemicals, non-pathogens, avirulent forms of pathogens, incompatible races of pathogens, or virulent pathogens under certain circumstances where infection is stalled owing to environmental conditions (Loon et al., 1998). Accumulation of salicylic acid (SA) and PR proteins is a characteristic of ISR (Kessmann et al., 1994; Ryals et al., 1996; Sticher et al., 1997; Uknes et al., 1992; Ward et al., 1991). Bacterial determinants of ISR include lipopolysaccharides (LPS), salicylic acid (SA), siderophores (under iron-limiting conditions), and iron-regulated factors (De Meyer and Höfte, 1997a, b; Leeman et al., 1995a, b; Maurhofer et al., 1994; Van Peer and Schippers, 1992; Van Wees et al., 1997). Specifically, studies of Duijff et al. (1997) and Leeman et al. (1995c) indicate that the specificity within the structure of lipid A–inner core–O-antigenic side chain (OA) of the LPS plays a major role in determining the induction of ISR in some rhizobacteria, but the exact mechanism involved is still unknown.

1.3 Applications of PGPB

To date, many PGPB strains of various genera have been isolated, characterized and studied in the laboratory, greenhouse and/or in the field. PGPB can be applied in agriculture, horticulture, forestry and environmental restoration, although the application in agriculture is most intensively studied (Reed and Glick, 2004). PGPB in agriculture can increase germination rates, root growth, yield (including grain), leaf area, chlorophyll content, magnesium content, nitrogen content, protein content, hydraulic activity, tolerance to drought, shoot and root weights, delayed leaf senescence and resistance to plant pathogens (Reed and Glick, 2004). Although the variability and inconsistency of results between laboratory, greenhouse and field studies has in the past limited the application of PGPB (Mishustin and Naumova, 1962), there have been many commercial PGPB products (Chet and Chernin, 2002; Reed and Glick, 2004). Interestingly, some

researchers are trying to achieve better promotion by co-inoculating plants with PGPB strains, non-PGPB microorganisms and organic and/or inorganic substances (Adesemoye et al., 2008; Jäderlund et al., 2008; Karakurt et al., 2009; Dardanelli et al., 2008). Unlike the application in agriculture, the aspects of biomass, seedling emergence and reduction in seedling transplant injury during the transfer from the nursery to the field rather than fruit and grain yield should be taken into account when considering using PGPB in forestry (Reed and Glick, 2004; Shishido and Chanway, 2000). Research in this field has been developed world-wide and many PGPB strains have been isolated (Reed and Glick, 2004). Another promising application of PGPB is in environmental remediation, such as phytoremediation. PGPB can relieve the inhibition stress to plants caused by flood or drought, salt, acidic or basic conditions, high or low temperature, and contamination by metals and other hazardous substances. Although the research in this field is currently limited (Reed and Glick, 2004), this activity continues to increase over time (Glick, 2010).

1.4 Two-dimensional electrophoresis (2-DE)

Proteomics, a term first coined by Peter James in 1997, is the large-scale study of proteins, particularly their structures and functions (Anderson and Anderson, 1998; Blackstock and Weir, 1999). With developments in biology, it becomes clear that gene study alone cannot give us enough information on the respective protein product. For example, many proteins are subject to posttranslational modifications (PTM), such as phosphorylation, glucosylation, ubiquitinylation, sumoylation, and many others (Mann and Jensen, 2001; Schweppe et al., 2003; Canovas et al., 2004). Those modifications play an important role in regulating protein functions and increasing the diversity and complexity of the proteome (Nouwens et al., 2000). In addition, in prokaryotes, protein expression may be regulated at the transcriptional, as well as translational, level. Thus, the use of proteomics is necessary to better understand a variety of cellular process.

A main strategy in proteomic studies is the comparison between two or more proteomes of different treatments to find out the proteins with changed expression levels. For example, to investigate the cold response of a bacterium strain, we can have two treatments of the same bacterium: one grows at optimal temperature and the other at a lower temperature. Then the proteins with changed expression levels, identified in a proteomic study, are quite possibly involved in the cold response of the organism. Currently, there are several available approaches used in proteomic studies, such as two-dimensional electrophoresis (2-DE) (Klose, 1975; O'Farrell, 1975), difference in-gel electrophoresis (DIGE) (Ünlü et al., 1997), cleavable isotope-coded affinity tags (cICAT) (Gygi et al., 1999), and isobaric tags for relative and absolute quantification (iTRAQ) (Ross et al., 2004).

The technique of 2-DE, was first developed in the 1970s for large-scale protein separation (Klose, 1975; O'Farrell, 1975), and is still the most widely used method in proteomic studies. This method consists of two sequential orthogonal separations of a protein mixture. After extraction from cells using appropriate methods, the protein samples are subject to a first dimension separation based on their different isoelectric points by isoelectric focusing (IEF). Then, in the second dimension, the proteins are separated according to their molecular masses by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The method of 2-DE can separate hundreds to thousands of proteins on a single gel, depending on the gel size. The well-isolated protein spots on the gel may be subjected to various subsequent analyses, such as Western blotting, visualization by pre-electrophoresis fluorescence labeling, post-electrophoresis staining with Coomassie blue, silver staining or SYPRO dyes, differential expression analysis, and identification by Edman degradation or mass spectrometry (MS) (Cheng et al., 2010).

Over the years, the quality of 2-DE, in terms of sensitivity, linearity, reliability and reproducibility, has been greatly improved by technical developments. Protein samples can be

enriched or pre-fractioned, or separated on narrow-range or ultra-narrow-range immobilized pH gradient strips to display more proteins, especially those of very low abundance (Corthals et al., 2000; Görg et al., 2009; Stasyk and Huber, 2004). Membrane proteins with a high level of hydrophobicity can be analyzed in 2-DE by incorporating thiourea, acetonitrile or detergents such as tetradecanoylamide-propyl-dimethyl ammonio-propane-sulforate in the sample buffer (Görg et al., 2009; Nouwens et al., 2000). The problems with highly alkaline proteins, such as ribosomal and nuclear proteins, can also be solved by adding isopropanol to the rehydration buffer and using pH gradients up to 12 (Görg et al., 1997, 1998, 1999, 2009; Hoving et al., 2002). Commercialized reagents and immobilized pH gradients (IPG), and more or less standardized procedures have increased the reliability and reproducibility of 2-DE (Görg et al., 1988, 1995, 2000, 2009).

1.5 Difference in-gel electrophoresis (DIGE)

Difference in-gel electrophoresis (DIGE), first termed as difference gel electrophoresis (DIGE) was originally introduced by Ünlü et al. (1997) on the basis of 2-DE. This method brings much greater sensitivity, accuracy and reproducibility, compared with the more traditional 2-DE methods.

GE Healthcare developed a commercialized Ettan™ DIGE system for this method which is now widely employed in proteomic studies. It uses three CyDye fluors (Cy2, Cy3 and Cy5) to label protein samples, followed by two-dimensional separation, thus allowing the separation of up to three different protein pools on the same isoelectric focusing (IEF) strip (first dimension) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (DIGE) gel (second dimension). Finally, each pool of proteins can be visualized independently by selecting specific excitation and emission wavelengths for the respective CyDyes when fluorescence scanning. By doing so, the method minimizes gel-to-gel variations, which have been one of the main problems with traditional 2-DE methods.

Another advantage of this system is that the protein spots on the final gel can be isolated directly for further identification e.g. using methods such as mass spectrometry. The CyDye fluors used in this system have an N-hydroxysuccinimide (NHS) ester reactive group and can covalently attach to the epsilon amino group of lysine via an amide linkage. The amount of these dyes that is used to label proteins is quite limited so that only 1-2% of the available lysine residues, approximately one lysine residue per protein molecule, is labeled. Thus, the method is referred to as 'minimal labeling'. As a result, the mass spectrometry data following DIGE separation is not affected by the labeling, as only 1 lysine digest site is lost per labeled protein.

1.6 *Pseudomonas putida* UW4

Pseudomonas putida UW4 was isolated from the rhizosphere of common reeds growing on the campus of the University of Waterloo (Waterloo, ON), based on its ability to utilize ACC as a sole source of nitrogen as well as to promote the growth of canola seedlings under gnotobiotic conditions (Cheng et al., 2007; Glick et al., 1995). At first, this bacterium was classified as *Pseudomonas sp.,* then as *Enterobacter cloacae* based on fatty acid profiles (Cheng et al., 2007; Shah et al., 1998), and subsequently, in 2005, Hontzeas et al. re-classified this strain as *P. putida* based on 16S rDNA sequence analysis and metabolic profiles. Recently, the entire genome of *P. putida* UW4 has been sequenced (Duan et al., unpublished results). The sequence data indicate that the bacterium has 7 16S rDNA genes, all of which are consistent with a designation of *P. putida.*

One mechanism used by *P. putida* UW4 to promote plant growth is the activity of 1-aminocyclopropane-1-carboxylate (ACC) deaminase to lower the concentration of ethylene in plants; and the ACC deaminase gene within *P. putida* UW4 as well as its transcriptional regulation have been studied (Shah et al., 1998; Grichko and Glick, 2001; Li and Glick, 2001; Hontzeas et al., 2004; Cheng et al., 2008). To date, there have been many studies showing that *P.*

putida UW4 is able to promote the growth of several kinds of plants alone or together with other microorganisms under various conditions (Grichko and Glick, 2001; Hontzeas et al., 2004; Cheng et al., 2007; Gamalero et al., 2008; Gamalero et al., 2010).

1.7 UnkG protein

In a proteomic study of bacterial proteins mediating the interaction between *Pseudomonas putida* UW4 and *Brassica napus* (canola), Cheng et al. (2009a), using two-dimensional difference in-gel electrophoresis, compared the proteomes of *P. putida* UW4 and the mutant strain *P. putida* UW4/AcdS$^-$ (which lacks ACC deaminase activity) after treating each strain with canola root exudates. Based on the results of these experiments, a function-unknown protein (UnkG) with an isoelectric point of 4.26 and a protein mass of 27, 000 Da, was identified that might be involved in mediating a portion of the interaction between *P. putida* UW4 and canola. Thus, it was observed that *P. putida* UW4 had a greater root-length-promoting ability when the *unkG* gene was knocked out. In contrast, root growth was inhibited relative to untreated plants when the *unkG* gene was over-expressed (Cheng et al., 2009a). The gene sequence encoding the UnkG protein has been determined (Appendix I) and similar sequences exist in some other *Pseudomonas* strains. To date, there has not been any study on the UnkG protein and there is no information as to what role this protein might play in the interaction between bacteria that contain this protein and plants.

1.8 Objective of this study

The objective of this study is to elaborate the role played by UnkG in the functioning of *P. putida* UW4, which may help us to better understand the mechanism(s) used by PGPB to promote the growth of plants. Thus, in this study we over-expressed UnkG in *P. putida* UW4 and then investigated any differences between the wild-type and the altered bacterial strains on

physiological, biochemical and proteomic levels, with the expectation that any differences observed would provide some information regarding the function of UnkG in *P. putida* UW4.

Chapter 2: Experimental Procedures

2.1 Bacterial strains, plasmids and culture conditions

All strains used in this study (Table 2-1) were from the culture collection of our lab. In most cases, *P. putida* UW4 wild-type strain and its transformant strains, *P. putida* UW4/pETP-*unkG* and *P. putida* UW4/pETP, were cultivated aerobically in Tryptic Soy broth (TSB; Fisher Scientific Co., Ottawa, ON) or on solid TSB medium at 30℃. For transformant strains, 50 µg/ml kanamycin was added to the medium as selective pressure. In addition, for *P. putida* UW4/pETP-*unkG*, the culture medium was supplemented with 1 mM isopropyl-ß-D-thiogalactoside (IPTG) to induce the expression of *unkG* on the plasmid. IPTG was added to medium before inoculation.

Escherichia coli DH5α (Hanahan, 1983) was used as the host strain for the construction and maintenance of recombinant plasmids. *E. coli* DH5α was cultivated aerobically in Luria-Bertani broth or solid medium (LB; Fisher Scientific Co., Ottawa, ON) at 37℃.

The DF minimal medium (Dworkin and Foster, 1958) used in this project was prepared as follows (Penrose and Glick, 2003): (1) the trace elements (10 mg H_3BO_3, 11.19 mg $MnSO_4 \cdot H_2O$, 124.6 mg $ZnSO_4 \cdot 7H_2O$, 78.22 mg $CuSO_4 \cdot 5H_2O$, and 10 mg MoO_3) were dissolved in 100 ml sterile distilled water and then stored in the refrigerator; (2) 100 mg $FeSO_4 \cdot 7H_2O$ was dissolved in 10 ml sterile distilled water and stored in the refrigerator; (3) all of the other ingredients including 4.0 g KH_2PO_4, 6.0 g Na_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 2.0 g glucose, 2.0 g gluconic acid, 2.0 g citric acid, 2.0 g $(NH_4)_2SO_4$ (in some cases, ACC was added to the final concentration of 3 mM instead of $(NH_4)_2SO_4$) and 0.1 ml of each of the solutions of trace elements and $FeSO_4 \cdot 7H_2O$ were dissolved in 1 l distilled water and autoclaved for 30 minutes.

Table 2- 1 Bacterial strains and plasmids used in this study.

[1] In that paper, *unkG* was named *hyp.*

Strain	Genotype	Reference
E. coli DH5α	*F80d/lacZ M15, recA1, endA1, gyr96, thi^{-1}, hsdR17, (rk^{-}, mk^{+}), supE44, relA1, (lacZYA-a rgF)U169*	Hanahan, 1983
P. putida		
UW4	Wild-type, PGPB, plasmid free	Glick et al., 1995
UW4/pETP	UW4 mutant, containing plasmid pETP	Cheng et al., 2009a[1]
UW4/pETP-*unkG*	UW4 mutant, containing plasimd pETP-*unkG*	Cheng et al., 2009a
Plasmid		
pGEM-T easy	Cloning vector, Amp^{R}	Promega
pET30a(+)	T7 terminator, 6xHis-tag, *lac*O, T7 promoter, Orf0, Km^{R}, f1 origin, pBR322 origin	Novagen
pUCP26	pUC18 derivative, *Escherichia-Pseudomonas* shuttle vector, Tet^{R}	West et al., 1994
pETP	pET30a(+) vector derivative with ori 1600 from pUCP26 inserted into SphI site, Km^{R}	Cheng et al., 2009a
pETP-*unkG*	pETP vector derivative with *unkG* fragment form *P. putida* UW4 genome inserted into NotI site, Km^{R}	Cheng et al., 2009a

2.2 Mutant strain construction

2.2.1 Isolation of total genomic DNA

A Wizard® Genomic Purification Kit (Promega Corporation, Madison, WI) was used to isolate genomic DNA from bacteria, following exactly the protocol for gram-negative bacteria suggested by the manufacturer. Bacteria were grown overnight in 5 ml of liquid medium in a shaking waterbath (200 rpm) at 37℃ for *E. coli* and 30℃ for *P. putida* UW4. Then a 1 ml aliquot of the bacterial culture was transferred to a 1.5 ml microcentrifuge tube and centrifuged at 16,000×g for 2 minutes. After removing the supernatant, 600 µl of Nuclei Lysis Solution (Promega Corp.) was added to the tube to resuspend the cells. The mixture was incubated at 80℃ for 5 minutes to lyse the cells, and then cooled to room temperature. Three µl of an RNase Solution (Promega) was added, mixed with the lysate and incubated at 37℃ for 30 minutes. To precipitate proteins, 200 µl of Protein Precipitation Solution (Promega) was added to the RNase-treated cell lysate and vortexed vigorously for 30 seconds. The sample was incubated on ice for 5 minutes and then centrifuged at 16,000×g for 3 minutes. The supernatant, which contains the genomic DNA, was transferred to a clean 1.5 ml microcentrifuge tube containing 600 µl of room temperature isopropanol. After gently mixing the contents, the thread-like genomic DNA was visible. Following centrifugation at 16,000×g for 2 minutes, the supernatant was carefully decanted. Then, 600 µl of 70% ethanol at room temperature was added to the tube and gently mixed with the genomic DNA by inverting the tube several times. The mixture was centrifuged at 16,000×g for 2 minutes and then carefully aspirated to remove the ethanol. The tube was left at room temperature with the cap open for 15 minutes to allow any remaining ethanol to evaporate. Finally, 100 µl of DNA Rehydration Solution (Promega) was added to the tube and incubated at

65℃ for 1 hour with periodic mixing. The genomic DNA solution was subsequently stored at -20℃ for future use.

2.2.2 Isolation of plasmid DNA

For plasmid DNA isolation, an EZ-10 Spin Column Plasmid DNA Miniprep Kit (Bio Basics Inc., Markham, ON) was used, following the manufacturer's protocol for purification of low copy number plasmids. The bacterium was grown in 5 ml liquid medium and then the culture was pelleted in a 1.5 ml microcentrifuge tube by centrifugation at 10,000×g for 2 minutes each time. After decanting the supernatant, 200 µl of Solution Ⅰ was added to the pellet, mixed well by vortexing for 5 seconds, and incubated at room temperature for 1 minute. Then, 400 µl of Solution Ⅱ was added to the mixture, mixed gently by inverting the tube 4-6 times, and incubated at room temperature for 1 minute. An aliquot of 700 µl of solution III was added, mixed gently and incubated at room temperature for 1 minute. After centrifuging at 10,000×g for 10 minutes, half of the supernatant was added to an EZ-10 column and kept at room temperature for 2 minutes. The sample was then centrifuged at 10,000×g for 2 minutes, the flow-through in the tube was discarded and the second half of the supernatant was added to the column which was centrifuged at 10,000×g for 2 minutes. After discarding the flow-through in the tube, 500 µl of Wash Solution was added to the column and centrifuged at 10,000×g for 2 minutes. The wash procedure was repeated once. After discarding the second flow-through in the tube, the column was centrifuged at 10,000 rpm for an additional 1 minute to remove any residual Wash Solution. The column was transferred to a clean 1.5 ml microcentrifuge tube and 50 µl of Elution Buffer was added to the column. After standing at room temperature for 2 minutes, the column was centrifuged at 10,000×g for 2 minutes. Finally, the plasmid DNA was stored at -20℃ for future use.

2.2.3 DNA amplification by PCR

The *unkG* gene (528 bp, Appendix I) was amplified from *P. putida* UW4 genomic DNA with primers: 5' ATGTTGAATGACCCGATTCC 3' (sense) and 5' CTAAACGTTTGGGTCACGCTT 3' (antisense). The PCR reaction mixture was prepared on ice as follows: 25 µl GoTaq® Green Master Mix (Promega), 2.5 µl of 20 pM of each primer, 100 ng genomic DNA of *P. putida* UW4 as template and deionized water to a final volume of 50 µl. The mixture was vortexed and centrifuged briefly. PCR amplification was carried out using a Mastercycler® Thermal Cyclers (Eppendorf) with the following program: one cycle of 95℃for 5 minutes; 30 cycles of 95℃for 1 minute, 58℃for 1 minute, and 72℃for 30 seconds; then one cycle of 72℃for 5 minutes. The PCR product was then subjected to gel electrophoresis.

2.2.4 Electrophoresis and gel extraction

Electrophoresis was used for verification and purification of PCR amplification, as well as restriction endonuclease digestion, products. The procedure was carried out following standard protocols (Sambrook and Russell, 2001). A 1% agarose gel was made by adding 1.2 g agarose into 120 ml 1×TAE buffer (40 mM Tris-acetate, 1 mM EDTA). The mixture was heated for about 3 minutes, using a microwave oven to melt the agarose, and then cooled at room temperature. Just before the gel solidified, 2 µl of ethidium bromide solution (10 mg/ml) was added and mixed well with the gel by gentle shaking. The gel mixture was poured into a mold, which already had an appropriate comb to form the sample slots in the gel, and left at room temperature for 30 minutes to solidify. A GeneRuler™ 1 kb DNA Ladder (Thermo Fisher Scientific Inc.) was used as a DNA sizing standard. After the samples were added to the slots in the gel, electrophoresis was carried out in 1×TAE buffer under 120 V voltage until samples or dyes migrated an appropriate distance through the gel. The gel was subsequently examined and photographed under UV light.

When necessary, the DNA band of the correct size was excised from the gel using a clean Ra20R blade and put into a clean 1.5 ml microcentrifuge tube. An EZ-10 Spin Column Gel Extraction Kit (Bio Basics Inc., Markham, ON) was used for gel extraction. Four hundred µl of Binding Buffer Ⅱ for each 100 mg of gel weight was added to the tube containing the gel slice and incubated at 60℃for about 10 minutes to let the gel slice dissolve completely. The mixture was transferred to the EZ-10 column and kept at room temperature for 2 minutes. It was then centrifuged at 10,000×g for 2 minutes and the flow-through was discarded. Five hundred µl of Wash Solution was added to the column and centrifuged at 10,000×g for 1 minute. The flow-through was discarded. The wash procedure was repeated once and then the column was centrifuged at 10,000×g for an additional minute to remove any residual Wash Solution. The column was transferred to a clean 1.5 ml microcentrifuge tube and 30 µl of Elution Buffer was added to the centre of the membrane inside the column. After incubating at room temperature for 2 minutes, the column was centrifuged at 10,000×g for 2 minutes to elute the DNA. The DNA solution was stored at -20℃ for future use.

2.2.5 Restriction endonuclease digestion and ligation

All restriction endonucleases and digestion buffers were purchased from Thermo Fisher Scientific Inc. The digestion system was made as follows: 5 µl of FastDigest® Green Buffer, two units of each enzyme, and 0.1-1 µg of DNA sample to a final volume of 50 µl. The mixture was briefly vortexed, centrifuged to mix and incubated at 37℃ for 2 hours, followed by appropriate thermal inactivation of the enzyme(s), as instructed by the manufacturer.

T4 DNA ligase and its buffer (Promega Corporation, Madison, WI) were used to carry out ligation. In a sterile 1.5 ml microcentrifuge tube, the 10 µl of ligation mixture contained 1 µl of Ligase 10× Buffer, 0.1-1 unit T4 DNA Ligase, both the vector DNA and insert DNA with the

optimal molar ratio 3:1 of insert to vector. The total DNA in the mixture was about 100-200 ng. The mixture was incubated at 4℃ overnight.

2.2.6 Transformation of *E. coli*

An aliquot of 100 µl competent *E. coli* cells in a 1.5 ml microcentrifuge tube was taken out from a -80℃ freezer and left on ice for 20 minutes to thaw. After incubating at 4℃ overnight, the above ligation mixture was added to the competent *E. coli* cells and mixed gently. The tube was incubated on ice for 30 minutes and then heat-shocked at 42℃ for 90 seconds. The tube was then returned to ice for another 5 minutes. Six hundred µl of room temperature LB liquid medium was added to the mixture and incubated at 37℃ for 90 minutes with shaking. After incubating, the tube was centrifuged at 5,000 × g for 2 minutes and 500 µl of supernatant was decanted. The pellet was resuspended in the remaining supernatant and plated evenly onto two LB agar plates containing appropriate selective antibiotics. The plates were incubated overnight at 37℃.

2.2.7 DNA sequencing

Plasmids and the sequencing primers were sent to the Molecular Biology Sequencing Facility at York University for DNA sequencing analysis. Cycle sequencing reactions were done with BigDye Terminator chemistry on an Applied Biosystems 3130xL DNA Sequencer.

2.2.8 Construction *P. putida* UW4/pETP–*unkG* and *P. putida* UW4/pETP

P. putida UW4/pETP–*unkG* was constructed to over-express *unkG* in *P. putida* UW4 while *P. putida* UW4/pETP served as a control to see whether any differences between wild-type *P. putida* UW4 and *P. putida* UW4/pETP–*unkG* were caused by introducing the plasmid (pETP) that carries *unkG.* Those two strains were constructed and confirmed by Cheng et al. (2009a). First,

the *Pseudomonas-Escherichia* shuttle expression vector pETP was constructed (Figure 2-1). Using a pair of primers: 5' ATAGCATGCTTCCGGCTCGTATGTTGTGT 3'(sense, PI) and 5' TGCAGGCATGCAAGCTTGGCA 3' (antisense, PO), the 1557-bp fragment containing ori 1600 was PCR amplified from pUCP26 (West et al., 1994). The primer PI has an incorporated SphI site while PO contains an original SphI site. The PCR fragment and pET30a (+) vector (Novagen) were digested with SphI and then ligated together. The resulting vector was named pETP (Figure 2-1).

The gene encoding UnkG was PCR amplified as previously described. Then the PCR product was cloned into pGEM-T easy vector (Promega Corporation, Madison, WI) to get pGEM-*unkG*. To ensure the correct cloning, sequencing was carried out at the Molecular Biology Sequencing Facility at York University (Toronto, ON).

Plasmid pGEM-*unkG* was digested with NotI, and the released *unkG* fragment was inserted into the NotI site of pETP. The resulting new plasmid was designated as pETP-*unkG*. Plasmid pETP-*unkG* was re-sequenced using primers: 5' GGGCTGAATTTCCTTTGACATG 3' (UnkGSF) and 5' CACGGGCAGAGCGGGATTTTTT 3' (UnkGSR) to make sure that it was constructed as desired. Plasmids pETP-*unkG* and pETP were introduced into wild-type *P. putida* UW4 by electroporation as described by Smith and Iglewski (1989) to get *P. putida* UW4/pETP-*unkG* and *P. putida* UW4/pETP, respectively.

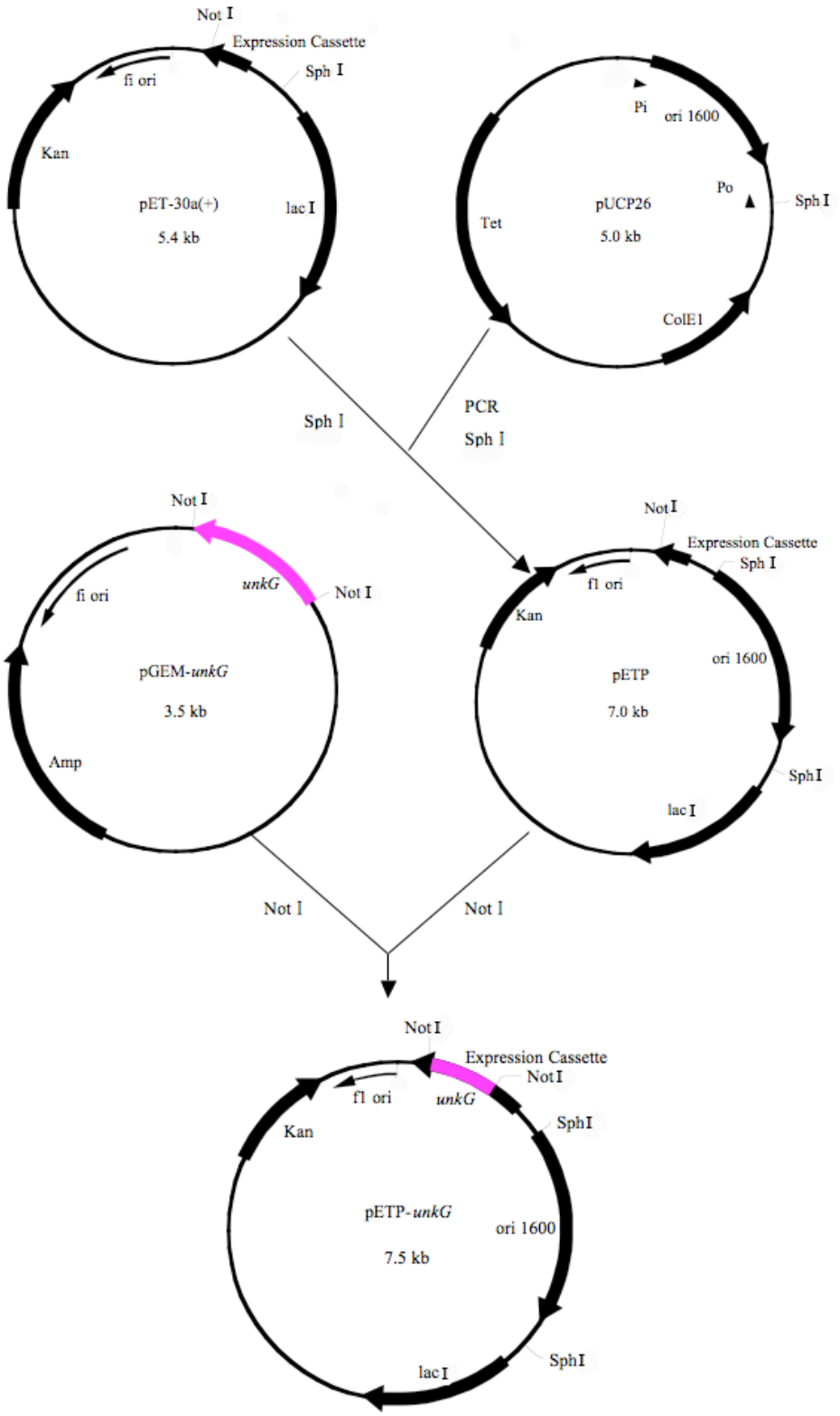


Figure 2- 1 Schematic representation of the procedure to construct pETP and pETP-*unkG*.

Western blotting was performed to confirm the expression of 6×His-tagged recombinant proteins in *P. putida* UW4/pETP-*unkG* but not in *P. putida* UW4/pETP. A mouse monoclonal His tag antibody (Novagen) and an HRP-conjugated anti-mouse IgG secondary antibody (Sigma, Aldrich Canada Ltd.) were used to detect the 6×His-tagged proteins. The immune-complexes were visualized by chemiluminescence using an ECL Plus Western blotting detection kit (GE Healthcare) (Cheng et al., 2009a).

2.3 Growth rate

Wild-type *P. putida* UW4 and *P. putida* UW4/pETP-*unkG* were grown in DF or TSB medium at three different temperatures, 15℃, room temperature (i.e. ~22℃) and 30℃. DF is a minimal medium while TSB serves as a rich medium. A temperature of 30℃ is optimal for bacterial growth. The seed culture was made by transferring a single colony to 5 ml liquid TSB (or DF) medium and growing overnight with shaking (200 rpm) at 30℃. Then, 100 µl of the seed culture was added to a flask containing 100 ml of fresh liquid TSB (or DF) medium. The culture was then grown at 15℃/room temperature/30℃ with shaking (200 rpm) and the optical density (OD) at 600 nm was measured every four hours using a Varian Cary 50 UV/Vis Spectrophotometer (Agilent Technologies Canada Inc.).

2.4 Biolog

GN2 MicroPlates™ (Biolog Inc., Hayward, CA) were used to test the ability of wild-type and transformed bacteria to utilize various carbon sources. First, the standard triple sugar iron (TSI) test and oxidase test were performed to determine whether the bacteria were enteric. Then a single colony growing on an agar plate was inoculated into a tube containing 5 ml TSB liquid medium and incubated at 30℃ with shaking (200 rpm) for 20 hours. A bacterial lawn of the

culture was made on the surface of a Bug agar plate using a sterile swab and incubated at 30℃ for 20 hours. The Bug agar plates were made as follows: Bug powder (Biolog Inc., Hayward, CA): 14.4g, maltose: 1g, agar: 6g, deionized water to 400 ml, autoclave and pour plates. As instructed by the manufacturer, an appropriate amount of the culture on the agar plate was transferred to 100 ml sterile GN/GP-IF solution (NaCl: 0.4%, pluronic F-68: 0.03%, gellan gum: 0.02%) and mixed to get a bacterial suspension of OD_{590} 0.2±0.01. The GN2 Microplate was inoculated using the bacterial suspension (150 µl/well). After incubating at 30℃ for 16-24 hours, the plates were scanned using Biolog MicroLog3 4.01C software (Biolog Inc., Hayward, CA).

2.5 Scanning electron microscopy (SEM)

Bacteria to be examined were grown overnight in 5 ml TSB medium at 30℃ with shaking (200 rpm). One ml of the culture was transferred to a clean 1.5 ml microcentrifuge tube and centrifuged at 5,000×g for 5 minutes. After decanting the supernatant, 1 ml sterile phosphate buffer pH 7.0 (0.2 M $NaH_2PO_4 \cdot 2H_2O$, 0.2 M $Na_2HPO_4 \cdot 2H_2O$) was added and mixed with cells. After standing at room temperature for 20 minutes, the tube was centrifuged at 5,000×g for 5 minutes. The above wash procedure was repeated twice. To fix the cells, 1 ml 2.5% glutaraldehyde in the above-mentioned phosphate buffer was added and mixed with the cells. The mixture was kept at room temperature for 1 hour. The cells were washed on a filter with phosphate buffer 3 times, each time for 5 minutes. Then the cells were dehydrated on the filter by washing with increasing concentrations of acetone, i.e. 20%, 50%, 70% and 100%. For the first two concentrations, the wash was done once, while for the others it was washed twice. Each wash was done for 10 minutes at room temperature. The cells were subjected to critical point drying and then coated with gold. Finally, the cell samples were observed using a scanning electron microscope (Model: S570; Hitachi Canada Ltd.). Here, we want to thank Dale Weber,

Department of Biology, University of Waterloo, Canada for his valuable help and instructions on sample preparation and SEM operation.

2.6 Growth pouch assay

Growth pouch assays were performed as described by Penrose and Glick (2003). The seed culture was made by inoculating one single colony into 5 ml liquid TSB medium followed by growth overnight at 30℃ with shaking (200 rpm). Fifteen µl of the seed culture was used to inoculate a 50 ml tube containing 15 ml fresh liquid TSB medium which was then incubated at the same conditions for 24 hours. The tube was centrifuged at 10,000×g, 4℃ for 10 minutes. After decanting the supernatant, the cells were washed by adding 5 ml sterile DF medium (without ACC or $(NH_4)_2SO_4$) to the tube and mixed by vortexing. The tube was centrifuged using the same condition as before, then the supernatant was decanted. The wash procedure was repeated twice. Finally, 7.5 ml sterile DF medium (without ACC or $(NH_4)_2SO_4$) and 45 µl 0.5 M ACC solution were added and mixed well with the cells. The tube was incubated at 30℃ with shaking (200 rpm) for 24 hours.

Meanwhile, growth pouches (Cyg seed germination pouch, Mega International, St. Paul, MN) were put on a rack and 20 ml of water was added to each pouch. The racks carrying the pouches were put into a plastic bin and the top of the bin was covered with aluminum foil. The bin with its contents as well as 4 L water in big flasks were autoclaved for 45 minutes.

The tubes containing the bacterial culture were centrifuged at 10,000×g, 4℃ for 10 minutes to remove the supernatant. Then, the cells were washed by adding 5 ml sterile 0.03 M $MgSO_4$ and mixed by gentle pipetting. The cells were centrifuged at 10,000×g, 4℃ for 10 minutes and the

supernatant was decanted. The wash procedure was repeated twice. A volume of 0.03 M $MgSO_4$ was added and mixed with the cells to obtain a bacterial suspension with a final OD_{600} 0.15.

About 100 *Brassica napus* var. Thunder canola seeds (Ontario seed company, Waterloo, ON) were put into a sterile Petri plate. The seeds were soaked in 70% ethanol for 1 minute followed by 1% sodium hypochlorite for 10 minutes. The seeds were washed thoroughly with sterile water five times and then they were treated with the above-mentioned bacterial suspension or 0.03 M $MgSO_4$ (as a control) at room temperature for 1 hour. After the treatment, 7 of the treated seeds were put onto the top of each growth pouch and 2 L sterile water was poured into the bin. The aluminum foil was put back on top of the bin and the pouches was incubated in a growth chamber (model: PGW36; Conviron Ltd., Winnipeg, MB) with the following conditions: mixed incandescent and fluorescent lighting (160 $\mu mol \cdot m^{-2} \cdot s^{-1}$; 400-700 nm), programmed for a 16-hour photoperiod, day-night cycle, with day and night temperatures of 22 and 18℃ respectively. On the third day, the aluminum foil was replaced with cling wrap. After six days, when the average root length was over 2 cm, the root length was measured. For each treatment, 50 (treated with *P. putida* UW4/pETP) to 59 (treated with the other three) seedlings were selectively counted.

2.7 Biochemical assays

2.7.1 Indole-3-acetic acid (IAA) assay

Using a modified version of the procedure of Patten and Glick (2002), the assay was performed to quantify the IAA production in the bacteria. The bacteria were cultivated in 5 ml liquid TSB medium at 30℃ with shaking (200 rpm) overnight. Then 20 µl of the culture was transferred to 5 ml liquid TSB medium containing either 200 µg/ml or 500 µg/ml L-tryptophan (Sigma-Aldrich Canada). The culture was incubated at 30℃ with shaking (200 rpm) for 42 hours. After incubation, the optical density of the culture was measured at 600 nm. Then 1.4 ml of the culture

was transferred to a 1.5 ml microcentrifuge tube and centrifuged at 6,000 ×g for 10 minutes to remove the cells. One ml of the supernatant or the same volume of a standard IAA solution (0, 0.5, 1, 5, 10, 15, 20, 25, 40 µg/ml IAA) was transferred to a 15 ml tube containing 4 ml of Salkowski's reagent (150 ml of concentrated H_2SO_4, 250 ml of deionized water, 7.5 ml of 0.5 M $FeCl_3 \cdot 6H_2O$; Gordon and Weber, 1951). After mixing vigorously by vortexing at high speed, the mixture was incubated at room temperature for 20 minutes followed by measuring the absorbance at 535 nm. The concentration in the samples was determined by comparing its absorbance with the standard curve. The experiment was done with 4 replicates for each treatment.

2.7.2 1-aminocyclopropane-1-carboxylate (ACC) deaminase assay

The method described by Penrose and Glick (2003) was followed for this assay. Bacteria were grown as described for the growth pouch assay. After incubation, the tube was centrifuged at 10,000×g, 4℃ for 10 minutes to remove the supernatant. The cells were washed by adding 5 ml 0.1 M Tris-HCl pH 7.6. After vortexing to mix cells with buffer, the tube was centrifuged using the same conditions to remove the supernatant. The washing procedure was repeated twice. One ml of the buffer was added to the tube to resuspend the cells. The mixture was transferred to a 1.5 ml microcentrifuge tube and centrifuged to remove the supernatant. Four hundred µl of 0.1 M Tris-HCl pH 8.5 and 20 µl toluene were added to the pellet and mixed with the cells by vortexing at high speed for 30 seconds. At this point, 100 µl aliquots of the "toluenized cells" were set aside and stored at 4℃ for protein assay at a later time.

In new 1.5 ml microcentrifuge tubes, 50 µl of "toluenized cells" were mixed with 5 µl of 0.5 M ACC solution. In addition, 50 µl of "toluenized cells" were mixed with 5 µl of deionized water as a negative control, and 50 µl of 0.1 M Tris-HCl pH 8.5 was mixed with 5 µl of 0.5 M ACC solution as a blank. The sample tubes were prepared in duplicate. The tubes were vortexed for 5

seconds and incubated at 30℃ for 30 minutes. After incubation, 500 µl of 0.56 M HCl was added and mixed with the cells. The tubes were centrifuged at 10,000×g for 10 minutes and 500 µl of the supernatant or standard α-ketobutyrate solution (0.1, 0.2, 0.4, 0.6, 0.8, 1 mM) was transferred to a clean glass tube containing 400 µl of 0.56 M HCl. One hundred and fifty µl of 2,4-dinitrophenylhydrazine (2,4-D) reagent (0.2% 2,4-D in 2 M HCl) was added to each glass tube and mixed by vortexing for 5 seconds. After incubating the tubes at 30℃ for 30 minutes, 1 ml of 2 M NaOH was added to each tube followed by measuring the absorbance at 540 nm.

The method of Bradford (1976) was used to determine the concentration of proteins in the toluenized cells. First, 40 µl of the cell solution was mixed with 160 µl of 0.1 M Tris-HCl of pH 8 and 200 µl 0.1 M NaOH in a 1.5 ml microcentrifuge tube. Each sample was done in duplicate. The tubes were sealed with parafilm and boiled in a water bath for 10 minutes. After cooling, 8 µl of the sample or a standard bovine serum albumin (BSA) solution (0.2, 0.4, 0.6, 0.8, 1.0 mg/ml) was mixed with 792 µl of deionized water and 200 µl of Bio-Rad protein dye reagent (Bio-Rad Laboratories (Canada) Ltd.). After mixing, the contents were incubated at room temperature for 5 minutes and the optical density value at 595 nm was measured. The protein concentration was determined by comparing the OD value with the BSA standard curve.

2.8 Proteomic study

2.8.1 Protein extraction

Fifty ml of liquid TSB medium was inoculated with 20 µl of bacterial seed culture prepared as described as earlier. For each sample of *P. putida* UW4/pETP-*unkG* (treated sample) and *P. putida* UW4/pETP (control sample), there were four replicates. The bacteria were cultivated at 30℃ with shaking (200 rpm) overnight. The following steps were carried out at 4℃. Cells were

harvested by centrifuging at 8,000×g, for 10 minutes to get cell pellet. The pellet was washed twice with cold water. After washing, the pellet was weighed and kept in liquid nitrogen for one minute followed by incubation at -80℃ for 30 minutes. The pellet was put on ice for 15 minutes to thaw and resuspended in about 10 ml (5 ml/g wet cell) of cold lysis buffer (30 mM Tris-HCl pH 8.5, 8 M urea, 4% CHAPS, adjusted to pH 8.5 with a final volume of 50 ml. Dithiothreitol was added to a final concentration of 10 mM just before use). The mixture was kept at 4℃ with shaking (100 rpm) for 1 hour and then subjected to sonication for five times, each time for 30 seconds, with a 30-second break after each sonication. The lysate was centrifuged at 10,000×g, for 10 minutes to sediment the cell debris. The supernatant was transferred to a clean 15 ml ultracentrifuge tube once the centrifugation was done and then centrifuged at 150,000×g (45,000 rpm in Beckman Ti 45 rotor) for 3 hour to sediment cell membranes. The final supernatant was distributed into 1.5 ml microcentrifuge tubes and stored at -20℃.

2.8.2 Sample preparation for difference in-gel electrophoresis

The concentration of the protein samples was measured using the method of Bradford as described above. Then, a specific amount of protein sample containing 200 µg protein was treated using a 2-D Clean-Up Kit (GE Healthcare). This kit is designed to reduce the conductivity, interfering substances and increase the concentration in the protein sample by selectively precipitating proteins. The protein sample was first distributed evenly into several 1.5 ml microcentrifuge tubes with each one containing 30-50 µl of the sample. The procedure for sample volumes of 1-100 µl (containing 1-10 µg protein per sample) was followed. Three hundred µl of Precipitant Solution was added to each tube and mixed well by vortexing or inversion. The tubes were incubated on ice for 15 minutes. Then 300 µl of Co-Precipitant Solution was added to the mixture and mixed by vortexing. The tubes were centrifuged twice at 12,000×g, 4℃ for 5 minutes to remove all of the supernatant. Forty µl of Co-Precipitant Solution was laid on top of

the pellet and incubated on ice for 5 minutes. The tubes were centrifuged as before to remove the supernatant. Twenty-five µl of deionized water was added on top of each pellet followed by 1 ml of Wash Buffer (pre-chilled for at least 1 hour at -20℃) and 5 µl of Wash Additive. The pellet was vortexed until it was fully dispersed. The tubes were incubated at -20℃ for at least 30 minutes with 30-second vortex every 10 minutes. After incubation, the tubes were centrifuged as before to remove the supernatant. The visible white pellet was air-dried for 3 minutes at room temperature and resuspended in 200 µl of lysis buffer (25 mM Tris-HCl of pH 8.0; 2 M thiourea; 7 M urea; 4% CHAPS; adjust pH to 8). The tubes were centrifuged using the same conditions and each supernatant was transferred to a new 1.5 ml microcentrifuge tube for further use.

2.8.3 Difference in-gel electrophoresis

Amersham CyDye™ DIGE Fluors (GE Healthcare) were used to label the protein samples. Both treated and control samples have four replicates, each containing 50 µg protein. Internal standards were prepared as four replicates with each having 25 µg protein of treated sample and 25 µg protein of control. Protein samples were divided into four groups, each having one internal standard, one protein sample of treated and one of control. Each protein sample was treated with 400 pmol of Cy2, Cy3 or Cy5 DIGE fluors according to the supplier's instructions. After labeling, the protein samples within each group were mixed and rehydration buffer (8 M urea, 2% CHAPS, 0.002% bromophenol blue, distilled water) was added to a final volume of 450 µl. Then 9 µl of 1 M dithiothreitol and 2.25 µl IPG buffer pH 4-7 (GE Healthcare) was added and mixed with the 450 µl mixture. The contents of each group was loaded on Immobiline DryStrips (pH 4-7, 24 cm; GE Healthcare) and incubated at room temperature for 14 hours. Since the majority of proteins in *P. putida* UW4 fall into the pH range of 4-7, the loss of proteins beyond the boundaries is deemed acceptable (Cheng et al., 2009a). The first dimension was run on an Ettan IPGphor Ⅱ system (GE Healthcare) using the following program: 500 V for 1 hour, gradient to

1,000 V for 3 hours, gradient to 3,000 V for 3 hours, constant voltage of 3,000 V for 2 hours, gradient to 8,000 V for 3 hours, constant voltage of 8,000 V for 10.5 hours, for a total of 108,000 V·hr, finally hold at 500 V. After the first dimension, the IEF strips were treated in 10 ml equilibration buffer (6 M urea, 2% SDS, 50 mM Tris-HCl pH 8.8, 30% Glycerol, 0.002% bromophenol blue) containing 10 mg/ml dithiothreitol for 20 minutes at room temperature, followed by another 20-minute treatment in the dark with 10 ml equilibration buffer containing 25 mg/ml iodoacetamide. The second dimension was carried out at 10℃ on 12% resolving gels for Tris-glycine SDS-polyacrylamide gel electrophoresis (66 ml distilled water, 80 ml 30% acrylamide mix, 50 ml 1.5 M Tris-HCl pH 8.8, 2 ml 10% SDS, 2 ml 10% ammonium persulfate, 80 µl TEMED). The gels were scanned on a Typhoon 9400 scanner (GE Healthcare) at three different wavelengths. Analysis of these gel pictures was performed by DeCyder V 6.0 software (GE Healthcare).

2.8.4 Mass spectrometry

To identify the protein spots of interest on DIGE gels, preparative gels were run using the same protein samples and running conditions except that 600-800 µg protein were used to get sufficient sample and the proteins were not labeled with CyDye fluors. After running, the gels were stained overnight in Coomassie blue G-250 stain solution, which was prepared as follows: (1) 55.5 ml 98% H_2SO_4 was added distilled water to a final volume of 2 L. Two g Coomassie blue G-250 was added and stired for at least 4 hours; (2) 88 g NaOH was dissolved in distilled water to final volume of 220 ml; (3) 310 ml 100% trichloroacetic acid was prepared; (4) the above solutions were mixed prior to use. Following staining, the gels were washed with distilled water several times until the washing waste was pale blue. Then the gels were scanned using a Typhoon 9400 scanner (GE Healthcare) at 633 nm wavelength without an emission filter. Finally, the gels were covered with distilled water and kept at 4℃ for up to 2 months.

After comparing the preparative gels with the DIGE gels, the corresponding protein spots on preparative gels were excised and put into clean 1.5 ml microcentrifuge tubes. The protein spots were treated as described by Cheng et al. (2009a). After washing with HPLC grade water three times, the protein spots were destained three times using 50 mM NH_4HCO_3 in 50% acetonitrile (ACN). The proteins were reduced and alkylated by incubating with 10 mM dithiothreitol in 100 mM NH_4HCO_3 at 50℃ for 30 minutes and 55 mM iodoacetamide in 100 mM NH_4HCO_3 for 30 minutes in the dark, respectively. The protein spots were first washed with 100 mM NH_4HCO_3 for 15 minutes and then dehydrated with acetonitrile. The protein spots were dried on a Savant SpeedVac for 20 minutes. Seven µl of trypsin solution (Promega Corporation, Madison, WI) containing 40 ng trypsin was added to each protein spot to attain a 1:100 to 1:10 ratio of trypsin:protein (w/w), followed by the addition of 50 µl of 50 mM NH_4HCO_3 before the mixture was digested at 37℃ for 18 hours. The peptides resulting from digestion were extracted by adding 5% formic acid in 50% ACN and vortexing. The tubes were briefly centrifuged and the supernatant was transferred to new 1.5 ml microcentrifuge tubes. The extraction procedure was repeated several times. Then, the extraction solution was concentrated using a Savant SpeedVac to a final volume of about 10 µl. The peptides were concentrated and purified using C-18 ZipTip® pipette tips (Millipore). The resulting peptide solution was analyzed by MS/MS using a Waters Micromass Ultima Q-TOF mass spectrometer. Protein identification using MS/MS data was performed with the aid of the PEAKS software V. 3.1 (Ma et al., 2003a) (Bioinformatics Solutions Inc., Waterloo, ON), which is a combination of auto *de novo* sequencing and database (MSDB and NCBI nr) searching. The following parameters were used: 0.2 Da for parental mass error, 0.1 Da for fragment mass error, trypsin as the digestion enzyme, one missed cleavage allowed, and with carbamidomethylation and methionine oxidation as fixed and variable modifications, respectively. The MASCOT MS/MS ion search and/or peptide-fingerprinting

algorithm (Perkins et al., 1999) was employed to confirm the identification results, using the same set of parameters.

Chapter 3: Results

3.1 Construction of mutant strains

3.1.1 PCR amplification of *unkG*

The *unkG* gene of *P. putida* UW4 (GenBank: EU514689.1), 528 bp in length, was PCR amplified using *P. putida* UW4 genomic DNA as a template. The primers that were used are shown schematically in Figure 3-1. The PCR products were then subjected to agarose gel electrophoresis to check their size and to purify them (Figure 3-2).

3.1.2 Confirmation of pETP and pETP-*unkG*

As described previously, the *unkG* PCR product was cloned into the pGEM-T easy vector and then released by NotI digestion. The resulting fragment, containing *unkG,* was incorporated into pETP, which was derived from pET-30a (+), to get pETP-*unkG.* After constructing, the plasmids pGEM-*unkG* and pETP-*unkG* were digested with appropriate restriction enzymes for confirmation purposes. Specifically, pGEM-*unkG* was digested with NotI to get two bands (Figure 3-3). The larger band, which corresponds to linear pGEM T easy vector, was about 3.0 kb and the smaller one, which corresponds to *unkG,* was approximately 530 bp. The confirmation of pETP-*unkG* was done by digesting with NotI to release two bands (Figure 3-4). Similarly, the larger band was about 7.0 kb, which corresponds to linear pETP vector and the smaller one was about 530 bp, which corresponds to *unkG.* DNA sequencing of pETP-*unkG* showed the insertion of *unkG* into pETP as desired, i.e. the start codon rather than stop codon of *unkG* was closer to the T7 promoter in the expression cassette. Furthermore, the expression of 6×Hig-tagged recombinant proteins in *P. putida* UW4/pETP-*unkG* was confirmed by Western blotting (Cheng et al., 2009a).

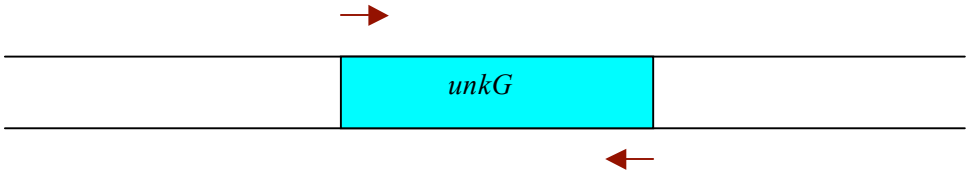


Figure 3- 1 Schematic view of the primers used to amplify *unkG* from *P. putida* UW4 genomic DNA. The arrows represent the primers.

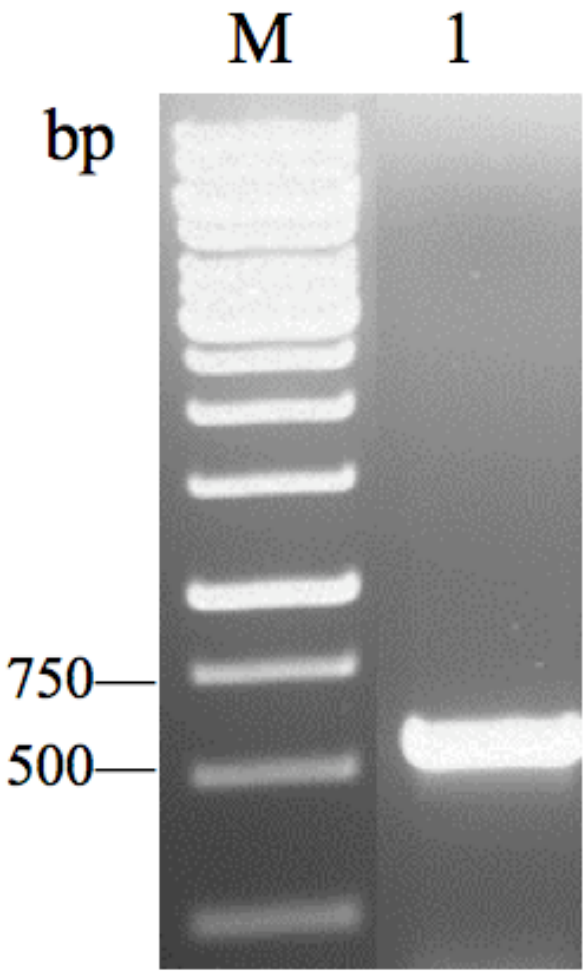
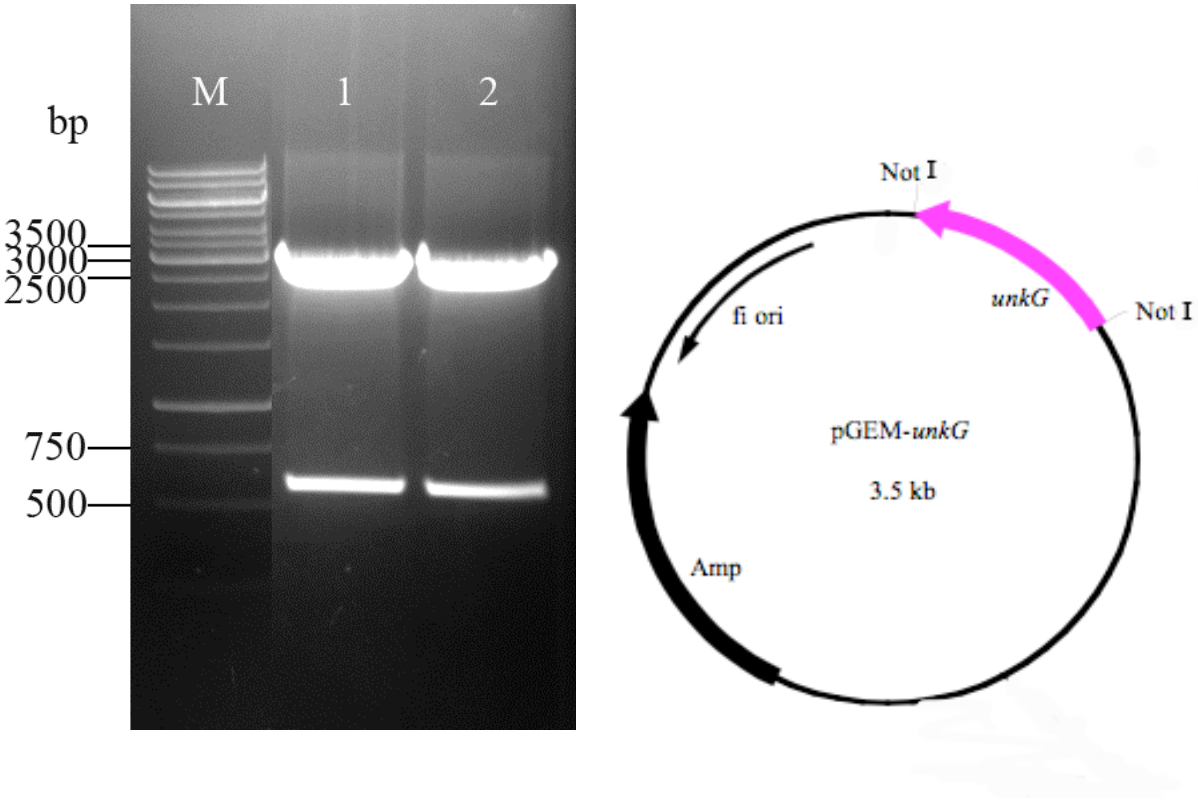
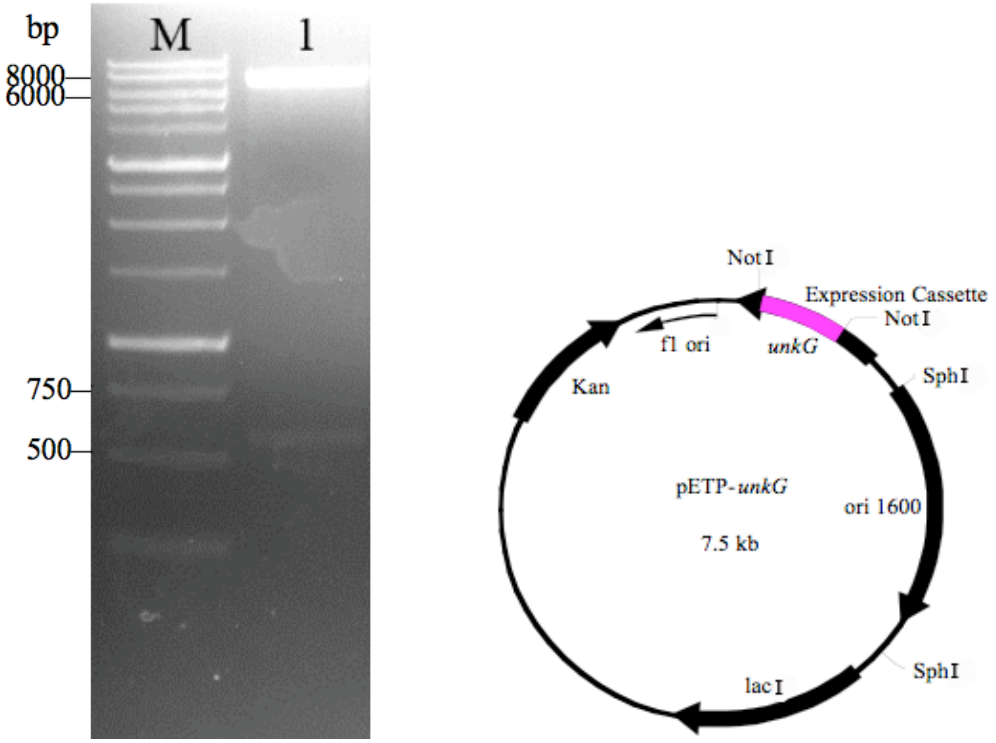


Figure 3- 2 Agarose gel electrophoresis of PCR product of *unkG*. M: 1kb DNA ladder. 1: PCR product of *unkG*.



A B

Figure 3- 3 Agarose gel electrophoresis of NotI-digested pGEM-*unkG* (A) and schematic map of pGEM-*unkG* (B). M: 1kb DNA ladder. 1 and 2: NotI-digested pGEM-*unkG*.



A B

Figure 3- 4 Agarose gel electrophoresis of NotI-digested pETP-*unkG* (A) and schematic map of pETP-*unkG* (B). M: 1kb DNA ladder. 1: NotI-digested pETP-*unkG*.

3.2 Growth rates

The growth rates of wild-type *P. putida* UW4 and *P. putida* UW4/pETP-*unkG* strains were measured at 15℃, room temperature (~ 22℃) and 30℃ in TSB (rich) and DF (minimal) media (Figures 3-5, 3-6, 3-7). The main purpose of this experiment was to see whether over-expression of UnkG has an effect on the growth of *P. putida* UW4 at different temperatures in either rich or minimal medium. Each growth curve was run in duplicate.

The results indicate that differences between wild-type *P. putida* UW4 and *P. putida* UW4/pETP-*unkG* strains were only observed when they were grown at 15℃ in DF medium. Under these conditions, it took 25 hours for wild-type *P. putida* UW4 to reach stationary phase and 40 hours for *P. putida* UW4/pETP-*unkG*. However, upon reaching stationary phase, the optical density of the wild-type *P. putida* UW4 culture was 1.6 while for *P. putida* UW4/pETP-*unkG* it was 2.0. In total, there were only small differences in the ability of the wild-type and transformant cells to proliferate.

3.3 Biolog tests

3.3.1 TSI and oxidase tests

These tests were to identify whether wild-type *P. putida* UW4 and *P. putida* UW4/pETP-*unkG* were enteric or non-enteric microbes. The results indicated that they were both non-enteric (Table 3-1). So the protocol for non-enteric microbes was followed when performing Biolog experiment.

3.3.2 Results of Biolog

Of the 95 carbon sources tested (Table 3-2), there were no differences between these two strains on this metabolic profile. The results indicated that over-expression of UnkG in *P. putida* UW4

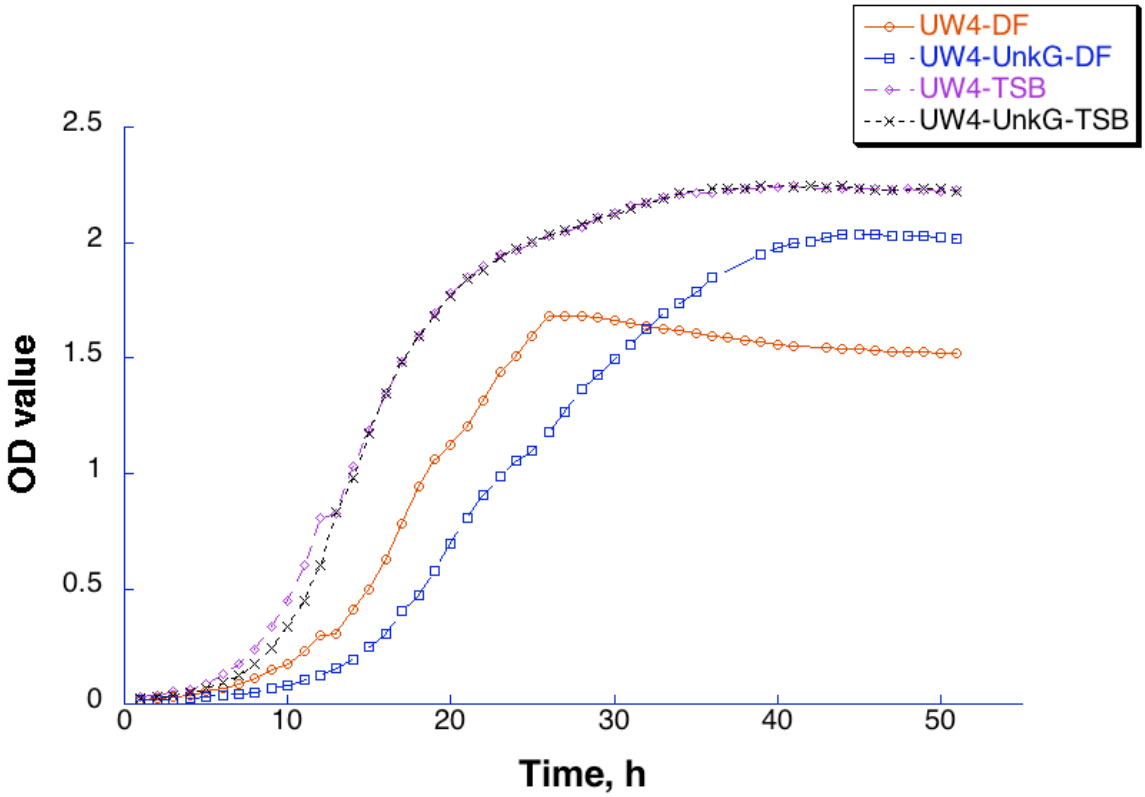


Figure 3- 5 Growth rates of *P. putida* UW4 and *P. putida* UW4/pETP-*unkG* at 15℃ in TSB and DF media. Optical density at 600 nm was measured very one hour. UW4: wild-type *P. putida* UW4; UW4-UnkG: *P. putida* UW4/pETP-*unkG*.

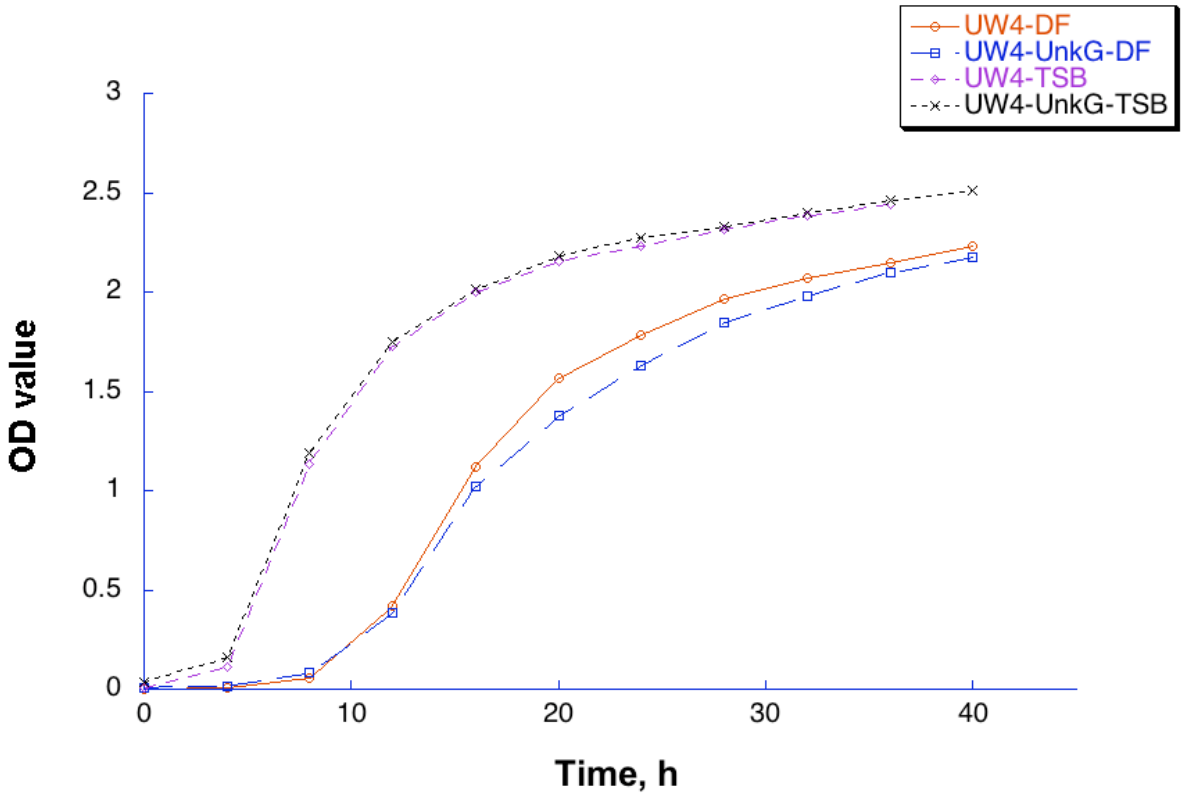


Figure 3- 6 Growth rates of *P. putida* UW4 and *P. putida* UW4/pETP-*unkG* at room temperature in TSB and DF media. Optical density at 600 nm was measured every four hours. UW4: wild-type *P. putida* UW4; UW4-UnkG: *P. putida* UW4/pETP-*unkG*.

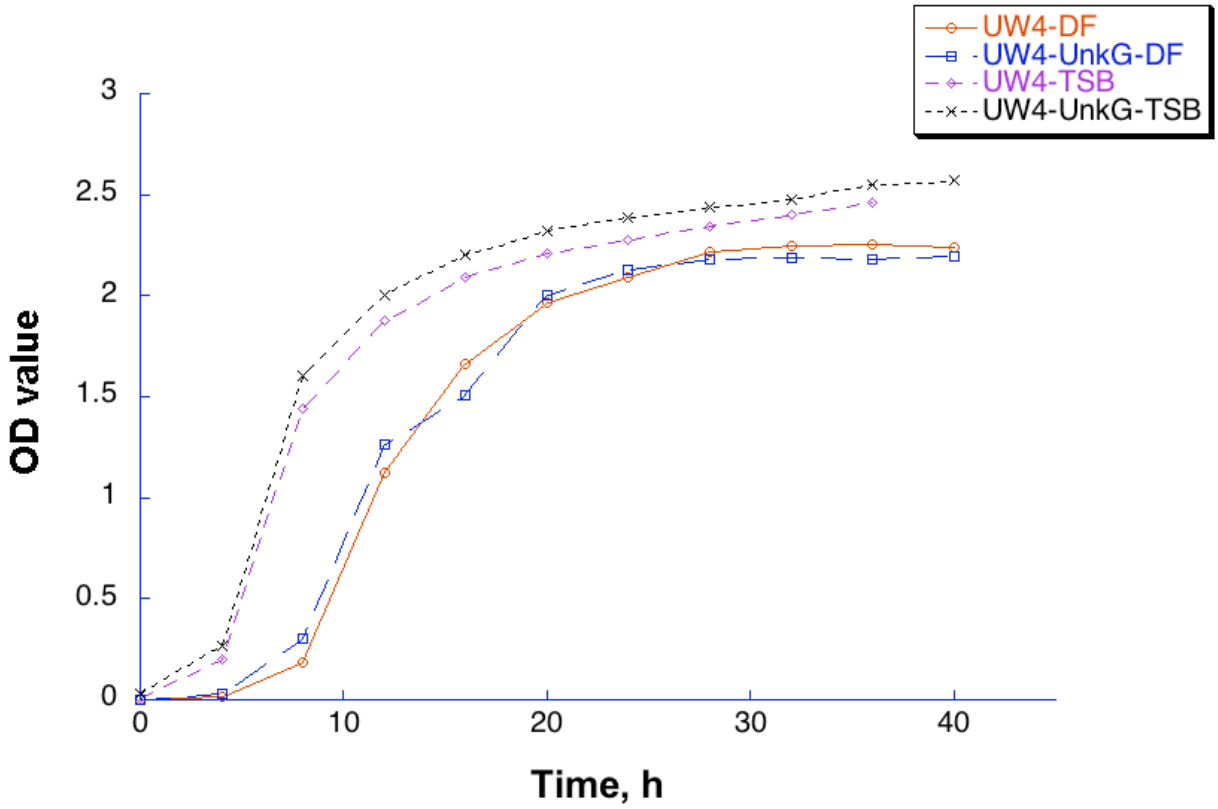


Figure 3- 7 Growth rates of *P. putida* UW4 and *P. putida* UW4/pETP-*unkG* at 30℃ in TSB and DF media. Optical density at 600 nm was measured every four hours. UW4: wild-type *P. putida* UW4; UW4-UnkG: *P. putida* UW4/pETP-*unkG*.

Table 3- 1 Results of oxidase test and triple sugar iron (TSI) test.

K: alkaline; +: positive; -: negative.

	Oxidase test	TSI test				Microbe type
		Slant	Butt (bottom)	H_2S	Gas	
P. putida UW4	+	K	K	-	-	Non-enteric
P. putida UW4/pETP-*unkG*	+	K	K	-	-	Non-enteric

Table 3- 2 Metabolic activities of *P. putida* UW4 and *P. putida* UW4/pETP-*unkG* on 95 carbon substrates tested on Biolog plate.

+: positive test; -: negative test; +/-: borderline.

Carbon source	*P. putida* UW4	*P. putida* UW4/pETP-*unkG*
α-Cyclodextrin	-	-
Dextrin	-	-
Glycogen	+/-	+/-
Tween 40	+	+
Tween 80	+	+
N-acetyl-D-galactosamine	-	-
N-acetyl-D-glucosamine	+	+
Adonitol	-	-
L-arabinose	+	+
D-arabitol	-	-
D-cellobiose	-	-
i-erythritol	-	-
D-fructose	+	+
L-fucose	-	-
D-galactose	+/-	-
Gentiobiose	-	-
α-D-glucose	+	+
m-inositol	-	-
α-D-lactose	-	-
Lactulose	-	-

Maltose	-	-
D-mannitol	-	-
D-mannose	+	+
D-melibiose	-	-
β-methyl-D-glucoside	-	-
D-psicose	-	-
D-raffinose	-	-
L-rhamnose	-	-
D-sorbitol	-	-
Sucrose	+	+
D-trehalose	-	-
Turanose	-	-
Xylitol	-	-
Pyruvic acid methyl ester	+	+
Succinic acid mono-methyl-ester	+	+
Acetic acid	+	+
Cis-aconitic acid	+	+
Citric acid	+	+
Formic acid	+/-	+/-
D-galactonic acid lactone	+	+
D-galacturonic acid	-	-
D-gluconic acid	+	+
D-glucosaminic acid	-	-
D-glucuronic acid	+	+
α-hydroxybutyric acid	-	-

β-hydroxybutyric acid	+	+
γ-hydroxybutyric acid	-	-
ρ-hydroxy phenylacetic acid	-	-
Itaconic acid	-	-
α-keto butyric acid	-	-
α-keto glutaric acid	+	+
α-keto valeric acid	-	+/-
D,L-lactic acid	+	+
Malonic acid	+	+
Propionic acid	+	+
Quinic acid	+	+
D-saccharic acid	+	+
Sebacic acid	-	-
Succinic acid	+	+
Bromosuccinic acid	+	+
Succinamic acid	+/-	+/-
Glucuron amide	+	+
L-alaninamide	+/-	-
D-alanine	+	+
L-alanine	+	+
L-alanyl-glycine	+	+
L-asparagine	+	+
L-aspartic acid	+	+
L-glutamic acid	+	+
Glycyl-L-aspartic acid	-	-

Glycyl-L-glutamic acid	-	-
L-histidine	+	+
Hydroxy-L-proline	+	+
L-leucine	+	+
L-ornithine	+	+
L-phenylalanine	-	-
L-proline	+	+
L-pyroglutamic acid	+	+
D-serine	-	-
L-serine	+	+
L-threonine	+	+
D,L-carnitine	+	+
γ –amino butyric acid	+	+
Urocanic acid	-	-
Inosine	+	+/-
Uridine	-	-
Thymidine	-	-
Phenyethyl-amine	-	-
Putrescine	+	+
2-aminoethanol	+	+
2,3-butanediol	-	-
Glycerol	+	+
D,L- α-glycerol phosphate	-	-
α-D-glucose-1-phosphate	-	-
D-glucose-6-phosphate	-	-

did not significantly change the metabolic activity toward these carbon sources. Moreover, when searching the profiles against the database, the top identity in the database was *Pseudomonas putida* Biotype B.

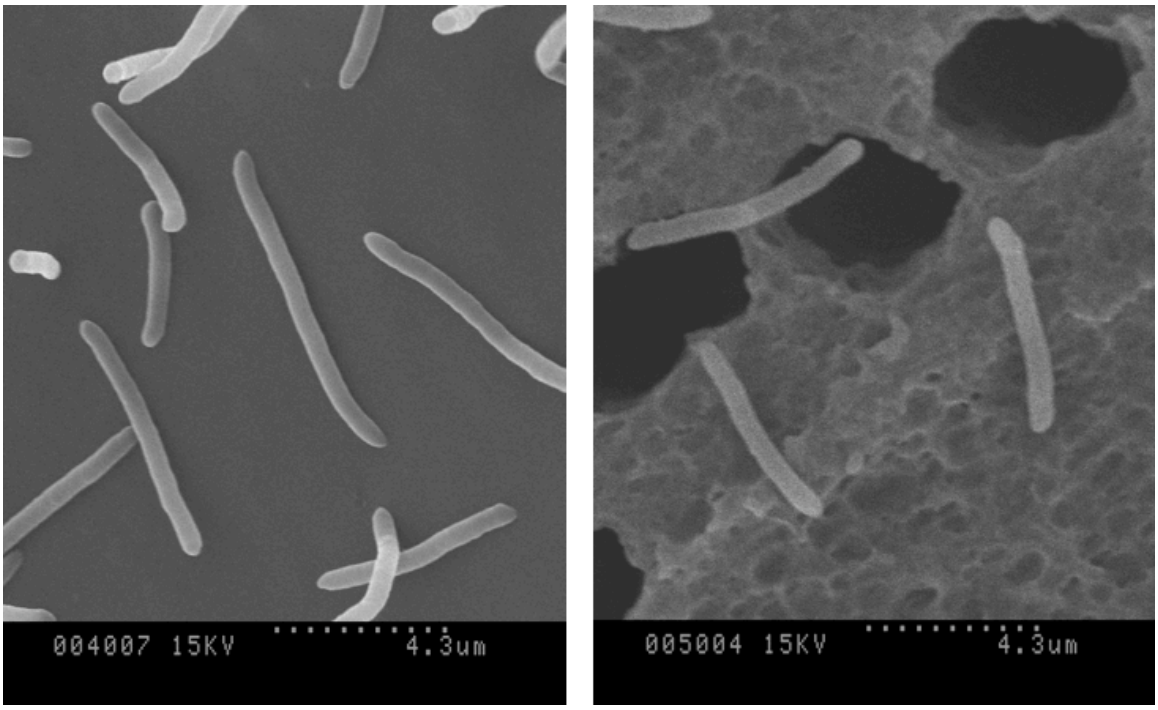
3.4 Scanning electron microscopy (SEM)

The cells of *P. putida* UW4/pETP-*unkG* and *P. putida* UW4/pETP were observed under scanning electron microscopy, using different magnifications. Typical images were shown in Figure 3-8. Ten cells of either strain were randomly selected and measured for length and diameter. On average, the cells of *P. putida* UW4/pETP-*unkG* (length: 5.77±0.54 μm; diameter: 0.68 μm) were slightly shorter and thicker than that of *P. putida* UW4/pETP (length: 5.94±0.23 μm; diameter: 0.60 μm). However, we cannot draw any conclusion as only a limited number of cells were observed. In addition, there was no significant difference in their morphology.

3.5 Growth pouch assay

After sterilization, canola seeds were treated separately with wild-type *P. putida* UW4, *P. putida* UW4/pETP-*unkG, P. putida* UW4/pETP and $MgSO_4$ (as a negative control). The seeds were incubated in growth pouches in a growth chamber for six days and then the root lengths were measured. All seedlings of each treatment that germinated within two days were used for root length measurements (i.e. 50~59 seedlings per treatment). The average root length and standard error for each treatment were calculated.

Figure 3-9 clearly indicates that compared with the other 3 treatments, *P. putida* UW4/pETP-*unkG* had a negative effect on the root-length elongation of canola seedlings while the wild-type *P. putida* UW4 had the greatest root growth-promoting ability. The transformant *P. putida* UW4/pETP with an empty plasmid vector promoted root growth to a significantly greater extent than *P. putida* UW4/pETP-*unkG* indicating that the observed inhibition of root growth by



A **B**

Figure 3- 8 Typical SEM images of *P. putida* UW4/pETP-*unkG* (A) and *P. putida* UW4/pETP (B). Bacteria were grown in TSB broth overnight. After imaging using a scanning electron microscope, ten cells of each strain were randomly selected to measure length and diameter. On average, the cells of *P. putida* UW4/pETP-*unkG* (length: 5.77±0.54 μm; diameter: 0.68 μm) were slightly shorter and thicker than that of *P. putida* UW4/pETP (length: 5.94±0.23 μm; diameter: 0.60 μm). The length from the first to the last dot is 4.3 μm.

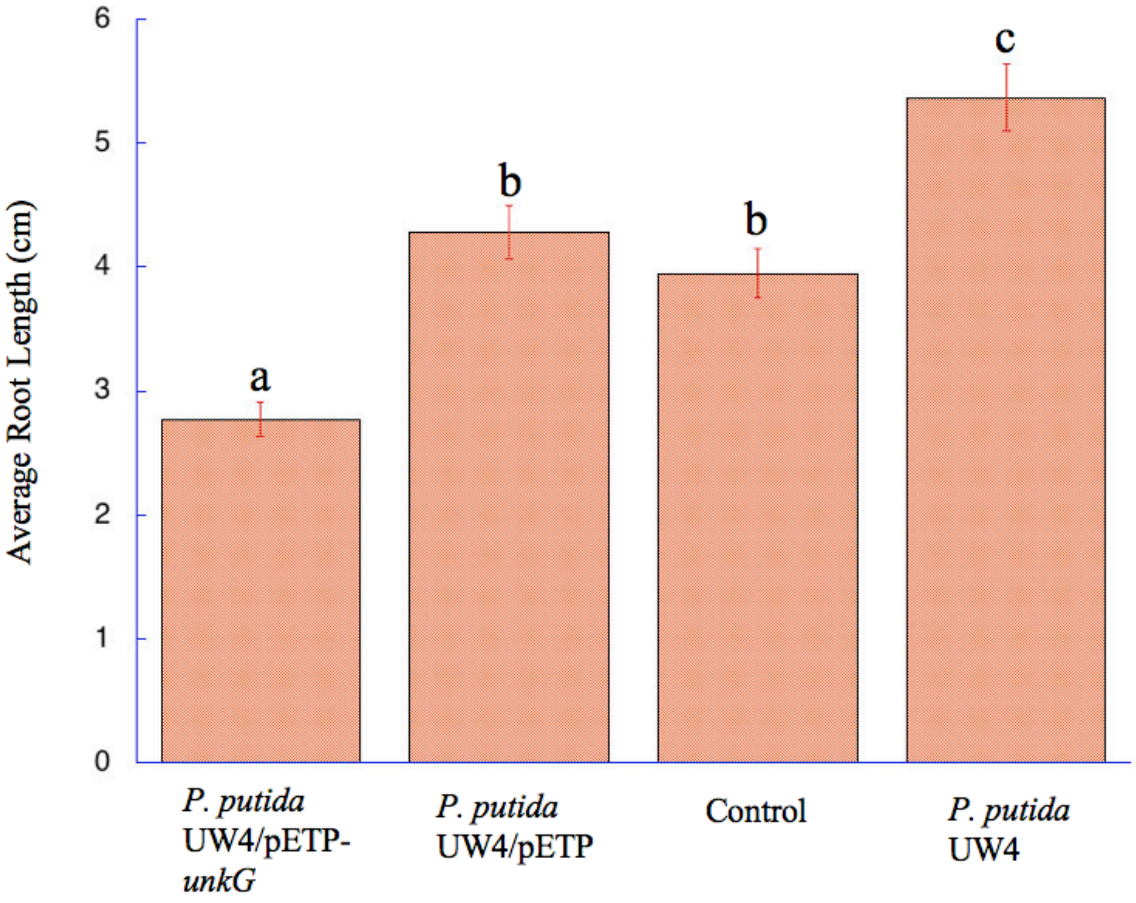


Figure 3- 9 Growth pouch assay of *P. putida* UW4 and its transformants. Root length was measured after 6 days of incubation. Error bars represent standard errors of the mean with 50 (for *P. putida* UW4/pETP) or 59 (for the other three treatments) plants per treatment. Letters represent the results of one-way ANOVA analysis of seedling root length. Statistical significance is indicated by a different letter.

P. putida UW4/pETP-*unk* was caused by over-expression of UnkG rather than by plasmid pETP.

3.6 Biochemical assays

3.6.1 Indole-3-acetic acid (IAA) assay

The amount of IAA secreted by wild-type and transformed *P. putida* UW4 into the cell broth was measured. Each strain was grown in liquid TSB medium containing 200 μg/ml or 500 μg/ml L-tryptophan, which is the substrate for IAA production. As Figure 3-10 shows, at both concentrations of L-tryptophan, wild-type *P. putida* UW4 produced the lowest level of IAA. However, the difference between the wild-type and the transformants was small, and the amounts of IAA produced by *P. putida* UW4/pETP-*unkG* and *P. putida* UW4/pETP were identical suggesting that overproduction of UnkG does not significantly affect the production of IAA.

3.6.2 1-Aminocyclopropane-1-carboxylate (ACC) deaminase assay

The ACC deaminase activity of *P. putida* UW4/pETP-*unkG, P. putida* UW4/pETP and wild-type *P. putida* UW4 was assayed (Figure 3-11). There were no significant differences among these three strains.

3.7 Proteomic study

3.7.1 Difference in-gel electrophoresis

The protein expression profiles of *P. putida* UW4/pETP-*unkG* and *P. putida* UW4/pETP were compared and analyzed. A total number of 1,839 protein spots were detected (Figure 3-12), out of which 43 (2.34%) proteins were increased and 41 (2.23%) were decreased in expression levels ($p \leq 0.05$, $|\text{Average Ratio R}| \geq 1$). Here, average ratios were calculated R = (treated/control) for up-regulated proteins, and R= - (control/treated) for down-regulated proteins, i.e. R = 2 and R = -2 represent a 2-fold up-regulation and a 2-fold down-regulation change, respectively. The protein

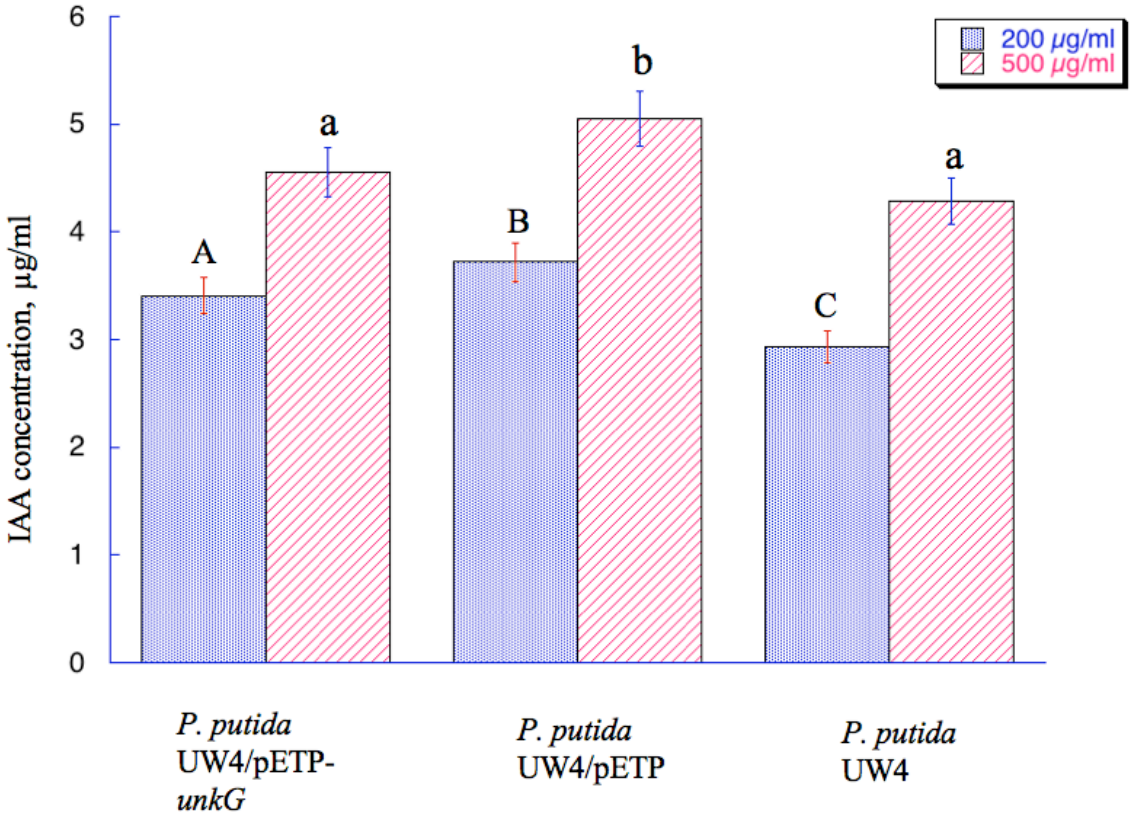


Figure 3- 10 IAA concentration of wild-type and transformed *P. putida* UW4 in liquid culture. 200 µg/ml and 500 µg/ml indicate the concentrations of the substrate L-tryptophan added to the growth medium. Error bars represent standard errors. All treatments were run in four replicates. Letters represent the results of one-way ANOVA analysis of IAA concentrations measured. Statistical significance is indicated by a different letter.

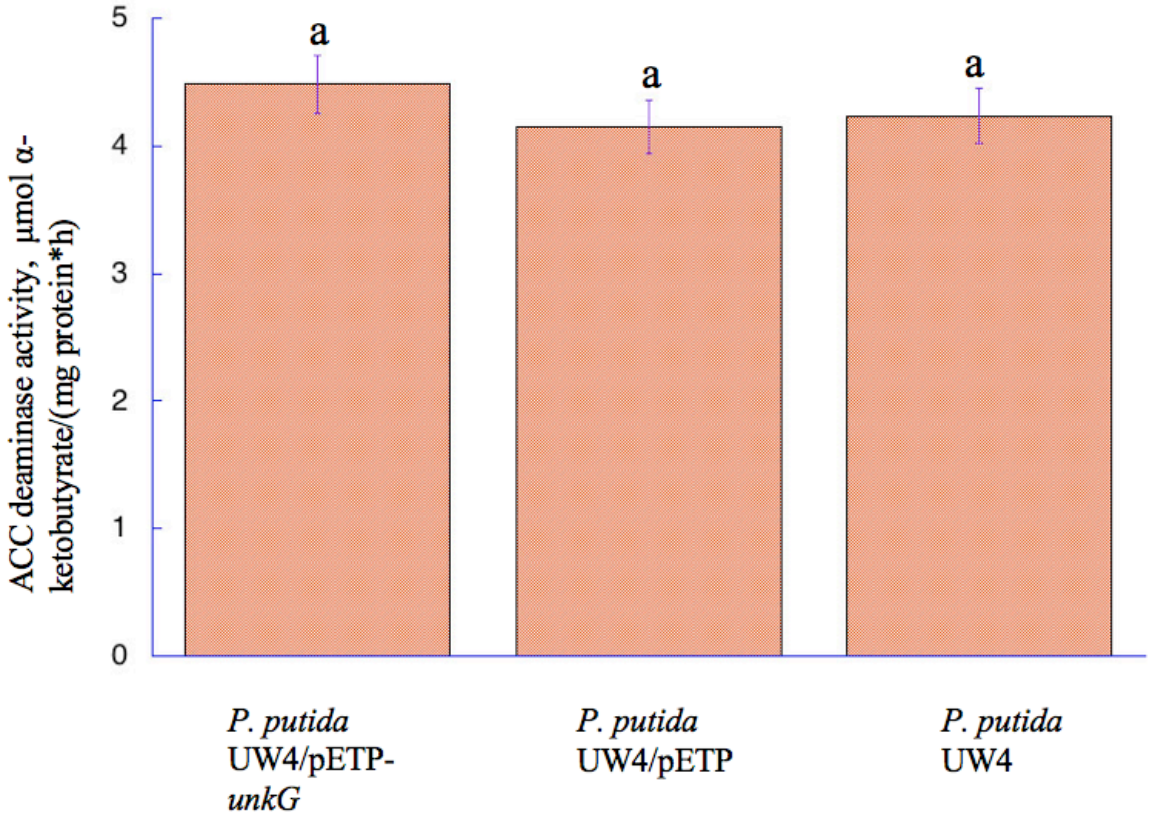


Figure 3- 11 ACC deaminase activity of wild-type and transformed *P. putida* UW4. Each treatment was run in triplicate. Error bars represent standard errors. Letters represent the results of one-way ANOVA analysis of ACC deaminase activity. Here, the same letter indicates that there is no significant difference among the three treatments.

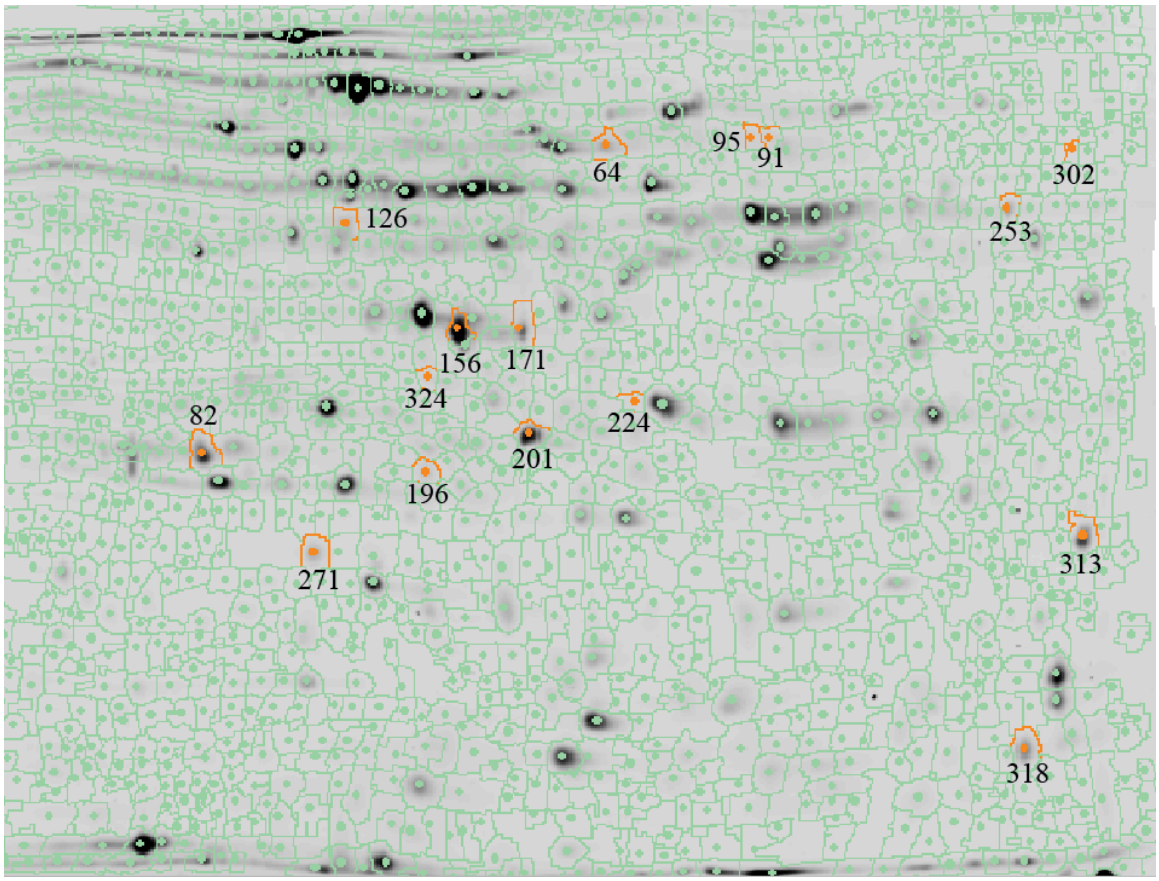


Figure 3- 12 A representative DIGE gel image and the 16 identified proteins. Each dot, either in green or red, represents a single protein on a DIGE gel. The red ones are the proteins identified in the following mass spectrometry study. The number represents the respective spot number found in Table 3-3.

spots with *p* no larger than 0.05 were considered statistically significant. The more strict False Discovery Rate method was used subsequently. In addition, it is worth noting that due to its low abundance, the fold change of UnkG is not reported in this study.

3.7.2 Mass spectrometry

Preparative polyacrylamide gels were made using the same protein samples as for the DIGE gels. Then, the preparative gels were compared with the DIGE gels and the corresponding protein spots with changed expression levels were excised and treated for mass spectrometry analysis. Due to the low abundance of many proteins, only 16 of the 84 protein spots with changed expression levels could be identified (Figure 3-12, Table 3-3). Two of them were identified by mass spectrometry (see Appendix Ⅱ for MS/MS data) and the others by comparison with proteome reference map of *P. putida* UW4, which was constructed by Cheng et al. (2009b). Of the identified 16 proteins, only spot 318 passed the False Discovery Rate method. The 16 identified protein spots were distributed into the following categories based on their cellular functions (Figure 3-13).

3.8 Phylogenetic analysis

Similar *unkG* gene sequences in different *Pseudomonas* strains were retrieved from National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/). The sequences were aligned using Seaview (v4.2.4) (Edgar, 2004) and manually edited using BioEdit (v7.0.5.3) (Hall, 1999). The GTR substitution model, as determined by jModelTest (v0.1.1) (Guindon and Gascuel, 2003; Posada, in press), was used in Seaview (v4.2.4) (Guindon and Gascuel, 2003) to construct a nucleotide phylogenetic tree. The nucleotide tree was constructed using the maximum likelihood method and 1000 bootstrap replicates. The result (Figure 3-14) indicates that compared with those in *P. putida* strains, the *unkG* gene in *P. putida* UW4 shares a higher similarity with those in *P. fluorescence* strains.

Table 3- 3 Identified protein spots with changed expression levels.

Spot numbering refers to numbers in the *P. putida* UW4 reference map.

Average ration R = (treated/control) for up-regulated proteins, and R= - (control/treated) for down-regulated proteins

Spot	*p* value	R	Function
Electron transporter			
318	1.1e-005	1.94	Transfers electrons from cytochrome c551 to cytochrome oxidase, copper ion binding
156	0.0032	1.28	Electron transfer flavoprotein, alpha subunit
Amino acid metabolism			
64	0.00058	1.54	Hydrolase arginine metabolism or hydrolase
171	0.0020	1.49	Dihydrodipicolinate synthase lysine metabolism
253	0.00060	-1.38	Glutaminase-asparaginase asparagines metabolism
ATP/GTP metabolism			
95	0.028	1.11	ATP-dependent Hsl protease ATP-binding subunit HslU
91	0.038	1.14	UDP-N-acetylmuramate--L-alanine ligase
126	0.036	-1.1	Cell division protein ftsZ GTP binding
82	0.027	-1.38	Glutaminyl-tRNA synthetase
Oxidoreductase			
201	0.015	1.19	Ferredoxin--NADP reductase
Transporter			
224	0.0040	-1.29	Arginine/ornithine ABC transporter, periplasmic arginine/ornithine binding protein

Transcription regulation			
196	0.029	1.14	Two component transcriptional regulator, Fis family
Fatty acid metabolism			
271	0.024	1.11	Biotin carboxyl carrier protein
Function unknown			
313	0.017	1.10	SubName: Full=YceI; Flags: Precursor
302	0.028	-1.47	iojap domain protein
324	0.00026	-1.32	Putative uncharacterized protein

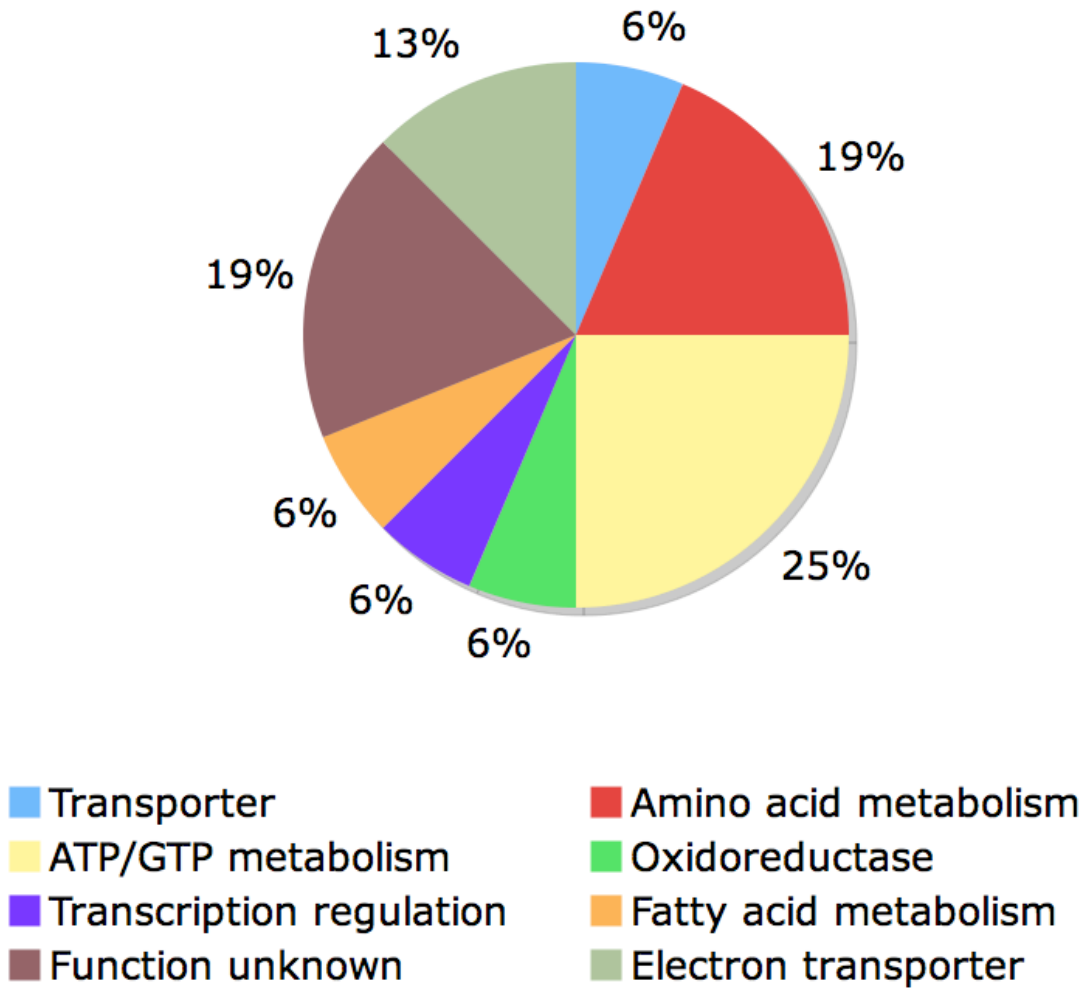


Figure 3- 13 Chart of the distribution of identified protein spots into functional categories. The percentages were calculated from the total number of protein spots identified (16).

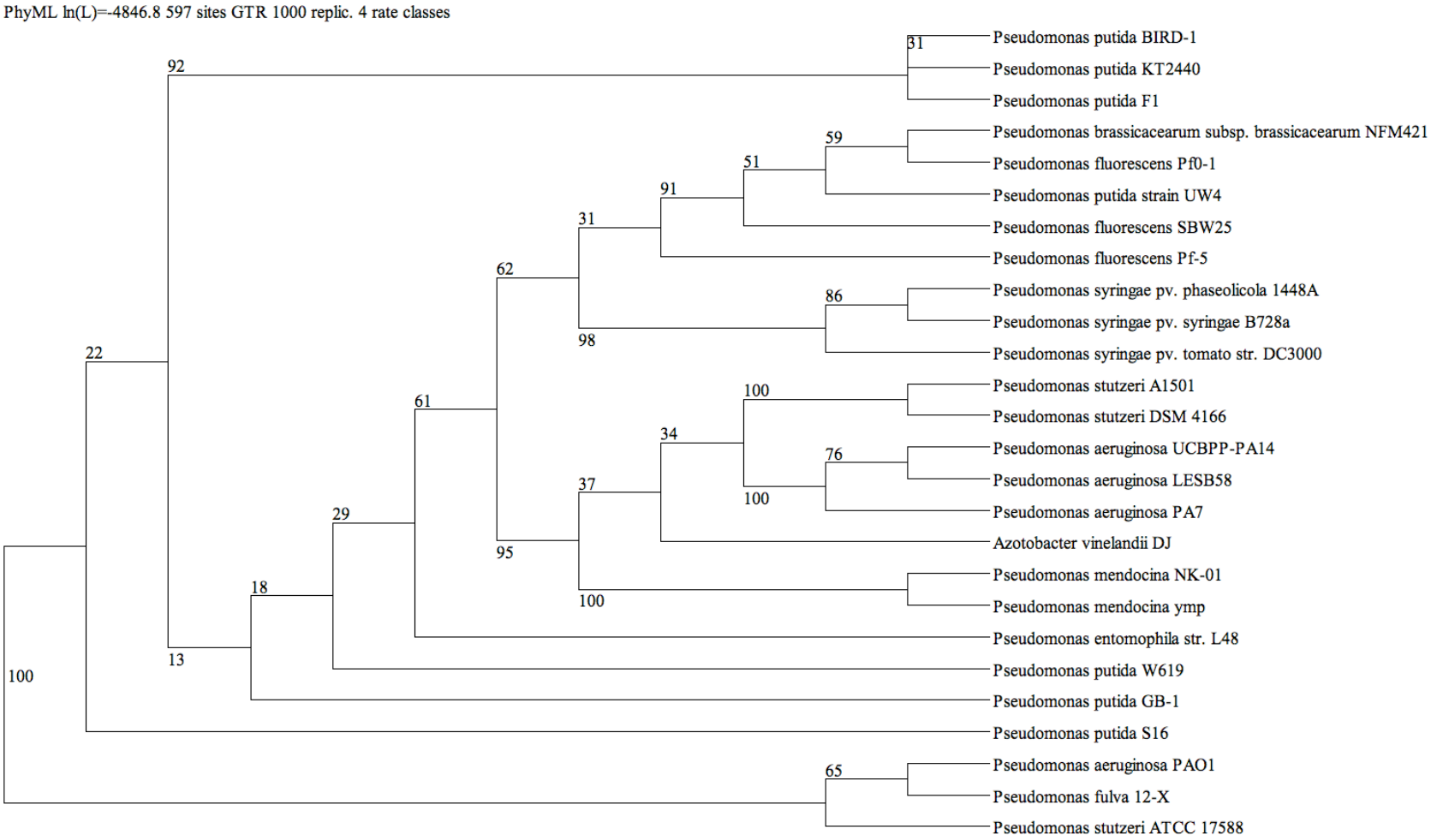


Figure 3- 14 Phylogeny tree of *unkG* genes in different *Pseudomonas* strains. The phylogeny tree was constructed using the maximum likelihood method and 1000 bootstrap replicates.

Chapter 4: Discussion

4.1 The shining future of PGPB

The fast growing world population has imposed a tremendous burden on the global agricultural community, urging highly efficient production of food using limited land fields (Zhu et al., 2007). Meanwhile, there is concern over food safety and environmental pollution related to the widespread use of chemical pesticides and fertilizers. One promising way to solve these problems is the application of PGPB to increase food productivity and quality (Glick et al., 1999). Over the years, studies in this field have identified many mechanisms used by PGPB to promote plant growth or to control plant pathogens. These exciting findings have facilitated the commercialization of PGPB. For example, many PGPB strains have been used to increase the yield and/or quality of various agronomically important crops, such as potato (Kloepper et al., 1980), cotton (Chen et al., 1994), soybean (Zhang et al., 1996); wheat (Amara and Dahdoh, 1997), corn (Pan et al., 1999), rice (Biswas et al., 2000), common bean (Figueiredo et al., 2008), etc. However, further studies are needed to give a more clear understanding on the mechanisms used by PGPB to promote plant growth and to control plant pathogens. Since in reality, a large number of microorganisms of different genera live together in the rhizosphere of plants and they form complex relationships with each other, new methods, possibly involving multidisciplinary approaches (Figueiredo et al., 2010), are urgently needed to be developed to study the relationships and mechanisms. There are two main problems associated with applications of PGPB: one is the poor ability of many PGPB strains, that behave well under laboratory conditions, to adapt to real environmental conditions. The other potential problem is the safety concern. In most cases, one specific PGPB strain is studied alone as a pure culture in the lab due to the complexity of introducing other microorganisms. However, without considering the interactions among various microorganisms and the influence of real environmental factors on

PGPB strains, a decrease in the apparent abilities of PGPB strains to promote plant growth is inevitable. On the other hand, we must make sure PGPB products will not cause any health or environmental problems before they are used in a field environment.

4.2 The need to construct *P. putida* UW4/pETP-*unkG* and *P. putida* UW4/pETP

A common strategy in protein function study is to construct mutant strains to over-express and/or to disrupt the protein-coding gene being studied. Then, by comparing the wild-type with the mutant strains, some information regarding protein function may be obtained. However, the construction of mutant strains often involves introducing foreign DNA sequences of various sizes into the wild-type strain, which may impose a metabolic load on the host. A metabolic load may be defined as "the portion of a host cell's resources - either in the form of energy such as ATP or GTP, or raw materials such as amino acids - that is required to maintain and express foreign DNA, as either RNA or protein, in the cell" (Glick, 1995b). Many factors, such as the amount of a foreign protein over-expressed, the copy number and size of the cloning vector, the metabolic state of the cell, the composition of the growth medium and the amount of dissolved oxygen in the growth medium, can affect the magnitude of metabolic load (Glick, 1995b). Metabolic load may cause many physiological changes in host cells, such as decreased growth rates, reduced production of various molecules, altered cell size and shape, etc (Glick, 1995b).

In this study, *unkG* gene was over-expressed by introducing plasmid pETP-*unkG,* which carried this gene, into wild-type *P. putida* UW4. A larger part of metabolic load, if any, was attributed to pETP vector but not *unkG.* In this case, in addition to *P. putida* UW4/pETP-*unkG, P. putida* UW4/pETP needed to be constructed to investigate the extent of metabolic load caused by the pETP vector.

The metabolic load theory may explain some of the results of this study. First, *P. putida* UW4/pETP-*unkG* grew more slowly than wild-type *P. putida* UW4 at 15℃ in DF medium. It is quite possible that introducing plasmid pETP-*unkG* imposed a metabolic load on *P. putida* UW4. However, it is worth pointing out that although it grew more slowly than the wild-type, *P. putida* UW4/pETP-*unkG* reached a higher optical density in stationary phase. In addition, at room temperature (~22℃) and 30℃, the metabolic load did not affect growth rates. Second, in the growth pouch root elongation assay, a small metabolic load may explain the slightly decreased root length promoting ability of *P. putida* UW4/pETP, compared with the wild-type strain. On the other hand, the decreased root length promoting ability of *P. putida* UW4/pETP-*unkG*, compared with *P. putida* UW4/pETP, was largely attributed to the effects of UnkG on plant growth. In this regard, over-expression of UnkG was deemed to be unlikely to cause such a big metabolic load. In support of this contention, IAA production and ACC deaminase activity in *P. putida* UW4 were not affected by over-expressing UnkG.

4.3 Summarization of the assays

To summarize, the growth pouch root elongation assay confirmed that over-expressing UnkG in *P. putida* UW4 decreased the root elongation-promoting ability of the host strain. Over-expressing UnkG did not significantly affect the morphology, size, IAA production, ACC deaminase activity or metabolic activities toward the 95 tested carbon substrates of *P. putida* UW4. One significant difference is that *P. putida* UW4/pETP-*unkG* grew more slowly than wild-type *P. putida* UW4 at 15℃ in DF medium. These assays provided little information on the role played by UnkG in the interactions between *P. putida* UW4 and plants. So proteomics was used as a more sensitive indicator to investigate the changes caused by over-expressing UnkG in *P. putida* UW4.

4.4 Proteomic study

With many technical improvements having been made in recent years, two-dimensional electrophoresis (2-DE) is the most widely used quantitative tool in proteomics (Jorrin-Novo et al., 2009; Kav et al., 2007). By incorporating an internal standard and fluorescently labeling protein samples, difference in-gel electrophoresis (DIGE) increases the productivity and eliminates the gel-to-gel difference among replicate 2-D gels (Alban et al., 2003; Minden et al., 2009; Timms and Cramer, 2008; Ünlü et al., 1997). However, the sensitivity of this technique can sometimes limit the effectiveness of this method. According to the preliminary sequence analysis, the whole genome of *P. putida* UW4 can encode 5556 proteins (Duan et al., unpublished results). However, in this study, only 1839 protein spots were detected on DIGE gels, and 16 out of the 83 protein spots with changed expression levels were identified by subsequent mass spectrometry. In another study, a proteome reference map containing 326 protein spots representing 275 different proteins was constructed for this strain (Cheng et al., 2009b). This incomplete coverage is a consequence of the low abundance as well as the loss or degradation of some proteins when handling protein samples. So more powerful methods and technical improvements need to be developed to increase the sensitivity of proteomic studies.

4.5 Possible role of each identified protein

In this study, 83 (4.51%) of the 1839 detected proteins in *P. putida* UW4/pETP-*unkG* were shown to have changed expression levels ($p \leq 0.05$, $| \text{Ratio} | \geq 1$), compared with those in *P. putida* UW4/pETP, which means that over-expressing UnkG in *P. putida* UW4 does not have a large impact on the protein expression profile of the bacterium. However, it is possible that even small changes in the expression of a few proteins can affect the ability of the bacterium (*P. putida* UW4) to promote root elongation or even plant growth. And, we can use this information to

better understand the role played by UnkG in the functioning of *P. putida* UW4 in its facilitation of plant growth.

Based on the functional annotation in the Swiss-Prot database, the 16 identified proteins were divided into 8 categories (Table 3-3, Figure 3-13). From the number of proteins in each of these categories, it is quite possible that over-expressing UnkG in *P. putida* UW4 may decrease the root elongation promoting ability of this bacterium by affecting proton/electron transportation, amino acid metabolism, and/or ATP/GTP (energy) metabolism.

Spot 64 was identified as arginine deiminase (EC 3.5.3.6), catalyzing the hydrolysis of L-arginine to citrulline and ammonium, which is the first step of the microbial L-arginine degradation pathway (Lu et al., 2004). The enzyme normally exists in the cytoplasm. Spot 171, identified as dihydrodipicolinate synthase (DHDPS, EC 4.2.1.52), catalyzes the chemical reaction L-aspartate 4-semialdehyde + pyruvate $\rightleftarrows$ (S)-2, 3-dihydropyridine-2, 6-dicarboxylate + 2 H_2O.

This enzyme plays a key role in the biosynthesis of (s)-lysine and is essential for bacterial growth (Kaur et al., 2011). Spot 253 was identified as glutaminase-asparaginase (EC 3.5.1.38), which catalyzes the hydrolysis of D and L isomers of glutamine and asparagine to respective dicarboxylates (Ortlund et al., 2000). This enzyme exists in many *Pseudomonas* strains, including a number of root-colonizing biocontrol strains (Sonawane et al., 2003). Since spots 64 and 171 were up-regulated and spot 253 down-regulated, over-expressing of UnkG may affect the plant growth-promoting ability of the bacterium by facilitating hydrolysis of D and L isomers of glutamine and asparagine as well as arginine while blocking the synthesis of (s)-lysine. The abnormal metabolism of these amino acids may in turn affect the synthesis and/or degradation of some important proteins or other molecules in the host.

In the "Electron transporter" group, spot 318 was identified as azurin, also named arsenate reductase (EC 1.20.98.1). The azurins are a group of small blue copper proteins each containing a single, type Ⅰ Cu center coordinated to a single polypeptide chain. This molecule is typically about 128-129 amino acid residues, with a molecular weight of approximately 14 kDa (Barber et al., 1993). In certain bacteria such as *Pseudomonas,* azurin acts as an electron carrier in the respiratory chain (Barber et al., 1993). *In vitro*, the azurin in *Pseudomonas putida* is also capable of oxidizing substrate reduced p-cresol methylhydroxylase (PCMH), a flavo-cytochrome c, and is believed to transfer electrons *in vivo* to the membrane-bound terminal cytochrome oxidase (Causer et al., 1984; Chen et al., 1998). Spot 156 corresponds to electron transfer flavoprotein (ETF), alpha subunit. ETFs, isolated as heterodimers consisting of αβ subunits, are flavin adenine dinucleotide (FAD) -containing proteins and serve as biological electron carriers between enzymes of mitochondrial and bacterial catabolic pathways, as well as their respective respiratory chains, and are thus involved in energy production and conversion (Thorpe, 1991; Roberts et al., 1996; Roberts et al., 1999; Bai et al., 2007). In *P. putida* UW4/pETP-*unkG,* spot 318 and 156 had a 1.94 and 12.8 fold increase, respectively, compared with that in *P. putida* UW4/pETP, Thus, there is the possibility that over-expressing UnkG in *P. putida* UW4 may affect the plant growth-promoting ability of the bacterium by interfering with electron/proton transfer.

Spot 201 was identified as Ferredoxin-NADP reductase (Fpr, EC 1.18.1.2). This enzyme is ubiquitous, monomeric, reversible and catalyzes the reaction of reduced ferredoxin, $NADP^+$ and H^+ to produce oxidized ferredoxin and NADPH, with FAD and flavin as cofactors (Yeom et al., 2009). In nonphotosynthetic organisms, such as most bacteria, Fpr mainly functions to provide reduced ferredoxin for various metabolic processes, including nitrogen fixation, terpenoid biosynthesis, steroid metabolism, oxidative stress response and iron-sulfur protein biosynthesis

(Aliverti et al., 2008). When over-expressing UnkG in *P. putida* UW4, this protein had a 1.19 fold increase, which unlikely affect the plant growth-promoting ability of the bacterium.

Spot 224 was identified as arginine/ornithine ABC transporter, which involves in the transportation of arginine and ornithine. This protein was down-regulated. Thus there is the possibility that over-expressing UnkG in *P. putida* UW4 deleteriously affects arginine/ornithine transfer, resulting in the loss of the bacterial ability to promote plant growth.

Spot 95 was identified as ATP-dependent Hsl protease, ATP-binding subunit HslU. HslVU (ClpQY) is the eubacterial counterpart of eukaryotic proteasomes (Bochtler et al., 2000). When in combination with the HslU protein in the ATP-bound state, the HslV protein degrades unneeded or damaged proteins. In *E. coli,* HslV is a 19-kDa protein similar to proteasome β subunits and HslU is a 50-kDa protein related to the ATPase ClpX (Rohrwild et al., 1996). In addition, a preliminary study of this system showed that HslV and HslU might form a complex in which ATP hydrolysis by HslU was essential for peptide hydrolysis by the proteasome-like component HslV (Rohrwild et al., 1996). When over-expressing UnkG, HslU had a 1.11-fold increase, possibly resulting in the consumption of more ATP for protein degradation. Spot 91 was identified as UDP-N-acetylmuramate-L-alanine ligase (EC 6.3.2.8), which catalyzes the following reaction:

ATP + UDP-N-acetylmuramate + L-alanine $\rightleftarrows$ ADP + phosphate + UDP-N-acetylmuramoyl-L-alanine.

This step is essential for bacterial cell wall biosynthesis as the first amino acid is added to the sugar moiety of the peptidoglycan precursor (Emanuele et al., 1996). It may serve as a novel target for antibiotic development (Jin et al., 1996). Over-expressing UnkG led to a 1.14-fold increase in the expression of this protein, which is unlikely to have a large impact on host

physiology. Spot 126 was identified as cell division protein FtsZ, GFP binding, which is a highly conserved cytoskeletal protein and a prokaryotic homologue of the eukaryotic cytoskeletal protein tubulin (Oliva et al., 2007). It plays a central role in prokaryotic cell division and is the first protein known to localize to the division site, where it polymerizes to form a dynamic ring structure known as a Z ring (Bi and Lutkenhaus, 1991; Stricker et al., 2002). The polymerization of FtsZs into a Z-ring allows the physical separation of daughter cells and depends on the property of FtsZ as a GTPase (Oliva et al., 2007; Paradis-Bleau et al., 2007). When over-expressing UnkG, this protein had a 1.1-fold decrease in expression, which could affect of cell division of the host, notwithstanding the fact that changes to cell morphology were not observed by SEM. Spot 82 was identified as glutaminyl-tRNA synthetase (GlnRS, EC 6.1.1.18). GlnRS catalyzes the aminoacylation of tRNA by the cognate glutamines and belongs to the class I aminoacyl-tRNA synthetase family (Uemura et al., 1988; Yamao et al., 1982). This enzyme, along with other aminoacyl-tRNA synthetases plays a central role in the process of protein biosynthesis (Yamao et al., 1982). A 1.38-fold decrease in expression of this protein, caused by over-expressing UnkG, may negatively affect protein synthesis in the host cells, which may impact on the loss of plant growth-promoting ability of *P. putida* UW4/pETP-*unkG* compared to the wild-type strain.

Spot 271 was identified as biotin carboxyl carrier protein. It is a component of acetyl-CoA carboxylase and serves as a carrier protein for biotin and carboxybiotin in the process of the ATP-dependent carboxylation of acetyl-CoA to form malonyl-CoA, which is the rate-regulating step of fatty acid biosynthesis (Brownsey et al., 2006). Over-expressing UnkG in *P. putida* UW4 caused a 1.11-fold increase in the expression of this protein and had only a small impact on the facilitating of the host.

It is worth noting that spot 196 is a two-component transcriptional regulator, Fis family, with a 1.14-fold increase when UnkG is over-expressed. A similar two-component system consisting of a sensor histidine kinase and a response regulator was identified in *Pseudomonas putida* KT2440 (Fernández-Piñar et al., 2008). According to their study, this system is a homolog of the *Pseudomonas aeruginosa* RoxS/RoxR system, which in turn belongs to the RegA/RegB family, existing in photosynthetic bacteria as a key regulatory element (Fernández-Piñar et al., 2008). In *P. putida* KT2440, this system is involved in redox signaling and cytochrome oxidase activity, as well as in expression of the cell density-dependent gene *ddcA,* which plays an important role in bacterial colonization of plant surfaces. Similarly, over-expression of UnkG may affect the ability of *P. putida* UW4 to colonize plant or seed surfaces. There is little information on the functions of the other three identified proteins, spot 313, 302 and 324, so their roles in the changed plant growth-promoting ability are unclear.

The phylogeny tree constructed in this study indicates that the *unkG* gene in *P. putida* UW4 shares a higher similarity with those in *P. fluorescence* strains, compared with those in *P. putida* strains. In addition, many proteins in *P. putida* UW4 are more similar to those in *P. fluorescence* than those in *P. putida,* despite the fact that the 16 SrRNA data are clear for *P. putida* (Duan et al., unpublished results).

In conclusion, over-expression of UnkG decreased the root growth-promoting ability of *P. putida* UW4, which could be the result of a detrimental effect of UnkG on plants. On the other hand, it appears that UnkG is acting to regulate a number of key cellular functions in a general way related to the energy balance of the bacterium so that overproduction of this protein acts to down regulate cell metabolism.

Appendix I

UncG encoding sequence (top, from start codon to stop codon) as well as amino acid sequence (bottom):

```
atg ttg aat gac ccg att cca cct cac gtt gac ccg cgc aaa ttg gct gat cgt ggc acc
 M   L   N   D   P   I   P   P   H   V   D   P   R   K   L   A   D   R   G   T

acc ctt caa ggt gaa atg ctg ctg gcc gat ttg gag aga ctc tgc gac ccg ctt tcc gac
 T   L   Q   G   E   M   L   L   A   D   L   E   R   L   C   D   P   L   S   D

aat gtc ggt acg gtg cag gct aaa ttc gtt ttt gaa cga gat gaa cgt aaa tct gtg gtc
 N   V   G   T   V   Q   A   K   F   V   F   E   R   D   E   R   K   S   V   V

atc cac agc ttt atc gac act gaa gtc aaa atg gtt tgc cag cgt tgt ctt gag ctg gtc
 I   H   S   F   I   D   T   E   V   K   M   V   C   Q   R   C   L   E   L   V

acc ctg ccg atc cat agc gaa tgc agt tac gct gtg gtg aag gag ggt gcg aat acc cag
 T   L   P   I   H   S   E   C   S   Y   A   V   V   K   E   G   A   N   T   Q

tcg ttg ccg aaa ggt tat gac gtg ctg gaa ctg ggc gaa gat cct ttg gat ctg cag tca
 S   L   P   K   G   Y   D   V   L   E   L   G   E   D   P   L   D   L   Q   S

ctg atc gag gag gag ctt ctg ctc gcc ttg ccc att gtg cct gct cat cat ccg gaa gaa
 L   I   E   E   E   L   L   L   A   L   P   I   V   P   A   H   H   P   E   E

tgc cag cag ccg gcg gga gca gat gag ccc gaa ccg agc gag gac gag gta acg cgg tcc
 C   Q   Q   P   A   G   A   D   E   P   E   P   S   E   D   E   V   T   R   S

aac ccg ttc agt gta ttg gcg cag tta aag cgt gac cca aac gtt tag
 N   P   F   S   V   L   A   Q   L   K   R   D   P   N   V   *
```

Appendix Ⅱ

Supplementary Table 1. MS information of identified protein spots.

Spot	Accession number	Protein identity	MW (KDa)	p*I*	Score %	Sequence Coverage %
156	Q4ZUF6_PSEU2	Electron transfer flavoprotein, alpha subunit	31.3	5.14	166	18[1]
64	Q3K7Y1_PSEPF	Arginine deiminase	51.8	5.52	106	10[2]

The protein identity, accession number, MW, p*I*, score, and sequence coverage were determined from the top MSDB database match using PEAKS software. Protein scores are derived from ions scores, which is -10*Log (P), as a non-probabilistic basis for ranking protein hits. P is the probability that the observed match is a random event.

[1] Amino acid sequence with matched peptides in bold and underlined.

1 M**TILVIAEHD** **NKVLAPATLN** **TVAAAAKIGG** **DIHVLVAGQG** **AGAVAEAAAK**

51 IAGVSKVLNA DNAAYAHQLP ENVAPLVAEL GAGYSHILAA ATSNGKNILP

101 RVAAQLDVDQ ISEIISVESA DTFKRPIYAG NAIATVQSTA AIKVITVRAT

151 GFDPVAAEGG SAAVEAVAAA HNAGTSSFVS EELAKSDRPE LTAAKIVVSG

201 GRGMQNGDNF K**HLYALADK**L GAAVGASRAA VDAGFVPNDM QVGQTGKIVA

251 PQLYIAVGIS GAIQHLAGMK DSKVIVAINK DEEAPIFQVA DYGLVADLFE

301 AVPELEKLV

[2] Amino acid sequence with matched peptides in bold and underlined.

1 MQLKDTQLFR QQAFIDGAWV DADNGQTIK**V** **NNPATGEILG** **TVPK**MGAAET

51 RRAIEAADKA LPAWRALTAK ERAGKLRRWF ELMIENQDDL ARLMTLEQGK

101 PLAEAKGEIV YAASFIEWFA EEAKRIYGDV IPGHQPDKRL IVIKQPIGVT

151 AAITPWNFPA AMITRKAGPA LAAGCTMVLK PASQTPFSAF ALAELAQRAG

201 IPKGVFSVVS GSAGDIGSEL TSNPIVRKLS FTGSTEIGRQ LMSECAKDIK

251 KVSLELGGNA PFIVFDDADL DKAVEGAIIS KYRNNGQTCV CANR**LYIQDS**

301 **VYDAFAEK**LK VAVAKLKIGN GLEEGTTTGP LIDEKAVAK**V** **QEHIADAVSK**

351 GATVLAGGKP MEGNFFEPTI LTNVPKDAAV AK**EETFGPLA** **PLFR**FKDEAD

401 VIAMSNDTEF GLASYFYARD LGRVFRVAEA LEYGMVGVNT GLISNEVAPF

451 GGIKASGLGR EGSKYGIEDY LEIKYLCLGI

References

Abeles F.B., Morgan P.W. and Saltveit M.E. 1992. In *Ethylene in Plant Biology*. 2nd edition. Eds. Abeles F.B., Morgan P.W. and Saltveit M.E. Academic Press, New York. pp. 50-51.

Adesemoye A.O., Torbert H.A. and Kloepper J.W. 2008. Enhanced plant nutrient use efficiency with PGPR and AMF in an integrated nutrient management system. *Can. J. Microbiol.* **54**, 876–886.

Alban A., David S.O., Bjorkesten L., Andersson C., Sloge E., Lewis S. and Currie I. 2003. A novel experimental design for comparative two-dimensional gel analysis: two-dimensional difference gel electrophoresis incorporating a pooled internal standard. *Proteomics*. **3,** 36-44.

Aliverti A., Pandini V., Pennati A., de Rosa M. and Zanetti G. 2008. Structural and functional diversity of ferredoxin-NADP(+) reductases. *Arch. Biochem. Biophys.* **474,** 283-291.

Amara M.A.T., Dahdoh M.S.A. 1997. Effect of inoculation with plant growth-promoting rhizobacteria (PGPR) on yield and uptake of nutrients by wheat grown on sandy soil. *Egypt J. Soil.* **37,** 467–484.

Anderson N.L., Anderson N.G. 1998. Proteome and proteomics: new technologies, new concepts, and new words. *Electrophoresis.* **19**, 1853–1861.

Anjaiah V., Koedam N., Nowak-Thompson B., Loper J.E., Hofte M., Tambong J.T. and Cornelis P. 1998. Involvement of phenazines and anthranilate in the antagonism of *Pseudomonas aeruginosa* PNA1 and Tn 5 derivatives toward *Fusarium spp*. and *Pythium. spp*. *Mol. Plant-Microbe Interact*. **11**, 847–854.

Babalola O.O., Osir E.O., Sanni A.I., Odhaimbo G.D. and Bulimo W.D. 2003. Amplification of 1-aminocyclopropane-1-carboxylic (ACC) deaminase from plant growth promoting rhizobacteria in Striga-infested soils. *Afri. J. Biotechnol*. **2**, 157–160.

Bai F., Li Y., Xu H., Xia H., Yin T., Yao H., Zhang L., Zhang X., Bai Y., Jin S. and Qiao M. 2007. Identification and functional characterization of *pfm,* a novel gene involved in swimming

motility of *Pseudomonas aeruginosa. Gene*. **401,** 19-27.

Banik S., Dey B.K. 1982. Available phosphate content of an alluvial soil is influenced by inoculation of some isolated phosphate-solubilizing microorganisms. *Plant Soil*. **69**, 353–364.

Barber M.J., Trimboli A.J. and McIntire W.S. 1993. The amino acid sequence of *Pseudomonas putida* azurin. *Arch. Biochem. Biophys.* **303,** 22-26.

Bardiya M.C., Gaur A.C. 1974. Isolation and screening of microorganisms dissolving low grade rock phosphate. *Folia. Microbiol.* **19**, 386–389.

Bar-Ness E., Chen Y., Hadar Y., Marschner H. and Romheld V. 1991. Siderophores of *Pseudomonas putida* as an iron source for dicot and monocot plants. In *Iron nutrition and interactions in plants*. Eds. Chen Y. and Hadar Y. Kluwer Academic Publishers, Dordrect, Netherlands. pp. 271-281.

Bar-Ness E., Hadar Y., Shanzer A. and Libman J. 1992. Iron uptake by plants from microbial siderophores. A study with 7-nitrobenz-2-Oxa-1, 3-diazole-des-ferrioxamine as fluorescent ferrioxamine B analog. *Plant Physiol*. **99**, 1329-1335.

Barraquio W.L., Segubre E.M., Gonzalez M.S., Verma S.C., James E.K., Ladha J.K. and Tripathi A.K. 2000. Diazotrophic *Enterobacteria*: What is their role in the rhizosphere of rice? In *The Quest for Nitrogen fixation in Rice.* Eds. Ladha J.K. and Reddy P.M. IRRI, Philippines. pp. 93-118.

Bashan Y., Holguin G. 1997. Azospirillum–plant relationships: environmental and physiological advances (1990–1996). *Can. J. Microbiol.* **43**, 103–121.

Bashan Y., Holguin G. 1998. Proposal for the division of plant growth-promoting rhizobacteria into two classifications: biocontrol-PGPB (plant growth-promoting bacteria) and PGPB. *Soil Biol. Biochem.* **30**, 1225-1228.

Bayliss C., Bent E., Culham D.E., MacLellan S., Clarke A.J., Brown G.L. and Wood J.M. 1997. Bacterial genetic loci implicated in the *Pseudomonas putida* GR12-2R3-canola mutualism: Identification of an exudates-inducible sugar transporter. *Can. J. Microbiol.* **47**, 809-818.

Belimov A.A., Hontzeas N., Safronova V.I., Demchinskaya S.V., Piluzza G., Bullitta S. and Glick B.R. 2005. Cadmium-tolerant plant growth-promoting rhizobacteria associated with the roots of Indian mustard (Brassica juncea L. Czern.). *Soil Bio. Biochem*. **37**, 241–250.

Belimov A.A., Safronova V.I., Sergeyeva T.A., Egorova T.N., Matveyeva V.A., Tsyganov V.E., Borisov A.Y., Tikhonovich I.A., Kluge C., Preisfeld A., Dietz K.J. and Stepanok V.V. 2001. Characterization of plant growth promoting rhizobacteria isolated from polluted soils and containing 1-aminocyclopropane-1-carboxylate deaminase. *Can. J. Microbiol*. **47**, 642–652.

Bi E.F., Lutkenhaus J. 1991. FtsZ ring structure associated with division in *Escherichia coli. Nature.* **354,** 161-164.

Bilal R., Malik K.A. 1987. Isolation and identification of a N_2-fixing zoogloea-forming bacterium from kallar grass histoplane. *J. Appl. Bacteriol.* **62**, 289-294.

Blackstock W.P., Weir M.P. 1999. Proteomics: quantitative and physical mapping of cellular proteins. *Trends Biotechnol*. **17**, 121–127.

Blaha D., Prigent-Combaret C., Mirza M.S. and Moënne-Loccoz Y. 2006. Phylogeny of the 1-aminocyclopropane-1-carboxylic acid deaminase-encoding gene acdS in phytobeneficial and pathogenic *Proteobacteria* and relation with strain biogeography. *FEMS Microbio. Ecol*. **56**, 455–470.

Bochtler M., Hartmann C., Song H.K., Bourenkov G.P., Bartunik H.D. and Huber R. 2000. The structures of HslU and the ATP-dependent protease HslU-HslV. *Science.* **403,** 800-805.

Boddey R.M., Reis V.M., Urquiaga S., daSilva L.G., dosReis F.B., Baldani J.I. and Dobereiner J. 1995. Biological nitrogen fixation associated with sugarcane and rice: Contributions and prospects for improvement. *Plant Soil.* **174**, 195-209.

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

Briat J.F. 1992. Iron assimilation and storage in prokaryotes. *J. Gen. Microbiol*. **138**, 2475-2483.

Brownsey R.W., Boone A.N., Elliott J.E., Kulpa J.E. and Lee W.M. 2006. Regulation of acetyl-

CoA carboxylase. *Biochem. Soc. Trans*. **34,** 223–227.

Burd G.I., Dixon D.G. and Glick B.R. 1998. A plant growth-promoting bacterium that decreases nickel toxicity in seedlings. *Appl. Environ. Microbiol.* **64**, 3663–3668.

Campbell B.G., Thomson J.A. 1996. 1-Aminocyclopropane-1-carboxylate deaminase genes from *Pseudomonas* strains. *FEMS Microbiol. Lett.* **138**, 207-210.

Canovas F. M., Dumas-Gaudot E., Recorbet G., Jorrin J., Mock H. P., Rossignol M. 2004. Plant proteome analysis. *Proteomics.* 4, 285–298.

Castignetti D., Smarrelli J. Jr. 1986. Siderophores, the iron nutrition of plants, and nitrate reductase. *FEBS Lett*. **209**, 147-151.

Causer M.J., Hopper D.J., McIntire W.S. and Singer T.P. 1984. Azurin from *Pseudomonas putida*: an electron acceptor for p-cresol methylhydroxylase. *Biochem. Soc. Trans.* **12,** 1131-1132.

Chen C., Bauske E.M., Musson G., Rodriguez-Kabaña R. and Kloepper J.W. 1994. Biological control of Fusarium on cotton by use of endophytic bacteria. *Biol. Control.* **5,** 83–91.

Chen Z., Barber M.J., McIntire W.S. and Mathews F.S. 1998. Crystallographic study of azurin from *Pseudomonas putida*. *Acta Cryst.* **D54,** 253-268.

Cheng Z., Duan J., Hao Y., McConkey B.J. and Glick B.R. 2009a. Identification of bacterial proteins mediating the interactions between *P. putida* UW4 and *Brassica napus* (canola). *Mol. Plant-Microbe Interact.* **22,** 686-694.

Cheng Z., Duncker B.P., McConkey B.J. and Glick B.R. 2008. Transcriptional regulation of ACC deaminase gene expression in *Pseudomonas putida* UW4. *Can. J. Microbiol.* **54,** 128-136.

Cheng Z., McConkey B.J. and Glick B.R. 2010. Proteomic studies of plant-bacterial interactions. *Soil Biol. Biochem.* **42**, 1673-1684.

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

Cheng Z., Woody O.Z., Song J., Glick B.R. and and McConkey B.J. 2009b. Proteome reference map for the plant growth-promoting bacterium *Pseudomonas putida* UW4. *Proteomics.* **9,** 4271-4274.

Chet I., Chernin L. 2002. Biocontrol, microbial agents in soil. In *Encyclopedia of Environmental microbiology.* Eds. Bitton G. John Willey and Sons Inc., New York. pp. 45-465.

Chin-A-Woeng T.F.C., Bloemberg G.V., Van der Bij A.J., Van der Drift K.M.G.M., Schripsema J., Kroon B., Scheffer R.J., Keel C., Bakker P.A.H.M., De Bruijn F.J., Thomas-Oates J.E. and Lugtenberg B.J.J. 1998. Biocontrol by phenazine-1-carboxamide producing *Pseudomonas chlororaphis* PCL1391 of tomato root rot caused by *Fusarium oxysporum* f.sp. radicis-lycopersici. *Mol. Plant-Microbe Interact*. **10**, 79–86.

Corthals G.L., Wasinger V.C., Hochstrasser D.F. and Sanchez J. 2000. The dynamic range of protein expression: a challenge fro proteomic research. *Electrophoresis*. **21**, 1104-1115.

Craven P.A, Hayasaka S.S. 1982. Inorganic phosphate solubilization by rhizosphere bacteria in a *Zostera marina* community. *Can. J. Microbiol*. **28**, 605–610.

Cronin D., MoenneLoccoz Y., Fenton A., Dunne C., Dowling D.N. and O'Gara F. 1997. Role of 2,4-diacetylphloroglucinol in the interactions of the biocontrol *Pseudomonas* strain F113 with the potato cyst nematode *Globodera rostochiensis*. *Appl. Environ. Microbiol*. **63**, 1357–1361.

Crowley D.E., Reid C.P.P. and Szaniszlo P.J. 1988. Utilization of microbial siderophores in iron acquisition by oat. *Plant Physiol*. **87**, 680-685.

Dardanelli M.S., Córdoba F.J.F., Espuny M.R., Carvajal M.A.R., Díaz M.E.S., Serrano A.M.G., Okon Y., Megías M. 2008. Effect of *Azospirillum brasilense* coinoculated with *Rhizobium* on *Phaseolus vulgaris* flavonoids and Nod factor production under salt stress, *Soil Biol. Biochem.* **40**, 2713–2721.

Darmwall N.S.**,** Singh R.B. and Rai R. 1989. Isolation of phosphate solubilizers from different sources. *Curr. Sci*. **58**, 570–571.

Das A.C. 1963. Utilization of insoluble phosphates by soil fungi. *J. Indian Soc. Soil Sci.* **11**, 203–

207.

De Meyer G, Höfte M. 1997a. Salicylic acid produced by the rhizobacterium *Pseudomonas aeruginosa* 7NSK2 induces resistance to leaf infection by Botrytis cinerea on bean. *Phytopathology.* **87**, 588–593.

De Meyer G, Höfte M. 1997b. Induction of systemic resistance by the rhizobacterium *Pseudomonas aeruginosa* 7NSK2 is a salicylic acid dependent phenomenon in tobacco. *IOBC Bull.* **21,** 117-122.

Dey K.B. 1988. Phosphate solubilizing organisms in improving fertility status. In *Biofertilizers: Potentialities and Problems.* Eds. Sen S.P, Palit P. Calcutta: Plant Physiology Forum, Naya Prokash. pp. 237–248.

Di Pietro A., Lorito M., Hayes C.K., Broadway R.M. and Harman G.E. 1993. Endochitinase from *Gliocladium virens*: isolation, characterization and synergistic antifungal activity in combination with gliotoxin. *Mol. Plant. Pathol.* **83**, 308-313.

Dobbelaere S., Croonenborghs A., Thys A., Vande Broek A.. and Vanderleyden J. 1999. Phytostimulatory effect of Azospirillum brasilense wild type and mutant strains altered in IAA production on wheat. *Plant Soil*. **212**, 155–164.

Duff R.B, Webley D.M. 1959. 2-Ketogluconic acid as a natural chelator produced by soil bacteria. *Chem. Ind*. 1376–1377.

Duijff B.J, Gianinazzi-Pearson V. and Lemanceau P. 1997. Involvement of the outer membrane lipopolysaccharides in the endophytic colonization of tomato roots by biocontrol *Pseudomonas fluorescens* strain WCS417r. *New Phytol.* **135**, 325–334.

Dworkin M., Foster J.W. 1958. Experiments with some microorganisms which utilize ethane and hydrogen. *J. Bacteriol.* **75,** 592-603.

Edgar R.C. 2004. MUSCLE: Multiple Sequence Alignment with High Accuracy and High Throughput. *Nucleic Acids Res*. **32,** 1792-1797.

Ei-Banna N., Winkelmann G. 1998. Pyrrolnitrin from *Burkholderia cepacia*: antibiotic activity

against fungi and novel activities against *streptomycetes*. *J. Appl. Microbiol.* **85**, 69-78.

Emanuele J.J. Jr, Jin H., Jacobson B.L., Chang C.Y., Einspahr H.M. and Villafranca J.J. 1996. Kinetic and crystallographic studies of *Escherichia coli* UDP-N-acetylmuramate: L-alanine ligase. *Protein Sci.* **5,** 2566-2574.

Fallik E., Sarig S. and Okon Y. 1994 Morphology and physiology of plant roots associated with *Azospirillum.* In *Azospirillum–Plant Associations*. eds. Okon Y. CRC Press, Boca Raton. pp. 77–85.

Fernández-Piñar R., Ramos J.L., Rodríguez-Herva J.J. and Espinosa-Urgel M. 2008. A two-component regulatory system integrates redox state and population density sensing in *Pseudomonas putida. J. Bacteriol.* **190,** 7666-7674.

Figueiredo M.V.B., Seldin L., Araujo F.F. and Mariano R.L.R. 2010. Plant growth promoting rhizobacteria: fundamentals and applications. *In* Plant growth and health promoting bacteria. Eds. Maheshwari D.k. Springer-Verlag Berlin, Heidelberg. pp. 21-43.

Frankenberger. W.T. Jr., Arshad M. 1995. Phytohormones in soil. Marcel Dekker, New York.

Ghosh S., Penterman J.N., Little R.D., Chavez R. and Glick B.R. 2003. Three newly isolated plant growth-promoting bacilli facilitate the growth of canola seedlings. *Plant Physiol Biochem*. **41**, 277–281.

Gamalero E., Berta G., Massa N., Glick B.R. and Lingua G. 2008. Synergistic interactions between the ACC deaminase-producing bacterium *Pseudomonas putida* UW4 and the AM fungus *Gigaspora rosea* positively affect cucumber plant growth. *FEMS Microbiol. Ecol.* **64**, 459-467.

Gamalero E., Berta G., Massa N., Glick B.R. and Lingua G. 2010. Interactions between *Pseudomonas putida* UW4 and *Gigaspora rosea* BEG9 and their consequences for the growth of cucumber under salt-stress conditions. *J. Appl. Microbiol.* **108,** 236–245.

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

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

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

Glick B.R. 2010. Using soil bacteria to facilitate phytoremediation. *Biotechnol. Adv.* **28,** 367-374.

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., 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. Overview of plant growth-promoting bacteria. In *Biochemical and genetic mechanisms used by plant growth promoting bacteria*. Eds. Glick B. R. Imperial College Press. London. pp. 1-10.

Goldstein A.H. 1994. Involvement of the quinoprotein glucose dehydrogenase in the solubilization of exogenous phosphates by gram-negative bacteria. In *Phosphate in Microorganisms: Cellular and Molecular Biology*. eds. Torriani-Gorini A, Yagil E. and Silver S. Washington, DC: ASM Press. pp. 197–203.

Goldstein A.H., Rogers R.D. and Mead G. 1993. Mining by microbe. *Bio/Technology.* **11,** 1250–1254.

Gordon S.A., Weber R.P. 1951. Colorimetric estimation of indole-acetic acid. *Plant Physiol.* **26,** 192-195.

Görg A., Boguth G., Obermaier C., Posch A. and Weiss W. 1995. Two-dimensional polyacrylamide gel electrophoresis with immobilized pH gradients in the first dimension (IPG-Dalt): the state of the art and the controversy of vertical versus horizontal systems. *Electrophoresis*. **16**, 1079-1086.

Görg A., Boguth G., Obermeier C. and Weiss W. 1998. Two-dimensional electrophoresis of proteins in an immobilized pH 4-12 gradient. *Electrophoresis*. **19**, 1516-1519.

Görg A., Drews O., Lück C., Weiland F. and Weiss W. 2009. 2-DE with IPGs. *Electrophoresis.* **30**, S122-S132.

Görg A., Obermeier C., Boguth G., Csordas A., Diaz J.J. and Madjar J.J. 1997. Very alkaline immobilized pH gradients for two-dimensional electrophoresis of ribosomal and nuclear proteins. *Electrophoresis*. **18**, 328-337.

Görg A., Obermaier C., Boguth G., Harder A., Scheibe B., Wildgruber R. and Weiss W. 2000. The current state of two-dimensional electrophoresis with immobilized pH gradients. *Electrophoresis*. **21,** 1037–1053.

Görg A., Obermaier C., Boguth G. and Weiss W. 1999. Recent developments in two-dimensional gel electrophoresis with immobilized pH gradients: wide pH gradients up to pH 12, longer separation distances and simplified procedures. *Electrophoresis*. **20**, 712-717.

Görg A., Postel W. and Günther S. 1988. Two-dimensional electrophoresis. The current stare of two-dimensional electrophoresis with immobilized pH gradients. *Electrophoresis.* **9**, 531-546.

Grichko V.P, Glick B.R. 2001. Amelioration of flooding stress by ACC deaminase-containing plant growth-promoting bacteria. *Plant Physiol. Biochem*. **39**, 11–17.

Guindon S., Gascuel O. 2003. A Simple, Fast, and Accurate Algorithm to Estimate Large Phylogenies by Maximum Likelihood. *Syst. Biol*. **52,** 696-704.

Gygi S.P., Rist B., Gerber S.A., Turecek F., Gelb M.H. and Aebersold R. 1999. Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat. Biotechnol.* **17,** 994-999.

Halder A.K, Mishra A.K, Bhattacharyya P. and Chakrabartty P.K. 1990. Solubilization of rock phosphate by *Rhizobium* and *Bradyrhizobium. J. Gen. Appl. Microbiol*. **36,** 81–92.

Hall T.A. 1999. BioEdit: A User-Friendly Biological Sequence Alignment Editor and Analysis Program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* **41,** 95-98.

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

Handelsman J., Stabb E.V. 1996. Biocontrol of soilborne plant pathogens. *Plant cell.* **8**, 1855-1869.

Honma M. 1993. Stereospecific reaction of 1-aminocyclopropane-1-carboxylate deaminase. In *Cellular and molecular aspects of the plant hormone ethylene*. Eds. Pech J.C., Latché A. and Balagué C. The Netherlands: Kluwer Academic Publishers. pp. 111–116.

Hontzeas N., Richardson A.O., Belimov A.A., Safranova V.I., Abu-Omar M.M. and Glick B.R. 2005. Evidence for horizontal gene transfer (HGT) of ACC deaminase genes. *Appl. Environ. Microbiol*. **71**, 7556–7558.

Hontzeas N., Zoidakis J., Glick B.R. and Abu-Omar M.M. 2004. Expression and characterization of 1-aminocyclopropane-1-carboxylate deaminase from the rhizobacterium *Pseudomonas putida* UW4: a key enzyme in bacterial plant growth promotion. *Biochim. Biophys*. *Acta.* **1703,** 11-19.

Hokeberg M., Wright S. A.I., Svensson M., Lundgren L.N. and Gerhardson B. 1998. Mutants of *Pseudomonas chlororaphis* defective in the production of an antifungal metabolite express reduced biocontrol activity. Abstract Proceedings ICPP98, Edinburgh, Scotland.

Hoving S., Gerrits B., Voshol H., Müller D., Roberts R.C. and van Oostrum J. 2002. Preparative two-dimensional gel electrophoresis at alkaline pH using narrow range immobilized pH gradients. *Proteomics*. **2**, 127-134.

Howell C.R., Beier R.C. and Stipanovic R.D. 1988. Production of ammonia by *Enterobacter cloacae* and its possible role in the biological control of Pythium preemergence damping-off by the bacterium. *Phytopathol.* **78,** 1075-1078.

Howell C.R., Stipanovic R.D. 1979. Control of *Rhizoctonia solani* on cotton seedlings with *Pseudomonas fluorescens* and with an antibiotic produced by the bacterium. *Phytopathol*. **69**, 480-482.

Illmer P, Schinner F. 1992. Solubilization of inorganic phosphates by microorganisms isolated from forest soil. *Soil Biol. Biochem*. **24**, 389–395.

Jacobson C.B., Pasternak J.J. and Glick B.R. 1994. Partial purification and characterization of 1-

aminocyclopropane-1-carboxylate deaminase from the plant growth promoting rhizobacterium *Pseudomonas putida* GR12-2. *Can. J. Microbiol*. **40**, 1019–1025.

James E.K., Gyaneshwar P., Barraqui W.L., Mathan N. and Ladha J.K. 2000. Endophytic diazotrophs associated with rice. In *The Quest for Nitrogen Fixation in Rice*. Eds. Ladha J.K. and Reddy P.M. IRRI, Philippines. pp. 119-140.

James E.K. Olivares F.L. 1997. Infection and colonization of sugarcane and other graminaceous plants by endophytic bacteria. *Cri. Rev. Plant Sci.* **17**, 77-119.

James P. 1997. Protein identification in the post-genome era: the rapid rise of proteomics. *Q. Rev. Biophys*. **30**, 279–331.

Jäderlund L., Arthurson V., Granhall U. and Jansson J.K. 2008. Specific interactions between arbuscular mycorrhizal fungi and plant growth-promoting bacteria: as revealed by different combinations. *FEMS Microbiol. Lett.* **287,** 174–180.

Jin H., Emanuele J.J.Jr., Fairman R., Robertson J.G., Hail M.E., Ho H., Falk P.J. and Villafranca J.J. 1996. Structural studies of *Escherichia coli* UDP-N-acetylmuramate:L-alanine ligase. *Biochem.* **35,** 1423-1431.

Jorrin-Novo J.V., Maldonado A.M., Echevarria-Zomeno S., Valledor L., Castillejo M.A., Curto M., Valero J., Sghaier B., Donoso G. and Redondo I. 2009. Plant proteomics update (2007-2008): Second-generation proteomic techniques, an appropriate experimental design, and data analysis to fulfill MIAPE standards, increase plant proteome coverage and expand biological knowledge. *J. Proteomics*. **72,** 285-314.

Jouanneau J.P., Lapous D. and Guern J. 1991. In plant protoplasts, the spontaneous expression of defense reactions and the responsiveness to exogenous elicitors are under auxin control. *Plant Physiol*. **96**, 459–466.

Kang Y., Carlson R., Tharpe W. and Schell M.A. 1998. Characterization of genes involved in biosynthesis of a novel antibiotic from *Burkholderia cepacia* BC11 and their role in biological control of *Rhizoctonia solani. Appl. Environ.Microbiol.* 3939-3947.

Karakurt H., Aslantas R., Ozkan G. and Guleryuz M. 2009. Effects of indol–3-butyric acid (IBA), plant growth promoting rhizobacteria (PGPR) and carbohydrates on rooting of hardwood cutting of MM106 Apple rootstock. *Afr. J. Agri. Res.* **4**, 60-64.

Katznelson H, Peterson E.A. and Rovatt J.W. 1962. Phosphate dissolving microoganisms on seed and in the root zone of plants. *Can. J. Bot.* **40**, 1181–1186.

Kaur N., Gautam A., Kumar S., Singh A., Singh N., Sharma S., Sharma R., Tewari R. and Singh T.P. 2011. Biochemical studies and crystal structure determination of dihydrodipicolinate synthase from *Pseudononas aeruginosa. Int. J. Biol. Macromol.* **48,** 779-787.

Kav N.N.V., Srivastava S., Yajima W. and Sharma N. 2007. Application of proteomics to investigate plant-microbe interactions. *Curr. Proteomics.* **4,** 28-43.

Kazan K., Manners J.M. 2009. Linking development to defense: auxin in plant-pathogen interactions. *Trends Plant Sci*. **14**, 373-382.

Keel C., Weller D.M., Natsch A., Défago G., Cook R.J. and Thomashow L.S. 1996. Conservation of the 2,4-diacetylphloroglucinol biosynthesis locus among *Fluorescent Pseudomonas* strains from diverse geographic locations. *Appl. Environ. Microbiol.* 552-563**.**

Kende H. 1993. Ethylene biosynthesis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **44**, 283-307.

Kessmann H., Staub T., Ligon J., Oostendorp M. and Ryals J. 1994. Activation of systemic acquired disease resistance in plants. *Eur. J. Plant Pathol.* **100**, 359–369.

Khalid A., Tahir S., Arshad M. and Zahir Z.A. 2004. Relative efficiency of rhizobacteria for auxin biosynthesis in rhizosphere and non-rhizosphere soils. *Aust. J. Soil. Res.* **42**, 921–926.

Klee H.J., Hayford M.B., Kretzmer K.A., Barry G.F. and Kishore G.M. 1991. Control of ethylene synthesis by expression of a bacterial enzyme in transgenic tomato plants. *Plant Cell*. **3**, 1187–1193.

Kloepper J.W., Schroth M.N. and Miller T.D. 1980. Effects of rhizosphere colonization by plant growth promoting rhizobacteria on potato plant development and yield. *Phytopathol.* **70,** 1078–1082.

Klose J. 1975. Protein mapping by combined isoelectric focusing and electrophoresis of mouse tissues. A novel approach to testing for induced point mutation in mammals. *Humangenetik.* **26**, 231-243.

Leeman M., Van Pelt J.A., Den Ouden F.M., Heinsbroek M., Bakker P.A.H.M. 1995a. Induction of systemic resistance by *Pseudomonas fluorescens* in radish cultivars differing in susceptibility to fusarium wilt, using a novel bioassay. *Eur. J. Plant Pathol.* **101**, 655–664.

Leeman M., Van Pelt J.A., Den Ouden F.M., Heinsbroek M., Bakker P.A.H.M. 1995b. Induction of systemic resistance against fusarium wilt of radish by lipopolysaccharides of *Pseudomonas fluorescens*. *Phytopathol.* **85**, 1021–1027.

Leeman M., Van Pelt J.A., Hendrickx M.J., Scheffer R.J., Bakker P.A.H.M., 1995c. Biocontrol of fusarium wilt of radish in commercial greenhouse trials by seed treatment with *Pseudomonas fluorescens* WCS374. *Phytopathol.* **85**, 1301–1305.

Leong J. 1986. Siderophores: their biochemistry and possible role in the biocontrol of plant pathogens. *Ann. Rev. Phytopathol*. **24**, 187-209.

Leyval C., Berthelin J. 1989. Interaction between *Laccaria laccata*, *Agrobacterium radiobacter* and beech roots: influence on P, K, Mg and Fe movilization from minerals and plant growth. *Plant Soil*. **117**, 103–110.

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

Ligon J.M., Hill D.S., Hammer P.E., Torkewitz N.R., Hofmann D., Kempf H.J. and van Pée K.H. 2000. Natural products with antifungal activity from *Pseudomonas* biocontrol bacteria. *Pest manage. Sci.* **56**, 688-695.

Lin T., Huang H., Shen F. and Young C. 2006. The protons of gluconic acid are the major factor responsible for the dissolution of tricalcium phosphate by *Burkholderia cepacia* CC-A174. *Bioresour. Technol.* **97,** 957-960.

Liu T.S., Lee L.Y., Tai C.Y., Hung C.H., Chang Y.S., Wolfram J.H., Rogers R. and Goldstein A.H. 1992. Cloning of an *Erwinia herbicola* gene necessary for gluconic acid production and enhanced mineral phosphate solubilization in *Escherichia coli HB101*: Nucleotide sequence and probable involvement in biosynthesis of the coenzyme pyrroloquinoline quinone. *J. Bacteriol.* **174,** 5814–5819.

Loon L.C. van, Bakker P.A.H.M. and Pieterse C.M.J. 1998. Systemic resistance induced by rhizosphere bacteria. *Annu. Rev. Phytopathol.* **36**, 453–483.

Louw H.A., Webley D.M. 1959. A study of soil bacteria dissolving certain phosphate fertilizers and related compounds. *J. Appl. Bacteriol.* **22**, 227–233.

Lu X., Galkin A., Herzberg O. and Dunaway-Mariano D. 2004. Arginine deiminase uses an active-site cysteine in nucleophilic catalysis of L-arginine hydrolysis. *J. Am. Chem. Soc.* **126,** 5374-5375.

Lucy M., Reed E. and Glick B.R. 2004. Applications of free living plant growth-promoting bacteria, *Anton. van Leeuwen.* **86**, 1-25.

Ma B., Zhang K., Hendrie C., Liang C., Li M., Doherty-Kirby A. and Lajoie G. 2003a. PEAKS: powerful software for peptide de novo sequencing by tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **17,** 2337-2342.

Ma W., Guinel F.C. and Glick B.R. 2003b. The Rhizobium leguminosarum bv. viciae ACC deaminase protein promotes the nodulation of pea plants. *Appl. Environ. Microbiol*. **69**, 4396–4402.

Ma W., Sebestianova S., Sebestian J., Burd G.I., Guinel F. and Glick B.R. 2003c. Prevalence of 1-aminocyclopropaqne-1-carboxylate in deaminase in *Rhizobia spp. Anton. Van Leeuwen.* **83**, 285–291.

Madhaiyan M., Poonguzhali S., Ryu J. and Sa T. 2006. Regulation of ethylene levels in canola (*Brassica campestris*) by 1-aminocycloprpane-1-carboxylate deaminase containing *Methylobacterium fjisawaense*. *Planta*. **224**, 268–278.

Mann M., Jensen O.N. 2001. Proteomic analysis of post-translational modifications. *Nature Biotechnol.* **21,** 255–261.

Malik K.A., Bilal R., Mehnaz S., Rasul G., Mirza M.S. and Ali S. 1997. Association of nitrogen fixing and plant growth promoting rhizobacteria (PGPR) with kallar grass and rice. *Plant Soil.* **194**, 37-44.

Maurhofer M., Hase C., Meuwly P., Métraux J.P. and Défago G. 1994. Induction of systemic resistance of tobacco to tobacco necrosis virus by the root-colonizing *Pseudomonas fluorescens* strain CHA0: influence of the *gacA* gene and of pyoverdine production. *Phytopathol.* **84**, 139–146.

Manulis S., Haviv-Chesner A., Brandl M.T., Lindow S.E. and Barash I. 1998. Differential involvement of indole-3-acetic acid biosynthetic pathways in pathogenicity and epiphytic fitness of *Erwinia herbicola pv. gypsophilae*. *Mol. Plant–Microbe Interact*. **11**, 634–642.

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

Mayak S., Tirosh T. and Glick B.R. 2004b. Plant growth-promotion bacteria that confer resistance to water stress in tomatoes and peppers. *Plant Sci*. **166**, 525–530.

McGrath J.W., Hammerschmidt F. and Quinn J.P. 1998. Biodegradation of phosphonomycin by *Rhizobium huakuii* PMY1. *Appl. Environ. Microbiol*. **64**, 356–358.

Minami R., Uchiyama K., Murakami T., Kawai J., Mikami K., Yamada T., Yokoi D., Ito H., Matsui H. and Honma M. 1998. Properties, sequence, and synthesis in *Escherichia coli* of 1-aminocyclopropane-1-carboxylate deaminase from Hansenula saturnus. *J. Biochem*. **123**, 1112–1118.

Minden J.S., Dowd S.R., Meyer H.E. and Stuhler K. 2009. Difference gel electrophoresis. *Electrophoresis.* **30,** S156-S161.

Mishustin E.N., Naumova A.N. 1962. Bacterial fertilizers: Their effectiveness and mode of action. *Mikrobiologiya* **31**, 543-555.

Nakayama T., Homma Y., Hashidoko Y., Mizutani J. and Tahara S. 1999. Possible role of xanthobaccins produced by *Stenotrophomonas sp.* strain SB-K88 in suppression of sugar beet damping-off disease. *Appl. Environ. Microbiol.* **55**, 4334-4339.

Neilands J.B. 1981. Microbial iron compounds. *Ann. Rev. Biochem*. **50**, 715-731.

Neilands J.B., Konopka K., Schwyn B., Coy M., Francis R.T., Paw B.H. and Bagg A. 1987. Comparative biochemistry of microbial iron assimilation. In *Iron transport in microbes, plants and animals*, Eds. Winkelmann G., van der Helm D. and Neilands J.B. Verlagsgesellschaft mbH, Weinheim, Germany. pp. 3-33.

Neilands J.B., Leong S.A. 1986. Siderophores in relation to plant growth and disease. *Annu. Rev. Plant Physiol*. **37**, 187-208.

Nouwens A.S., Cordwell S.J., Larsen M.R., Molloy M.P., Gillings M., Willcox M.D.P. and Walsh B.J. 2000. Complementing genomics with proteomics: the membrane subproteome of *Pseudomonas aeruginosa* PAO1. *Electrophoresis*. **21**, 3797-3809.

O'Farrell P.H. 1975. High resolution two dimensional electrophoresis of proteins. *J. Biol. Chem.* **250**, 4007-4021.

Okon Y., Bloemberg G.V., Lugtenberg B.J.J. 1998. Biotechnology of biofertilization and phytostimulation. In *Agricultural Biotechnology*, Eds. Altman A. New York: Marcel Dekker. pp. 327–349.

Oliva M.A., Trambaiolo D. and löwe J. 2007. Structural insights into the conformational variability of FtsZ. *J. Mol. Biol.* **373,** 1229-1242.

Ordentlich A., Elad Y. and Chet I. 1988. The role of chitinase of *Serratia marcescens* in the biocontrol of *Sclerotium rolfsii. Phytopathol*. **78**, 84-88.

Ortlund E., Lacount M.W., Lewinski K. and Lebioda L. 2000. Reactions of *Pseudomonas* 7A glutaminase-asparaginase with diazo analogues of glutamine and asparagines result in unexpected covalent inhibitions and suggests an unusual catalytic triad Thr-Tyr-Glu. *Biochem.* **39,** 1199-1204.

Ostwal K.P., Bhide V.P. 1972. Solubilization of tricalcicum phosphate by soil *Pseudomonas*. *Indian J. Exp. Biol.* **10**, 153–154.

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

Pal K.K., Gardener B.M. 2006. Biological Control of Plant Pathogens. In *The Plant Health Instructor*. DOI: 10.1094/PHI-A-2006-1117-02.

Palumbo J.D., Yuen G.Y., Jochum C.C., Tatum K. and Kobayashi D.Y. 2005. Mutagenesis of beta-1,3-glucanase genes in *Lysobacter enzymogenes* strain C3 results in reduced biological control activity toward Bipolaris leaf spot of tall fescue and Pythium damping-off of sugar beet. *Phytopathol.* **95**, 701-707.

Paradis-Bleau C., Beaumont M., Sanschagrin F., Voyer N. and Levesque R.C. 2007. Parallel solid synthesis of inhibitors of the essential cell division FtsZ enzyme as a new potential class of antibacterials. *Bioorg. Med. Chem.* **15,** 1330-1340.

Patten C.L., Glick B.R. 1996. Bacterial biosynthesis of indole-3-acetic acid. *Can. J. Microbiol.* **42**, 207–220.

Patten C.L., 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., Glick B.R. 2001. Levels of 1-aminocyclopropane-1-carboxylic acid (ACC) in exudates and extracts of canola seeds treated with plant growth-promoting bacteria. *Can. J. Microbiol.* **47**, 368-372.

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

Perkins D.N., Pappin D.J., Creasy D.M. and Cottrell J.S. 1999. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis.* **20,** 3551-3567.

Posada D. In press. jModelTest: Phylogenetic Model Averaging. Molecular Biology and Evolution.

Raaijmakers J.M., Vlami M. and de Souza J.T. 2002. Antibiotic production by bacterial biocontrol agents. *Anton. van Leeuwen.* **81**, 537-547.

Raghu K., MacRae I.C. 1966. Occurrence of phosphate-dissolving microorganisms in the rhizosphere of rice plants and in submerged soils. *J. Appl. Bacteriol.* **29**, 582–586.

Reinhold-Hurek B., Hurek T., Gillis M., Hoste B., Vancannyt M., Kersters K. and De Ley J. 1993. *Azoarcus* gen. nov., nitrogen-fixing proteobacteria associated with roots of kallar grass (*Leptochloa fusca* L. Kunth) and description of two species, *Azoarcus indigens* sp. nov., and *Azoarcus communis* sp. nov. *Int. J. Syst. Bacteriol.* **43**, 574-584.

Roberts D.L., Frerman F.E. and Kim J.J. 1996. Three-dimensional structure of human electron transfer flavoprotein to 2.1-Å resolution. *Proc. Natl Acad. Sci.* **93,** 14355–14360.

Roberts D.L., Salazar D., Fulmer J.P., Frerman F.E. and Kim J.J.P. 1999. Crystal structure of *Paracoccus denitrificans* electron transfer flavoprotein and electrostatic analysis of a conserved flavin binding domain. *Biochem.* **38,** 1977–1989.

Rodríguez H., Fraga R. 1999. Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnol. Adv.* **17**, 319–339.

Rohrwild M., Coux O., Huang H.C., Moerschell R.P., Yoo S.J., Seol J.H., Chung C.H. and Goldberg A.L. 1996. HslV-HslU: A novel ATP-dependent protease complex in *Escherichia coli* related to the eukaryotic proteasome. *Proc. Natl. Acad. Sci.* **93,** 5808–5813.

Ross P.L., Huang Y.N., Marchese J.N., Williamson B., Parker K., Hattan S., Khainovski N., Pillai S., Dey S., Daniels S., Purkayastha S., Juhasz p., Martin S., Bartlet-Jones M., He F., Jacobson A. and Pappin D.J. 2004. Multiplexed protein quantitation in saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. *Mol. Cell. Proteomics*. **3,** 1154-1169.

Ryals J.A., Neuenschwander U.H., Willits M.G., Molina A. and Steiner H.Y. 1996. Systemic acquired resistance. *Plant Cell.* **8**, 1809–1819.

Salih H.M., Yahya A.Y., Abdul-Rahem A.M. and Munam B.H. 1989. Availability of phosphorus in a calcareus soil treated with rock phosphate or superphosphate as affected by phosphate dissolving fungi. *Plant Soil*. **120**, 181–185.

Sambrook J., Russell D.W. 2001. In *Molecular Cloning: a laboratory manual.* 3rd edition. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, New York. pp. 5.1-5.17.

Schweppe R.E., Haydon C.E., Lewis T.S., Resing K.A. and Ahn N.G. 2003. The characterisation of protein post-translational modifications by mass spectrometry. *Acc. Chem. Res.* 36, 453–461.

Sequeira L. 1965. Origin of indoleacetic acid in tobacco plants infected by *Pseudomonas solanacearum*. *Phytopathol*. **55**, 1232–1236.

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

Sheehy R.E., Honma M., Yamada M., Sasaki T., Martineau B. and Hiatt W.R. 1991. Isolation, sequence, and expression in Escherichia coli of the Pseudomonas sp. strain ACP gene encoding 1-aminocyclopropane-1-carboxylate deaminase. *J. Bacteriol*. **173**, 5260–5265.

Shinshi H., Mohner D. and Meins F.Jr. 1987. Regulation of a plant pathogenesis-related enzyme: inhibition of chitinase and chitinase mRNA accumulation in cultured tobacco tissues by auxin and cytokinin. *Proc. Natl. Acad. Sci.* **84**, 89–93.

Shishido M., Chanway C.P. 2000. Colonization and growth of outplanted spruce seedlings pre-inoculated with plant growth-promoting rhizobacteria in the greenhouse. *Can. J. For. Res.* **30**, 848-854.

Smith A.W., Iglewski B.H. 1989. Transformation of *Pseudomonas aeruginosa* by electroporation. *Nucleic Acids Res*. **17,** 10509.

Sonawane A., Klöppner U., Derst C. and Röhm K. 2003. Utilization of acidic amino acids and their amides by *Pseudomonas*: role of periplasmic glutaminase-asparaginase. *Arch Microbiol.* **179,** 151-159.

Spaepen S., Vanderleyden J. and Remans R. 2007. Indole-3-acetic acid in microbial and microorganism-plant signaling. *FEMS Microbiol. Rev.* **31**, 425–448

Sperberg J.I. 1958. The incidence of apatite-solubilizing organisms in the rhizosphere and soil. *Aust. J. Agric. Res.* **9**, 778-782.

Stasyk T., Huber L.A. 2004. Zooming in: fractionation strategies in proteomics. *Proteomics*. **4**, 3704-3716.

Sticher L., Mauch-Mani B. and Métraux J.P. 1997. Systemic acquired resistance. *Annu. Rev. Phytopathol.* **35**, 235–270.

Stricker J., Maddox P., Salmon E.D. and Erickson H.P. 2002. Rapid assembly dynamics of the *Escherichia coli* FtsZ-ring demonstrated by fluorescence recovery after photobleaching. *Proc. Natl. Acad. Sci.* **99,** 3171-3175.

Sundara R.W.V.B., Sinha M.K. 1963. Phosphate dissolving microorganisms in the soil and rhizosphere. *Indian J. Agric. Sci*. **33**, 272–278.

Taiz L., Zeiger E. 1998. Plant Physiology. Sinauer Associates, Sunderland, MA.

Teale W.D., Paponov I.A. and Palme K. 2006. Auxin in action: signalling, transport and the control of plant growth and development. *Nat. Rev. Mo.l Cell Biol.* **7,** 847–859.

Thomashow L.S., Weller D.M. 1988. Role of phenazine antibiotic from *Pseudomonas fluorescens* in biological control of *Gaeumannomyces graminis var. tritici*. *J. Bacteriol*. **170**, 3499–3508.

Thorpe C. 1991. The reductive half-reaction in acyl-CoA oxidase from *Candida tropicalis*: interaction with acyl-CoA analogues and an unusual thioesterase activity. *Arch. Biochem. Biophys*. **286,** 504–510.

Timms J.F., Cramer R. 2008. Difference gel electrophoresis. *Proteomics.* **8,** 4886-4897.

Uchiumi T., Ohwada T., Itakura M., Mitsui H., Nukui N., Dawadi P., Kaneko T., Tabata S., Yokoyama T., Tejima K., Saeki K., Omori H., Hayashi M., Maekawa T., Sriprang R., Murooka Y., Tajima S., Simomura K., Nomura M., Suzuki A., Shimoda Y., Sioya K., Abe M. and

Minamisawa K. 2004. Expression islands clustered on the symbiosis island of the *Mesorhizobium loti* genome. *J. Bacteriol*. **186**, 2439–2448.

Uemura H., Conley J., Yamao F., Rogers J., Soell D.G. 1988. *Escherichia coli* glutaminyl-tRNA synthetase: a single amino acid replacement relaxes rRNA specificity. *Protein Seu. Data Anal.* **1,** 479-485.

Uknes S., Mauch-Mani B., Moyer M., Potter S., Williams S., Dincher S., Chandler D., Slusarenko A., Ward E. and Ryals J. 1992. Acquired resistance in *Arabidopsis*. *Plant Cell.* **4**, 645– 456.

Ünlü M., Morgan M. E., Minden J. S. 1997. Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. *Electrophoresis*. **18,** 2071–2077.

Van Peer R., Schippers B. 1992. Lipopolysaccharides of plant-growth promoting *Pseudomonas* sp. strain WCS417r induce resistance in carnation to fusarium wilt. Neth. *J. Plant Pathol.* **98**, 129–139.

Van Wees S.C.M., Pieterse C.M.J., Trijssenaar A., Van 't Westende Y., Hartog F. and Van Loon L.C. 1997. Differential induction of systemic resistance in *Arabidopsis* by biocontrol bacteria. *Mol. Plant-Microbe Interact.* **10,** 716-724.

Vincent M.N., Harrison L.A., Brackin J.M., Kovacevich P.A., Murkerji P., Weller D.M. and Pierson E.A. 1991. Genetic analysis of the antifungal activity of a soilborne *Pseudomonas aureofaciens* strain. *Appl. Environ. Microbiol*. **57**, 2928–2934.

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 Y., Brown H.N., Crowley D.E. and Szaniszlo P.J. 1993. Evidence for direct utilization of a siderophore, ferrioxamine B, in axenically grown cucumber. *Plant Cell Environ*. **16**, 579-585.

Ward E.R., Uknes S.J., Williams S.C., Dincher S.S., Wiederhold D.L., Alexander D.C., Ahl-Goy P., Metraux J.P. and Ryals J.A. 1991. Coordinate gene activity in response to agents that induce systemic acquired resistance. *Plant Cell.* **3**, 1085–1094.

West S.E., Schweizer H.P., Dall C., Sample A.K. and Runyen-Janecky L.J. 1994. Construction of improved *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19 and sequence of the region required for their replication in *Pseudomonas aeruginosa. Gene.* **148,** 81-86.

Whipps J.M. 1990. Carbon utilization. In *The Rhizosphere*. Eds. Lynch J.M. Wiley Interscience, Chichester, UK. pp. 59-97.

Woodward A.W., Bartel B. 2005. Auxin: regulation, action, and interaction. *Ann. Bot*. **95**, 707–735.

Xie H., Pasternak J.J. and Glick B.R. 1996. Isolation and characterization of mutants of the plant growth-promoting rhizobacterium *Pseudomonas putida* GR-12 that overproduce indoleacetic acid. *Curr. Microbiol.* **67**, 4999-5009.

Yamada T. 1993. The role of auxin in plant-disease development. *Annu. Rev. Phytopathol.* **31,** 253–273.

Yamao F., Inokuchi H., Cheung A., Ozeki H., Soell D.G. 1982. *Escherichia coli* glutaminyl-tRNA synthetaseI. Isolation and DNA sequence of the glnS gene. *J. Biol. Chem.* **257,** 11639-11643.

Yeom J., Jeon C.O., Madsen E.L. and Park W. 2009. Ferredoxin-$NADP^+$ reductase from *Pseudomonas putida* functions asa ferric reductase. *J. Bacteriol.* **191,** 1472-1479.

Zhang F., Dashti N., Hynes R.K. and Smith D.L. 1996. Plant growth-promoting rhizobacteria and soybean [*Glycine max* (L.) Merr.]. Nodulation and fixation at suboptimal root zone temperatures. *Ann. Bot*. **7,** 453–459.

Zhu C., Naqvi S., Gomez-Galera S., Pelacho A.M., Capell T. and Christou P. 2007. Transgenic strategies for the nutritional enhancement of plants. *Trends Plant Sci.* **12,** 548-555.