# MOLECULAR BASIS OF *PSEUDOMONAS*-HOST INTERACTIONS IN BIOCONTROL OF PHYTOPATHOGENS

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**A Thesis Submitted in Partial Fulfillment of the Requirements for the**

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# หลักการพื้นฐานทางความสัมพันธ์ระดับโมเลกุลระหว่าง *Pseudomonas* และพืชอาศัยในการควบคุมเชื้อก่อโรคในพืช

นางสาวพฤกษา หล้าวงษา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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**MOLECULAR BASIS OF *PSEUDOMONAS*-HOST INTERACTIONS IN BIOCONTROL OF PHYTOPATHOGENS**

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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พฤกษา หล้าวงษา : หลักการพื้นฐานทางความสัมพันธ์ระดับโมเลกุลระหว่าง *Pseudomonas* และพืชอาศัยในการควบคุมเชื้อก่อโรคในพืช (MOLECULAR BASIS OF *PSEUDOMONAS*-HOST INTERACTIONS IN BIOCONTROL OF PHYTOPATHOGENS) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.หนึ่ง เตียอำรุง, 193 หน้า.

วัตถุประสงค์ของงานวิจัยนี้เพื่อศึกษาความหลากหลายของประชากรแบคทีเรีย *Pseudomonas* spp. ที่ช่วยส่งเสริมการเจริญเติบโตของพืช (plant growth promoting *Pseudomonas* spp.) จากดินบริเวณรอบรากข้าว และข้าวโพด ใช้เทคนิค amplified rDNA restriction analysis (ARDRA) ตรวจสอบความหลากหลายทางจีโนไทป์พบว่าสามารถจัดจำแนก *Pseudomonas* ได้ 2 กลุ่ม ตามชนิดของพืช โดยความสามารถของ *Pseudomonas* ในการควบคุมโดยชีววิธี และส่งเสริมการเจริญเติบโตของพืชทดสอบโดยการตรวจสอบคุณสมบัติทางชีววิทยาต่อเชื้อรา และแบคทีเรียก่อโรคในพืช ความสามารถในการสร้าง indole-3-acitic acid (IAA) และความสามารถในการใช้แหล่งคาร์บอน พบว่า *Pseudomonas* ที่ถูกคัดแยกมาจากดินบริเวณรอบรากข้าวนั้นมีความสามารถในการสร้าง IAA และความสามารถในการยับยั้งการเจริญเติบโตของเชื้อรา และแบคทีเรียก่อโรคในพืชสูงกว่า *Pseudomonas* ที่ถูกคัดแยกมาจากดินบริเวณรอบรากข้าวโพด การทดลองนี้บ่งชี้ว่ามี *Pseudomonas* หลากหลายไอโซเลตที่มีศักยภาพในการส่งเสริมการเจริญเติบโตของพืช และช่วยควบคุมโรคพืชทั้งในข้าว และข้าวโพด ในการทดลองต่อไปแบคทีเรีย *Pseudomonas fluorescens* สายพันธุ์ R31 ซึ่งคัดแยกมาจากดินบริเวณรอบรากข้าวในประเทศไทยที่มีความสามารถในการสร้าง IAA และมีความสามารถในการควบคุมเชื้อก่อโรคพืชนั้นได้ถูกนำมาทดสอบหาความสามารถในการสร้างสารประกอบทุติยภูมิ เช่น pyoluteorin, pyrrolnitrin, hydrogen cyanide และ 2,4-diacetylphloroglucinol (DAPG) จากนั้น ความสามารถในการผลิต DAPG ของสายพันธุ์ R31 ถูกทดสอบโดยเปรียบเทียบกับ *P. fluorescens* สายพันธุ์ F113 ซึ่งเป็นสายพันธุ์ที่ผลิต DAPG ซึ่งคัดแยกมาจากดินบริเวณรอบรากต้นชูการ์บีทในประเทศไอร์แลนด์ ในการทดลองนี้แผนภูมิต้นไม้ที่สร้างจากยีน *phlD* และยีน housekeeping ถูกสร้างขึ้นเพื่อหาวิวัฒนาการของสายพันธุ์ R31 และการผลิต DAPG ของสายพันธุ์ R31 ถูกทดสอบโดยตรงด้วย HPLC และโดยอ้อมด้วยการตรวจสอบการแสดงออกของยีนรายงานผล *phlA-gfp* ร่วมกับ RT-PCR ของยีน *phlA* ผลการทดลองแสดงให้เห็นว่าความสามารถในการผลิต DAPG ของสายพันธุ์ R31 นั้นต่ำกว่าสายพันธุ์ F113 ซึ่งสอดคล้องกับการแสดงออกของยีน *phlA-gfp* เป็นที่น่าสนใจว่าลำดับ และทิศทางโอเปอรอน *phl* ของสายพันธุ์ R31 นั้นเป็นไปในทิศทางเดียวกันกับสายพันธุ์ F113 แต่อย่างไรก็ตามพบว่ามีจำนวนเบสแตกต่างกันในส่วนของ *phlA-phlF* intergenic ซึ่งจากข้อมูลดังกล่าวแสดงว่ากิจกรรมของโปรโมเตอร์ของ

ยีน *phlA* (*phlA* promoter) นั้นไม่ได้เป็นตัวชี้วัดที่ดีสำหรับการผลิต DAPG และอาจจะเกี่ยวข้องกับการที่สายพันธุ์ R31 ผลิต DAPG ได้ในระดับต่ำ นอกจากนี้อิทธิพลของ exudates ในแต่ละชนิดของพืชเจ้าบ้านยังถูกนำมาทดสอบถึงผลกระทบต่อยีน *phlA* ด้วย โดยพบว่าชนิดของพืชเจ้าบ้านเป็นปัจจัยหลักที่ส่งผลกระทบต่อการแสดงออกของยีน *phlA* ในสายพันธุ์ R31 เมื่อมีการเติม exudates จากรากข้าว นอกจากนั้นพบว่าสายพันธุ์ R31 และสายพันธุ์ F113 แสดงผลในการเป็นปรปักษ์กับเชื้อราก่อโรคพืช *Pythium* spp. ในต้นข้าว ยิ่งไปกว่านั้นการประยุกต์ใช้สายพันธุ์ R31 กับเมล็ดข้าวนั้นยังช่วยเพิ่มความสูงของต้นข้าว น้ำหนักแห้งต้น และรากข้าว ในขณะเดียวกันการประยุกต์ใช้สายพันธุ์ F113 กับเมล็ดข้าวนั้นให้ค่าที่ไม่แตกต่างกันอย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับชุดควบคุม

สาขาวิชาเทคโนโลยีชีวภาพ
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ลายมือชื่อนักศึกษา พฤกษา หล้าวงษา
ลายมือชื่ออาจารย์ที่ปรึกษา
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม

PHRUEKSA LAWONGSA : MOLECULAR BASIS OF *PSEUDOMONAS*-HOST INTERACTIONS IN BIOCONTROL OF PHYTOPATHOGENS. THESIS ADVISOR : ASSOC. PROF. NEUNG TEAUMROONG, Dr. rer. nat., 193 PP.

PLANT GROWTH PROMOTING RHIZOBACTERIA/*PSEUDOMONAS*/ 2,4-DIACETYLPHLOROGLUCINOL (DAPG)/RICE/MAIZE

The objective of this experiment was to investigate bacterial diversity in a wetland rhizosphere soil under rice (*Oryza sativa*) cultivation and a desiccated rhizosphere soil under maize (*Zea mays*) cultivation of plant growth promoting *Pseudomonas* spp. The genotypic diversity of isolates was determined on a basis of amplified rDNA restriction analysis (ARDRA). This analysis showed that both plant species selected for two distinct populations of *Pseudomonas*. The actual biocontrol of these strains was confirmed by bioassays on fungal and bacterial plant pathogens, and the plant growth promotion abilities of these strains were confirmed by indole-3-acetic acid (IAA) production and carbon source utilization. The ability to produce IAA and antagonistic activity of a selected group of pathogens of rhizosphere *Pseudomonas* was higher than maize rhizosphere *Pseudomonas*. This work clearly identified a number of isolates having the potential for being used as plant growth promotion and biocontrol agents on rice and maize. In the following experiment, *Pseudomonas fluorescens* R31 was isolated from rice rhizosphere in Thailand. This strain which had the ability to produce IAA and to control plant pathogens was selected to screen for the production of the secondary metabolites such as pyoluteorin, pyrrolnitrin, hydrogen cyanide and 2,4-diacetylphloroglucinol (DAPG). Then, DAPG

production by strain R31 was investigated in comparison with the DAPG production by DAPG-produced *P. fluorescens* strain F113 which was isolated from sugar beet rhizosphere in Ireland. In this study, the *phlD* gene tree and housekeeping genes tree were constructed to uncover the evolution of strain R31. DAPG production by strain R31 was investigated directly by high performance liquid chromatography (HPLC), and indirectly by quantifying the expression of a *phlA-gfp* reporter gene fusion and also by RT-PCR of *phlA*. The results revealed that DAPG produced by strain R31 was lower than that by strain F113, which is similar to the *phlA-gfp* expression. Interestingly, the same orientation of each gene in *phl* operon of strain R31 was found whereas many different nucleotides between strain R31 and F113 in *phlA-phlF* intergenic region were discovered. This information demonstrated that *phlA* promoter activity was not a good indicator of DAPG production and associated with the presence of low level of DAPG production by strain R31. Moreover, the effect of plant host exudates on *phlA* gene revealed that the host cultivar had a major influence on *phlA* gene expression in strain R31 in the rice exudates amendment. Furthermore, the result of *in vivo* antagonistic activity illustrated that *Pythium* spp. was suppressed by strains R31 and F113. In addition, the application of strain R31 on rice seeds showed significant increase in plant height, shoots dry weight and roots dry weight of rice while the application of strain F113 on rice seeds showed no significant differences when compared to those of the control.

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# LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| bp | = | base pair(s) |
| cDNA | = | complementary deoxyribonucleic acid |
| DAPG | = | 2,4-diacetylphloroglucinol |
| DNA | = | deoxyribonucleic acid |
| FRU | = | fructose |
| GFP | = | green fluorescent protein |
| GLU | = | glucose |
| Gm | = | gentamycins |
| GLY | = | glycerol |
| h | = | hour |
| HPLC | = | high performance liquid chromatography |
| kb | = | kilo base pair(s) |
| kDa | = | kilo dalton |
| Km | = | kanamycin |
| MAPG | = | monoacetylphloroglucinol |
| min | = | minute |
| °C | = | degree celcius |
| OD | = | optical density |
| PCR | = | polymerase chain reaction |
| PGPR | = | plant growth promoting rhizobacteria |

# LIST OF ABBREVIATIONS (Continued)

| | | |
|---|---|---|
| RNA | = | ribonucleic acid |
| rRNA | = | ribosomal ribonucleic acid |
| RT-PCR | = | reverse transcription polymerase chain reaction |
| SDS | = | sodium dodecyl sulfate |
| ST-PCR | = | semi random-two step polymerase chain reaction |
| SUC | = | sucrose |
| SUCC | = | succinate |
| TAE | = | tris-acetate-EDTA |
| Tc | = | tetracycline |
| UV | = | ultraviolet |
| w/v | = | weight by volume |

# CHAPTER I

# INTRODUCTION

The use of chemical fertilizers and pesticides has caused an incredible harm to the environment. These agents are both hazardous to animals and humans. They may persist and accumulate in natural ecosystems. An answer to this is by replacing the use of chemicals with biological approaches, which are considered more environmentally friendly in the long term. One of the biological approaches for the control of different phytopathogenic agents is the use of biocontrol plant growth promoting rhizobacteria (PGPR), which is capable of suppressing or preventing the phytopathogen damage.

The biocontrol agents, which are best characterized at the molecular level, belong to the bacteria genus *Pseudomonas*. They can be utilized in low-input sustainable agricultural applications, such as biocontrol, on account of their ability to synthesize secondary metabolites with antibiotic properties (Franks et al., 2006). These secondary metabolites include 2,4-diacetylphloroglucinol (DAPG), phenazine (Phz), pyrrolnitrin, oomycin A, viscosinamide, pyoluteorin and hydrogen cyanide (HCN). Among these secondary metabolites, DAPG has received the particular attention because of its production by a wide range of pseudomonads used for the biological control of root diseases (Dowling and O'Gara, 1994; Keel et al., 1996; Sharifi-Tehrani et al., 1998). These root diseases include take-all of wheat caused by *Gaeumannomyces graminis* var. *tritici* (Keel et al., 1992), fusarium wilt of tomato

(Tamietti et al., 1993), black root of tobacco caused by *Thielaviopsis basicola* (Stutz et al., 1986) and damping-off of sugar beet caused by *Pythium ultimum* (Fenton et al., 1992; Shanahan et al., 1992). In addition to its antifungal activity, DAPG has some antiviral properties (Tada et al., 1990), and also inhibits the growth of soft-rotting bacteria and cyst nematodes of potato (Cronin et al., 1997).

Plant roots influence soil-borne microbial communities via several mechanisms, including excretion of specific organic compounds, competition for nutrients, and providing a solid surface for attachment. It was previously demonstrated that an effective biological control strain isolated from one region may not perform as effectively in other soils or plants (Kiely et al., 2006). Anti-microbial and secondary metabolite produced by colonizing bacteria were also been shown to be influenced by certain carbon sources (Casey et al., 1998).

For these reasons, it is important to screen for *Pseudomonas* spp. in different environments to understand the role that the rhizosphere plays in the selection of the diversity of *Pseudomonas* spp.. The study of genotypic and phenotypic diversity of *Pseudomonas* spp. and their plant growth-promoting potential is important not only for understanding their ecological role in the rhizosphere and the interaction with plants, but also for any biotechnological applications (Berg et al., 2002).

The purpose of this study was to investigate bacterial populations of the wetland soil of rice (*Oryza sativa* L.) and desiccated soil of maize (*Zea mays* L.) on the abundance and diversity of plant growth-promoting *Pseudomonas* spp. *Pseudomonas* isolates were selected from both plant hosts and profiled using amplified 16S rDNA restriction analysis (ARDRA). These isolates were further screened for phenotypes associated with the production of plant growth promotions

substances, such as, indole-3-acetic acid (IAA) and the antagonistic activity of soil-borne phytopathogenic bacteria and fungi. Subsequently, fluorescent pseudomonads were selected to examine the ability to produce secondary metabolites with antibiotic properties, and gene involving in the antibiotic production was investigated. Besides the expression of antibiotic biosynthetic gene was observed by fusing the antibiotic structural gene to a reporter gene and monitoring its expression using green fluorescent protein (GFP), and RT-PCR (reverse transcription-polymerase chain reaction) was also done. *Pseudomonas* spp. carrying the reporter construct was assayed for reporter activity in different carbon sources. Alternatively, root exudates was collected and tested for stimulation of reporter expression *in vitro* and *in vivo* antagonistic activity against *Pythium* spp. was also investigated.

**Research objectives**

a) To isolate and characterize *Pseudomonas* spp. from rice and maize rhizospheres.
b) To investigate the antagonistic activity of *Pseudomonas* spp. from rice and maize rhizospheres against phytopathogens.
c) To examine 2,4-diacetylphloroglucinol (DAPG) production and investigate the expression of gene that involved in DAPG production of *Pseudomonas* spp. isolates.

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# CHAPTER II

# LITERATURE REVIEWS

## 2.1 Plant Growth Promoting Rhizobacteria (PGPR) and *Pseudomonas* spp.

The rhizosphere, volume of soil surrounding roots and influenced chemically, physically and biologically by the plant root, is a highly favorable habitat for the proliferation of microorganisms and exerts a potential impact on plant health and soil fertility (Sorensen, 1997). Root exudates rich in amino acids, monosaccharides and organic acids, serve as the primary source of nutrients, and support the dynamic growth and activities of various microorganisms within the vicinity of the roots. These root-colonizing microorganisms could be free-living, parasitic or saprophytic and their diversity remains dynamic with a frequent shift in community structure, and species abundance.

An important group of these microbial communities that exerts beneficial effects on plant growth upon root colonization were first defined by Joseph Kloepper and Milton Schroth and termed as plant growth-promoting rhizobacteria (PGPR) (Kloepper and Schroth, 1978). Bacteria of diverse genera were identified as PGPR of which *Bacillus* and *Pseudomonas* spp. are predominant. These PGPR can be classified according to their beneficial effects. They can have an impact on plant growth and development in two different ways: indirectly or directly. The indirect promotion of plant growth occurs when these bacteria decrease or prevent some of the

deleterious effects of a phytopathogenic organism by any one or more of several different mechanisms. On the other hand, the direct promotion of plant growth by PGPR generally entails providing the plant with a compound that is synthesized by the bacterium or facilitating the uptake of nutrients from the environment (Glick et al., 1999). For instance, biofertilizers can fix nitrogen, which can subsequently be used by the plant, thereby improving plant growth when the amount of nitrogen in the soil is limiting. Phytostimulators can directly promote the growth of plants, usually by the production of phytohormones (such as auxin) and volatile growth stimulants (such as ethylene and 2,3-butanediol). Biocontrol agents are able to protect plants from infection by phytopathogenic organisms (Bloemberg and Lugtenberg, 2001; Haas and Defago, 2005; Vessey, 2003). In other PGPR, which are sometimes called biopesticides, the biocontrol aspect is most conspicuous. These PGPR, which mostly belong to *Pseudomonas* and *Bacillus* spp., are antagonists of recognized root pathogens (Haas and Defago, 2005) and the biocontrol agents that are best-characterized at the molecular level belong to the genus *Pseudomonas* (Bloemberg and Lugtenberg, 2001).

## 2.2 Taxonomy of *Pseudomonas*

Members of the genus *Pseudomonas* are rod-shaped Gram-negative bacteria that are characterized by metabolic versatility, aerobic respiration (some strains also have anaerobic respiration with nitrate as the terminal electron acceptor and/or arginine fermentation), motility owing to one or several polar flagella, and a high genomic G+C content (59–68%) (Haas and Defago, 2005).

This genus is heterogeneous and harbors plant, animal and human pathogenic species, including *P. aeruginosa*, *P. plecoglossicida*, *P. tolaasii*, and *P. syringae*. The genus *Pseudomonas* covers one of the most diverse and ecologically significant groups of bacteria. Members of the genus are found in large numbers in a wide range of environmental niches, such as terrestrial and marine environments, as well as in association with plants and animals. This almost universal distribution of *Pseudomonas* suggests a remarkable degree of genomic diversity and genetic adaptability.

At first, the genus *Pseudomonas* was described that it included polarly flagellated strictly aerobic rods with a respiratory type of metabolism in which oxygen is used by Migula (1894). Based at that time on the shape and general appearance of bacteria, the genus was very heterogeneous, and a large number of species of the genus was reported. In 1984, over 100 species of the genus were listed, and the characteristics of these species were described in Bergey's Manual of Systematic Bacteriology (Palleroni, 1984).

During the first half of the 20$^{th}$ century, the physiological diversity of these organisms was discovered. In addition to these, other criteria that include microbe interactions with higher organisms such as pathogenicity were considered of doubtful value as a taxonomic criterion since the beginning of bacteriology (Palleroni, 2003).

In the 1923 edition, a chapter focusing on genus *Pseudomonas* was already included, and the classification of the species was based on phenotypic characteristics. In any case, since its discovery the genus *Pseudomonas* was formed by a large number of species increasing with time to reach alarming proportions (Palleroni, 2008).

In the 1960s, the development of methodology to extract DNA from cells and the discovery of DNA renaturation by Marmur (Marmur, 1961) made the first studies on DNA homologies and genetic comparisons applied to bacterial taxonomy possible. Studies of relationships among *Pseudomonas* species according to DNA base composition and DNA–DNA hybridization were carried out confirming the phenotypic classification (Colwell et al., 1965; Colwell and Mandel, 1964; Johnson and Ordal, 1968).

However, only the G + C content was included for all species in Bergey's Manual edition of 1974. In this edition, the genus was included in the Family *Pseudomonadaceae* (Doudoroff and Palleroni, 1974). It included Gram negative aerobic and motile rods with some denitrifying species and the description of only 29 species of genus *Pseudomonas* was given, although a long list of species without phenotypic description was added to them.

The species with phenotypic description were distributed into two groups according to their physiological characteristics: group I including the species that do not require growth factors and group II for those requiring growth factors. Group I was divided into two subgroups depending on the accumulation or absence of poly-β-hydroxybutyrate. The production of fluorescent pigments, arginine dihydrolase and denitrification and the use of DL-arginine or betatine were the criteria for species grouping within these subgroups.

The classification based on data obtained from the measurements of RNA–DNA relatedness in genus *Pseudomonas* was included in 1984 in the first edition of Bergey's Manual of Systematic Bacteriology (Palleroni, 1984) although the number of species and its phenotypic classification were maintained. In the past two decades,

polyphasic taxonomic studies, especially using methods for analyzing micro-organisms at the molecular level, have played a crucial role in improving the classification of the pseudomonads. The deep changes in bacterial taxonomy undergone from the 1980s originated from the proposal of Woese to classify and identify them in agreement with the sequence of their ribosomic RNA (Woese et al., 1984). Nevertheless this new identification scheme was not yet considered in the edition of Bergey's Manual of Determinative Bacteriology of 1994 in which the species differentiation was based on phenotypic characteristics.

The phylogenetic classification of bacteria based on their 16S rRNA genes led to the establishment of the three subdivisions of the later called Proteobacteria, ''alpha'', ''beta'' and ''gamma'' (Woese et al., 1984). Other reclassifications made in the late 80s on the basis of the DNA homologies of several strains with different strains of the *Pseudomonas* sensu stricto cluster.

From the 1990s onwards, the routinary sequencing of 16S rRNA gene of all the known bacteria started, partial at the beginning and later complete sequences were and are currently being deposited in public databases. The sequencing of the 16S rRNA gene together with the development of mathematical models for construction of trees representing the similarity of the sequences allowed the phylogenetic classification of prokaryotes.

In the 2000s decade, the most detailed taxonomic revision of genus *Pseudomonas* through the analysis of 16S rRNA gene sequences was performed by Anzai et al., 2000. Reclassifications continued from the 2000 onwards and several *Pseudomonas* species changed to different new genera belonging to different classes

of Proteobacteria. All these changes have been recorded in the current edition of Bergey's Manual of Systematic Bacteriology edited in the year 2005.

Currently the most modern techniques for analysis of biomolecules are being applied to *Pseudomonas* taxonomy. Despite the relevance of these chemotaxonomic approaches together with phenotypic and ecological studies, it is clear that gene sequencing studies have provided the greatest advances in taxonomy of bacteria, including *Pseudomonas*. The ribosomal genes have special characteristics that other genes lack. They are present in all organisms having the same essential function for life (protein synthesis) and are present since the beginning of the evolution. The 16S rRNA gene became the key molecule in which the classification of prokaryotes has been based, including the species of genus *Pseudomonas* (Anzai et al., 2000; Palleroni, 2005).

Therefore in the last decade other gene sequences have been used as phylogenetic molecular markers in taxonomic studies such as the ''housekeeping'' genes *recA* (recombinase A), *atpD* (ATP synthase F1, β-subunit), *gyrB* (DNA gyrase β-subunit), *rpoB* (RNA polymerase β-subunit), *rpoD* (RNA polymerase σ-subunit), *groEL* (chaperonin) etc., whose usefulness for species differentiation has been demonstrated in genus *Pseudomonas* (Hilario et al., 2004). Nevertheless, the analysis of housekeeping genes is not commonly used yet in *Pseudomonas* species description and only *gyrB*, *rpoB*, and *rpoD* have been included in the recent description of *P. xiamenensis* (Lai and Shao, 2008).

All these phenotypic, chemotaxonomic and genotypic studies aim to overcome the problem that bacteria cannot be classified on the basis of the sexual reproduction criteria commonly used in non-microscopic eukaryotes. The selection of the minimal

characteristics (named minimal standards) necessary for species delineation and description are chosen for each bacterial genus by a Committee formed by experts in the given genus (Peix et al., 2009). The Subcommittee on the taxonomy of *Pseudomonas* and related genera has worked for years to standardize the methods used in the taxonomy of the genus. Nevertheless, after the 2002 meeting of this Subcommittee, the minimal standards for species description in genus *Pseudomonas* have not been defined yet (De Vos and Yabuuchi, 2002). Therefore, the description of new species in this genus must address the general minimal standards for bacterial species definition (Stackebrandt et al., 2002). They mandate include 16S rRNA sequencing, DNA-DNA hybridization, fatty acid analysis and phenotypic characterization for new species and/or subspecies description.

In recent years there has been much success in obtaining biological control of plant pathogens using bacterization techniques (Howell and Stipanovic, 1980; Iavicoli et al., 2003; Siddiqui and Shahid Shaukat, 2003; Weller and Cook, 1983). Bacteria used, as inoculants are mostly devoted to *P. fluorescens* obtained from soils and plant surfaces. The bacteria belonging to these species could potentially be used for environmental and agricultural purposes. *P. fluorescens* is a gram negative, rod-shaped bacterium, and has multiple flagella. It is an obligate aerobe but certain strains are capable of using nitrate instead of oxygen as a final electron acceptor during cellular respiration. It secretes a soluble fluorescent pigment called pyoverdin, which is a type of siderophore. Some of these bacteria produce metabolites, which chelate the environmental iron thus making it unavailable to pathogens (De Weger et al., 1986). To date a number of these diseases suppressive antibiotic compounds have been characterized chemically and include phenazines (Chin-A-Woeng et al., 1998;

Thomashow and Weller, 1988), pyrrole type antibiotics (Burkhead et al., 1994), and pyo-compounds (Yuan et al., 1998). A small number of antibiotics like compounds that do not contain nitrogen have also been isolate from fluorescent pseudomonads (Kanda et al., 1975), one of these metabolites 2,4-diacetylphloroglucinol (DAPG), is a major factor controlling a range of plant pathogens (de Souza et al., 2003; Landa et al., 2002; Validov et al., 2005). Bacteria that produce DAPG play a key role in agricultural environments, and their potential for use in sustainable agriculture is promising.

## 2.3 Roles of *Pseudomonas* as PGPR

Pseudomonads possess many traits that make them well suited as biocontrol and growth-promoting agents (Weller, 2007). There are several ways in which different plant growth promoting *Pseudomonas* have been reported to directly and indirectly facilitate the proliferation of their plant hosts.

**a) Indirect mechanisms including:**

1. Antibiotic production
2. Induced systemic resistance
3. Competition for sites on the root

**b) Direct mechanisms including:**

1. Production of phytohormones
2. Nitrogen fixation
3. Sequestering iron by siderophores
4. Solubilization of phosphorus

### 2.3.1 Indirect mechanisms

The indirect promotion of plant growth occurs when these bacteria decrease or prevent some of the deleterious effects of a phytopathogenic organism by any one or more of several different mechanisms. It consists of i) antibiotic production, ii) induced systemic resistance, and iii) competition for sites on the root.

#### 2.3.1.1 Antibiotic production

Among the variety of *Pseudomonas* species inhabiting the rhizosphere, certain strains of fluorescent pseudomonads have received particular attention because of their potential to control seed- and soilborne pathogenic fungi and oomycetes (Keel et al., 1992; Keel et al., 1996a; Raaijmakers and Weller, 2001). Their beneficial effects on plant health have been mainly attributed to active exclusion of pathogens from the rhizosphere through the secretion of a diverse array of antimicrobial metabolites (Haas and Keel, 2003; Handelsman and Stabb, 1996; Raaijmakers et al., 2002; Thomashow and Weller, 1996).

Most of the identified *Pseudomonas* biocontrol strains produce anti-fungal metabolites, of which DAPG, phenazines, pyrrolnitrin, pyoluteorin and volatile hydrogen cyanide are the most frequently detected classes. However, new anti-fungal metabolites belonging to the class of cyclic lipopeptides, such as viscosinamide (Nielsen et al., 1999) and tensin (Nielsen et al., 2001) have been discovered (Figure 2.1).

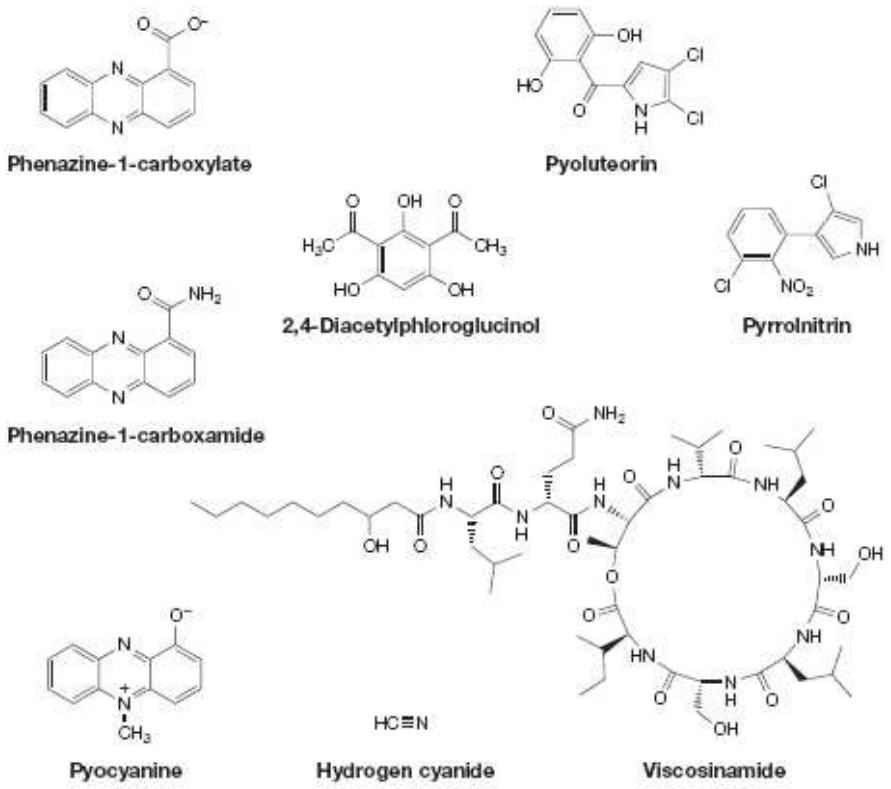


**Figure 2.1** The antibiotic compounds produced by fluorescent pseudomonads that are relevant for biocontrol. The phenazines, phloroglucinols, pyoluteorin, pyrrolnitrin and cyclic lipopeptides are all diffusible, whereas hydrogen cyanide is volatile (Haas and Defago, 2005).

Fluorescent pseudomonads that produce the polyketide antibiotic DAPG are an important group of PGPR that suppress root and seedling diseases on a variety of crops. Examples include *P. fluorescens* CHA0 that suppresses black root rot of tobacco (Stutz et al., 1986), take-all of wheat (Keel et al., 1992) and Fusarium wilt and crown and root rot of tomato (Duffy and Defago, 1997; Tamietti et al., 1993), *Pseudomonas* sp. F113 that suppresses damping-off of sugar beet (Fenton et al., 1992; Shanahan et al., 1992), and *P. fluorescens* Q2-87 (Harrison et al., 1993; Pierson and Weller, 1994) and Q8r1-96 (Raaijmakers and Weller, 1998) that suppress

take-all of wheat. Strains of *P. fluorescens* that produce DAPG also have a key role in the natural biological control of take-all known as take-all decline (Raaijmakers et al., 1999; Raaijmakers and Weller, 1998; Raaijmakers et al., 1997). However, the mode of action of DAPG has not been elucidated yet.

The DAPG inhibits a wide range of fungi and bacteria, and its importance in biocontrol activity has been demonstrated conclusively by genetic approaches (Thomashow, 1996) and direct isolation from the rhizosphere environment (Bonsall et al., 1997; Duffy and Defago, 1997; Raaijmakers et al., 1999; Raaijmakers and Weller, 1998).

#### 2.3.1.2 Induced systemic resistance

A few strains of rhizobacteria activate plant defense responses against a broad spectrum of plant pathogens, termed as induced systemic resistance (ISR). Rhizobacteria-mediated ISR has been demonstrated in many plant-pathogen systems wherein the bacterium and the challenging pathogen remained spatially separated, and these observations indicate that ISR is genetically determined (Pieterse et al., 2001).

Many effective biocontrol PGPR elicit ISR, irrespective of antibiotic production (Ongena et al., 2004; Ton et al., 2002; Zehnder et al., 2001). The effects of three different strains of *Pseudomonas* spp. mediating ISR in *Arabidopsis thaliana* have been investigated through transcriptome analysis of plants with roots that were colonized by one of these strains (*P. fluorescens* WCS417r, *P. thivervalensis* or *P. fluorescens* CHA0). In each instance, the transcript levels in the leaves were not markedly changed compared with the uninoculated control, and

systemic responses that are typically seen after attack by necrotizing pathogens did not occur during ISR (Cartieaux et al., 2003; Verhagen et al., 2003). In DAPG– mutants of *P. fluorescens* CHA0 are less effective than the wild-type bacteria in protecting *Arabidopsis* from the leaf pathogen *Peronospora parasitica* and application of DAPG to the roots triggers ISR to this pathogen (Iavicoli et al., 2003).

#### 2.3.1.3 Competition for sites on the root

PGPR compete with deleterious microorganisms and pathogens for limited available nutrients in root exudates and suitable colonization niches, and finally out number them. Populations of PGPR established on the plant roots could act as a sink for the available nutrients and limit the nutrient availability for pathogen stimulation and its subsequent root colonization. This mechanism is most often used by fluorescent pseudomonads due to their nutritional versatility, and because of their high growth rates in the rhizosphere (Walsh et al., 2001).

Apart from root colonization, the PGPR should be able to compete for nutrients with native microbial populations in the rhizosphere for successful elimination of the pathogens. Siderophore production by PGPR, sequester most of the available $Fe^{3+}$ in the rhizosphere and force the pathogens for iron starvation, thus is a major contributor for pathogen suppression (O'Sullivan and O'Gara, 1992).

The proposed mechanism for siderophore-mediated disease suppression by fluorescent pseudomonads is illustrated in Figure. 2.2. Fluorescent siderophores, which have a very high affinity for ferric iron, are secreted during growth under low-iron conditions. The resulting ferric-siderophore complex is

unavailable to other organisms, but the producing strain can utilize this complex via a very specific receptor in its outer cell membrane (Buyer and Leong, 1986). In this way, fluorescent *Pseudomonas* strains may restrict the growth of deleterious bacteria and fungi at the plant root (Loper and Buyer, 1991). Suppression of Fusarium wilt of radish by *Pseudomonas* strain WCS358 through siderophore mediated competition for iron (Costa and Loper, 1994) is also example.

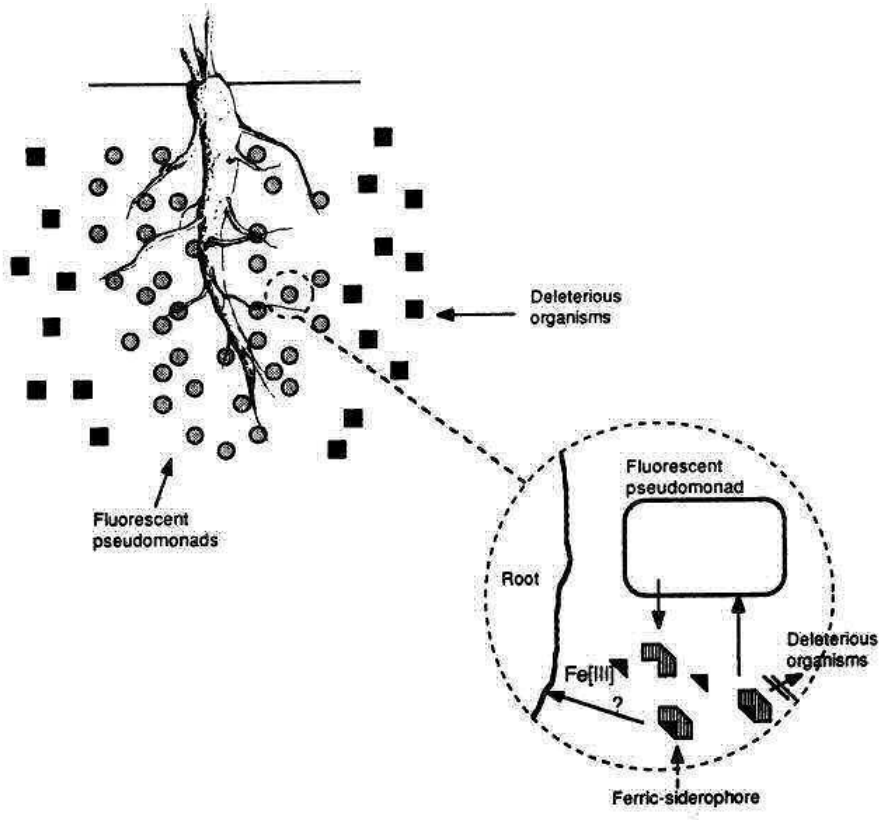


**Figure 2.2** Model for suppression of root pathogens by siderophores from fluorescent pseudomonads. The growth of deleterious organisms is restricted by the unavailability of the growth-limiting ferric iron (O'Sullivan and O'Gara, 1992).

### 2.3.2 Direct mechanisms

The direct promotion of plant growth by PGPR generally entails providing the plant with a compound that is synthesized by the bacterium or facilitating the uptake of nutrients from the environment. Direct mechanisms have reported in plant growth promotion including: i) production of phytohormones, ii) nitrogen fixation, iii) sequestering iron by siderophores, and iv) solubilization of phosphorus.

#### 2.3.2.1 Production of phytohormones

Auxin, IAA is a quantitatively important phytohormone produced by PGPR, and treatment with auxin-producing rhizobacteria also increased some of the plant growth (Vessey, 2003).

Many plant-beneficial *Pseudomonas* strains, isolated from the rhizosphere, produce IAA which helps in stimulating plant growth (Loper and Schroth, 1986). Rapid establishment of roots, whether by elongation of primary roots or by proliferation of lateral and adventitious roots, is advantageous for young seedlings as it increases their ability to anchor themselves to the soil and to obtain water and nutrients from their environment, thus enhancing their chances for survival (Patten and Glick, 2002). In bacteria, several pathways have been described leading from L-tryptophan (Trp) to IAA (Figure 2.3).

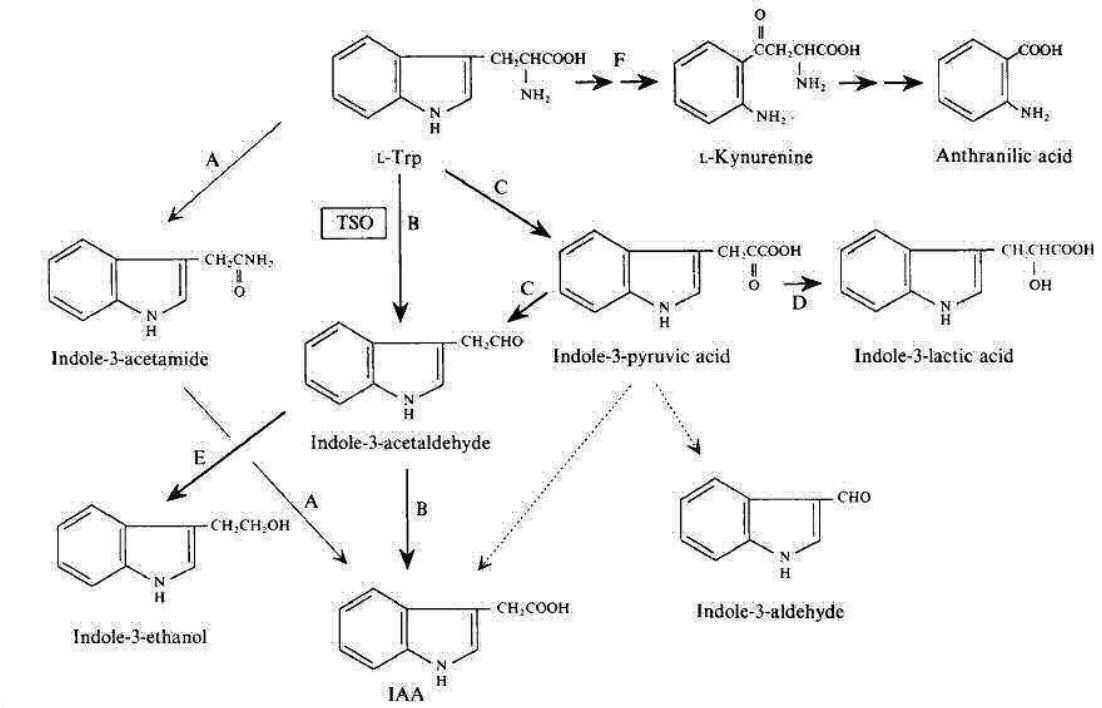


**Figure 2.3** Bacterial pathways leading from Trp to IAA: A, indoleacetamide pathway of *P. syringae* subsp. *savastanoi*, catalysed by Trp-2-mono-oxygenase and indoleacetamide hydrolase; B, TSO and indoleacetaldehyde dehydrogenasecatalysed formation of IAA; C, indolepyruvic acid pathway initiated by L-TT transaminase; D, enzymic formation of indolelactate; E, indole-ethanol dehydrogenase; F, ring cleavage pathway initiated by Trp-2,3-dioxygenase leading to kynurenine and anthranilic acid (Oberhansli et al., 1991).

A positive correlation was observed between L-tryptophan dependent auxin production by different PGPR strains and their ability to increase the grain yield, and number of branches and pods per plant in *Brassica* spp. (Asghar et al., 2002). It was further supported by the positive relation between auxin production

by PGPR and the increase in number of branches and oil content in *B. napus* treated with these PGPR (Asghar et al., 2004).

#### 2.3.2.2 Nitrogen fixation

In 1954, nitrogen fixation by *Pseudomonas*-like soil bacteria has been reported (Anderson, 1955). However, nitrogen fixation ability within the genus is poorly understood. It was long believed that there were no nitrogen-fixers among strains of the genus *Pseudomomas* (Young, 1992) but recent studies have demonstrated that several strains, classified as *Pseudomonas stutzeri*, do have the ability to fix nitrogen (Desnoues et al., 2003; Krotzky and Werner, 1987). Among them, *P. stutzeri* A15 (A1501), isolated from rice paddies in China, was studied in detail to determine the conditions under which nitrogen fixation occurred and the structure of the nitrogenase genes involved (Desnoues et al., 2003).

The consistent with this assumption, most of the strains described as putative nitrogen-fixing *Pseudomonas* were later reassigned to genera in the α- and β-Proteobacteria (Chan et al., 1994). It now seems that several strains unambiguously classified as true *Pseudomonas* spp. can be added to the list of nitrogen fixers, on the basis of physiological properties, nitrogenase assays, phylogenetic studies and detection of *nifH* DNA by hybridization or PCR amplification (Chan et al., 1994; Vermeiren et al., 1999). Two nitrogen-fixing isolates belong to the species *P. stutzeri*. *P. stutzeri* CMT.9.A was isolated from the roots of sorghum (Krotzky and Werner, 1987), whereas strain A15 originated from rice paddies in China (You et al., 1991). Strain A15 was initially identified as an *Alcaligenes faecalis*, and was later reassigned to *P. stutzeri* (Vermeiren et al., 1999).

#### 2.3.2.3 Sequestering iron by siderophores

Iron is essential for life. With rare exceptions, living organisms, whether animals, plant or microbes, require iron as a component of proteins involved in important processes such as respiration, photosynthesis and nitrogen fixation. Despite the abundance of this element on the earth's surface, soil organisms such as plants and microbes have difficulty obtaining enough iron to support their growth because iron in soil is largely present as insoluble, ferric hydroxides, which cannot be readily transported into cells. To solve the iron problem, bacteria, fungi and some plants secrete small, specialized iron-binding molecules called siderophores into the soil to scavenge iron. Once bound, soluble iron-siderophore complex is taken up by specific receptors on the soil exposed surfaces of these organisms; following reduction to the ferrous state, the iron is released from the siderophore (Glick et al., 1999).

Siderophores are low molecular weight molecules, usually less than 1 kDa, with three functional, or iron-binding, groups connected via a flexible backbone. Each functional group presents two atoms of oxygen, or less commonly, nitrogen, that bind to iron. In general, catecholates-type siderophores are typical to bacteria (Figure 2.4)

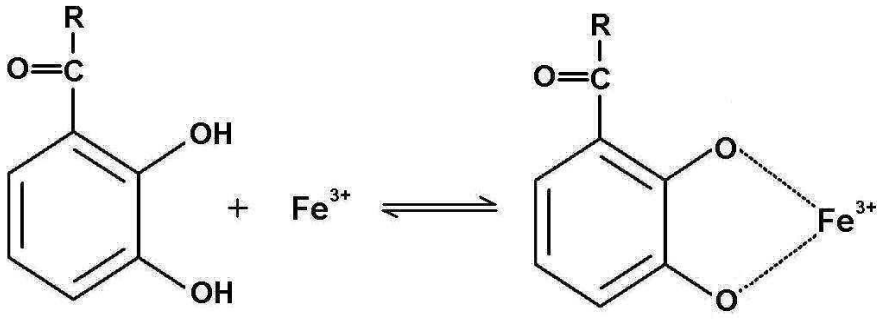


**Figure 2.4** Iron binding group (catecholate group) of microbial siderophores (adapt from Glick et al., 1999).

It is known that many bacteria, including *Pseudomonas* spp., react to limiting $Fe^{3+}$ concentrations by inducing a high-affinity iron uptake system (Braun, 1985; Neilands, 1982) consisting of siderophores, $Fe^{3+}$-chelating molecules, and outer membrane receptor proteins with a high affinity for the matching $Fe^{3+}$-siderophore complex (De Weger et al., 1986).

For some plant growth promoting *Pseudomonas* spp., it has been shown that the production of siderophores during iron starvation on laboratory media was accompanied by growth inhibition of other microorganisms. Neither this antagonistic activity to other microorganisms nor siderophore production was observed when the $Fe^{3+}$ supply was sufficient (Geels and Schippers, 1983). The following scenario was proposed to account for the enhancement of plant growth by the *Pseudomonas* spp. (Kloepper et al., 1980).

After the inoculation of seeds, the *Pseudomonas* bacteria rapidly colonize the roots of the developing plant. The limiting $Fe^{3+}$ concentration in the soil induces the high-affinity iron uptake system.

The siderophores bind $Fe^{3+}$, and as uptake of this $Fe^{3+}$-siderophore complex requires a very specific uptake mechanism, this binding makes this essential element unavailable for many other rhizomicroorganisms. These microorganisms, including deleterious species, then are unable to obtain sufficient iron for optimal growth since they produce either no siderophores at all or less efficient ones. Thus the population of deleterious microorganisms is reduced, creating a favorable environment for the development of the plants (De Weger et al., 1986).

#### 2.3.2.4 Solubilization of phosphorus

Phosphorous, next to nitrogen, is the second important macronutrient required for plant growth. Even in phosphorous rich soils, most of the P is in insoluble form - iron and aluminium phosphates in acidic soils, and calcium phosphates in alkaline soils, rendering a small proportion (~0.1%) is available to plants (Stevenson and Cole, 1999). Phosphate solubilizing bacteria (PSB) secrete organic acids and phosphatases to convert the insoluble phosphates into soluble monobasic ($H_2PO^{4-}$) and dibasic ($HPO_4^{2-}$) ions, a process referred to as mineral phosphate solubilization (MPS). MPS in the rhizosphere leads to an increase in the phosphorous available to plants and in turn the plant uptake (Gyaneshwar et al., 2002).

Phosphate solubilizing bacteria are ubiquitous (Gyaneshwar et al., 2002), and *Bacillus*, *Enterobacter*, *Erwinia* and *Pseudomonas* spp. are among the most potent strains. PSB are common in rhizospheres of crop plants and few examples of beneficial association of phosphate solubilizing PGPR and plants include *P. chlororaphis* or *P. putida* and soybean (Cattelan et al., 1999). The phosphate

solubilizing ability of PGPR in natural habitats, in turn, depends on the available nutrients - carbon and nitrogen sources, and metal ions (Kim et al., 1998).

The most important among the various organic acids that solubilize inorganic phosphates is β-keto-gluconic acid, a secondary oxidation product of glucose. The oxidation of glucose to gluconic acid and β-ketogluconic acid is catalyzed by glucose dehydrogenase (GDH) located on the outer leaf or cytoplasmic membrane. Mutants of phosphate solubilizing *Enterobacter asburiae*, deficient in GDH activity, failed to release phosphate from alkaline soils indicating the essential role of GDH in MPS (Gyaneshwar et al., 1999).

## 2.4 *Pseudomonas* and phloroglucinol antibiotics

Phloroglucinol (Phl) antibiotics are phenolic metabolites produced by bacteria and plants (Bangera and Thomashow, 1996; Bangera and Thomashow, 1999; Singh and Bharate, 2006; Thomashow, 1996). Three types of phloroglucinols are known to be produced by *Pseudomonas* species: monoacetylphloroglucinol (MAPG), DAPG, and triacetylphloroglucinol (TAPG) (Figure 2.5), the latter only present in trace amounts (Bangera and Thomashow, 1999; Raaijmakers et al., 2002; Schnider-Keel et al., 2000; Shanahan et al., 1992).

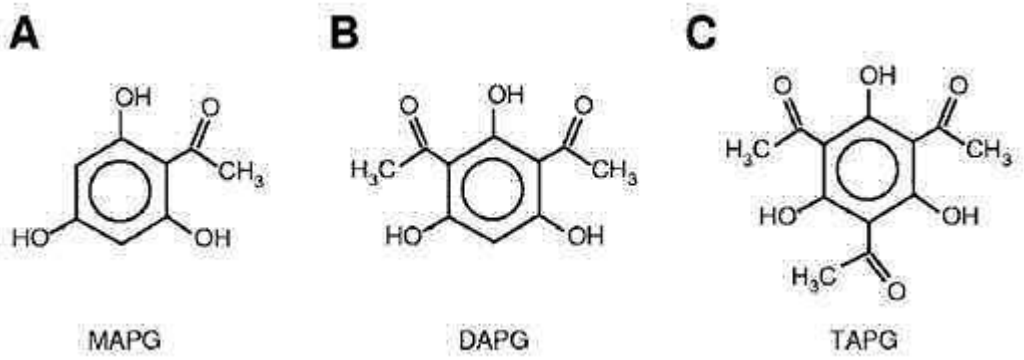


**Figure 2.5** Structures of three acylphloroglucinols produced by fluorescent pseudomonads. (A) MAPG; (B) DAPG; (C) TAPG (adapted from Bottiglieri and Keel, 2006).

DAPG is thought to be derived from monoacetylphloroglucinol (MAPG), and an acetyltransferase activity capable of converting MAPG to DAPG has been described in *Pseudomonas* sp. strain F113 (Shanahan et al., 1992). No precursors of MAPG have yet been identified, but the hydroxyl groups at alternating positions on the phloroglucinol ring are consistent with biosynthesis via a polyketide mechanism.

## 2.5 Biosynthesis and regulation of DAPG

The compound 2,4-diacetylphloroglucinol (DAPG), is produced by certain plant-associated fluorescent *Pseudomonas* species of worldwide origin (Keel et al., 1996a; Thomashow and Weller, 1995). The DAPG-biosynthetic locus includes the four biosynthetic genes *phlACBD* (Figure 2.6).

The genes required for the biosynthesis of DAPG have been identified in several fluorescent *Pseudomonas* species, including *P. fluorescens* strains Q2-87, F113, CHA0 and Pf-5 (Bangera and Thomashow, 1999; Delany et al., 2000; Fenton et

al., 1992; Paulsen et al., 2005; Schnider-Keel et al., 2000). DAPG is produced until the early stationary growth phase (Abbas et al., 2004; Baehler et al., 2005; Brodhagen et al., 2004; Pechy-Tarr et al., 2005; Schnider-Keel et al., 2000). Thereafter, it appears to be degraded by the producing bacterium, with MAPG temporarily accumulating as an intermediate product of the degradation process (Schnider-Keel et al., 2000).

In addition, DAPG is an autoregulator, positively influencing its own biosynthesis (Abbas et al., 2002; Schnider-Keel et al., 2000). The structural genes, *phlA*, *phlC*, *phlB*, and *phlD*, are transcribed as a single operon (*phlACBD)* (Bangera and Thomashow, 1996; Bangera and Thomashow, 1999; Delany et al., 2000). The operon *phlACBD* encodes enzymes responsible for both MAPG and DAPG biosynthesis (Bangera and Thomashow, 1999; Delany et al., 2000; Mavrodi et al., 2001; Schnider-Keel et al., 2000). Protein PhlD shows structural similarities with plant chalcone synthase, also called polyketide synthase type III (Bangera and Thomashow, 1999). (Zha et al., 2006) demonstrated that PhlD belongs to the type III PKS family and exhibits a relatively broad substrate specificity compared to other members of this family. In addition to its ability to produce phloroglucinol it can produce a great diversity of products thereby expanding the existing reservoir of polyketides. PhlD plays an essential role in the two proposed routes for the biosynthesis of the DAPG: biosynthesis of DAPG from glucose (Achkar et al., 2005) and synthesis of MAPG, the precursor of DAPG, from acetoacetyl-CoA (Bangera and Thomashow, 1999). PhlA, PhlC, and PhlB are necessary and sufficient for the transacetylation of MAPG to produce DAPG (Bangera and Thomashow, 1999; Shanahan et al., 1992).

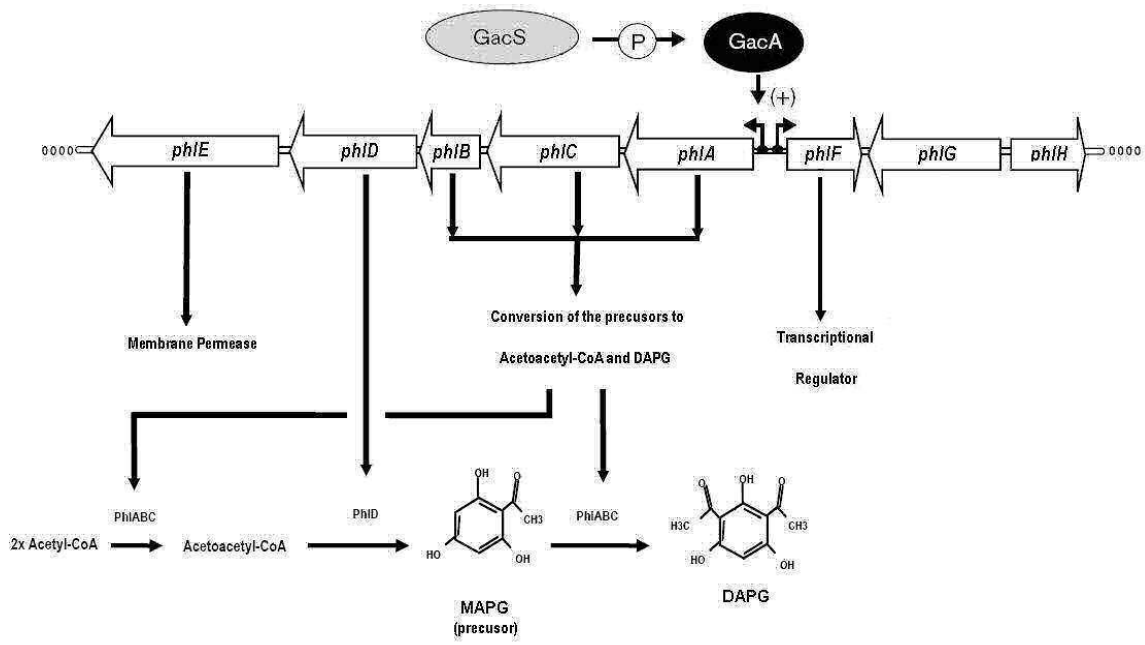


**Figure 2.6** Positive autoregulation of the biocontrol factors DAPG in fluorescent pseudomonads. Organization of the DAPG gene cluster in *P. fluorescens* CHA0. White arrows indicate direction of transcription. Oval symbols designate proteins (dark shading, transcriptional regulators; light shading, other regulatory proteins). P, phosphotransfer; (+), putative positive control (Haas et al., 2000; Rezzonico et al., 2004).

The *phlE* gene located immediately downstream of the *phlACBD* operon, encodes a putative permease (Bangera and Thomashow, 1996; Bangera and Thomashow, 1999; Delany et al., 2000) suggested to be a member of the major facilitator superfamily with 12 transmembrane segments (TMS) (Bangera and Thomashow, 1999) which appears to be involved in DAPG resistance (Abbas et al., 2004). They propose that the role of phlE and general stress tolerance is to export

toxic intermediates of PHL degradation from the cells. The *phlF*, encodes a pathway-specific transcriptional repressor of the DAPG biosynthetic operon, is located upstream of the *phlACBD* operon and is transcribed in the opposite direction (Abbas et al., 2002; Bangera and Thomashow, 1999; Schnider-Keel et al., 2000). The repression by the TetR-like regulator PhlF is due to its interaction with specific binding sites in the *phlA* promoter region (Abbas et al., 2002; Haas and Keel, 2003). DAPG acts as the signal that dissociates PhlF from the *phlA* promoter, thereby autoinducing its own biosynthesis (Abbas et al., 2002; Haas and Keel, 2003; Schnider-Keel et al., 2000). PhlF overexpression causes transcriptional repression of the biosynthetic *phlACBD* gene cluster (Delany et al., 2000), whereas inactivation of the *phlF* gene results in enhanced *phl* expression and DAPG synthesis at low cell densities. The derepressing signal has been identified in culture supernatants of *P. fluorescens* CHA0: it is DAPG, the end product of the pathway. Depression by DAPG requires a functional PhlF protein. It is thought that an interaction between DAPG and PhlF prevents repression of DAPG biosynthesis by PhlF (Schnider-Keel et al., 2000). The *phlH* gene is located downstream of *phlF* encodes a second TetR-like regulator involved in pathway-specific control of DAPG synthesis (Schnider-Keel et al., 2000). However, the precise role of PhlH remains to be elucidated. In *P. fluorescens* CHA0, the *phlG* gene is located between *phl*F and *phl*H (Figure 2.6). Bottiglieri and Keel (2006) assigned a function to PhlG as a hydrolase specifically degrades DAPG to equimolar amounts of mildly toxic MAPG and acetate and demonstrated that the expression of the phlG gene is negatively controlled by the pathway-specific regulators PhlF and PhlH and positively affected by the two-component regulatory system GacS/GacA. The enzymatic activity of PhlG appears to be highly specific for

its substrate DAPG (Bottiglieri and Keel, 2006). The available genetic data from other DAPG-producing pseudomonads (Pf-5, Q2-87, and F113) indicated that the phlG gene is not unique to strain CHA0, but appears to be commonly associated with the DAPG biosynthetic locus.

## 2.6 Quantitative analysis of phloroglucinols

Phloroglucinols have previously been investigated chromatographically by using gas-liquid chromatography (Pyysalo and Widen, 1979) and high-performance liquid chromatographic (HPLC) (Widen et al., 1980). However, HPLC-based method could detect an antibiotic both qualitatively and quantitatively in situ. Such a method was recently employed by Thomashow et al., 1990 for the detection of phenazine carboxylic acid in soil. Usually, antibiotics are recovered by extraction with organic solvents and purified by solid-phase extraction (e.g., Sep-Pak cartridges) and reverse-phase HPLC. Chemical identification is then based on the retention time in HPLC and on spectral properties (UV/visible absorbance, fluorescence when appropriate, and mass spectrometry) (Thomashow et al., 1990). This procedure has been applied to detect PCA, DAPG, pyrrolnitrin, pyoluteorin, and viscosinamide produced by pseudomonads in the rhizosphere (Bonsall et al., 1997; Glandorf et al., 2001; Keel et al., 1992; Maurhofer et al., 1995; Raaijmakers et al., 1999; Thomashow et al., 1990; Thrane et al., 2000). HPLC assay was developed to detect it quantitatively in growth culture media and soil. Shanahan et al., 1992 used HPLC assay to test the effect of different environmental conditions on the production of DAPG by *P. fluorescens* F113 and proposed that plant exudates composition and the ability of different carbon

sources to induce a particular antibiotic are essential in selecting suitable bacterium-plant combinations for biocontrol purposes.

Sensitive methods have been developed by which the expression of specific traits of biocontrol agents, such as antibiotic production, can be measured in natural environments. One approach involves the use of reporter gene systems (Chin-A-Woeng et al., 1998; Lindow, 1995). One of the disadvantages of this approach in studying in situ antibiotic production is that it does not provide an accurate measure of the amount of the antibiotic produced. Analytical techniques like thin layer chromatography and HPLC are now being used to detect and quantify antibiotics produced by microorganisms in situ (Thomashow et al., 1997). The versatility, resolving capability, and quantitative accuracy of HPLC make it one of the best direct methods to study the production of antibiotics in situ. HPLC has been successfully used for the in situ detection of a variety of antibiotics including phenazine-1-carboxylic acid (Thomashow et al., 1990), herbicolin A (Kempf et al., 1993), pyrrolnitrin (Burkhead et al., 1994; El-Banna and Winkelmann, 1998), gliotoxin (Lumsden et al., 1992), and DAPG (Bonsall et al., 1997; Keel et al., 1992). Reporter gene systems are widely used as a marker to monitor populations of introduced strains but also may provide information on the transcriptional activity of specific antibiotic biosynthetic genes.

The expression of antibiotic biosynthetic genes can be monitored *in situ* by fusing the antibiotic structural gene(s) to a reporter gene and monitoring its expression, e.g., *lacZ* (β-galactosidase), *luxAB* (luciferase), or *inaZ* (icenucleation protein). Bacteria carrying the reporter construct are recovered from the rhizosphere and assayed for reporter activity. Alternatively, root and seed exudates are collected

and tested for stimulation of reporter expression *in vitro*. These experiments showed that PCA, DAPG and lipopeptide genes are expressed *in situ* (Georgakopoulos et al., 1994; Koch et al., 2002; Kraus and Loper, 1995; Notz et al., 2002; Notz et al., 2001; Pierson et al., 1998; Seveno et al., 2001; Wood et al., 1997).

Unfortunately, reporter gene systems generally do not provide an accurate measure of the amount of the antibiotic produced *in situ*. The versatility, resolving capability, and quantitative accuracy of HPLC make it one of the best direct methods to study the production of antibiotics *in situ*.

Recently, unstable variants of green fluorescent protein (GFP) have been used as reporters; the corresponding *gfp* genes are fused to bacterial genes of interest, allowing gene expression to be observed *in situ* (Andersen et al., 2001; Andersen et al., 1998; Leveau and Lindow, 2001; Leveau and Lindow, 2002; Ramos et al., 2000). Using unstable GFP variants, the expression of biosynthetic genes for DAPG, pyoluteorin and phenazine-1- carboxamide could be visualized in the rhizosphere of wheat and tomato. In contrast to conventional reporters such as the stable enzyme β-galactosidase, which can only monitor increases in gene expression, unstable GFP can report both “on” and “off” states (Haas and Keel, 2003).

## 2.7 Methods used to study phenotypic and genotypic diversity of *Pseudomonas*

The genus *Pseudomonas* is one of the most diverse gram negative bacterial genus, isolated from sources ranging from plants to soils and water of this genus are straight or slightly curved rods, motile by means of polar flagella. *Pseudomonas* is characterized by their ability to grow in simple media at the expense of a great variety

of simple organic compounds, without needing organic growth factors. King's B media is an optimal for most species of *Pseudomonas* isolation. One of the most successful media for the detection of *Pseudomonas* is *Pseudomonas* isolation agar which is modified medium based on the formulation of medium A by (King et al., 1954). Peptic digest of animal tissue provides the carbon and nitrogen necessary for bacterial growth. Glycerol serves as carbohydrate source. Magnesium is a cofactor for many metabolic reactions and together potassium sulfate stimulate phocyanin (blue-green pigment) production for the improved detection and differentiation of *Pseudomonas* species. Irgasan is an antibiotic and selective inhibits gram-positive and gram-negative bacteria other than *Pseudomonas* spp. Agar is the solidifying agent.

A variety of phenotypic and genotypic methods are employed for microbial typing, identification and classification. DNA-based typing may involve specific or aspecific PCR amplification, restriction enzyme digestion and always fragment length analysis. PCR-based typing methods have a widespread and important role in studies of environmental, microbial diversity, agricultural, medical and industrial microbial ecology. This method enables scanning of part of, or the entire, microbial genome structure. The typing methods yield banding or fingerprint profiles that are generally amenable to computer-assisted analysis and comparative typing or database-mediated identification of bacteria (Rademaker et al., 2005).

Phenotypic analysis is essential for obtaining a putative identification, and both commercially available biochemical assimilation kits (such as API strip tests, BioMerieux Inc,: or GN Microplates, Biolog Inc.) and immunodiagnostic assays can be used for characterization. Although biochemical assays are based on carbon assimilation, the production of enzyme intermediates in these tests may not effectively

discriminate between isolates of close relatedness (i.e. at the sub-species/pathovar level); however, they do result in a putative identification to aid further phenotypic and molecular analyses (Franks et al., 2006).

A range of techniques has been developed to examine the genotypic diversity in soil microbial communities. These include denaturing or temperature gradient gel electrophoresis (DGGE/TGGE) (Heuer et al., 1997; Muyzer et al., 1993; Muyzer and Smalla, 1998), amplified rDNA restriction analysis (ARDRA) (Massol-Deya et al., 1995), terminal restriction fragment length polymorphism (T-RFLP) (Liu et al., 1997), single-strand conformational polymorphism (SSCP) (Schmalenberger and Tebbe, 2002), and ribosomal intergenic spacer length polymorphism (RISA) (Ranjard et al., 2001). Numerous studies indicate that ARDRA can be applied at the present time to differentiate between bacterial and eukaryotic species, and that it can be used as a tool in the study of complex microbial communities as a rapid classification method preceding more profound taxonomic studies, and also as a preliminary phylogenetic tool. It is a simple method based on restriction endonuclease digestion of the amplified bacterial 16S rDNA. In ARDRA, specific (or moderately degenerate) PCR primers targeting highly conserved domains in the sequences of *rrs* and *rrl* genes are used to amplify partial or nearly full-length 16S, 23S or intergenic spacer rDNA fragments. The high specificity and low degeneracy of these primers makes the amplification of the selected targets possible from different sources, including liquid or plate cultures, without a previous DNA purification step. The PCR products are generally single bands of a well-defined size making the analysis of the specificity and efficiency of the amplification reaction a straightforward and rapid undertaking**,** using standard agarose gel electrophoresis (Rademaker et al., 2005).

Instead of full sequence determination, cluster analysis of the combination of ARDRA patterns obtained with different restriction enzymes has been used successfully for phylogenetic analysis and/or taxonomic classification of microorganisms. For numerical analysis of ARDRA patterns, correct scoring and combination of the restriction fragments obtained with the different restriction enzymes is a crucial factor. Although this step can be done manually and visually, it is preferable to use appropriate software for this purpose. Despite some pitfalls introduced by numerical analysis of banding patterns, it seems that ARDRA can be used as a rapid technique to study phylogenetic relationships between closely related species, and to study phylogenetic relationships which correspond with the genus level or with rRNA groups within a phylogenetically diverse genus. On the other hand, phylogenetic relationships situated between these two boundary levels are often not reflected appropriately by ARDRA. On the other hand, ARDRA has been widely used in the analysis of mixed bacterial populations from different environments. Although it gives little or no information about the type of micro-organisms present in the sample, it can be used for a quick assessment of diversity and genotypic relatedness between isolates, as in the case of its application to Xanthomonads (Nesme et al., 1995). The simplicity and the general applicability of ARDRA make it possible to implement this technique in most laboratories with basic molecular biology equipment.

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# CHAPTER III

# MOLECULAR AND PHENOTYPIC CHARACTERIZATION OF POTENTIAL PLANT GROWTH PROMOTING *PSEUDOMONAS* FROM RICE AND MAIZE RHIZOSPHERES

## 3.1 Abstract

Beneficial plant-microbial interactions in the rhizosphere can result in the promotion of plant health and development. The most studied species of plant growth promoting rhizobacteria (PGPR) is *Pseudomonas*, which has received particular attention because of their potential as biocontrol agents, biofertilizers and plant protectors. It is well established that different plant species can select for specific microbial populations in the rhizosphere. In this study, *Pseudomonas* species were isolated from rhizospheres of two plant hosts: rice (*Oryza sativa*) and maize (*Zea mays*). The genotypic diversity of isolates was determined on basis of amplified rDNA restriction analysis (ARDRA). This analysis showed that both plant hosts were selected for two distinct populations of *Pseudomonas*. The actual biocontrol and plant promotion abilities of these strains was confirmed by bioassays on fungal (*Verticillum* sp., *Rhizoctonia solani* and *Fusarium* sp.) and bacterial (*Ralstonia solanacearum* and *Bacillus subtilis*) plant pathogens, as well as indole-3-acetic acid (IAA) production and carbon source utilization. This analysis showed that rice selected for a group of *Pseudomonas* with a higher ability to produce IAA and inhibition to some group of

plant pathogens. This work clearly identified a number of isolates which are potential for use as plant growth promoting and biocontrol agents on rice and maize.

## 3.2 Introduction

Modern agriculture is heavily dependent on the application of chemical inputs, including fertilizers and pesticides. Because of concerns regarding both human health and environmental protection, viable alternatives to these chemicals are being sought (Franks et al., 2006; Morrissey et al., 2004). It has been long recognized that many naturally occurring rhizospheric bacteria and fungi are antagonistic toward crop pathogens and, as a result, may offer a viable substitute to the use of these chemicals.

Of particular interest are the soil-borne pseudomonads, which can be utilized in low-input sustainable agriculture applications such as biocontrol. Numerous *Pseudomonas* strains display plant growth promoting activities and have been reported their widespread distribution in soil, ability to colonize the rhizospheres of host plants, and ability to produce a range of compounds antagonistic to a number of serious plant pathogens (Rodriguez and Pfender, 1997; Ross et al., 2000). The study of phenotypic and genotypic diversity of *Pseudomonas* spp. and their plant growth promoting potential is important not only for understanding their ecological role in the rhizosphere and the interaction with plants, but also for any biological control application (Berg et al., 2002).

*Pseudomonas* spp. is common soil bacteria easily cultured from most agricultural soils and rhizospheres (Morrissey et al., 2004). They have been studied intensively because of their ability to promote plant growth, either by directly stimulating the plant or by suppressing root pathogens. (Moenne-Loccoz et al., 2001)

studied the culturable fluorescent pseudomonad community associated with the roots of field-grown sugar beet seedlings using ARDRA, which suggested that the root-associated *Pseudomonas* community was flexible to the change that may be caused by adding a similar inoculant. (Bakker et al., 2002) further confirmed this using ARDRA by showing effects of biocontrol agents on the entire bacterial community in the rhizosphere of wheat.

To date studies in biological control have focused mainly on fluorescent pseudomonads, producing the fluorescent pigments and siderophores like pyoverdin (Haas and Defago, 2005), because of their metabolic versatility, their excellent root colonization ability and antimicrobial metabolites (O'Sullivan and O'Gara, 1992).

Plant roots influence soil-borne microbial communities via several mechanisms, including excretion of specific organic compounds, competition for nutrients, and providing a solid surface for attachment. It has been previously been demonstrated that an effective biological control strain isolated from one region may not perform as effectively in other soil or plant (Kiely et al., 2006). For this reason it has become important to screen different environments to understand the role that rhizosphere play in selected for bacterial populations.

The purpose of this study was to investigate bacterial populations of the wetland soil of rice (*Oryza sativa*) and desiccated soil of maize (*Zea mays*) on the abundance and diversity of plant growth promoting *Pseudomonas* spp. *Pseudomonas* isolates were selected from both plant host and profiled using amplified 16S rDNA restriction analysis (ARDRA). These isolates were further screened for phenotypes associated with plant growth promotion, such as, indole-3-acetic acid (IAA) production and the antagonistic activity of soil-borne disease bacteria and fungi.

## 3.3 Materials and methods

### 3.3.1 Soil sample collection and isolation of bacterial strains

Rhizosphere samples were collected from two different crops, rice (*Oryza sativa*) and maize (*Zea mays*) in Nakhon Ratchasima province, Thailand. The intact root systems were collected. Loosely adhering soils were shaken and detached from the roots and discarded. These 1 g root portions with just a layer of closely adhering rhizosphere soil was then transferred to 10 ml sterilized water and vigorously shaken for 10-15 min. The suspensions from all samples were serially diluted up to $10^{-6}$ with three replications for each sample. From $10^{-1}$ to $10^{-6}$ dilutions, 100 µl of each dilution was spread on *Pseudomonas* isolation agar and *Pseudomonas* selective medium based on King's medium B (KMB) (King et al., 1954). Three replicate plates were incubated for overnight at 28°C. All *Pseudomonas* strains isolated in this study were then kept at -80°C after an addition of glycerol to a final concentration of 40% (v/v). All strains were determined for fluorescent pigment production under UV light (Sharifi-Tehrani et al., 1998) and routinely maintained on KMB agar.

### 3.3.2 Total genomic DNA isolation

*Pseudomonas* isolates were grown in a nutrient broth and incubated at 28°C for overnight. Bacterial cells were harvested by centrifugation at 5,000 x g for 5 min and washed twice in 500 µl of TEN buffer (50 mM Tris, 20 mM disodium EDTA, and 50 mM NaCl, pH 8.0). Cell lysates were prepared by mixing the cell pellet with 200 µl of 20% (w/v) sucrose in TEN buffer to this 20 µl of 2 mg/ml of lysozyme and 20 µl of 10 mg/ml of RNase was added. Cell mixtures were incubated

at 37°C for 60 min. Then 75 µl of 5 M NaCl and 100 µl of 10% SDS were added before gently mixed. The solution was purified twice by using phenol: chloroform: isoamyl-alcohol (25:24:1, by vol.). The upper phase was collected and precipitated by using isopropanol and 3 M sodium acetate. DNA pellet was resuspended in sterilized deionized-water and total genomic DNA was kept at -20°C before use (Sambrook and Russell, 2001).

### 3.3.3 Identification of the genus *Pseudomonas*

16S rDNA region was amplified using the primers of *Pseudomonas* genus-specific 16S rRNA gene. The forward primer Ps-for (20-mer [5'-GGTCTGAGAGGATGATCAGT-3']) and reverse primer Ps-rev (18-mer [5'-TTAGCTCCACCTCGCGGC-3']) (Cirvilleri et al., 2005; Rangarajan et al., 2002) were used to amplify the total genomic DNA. The total volume of the reaction mixture was 25 µl containing 10-50 ng of template DNA, 10X reaction buffer, 2.5 mM dNTPs, 20 pmol of each primer, and 1 U *taq* DNA polymerase (invitrogen). PCR amplification was performed as detailed in previously study (Rangarajan et al., 2002). To estimate the product size, the products were run on 1% agarose gels along with 1 kb ladder as marker and stained with ethidium bromide. The *Pseudomonas* type strain, *P. fluorescens* 96.578 obtained from soil microbiology research group, Department of Agriculture, Thailand (DOA) was used as references.

### 3.3.4 Phenotypic characterization and carbon source utilization

Carbon sources solution were filtered sterilized and added at 0.1% (w/v) final concentration to LG medium (Lipman, 1904) plus 1 ml of 1M $KNO_3$ pH

7.2. *Pseudomonas* strains were streaked onto the medium and incubated at 28°C for 3 days. Growth was compared to the carbon source-free medium. The carbon substrates tests included myo-inositol, arabinose, glucose, sorbitol, mannitol, sucrose, lactose, xylose, glycerol, fructose oxidase test, and the ability to grow in nitrogen free medium (Desnoues et al., 2003).

### 3.3.5 Indole-3-acetic acid (IAA) production

IAA production was determined by adding 2 ml of 0.01 M $FeCl_3$ in 35% $HClO_4$ into 1 ml of Tris-TMRT (D-mannitol 10 g, yeast extract 0.2 g, $CaCl_2 \times 2H_2O$ 0.2 g, $MgSO_4 \times 7H_2O$ 0.25 g, Tris-base 1.21 g, L-tryptophane 0.061 g/l, pH 6.8) culture broth after being incubated at 28°C for 10 days. The mixture was incubated in the dark at 30°C for 30 minutes. Results were compared with positive control of 1 g of IAA in distilled water and ethanol (1:1, by vol. for 1.0 ml) (Nuntagij et al., 1997).

### 3.3.6 Biological control assay

An inhibition of phytopathogens by the *Pseudomonas* strains on PDA (Potato dextrose agar) plate for pathogenic fungi and NA plate for pathogenic bacteria was performed as detailed in previously study (Keel et al., 1996b). Briefly fungi and bacteria were grown overnight in LG broth, and 10 ml of each culture was spotted 2 cm from the edge of the plate (four spots per plate) and 0.1-0.3-cm square plug from a culture of *Verticillum* sp., *Rhizoctonia solani*, *Fusarium* sp. and *Ralstonia solanacearum* were placed at the center of the plate. The results were assessed after 3 days by measuring the distance between the edges of the bacterial colony and the

fungal mycelium. An inhibition of *Bacillus subtilis* by the *Pseudomonas* strains on SA (Fenton et al., 1992) plates containing 100 µmol $l^{-1}$ of $FeCl_3$ was performed. *B. subtilis* was grown overnight in LB at 37 °C by shaking and was sprayed onto the plates contain test strains. Results were assessed after overnight incubation. A zone of inhibition of *Bacillus* around the test strain is indicative of a positive result.

### 3.3.7 Amplified rDNA restriction analysis (ARDRA)

The 16S rDNA universal primers fD1 (5' - AGA GTT TGA TCC TGG CTC AG - 3') and rD1 (5' - AAG GAG GTG ATC CAG CC - 3') (Weisburg et al., 1991) were used to amplify a 1.5-kb internal region of the 16S rRNA gene. This primer pair was capable of amplifying nearly full-length 16S ribosomal DNA from a wide variety of bacterial taxa. Amplification was performed as previously described (Picard et al., 2004). Restriction analysis was performed with 5 µl of amplified product and 10 µl of restriction buffer containing 2 U of either the restriction enzymes *Alu*I, *Hinf*I, *Msp*I or *Rsa*I. After a 3 h digestion at the appropriate temperature, the enzyme was inactivated by heating the preparations at 70 °C for 15 min. For each isolate, PCR amplification and restriction analysis were performed at least three times. Calculation of the pair-wise coefficients of similarity was based on the presence or absence of bands. A cluster analysis with the UPGMA algorithm was performed with the NTSYS-pc numerical taxonomy and multivariate analysis system (Raaijmakers and Weller, 2001).

## 3.4 Results and discussion

### 3.4.1 Isolation and preliminary investigation of the genus *Pseudomonas*

The number of cultivable bacteria isolates obtained on *Pseudomonas* isolation agar was significantly different between rice and maize rhizospheres. It is obvious that 103 out of 138 total bacterial isolates were obtained from rice rhizosphere and 35 isolates were obtained from maize rhizosphere. Amplification of a 16S rRNA gene was approximately 950 bp to 1 kb, from all isolates. It is confirmed that each isolate was closely related to pseudomonads. Isolates were designated with a strain number and an alphabetical prefix denoting the site from which it was obtained (R for rice and M for maize) (Figure 3.1).

### 3.4.2 Genotypic characterization

The cluster dendogram of ARDRA analysis of pseudomonads isolates obtained from rice and maize rhizospheres is illustrated in Figure 3.1. One hundred and thirty eight isolates selected at random but representing each field site were analyzed by ARDRA. Digestion of amplified 16S rDNA with four restriction enzymes revealed 4 main clusters of ARDRA dendogram after hierarchical cluster analysis by Nei's genetic similarity statistic. The rhizospheres of rice and maize clearly selected from different soil bacterial ribotypes. ARDRA group 1 contained isolates found in maize rhizosphere except one isolate, R24, was found in rice rhizosphere.

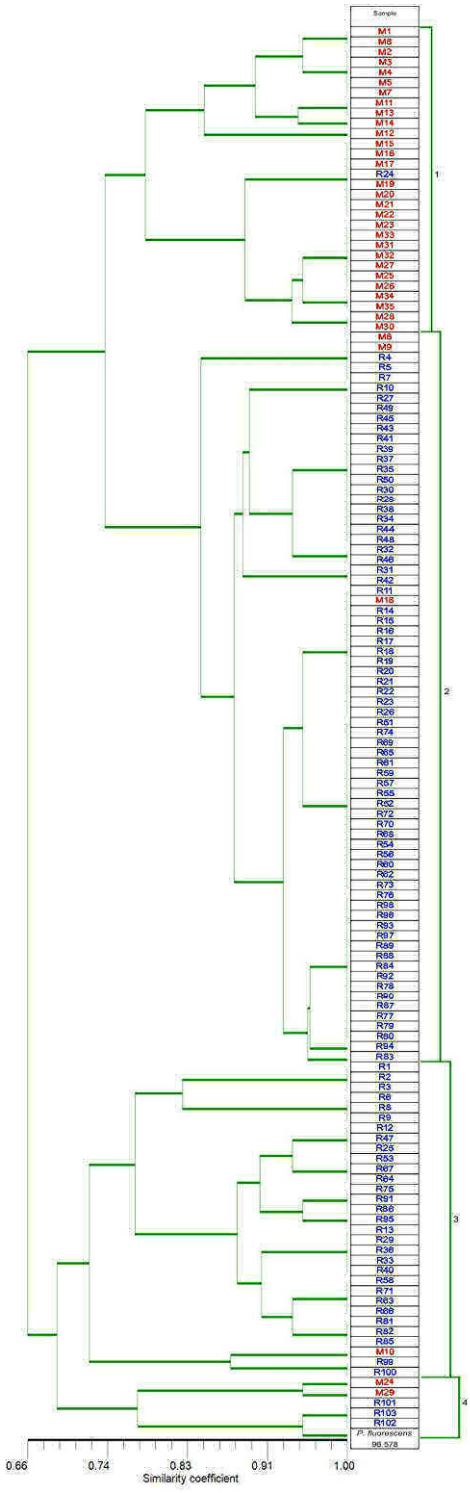


**Figure 3.1** Cluster dendogram, based on ARDRA analysis with four restriction enzymes (*Alu*I, *Hinf*I, *Msp*I, *Rsa*I) of *Pseudomonas* isolates that were isolated from maize and rice rhizosphere.

### 3.4.3 Phenotypic characterization of isolate for plant protection and biocontrol traits

The pseudomonads isolates were further analyzed for phenotypic traits associated with biocontrol and plant promotion. Firstly, their ability to produce IAA in presence of L-tryptophan as precursor was tested. One hundred and two isolates showed the ability to produce IAA, 17.14% of isolates obtained from maize rhizosphere and 93.2% of isolates obtained from rice rhizosphere. The pseudomonads isolates from rice rhizosphere, R4, R8, R31 and R35, were the most productive IAA while 36 isolates have no IAA produced. Interestingly, all pseudomonads isolates in cluster 1 did not show the ability to produce IAA except strain M22 (Table 3.1). The percentage of pseudomonads isolates obtained from rice rhizosphere which showed the ability to produce IAA were almost four-fold higher than pseudomonads isolates obtained from maize rhizosphere which showed the ability to produce IAA (Figure 3.2).

Sixty four isolates showed the ability to produced fluorescence under UV light, 17.14% of isolates obtained from maize rhizosphere and 56.31% of isolates were obtained from rice rhizosphere. Most isolates in cluster 1 have no ability in fluorescence production, but most of isolates in cluster 2, 3 and 4 are capable to produce fluorescence. The percentage of pseudomonads isolates obtained from rice rhizosphere which showed the ability to produce fluorescence were almost three-fold higher than pseudomonads isolates obtained from maize rhizosphere which showed the ability to produce fluorescence (Figure 3.2).

Eighty nine isolates showed the ability to grow in nitrogen-free medium, 14.28% of isolates obtained from maize rhizosphere and 81.55% of the

isolates obtained from rice rhizosphere. Most isolates in cluster 1 cannot grow in nitrogen-free medium, but most isolates in cluster 2, 3 and 4 can grow in nitrogen-free medium. Pseudomonads isolates obtained from rice rhizosphere showed higher in the ability to grow in nitrogen-free medium than pseudomonads isolates obtained from maize rhizosphere (Figure 3.2).

One hundred and eight isolates showed oxidase test positive, 77.14% of isolates obtained from maize rhizosphere and 52.43% of isolates obtained from rice rhizosphere. Most isolates in cluster 1 showed oxidase test positive, but most isolates in cluster 2, 3 and 4 showed oxidase test negative (Table 3.1, Figure 3.2).

**Table 3.1** Phenotypic characteristics of *Pseudomonas* isolates that were isolated from maize and rice rhizospheres (cluster 1).

| Sample | IAA production | Oxidase test | fluorescence production | Growth in N-free medium | Myo-inositol | Arabinose | Glucose | Sorbitol | Mannose | Sucrose | Lactose | Xylose | Glycerol | Fructose | *Verticillum* sp. | *Rhizoctonia solani* | *Fusarium* sp. | *Ralstonia solanacearum* | *Bacillus subtilis* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **M1** | - | + | + | - | - | - | + | - | - | - | - | + | + | - | - | - | - | - | + |
| **M8** | - | + | - | - | + | + | + | + | + | + | - | + | + | - | - | - | - | - | - |
| **M2** | - | + | - | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | + |
| **M3** | - | + | - | - | + | - | + | - | - | - | - | - | + | - | - | - | - | - | - |
| **M4** | - | + | - | - | + | - | + | - | - | - | - | - | + | - | - | - | - | - | - |
| **M5** | - | + | + | - | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **M7** | - | + | - | - | + | + | + | + | + | + | - | + | + | - | - | - | - | - | - |
| **M11** | - | + | - | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | + |
| **M13** | - | + | - | + | + | + | + | + | + | - | + | + | + | + | + | - | - | - | + |
| **M14** | - | + | - | - | + | + | + | + | + | - | + | + | - | + | + | - | - | - | - |
| **M12** | - | + | - | - | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **M15** | - | + | - | - | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **M16** | - | + | - | - | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **M17** | - | + | - | - | - | - | + | + | + | - | - | - | - | - | - | - | - | - | - |
| **R24** | + | + | - | + | - | + | + | - | - | - | - | - | - | - | - | - | + | - | - |
| **M19** | - | + | - | - | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **M20** | - | + | - | - | + | + | + | + | + | + | - | + | + | + | - | - | - | - | - |
| **M21** | - | + | - | - | + | + | + | + | + | + | + | - | + | + | - | - | - | - | - |
| **M22** | + | + | - | + | + | + | + | + | + | + | - | - | + | + | - | - | + | - | - |
| **M23** | - | + | - | - | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - |
| **M33** | - | - | - | - | + | + | + | + | + | + | - | + | + | + | - | - | - | - | - |

**Table 3.1** (Continued).

| Sample | IAA production | Oxidase test | fluorescence production | Growth in N-free medium | Myo-inositol | Arabinose | Glucose | Sorbitol | Mannose | Sucrose | Lactose | Xylose | Glycerol | Fructose | *Verticillum* sp. | *Rhizoctonia solani* | *Fusarium* sp. | *Ralstonia solanacearum* | *Bacillus subtilis* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **M31** | - | - | - | - | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **M32** | - | - | - | - | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **M27** | - | + | - | - | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **M25** | - | + | - | - | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **M26** | - | + | - | - | + | + | + | + | + | + | + | + | - | + | - | - | - | - | - |
| **M34** | - | - | - | - | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **M35** | - | - | - | + | - | - | + | + | - | - | - | - | - | - | - | - | - | - | - |
| **M28** | - | + | - | - | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **M30** | - | + | - | - | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |

Key: +, positive result (Production of IAA, oxidase test positive, fluorescence detected, grow in N-free medium, grow on a variety of carbon sources, zone of inhibition detected)

-, negative result (No production of IAA, oxidase test negative, no fluorescence detected, not growing on a variety of carbon sources, no zone of inhibition detected)

**Table 3.2** Phenotypic characteristics of *Pseudomonas* isolates that were isolated from maize and rice rhizospheres (cluster 2).

| Sample | IAA production | Oxidase test | fluorescence production | Growth in N-free medium | Myo-inositol | Arabinose | Glucose | Sorbitol | Mannose | Sucrose | Lactose | Xylose | Glycerol | Fructose | *Verticillum* sp. | *Rhizoctonia solani* | *Fusarium* sp. | *Ralstonia solanacearum* | *Bacillus subtilis* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **M6** | + | + | + | - | + | + | + | + | + | + | + | + | + | + | - | + | - | - | - |
| **M9** | + | + | - | - | + | + | + | + | + | + | - | + | + | + | - | - | - | - | - |
| **R4** | + | - | - | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | + |
| **R5** | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **R7** | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **R10** | + | - | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **R27** | + | - | + | - | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **R49** | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - |
| **R45** | + | + | - | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **R43** | + | + | - | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **R41** | + | - | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **R39** | + | - | - | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | + |
| **R37** | + | - | - | - | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - |
| **R35** | + | - | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | + |
| **R50** | + | + | - | + | + | + | + | + | + | + | + | + | + | + | - | + | - | - | + |
| **R30** | + | - | + | - | + | + | + | + | + | + | + | + | + | + | + | - | + | - | - |
| **R28** | + | + | + | - | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **R38** | + | + | - | - | + | + | + | + | + | + | + | + | + | + | - | - | + | - | - |
| **R34** | + | - | - | - | - | + | + | - | - | + | + | + | + | + | - | - | - | - | + |

**Table 3.2** (Continued).

| Sample | IAA production | Oxidase test | fluorescence production | Growth in N-free medium | Myo-inositol | Arabinose | Glucose | Sorbitol | Mannose | Sucrose | Lactose | Xylose | Glycerol | Fructose | *Verticillum* sp. | *Rhizoctonia solani* | *Fusarium* sp. | *Ralstonia solanacearum* | *Bacillus subtilis* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **R44** | + | - | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **R48** | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | + | - | - | - |
| **R32** | + | - | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **R46** | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **R31** | + | + | + | + | + | - | + | + | + | + | - | + | + | + | - | - | - | - | + |
| **R42** | - | + | - | + | + | + | + | + | + | + | + | - | + | - | - | - | - | + | + |
| **R11** | + | + | + | - | + | + | + | + | + | + | + | + | + | + | - | - | - | - | + |
| **M18** | + | - | + | - | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **R14** | + | - | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **R15** | + | - | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **R16** | + | + | - | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **R17** | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | + | - | + | - |
| **R18** | + | - | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | + | - |
| **R19** | + | + | - | - | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **R20** | + | + | + | - | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **R21** | + | + | + | - | + | + | + | + | + | + | + | + | + | + | - | - | - | - | + |
| **R22** | + | + | + | - | + | + | + | + | + | + | + | + | + | + | - | - | + | - | - |
| **R23** | + | - | - | + | + | + | + | + | + | + | + | + | + | + | - | - | - | + | - |
| **R26** | + | - | - | + | + | + | + | + | + | - | - | - | - | + | - | - | - | - | - |
| **R51** | + | - | - | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | + |
| **R74** | + | - | + | + | + | + | + | + | + | - | + | + | + | + | - | - | - | + | - |

**Table 3.2** (Continued).

| Sample | IAA production | Oxidase test | fluorescence production | Growth in N-free medium | Myo-inositol | Arabinose | Glucose | Sorbitol | Mannose | Sucrose | Lactose | Xylose | Glycerol | Fructose | *Verticillum* sp. | *Rhizoctonia solani* | *Fusarium* sp. | *Ralstonia solanacearum* | *Bacillus Subtilis* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **R65** | + | + | + | + | - | + | + | - | - | - | - | - | - | - | - | - | - | + | - |
| **R61** | + | - | + | + | + | + | + | + | + | + | + | + | + | + | - | + | - | - | - |
| **R59** | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - |
| **R57** | + | - | - | - | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **R55** | + | - | + | + | - | - | + | - | + | - | - | - | - | - | + | - | - | + | - |
| **R52** | + | - | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **R72** | + | - | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **R70** | + | - | + | - | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **R68** | + | - | + | - | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **R54** | + | - | - | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - |
| **R56** | + | - | + | + | - | + | + | - | + | - | - | - | - | - | + | - | - | + | - |
| **R60** | + | - | + | + | + | + | + | + | + | + | + | + | + | + | - | + | - | + | - |
| **R62** | + | - | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **R73** | + | + | + | + | + | + | + | + | + | - | - | - | - | + | - | - | - | + | + |
| **R76** | + | - | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **R98** | + | - | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **R96** | + | - | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | + |
| **R93** | + | - | - | - | + | + | + | + | + | + | + | + | + | + | - | - | - | - | + |

**Table 3.2** (Continued).

| Sample | IAA production | Oxidase test | fluorescence production | Growth in N-free medium | Myo-inositol | Arabinose | Glucose | Sorbitol | Mannose | Sucrose | Lactose | Xylose | Glycerol | Fructose | *Verticillum* sp. | *Rhizoctonia solani* | *Fusarium* sp. | *Ralstonia solanacearum* | *Bacillus subtilis* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **R97** | + | - | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | + |
| **R89** | + | - | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | + | - |
| **R88** | + | - | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **R84** | + | + | + | + | - | + | + | - | + | - | - | - | - | - | - | - | - | - | - |
| **R92** | + | - | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **R78** | + | - | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **R90** | + | - | - | + | + | + | + | + | + | + | + | + | + | + | - | + | - | - | + |
| **R87** | + | + | - | + | + | + | + | + | + | + | + | + | + | + | - | + | - | - | - |
| **R77** | + | - | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **R79** | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - | + | - |
| **R80** | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **R94** | - | - | - | + | + | + | + | + | + | + | - | + | + | + | - | - | - | - | + |
| **R83** | + | - | - | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |

Key: +, positive result (Production of IAA, oxidase test positive, fluorescence detected, grow in N-free medium, grow on a variety of carbon sources, zone of inhibition detected)

-, negative result (No production of IAA, oxidase test negative, no fluorescence detected, not growing on a variety of carbon sources, no zone of inhibition detected) Data includes a control typed strain: *P. fluorescens* 96.578.

**Table 3.3** Phenotypic characteristics of *Pseudomonas* isolates that were isolated from maize and rice rhizospheres (cluster 3).

| Sample | IAA production | Oxidase test | fluorescence production | Growth in N-free medium | Myo-inositol | Arabinose | Glucose | Sorbitol | Mannose | Sucrose | Lactose | Xylose | Glycerol | Fructose | *Verticillum* sp. | *Rhizoctonia solani* | *Fusarium* sp. | *Ralstonia solanacearum* | *Bacillus subtilis* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **R1** | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **R2** | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - |
| **R3** | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **R6** | + | + | - | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - |
| **R8** | + | - | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **R9** | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - |
| **R12** | + | + | - | + | + | + | + | + | + | + | + | + | + | + | - | - | - | + | + |
| **R47** | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - |
| **R25** | - | - | - | + | + | + | + | + | + | + | + | + | + | + | - | - | - | + | - |
| **R53** | - | + | - | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | + |
| **R67** | - | + | - | - | + | + | + | + | + | + | - | + | + | + | - | - | - | + | - |
| **R64** | + | + | - | + | + | + | + | + | + | + | + | + | + | + | - | - | - | + | + |
| **R75** | + | + | - | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | + |
| **R91** | + | - | - | + | + | + | + | + | + | + | + | + | + | + | - | - | - | + | + |
| **R86** | + | + | - | + | + | + | + | + | + | + | + | - | + | + | - | - | - | - | + |
| **R95** | + | - | - | - | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **R13** | - | + | - | + | + | + | + | + | + | + | - | + | + | + | - | - | - | + | + |
| **R29** | + | + | - | - | + | + | + | + | + | + | + | + | + | + | - | - | - | + | + |
| **R36** | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - | + | + |

**Table 3.3** (Continued).

| Sample | IAA production | Oxidase test | fluorescence production | Growth in N-free medium | Myo-inositol | Arabinose | Glucose | Sorbitol | Mannose | Sucrose | Lactose | Xylose | Glycerol | Fructose | *Verticillum* sp. | *Rhizoctonia solani* | *Fusarium* sp. | *Ralstonia solanacearum* | *Bacillus subtilis* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **R33** | + | + | - | + | + | + | + | + | + | + | - | + | + | - | - | - | - | + | + |
| **R40** | + | + | - | + | + | + | + | + | + | + | + | + | + | + | - | - | - | + | + |
| **R58** | + | + | - | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | + |
| **R71** | + | + | - | + | + | + | + | + | + | + | + | + | + | + | - | - | - | + | + |
| **R63** | + | + | - | + | + | + | + | + | + | + | + | + | + | + | - | - | - | + | + |
| **R66** | + | + | - | + | + | + | + | + | + | + | + | + | + | + | - | - | - | + | + |
| **R81** | + | + | - | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | + |
| **R82** | + | + | - | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | + |
| **R85** | + | + | - | + | + | + | + | + | + | + | + | + | + | + | + | - | - | + | + |
| **M10** | - | + | - | - | - | - | + | - | + | - | + | + | - | + | - | - | - | - | + |
| **R99** | + | + | - | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | + |
| **R100** | + | + | - | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |

Key: +, positive result (Production of IAA, oxidase test positive, fluorescence detected, grow in N-free medium, grow on a variety of carbon sources, zone of inhibition detected)

-, negative result (No production of IAA, oxidase test negative, no fluorescence detected, not growing on a variety of carbon sources, no zone of inhibition detected) Data includes a control typed strain: *P. fluorescens* 96.578.

**Table 3.4** Phenotypic characteristics of *Pseudomonas* isolates that were isolated from maize and rice rhizospheres (cluster 4).

| Sample | IAA production | Oxidase test | fluorescence production | Growth in N-free medium | Myo-inositol | Arabinose | Glucose | Sorbitol | Mannose | Sucrose | Lactose | Xylose | Glycerol | Fructose | *Verticillum* sp. | *Rhizoctonia solani* | *Fusarium* sp. | *Ralstonia solanacearum* | *Bacillus subtilis* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **M24** | + | - | + | - | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - |
| **M29** | + | - | + | - | + | + | + | + | + | + | + | + | + | + | - | - | - | - | + |
| **R101** | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **R103** | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| **R102** | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - |
| ***P. fluorescens* 96.578** | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |

Key: +, positive result (Production of IAA, oxidase test positive, fluorescence detected, grow in N-free medium, grow on a variety of carbon sources, zone of inhibition detected)

-, negative result (No production of IAA, oxidase test negative, no fluorescence detected, not growing on a variety of carbon sources, no zone of inhibition detected) Data includes a control typed strain: *P. fluorescens* 96.578.

Differences in utilization of carbon sources between pseudomonad isolates obtained from maize and rice rhizosphere were summarized in Figure 3.1 and 3.2. All pseudomonads isolates in ARDRA group 4 showed ability to grow on all ten sources of substrate (Table 3.1). However, not all of the isolates used the same set of substrates. Ten isolates, M1, M3, M4, M17, M35, R24, R55, R56, R65 and R84, grew significantly on fewer substrates than the other isolates. Pseudomonads isolates obtained from maize rhizosphere showed lower reduced ability to utilize a variety carbon sources than pseudomonads isolates obtained from rice rhizosphere except one that is glucose (Figure 3.2).

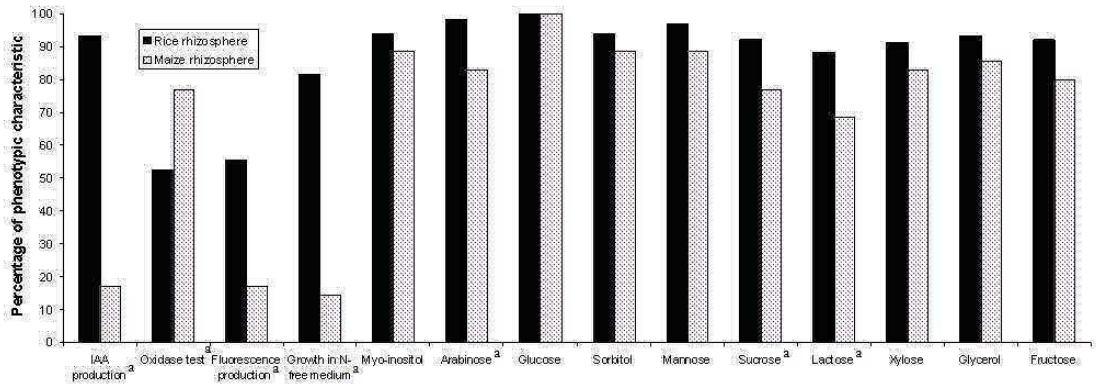


**Figure 3.2** Percentage of phenotypic characteristic at each sampling site which was obtained by dividing the number of isolates from each site represented in a particular group by the total number of isolates tested from that site.

Key: [a], There was significantly difference between isolates from rice rhizosphere and maize rhizosphere ($p < 0.05$, T-test).

### 3.4.4 Plant protection assays

In this study *Verticillum* sp., *Rhizoctonia solani, Fusarium* sp., *Ralstonia solanacearum* and *Bacillus subtilis* were used for biocontrol assay because they are considered to be widely distributed and destructive plant pathogens in agriculture. *Verticillum* sp. and *Ralstonia solanacearum* cause wilt of a wide range of broad hosts (NSW Department of Environment & Climate Change, 2007). *Rhizoctonia solani* and *Fusarium* sp. cause sheath blight diseases (Inagaki, 1998) and sheath rot disease in rice (Abbas et al., 1998). *Bacillus subtilis* cause seed rot-seedling blight disease in maize.

*In vitro* antagonistic capability experiments with *Verticillum* sp., *Rhizoctonia solani, Fusarium* sp., *Ralstonia solanacearum* and *Bacillus subtilis* revealed that 53.62 % of the 138 isolates tested detectable antifungal activity, 31.42% of isolates obtained from maize rhizosphere and 61.16% of isolates obtained from rice rhizosphere. The biocontrol assay results indicated that pseudomonad isolated from rice showed a higher ability to control bacterial and fungal root pathogens than pseudomonad isolates obtained from maize. Comparing to all fluorescent pseudomonad isolates obtained from rice and maize rhizosphere which had the ability to inhibit phytopathogens, the fluorescent pseudomonad isolates obtained from rice rhizosphere showed the antagonistic activity of 91.67%, 85.71%, 100%, 100% and 77.78% against *Verticillum* sp., *Rhizoctonia solani, Fusarium* sp., *Ralstonia solanacearum* and *Bacillus subtilis*, respectively. However the fluorescent pseudomonad isolates obtained from maize rhizosphere showed a much lower antagonistic activity against the pathogens with 16.67%, 16.67%, 0%, 0% and 33.33% against *Verticillum* sp., *Rhizoctonia solani, Fusarium* sp., *Ralstonia solanacearum*

and *Bacillus subtilis*, respectively. Seventy four isolates have the ability to control plant pathogens and sixty four isolates gave no control of plant pathogen. It is of some note that this result from maize isolates is conflicting to data already published (Nielsen et al., 1998), as fluorescent Pseudomonads have correlated with the level of pathogen antagonistic activity but in our study with the low number of isolates taken direct correlation is difficult. Previously, (Berg et al., 2002) explored the effect of different plant species on the abundance and diversity of bacteria antagonistic to plant pathogens, isolated originating from the rhizospheres of three host plants of *Verticillium dahliae*-strawberry, potato, and oilseed rape and from soil were analyzed for their antagonistic properties. Plants can have strong effects on soil microbial communities viewed from the functional perspective. The proportion of isolates with antagonistic activities was the highest for the strawberry rhizosphere (9.5%), followed by oilseed rape (6.3%), potato (3.7%), and bulk soil (3.3%). Hence, plants affect their associated communities also in a functional way.

Four isolates from rice rhizosphere in ARDRA group 3 and 4, R2, R36, R85 and R102, were the most antagonistic against all pathogens, while the other isolates displayed various degrees of antagonistic activity. Figure 3.3 shows that there was a significant difference between isolates from rice and maize rhizosphere in terms of biological control against *Ralstonia solanacearum* and *Bacillus subtilis*. Interestingly, none of the pseudomonads isolated from maize rhizosphere showed antagonistic activity against *Ralstonia solanacearum* and 50% of antagonistic isolates against *Bacillus subtilis* were found in ARDRA group 3 (Figure 3.3, 3.4).

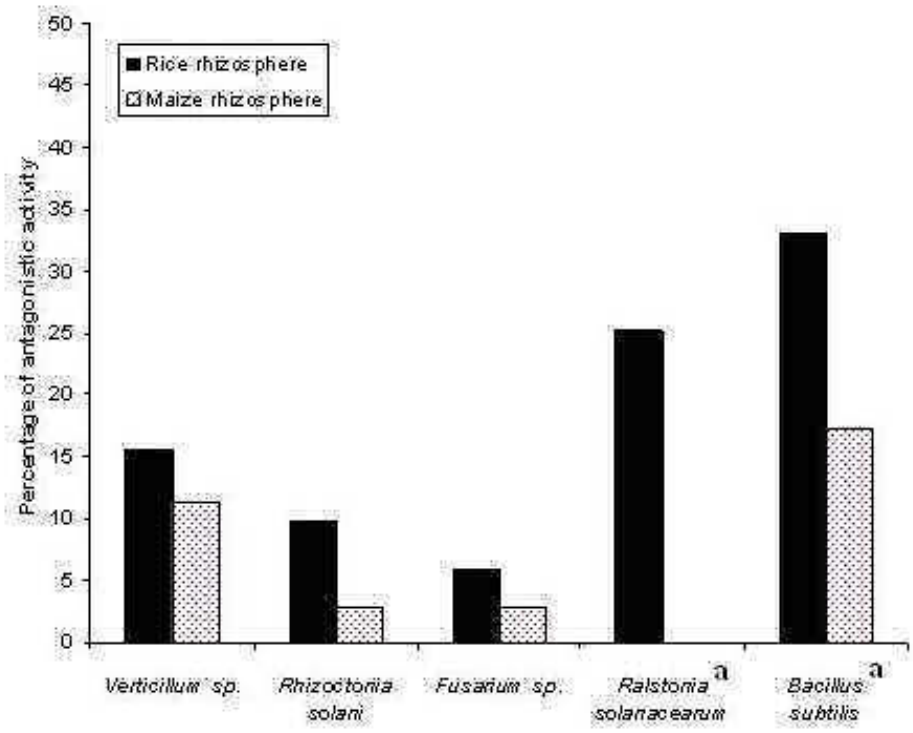


**Figure 3.3** Percentage of antagonistic activity at each sampling site which was obtained by dividing the number of isolates from each site represented in a particular group by the total number of isolates tested from that site.

Key: [a], There was significantly difference between isolates from rice rhizosphere and maize rhizosphere ($p < 0.05$, T-test).


**Figure 3.4** Antagonistic activity of *Bacillus subtilis* by pseudomonad isolate R31 compared with *P. fluorescens* F113 which was carried out on 9 cm diameter agar plate.

## 3.5 Conclusion

The genotypic diversity of one hundred and thirty eight *Pseudomonas* isolates that were isolated from rice and maize rhizospheres in Thailand was determined on basis of ARDRA. The result indicated that the rhizospheres of rice and maize clearly selected from different soil bacterial ribotypes. Moreover, The percentage of pseudomonad isolates obtained from rice rhizosphere which showed the ability to produce IAA were higher than pseudomonad isolates obtained from maize rhizosphere which showed the ability to produce IAA. Additionally, the percentage of pseudomonad isolates obtained from rice rhizosphere which showed the ability to produce fluorescence were almost threefold higher than pseudomonad isolates with the same property obtained from maize rhizosphere. Pseudomonad isolates obtained from maize rhizosphere showed lower reduced ability to utilize a variety carbon sources than pseudomonad isolates obtained from rice rhizosphere, except one that is

glucose. Furthermore, the biocontrol assay results indicated that pseudomonad isolated from rice showed a higher ability to control bacterial and fungal root pathogens than pseudomonad isolates obtained from maize. This information indicated that ARDRA of cultured bacteria from different communities can be used to estimate bacterial diversity. However, it is becoming important to map these pseudomonad populations in order to select “consortia” of bacteria for biological control for each plant species.

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# CHAPTER IV

# INVESTIGATION OF 2,4-DIACETYLPHLOROGLUCINOL (DAPG) PRODUCTION OF *PSEUDOMONAS FLUORESCENS* ISOLATED FROM RICE RHIZOPSHERE IN THAILAND

## 4.1 Abstract

2,4-diacetylphloroglucinol (DAPG) produced by fluorescent *Pseudomonas* plays key roles in the suppression of various soilborne plant pathogens. However, the performance of this biocontrol agent varies depending on the environment and host plant species. In this study, 16S rRNA gene tree and concatenated housekeeping genes tree were constructed to uncover the evolution of *Pseudomonas fluorescens* R31 that was isolated from rice rhizosphere in Thailand compared with the reference strain *P. fluorescens* F113. In addition, the ability to produce DAPG by strain R31 was investigated directly by high performance liquid chromatography (HPLC), and indirectly by quantifying the expression of a *phlA-gfp* reporter gene fusion. The results revealed that DAPG produced by strain R31 was lower than that by strain F113, which is similar to the *phlA-gfp* expression. Interestingly, the same orientation of each gene in *phl* operon of strain R31 was found whereas many different nucleotides between strain R31 and F113 in *phlA-phlF* intergenic region were discovered. This information appears that *phlA* promoter activity is not a good

indicator of DAPG production and may be associated with the presence of low level of DAPG production by strain R31. Moreover, the determination of the effect of plant host exudates on *phlA* gene was involved. This study revealed the host cultivar had a major influence on *phlA* gene expression in strain R31 in the rice exudates amendment. Furthermore, the result of *in vivo* antagonistic activity showed that *Pythium* spp. was suppressed by strains R31 and F113. The application of strain R31 to rice seeds showed significantly increased plant height, shoots dry weight and roots dry weight of rice while the application of strain F113 to rice seeds showed no significant difference when compared to control. Identification and understanding of these finding will provide a step on the way to overcome inconsistent performance in agricultural application.

## 4.2 Introduction

Agriculture over the past few decades is heavily dependent on the application of chemical inputs, including fertilizer and pesticides. However, many chemical pesticides are very toxic and thus result in contamination of environment. Biological control is thus being considered as an alternative or a supplemental way of reducing the use of chemicals in agriculture (Compant et al., 2005; De Weger et al., 1995; Gerhardson, 2002; Postma et al., 2003; Welbaum et al., 2004).

Among biocontrol agents, *P. fluorescens* producing the polyketide antibiotic DAPG are an important group of plant growth promoting rhizobacteria (PGPR) that suppress root and seedling diseases on a variety of crops. Examples included *P. fluorescens* CHA0 that suppresses black root rot of tobacco (*Thielaviopsis basicola*) (Stutz et al., 1986), take-all of wheat (*Gaeumannomyces graminis* var. trici) (Keel et

al., 1992), Fusarium wilt and crown and root rot of tomato (*Fusarium oxysporum*) (Duffy and Defago, 1997; Tamietti et al., 1993), *P. fluorescens* Q2-87 (Harrison et al., 1993; Pierson and Weller, 1994) and Q8r1-96 (Raaijmakers and Weller, 1998) suppress take-all of wheat (*G. graminis* var. trici) and *P. fluorescens* F113 that suppresses damping-off of sugar beet (*Pythium ultimum*) (Fenton et al., 1992; Schnider et al., 1995; Shanahan et al., 1992). de Souza et al., 2003 reported that DAPG causes membrane damage to *Pythium* spp. and is particularly inhibitory to zoospores of this oomycete.

Thus, introduction of *P. fluorescens* as a biocontrol agent offers a promising alternative to manage soilborne plant pathogens. However, the production of an antimicrobial compound varies among cultivars of the same species, and this has hampered commercialization (Notz et al., 2001). The studies of the ability to produce antibiotic DAPG and their plant growth promoting potential are important not only for understanding their ecological roles in the rhizosphere and their interaction with plants, but also for any biotechnological applications.

The DAPG-biosynthetic locus includes the four biosynthetic genes *phlACBD*. The structural genes, *phlA*, *phlC*, *phlB*, and *phlD*, are transcribed as a single operon (*phlACBD)* (Bangera and Thomashow, 1996; Bangera and Thomashow, 1999) which encodes enzymes responsible for the biosynthesis of both DAPG and its potential precursor, monoacetylphloroglucinol (MAPG) (Bangera and Thomashow, 1996; Bangera and Thomashow, 1999; Mavrodi et al., 2001; Schnider-Keel et al., 2000; Schnider et al., 1995). PhlD plays an essential for the biosynthesis of the DAPG. PhlA, PhlC and PhlB are necessary and sufficient for the transacetylation of MAPG to produce DAPG (Bangera and Thomashow, 1999; Dwivedi and Johri, 2003). The *phlE*

gene located downstream of the *phlACBD* operon encodes a putative permease (Abbas et al., 2004; Bangera and Thomashow, 1999) which is involved in general stress tolerance and exports toxic intermediates of DAPG degradation from the cells (Abbas et al., 2004).

DAPG biosynthesis is regulated by the pathway specific repressor PhlF, which binds to the *phlA* promoter and represses the expression of DAPG biosynthetic operon at the transcriptional level (Delany et al., 2000). The *phlA–phlF* intergenic region displays a complex organization where *phlA* is transcribed from a $\sigma^{32}$ RNA pol-dependent promoter that overlaps the promoter of the divergently transcribed *phlF* gene. Another specific sequence of 30 bp, known as *phlO*, is located downstream of *phlA*. Interaction of PhlF repressor protein with this sequence results in repression. This signifies that the repression occurs by inhibition of promoter clearance (Abbas et al., 2002; Bangera and Thomashow, 1996; Schnider et al., 1995). The *phlG* gene located between *phlF* and *phlH* encodes a hydrolase specifically degrades DAPG to equimolar amounts of mildly toxic MAPG and acetate (Bottiglieri and Keel, 2006). The *phlH* gene encodes a second TetR-like regulator involved in pathway-specific control of DAPG synthesis (Bottiglieri and Keel, 2006; Schnider-Keel et al., 2000). However, the precise role of PhlH remains to be elucidated.

In this study, *P. fluorescens* R31 that was isolated from rice (*Oryza sativa*) rhizosphere in Thailand that has the ability to produce indole-3-acetic acid (IAA) and to control plant pathogenic fungi was selected (Lawongsa et al., 2008). The phylogenetic trees based on 16S rRNA gene and the concatenated housekeeping genes of strain R31 were evaluated. Strain R31 was screened for secondary

metabolites production and the antagonistic activity of pathogenic bacteria and fungi. Moreover, DAPG production by strain R31 was investigated.

The influence of host plant exudates on DAPG gene expression using the reporter gene, which is *gfp* transcriptional fusion to the structural gene *phlA* which is the promoter gene in the *phlACBD* cluster (Bangera and Thomashow, 1999; Haas and Keel, 2003) was evaluated. Moreover, RT-PCR was used to detect the expression of *phlA* promoter in strains R31 and F113. In addition, the phylogenetic groups based on their *phlD*, a key gene in the biosynthesis of the antibiotic and highly conserved in nature (De La Fuente et al., 2006; Keel et al., 1996; Ramette et al., 2001) was also examined. These results will facilitate in overcoming existing limitations in the understanding of plant – microbe interactions in terms of DAPG production of strain R31. In this work, *P. fluorescens* F113 isolated from sugar beet (*Beta vulgaris*) rhizosphere in Ireland was used as the reference strain to compare with strain R31.

## 4.3 Materials and methods

### 4.3.1 Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmid used in this study are listed in Table 4.1. Isolation of rice rhizosphere strain R31 was carried out by serial dilution. Nutrient agar and *Pseudomonas* isolation agar (Sigma-Aldrich) were used. Strain R31 was identified on the basis of growth characteristic, microscopy and biochemical test. The strains of *Pseudomonas fluorescens* were maintained on Luria-Bertani (LB) agar (Sambrook and Russell, 2001) at 4°C and grown at 28°C overnight with shaking at 150 rpm. Growth media were LB, sucrose asparagine (SA) medium (Fenton et al., 1992; Scher and Baker, 1982) supplemented with 100 μM $FeCl_3$ and M9 minimal

medium (Sambrook and Russell, 2001). *Escherichia coli* DH5α was grown at 37°C in LB broth. Antibiotics were used as the following concentrations: for *P. fluorescens*, gentamycin 30 μg/ml; for *E. coli* gentamycin 30 μg/ml and kanamycin 25 μg/ml.

**Table 4.1** Bacterial strains and plasmids.

| Bacterial strain or plasmid | Description | References |
|---|---|---|
| **Bacterial strains** | | |
| *Pseudomonas fluorescens* F113 | Wild-type; $PHL^+$, Source; Sugar beet, Location; Ireland | (Shanahan et al., 1992) |
| R31 | Wild-type; $PHL^+$, Source; Rice, Location; Thailand | This study |
| *Escherichia coli* DH5α | ϕ80*lacZ*dM15 Δ (*lacZYA-arg*F) U169 *recA1 endA1 hsdR17 supE44 thi-1gyrA96 relA1* | (Sambrook et al., 1989) |
| Plasmids | | |
| pPROBE-GT (Figure 4.1) | Broad-host-range promoter-probe vector, $Gm^r$ *mob gfp* | (Miller et al., 2000) |
| pRK2013 | Helper plasmid; $Tra^+$ $Mob^+$ ColE1 $Km^r$ | (Flgurski and Helinski, 1979) |

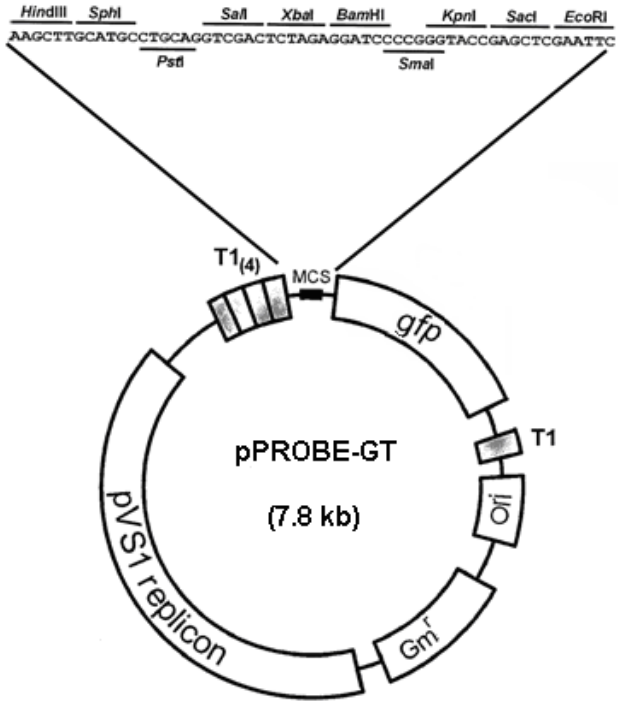


**Figure 4.1** Physical map of plasmid pPROBE-GT containing a gentamicin resistance gene, a common cassette that has four tandem copies of the T1 terminator ($T1_{(4)}$; shaded boxes) from the *E. coli rrnB1* operon (Brosius et al. 1981), a multicloning site (solid box) containing the pUC18 polylinker (*Hin*dIII → *Eco*RI), the *gfp* reporter gene, and a single *rrnB* T1 terminator (T1; shaded box) (Miller et al., 2000).

### 4.3.2 Morphological and biochemical characterization

*Pseudomonas* isolates were scored for gram-straining, carbon source assimilation and others biochemical characteristics according to Bergey's manual of Systematic Bacteriology. Biochemical assays were done by standard method of API identification. Utilization of additional carbon substrates was analyzed using the basal medium supplemented with a single organic substance in a final concentration of 1%

(w/v). Oxidase activity was determined from the oxidation of tetramethyl-p-phenylenediamine. Oxidase reagent (1% N,N,NN,NN-tetramethyl-p-phenylenediamine) was dropped on a piece of filter paper in a petri dish. Bacterial colonies were smeared across the wet paper with a toothpick. A positive reaction was scored from a dark purple color that develops in 10 seconds (Ewing and Johnson, 1960). The organism that remained colorless or turned purple after 10 seconds was scored as negative reaction. Catalase activity was determined by 3% $H_2O_2$. A small amount of a fresh bacterial colony was placed on a clean glass slide. Solution of 3% hydrogen peroxide was dropped on the colony. Bubble production was identified as a positive reaction. The method to detect siderophore production was based on that described by (Schwyn and Neilands, 1987). Chrome azurol S (CAS) plates were used to screen *Pseudomonas* of the organism for production of siderophores. Orange halos around the colonies are indicative of siderophore activity. IAA production was determined by adding 2 ml of 0.01 M $FeCl_3$ in 35% $HClO_4$ into 1 ml of Tris-TMRT (D-mannitol 10 g, yeast extract 0.2 g, $CaCl_2 \times 2H_2O$ 0.2 g, $MgSO_4 \times 7H_2O$ 0.25 g, Tris-base 1.21 g, L-tryptophane 0.061 g/l, pH 6.8) culture broth after being incubated at 28°C for 10 days. The mixture was incubated in the dark at 30°C for 30 minutes. Results were compared with positive control of 1 g of IAA in distilled water and ethanol (1:1, by vol. for 1.0 ml) (Nuntagij et al., 1997). Phosphate solubilization was tested on NBRIP plate. The halo and colony diameters were measured after 7 days of the incubation of plates at 28 °C.

### 4.3.3 Total genomic DNA isolation

*Pseudomonas* isolates were grown in a nutrient broth at 28°C overnight. Bacterial cells were harvested by centrifugation at 5,000 x g for 5 min and washed twice in 500 µl of TEN buffer (50 mM Tris, 20 mM disodium EDTA, and 50 mM NaCl, pH 8.0). Cell lysates were prepared by mixing the cell pellet with 200 µl of 20 % (w/v) sucrose in TEN buffer to this 20 µl of 2 mg/ml of lysozyme, and 20 µl of 10 mg/ml of RNase was added. Cell mixtures were incubated at 37°C for 60 min. Then, 75 µl of 5 M NaCl and 100 µl of 10 % SDS were added before gently mixed. The solution was purified twice by using phenol: chloroform: isoamyl-alcohol (25:24:1, by vol.). The upper phase was collected and precipitated by using isopropanol and 3 M sodium acetate. DNA pellet was resuspended in sterilized deionized-water and total genomic DNA was kept at -20°C before used (Lawongsa et al., 2008; Sambrook and Russell, 2001).

### 4.3.4 Phylogenetic analysis

Sequences of the genus tree based on 16S rRNA gene, the four concatenated genes (16S rRNA gene, *gyrB*, *groEL* and *rpoD*) and *phlD* were aligned using ClustalW multiple-sequence alignment program (Thompson et al., 1994). Further sequence analysis was performed using the Molecular Evolutionary Genetics Analysis (MEGA) version 4 program (Tamura et al., 2007). Phylogenetic trees for the *phlD* gene, 16S rRNA gene and concatenated housekeeping genes were constructed using the neighbor-joining method (Saitou and Nei, 1987) with the Jukes-Cantor distance method (Steel and Fu, 1995) for all sites. Bootstrap tests using 1,000

replicates were performed to test the robustness of each phylogeny (Felsenstein, 1985).

### 4.3.5 Genome sequences and nucleotide sequence accession numbers

The complete nucleotide sequences and annotations of the *P. fluorescens* Pf-5 (Paulsen et al., 2005) and *P. fluorescens* Pf0-1 genomes used in this project were retrieved and downloaded from the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/genomes). The nucleotide sequences of *P. fluorescens* R31 that were determined in this study have been deposited in the GenBank database under accession numbers GU475123 (16S rRNA gene), GU181357 (*phlD*), GU475124 (*groEL*), GU190379 (*gyrB*) and GU190381 (*rpoD*).

### 4.3.6 Multilocus sequence analysis

Multilocus sequence analysis was performed using primer pairs designed for PCR amplification of 16S rRNA gene and three housekeeping genes (*gyrB, groEL*, and *rpoD*), one gene from the *phl* cluster, *phlD* and also 16S rRNA gene for genus tree (Table 4.2). Following PCR product amplification, PCR purification was performed using the PCR extraction kit protocol from Promega or the gel extraction kit protocol from Qiagen and sequencing was performed by AGOWA sequencing center (Berlin, Germany).

**Table 4.2** Primers used for PCR amplification and DNA sequencing.

| Function and primers | Sequence (5' to 3') | Product size (kb) | References |
|---|---|---|---|
| Genes involve in DAPG production | | | |
| *phlA* | | 0.418 | (Rezzonico et al., 2003) |
| phlA-1f | TCAGATCGAAGCCCTGTACC | | |
| phlA-1r | GATGCTGTTCTTGTCCGAGC | | |
| *phlD* | | | |
| Phl2a | GAGGACGTCGAAGACCACCA | 0.745 | (Raaijmakers et al., 1997) |
| Phl2b | ACCGCAGCATCGTGTATGAG | | |
| Pyoluteorin production | | | |
| *PltB* | | | |
| PltBf | CGGAGCATGGACCCCCAGC | 0.773 | (Mavrodi et al., 2001) |
| PltBr | GTGCCCGATATTGGTCTTGACCGAG | | |
| Pyrrolnitrin production | | | |
| *prnC* | | | |
| PrnCf | CCACAAGCCCGGCCAGGAGC | 0.719 | (Mavrodi et al., 2001) |
| PrnCr | GAGAAGAGCGGGTCGATGAAGCC | | |
| ST-PCR Degenerate primers | | | |
| 2a | GGCCACGCGTCGACTAGTACN(10)AGAG | | (Manoil, 2000) |
| 2b | GGCCACGCGTCGACTAGTACN(10)ACGCC | | (Manoil, 2000) |
| 2c | GGCCACGCGTCGACTAGTACN(10)GATAT | | (Chun et al., 1997) |
| 4 | GGCCACGCGTCGACTAGTAC | | (Chun et al., 1997) |
| Specific primers for ST-PCR | | | |
| STD2 | GTTCTCCTCGCTGTGCTACC | | This study |
| STD5 | GACAAGGCCGTGATGAACTC | | This study |
| STA1 | CATCTCCAGGATCACCGC | | This study |
| STA2 | GTGTTGCGGTTGATGGTGT | | This study |
| STA5 | AACTCGGCGATCAGGTTGT | | This study |
| STF1 | GGTGTACGAGAAGGAAAGCG | | This study |
| STF2 | TCGGTAGCCGTGGTTACTTC | | This study |
| ST-GH1 | TCCTTGAGTTCTTCCAGCGT | | This study |
| ST-GH2 | CTCTACCGCGAGTTTTCTGG | | This study |
| G1 | AAACCAGAGCATGTAGCGCT | | This study |
| G2 | AGAAGCTGTTGCCGTAGAGC | | This study |
| E1 | TATCAGGACCTCGGCAACAT | | This study |
| E2 | GAAACACGCATGGTGATCCT | | This study |

**Table 4.2** (continued).

| Function and primers | Sequence (5' to 3') | Product size (kb) | References |
|---|---|---|---|
| Housekeeping genes | | | |
| gyrBF | CGGTAAGTT(CT)GACGACAACTC | 0.75 | (Moynihan et al., 2009) |
| gyrBR | CAGGAAGTCGGAGAAGTAC | | |
| groELF | GATGCCCATTTCGATCATG | 0.67 | (Moynihan et al., 2009) |
| groELR | GAACAACATGCGTGGCATC | | |
| rpoDF | GAAATCGCCAAGCGTATCG | 0.74 | (Moynihan et al., 2009) |
| rpoDR | ATCGACATGCGACGGTTG | | |
| 27F (16S rRNA gene) | AGAGTTTGATCCTGGCTCAG | 1.5 | (Lane et al., 1985) |
| 1492R (16S rRNA gene) | GGTTACCTTGTTACGACTT | | |
| *phlA-gfp* expression and RT-PCR experiment | | | |
| R31 inter F | CGCAGATCTGTGAGCGTGTTCGAGTCAAG | 0.705 | This study |
| R31 inter R | GCGGGTACCAGGGTGATCACATCCTCGTC | | This study |
| F113 inter F | CGCAGATCTGAGCTCCGAGATGGTTTACG | 0.64 | This study |
| F113 inter R | GCGGGTACCATGACGTCTTCATCGGGTTG | | This study |

### 4.3.7 Sequence analysis and homology searching

Comparisons of nucleotide sequences and deduced nucleotide sequences were performed by the BLAST algorithms to search the data bases maintained by the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/).

### 4.3.8 Biological control assay

An inhibition of phytopathogens by the *Pseudomonas* strains on Potato dextrose agar (PDA) plates was performed as detailed in previous studies (Lawongsa et al., 2008). Bacterial suspension of strains F113 and R31 was spot 2 cm from the edge of the plate, and 0.1-0.3 cm square plug from a culture of *Pythium* spp. and *Fusarium* spp. was placed at the center of the plate. The results were assessed after 3 days by measuring the distance between the edges of the bacterial colony and the fungal mycelium. An inhibition of *Bacillus subtilis* by the *Pseudomonas* strains on SA plates containing 100 μM $FeCl_3$ was performed. *B. subtilis* was grown overnight in LB at 37°C by shaking and sprayed onto the plates containing test strains. Results were assessed after overnight incubation. A zone of inhibition of *Bacillus* around the test strain is indicative of a positive result (Fenton et al., 1992).

### 4.3.9 Detection of pyoluteorin and pyrrolnitrin biosynthetic loci

Primers PltBf2 and PltBr were used for detection of the pyoluteorin biosynthetic locus of *pltB.* Primers PrnCf and PrnCr were used for detection of the pyrrolnitrin biosynthetic locus of *prn*C (Table 4.2). PCR amplification was carried out in 25-μl reaction mixture containing 1× *Taq* DNA polymerase buffer, 200 μM each of

dATP, dTTP, dGTP, and dCTP, 20 pmol of each primer, 1.5 mM $MgCl_2$, and 0.06 units of Go*Taq* DNA polymerase (Promega). The PCR cycling program consisted of initial denaturation at 94°C for 2 min followed by 29 cycles of 94°C for 1 min, 58°C for 45 s, and 72°C for 1 min. The amplification products were electrophoresed in 1% agarose gels in 1× TAE buffer for 40 min at 100 Volt at room temperature, stained with ethidium bromide, and photographed under UV light (Mavrodi et al., 2001).

### 4.3.10 Detection of hydrogen cyanide production

The production of hydrogen cyanide (HCN) was measured as described by Castric, 1975. Whatman 3MM paper was soaked in a chloroform solution containing copper (II) ethyl acetoacetate (5 mg/ml) and 4,4'-methylene-bis-(N,N-dimethylaniline) (5 mg/ml), and subsequently dried and stored in the dark. A piece of paper was placed in the lid of a Petri dish in which bacteria had been placed on SA agar. The Petri dish was incubated overnight at 28 °C. Production of HCN by the bacteria was indicated by blue coloration of the paper (Castric, 1975).

### 4.3.11 Detection of genes involved with DAPG production by PCR

Standard PCR mixtures and PCR conditions were employed: typically, an initial denaturation step at 96°C for 3 min followed by 30 cycles of denaturation at 94°C for 30 s, 30 s of primer annealing with temperatures dependent on the primer pairs used, and an extension of 1 kb per min at 72°C. DAPG genes were determined using *phlD* gene specific primers, Phl2a and Phl2b (Raaijmakers et al., 1997), and *phlA* gene specific primer, phlA-1f and PhlA-1r (Rezzonico et al., 2003). For strain R31, additional analysis of the genomic of *phl* genes was performed using a

semirandom, two-step PCR protocol (ST-PCR) (Chun et al., 1997). Degenerated primers were used for the first PCR step. After completion of the first PCR, the 20 μl reaction was diluted with 80 μl of water, and 1 μl was used for the second PCR with specific primers. The orientation and annotation of the genomic context of the *phl* genes of strain R31 were performed using Vector NTI Advance software package from Invitrogen. The primers using in this study were listed in Table 4.2.

### 4.3.12 Production of DAPG

The levels of DAPG and MAPG produced by strains F113 and R31 were quantified by HPLC-based technique (Shanahan et al., 1992). Strains F113 and R31 ($OD_{600nm}$ = 0.01) were inoculated into SA broth and incubated at 28°C. Cell samples were collected at 4, 8, 12, 16, 20 and 24 hours after inoculation. A cell culture was centrifuged at 5,000 x g for 5 min, and phloroglucinols (MAPG and DAPG) in the supernatant was purified by filtration through a Sep-Pak$C_{18}$ cartridge. The cartridge was flushed twice with 10 ml of methanol and then 10 ml of HPLC grade water. Then, 3 ml of filter-sterilized culture supernatant was injected onto the cartridge, which was washed with 10 ml of HPLC grade water and then with 10 ml of methanol to elute phloroglucinols. The eluate is filtered (0.45 μm pore size) and 20 μl aliquots were injected into the HPLC system. Samples were run at a flow rate of 1.0 ml/min and detected at a wavelength 296 nm over 35 by C18 column reverse phase HPLC (4.6 mm×250 mm). The extracellular phloroglucinols were extracted from the culture supernatant.

To extract cell-associated phloroglucinols, cells were broken by French press, 3 passages for each sample. The broken cells were centrifuged at 10,000 x g for 5 min, and phloroglucinols were extracted from the supernatant. In order to determine the effect of different carbon sources on DAPG production of strain R31 compared with strain F113, both strains were inoculated on M9 minimal medium supplemented with different carbon sources (0.4 % of sucrose, succinate, glucose, fructose and glycerol) and incubated at 28°C for 16 hours. Subsequently, DAPG is extracted from the supernatant according to the procedure described above.

### 4.3.13 Root Exudates Collection

Root exudates were collected separately from sugar beet (*Beta vulgaris*), maize (*Zea mays*) and rice (*Oryza sativa*) grown in gnotobiotic microcosms in Magenta GA-7 vessels (Sigma) using the method described by previous studies (Kato-Noguchi and Kanesawa, 2003; Mark et al., 2005). Plant and control microcosms were arranged in a replicate randomized block design and maintained for 16 days at 12°C in a 16-h light/8-h dark regime for sugar beet and maize, for 16 days at 25°C in a 12-h light/12-h dark regime for rice (Kato-Noguchi and Kanesawa, 2003). After this time, shoots were removed and root exudates were collected via vacuum filtration. Each vessel was rinsed with 5 ml of sterile distilled water, and the total volume obtained was 10 ml. Root exudates were filter-sterilized (0.22-μm filter, Millipore) and stored at -20°C in the dark until used.

### 4.3.14 Construction of transcriptional *phlA-gfp* fusions

To characterize the transcriptional activity of the *phlA* genes, pPROBE-GT (Miller et al., 2000) was used to construct bearing *gfp* transcriptionally fused with *phlA* (Figure 4.1). Plasmid DNA isolations were performed using the Qiagen plasmid mini kit. The restriction endonucleases and T4 DNA ligase (New England BioLabs) were used according to the manufacturer's specifications. Plasmids were mobilized into *E. coli* strain DH5α and *P. fluorescens* by triparental mating using the helper plasmid pRK2013 (Flgurski and Helinski, 1979). The kinetics of expression of biosynthetic fusions in different backgrounds was monitored by the emission of green fluorescence. Strain F113 and strain R31 cells were grown overnight with shaking (150 rpm) in 100-ml flasks containing 25 ml of SA minimal medium. In order to determine the effect of different carbon sources on *phlA* expression of strain R31 compared with strain F113, these two strains were grown overnight in M9 minimal medium supplemented with different carbon sources (0.4 % of sucrose, succinate, glucose, fructose and glycerol) at 28°C with shaking. Two percent of sugar beet exudates or rice exudates were applied into M9 minimal medium (Sambrook and Russell, 2001) to determine if host plant exudates affect *phlA-gfp* expression in *P. fluorescens* strain F113 and strain R31. All measurements were performed in triplicate with six time course experiments. Then, samples were taken from cultures at the indicated times. OD at 600 nm (growth) and green fluorescence (excitation at 480 nm and emission at 520 nm) was measured with a Fluostar fluorescence microplate reader (BMG Labtechnologies) throughout the exponential and stationary growth phases. For each individual measurement, the green fluorescence value was divided by the corresponding OD at 600 nm value giving the

specific fluorescence of the cells expressed as relative fluorescence units (RFU). The green fluorescence emitted by cells of wild-type strains R31 and F113 without gfp reporter fusion was determined for background correction. The expressions of *phlA-gfp* were carried out using the microtitre trays and $OD_{600nm}$/fluorescent plate counter.

### 4.3.15 Expression analysis of the *phlA* operon in strains R31 and F113

RT-PCR analysis was used to quantify the expression of the *phlA* promoter. RNA was extracted from the cell pellets (RNeasy Mini Kit, Qiagen Sciences) and cDNA synthesized (ImProm-II Reverse Transcription System, Promega) was followed by RT-PCR. Total RNAs (1 μg) from strains R31 and F113 at 4, 8, 12, 16, 20 and 24 hours after inoculation in SA medium were subjected to RT-PCR with specifically designed primers, R31 inter F and R31 inter R for strain R31 and F113 inter F and F113 inter R for strain F113. The 16S rRNA gene was used as a control for standardized RNA levels.

### 4.3.16 Statistical analysis

Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) at 5 % level using the statistical package of the social sciences (SPSS) version 17.0. The data presented are means standard deviation (SD) of three replicates.

### 4.3.17 Rice and soil preparation for the assay of *in vivo* antagonistic activity

The cultivated rice (*Oryza sativa* cultivar Pathum Thani 1) was obtained from Pathum Thani Rice Research Center, Pathum Thani, Thailand. The rice seeds were surface sterilized with 70% ethanol for 1 min and shaken in 10% (w/v) NaOCl solution for 30 min. Seeds were then washed three times with sterilized distilled water with shaking (15 min each) (Prakamhang et al., 2009). Surface sterilized seeds were gnotobiotically germinated on sterilized wet tissue paper. After 3 days, rice seedlings in each treatment were transferred into pots (cm in diameter, 60 cm high) containing autoclaved soil. Three rice seeds were planted to a depth of 1 cm in each pot. Three replications for each treatment were done. Rice pots were placed in growth chambers maintained at 25 °C (± 1 °C) with 12 hours light, 12 hours darkness (Kato-Noguchi and Kanesawa, 2003).

### 4.3.18 Preparation of bacterial inocula and seed treatment for the assay of *in vivo* antagonistic activity

Cells of antagonistic bacteria for use in the assay of *in vivo* antagonistic activity were grown in King's Medium B broth (KMB) (King et al., 1954) to late exponential phase at 28°C with shaking at 150 rpm overnight. Cells were then harvest by centrifugation (5000 rpm/min for 5 min), washed twice and resuspended in 0.5% sterile NaCl solution (Kazempour, 2004). The rice seeds were soaked with bacterial suspension that was adjusted to about $10^8$ colony forming units (CFU/ml) for each experiment and applied with *Pythium* spp. ($1 \times 10^6$ spores/ml) (Timms-Wilson et al., 2000). Control treatments were inoculated in sterile distilled

water and seedling with disease symptoms were recorded 4 weeks after planting. Plants were arranged in a randomized completed block design with three replications. Percentage of disease incidence was assessed 4 weeks after plant. Rice plants were sampled 4 weeks after planting to measure for plant height, shoot and root dry weight.

## 4.4 Results and discussion

### 4.4.1 Biochemical characteristics of *Pseudomonas* sp. strain R31

*Pseudomonas* spp. strain R31 was firstly characterized by biochemical test. *Pseudomonas* spp. strain R31 could utilize glucose, sucrose, mannose, mannital, fructose, glycerol, succinate, gluconate, caprate, malate, citrate, and phenyl-acetate, but could not utilize esculin, arabinose, and maltose as a sole carbon source. Moreover, *Pseudomonas* sp. R31 had the ability to produce IAA and siderophore and had the potential to solubilize phosphate. Characteristic of the specie and *Pseudomonas* five biovars were presented in Table 4.3. Identification results showed *Pseudomonas* sp. strain R31 was not able to grow at 41 °C which indicated that *Pseudomonas* sp. strain R31 was not assigned in *P. aeruginosa* group and it was also not designated in *P. putida* group due to gelatin liquefaction assay. Moreover, identification results showed *Pseudomonas* sp. strain R31 could assimilate sucrose whilst it could not assimilate arabinose and phenylacetate which identified as *P. fluorescens* biovar V. (Sands and Rovira, 1971) found that strains of biovar V are very common in soils. The biovar is very heterogeneous in its nutritional properties, and may consist of strains that have lost one or more of the properties considered to be of diagnostic importance in differentiating among the better characterized biovars.

**Table 4.3** Biochemical characteristic of *Pseudomonas* sp. R31 and biovar I-V of *Pseudomonas* (Palleroni, 2005). d, 11-89% of the strains are positive.

| **Characteristics** | ***Pseudomonas* sp. R31** | ***P. fluorescens* Biovar I** | ***P. fluorescens* Biovar II** | ***P. fluorescens* Biovar III** | ***P. fluorescens* Biovar IV** | ***P. fluorescens* Biovar V** |
|---|---|---|---|---|---|---|
| Gram strain | Negative | Negative | Negative | Negative | Negative | Negative |
| Oxidase test | + | + | + | + | + | + |
| Catalase test | + | + | + | + | + | + |
| Fermentative of acid from glucose | - | - | - | - | - | - |
| Arginine dihydrolase | + | + | + | + | + | + |
| Urease production | - | - | - | - | - | - |
| Hydrolysis of esculin | - | - | - | - | - | - |
| Gelatin liquefaction | + | + | + | + | + | + |
| β-galactosidase production | - | - | - | - | - | - |
| Glucose assimilation | + | + | + | + | + | + |
| Sucrose assimilation | + | + | + | - | + | d |
| Arabinose assimilation | - | + | + | d | + | d |
| Mannose assimilation | + | + | + | + | + | d |
| Mannitol assimilation | + | + | + | d | + | d |
| Fructose assimilation | + | + | d | + | + | + |
| Maltose assimilation | - | - | - | - | - | - |
| Glycerol assimilation | + | + | + | + | + | + |
| Succinate assimilation | + | + | + | + | + | + |
| Gluconate assimilation | + | + | + | + | + | + |
| Caprate assimilation | + | + | + | + | + | + |
| Malate assimilation | + | + | + | + | + | + |
| Citrate assimilation | + | + | + | + | + | + |
| Phenyl-acetate assimilation | + | - | - | d | - | d |
| Growth at 41 °C | - | - | - | - | - | - |

### 4.4.2 Phylogenetic tree of 16S rRNA gene and four housekeeping genes

In order to investigate the relationship of strain R31 among genus *Pseudomonas*, a phylogenetic tree based on 16S rRNA gene was constructed (Figure 4.2). The examination of the genus tree based on 16S rRNA gene showed R31 was grouped into a cluster with the various strain of *Pseudomonas fluoresccens*. In this cluster, a lineage of the isolated strains was separated from the group of *Pseudomonas putida* with the bootstrap support of 77% bootstrap value. The distance between strain R31 and strain F113 was supported by an 88% bootstrap value. Moreover, the 16S rRNA gene sequencing showed that strain R31 was close with *P. fluorescens* Pf-5 and, *P. fluorescens* CHA0 for 100% identities and 99% coverage when BLAST searches was carried out. However, the ribosomal RNA genes sequence analysis does not always allow species identification. Thus, the analysis of several housekeeping genes could be useful to improve the reliability of the phylogenies (Stackebrandt et al., 2002).

The relationship among the seven *P. fluorescens* strains; Pf-5, CHA0, PGNL1, F113, SBW25, Pf0-1, and R31 was investigated by constructing a phylogenetic tree using housekeeping gene tree. Strain R31 showed *groEL* sequence identities of 99% to *P. otitidis* with 93% coverage, 80-81% to *P. aeruginosa*, and 79% to *P. mendocina* ymp with 94% coverage. The phylogenetic tree using *rpoD* gene tree revealed that the distance between strain R31 and the group of *P. aeruginosa* was supported by 81% bootstrap value (Figure 4.3). The distance between strain R31 and *P. mendocina* was supported by 87% bootstrap value. The distance between strain R31 and the group of *P. fluorescens* was supported by 100% bootstrap value (Figure 4.3). The *gyrB* gene sequencing showed that strain R31 was closely related to *P.*

*otitidis* for 98% identities and 99% coverage and *P. mendocina* ymp for 89% identities and 99% coverage. The phylogenetic tree using *gyrB* gene tree revealed that the distance between strain R31 and the group of *Pseudomonas* spp. was supported by 100% bootstrap value (Figure 4.4). On the basis of *groEL* sequence similarity, levels of sequence similarity between the isolated strains and the other relative species were very low (less than 81%). They showed *groEL* sequence identities of 80-81% to *P. aeruginosa*, and 80% to *P. stutzeri*. The phylogenetic tree using *groEL* gene tree revealed that the distance between strain R31 and the group of *P. mendocina* was supported by 93% bootstrap value (Figure 4.5).

The relationship among the seven *P. fluorescens* strains; Pf-5, CHA0, PGNL1, F113, SBW25, Pf0-1 and R31 was also investigated by constructing a phylogenetic tree using the concatenated sequence of four genes (16S rRNA gene, *gyrB, groEL* and *rpoD*). The examination of the concatenated housekeeping genes tree showed R31 was completely distinguished from the *P. fluorescens* clade. The distance between strain R31 and the group of *P. fluorescens* including strain F113 and *P. putida* was supported by a 100% bootstrap value. Moreover, the concatenated phylogenetic tree result revealed that the distance between strain R31 and the group of *P. mendocina* and *P. aeruginosa* was supported by 71% bootstrap value (Figure 4.6). An essential prerequisite for a detailed investigation of the roles and evolution of pseudomonads is an accurate system of classification and identification. However, the degree of resolution obtained with 16S rRNA sequence analysis is not sufficiently discriminatory to permit resolution of intrageneric relationships because of the extremely slow rate of evolution of 16S rRNA. (Yamamoto and Harayama, 1998) observed that the genetic distances in the variable regions of 16S rRNAs correlated

poorly with the synonymous distances in the *gyrB* and *rpoD* genes. Molecular phylogeny deduced from a single locus may be unreliable due to the stochastic nature of base substitutions or to rare horizontal gene transfer events. Therefore, the different placement of the isolated strains in individual gene tree analyses may be due to different evolutionary histories of the genes used for analysis.

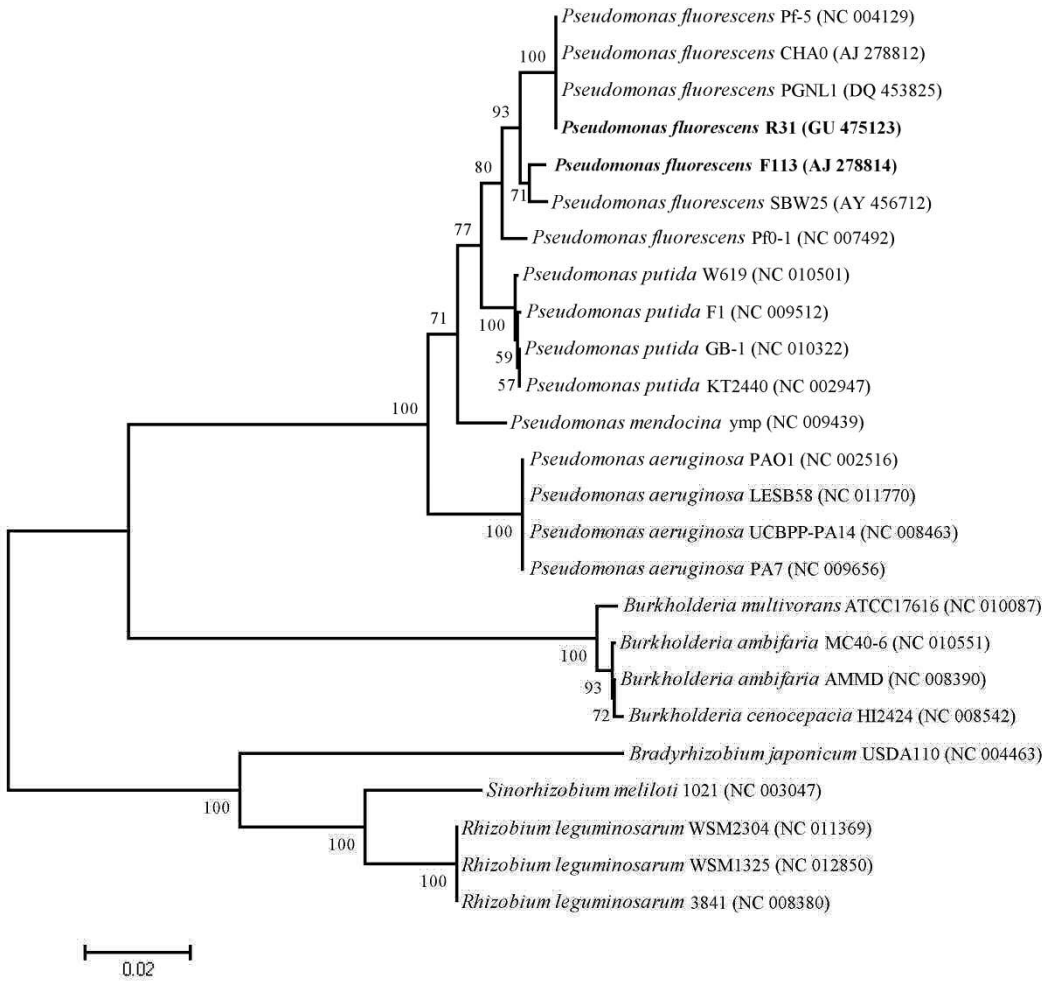


**Figure 4.2** Phylogenetic tree based on 16S rRNA gene using *Burkholderia ambifaria* AMMD, MC40-6, *Burkholderia cenocepacia* HI2424, *Burkholderia multivorans* ATCC17616, *Bradyrhizobium japonicum* USDA110, *Sinorhizobium meliloti* 1021, *Rhizobium leguminosarum* 3814, WSM1325 and WSM2304 as outgroup inferred by the neighbor-joining. Bootstraps of 1,000 replicates were used and shown at the branch nodes on the phylogenetic tree. Bar, 0.02% estimated substitutions.

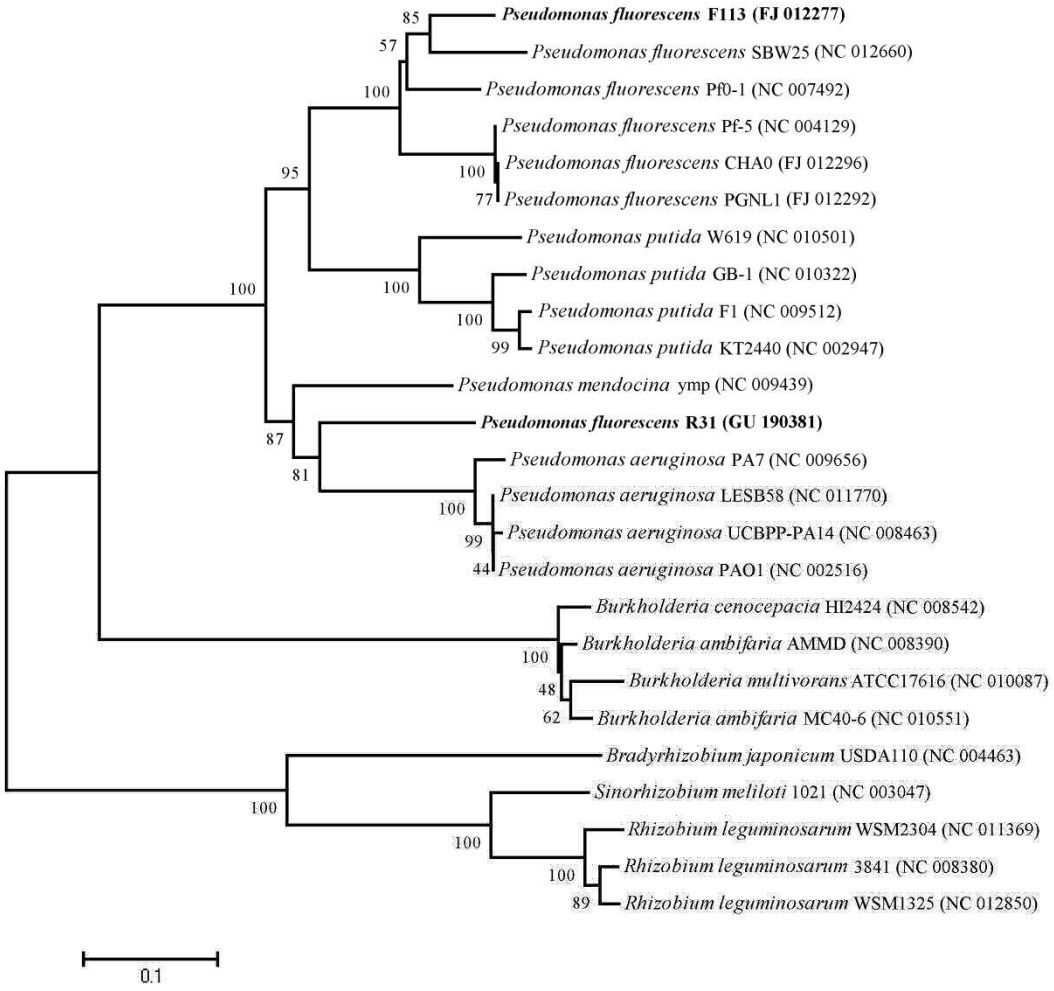


**Figure 4.3** Phylogenetic tree based on *rpoD* gene using *Burkholderia ambifaria* AMMD, MC40-6, *Burkholderia cenocepacia* HI2424, *Burkholderia multivorans* ATCC17616, *Bradyrhizobium japonicum* USDA110, *Sinorhizobium meliloti* 1021, *Rhizobium leguminosarum* 3814, WSM1325 and WSM2304 as outgroup inferred by the neighbor-joining. Bootstraps of 1,000 replicates were used and shown at the branch nodes on the phylogenetic tree. Bar, 0.1% estimated substitutions.

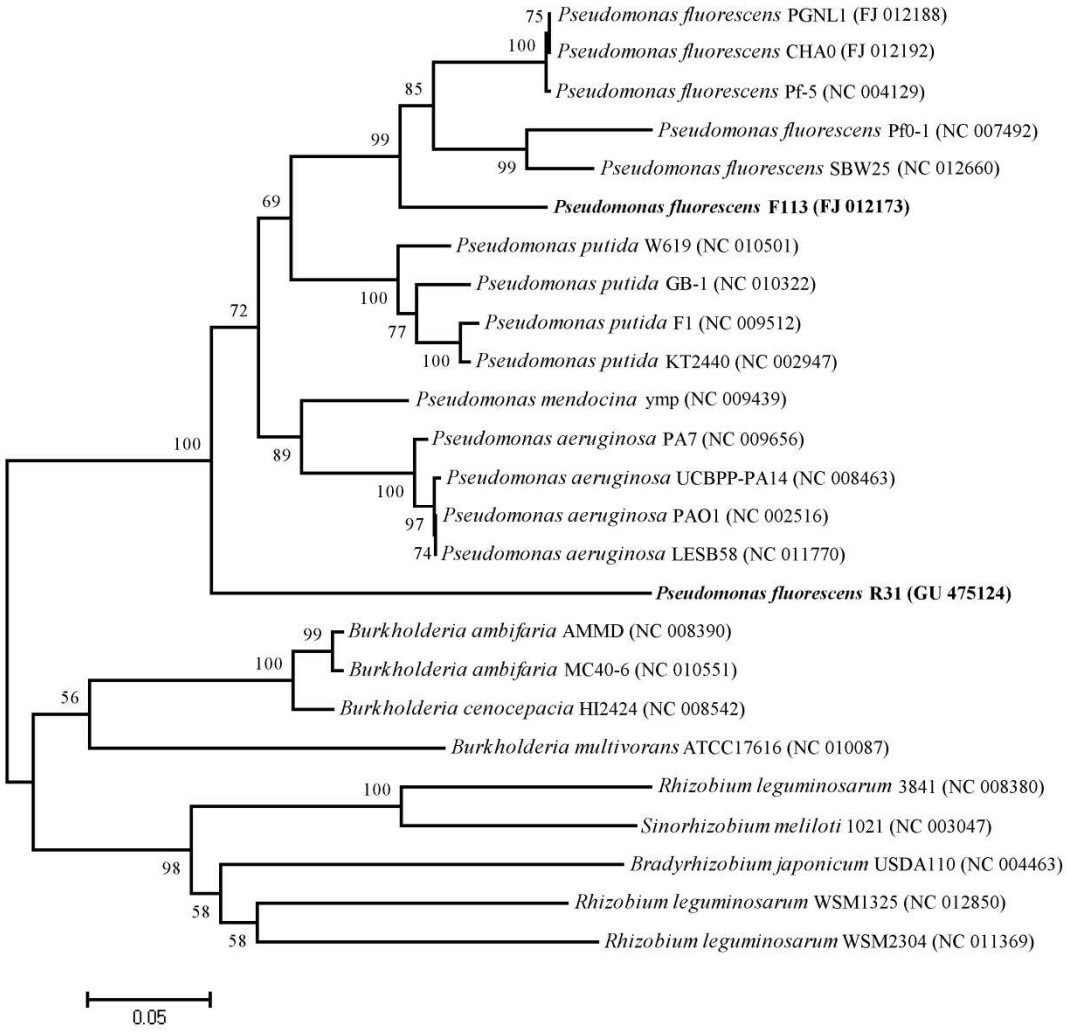


**Figure 4.4** Phylogenetic tree based on *groEL* gene using *Burkholderia ambifaria* AMMD, MC40-6, *Burkholderia cenocepacia* HI2424, *Burkholderia multivorans* ATCC17616, *Bradyrhizobium japonicum* USDA110, *Sinorhizobium meliloti* 1021, *Rhizobium leguminosarum* 3814, WSM1325 and WSM2304 as outgroup inferred by the neighbor-joining. Bootstraps of 1,000 replicates were used and shown at the branch nodes on the phylogenetic tree. Bar, 0.05% estimated substitutions.

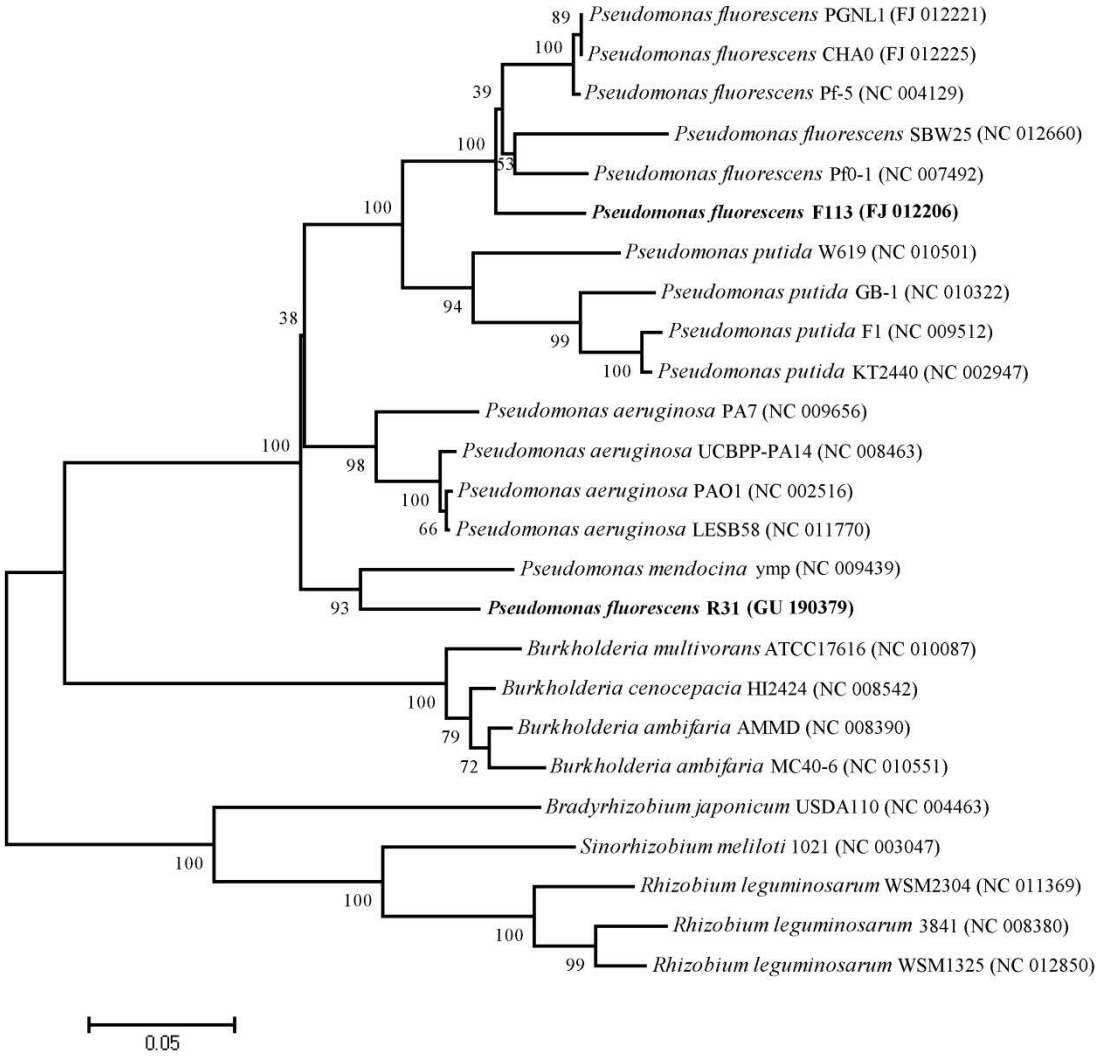


**Figure 4.5** Phylogenetic tree based on *gyrB* gene using *Burkholderia ambifaria* AMMD, MC40-6, *Burkholderia cenocepacia* HI2424, *Burkholderia multivorans* ATCC17616, *Bradyrhizobium japonicum* USDA110, *Sinorhizobium meliloti* 1021, *Rhizobium leguminosarum* 3814, WSM1325 and WSM2304 as outgroup inferred by the neighbor-joining. Bootstraps of 1,000 replicates were used and shown at the branch nodes on the phylogenetic tree. Bar, 0.05% estimated substitutions.

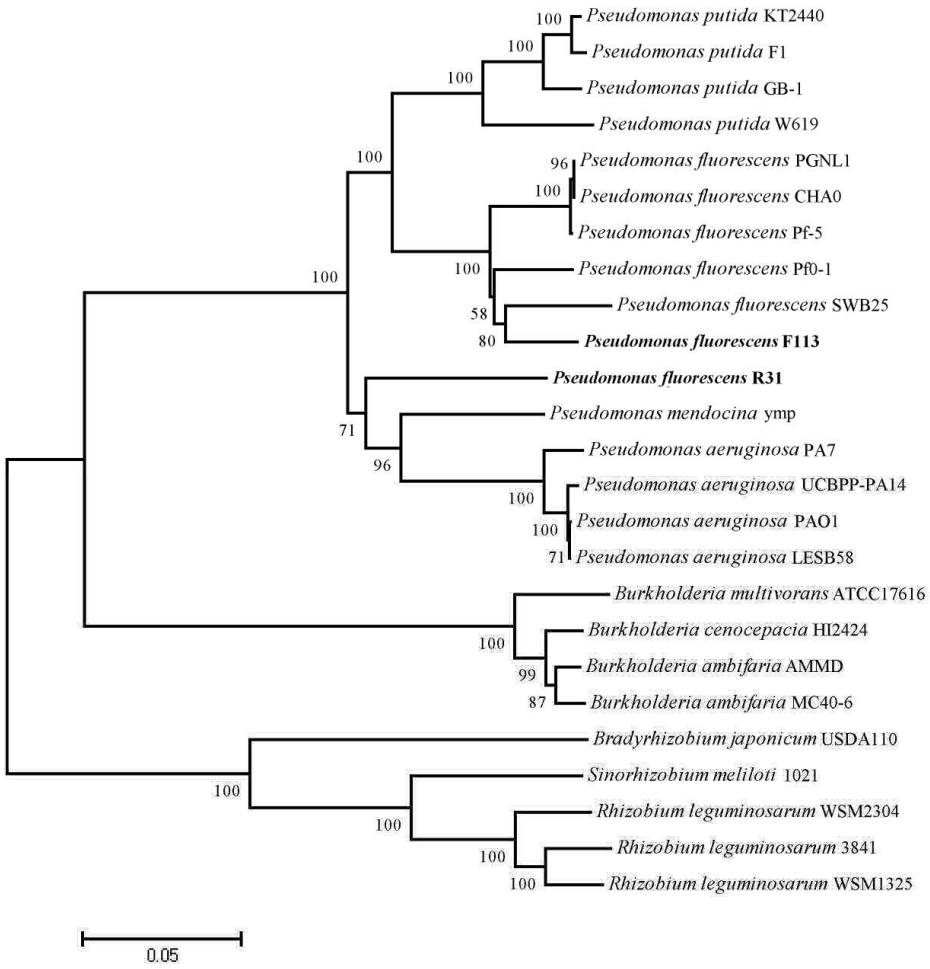


**Figure 4.6** Phylogenetic tree based on the concatenated sequence data for the genes 16S rRNA, *gyrB*, *groEL* and *rpoD* using *Burkholderia ambifaria* AMMD, MC40-6, *Burkholderia cenocepacia* HI2424, *Burkholderia multivorans* ATCC17616, *Bradyrhizobium japonicum* USDA110, *Sinorhizobium meliloti* 1021, *Rhizobium leguminosarum* 3814, WSM1325 and WSM2304 as outgroup inferred by the neighbor-joining. Bootstraps of 1,000 replicates were used and shown at the branch nodes on the phylogenetic tree. Bar, 0.05% estimated substitutions.

### 4.4.3 Detection of secondary metabolites

Most biocontrol strains of *Pseudomonas* spp. with a proven effect in plant bioassays produce one or several antibiotic compounds. Pyoluteorin is composed of a resorcinol ring, derived through polyketide biosynthesis (Nowak-Thompson et al., 1997). It is an antibiotic that inhibits oomycete fungi, including the plant pathogen *Pythium ultimum*, and suppresses plant diseases caused by this fungus (Howell and Stipanovic, 1980). The pyoluteorin biosynthetic gene cluster *pltLABCDEFG* is required for pyoluteorin biosynthesis (Nowak-Thompson et al., 1999). In this experiment, strain R31 was screened for the presence of pyoluteorin. *P. fluorescens* Pf-5 and CHA0 were used to be the positive control. The PCR result showed no PCR product was amplified from strain R31 with the PltBf2 and PltBr primers that amplified the predicted 773-bp fragment for detection of the pyoluteorin biosynthetic locus of *pltB* (Figure 4.7).

Pyrrolnitrin is a secondary metabolite derived from tryptophan and has strong antifungal activity (Zhou et al., 1992). DNA region confers the ability to produce pyrrolnitrin contains four genes, *prnABCD*, each of which is required for pyrrolnitrin production. Pyrrolnitrin has been described as an inhibitor of fungal respiratory chains (Tripathi and Gottlieb, 1969) and synthetic analogues of pyrrolnitrin have been developed for use as agricultural fungicides (Ligon et al., 2000). In this experiment, strain R31 was screened for the presence of pyrrolnitrin. *P. fluorescens* Pf-5 and CHA0 were used to be the positive control. The PCR result showed no PCR product was amplified from strain R31 with the PrnCf and PrnCr primers that amplified the predicted 719-bp fragment of *prnC* (Figure 4.7).

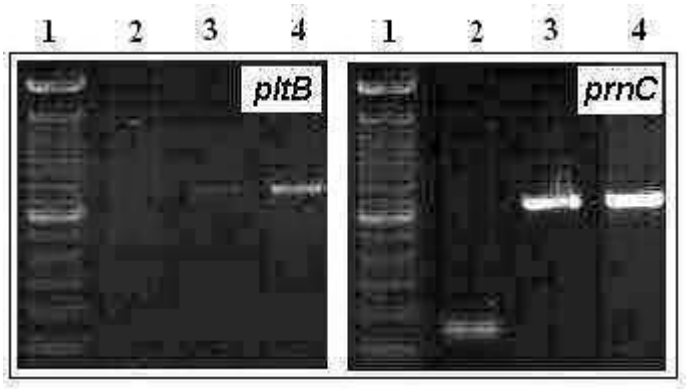


**Figure 4.7** PCR analysis of genes involved in pyoluteorin (*pltB*) and pyrrolnitrin (*prnC*) production. Lane 1, 100 bp ladder (Invitrogen); lane 2, R31; lane 3, Pf-5; lane 4, CHA0.

DAPG is the best-known phloroglucinol compound in a family of related molecules that includes MAPG and uncharacterized condensation products of DAPG and MAPG (Heeb et al., 2002). DAPG causes membrane damage to *Pythium* spp. and is particularly inhibitory to zoospores of this oomycete (de Souza et al., 2003). In this experiment, strain R31 was screened for the presence of DAPG. *P. fluorescens* Pf-5, CHA0, and F113 were used to be the positive control. The result showed positive amplification PCR product was revealed by strain R31 using primers Phl2a and Phl2b that amplified the predicted 745-bp fragment of *phlD* (Figure 4.8) and primers phlA-1f and phlA-1r that amplified the predicted 418-bp fragment (Figure 4.8).

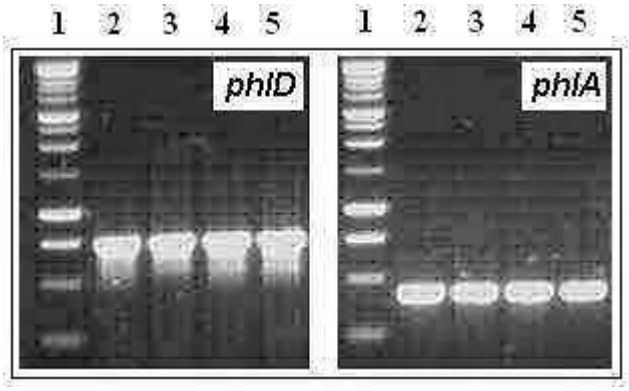


**Figure 4.8** PCR analysis of genes involved in DAPG production (*phlD* and *phlA*). Lane 1, 1 kb ladder (Promega); lane 2, R31; lane 3, Pf-5; lane 4, CHA0; lane 5, F113.

HCN is a volatile compound which plays a role in biological control of some soilborne diseases (Haas and Defago, 2005). The cyanide ion derived from HCN is a potent inhibitor of many metalloenzymes, especially copper-containing cytochrome *c* oxidases (Blumer and Haas, 2000). In this experiment, strain R31 was screened for the presence of HCN. *P. fluorescens* Pf-5 and F113 were used to be the positive control for HCN detection. Strain R31 showed negative result of hydrogen cyanide production (Figure 4.9).

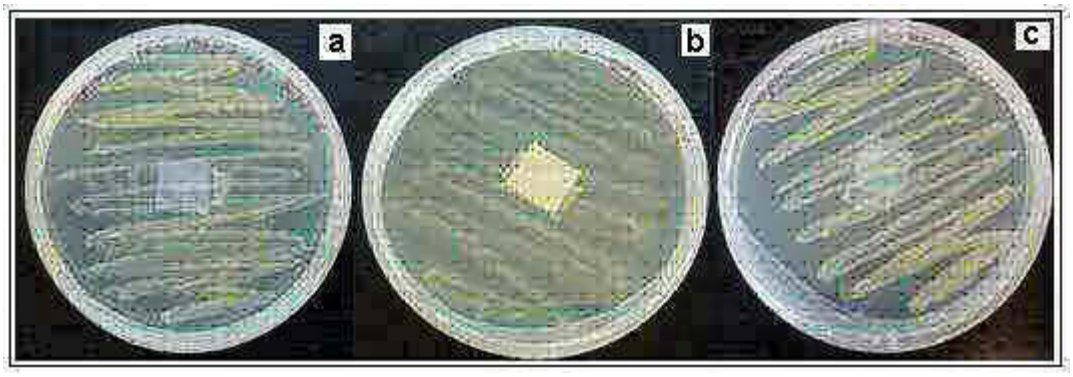


**Figure 4.9** Hydrogen cyanide production assay of strains Pf-5 (a), R31 (b), and F113 (c).

### 4.4.4 Phylogenetic tree of the *phlD* gene

In order to investigate the relationship among *phl*-positive *P*seudomonas isolates including strain R31, a phylogenetic tree using *phlD* sequence was constructed (Figure 4.10). The examination of the *phl*-positive strains on *phlD* gene tree showed four major lineages. The first lineage comprises 12 strains related to strain F113; the second lineage comprises two strains, Q2-1 and Q1-87; the third consists of strain R31 and KH-1; and the fourth comprises five isolates related to strain Pf-5. Interestingly, strain R31, which was isolated from rice rhizosphere in Thailand, was the most closely related to KH-1, which was isolated from rice rhizosphere in India, among the four clades. To determine the homology of phlD within *phl*-positive *Pseudomonas* isolates, the sequence alignment of the translated amino acid sequences of *phlD* gene of the representative of each clade (F113, Pf-5, Q2-1, KH-1) was aligned (Figure 4.11). This finding indicated that there is a highly conserved sequence in this gene. The highest pairwise alignment score with strain R31 is strain KH-1 (95% identities) (Figure 4.12), strain Pf-5 (85% identities) (Figure

4.13), strain F113 (79% identities) (Figure 4.14), and strain Q2-1 (79% identities) (Figure 4.15), respectively.

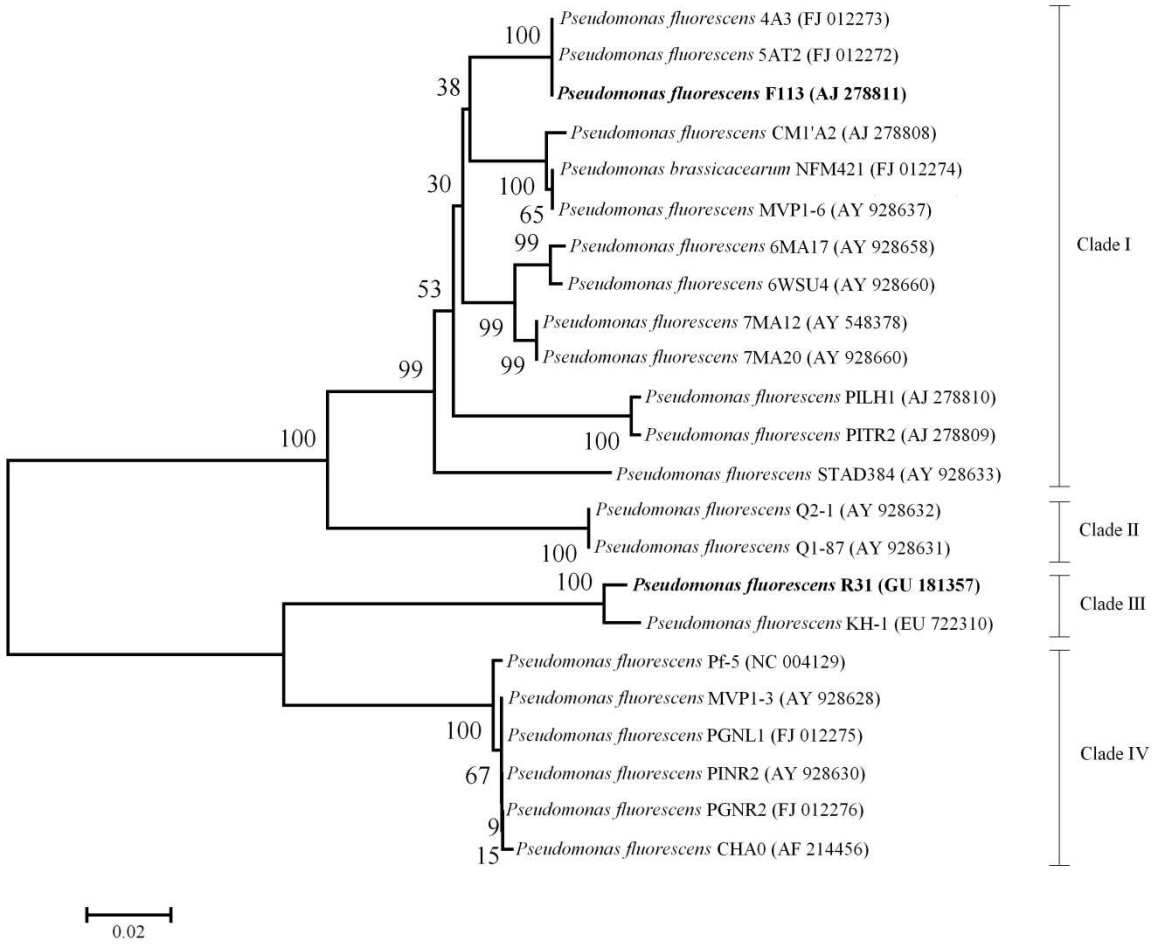


**Figure 4.10** Phylogenetic tree of *phlD* gene consisting of 23 *phl*-positive *Pseudomonas* strains inferred by the neighbor-joining. Bootstraps of 1,000 replicates were used and shown at the branch nodes on the phylogenetic tree.


**Figure 4.11** Alignment of the protein sequences of the *phlD* gene identified in *P. fluorescens* Pf-5, F113, Q2-1, and KH-1 lineages.

```
Query  1    SLAAARQALDNAGLEPEDVRMVIVTSCTGFMMPSLTAHLINDLGLPNSTIQLPIAQLGCV  60
            S AAARQA DNAGL+PEDVRMVIVTSCTGFMMPSLTAHLINDLGLPNSTIQLPIAQLGCV
Sbjct  80   SSAAARQAPDNAGLKPEDVRMVIVTSCTGFMMPSLTAHLINDLGLPNSTIQLPIAQLGCV  139

Query  61   AGAAAINRAHDFATLKADNHVLIVSLEFSSLCYQPDDTKLHSFISAALFGDAVSACVLRA  120
            AGAAAINRAHDFATLKADNHVLIVSLEFSSLCYQPDDTKLHSFISAALFGDAVSACVLRA
Sbjct  140  AGAAAINRAHDFATLKADNHVLIVSLEFSSLCYQPDDTKLHSFISAALFGDAVSACVLRA  199

Query  121  DDKAKGFRIKATDSFFLPKSEHFIKYDVKDTGFHFTLDKAVMNSIKDVAPVIERLNQAGY  180
            DD+AKGFRIKATDSFFLPKSEHFIKYDVKDTGFHFTLDKAVMNSIKDVAPVIERLNQAGY
Sbjct  200  DDEAKGFRIKATDSFFLPKSEHFIKYDVKDTGFHFTLDKAVMNSIKDVAPVIERLNQAGY  259

Query  181  QQNCAQNDFFIFHTGGRKILDELVRHLDLPSDRVLALAHQSL  222
            QQNCAQNDFFIFHTGGRKILDELVRHLDLPS+RV +L+  SL
Sbjct  260  QQNCAQNDFFIFHTGGRKILDELVRHLDLPSERV-SLSRTSL  300
```

**Figure 4.12** Blast search analysis of PhlD of *P. fluorescens* R31 shows homologies with PhlD of *P. fluorescens* KH-1 with 95% identities and 97% positives. Abbreviations are: + conservative substitution amino acid, -; insertion or deletion of amino acid.

```
Query  1    MSTLCLPHVMFPQHKITQQQMVDHLENLHADHPRMALAKRMIANTEVNERHLVLPIDELA  60
            MSTLC PHV+FPQHKITQQQM++HLENLH DHPRMALAKRMI NTEVNERHLVLPIDELA
Sbjct  1    MSTLCQPHVLFPQHKITQQQMIEHLENLHGDHPRMALAKRMILNTEVNERHLVLPIDELA  60

Query  61   VHTGFTHRSIVYEREARQMSSAAARQAIENAGLQISDIRMVIVTSCTGFMMPSLTAHLIN  120
            +HTGFTHRSIVYER AR+MSSAAARQA +NAGL+  D+RMVIVTSCTGFMMPSLTAHLIN
Sbjct  61   IHTGFTHRSIVYERAAREMSSAAARQAPDNAGLKPEDVRMVIVTSCTGFMMPSLTAHLIN  120

Query  121  DLALPTSTVQLPIAQLGCVAGAAAINRANDFARLDARNHVLIVSLEFSSLCYQPDDTKLH  180
            DL LP ST+QLPIAQLGCVAGAAAINRA+DFA L A NHVLIVSLEFSSLCYQPDDTKLH
Sbjct  121  DLGLPNSTIQLPIAQLGCVAGAAAINRAHDFATLKADNHVLIVSLEFSSLCYQPDDTKLH  180

Query  181  AFISAALFGDAVSACVLRADDQAGGFKIKKTESYFLPKSEHYIKYDVKDTGFHFTLDKAV  240
            +FISAALFGDAVSACVLRADD+A GF+IK T+S+FLPKSEH+IKYDVKDTGFHFTLDKAV
Sbjct  181  SFISAALFGDAVSACVLRADDEAKGFRIKATDSFFLPKSEHFIKYDVKDTGFHFTLDKAV  240

Query  241  MNSIKDVAPVMERLNYESFEQNCAHNDFFIFHTGGRKILDELVMHLDLASNRVSQSRSSL  300
            MNSIKDVAPV+ERLN   ++QNCA NDFFIFHTGGRKILDELV HLDL S RVS SR+SL
Sbjct  241  MNSIKDVAPVIERLNQAGYQQNCAQNDFFIFHTGGRKILDELVRHLDLPSERVSLSRTSL  300

Query  301  SEAGNIASVVVFDVLKRQFDSNLNRGDIGLLAAFGPGFTAEMAVGEWTA  349
            SEAGNIASVVVFDVL+RQFDS+   G  GLLAAFGPGFTAEMA+GEW A
Sbjct  301  SEAGNIASVVVFDVLRRQFDSSPKPGSKGLLAAFGPGFTAEMALGEWAA  349
```

**Figure 4.13** Blast search analysis of PhlD of *P. fluorescens* R31 shows homologies with PhlD of *P. fluorescens* Pf-5 with 85% identities and 91% positives. Abbreviations are: + conservative substitution amino acid, -; insertion or deletion of amino acid.

```
Query  1    RSIVYEREARRMSSIAARQAIENAGLTTDDIRMVAVTSCTGFMMPSLTAHLINDLGLRTS  60
            RSIVYER AR MSS AARQA +NAGL  +D+RMV VTSCTGFMMPSLTAHLINDLGL  S
Sbjct  68   RSIVYERAAREMSSAAARQAPDNAGLKPEDVRMVIVTSCTGFMMPSLTAHLINDLGLPNS  127

Query  61   TVQLPIAQLGCVAGAAAINRANDFASRAPDNHVLIVSLEFSSLCYQPQDTKLHAFISAAL  120
            T+QLPIAQLGCVAGAAAINRA+DFA+   DNHVLIVSLEFSSLCYQP DTKLH+FISAAL
Sbjct  128  TIQLPIAQLGCVAGAAAINRAHDFATLKADNHVLIVSLEFSSLCYQPDDTKLHSFISAAL  187

Query  121  FGDAVSACVMRADDQAPGFKIAKTGSYFLPDSEHYIKYDVKDSGFHFTLDKAVMNSIKDV  180
            FGDAVSACV+RADD+A GF+I  T S+FLP SEH+IKYDVKD+GFHFTLDKAVMNSIKDV
Sbjct  188  FGDAVSACVLRADDEAKGFRIKATDSFFLPKSEHFIKYDVKDTGFHFTLDKAVMNSIKDV  247

Query  181  APMMEELNYETFNQHCAQNDFFIFHTGGRKILDELVLQLXLEPGRVAQSRDSLSEAGNIA  240
            AP++E LN   + Q+CAQNDFFIFHTGGRKILDELV  L L   RV+ SR SLSEAGNIA
Sbjct  248  APVIERLNQAGYQQNCAQNDFFIFHTGGRKILDELVRHLDLPSERVSLSRTSLSEAGNIA  307

Query  241  SVVVFHVL  248
            SVVVF VL
Sbjct  308  SVVVFDVL  315
```

**Figure 4.14** Blast search analysis of PhlD of *P. fluorescens* R31 shows homologies with PhlD of *P. fluorescens* F113 with 79% identities and 87% positives. Abbreviations are: + conservative substitution amino acid, -; insertion or deletion of amino acid.

```
Query  1    REARSMSSIAARQAIENAGLTTDDIRMVAVTSCTGFMMPSLTAHLINDLGLRTSTVQLPI  60
            R AR MSS AARQA +NAGL  +D+RMV VTSCTGFMMPSLTAHLINDLGL  ST+QLPI
Sbjct  74   RAAREMSSAAARQAPDNAGLKPEDVRMVIVTSCTGFMMPSLTAHLINDLGLPNSTIQLPI  133

Query  61   AQLGCVAGAAAINRANDFASLSPDNHALIVSLEFSSLCYQPQDTKLHAFISAALFGDAVS  120
            AQLGCVAGAAAINRA+DFA+L  DNH LIVSLEFSSLCYQP DTKLH+FISAALFGDAVS
Sbjct  134  AQLGCVAGAAAINRAHDFATLKADNHVLIVSLEFSSLCYQPDDTKLHSFISAALFGDAVS  193

Query  121  ACVMRADDKAPGFKIAKTGSYFLPDSEHYIKYDVKDSGFHFTLDKAVMNSIKDVAPMMEE  180
            ACV+RADD+A GF+I  T S+FLP SEH+IKYDVKD+GFHFTLDKAVMNSIKDVAP++E
Sbjct  194  ACVLRADDEAKGFRIKATDSFFLPKSEHFIKYDVKDTGFHFTLDKAVMNSIKDVAPVIER  253

Query  181  LNFETFNQHCAQNDFFIFHTGGRKILDELVLQLDLEPGRVAQSRDSLSEAGNIAS  235
            LN   + Q+CAQNDFFIFHTGGRKILDELV  LDL   RV+ SR SLSEAGNIAS
Sbjct  254  LNQAGYQQNCAQNDFFIFHTGGRKILDELVRHLDLPSERVSLSRTSLSEAGNIAS  308
```

**Figure 4.15** Blast search analysis of PhlD of *P. fluorescens* R31 shows homologies with PhlD of *P. fluorescens* Q2-1 with 79% identities and 87% positives. Abbreviations are: + conservative substitution amino acid, -; insertion or deletion of amino acid.

### 4.4.5 Production of 2,4-diacetylphloroglucinol over 24 hours and antagonistic activity

Prior to investigating the level of DAPG and MAPG produced by strain R31 and F113, biological control properties were carried out. Two phytopathogenic fungi, *Pythium* sp., *Fusarium* sp., and a DAPG-sensitive indicator bacterium, *B. subtilis* (Compant et al., 2005; Mavrodi et al., 2001), were used as tested strains. The antagonistic activity from both strains R31 and F113 was summarized in Table 4.4. The *in vitro* antagonism experiments with *Pythium* sp., *Fusarium* sp. and *B. subtilis* (Figure 4.16) revealed that strain F113 showed a stronger antagonistic activity than strain R31.

**Table 4.4** The measurement of diameter of fungal inhibition ring (cm) of *P. fluorescens* R31 against *Pythium* sp., *Fusarium* sp. and *B. subtilis*.

| **Pathogens** | **Diameter of fungal inhibition ring (cm)** | |
|---|---|---|
| | **R31** | **F113** |
| *Pythium* sp. | 1.4±0.05 | 1.4±0.05 |
| *Fusarium* sp. | 0 | 1.7±0.20 |
| *Bacillus subtilis* | 2.0±0.17 | 3.8±0.15 |

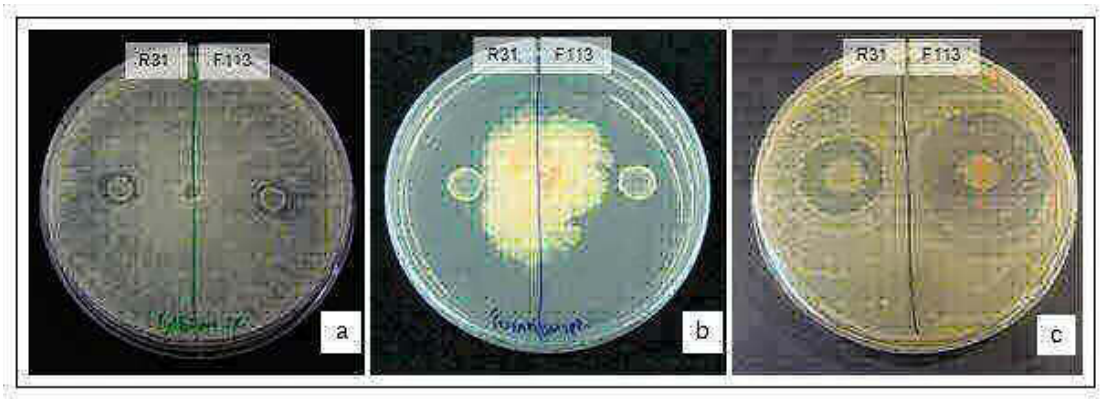


**Figure 4.16** *In vitro* antagonistic activity against *Pythium* sp. (a), *Fusarium* sp. (b) and *B. subtilis* (c) by *P. fluorescens* R31 and *P. fluorescens* F113.

Thus, quantification of DAPG was further studied by high-pressure liquid chromatography analysis. When strain F113 and strain R31 were grown in SA minimal medium, extracellular DAPG production over the 24 h of growth was significantly higher in strain F113 when compared to strain R31 (Figure 4.17). The amounts of extracellular DAPG production by strain F113 ranged from 3.48 to 49.64 mg/l whilst the amounts of extracellular DAPG production by strain R31 ranged from 0.36 to 7.66 mg/l (Figure 4.17). The highest concentration of extracellular DAPG was found at 16 hours for both strains, thereafter, extracellular DAPG concentrations in the medium were steadily decreased.

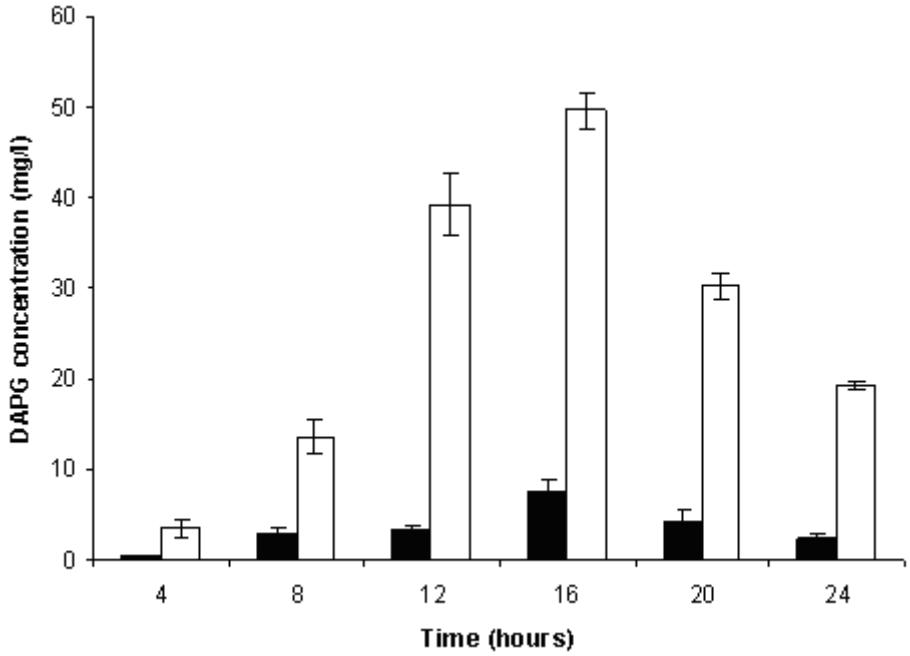


**Figure 4.17** Time course of extracellular DAPG production. Samples from cultures of *P. fluorescens* R31 (Black) and *P. fluorescens* F113 (white) grown in SA minimal medium were taken at the indicated times and DAPG concentration was quantified by HPLC. Means ± the standard deviations from three independent experiments are shown.

Besides the investigation of DAPG production, its precursor, MAPG, was also investigated to determine the relation of these two phloroglucinols in both strains (Figure 4.18). The production level of extracellular MAPG in strain R31 was lower than in strain F113. The amounts of extracellular MAPG in strain R31 was lower than 1 mg/l at any of the time points.

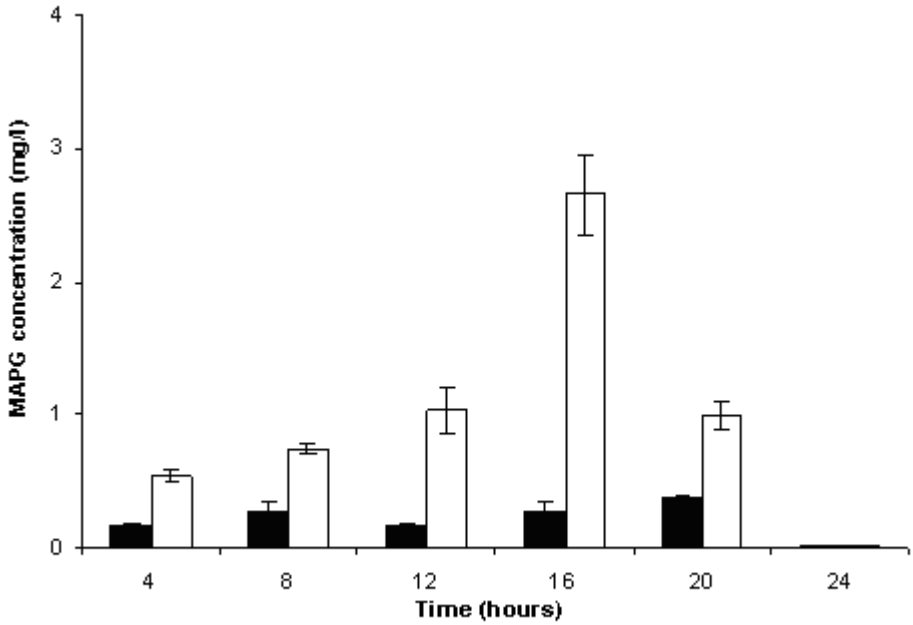


**Figure 4.18** Time course of extracellular MAPG production. Samples from cultures of *P. fluorescens* R31 (Black) and *P. fluorescens* F113 (white) grown in SA minimal medium were taken at the indicated times and DAPG concentration was quantified by HPLC. Means ± the standard deviations from three independent experiments are shown.

To investigate whether MAPG and DAPG were accumulated inside the cell, a time-course experiment monitoring the production of cell-associated phloroglucinols (DAPG and MAPG) in strain R31 and strain F113 (Figure 4.19 and 4.20) was performed.

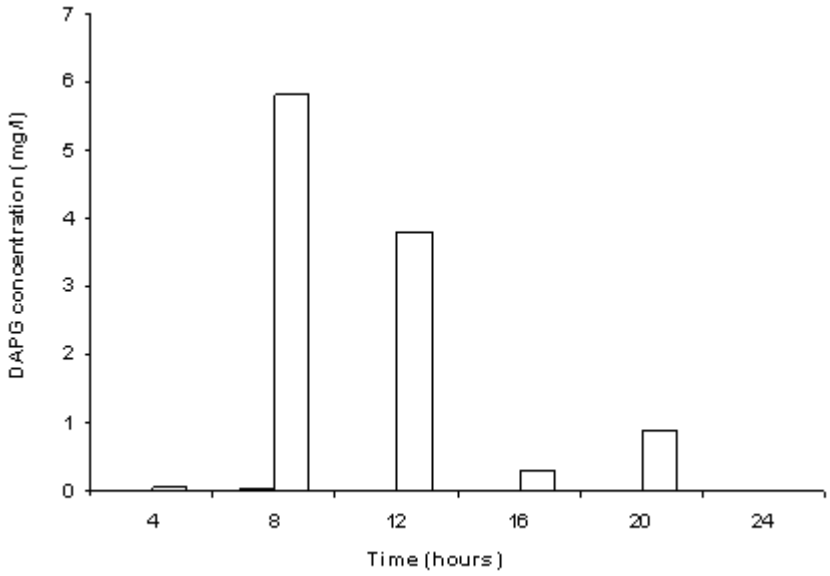


**Figure 4.19** Time course of cell-associated DAPG production. Samples from cultures of *P. fluorescens* R31 (Black) and *P. fluorescens* F113 (white) grown in SA minimal medium were taken at the indicated times and DAPG concentration was quantified by HPLC. Means ± the standard deviations from three independent experiments are shown.

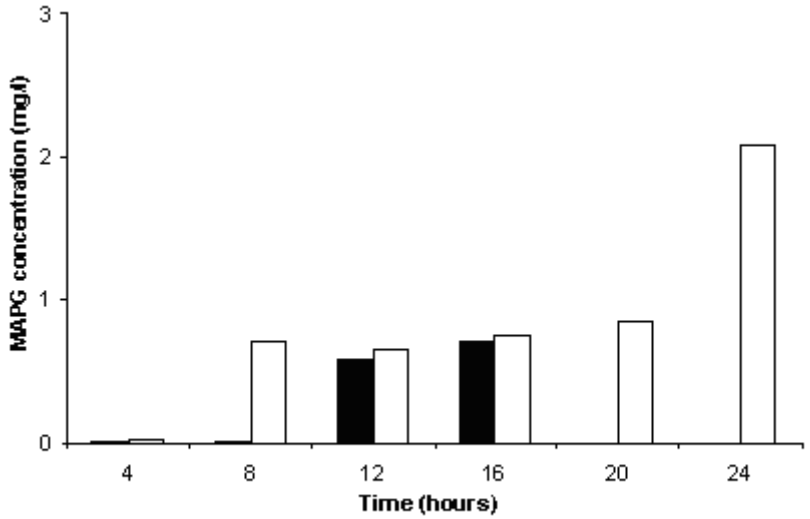


**Figure 4.20** Time course of cell-associated MAPG production. Samples from cultures of *P. fluorescens* R31 (Black) and *P. fluorescens* F113 (white) grown in SA minimal medium were taken at the indicated times and DAPG concentration was quantified by HPLC. Means ± the standard deviations from three independent experiments are shown.

The results showed that cell-associated DAPG produced by strain R31 was very low. The amount of cell-associated DAPG produced by strain R31 was not higher than 0.02 mg/l, while the amount of cell-associated DAPG produced by strain F113 reached 5.8 mg/l (Figure 4.19). In addition, the amounts of cell-associated MAPG produced by both strains were likely the same amount at 12 and 16 hours (Figure 4.20), implying that these two strains should produce a similar amount of extracellular DAPG at 12 and 16 hours. However, in fact, the result of extracellular DAPG produced by strain F113 showed 5 folds higher than strain R31. Interestingly,

the pattern of extracellular DAPG production by strain R31 was more similar to that by strain F113 than strain Pf-5. The highest DAPG concentration produced by strain R31 and F113 was found at 16 hours and then steadily declined (Delany et al., 2000), but the highest DAPG concentration was detected at 72 hours for Pf-5 (Hultberg and Alsanius, 2008). After 24 hours, DAPG concentration produced by strain Pf-5 was 11.0 mg/l and the concentration increased during the following 48 hours to 100.7 mg/l (Hultberg and Alsanius, 2008), while the maximum concentration of DAPG recovered by strain F113 was 49.6 mg/l, and by strain R31 was 7.7 mg/l at 16 h growth and then steadily declined.

### 4.4.6 The effect of carbon sources on DAPG production

The effect of various carbon sources on DAPG production by both of strains R31 and F113 was determined. The results indicated that different carbon sources influenced the production of DAPG by both strains. DAPG produced by strain F113 was higher than that by strain R31 in any carbon sources (Figure 4.21 and 4.22). The amount of DAPG produced by strain F113 ranged from 0.6 to 64 mg/l, whilst strain R31 produced DAPG level less than 0.25 mg/l in any carbon sources. The amount of DAPG produced by strain R31 ranged from 0.01 to 0.24 mg/l. Succinate was the best carbon source for strain R31 to produce DAPG which was 0.24 mg/l. On the other hand, sucrose was the best carbon source for strain F113 to produce DAPG which was 63.77 mg/l. Moreover, the presence of glucose and fructose (0.005 mg/l and 0.009 mg/l, respectively) promoted low yields of DAPG produced by strain R31, whereas low yield of DAPG was observed in succinate and glycerol (0.63/1.27 mg/l, respectively) by strain F113. DAPG produced by strain F113 was enhanced by

sucrose and depressed by succinate. This result supported a previous study by Shanahan et al., 1992. Whereas nutrient broth amended with 2% glucose (NBglu) has been previously shown to be superior for DAPG production by strain Pf-5 (Nowak-Thompson et al., 1994) and in strain CHA0, DAPG production was dramatic increased when glucose was added to dilute NBY broth (Duffy and Defago, 1999) and in strain S272, highest DAPG yield was obtained with ethanol as the sole source of carbon (Yuan et al., 1998). In general, DAPG is synthesized by condensation of three molecules of acetyl-CoA with one molecule of malonyl-CoA to produce the precursor, MAPG, which is subsequently transacetylated to generate DAPG (Bangera and Thomashow, 1999; Dwivedi and Johri, 2003). However, an alternative route to synthesize phloroglucinol in a single microbe-catalyzed step from glucose was described (Achkar et al., 2005), thus it is possible that the bacteria can convert the other carbon sources to malonyl-CoA and synthesize phloroglucinol.

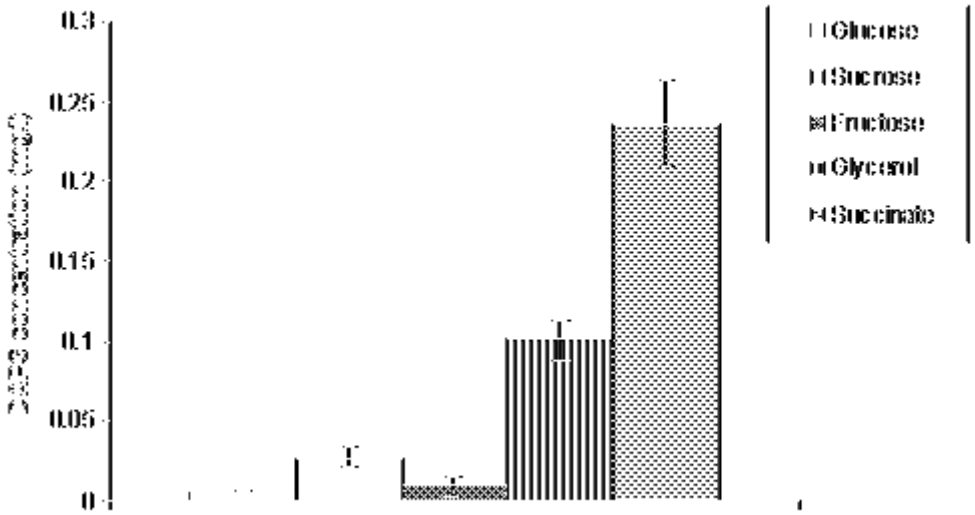


**Figure 4.21** Effect of carbon sources on DAPG production by *P. fluorescens* R31. Samples were grown in M9 minimal medium with different carbon sources (glucose, sucrose, fructose, glycerol and succinate) and were taken at 16 hours after inoculation and DAPG concentration was quantified by HPLC. Means± the standard deviations from three independent experiments are shown.

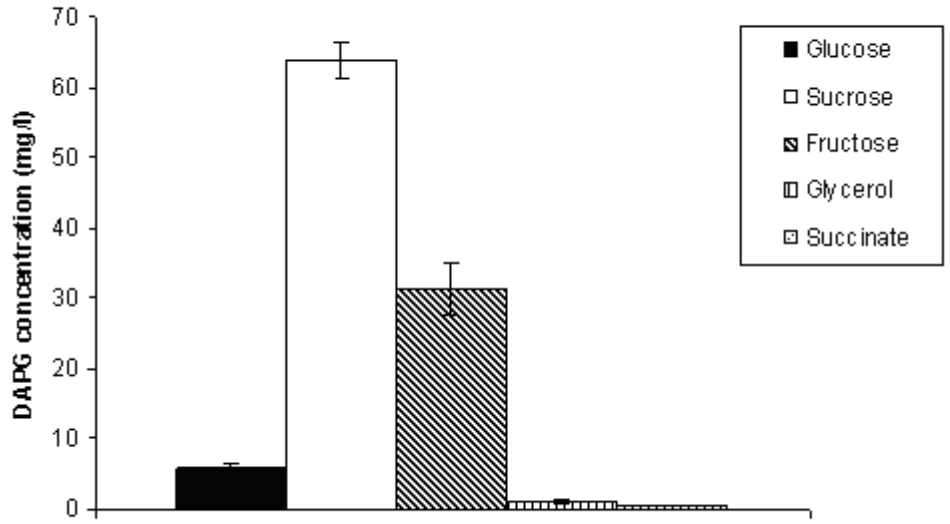


**Figure 4.22** Effect of carbon sources on DAPG production by *P. fluorescens* F113. Samples were grown in M9 minimal medium with different carbon sources (glucose, sucrose, fructose, glycerol and succinate) and were taken at 16 hours after inoculation and DAPG concentration was quantified by HPLC. Means± the standard deviations from three independent experiments are shown.

### 4.4.7 The *phlA* expression in strains F113 and R31

A time course experiment monitoring the expression of *phlA* was performed using *gfp* transcriptional fusions. Strain F113 and strain R31 containing the *phlA-gfp* transcriptional fusion carried by pPROBE-GT were grown independently on SA minimal medium and analyzed by the emission level of green fluorescence (Figure 4.23). The *phlA–gfp* fusion in strain F113 gradually increased the expression level during the exponential and early stationary growth phases reaching maximum

levels of almost 25,000 RFU/$OD_{600nm}$. In strain R31, *phlA* expression was lower than in strain F113 at any of the time points and reached maximum levels of almost 12,500 RFU/$OD_{600nm}$ likewise the *phlA–gfp* fusion in strain CHA0, reaching maximum levels of 30,000 RFU at $OD_{600nm}$ = 1 (Baehler et al., 2005). On the other hand, strain R31 showed two-times lower of *phlA-gfp* expression when compared to strain F113 over 24 hours at $OD_{600nm}$ = 1 in SA minimal medium. This result confirms our previous finding of the quality of DAPG by *Bacillus* assay and quantity of DAPG by HPLC of strain R31 that showed the lower level when compared to strain F113. The level of expression of *phlA* of strain R31 in comparison with strain F113 was confirmed by RT-PCR. As shown in Figure 8b, the *phlA* was expressed, although at high level in strain F113, whereas low expression of *phlA* of strain R31 was detected. These data also showed similar to the time course experiment monitoring the expression of *phlA* that was performed using *gfp* transcriptional fusions result (Figure 4.24).

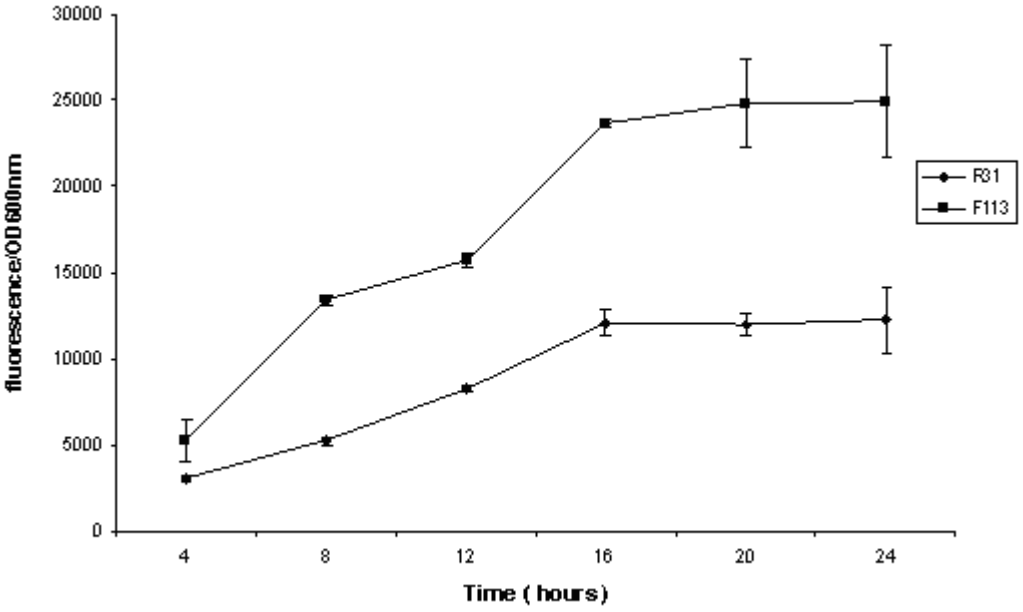


**Figure 4.23** Time course of expression of the *phl* operon. The transcriptional fusions *phlA-gfp* (pPROBE-GT) was introduced into *P. fluorescens* R31 (black) and *P. fluorescens* F113 (white). Cultures were grown on SA minimal medium. Expression of the fusions was assessed by measuring emission levels of green fluorescence. Triplicate cultures were assayed. The standard deviations are represented with error bars.

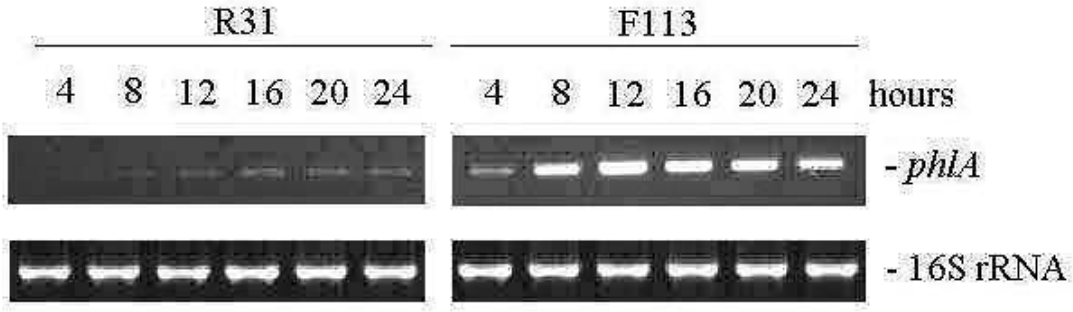


**Figure 4.24** Expression analysis of the *phlA* promoter. Total RNAs from *P. fluorescens* R31 and *P. fluorescens* F113 at 4, 8, 12, 16, 20 and 24 hours after inoculation in SA medium were subjected to RT-PCR with primers to amplify the *phlA* fragment. The 16S rRNA RT-PCR was used as a control.

### 4.4.8 The effect of carbon sources on *phlA-gfp* expression

To investigate the effect of carbon sources on *phlA* expression and to determine the relationship between DAPG production and *phlA* expression, strain F113 and strain R31 containing the *phlA-gfp* transcriptional fusion carried by pPROBE-GT were grown independently on M9 minimal medium supplemented with different carbon sources, and analyzed by the emission level of green fluorescence. As shown in Figure 4.25, the *phlA-gfp* expression by strain R31 was still lower than that by strain F113 in any type of carbon sources. Moreover, the *phlA-gfp* expression in strain F113 was high in the presence of sucrose and succinate. On the other hand, *phlA-gfp* expression in strain R31 was high in the presence of succinate and fructose. Shanahan et al., 1992 found that DAPG production increased in the presence of sucrose, fructose, and mannitol, whereas yields of DAPG were reduced when cells

were incubated in glucose or succinate. However, the result of *phlA-gfp* expression in figure 2.5 showed the *phlA-gfp* expression in M9 minimal medium supplemented with succinate by strain F113 was a second highest among the other carbon sources except for sucrose at 16, 20, and 24 hours. This result was consistent with (Baehler et al., 2005). They found that the expression of the *phlA-gfp* fusions may not always be affected by the lack of DAPG, as *phlA* expression in mutant strain did not significantly differ from expression of this gene in the wild-type. Besides the investigation of *phlA-gfp* expression, growth curves of both strains were determined (Table 4.5 and Figure 4.26).

In Table 4.5, strain R31 showed better growth when compared to strain F113 in any carbon sources. In the presence of sucrose, strain R31 reached maximum growth at $OD_{600nm}$ = 1, while strain F113 reached maximum growth at $OD_{600nm}$ = 0.84 at 24 hours. In the presence of glucose, strain R31 reached maximum growth at $OD_{600nm}$ = 0.85 while strain F113 reached maximum growth at $OD_{600nm}$ = 0.59 at 24 hours. In the presence of fructose, strain R31 reached maximum growth at $OD_{600nm}$ = 0.58 whilst strain F113 reached maximum growth at $OD_{600nm}$ = 0.51 at 24 hours. In the presence of glycerol, strain R31 reached maximum growth at $OD_{600nm}$ = 0.83 while strain F113 reached maximum growth at $OD_{600nm}$ = 0.6 at 24 hours. In the presence of succinate, strain R31 reached maximum growth at $OD_{600nm}$ = 0.53 while strain F113 reached maximum growth at $OD_{600nm}$ = 0.43 at 8 hours. Secondary metabolism is brought on by exhaustion of a nutrient, biosynthesis or addition of an inducer, and/or by a growth rate decrease (Demain, 1998) and the growth rates and secondary metabolite production were different for the various strains (Minas et al.,

2000). Therefore, it is possible that better growth use less carbon into secondary metabolites production.

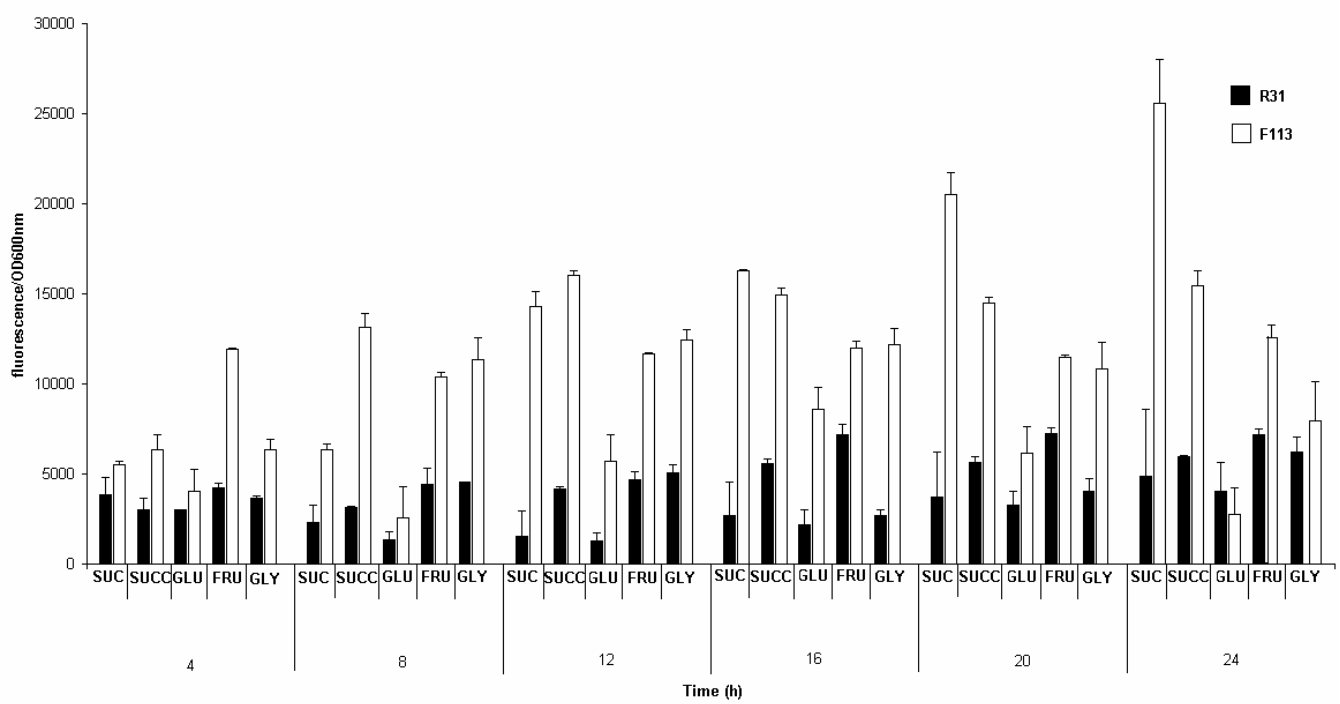


**Figure 4.25** Effect of carbon sources (glucose, sucrose, fructose, glycerol and succinate) amendment on the *phlA-gfp* expression of *P. fluorescens* R31 (black) and *P. fluorescens* F113 (white) in M9 minimal medium. Triplicate cultures were assayed. The standard deviations are represented with error bars. Abbreviations: SUC, sucrose; SUCC, succinate; FRU, fructose; GLU, glucose and GLY, glycerol. Expression of the fusions was assessed by measuring emission levels of green fuorescence.

**Table 4.5** Optical density of *P. fluorescens* strains R31 and F113**;** the absorbance was measured at 600 nm.

| **Treatment (strain, time)** | **Sucrose** | **Succinate** | **Glucose** | **Fructose** | **Glycerol** |
|---|---|---|---|---|---|
| R31, o h | 0.07±0.00 | 0.07±0.00 | 0.06±0.00 | 0.07±0.00 | 0.07±0.00 |
| R31, 4 h | 0.13±0.01 | 0.20±0.01 | 0.12±0.01 | 0.08±0.01 | 0.08±0.00 |
| R31, 8 h | 0.45±0.00 | 0.48±0.02 | 0.38±0.01 | 0.12±0.01 | 0.25±0.01 |
| R31, 12 h | 0.70±0.01 | 0.54±0.01 | 0.57±0.00 | 0.23±0.01 | 0.45±0.01 |
| R31, 16 h | 0.85±0.01 | 0.49±0.01 | 0.71±0.01 | 0.35±0.01 | 0.63±0.01 |
| R31, 20 h | 0.94±0.01 | 0.45±0.01 | 0.80±0.01 | 0.48±0.01 | 0.76±0.00 |
| R31, 24 h | 0.98±0.02 | 0.40±0.01 | 0.86±0.02 | 0.58±0.02 | 0.83±0.01 |
| F113, o h | 0.07±0.00 | 0.07±0.00 | 0.07±0.00 | 0.07±0.00 | 0.07±0.00 |
| F113, 4 h | 0.08±0.01 | 0.11±0.00 | 0.07±0.00 | 0.07±0.00 | 0.05±0.00 |
| F113, 8 h | 0.30±0.00 | 0.39±0.01 | 0.18±0.02 | 0.11±0.01 | 0.09±0.01 |
| F113, 12 h | 0.55±0.01 | 0.39±0.00 | 0.40±0.01 | 0.23±0.01 | 0.28±0.01 |
| F113, 16 h | 0.72±0.04 | 0.35±0.00 | 0.51±0.01 | 0.35±0.01 | 0.45±0.01 |
| F113, 20 h | 0.81±0.03 | 0.32±0.00 | 0.58±0.01 | 0.46±0.01 | 0.55±0.01 |
| F113, 24 h | 0.84±0.05 | 0.28±0.00 | 0.61±0.01 | 0.51±0.02 | 0.60±0.00 |
| % CV | 0.86 | 2.26 | 1.028 | 1.426 | 1.021 |

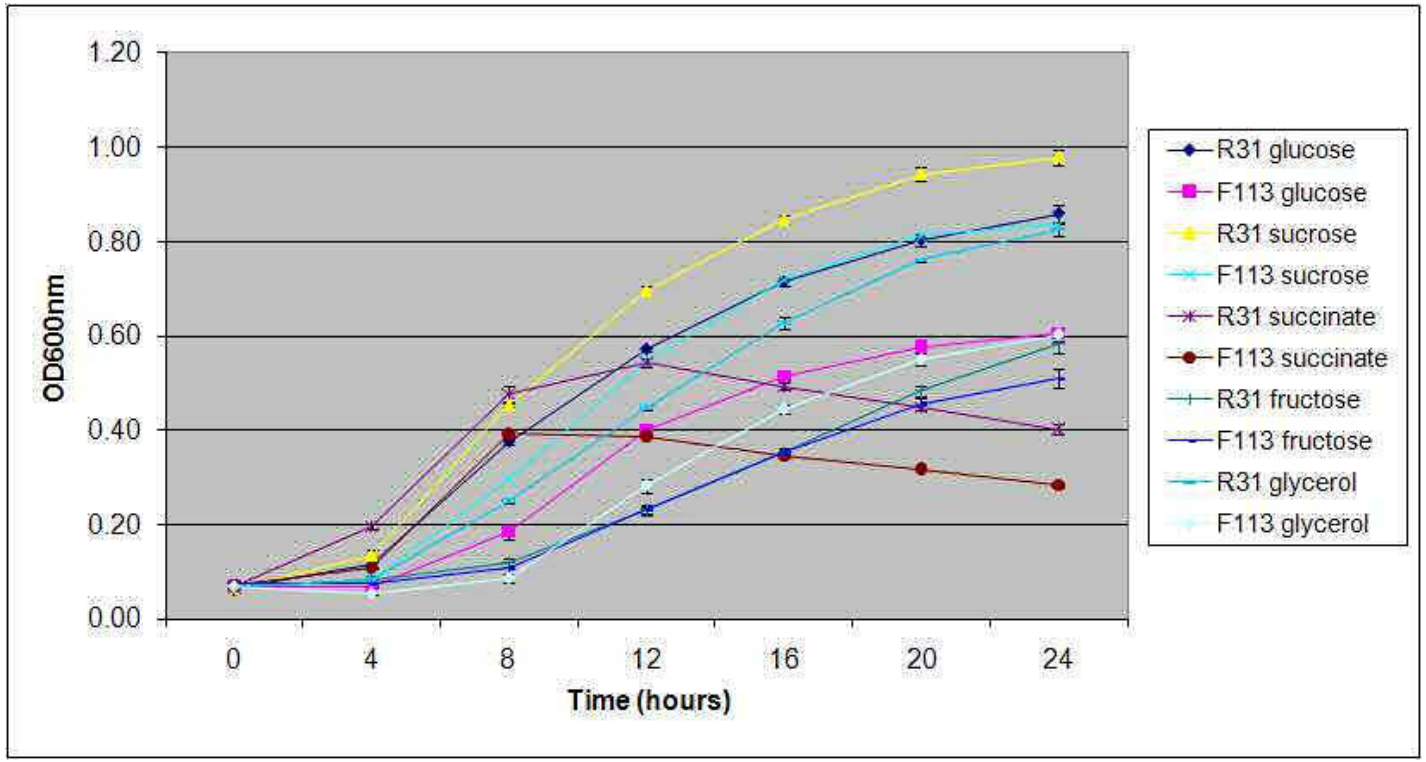


**Figure 4.26** The absorbance was measured at 600 nm. Triplicate cultures were assayed. The standard deviations are represented with error bars.

### 4.4.9 The effect of plant exudates amendment on *phlA-gfp* expression

Since the strain R31 was originally isolated from rice rhizosphere while strain F113 was isolated from sugar beet rhizosphere, therefore the effect of host plant exudates on *phlA* expression of both strains was examined. As shown in Figure 4.27, the presence of rice exudates amendment in M9 minimal medium had a strong influence on the expression of a *phlA-gfp* reporter gene fusion in strain R31 when compared to the presence of sugar beet exudates or maize exudates. Taken together, strain F113 showed higher expression of *phlA* in all plant exudates, even these exudates are from non host plants. Regarding strain R31 and F113 was isolated from the different host plant rhizosphere consequently, root exudates amendment was remarkable to examine whether root exudates are involved in DAPG production. The most obvious explanation for differences in DAPG production between plant species and crop cultivars is the differences in the quantity and/or quality of root exudates. Previous studies suggested that the production of antimicrobial compounds by biocontrol bacteria is modulated by the total concentration and the type of carbon source, nitrogen, amino acids, and other compounds found abundantly in plant root exudates but absent in bulk soil (Duffy and Defago, 1999; Fiddaman and Rossall, 1994; Milner et al., 1996; Slininger and Jackson, 1992).

This explanation supported the result in figure 4.27 which reveals that strain R31 and strain F113 provided the highest level of *phlA-gfp* expression when their host plant exudates were amended. Bais et al. (2006) reviewed that root exudates are critical to the development of associations between some parasitic plants and their hosts, and may play important indirect roles in resource competition by altering soil chemistry, soil processes, and microbial populations. This finding supports the result in this study that host cultivar had a major influence on *phlA* gene expression in strain R31 in the rice exudates amendment.

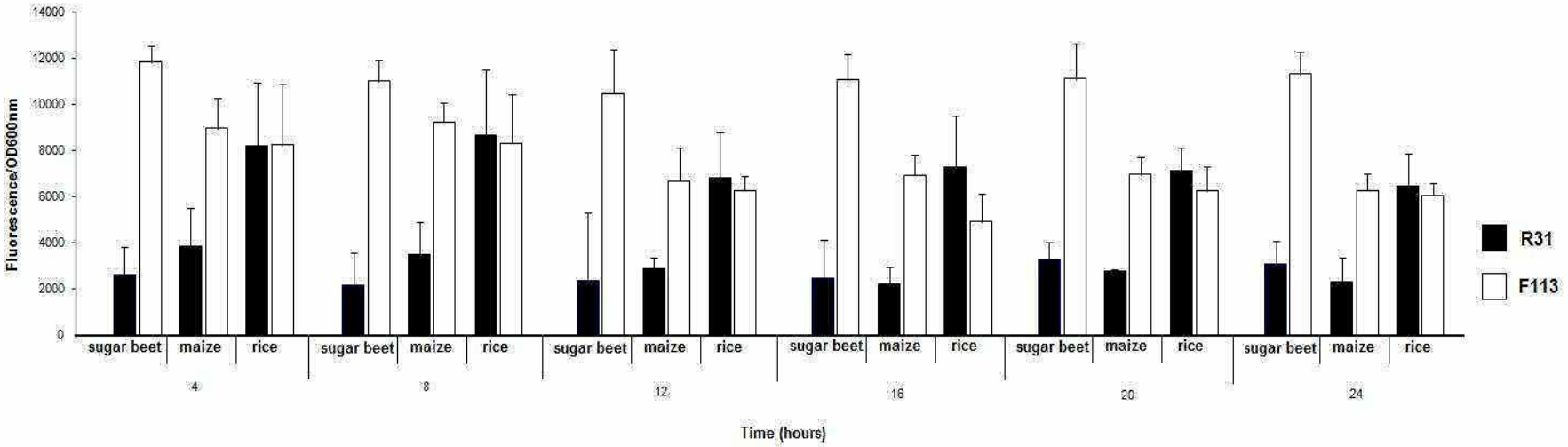


**Figure 4.27** Effect of root exudates amendment on the *phlA-gfp* expression of *P. fluorescens* R31 (black) and *P. fluorescens* F113 (white) in M9 minimal medium. Expression of the fusions was assessed by measuring emission levels of green fluorescence. Triplicate cultures were assayed. The standard deviations are represented with error bars.

### 4.4.10 Characterization of the *phl* operon and analysis of the organization of the *phlA-phlF* intergenic region of strain R31

Since gene missing might caused the low level of DAPG production, thus ST-PCR was used to determine the context of *phl* genes and investigate whether *phl* gene is missing in strain R31. The result showed *phl* genes involved in DAPG synthesis were present in strain R31 (*phlH, phlG, phlF, phlA, phlC, phlB, phlD, phlE*). The same orientation of each gene in *phl* operon of strain R31 was depicted in Figure 4.28. Although the orientation of *phl* genes in strain R31 and F113 was identical, the alignment of nucleotide sequences of *phlA-phlF* intergenic region of strain R31 compared to the other strains showed that *phlA-phlF* intergenic region of strain R31 was more similar to those of strains Pf-5 and CHA0 than strain F113. The big insert in *phlA-phlF* intergenic region of strain R31 is the same as that in strain Pf-5 and CHA0. However, these sequences were not found in strain F113. In strain R31, the intergenic region upstream of *phlA* encompasses 449 nucleotides while in strain Pf-5 and CHA0, the intergenic region upstream of *phlA* encompasses 462 nucleotides (Schnider-Keel et al., 2000), and in strain F113, the intergenic region upstream of *phlA* encompasses 417 nucleotides (Abbas et al., 2002). Moreover, the divergently oriented *phlF* gene appears to start at a GTG codon in strain R31, CHA0 (Schnider-Keel et al., 2000) and Pf-5, but the divergently oriented *phlF* gene appears to start at a ATG codon in strain F113 (Abbas et al., 2002). Detailed analysis of the strain R31 *phlA-phlF* intergenic region indicated that a putative $\sigma^{70}$ -10 element (TAGGAT) and a conserved -35 element (TTGACA) of strain R31 were identified (Figure 4.29). The sequences of the two promoter elements are completely conserved in strain F113 (AF497760) and two other strains of *P. fluorescens*, Q2-87 (U41818) and strain CHA0 (AF207529).

Moreover, the operator sequence *phO* of strain R31 was similar to strain F113. However, *phlA-phlF* intergenic region of strain R31 indicated the presence of many different nucleotides, only 34% identities between strain R31 and F113 in *phlA-phlF* intergenic region was found. The regulation of the production of secondary metabolites such as antifungals is operated in bacteria through various mechanisms acting at transcriptional and posttranscriptional levels. Besides this finding appears that *phlA* promoter activity is not a good indicator of DAPG production but, also post transcriptional regulation may be associated with the presence of low level of DAPG production by strain R31. In strain CHA0, Gac/Rsm signal transduction pathway, which operates essentially at a post-transcriptional level quorum sensing pathway is initiated by the GacS/GacA two-component system, has a major role in positively regulating biocontrol factor expression in various host-pathogen systems (Laville et al., 1992; Siddiqui et al., 2005). The importance of the GacS/GacA pathway is supported by studies on *gacS* and *gacA* mutants of *P. fluorescens* BL915 and *Pseudomonas* sp. PCL1171 which have lost biocontrol properties (Ligon et al., 1999; van den Broek et al., 2003).

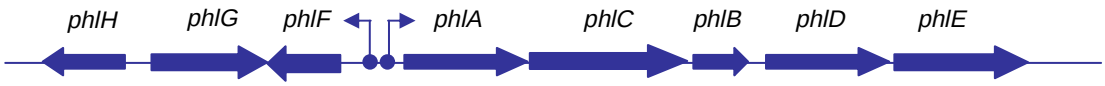


**Figure 4.28** Organization of the 2,4-diacetylphloroglucinol (DAPG) gene cluster in *P. fluorescens* R31. Arrows indicate direction of transcription.

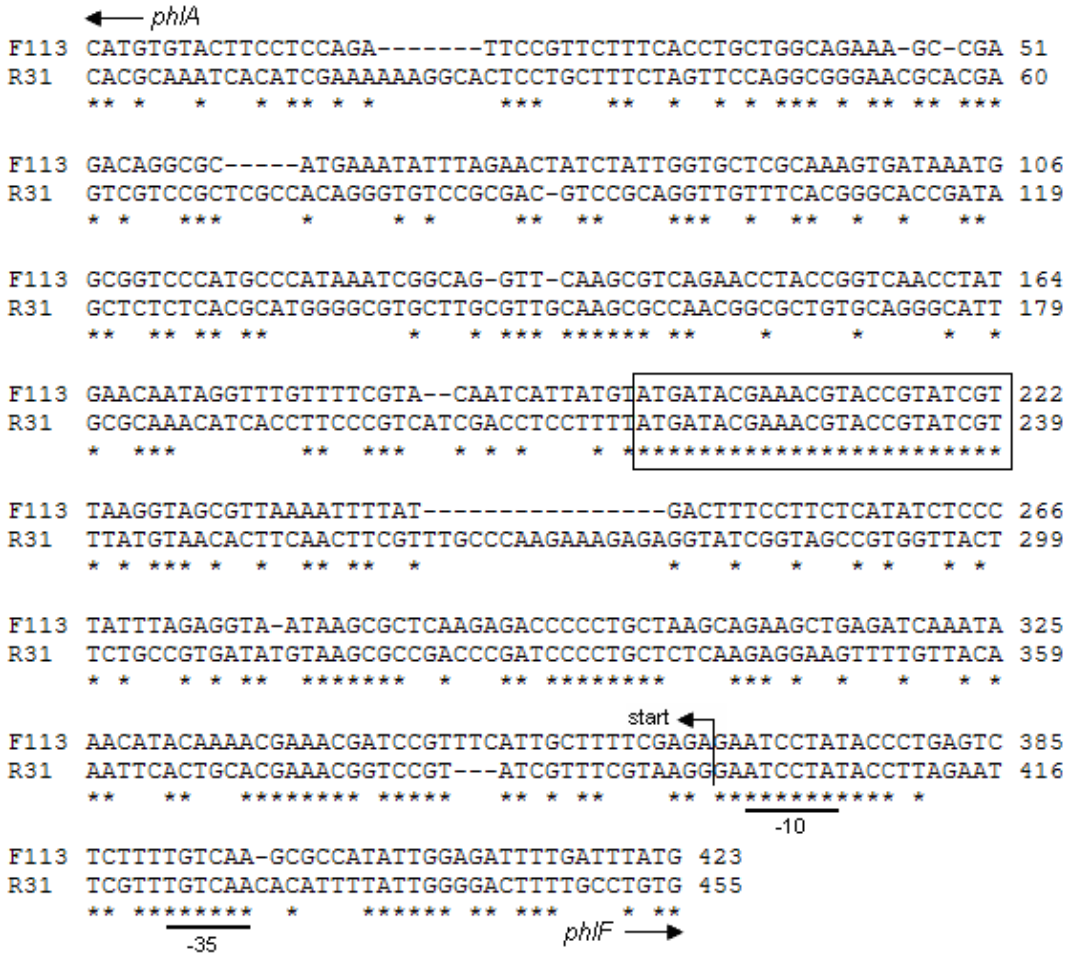


**Figure 4.29** Sequence alignment and organization of the *phlA-phlF* intergenic region. *phlA* and *phlF* indicate the start of the *phlA* and *phlF* open reading frames, respectively. Start indicates the transcriptional start of the *phlACBD* operon. The putative $\sigma^{70}$ -10 and -35 elements for *phlA* are underlined. The PhlF binding site, *phO*, is indicated in box.

### 4.4.11 The assay of *in vivo* antagonistic activity

After two weeks, rice seedling inoculated with strains R31 and F113 showed damping-off symptoms. An initial symptom was developed as lesions on sheaths of lower leaves near the water line. After 4 weeks, the disease intensity in the

treatment inoculated with *Pythium* spp. and strain R31 was 58.3 % while the disease intensity in strain F113 was 66.7 %. However, there is no significant difference in the disease intensity between applications of strain R31 and strain F113 against *Pythium* symptom (Figure 4.30 and Table 4.5). These results indicated and confirmed the result of *in vitro* antagonistic activity that the colonization of roots by *Pythium* spp. was suppressed by both strains R31 and F113. Additionally, there is no significant difference in plant height, shoots dry weight and roots dry weight of rice between applications of strain R31 and strain F113 against *Pythium* spp. Interestingly, the application of strain R31 to rice seeds showed significantly increased plant height, shoots dry weight and roots dry weight of rice when compared with control while the application of strain F113 to rice seeds showed no significant difference when compared to control.

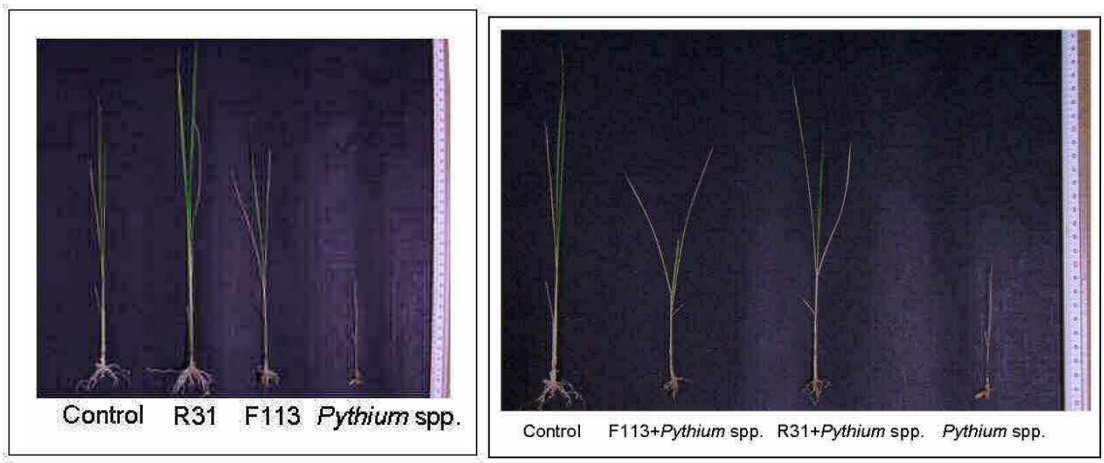


**Figure 4.30** *In vivo* antagonistic activity of *P. fluorescens* strain R31 and strain F113 against *Pythium* spp.

**Table 4.6** Disease incidence, plant height, dry weight of shoots and roots of rice inoculated with *P. fluorescens* strain R31 grown in the presence of *Pythium* spp.

| Sample | % incidence | Plant height (cm) | Shoot dry mass (mg) | Root dry mass (mg) |
|---|---|---|---|---|
| Control | 0 [c] | 21.70±1.57 [bc] | 32.47±2.50 [b] | 11.44±0.30 [b] |
| R31 | 0 [c] | 28.77±2.81 [a] | 56.57±6.14 [a] | 22.22±3.89 [a] |
| F113 | 0 [c] | 22.40±1.44 [b] | 33.00±7.05 [b] | 10.15±3.21 [b] |
| *Pythium* spp. | 100±0.00 [a] | 9.40±3.65 [d] | 7.90±0.98 [d] | 2.17±0.75 [c] |
| R31 + *Pythium* spp. | 58.3±14.43 [b] | 18.77±2.85 [bc] | 23.57±5.95 [c] | 5.56±0.70 [c] |
| F113 + *Pythium* spp. | 66.7±14.43 [b] | 17.67±1.59 [c] | 22.57±1.05 [c] | 5.82±0.98 [c] |
| % CV | 22.22 | 12.46 | 15.94 | 20.36 |

* Means in columns followed by different letters are significantly different at 5 % level according to the Duncan's Multiple Range Test (DMRT)

Root exudates represent an important source of nutrients for microorganisms in the rhizosphere and seem to participate in early colonization inducing chemotactic responses of rhizospheric bacteria. Moreover, anti-microbial and secondary metabolite production by colonising bacteria has been shown to be influenced by certain carbon sources. (James Jr and Gutterson, 1986) found that production of an antimicrobial metabolite by *Pseudomonas fluorescens* HV37a required the presence of glucose but that production of two other biologically active metabolites by the same strain was inhibited by glucose. When Shanahan et al., 1992 investigated the influence of different carbon sources on the production of DAPG, it was found that DAPG production increased in the presence of fructose, sucrose and mannitol,

whereas yields of DAPG were reduced when cells were incubated in glucose or succinate. A number of studies showed that the composition of plant root exudate varies with plant species, and with other extrinsic factors such as soil type and available nutrients in soil (Aulakh et al., 2001; Bacilio-Jimenez et al., 2003; Casey et al., 1998; Kerdchoechuen, 2005). These exudates are readily available as nutrients for microorganisms and are considered to be one of the main reasons for increased microbial numbers and activity in the rhizosphere. This study indicated that the composition and concentration of sugars and amino acids from rice root exudates, exerts great attraction for indigenous strains and root exudate is major determinant which organize colonization of bacteria at root surface. The development of the indigenous bacteria that isolated from their host plant is an important criterion for production a good quality of the biocontrol inoculants.

## 4.5 Conclusion

Introduction of biocontrol agent to agriculture requires appropriate and compatible PGPR for the goal of making agriculture more sustainable. Moreover, an understanding of how biocontrol bacteria regulate the expression of genes involved in the inhibition of pathogens is important for predicting the optimum environmental conditions of the bacteria to produce antagonistic compounds. In an attempt to understand the interaction of *Pseudomonas*-host in biocontrol of phytopathogens, *Pseudomonas* strains were isolated from rice and maize rhizospheres at Nakhon Ratchasima, north-eastern region of Thailand. One hundred and thirty eight isolates were screened for DAPG-producing *Pseudomonas*. The strain R31 that was isolated from rice rhizosphere was selected for isolation of genes for DAPG production. The

characterization of this isolate using full-length 16S rRNA gene sequencing and biological assay demonstrated that it belonged to the species *P. fluorescens*.

The result of DAPG production using HPLC showed that the ability to produce DAPG by *P. fluorescens* strain F113 was higher than by *P. fluorescens* strain R31, which is similar to the *phlA-gfp* expression. However, the same orientation of each gene in *phl* operon of strain R31 was found, whereas big insert in *phlA-phlF* intergenic region of strain R31 was discovered. The *phlD* tree suggested that *P. fluorescens* strain R31 was the most closely related to *P. fluorescens* strain KH-1 that was isolated from rice rhizosphere in India. Nevertheless, the housekeeping genes tree indicated that *P. fluorescens* strain R31 was more closely related to the other *Pseudomonas* species than species *P. fluorescens*. This information suggested that the horizontal gene transfer may be associated with the presence of *phlD* gene in strain R31 including the presence of low level of DAPG production.

Interestingly, the host cultivar had a major influence on *phlA* gene expression in strain R31 in the rice exudates amendment. Moreover, the results of *in vitro* and *in vivo* antagonistic activity showed that the colonization of rice roots by *Pythium* spp. that caused damping-off and root rot was suppressed by strain R31 and F113. The application of strain R31 to rice seeds showed significantly increased plant height, shoots dry weight and roots dry weight of rice when compared with control while the application of strain F113 to rice seeds showed no significant difference when compared to control. This information indicated that the ability to colonize rice roots is variable between rhizobacteria, being these characteristics a reflection for their ability to compete for ecological niches in the rhizosphere. Moreover, the exertion an

appropriate biological control of soil borne fungi relies on their ability to colonize roots efficiently, otherwise, their biocontrol character would be unusable.

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# CHAPTER V

# OVERALL CONCLUSIONS

Plant species can have an effect on the diversity of the root-associated microflora. This experiment provides the evidence that the amplified rDNA restriction analysis (ARDRA) can typically estimated the bacterial genotypic diversity of *Pseudomonas* spp. that was isolates from different plant species. The result indicated that the rhizospheres of rice and maize clearly selected from different soil bacterial ribotypes by ARDRA. The effect of plant species on microbial communities and on populations of bacterial genera has been documented. For example, the composition of populations of *Pseudomonas* spp. may vary significantly within the rhizosphere of different plant species cultivated in the same soil. This experiment provides the evidence that plant species variation was shown to significantly influence the community structure of *Pseudomonas.*

Furthermore, the effect of plant species on the activity of indigenous bacterial populations that share a specific antagonistic trait, such as the DAPG-producing *Pseudomonas* spp. has addressed in this study. The ability to produce DAPG by *P. fluorescens* strain R31 that was isolated from rice rhizosphere in Thailand was lower than by *P. fluorescens* strain F113 that was isolated from sugar beet rhizosphere in Ireland, which is similar to the *phlA-gfp* expression. However, the same orientation of each gene in *phl* operon of strain R31 was found.

This information indicated that it appears that *phlA* promoter activity is not a good indicator of DAPG production. However, the experiment of the effect of plant root exudates amendment on *phlA-gfp* expression demonstrated that the host cultivar had a major influence on *phlA* gene expression in strain R31 in the rice exudates amendment. Additionally, the results of *in vitro* and *in vivo* antagonistic activity showed that the colonization of roots by *Pythium* spp. was suppressed by strain R31 and F113.

Interestingly, the application of strain R31 to rice seeds showed significantly increased plant height, shoots dry weight and roots dry weight of rice when compared with control while the application of strain F113 to rice seeds showed no significant difference when compared to control. Identifying different genetic traits that have evolved in microorganisms to compete successfully in diverse rhizosphere environments may allow maximizing root colonization and disease suppression. Knowledge of such genetic traits involved in host preference of the antagonistic bacteria will help to identify strains that are effectively adapted to specific host-pathogen systems.

# APPENDIX

# GROWTH MEDIA AND N-FREE NUTRIENT SOLUTION

## 1 Tryptone-yeast extract (TY) medium

*Constituents per liter*:

| | | |
|---|---|---|
| Tryptone | 5.0 | g |
| Yeast extract | 3.0 | g |
| $CaCl_2.2H_2O$ | 0.65 | g |

*Preparation*:

Dissolve these ingredients in 1 l of distilled water.

Add 15 g of agar if a solid medium is required.

Adjust pH to 6.8 – 7.0.

Autoclave at 121°C for 15 min.

## 2 Luria-Bertani (LB) medium

*Constituents per liter*:

| | | |
|---|---|---|
| Tryptone | 10.0 | g |
| Yeast extract | 5.0 | g |
| NaCl | 5.0 | g |

*Preparation*:

Dissolve these ingredients in 1 l of distilled water.

Add 15 g of agar if a solid medium is required.

Adjust pH to 6.8 – 7.0.

Autoclave at 121°C for 15 min.

## 3 Sucrose asparagine (SA) medium

*Constituents per liter*:

| | | |
|---|---|---|
| Sucrose | 20 | g |
| L-asparagine | 2 | g |
| $K_2HPO_4$ | 1 | g |

*Preparation*:

Dissolve these ingredients in 1 l of distilled water.

Add 15 g of agar if a solid medium is required.

Adjust pH to 6.8-7.0.

Autoclave at 121°C for 15 min.

*Post-autoclave*:

Add 2 ml/l 1 M $MgSO_4$ (sterile)

Add 100 µl/l 100 µM $FeCl_3$ (sterile)

## 4 LG medium

*Constituents per liter*:

| | | |
|---|---|---|
| Glucose | 10 | g |
| $KH_2PO_4$ | 0.15 | g |
| Yeast extract | 0.2 | g |

*Solution*:

| | | |
|---|---|---|
| $K_2HPO_4$ | 0.5 | ml of 100 g/l stock solution |
| $MgSO_4.7H_2O$ | 1 | ml of 200 g/l stock solution |

| | | |
|---|---|---|
| $CaCl_2.2H_2O$ | 1 | ml of 20 g/l stock solution |
| $FeCl_3.6H_2O$ | 1 | ml of 10 g/l stock solution |
| $Na_2MoO_4.2H_2O$ | 1 | ml of 2 g/l stock solution |

*Preparation*:

Dissolve these ingredients in 1 l of distilled water.

Add 15 g of agar if a solid medium is required.

Adjust pH to 6.8-7.0.

Autoclave at 121°C for 15 min.

## 5 M9 salt solution (5X)

*Constituents per liter*:

| | | |
|---|---|---|
| $NaHPO_4.7H_2O$ | 64 | g |
| $KH_2PO_4$ | 15 | g |
| NaCl | 2.5 | g |
| $NH_4Cl$ | 5 | g |

*Preparation*:

Dissolve these ingredients in 1 l of distilled water.

Add 15 g of agar if a solid medium is required.

Adjust pH to 7.0 – 7.4.

Autoclave at 121°C for 15 min.

## 6 M9 minimal medium

*Constituents per liter*:

| | | |
|---|---|---|
| M9 salt solution (5X) | 200 | ml |

| | | |
|---|---|---|
| 1 M $MgSO_4$ (sterile) | 2 | ml |
| 20 % glucose or other carbon source (filter sterile) | 20 | ml |

*Preparation*:

Adjust these ingredients in 1 l of autoclaved distilled water.

## 7 NBRIP (National Botanical Research Institute's phosphate) growth medium agar

*Constituents per liter*:

| | | |
|---|---|---|
| Glucose | 10 | g |
| $Ca_3(PO_4)_2$ | 5 | g |
| $MgCl_2.6H_2O$ (sterile) | 5 | g |
| $MgSO_4.7H_2O$ (sterile) | 0.25 | g |
| KCl | 0.2 | g |
| $(NH_4)_2SO_4$ | 0.1 | g |
| Bromo-phenol-blue | 0.025 | g |

*Preparation*:

Dissolve these ingredients in 1 l of distilled water.

Adjust pH to 6.8-7.0.

Add 15 g of agar if a solid medium.

Autoclave at 121°C for 15 min.

## 8 Chromazural S (CAS) medium

Dye A: 60.5 mg of Chrome A in 50 ml distilled water

10 ml of $FeCl_2$ stock (1 mM $FeCl_2.6H_2O$ in 10 ml of 1N HCl)

72.9 mg HDTMA dissolved in 40 ml distilled water

| | | | |
|---|---|---|---|
| Medium B: | $Na_2HPO_4$ | 3.5 | g/l |
| | $KH_2PO_4$ | 0.3 | g/l |
| | Asparagine | 4 | g/l |
| | Pipes buffer | 20 | g/l |
| | Purified agar | 15 | g/l |
| | Distilled water | 90 | ml |
| | Adjust pH to 6.8-7.0 with NaOH. | | |

*Preparation*:

Prepare dye A to a final volume of 100 ml and autoclave.

Prepare medium B to a final volume of 900 ml and autoclave.

Add dye A to medium B plus $MgSO_4$ 0.5 g/l.

## 9 N-free nutrient solution

| Stock solution | Chemical | g/l |
|---|---|---|
| 1 | $CaCl_2.2H_2O$ | 294.1 |
| 2 | $KH_2PO_4$ | 136.1 |
| 3 | $FeC_6H_5O_7.3H_2O$ | 6.7 |
| | $MgSO_4.7H_2O$ | 123.3 |
| | $K_2SO_4$ | 87.0 |
| | $MnSO_4.H_2O$ | 0.338 |
| 4 | $H_3BO_3$ | 0.247 |
| | $ZnSO_4.7H_2O$ | 0.288 |
| | $CuSO_4.5H_2O$ | 0.100 |

| | |
|---|---|
| $CoSO_4.7H_2O$ | 0.056 |
| $Na_2MoO_2.2H_2O$ | 0.048 |

*Preparation*:

Prepare stock solutions by using warm water to get the ferric citrate into the solution.

Make 10 liters of full-strength plant culture solution as follows.

To 5 liters of water, add 5 ml of each stock solution and mix.

Dilute to 10 liters by adding another 5 liters of water.

Adjust pH to 6.6-6.8 with 1N NaOH.

For plus-N controls treatment; add 0.5 g $KNO_3$ per liter (0.05%), giving N concentration of 70 ppm.

# BIOGRAPHY

Miss Phrueksa Lawongsa was born on 19 May, 1982 in Nakhon Ratchasima, Thailand. She received the Bachelor's degree of Science (Agriculture) ($2^{nd}$ Honor), major in Soil Science, Khon Kaen University in 2002. In 2003, she has pursued her Doctoral degree in School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima and received a scholarship from the Royal Golden Julibee (RGJ) grant of the Thailand Research Fund in 2003. She had presented posters in the topic of "Diversity of *Pseudomonas* species isolated from rice and maize rhizospheres" in The $6^{th}$ National Graduate Research Conference, $13^{th}$-$14^{th}$ October 2006 at Chulalongkorn University, Bangkok and "Phenotypic and molecular characterization of potential plant growth promoting *Pseudomonas* from rice and maize rhizospheres" in The $162^{nd}$ Society for General Microbiology Spring Meeting $31^{st}$ March-$3^{rd}$ April 2008 at Edinburgh International Conference Centre, Scotland. She gave an oral presentation of her work in the topic of "Examination of 2,4-diacetylphloroglucinol (DAPG) produced by *Pseudomonas* sp. R31 isolated from rice rhizosphere in Thailand" in RGJ-Ph.D. Congress XI, $1^{st}$-$3^{rd}$ April 2010 at Jomtien Palm Beach Resort, Pattaya. Her work has been published in World Journal of Microbiology & Biotechnology in the topic of "Molecular and phenotypic characterization of potential plant growth promoting *Pseudomonas* from rice and maize rhizospheres" of volume 24, page 1877-1884 in 2008.