UNIVERSITI PUTRA MALAYSIA

IDENTIFICATION OF A PLANT GROWTH PROMOTING RHIZOBACTERIA, BACILLUS SPHAERICUS (UPMB10), USING PCR-BASED DNA FINGERPRINTING TECHNIQUE

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Chairman: **Halimi Mohd Saud, Ph.D.**

Faculty: **Agriculture**

One of the major constraints in increasing crop yield is the supply of nutrient and nitrogen is obviously the main limiting nutrient. Biological nitrogen fixation is believed to have a great potential to contribute to productive and sustainable agricultural system for the tropics. *Bacillus sphaericus* UPMB10 is a locally isolated plant growth promoting rhizobacteria that has the ability to fix atmospheric nitrogen efficiently and has been proven to promote the growth of vegetable soybean, oil palm and bananas. When UPMB10 is applied to the soil as a biofertilizer, it is very important to have a method of identifying and monitoring the effectiveness of the introduced inoculant. Polymerase chain reaction based DNA fingerprinting, a DNA polymorphism assay based on the amplification of random DNA segment with single primers of arbitrary nucleotide sequence, was employed to detect UPMB10 used as an inoculant. DNA polymorphism simply detects DNA segments, which are amplified from one individual bacterium but not others and the

polymorphisms function as genetic markers. PCR amplification does not require the culturing of the bacterial strains and since it is capable of amplifying unique sequences in the midst of a myriad of DNA sequences, it has the potential to identify specific strains found within the soil. The objectives of this study were to identify suitable primers for identification and distinguishing UPMB10 when applied as a biofertilizer.

In this study, the use of single random primers of 10 nucleotides length, selected in the absence of target sequence information of genomic DNA of UPMB10, has been shown to be effective in producing DNA amplifications that provided DNA polymorphic fingerprints which are unique to the individual organism. Twenty primers, from OPA-01 to OPA-20, were tested. Based on the basic of large range of fragment sizes and a small number of minor fragments, four primers namely OPA-05, OPA-09, OPA-10 and OPA-16 with nucleotide sequences of 5'-AGGGGTCTTG-3', 5'-GGGTAACGCC-3', 5'-GTGATCGCAG-3' and 5'-AGCCAGCGAA-3' respectively were selected.

When two, three and four primers were used, UPMB10 produced more than four fingerprints, ranging between 250 to 3000 bp, with different DNA polymorphism from:

(1) *Bacillus sphaericus* ATCC 33203, ATCC 14577 and ATCC 2362; the homology among the four strains was 11 – 25%,

(2) *Paenibacillus polymyxa* ATCC 15970 and *Paenibacillus macerans* ATCC 8244, which possess the general characteristics of the genus *Paenibacillus* based on the recently described *Bacillus* species,

(3) UPMB11, UPMB12, UPMB13 and UPMB14 (local isolates of PGPR). Strains UPMB11, UPMB12 and UPMB13 can be classified as belonging to the same species group since very similar bandings were produced, but UPMB14 was quite different from the rest. All the bandings were within the range of 320-3500 bp.

In conclusion, PCR-based DNA fingerprinting can produce accurate, faster and reproducible DNA fingerprint patterns for maximum discrimination of UPMB10 strain using the appropriate primers, OPA-09, OPA-10 and OPA-16 under optimised PCR conditions and showed clear differences in the DNA polymorphism by the presence or absence of bands. These results showed a way of distinguishing UPMB10 from other bacterial strains and have the potential for identifying this strain when used as an introduced inoculant in the soil.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENGENALPASTIAN RHIZOBAKTERIA PENGGALAK PERTUMBUHAN POKOK, *BACILLUS SPHAERICUS* (UPMB10), MENGGUNAKAN TEKNIK DNA 'FINGERPRINTING' ATAU CETAKAN JARI BERDASARKAN PCR

Oleh

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Salah satu daripada kekangan utama dalam peningkatan hasil tanaman ialah bekalan nutrien dan nitrogen ialah satu-satunya nutrien yang amat terhad. Pengikatan nitrogen biologi berpotensi dalam sistem pengeluaran dan kelestarian pertanian di kawasan tropika. *Bacillus sphaericus* UPMB10 ialah satu asingan PGPR (rhizobakteria penggalak pertumbuhan pokok) tempatan yang boleh mengikat nitrogen dari atmosfera dengan berkesan dan telah terbukti mampu meningkatkan tumbesaran kacang soya, kelapa sawit dan pisang. Apabila UPMB10 digunakan sebagai sejenis 'biofertilizer' atau biobaja, adalah sangat penting untuk mengenalpasti dan memantau kesannya sebagai sejenis inokulan. DNA 'fingerprinting' atau cetakan jari berdasarkan PCR iaitu asai polimorfisme DNA berasaskan amplifikasi cebisan/serpihan rawak DNA dengan satu primer yang mempunyai jujukan nukleotida rawak telah digunakan untuk mengesan UPMB10 yang digunakan sebagai inokulan. Polimorfisme DNA

mengenalpasti hanya serpihan DNA yang diamplifikasi daripada satu jenis bakteria sahaja dan bukan daripada bakteria yang lain, oleh itu polimorfisme tersebut berperanan sebagai penanda genetik. Amplifikasi PCR tidak memerlukan pembiakan bakteria kerana ia mampu mengamplifikasi jujukan yang unik di antara jujukan DNA yang banyak, jadi, ia berpotensi untuk mengenalpasti strain khusus di dalam tanah. Objektif kajian ini ialah mengenalpasti primer yang sesuai untuk mengenalpasti dan membezakan UPMB10 apabila digunakan sebagai biobaja.

Dalam kajian ini, primer tunggal sepanjang 10 nukleotida yang terpilih telah digunakan kerana ketiadaan maklumat jujukan sasaran DNA genomik UPMB10. Primer ini telah membuktikan keberkesanannya dalam menghasilkan amplifikasi DNA dan seterusnya polimorfisme 'cetakan jari DNA' yang unik kepada setiap sel individu. 20 primer, dari OPA-01 hingga OPA-20 telah diuji. Berdasarkan kepada julat ukuran serpihan yang besar dan bilangan kecil fragmen bersaiz kecil, 4 primer iaitu OPA-05, OPA-09, OPA-10 dan OPA-16 dengan jujukan nukleotida masing-masing 5'-AGGGGTCTTG-3', 5'-GGGTAACGCC-3', 5'-GTGATCGCAG-3' dan 5'-AGCCAGCGAA-3' telah dipilih untuk kajian selanjutnya.

Daripada empat primer yang telah digunakan, UPMB10 menghasilkan lebih daripada empat cetakan jari berukuran di antaran 250 hingga 3000 bp yang mempunyai polimorfisme DNA yang berlainan daripada:

(1) *Bacillus sphaericus* ATCC 33203, ATCC 14577 dan ATCC 2362, dan didapati bahawa homologi antara keempat-empat strain ialah 11 – 25%,

(2) *Paenibacillus polymyxa* ATCC 15970 dan *Paenibacillus macerans* ATCC 8244, berdasarkan penghuraian baru spesis *Bacillus* yang menyerupai sifat-sifat umum genus *Paenibacillus*,

(3) UPMB11, UPMB12, UPMB13 dan UPMB14 (asingan PGPR tempatan). Strain-strain UPMB11, UPMB12 and UPMB13 boleh dikelaskan dalam satu kumpulan spesis yang sama sebab jalur DNA yang diperolehi hampir serupa tetapi bukan UPMB14. Kesemua saiz jalur-jalur adalah di antara 320 hingga 3500 bp.

Sebagai kesimpulan, cetakan jari DNA berdasarkan PCR mampu menghasilkan corak cetakan jari DNA yang tepat, cepat dan berulangan bagi membezakan strain UPMB10 dengan menggunakan primer bersesuaian. OPA-09, OPA-10 dan OPA-16 dalam keadaan PCR optimum dan boleh menunjukkan perbezaan polimorfisme DNA dengan jelas berasaskan kehadiran atau ketiadaan jalur. Keputusan kajian menunjukkan satu kaedah membezakan UPMB10 daripada strain bakteria lain dan berpotensi untuk mengenalpasti strain tersebut apabila digunakan sebagai inokula di dalam tanah.

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This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Master of Science.

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LIST OF ABBREVIATIONS

Symbol	Description
BNF	biological nitrogen fixation
bp	base pairs
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphates
KCl	potassium chloride
Mg^{2+}	magnesium ion
$MgCl_2$	magnesium chloride
N	nitrogen
PCR	polymerase chain reaction
PGPR	plant growth promoting rhizobacteria
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SEM	scanning electron microscopy
TE	tris-EDTA

CHAPTER 1

INTRODUCTION

The dramatic increase in agricultural and industrial productivity worldwide has created severe environmental problems. Biological agents, such as bacteria with beneficial properties, could serve to remediate contaminated environments like soil and sediment or substitute chemicals in pesticides (biological control). Bacteria have already been introduced into soil to promote plant growth (Gaskins *et al.*, 1985; De Freitas and Germida., 1992; Lugtenberg and de Weger, 1992, Shamsuddin *et al.*, 2000), for pest control (Schroth *et al.*, 1984; Keel *et al.*, 1990; Lugtenberg and de Weger, 1991) and for the degradation of a variety of polluting compounds (Alexander, 1981; Brunner *et al.*, 1985; Pipke *et al.*, 1992).

The agricultural use of plants and animals in open environments has been an established traditional practice. Pure bacterial cultures have been used for agricultural purposes for almost a century (Marco *et al.*, 1994). The use of microbes for agricultural purposes extends over a considerable area (Table 1, Appendix A) and is expected to increase in the framework of land managerial practices such as sustainable agriculture and organic farming.

Bacteria isolated from the environment, which are potentially better adapted to ecological stresses, can be genetically altered for specific environmental purposes (Griffiths, 1981; Lindow, 1985; Sk∅t *et al.*, 1990; Doyle *et al.*, 1991; Ramos *et al.*, 1991; van Fisas *et al.*, 1991; Waalwijk *et al.*, 1991; Duque *et al.*, 1992; Fenton *et al.*, 1992; Shamsuddin *et al.*, 2000). The use of recombinant microorganisms in large-scale field trials is, however, still restricted. This is due to a lack of knowledge on the fate of the microorganism and/or the heterologous DNA and its possible ecological and health effects as a consequence of the release (Reanney, 1977; Griffiths, 1981; Levin and Strauss, 1990; Wellington and van Elsas, 1992).

Microorganisms are introduced into the soil and rhizosphere to improve plant growth through nitrogen fixation, formation of mycorrhizae, direct growth stimulation and biological control of disease. There is a need to be able to monitor introduced organisms specifically, to study their fate in the environment and thereby to help explain the success or failure of the inoculation. Besides that there is also a need to monitor the fate of genetically manipulated organisms after they are released into the environment. There is now a wide variety of methods to track microbes in the soil. In addition to the traditional antibiotic resistance and selective plating methods, we can use introduced marker genes, different types of immunological methods and specific DNA and RNA probes. The advent of the polymerase chain reaction (PCR) technique allows for development of

a faster technique for the analysis of polymorphisms. A PCR results in the amplification of a specific DNA sequence and is performed by an *in vitro* replication system. The PCR-fingerprinting technique is fast and easy to perform and gives as much or more information than other methods.

A locally isolated bacteria, *Bacillus sphaericus* (UPMB10), has been shown to have the ability as a plant-growth promoting rhizobacteria (PGPR) namely to fix atmospheric nitrogen efficiently and to promote the growth of vegetable soybean, oil palm and bananas (Mia *et al.*, 1999; Shamsuddin *et al.*, 1999a; Shamsuddin *et al.*, 1999b; Shamsuddin *et al.*, 2000). However, a genetic marker for this plant growth promoting rhizobacteria has not been established. Thus this research project was carried out with the following objectives:

(1) To identify arbitrary primers as probes for distinguishing different species of plant-growth promoting rhizobacteria (PGPR).

(2) To distinguish and identify a specific strain of plant-growth promoting rhizobacteria, *Bacillus sphaericus* (UPMB10), using DNA fingerprinting technique.

CHAPTER 2

LITERATURE REVIEW

It is widely established that substantial amounts of nitrogen are fixed by different species of indigenous bacteria under the tropical agriculture system, in particular the cultivation of soybeans, lowland rice, sugarcane, cereals, oil palm and pasture grasses, where little or no fertiliser is used (Döbereiner *et al.*, 1972; van Berkum and Bohlool, 1980; Boddey and Döbereiner, 1982; Claudine *et al.*, 1992, Döbereiner, 1997). The association of plants with these N_2-fixing bacteria, without formation of differentiated structures such as root nodules, is designated as associative symbiosis.

The bacterial species involved in associative symbiotic ecosystems have been isolated, and for most of them little information on their biological characteristics are available. Therefore, arrays of molecular genetic methodologies to examine soil microorganisms at the molecular level were developed (Neale *et* al., 1990; Maarten *et al.*, 1994). Specific molecular analyses are based on the structure of the nucleic acids and the intricate mechanisms that synthesize specific microbial compounds at the genetic level. Nucleic acids are either deoxyribonucleic acid (DNA) or ribonucleic acids (RNA). Double-stranded DNA is the basis for two

extremely important molecular analyses - gene probes and polymerase chain reaction (PCR) (Claudine *et al*., 1992; Ian and Karen, 1998).

PCR-based DNA fingerprinting technology opens new vistas in population dynamics since the use of multiple probes allows for many genomic loci to be rapidly investigated. The use of single random primers, selected in the absence of target sequence information, were shown to be effective in producing DNA amplifications that provide fingerprints which are unique to individual organisms (Williams *et al*., 1990). Hans-Volker and Rein (1994) have provided an outline of several currently available DNA fingerprinting methods such as southern blot and hybridisation, PCR-amplification of polymorphic DNA, ribotyping of bacterial strains, fingerprinting by arbitrarily primed PCR, fingerprinting by tRNA consensus primed PCR, and automated analysis of fingerprints for the typing of bacterial strains and species. In microbial ecology, PCR-fingerprinting is the conventional technique that could be used for species identification in the future, because large numbers of samples can be processed in a relatively short time, and no species-specific DNA probes are needed.

2.1 Associative Nitrogen-Fixing Bacteria

The association of plants with these N_2-fixing bacteria, without formation of differentiated structures, is designated as associative symbiosis. Nitrogen fixation is unique only to bacteria; animals and plants

involved in fixing atmospheric nitrogen must do so in association with bacteria. An understanding of biological nitrogen fixation (BNF) is essential to elucidate the dynamics of the global nitrogen cycle (Gary *et al*., 1992).

2.1.1 Using Atmospheric Nitrogen

The element nitrogen is an essential component of all living things, being the basis of proteins and the genetically important nucleic acids. It is an unfortunate quirk of nature, therefore, that very few living organisms have the ability to utilize the enormous reservoir of nitrogen, nearly 80% in the earth's atmosphere. The nitrogen cycle has been described as being second in importance only to the carbon cycle.

The nitrogen cycle is obviously of considerable importance in the biosphere. The N_2 fixation component demonstrates tremendous potential for replacing chemical nitrogen fertilizers by biologically fixed nitrogen from the *Rhizobium*-legume symbiosis or association with non-legumes (Claudine *et al*., 1992).

Indeed, it is the environmental implication of the over liberal use of fertilizer nitrogen coupled with the need to reduce crop input costs that has given the study of biological nitrogen fixation (BNF) an impetus in recent years. The global economic and environmental pressures also coincided with significant developments in the field of molecular biology and, in

particular, new understanding of the genetic control of nitrogen fixing bacteria. Given the potential rewards from an efficient symbiotic association, it is imperative that both components of the micro and macro-symbionts are considered and the limiting factors to the host and bacteria fully understood. The vast literature on the practice of legume inoculation with strains of rhizobia provided a good insight into the implications of these interactions. These include reports on legume inoculant production, standards and quality control (Anon, 1991; Thompson, 1991).

2.1.2 Nitrogen-fixing Bacteria

Members of the plant and animal kingdoms are eukaryotes, a group of living organisms that cannot convert the stable gas nitrogen – of which nearly 80% of the atmosphere is comprised – into a biologically useful form (Janet and Peter, 1990). Organisms that can utilize atmospheric nitrogen all belong to a group known as prokaryotes. This group has now been divided into two sub-groups: eubacteria and archaebacteria, which differ from each other at least as much as either differs from the eukaryotes (Wooese, 1987). All organisms, which reduce N_2 to ammonia, do so with the aid of an enzyme complex *nitrogenase*. This intricate enzymatic system depends upon various complex biochemical and physiological processes.

There are a number of free-living N_2-fixing bacteria isolated from the rhizosphere. Since the initial discovery of *Azotobacter paspali-Paspalum notatum* association (Döbereiner *et al.*, 1973), the other new bacterial genera and species include *Azospirillum*, *Klebsiella*, *Bacillus*, *Acetobacter*, *Herbaspirillum* and *Pseudomonas*-like bacteria (Claudine *et al.*, 1992).

2.2 Biofertilizer

The utilisation of biological nitrogen fixation (BNF) has been introduced since the beginning of the 20th century (Watanabe *et al.*, 1992). Biofertilizer is a microbial fertilizer carefully prepared by growing a group of beneficial microorganisms (bacteria and / or fungi) and incorporated aseptically into suitable sterile carrier mediums, such as peat, lignite, charcoal or etc. to make it easy to apply into the soil (Ajay, 1999; National Research Development Corporation, 1999). Presently, most of the biofertilizers available in some global markets are functional which implies that with chemical nitrogen or phosphorus they have been testified. In India, most of the biofertilizers have 5, 7 and 11 times higher nitrogen, phosphate and potassium contents, respectively, in relation to a normal fertile soil (Ghatnekar and Kavian, 1999).

One of the major constraints in increasing crop yields is the supply of nutrients. The most limiting nutrient is nitrogen (Ghatnekar and Kavian,