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Molecular Characterization of *Pseudomonas aeruginosa* UPM P3 from Oil Palm Rhizosphere

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Abstract: Problem statement: *Pseudomonas aeruginosa* has been used in agriculture as biological agents. It has shown substantial control of a variety of soil-borne plant pathogens including *Macrophomina phaseolina*, *Botrytis cinerea*, *Rhizoctonia solani*, *Colletotrichum truncatum*, *Pythium*, *Fusarium* and others. Species aggregate of *Pseudomonas aeruginosa* strain UPM P3 was shown to have potential as a biocontrol agent against *Ganoderma boninense*, the causal agent of Basal Stem Rot (BSR) of oil palm. However, *P. aeruginosa* is also an opportunistic pathogen. It typically infects the pulmonary tract, urinary tract, burns, wounds and also causes other blood infections. The objective of this study was to carry out DNA fingerprinting for strain differentiation to differentiate between pathogenic and non-pathogenic forms of *P. aeruginosa* strain UPM P3. **Approach:** Genotype characterization was carried out by amplification of the recA gene using specific primers, purified using QIA Quick PCR purification Kit and sent for sequencing. Multiple sequence alignments were performed on the selected closely related sequence accessions using CLUSTAL W software. The recA gene was used for phylogenetic and PCR-RFLP studies. **Results:** From the phylogenetic tree, UPM P3 has more than 90% similarity with *Pseudomonas aeruginosa* strains: PAM7, PAO1, UCBPP-PA14 and PA7. UPM P3 was further digested with restriction enzymes; PvuII, BsrI, ZraI, FokI and SgrAI. RFLP results showed that strain UPM P3 has close similarity with strain PAO1 of *Pseudomonas aeruginosa*. **Conclusion:** Strain PAO1 is commonly associated with strains of medical, human or plant pathogens and agricultural environment. Common habitats include soil, hosts, aquatic environment and wastewater and also a common contaminant of public places. Thus the use of *Pseudomonas aeruginosa* strain UPMP3 as a biological control candidate in agriculture has to be monitored.

Key word: *Pseudomonas aeroginusa*, recA gene, PAO1, RFLP, UPM P3, Basal Stem Rot (BSR), *Ganoderma boninense*

INTRODUCTION

Pseudomonas is a genus of gram- negative, nonspore forming, rod-shaped bacteria. They are commonly found in soil, water and decaying matter and including some species that are plant and animal pathogens. *Pseudomonas* species typically give a positive result to the oxidase and catalase tests. The genus demonstrates a great deal of metabolic diversity and consequently are able to colonise a wide range of niches. Certain members of the *Pseudomonas* genus have been applied to seeds or applied directly to soils as a way of controlling the growth or establishment of crop pathogens. The biocontrol properties of *P. fluorescens* strains CHA0 or Pf-5, for example have biocontrol properties, protecting the roots of some plant species against parasitic fungi such as *Fusarium* or *Pythium*, as well as some phytophagous nematodes[1]. It is not clear exactly how the plant growth promoting properties of *P. fluorescens* are achieved; theories include that the bacteria might induce systemic resistance in the host plant, so it can better resist attack by a pathogen, or the bacteria might out compete other (pathogenic) soil microbes, by siderophores giving a competitive advantage at scavenging for iron or the bacteria might produce compounds antagonistic to other soil microbes, such as phenazine-type antibiotics or hydrogen cyanide[2]. *Pseudomonas aeruginosa* strain UPM P3, was isolated from the roots and rhizospheres of oil palm (*Elaeis guineensis*) and their potential as biocontrol agent against *Ganoderma boninense*, causal pathogen of Basal Stem Rot (BSR) of oil palm and *Colletotrichum truncatum* anthracnose of soybean has been established. However, *P. aeruginosa* is also an

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opportunistic pathogen of plants[3] and humans[4]. Opportunistic infections are infections caused by organisms that usually do not cause disease in a person with a healthy immune system, but can affect people with a poorly functioning or suppressed immune system.

Restriction Fragment Length Polymorphism (RFLP) analysis of the PCR-amplified fragment of recA gene is an appropriate technique for the differentiation and characterization of micro-organisms on the basis of their phylogenetic relationships[5] and regarded as the most sensitive method for strain identification[6]. Analysis of recA has also proven very useful in molecular systematic among closely related bacteria[7] RFLP analysis of PCR-amplified recA demonstrated sufficient nucleotide sequence variation to enable separation of strains of all five *B. cepacia* complex genomovars[8]. PCR-RFLP of recA gene also was applied for the characterization of *Erwinia amlyovora* and *Erwinia* strains[9]. RFLP analysis via polymerase chain reaction involves amplifying DNA using PCR, digest PCR products with restriction enzyme (s), separate fragments by size using gel electrophoresis and score data. The goal was to determine whether molecular markers based on PCR-RFLP analysis of recA gene can be effectively used for the differentiation of *Pseudomonas aeruginosa* strain UPM P3.

MATERIALS AND METHODS

Genomic DNA extraction from bacteria: DNA extraction was carried out following the Cetyltrimethyl Ammonium Bromide (CTAB) method. Ten colonies of UPM P3 was inoculated into 10 mL nutrient broth and incubated at 28±2°C overnight. One mL of the overnight culture was transferred into a 1.5 mL eppendorf tube and centrifuge for 30 sec at 13,000 rpm. The bacteria cells were collected by discarding the supernatant and resuspended in 567 µL TE buffer (10 mM Tris-HCL, pH 7.4, 1 mM EDTA and 1 L distilled water), mixing well by vortexing. Then 30 µL of 10% SDS, 3 µL of NAOAC (sodium acetate) pH 5.2, 100 µL of 5 M NACL and 80 µL CTAB-NACL were added to a total volume of 780 µL and mixed well before incubating for 10 min in waterbath at 65°C. An equal volume (780 µL) of chloroform/isoamyl alcohol (24:1) was added to the mixture and centrifuged at 13000 rpm for 5 min to separate the phases. The clear supernatant was transferred into a new eppendorf tube and the aqueous DNA layer was again extracted using phenol/chloroform/isoamyl alcohol (25:24:1). This step was repeated 3 times and the supernatant pooled. The clear supernatant was transferred into new eppendorf tube and 400 µL of isopropanol was added to precipitate the nucleic acid. Finally the DNA was washed with 200 µL of 75% of cooled ethanol and dried at room temperature (28±2°C) before dissolving in 100 µL Sterile Distilled Water (SDW) and kept at -20°C for further analysis. Electrophoresis was run for identifying the nucleic acids after DNA extraction in 1% of agarose gel and 1% of TBE (Tris base, boric acid, 0.5 M EDTA solution, 1L ddH2O, pH 8.0). The products were mixed with loading dye buffer (MBI Fermentas) in 5:1 ratio and subjected to electrophoresis at 70 volts for 1 h and 45 min. DNA ladder 100 bp (MBI Fermentas) was used as marker. The gel was stained in ethidium bromide solution and the bands visualized and photographed using BioRad Gel Doc 2000.

Nucleotide sequence analysis: The PCR products was purified using a commercial kit (QIA Quick PCR purification kit (Qiagen, Valencia, CA), according to manufacturer's instruction. After purification, the PCR products were sent for sequencing services. The recA gene sequences were aligned using BioEdit software versions 7.0.8 (http://www.mbio.nscu.edu/bioEdit/bioEdit) and searched for sequence similarity to other sequences which are available in the NCBI database at http://www.ncbi.nih.gov using Basic Local Alignment Search Tool (BLAST) algorithm. Multiple sequence alignments were performed on the selected closely related sequence accessions available using CLUSTAL W software (http://workbench.sdsc.edu/).

Primer design and PCR amplification: Oligonucleotide primers were rec-AS (5'-atggacgagaacaagaagcg-3’, 20 mer) and recA-AS (5'-tcaatcggcttcggcgtca-3'; 19mer) were designed on the basis of the sequence of *P. aeruginosa* recA gene available in Gene Bank. Amplification reaction for UPM P3 was performed in 25 µL of total volume containing 2 µL of DNA as a template, 2.5 µL of 10×PCR buffer (Fermentas), 1.5 µL of 25 mM MgCl2 (Fermentas), 0.2 µL of 10 mM dNTPs, 0.1 µL primer oligonucleotides, 0.1 µL Taq polymerase (Fermentas) and 18.5 µL of sterilized distilled water. The amplification was performed in a Thermal Cycler (Biometra®,T3 thermocycler) (Syngene, UK) programmed for pre-denaturing of 3 min at 94°C, 30 cycles of 1min at 94°C, 1min at 58°C and 2 min at 72°C. After a final extension of 10 min at 72°C, the samples were cooled to 4°C.

Phylogenetic analysis: Phylogenetic analysis was done based on the nucleotides of recA gene using draw tree

software provided by the Biology Workbench Program (http://workbench.sdsc.edu/).

Restriction fragment length polymorphism analysis: The amplified DNA fragment of recA gene were digested with restriction endonucleases (PvuII, BsrI, ZraI, FokI and SgrAI) selected on the basis of nucleotide sequence of the *Pseudomonas aeruginosa* strains and UPM P3. 10 µL of PCR product combined with 2 µL restriction enzymes of BsrI and SgrAI and 1 µL of diluted PvuII, ZraI and FokI with 2 µL buffer for BsrI and SgrAI and 5 µL buffer for PvuII, ZraI and FokI. The reactions were incubated at 37°C for PvuII, ZraI, FokI and SgrAI and 65°C for BsrI. Digested products were then separated on 1.2% agarose gel. The size of RFLP bands were measured with Gene Tool software (Syngene, UK).

RESULTS

Total genomic DNA of *Pseudomonas aeruginosa* (UPM P3) was successfully extracted using the Cetyltrimethyl Ammonium Bromide (CTAB) method.

PCR amplification of recA gene: Primers recA-*S* and recA-*AS* were designed based on recA gene sequence of *Pseudomonas aeruginosa*. The expected product size (1041bp) for PCR implication of P. *aeruginosa* recA gene was shown (Fig. 1).

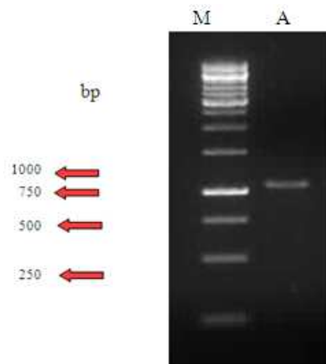

Fig. 1: Amplification of recA gene (A) for *Pseudomonas aeruginosa* (UPM P3). Size of DNA ladder (M) used is 1 KB

BLAST result: There was 99% similarity between strain UPM P3 and PAO1 and 98% similarity between strains UPM P3, PAM7, UCBPP_PA14 and PA7. Sequence of *Pseudomonas aeruginosa* strain UPM P3 has been submitted to NCBI Gen Bank. The Gen Bank accession numbers is GQ183951. Strains of *P. aeruginosa* from BLAST result used for comparison was as shown in Table 1.

Phylogenetic analysis: Phylogenetic analysis carried out on the nucleotides of recA gene resulted in the Phenogram for *P. aeruginosa* (Fig. 2). In this cluster UPM P3 was closer to PAO1. Strain UCBPP-PA14 however, was related with plant pathogen, whereas strain PA7 and PAO1 were related to medical, human, animal, plant pathogen and also implicated with agricultural uses. Habitats of most isolates are hosts, plant and soil. Diseases related with strain PA7: Opportunistic infection, PAO1: Nocosomial infection, UCBPP-PA14: Opportunistic infection and Nocosomial infection. Strains PA7 andPA14 UCBPP-PA14 were isolated from clinical isolates. However all of these strains are also common contaminants in the public place.

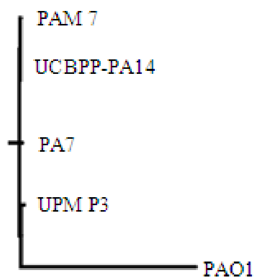

Fig. 2: PHYLIP rooted tree: Phenogram for *Pseudomonas aeruginosa* strain UPM P3

Table 1: Strains *Pseudomonas aeruginosa* from BLAST result used as comparison for the phylogenetic analysis of recA sequences

Gen bank accession numbers	Description	e-value
AE004091.2	PAO1	0.0
CP000438.1	UCBPP-PA14	0.0
X52261.1	PAM 7	0.0
CP000744.1	PA7	0.0

Table 2: Size of RFLP bands of recA gene of *Pseudomonas aeruginosa* strains with the standard sequence available in NCBI in comparison with UPM P3 based on Gene Tool software (Syngene, UK)

Restriction enzyme	UCBPP-14	PAM_7	PA7	PAO1	UPM P3
PvuII	975-66	1118-88	*	*	*
BsrI	956-85	*	842-199	956-69-16	959-87-10
ZraI	*	*	*	*	*
FokI	655-294-92	798-294-111	655-294-92	659-382	665-381
SgrAI	416-358-267	501-438-267	358-267-233-183	412-362-267	413-363-264

*: RFLP bands not detected

RFLP analysis of recA gene: RFLP patterns of amplified recA gene of UPM P3 digested with enzymes PvuII, BsrI, ZraI, FokI and SgrAI were compared with the standard recA sequence of *Pseudomonas aeruginosa* strains, available in NCBI. Results showed that strain UPM P3 had close similarity with the strain PAO1 (Table 2).

DISCUSSION

Pseudomonas aeruginosa is an opportunistic human pathogen, most commonly affecting immuno compromised patients, such as those with cystic fibrosis[10]. Environmental *P. aeruginosa* isolates have been considered as potential biological control agents or inducers of systemic acquired resistance and also somestrains have been reported as plant-growth promoting rhizobacteria[11,12]. PCR and sequencing of recA gene generated the phylogenetic tree for UPM P3. There was 99% similarity (based on the BLAST) between UPM P3 and PAO1 and 98% similarity between UPM P3, UCBPP-PA14 andPAM7and PA7. To differentiate the strain of UPM P3, RFLP fingerprinting was carried out using restriction enzymes. RFLP fingerprinting showed that strain UPM P3 had close similarity with the strain PAO1. PAO1 which have been considered as contaminant in hospitals can be opportunistic human pathogens. PAO1 has 6.3 million base pairs, which is the largest bacterial genome sequenced and the sequence provides insights into the basis of the versatility and intrinsic drug resistance of *P. aeruginosa*. Consistent with its large genome size and environmental adaptability, *P. aeruginosa* contains the highest proportion of regulatory genes observed for a bacterial genome and a large number of genes involved in the catabolism, transport and efflux of organic compounds as well as for potential chemo taxis systems. *Pseudomonas aeruginosa* PAO1 paralyzes rapidly and killed the nematode *Caenorhabditis elegans*[13]. Hydrogen cyanide is the sole or primary toxic factor produced by this strain that is responsible for killing of the nematode. Because of the potential of this strain causing opportunistic infections in humans, strain UPM P3 has to be used with specific guidelines for agricultural purposes. Thus strain UPM P3 has the potential to be used as a biocontrol agent against *Ganoderma boninense*.

CONCLUSION

Genotype characterization of recA of *P. aeruginosa* strain UPM P3 showed 90% similarity with PAO1 and further confirmed with RFLP results using restriction enzymes PvuII, BsrI, ZraI, FokI and SgrAI.

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