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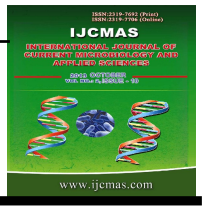

Original Research Article

Molecular studies on chitinase producing, biofilm forming *Pseudomonas putida* isolated from fish waste dumping yards

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A B S T R A C T

Dual action of the *Pseudomonas putida* forming antibiotic and biofilm in same species is useful for the plants against the fungal pathogens. *lap*D, a gene which is located flanking to the *lap*A gene, which is possible role in controlling *lap*A protein secretion and thus plays a major role in biofilms formation in *Pseudomonas* sp. Pyocyanin is a bioactive compound, produced by majority of the *Pseudomonas* sp. In this study, the screening of *lap*D (biofilm gene) gene and *phz*S (pyocyanin gene) among the *P. putida* isolated from fish waste dumping locations. Being a potential biofilm former, all the isolates of *P. putida* were tested for the presence of *lap*D gene, since it role is highly significant in biofilm formation and *phz*S gene for it antibiotic activity. Among the 15 isolates tested, only one is positive for *lap*D gene, while 4 isolates were having pyocyanin gene (*phz*S).

Keywords

P. putida; biofilm; pyoyanin; *lap*D; *phz*S.

Introduction

Pseudomonas putida is frequently isolated from the rhizosphere soil and many strains promote plant-growth, exhibit antagonistic activities against plant pathogens and also have the capacity to degrade various pollutants in different levels. Factors that appear to contribute to the rhizosphere fitness are the ability of the organism to form biofilms and the utilization of cell-to-cell-communication systems (quorum sensing, QS) to co-ordinate the expression of certain phenotypes in a cell density dependent manner (Arevalo *et al*., 2005). Genus *Pseudomonas* is distinguished by their colourful secondary metabolite, known as Phenazine. Pyocyanin is one of the phenazine derivatives, which is a bluish green pigment, mainly produced by *P. aeruginosa.* Pyocyanin is synthesized from chorismate through a series of complex steps mediated by gene products encoded by two homologous core loci (PhzA1B1C1D1E1F1G1 and PhzA2B2 C2D2E2F2G2) responsible for synthesis of Phenazine-1-carboxamide (PCA) and three additional genes (*phz*M, *phz*S and *phz*H) encoding unique enzymes involved in conversion of Phenazine-1-carboxamide (PCA) to pyocyanin, (Mavrodi *et al*., 2001). *PhzM* encodes a 334 residue

protein with a calculated molecular mass of 36.4 kDa, while *phz*S encodes a protein of 43.6 kDa, which is similar to bacterial monooxygenases. Polypeptides corresponding to all the *phz* genes were identified by analysis of recombinant plasmids by Mavrodi *et al*., (1998). The *phz*M gene was located upstream of the *phz*A1B1C1D1E1F1G1 operon and transcribed divergently, that gene encodes a 334 amino acids protein (Mavrodi *et al.*, 2001). Functional analysis revealed that the *phz*M gene product is involved in the production of pyocyanin. Movrodi *et al*., (2001) suggests that two steps are involved in the synthesis of pyocyanin from PCA. In the first step, catalyzed by the SAM-dependent methyltransferase *phz*M, PCA is converted to 5-methylphenazine-1-carboxylic acid betaine. The second step, catalyzed by the NADH (or NADPH)-dependent flavoprotein monooxygenases, *phz*S involves hydroxylamine decarboxylation of 5-methylphenazine-1-carboxylic acid betaine to pyocyanin. Pyocyanin is having effective antimicrobial properties against various pathogens. Antibacterial activities against *Staphylococcus* and *Vibrio* sp. (Arun *et al.,* 1977) and antifungal activity against the *Candida albicans* and *Aspergillus fumigates* (Cusmanao, 1975; Kerr *et al*., 1999) have been reported.

Biofilm conception is hypothesized to facilitate the development of a "micro niche" (Costerton *et al*., 1999) that protects bacteria against various physical and chemical stresses and confers resistance to deleterious agents such as antibiotics and detergents. There is a notable trend toward the identification of pathogenic surface-attached organisms in such diverse genera as *Pseudomonas*, *Vibrio, Escherichia*, *Salmonella*, *Listeria*, *Streptococcus, Staphylococcus* and *Mycobacteria* (Luanne Hall-Stoodley 2002).

Hinsa and Toole (2006) suggest that *lap*D, an inner-membrane protein, modulates the secretion of the LapA protein. *lap*D contains signature sequences of proteins that catalyze the synthesis and degradation of cyclic di-GMP. The identification of an ORF mapping adjacent to the gene lapAEBC locus designated *lap*D gene, which is conditionally required for biofilm formation. *lap*D gene which is located flanking to the *lap*A gene, which is possible role in controlling LapA protein secretion and plays a major role in biofilm formation among *Pseudomonas* sp., On the other hand, *lap*D is an inner-membrane protein required by *P. fluorescens* for biofilm formation and maintenance of the adhesion for *lap*A on the cell surface (O'Toole and Kolter, 1998).

A mutation in *lapD* results in a conditional biofilm, this biofilm phenotype is exacerbated when biofilm formation is assayed in a flow-cell system (O'Toole *et al*., 2006). *lap*D utilizes an inside-out signaling mechanism: binding c-di-GMP in the cytoplasm and communicating this signal to the periplasm via its periplasmic domain (Newell *et al*., 2009; Newell *et al*., 2011). Studies on *P. putida* biofilm have suggested that the transmembrane protein lapD, which controls the activity of the periplasmic protease *lap*G (Gjermansen *et al*., 2010).

P. putida can respond rapidly to the presence of root exudates in soils, converging at root colonization sites and establishing stable biofilms (Urgel *et al*., 2002). *P. putida* has been widely used for the biotechnology allocation, biofilm formation by as *P. putida* was first studied

by Shrove *et al*. (1991) by degradation of toluene, the strain *P. putida* KT2440 is a model bacteria used in lab for the plant bacterial interactions and biofilms formation (Molina *et al*., 2000). In *P. putida* KT2440, about 43 genes are involved in synthesis and degradation of c-di-GMP (Matilla *et al*., 2011).

These findings suggest that the lapA protein may function as an adhesion during plant attachment and biofilm formation. The *lap*A among *P. fluorescens* and *P. putida* plays an important in initial attachment of microbial cells to surfaces (Hinsa *et al*., 2003; Urgel *et al*., 2000). *P. putida* is a metabolically versatile saprophytic soil bacterium that has been certified as a biosafety host for the cloning of foreign genes. Analysis of the genome gives insight into the non-pathogenic nature of *P. putida* and points to potential new applications in agriculture, biocatalysis, bioremediation and bioplastic production (Nelson *et al*., 2003). This study showed that bacterial biofilms were highly heterogeneous, covering about 40% of the roots. This study aims to isolate the *P. putida* in the fish scales contaminated soil which is able to produce biofilms and pyocyanin by PCR amplification.

Materials and Methods

Collection of samples

Samples from fish waste dumping yards have been considered as the best source for the incidence of *Pseudomonas* sp. About 30 samples from the yards of fish waste dumping locations were collected from Coimbatore and Nagercoil (Tamil Nadu state) and Palakkad (Kerala State). Standard procedures have been adopted for the enumeration of *P. putida* from the collected samples.

Sample collection and processing

The samples were collected in pre-sterile polythene covers, using a sterile spatula and transported to the laboratory for the further analysis. About 1g of sample was weighed and serially diluted up to 10^{-9} dilution. One ml of the respective dilutions were plated onto the specific media, the King's B media and incubated at 37 ^{o}C overnight. The selected colonies were picked and streaked onto a King's B agar plate to get pure individual colonies. King's B medium which contains 20 g of peptone 1.5 g of magnesium sulphate, 1.5g of Dipotassium hydrogen phosphate, 10ml of glycerol and 1.5 g of agar for 1000 ml of water with pH 7.

Preliminary identification of bacterial isolates

Gram's staining, motility determination test, indole test, urease, MR, VP, TSI, catalase, glucose, lactose, maltose, sucrose tests were done for the preliminary identification of the strain. Then the strain was subjected to molecular characterization for identification of the specific gene.

Molecular characterizations

Extraction of DNA (CTAB/ NaCl solution (10% CTAB in 0.7 M NaCl)).

Molecular characterization of the strain is done by extraction of genomic DNA. The DNA was extracted by CTAB/ NaCl solution (10% CTAB in 0.7 M NaCl and washed precipitate with 70% ethanol. Remove supernatant and briefly dry pellet in lyophilizer. Resuspend pellet in 100µl TE buffer (Murray and Thompson, 1980).

In 1974, Sanger, designed a procedure for sequencing the DNA similar to the natural

process of DNA replication. DNA sequencing enables us to perform a thorough analysis of DNA because it provides us with the most basic information of the sequence of all nucleotides. With this knowledge, comparisons are made between homologous genes across species. Identification of strain was done by 16S rRNA sequence analysis. PCR amplification of 16S rRNA gene of *Pseudomonas* sp., was performed to determine the presence of *Pseudomonas* sp., in the template DNA by using the universal forward and reverse primers. Forward: UNI-F: AGAGTTT GATCATG GCTCAG, UNI-R: TACGGCTA CCTTG TTACGACTT.

PCR

The polymerase chain reaction (PCR) is an enzyme catalyzed biochemical reaction in which stranded DNA (Mullis, 1990). The PCR reaction was performed in 0.2 ml micro centrifuge PCR tube by adding 1µl of template DNA, 1 µl of forward primer, 1 µl of reverse primer, 5 µl of 2x PCR mix and 2 µl of nuclease free water for 10 µl reactions and homogenized by quick spin. The reaction was performed with initial heating at 94 ^{o}C for 7 min, 35 cycles of denaturation at 94 ^{o}C for 1 min, annealing at 54 ^{o}C for 1 min, extension at 72 ^{o}C for 1 min, followed by final extension at 72 ^{o}C for 10 min. The reaction tubes were cooled and a small aliquot of PCR product was run on agarose gel electrophoresis along with the DNA molecular weight marker to analyze the product.

PCR amplification of *lap*D gene

PCR amplification of *lap*D gene was done by using primers forward: 5'-GTTCCTGGTGGTTGCCTT- 3' and reverse: 5'-AATCGCTTGTCACTTTCC-3'. The reaction was performed with initial heating at 94 ^{o}C for 7 min, 35 cycles of denaturation at 94 ^{o}C for 1 min, annealing at 53 ^{o}C for 1 min, extension at 72 ^{o}C for 1 min, followed by final extension at 72 ^{o}C for 10 min. The reaction tubes were cooled and a small aliquot of PCR product was run on agarose gel electrophoresis along with the DNA molecular weight marker to analyze the expected amplicon size was 875 bp.

PCR amplification of *phzS* gene

Amplification of *phz*S gene was done by using primers forward: 5'-TCTGTCTGTTCCTGGTGGTT-3' reverse:5'-GCAGGTCAATACTGTGAG-3'. The reaction was performed with initial heating at 94^{o}C for 7 min, 35 cycles of denaturation at 94^{o}C for 1 min, annealing at 53^{o}C for 1 min, extension at 72^{o}C for 1 min, followed by final extension at 72^{o}C for 10 min. The reaction tubes were cooled and a small aliquot of PCR product was run on agarose gel electrophoresis along with the DNA molecular weight marker to analyze the expected amplicon size of 1750bp units.

Result and Discussion

Biochemical identification

Under morphological analysis and biochemical test the bacterial genera was conformed to be *Pseudomonas* sp. and for molecular characterizations leads to the conformation of species *P. putida.* Out of 60 samples from fish waste dumping sites, 30 samples were found to be positive for the biochemical analysis.

Visualization of PCR products

The amplified PCR products was separated using agarose gel

electrophoresis and the amplicon size was determined as 1.4 kb of about 1330 bp (Rathanakumari *et al*., 2012) and this was similar to that of the expected size. This molecular identification gives positive results for *P. putida.* Of 30 *Pseudomonas* species isolates, the 16S rRNA PCR confirms the presence of 15 *P. putida.* The results on the AGE were shown in the figure 1. It is compared with the specific markers.

PCR amplification of *lap*D gene

Of 15 isolates tested only one isolate of *P. putida* was positive for *lap*D gene with the expected amplicon size of 875 bp. It is compared with the specific markers (figure 2).

PCR amplification of *phz*S gene

Of the 15 strains of *P. putida*, only four strains were positive for *phz*S gene (figure 3).

The soil sample are chitin rich and the *Pseudomonas* which was isolated from chitin containing soil sample has the characteristics of chitin degradation as Shuba *et al.* (2010) reported chitinolytic properties of *Pseudomonas* species. Rathanakumari *et al*. (2012) isolated and confirmed the isolates as *P. putida* using 16S rRNA PCR technique. Same primers were used in the present investigation and we observed that only 15 strains were confirmed as *P. putida*.

Since the genus *Pseudomonas* is a potential biofilm former, we have concentrated on the biofilm formation potential of our isolates. The *lap*D gene is the biofilm regulatory gene, which help the organism to form biofilm on many of the surfaces. Hinsa and Toole (2006) worked on *P. fluorescence* and reported the presence of *lap*D among their isolates. They also suggested that that the *lap*D gene is responsible for the attachment of the bacteria for the formation of biofilm. Newell *et al*. (2009) also stated that *lap*D, a large adhesion protein, which is located in the inner membrane of the bacteria and required for the attachment of bacteria via *lap*A gene. In another Study, Urgel *et al*. (2008) reported that the lap proteins are important in *P. putida*, to adhere to the seeds.

In the present investigation, all the 15 isolates were subjected for the amplification of *lap*D gene. Surprisingly, only one isolate was found to have the gene in it (Figure 2). *P. putida* a PGPR bacterium with biofilm is useful for the growth of the plant. Richard (2011) reported that the adhesion of *Pseudomonas* sp. to the plant roots is supported by the *lap*D gene. This will helps the organism to establish a strong base on / in the plant or plant's related materials. Biofilm is one of the potential characters of interest and this will help the organism and the host in many different ways. Since *P. putida* is a well-established PGPR group of bacteria, definitely the establishment of biofilm will be much beneficial to the plant and the bacteria as well.

In addition to the biofilm formation, many of the species of *Pseudomonas* are capable of producing bio-active compounds. One among them is pyocyanin. This is a fluorescent green colour pigment which is having antimicrobial activity too (Pierson *et al*., 2010). Mavrodi *et al*. (2001) reported that gene *phz*S for the production of pyocyanin. They also stated that the gene *phz*S is similar to bacterial monooxygenases.

Figure. 1 Amplification of 16S rRNA for *P. putida* in the gel where the markers with 1500 bp units and the final gel shows the amplification of the *P. putida* shows the amplicon size of 1400 bp units

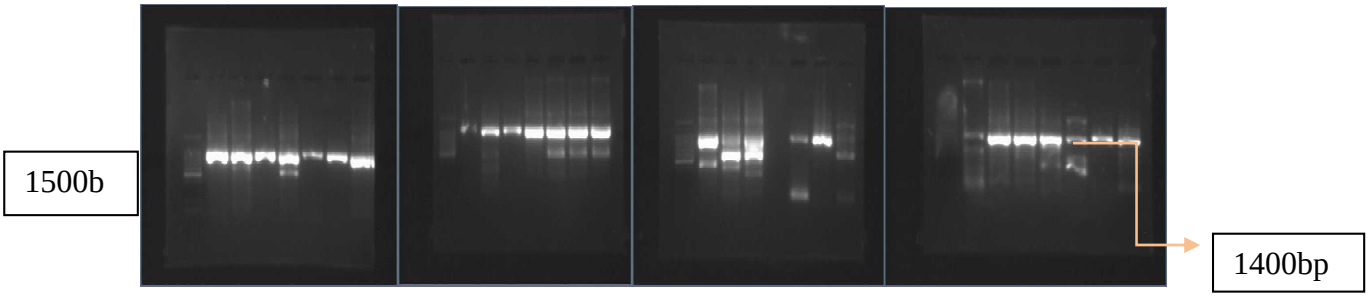


Figure. 2 Amplification of *lap*D gene with the primers and this is in the AGE compared with the markers for its specific amplicon size.

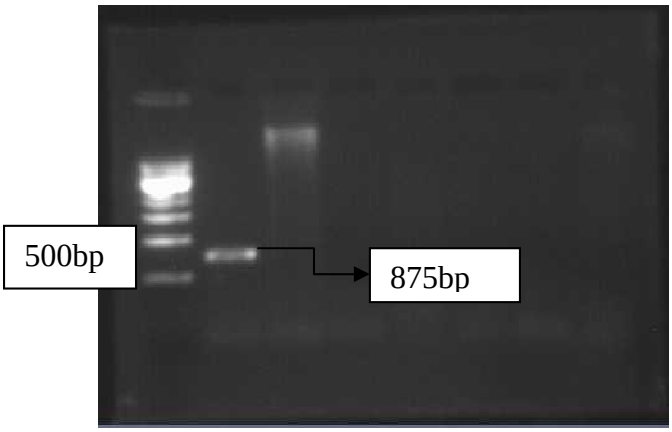
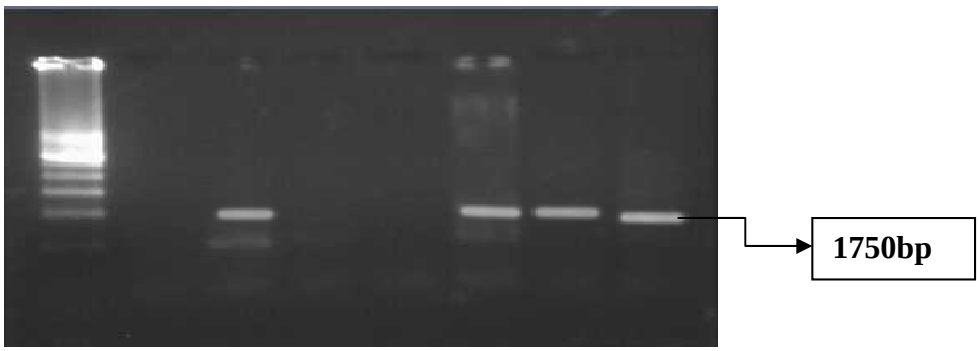


Figure.**3** Amplification of *phz*S gene with the primers and this is in the AGE compared with the markers for its specific amplicon size



Thomashow *et al*. (2006) used *phz*S primers and amplified 1215 bp of DNA encoding *phz*S gene from *P. aeruginosa* and also crystallized their enzymes responsible for the formation of the gene and relived the importance of the pyocyanin. Thomashow *et al*. (2011) proved that the presence of *phz*S gene is essential for the production pyocyanin. He insertionally inactivated the gene *phz*M and he developed a pyocyanin deficient phenotype. Pyocyanin producing *P. aeruginosa* environmental isolates have been recognized as putative biological control agents against phytopathogenic fungi and bacteria in agriculture fields (Rangarajan *et al*., 2003). Pyocyanin is bactericidal and against the different plant pathogens like *Rhizoctonia solani* (Pierson *et al*., 2010).

All the 15 strains were subjected to the amplification of pyocyanin gene (*phz*S) since pyocyanin is said to be involving in the biofilm formation. The pyocyanin can facilitate biofilm formation via an iron-independent pathway (Wang *et al*., 2011). It was observed that only four strains were positive for this gene. Because of the presence of this *phz*S, the biofilm formation by the *P. putida* may be enhanced. This will helps the organisms in different ways, during and after the formation of biofilm.

Several strains of *P. chlororaphis* have been shown to be efficient root colonizers capable of producing a variety of antifungal substances including phenazine-1-carboxamide (PCN) (Bloemberg *et al*., 2001). Since four of our isolates have been containing the *phz*S gene, these organisms could protect not only themselves, but also the plants from infections caused by various microorganisms.

We also conclude that the *phz*S gene is responsible for the synthesis of pyocyanin. The presence of *phz*S gene is confirmed by using specific primers. We took all 15 bacterial strains for amplification finally we found that 4 isolates produces pyocyanin. *P. putida* with dual characters of biofilm formation and pyocyanin production is useful for the plant against pathogens and can increase the disease resistant.

Biofilm formation by a non pathogenic and PGPR organisms like *P. putida* is useful for the plants. *P. putida* is known to respond rapidly to the presence of root exudates in the soil converging at the root colonization sites and established a stable biofilms. If biofilm formed in the root nodules and it also produces pyocyanin it is helpful for the plants against variety of the pathogens. Such kind of strains could be effectively used as a potential biofertilizers.

The *lap*D is an important gene for the formation of the adhesion for stable biofilm by *Pseudomonas* sp., This shall be easily confirmed by the amplification of the specific gene. Strains with these characters could be much useful in the production / formulation of biofertilizers. Commercialization of the bacterium as a biofertilizer for the large scale can be done in future. Before that one has to go for variety of studies such as compatibility, application and field trails.

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