Research Article

PCR based DNA fingerprinting of mercury resistant and nitrogen fixing *Pseudomonas* spp.

Sumaira Tariq, Aatif Amin* and Zakia Latif

Department of Microbiology and Molecular Genetics, University of the Punjab, Lahore-54590, Pakistan.

*Corresponding author's email: aatifamin93@gmail.com Phone: +92-42-35952811, Fax: +92-42-99230481

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Abstract

Mercury resistant *Pseudomonas* strains were isolated from polluted water, rhizospheric soil and root nodules of different plant species. Selected bacterial isolates showed potential to produce fluorescence under UV light and to tolerate heavy metal stress of 10-20 µg/ml HgCl₂. Biochemical characterization revealed all selected bacterial isolates belong to genus *Pseudomonas*. A total of 27 bacterial isolates were evaluated for bioremediation of mercury due to their ability to produce H₂S and fix nitrogen. Genomic DNA of 12 *Pseudomonas* strains was isolated and their genetic diversity was evaluated using SSR (GACA)₄ microsatellite fingerprinting. On the basis of banding pattern of PCR amplified products, a phylogenetic tree was constructed using Minitab 13 software which showed 71%, 46% and 44% homology of SZ-30, SZ-6 and SZ-8 with strains of cluster 1 and SZ-16 respectively. Results exhibited significant diversity among selected *Pseudomonas* strains.

Key Words: Microsatellite-primed PCR; Fluorescent *Pseudomonads*; Hg-resistance.

Introduction

The microbial diversity is the current frontier in biology which still needs much exploration. It is the largest reservoir comprising majority of earth's species on earth which is under intensive investigation. Microbial diversity is very important area of research by fact that microorganism play pivotal role in many processes of the biosphere like recycling of nutrients and detoxification of many environmental pollutants [1, 2]. The sustainable development due the industrial and commercial applications of microbes is

totally dependant on proper exploration of microbial diversity. The heterogeneity potential of microorganisms leads to novel bioremediation strategies of toxic pollutants under the umbrella of biotechnology [3].

Plant microbe interaction is very beneficial and detrimental process for proper maintenance of soil texture, plants and microbes respectively [4]. Such free living bacteria benefit to plants by (1) production of phytohormones like auxins and derivatives of IAA (2) N₂ fixation by asymbiotic processes (3) antagonistic activity against pathogenic bacteria of plants

by producing protease, chitinase, hydrogen cyanide, antibiotics and siderophores (4) solubilization of phosphate minerals and termed as plant growth promoting bacteria (PGPR). PGPR include species of genera like *Bacillus*, *Pseudomonas*, *Arthrobacter* and *Azotobacter* and have been directly used for enhancement of plant growth and performance [5, 6].

The applications of fluorescent *Pseudomonads* (FLPs) used as PGPR require proper understanding of plant microbe interaction (PMI) and factors including biotic and abiotic which affect this relationship. Modern techniques such as immunofluorescence microscopy and reporter genes have markedly improved the knowledge about concise application of such *Pseudomonas*. Over last few years, a significant emphasis has been given to molecular marking for detection of microbial diversity in soil samples [7]. The use of repetitive DNA sequences for specific DNA fingerprinting (SSR) has become a famous and the most reliable technique due to its precise sensitivity, specificity and simplicity to differentiate the most closely related strains of microbes [8, 9].

Nevertheless it is still urgent need to study the plant response to inoculate plant growth promoting bacteria by characterization of the rhizospheric microbial populations and selection of successful strains that enhance plant growth and suppress diseases. Recent study in the area of quorum sensing systems in antifungal metabolite production, the intra- and inter-species signaling and characterization of promoters are provided new approaches for the development of new biological control agents [10].

In the present study a novel approach was applied to characterize *pseudomonads* population by SSR microsatellite fingerprinting, isolated from different samples such as polluted water, rhizospheric soil and

root nodules of six plant species, viz., *Cycus revolute* (Sago Palm), *Sesbania concolor* (Tingin), and *Iresine herbstii* (Lal jhari), *Sesamum indicum* (Till), *Vigna radiate* (Mongo bean), *Solanum tuberosum* (Potato) and *Vigna unguiculata* (Lobia); that plants were denizen of University of the Punjab, Lahore, Pakistan.

Material and Methods

Sample Collection

Mercury resistant *Pseudomonas* strains were isolated from root nodules of six plant species, viz., *Cycus revolute* (Sago Palm), *Sesbania concolor* (Tingin), and *Iresine herbstii* (Lal jhari), *Sesamum indicum* (Till), *Vigna radiate* (Mongo bean) and *Vigna unguiculata* (Lobia) collected from different areas of University of the Punjab, Lahore, Pakistan. For the isolation of bacterial isolates, the nodule surfaces of selected plants were sterilized with 0.1% HgCl_2 for 10 min. Fine thick nodules (1 g) were separated and crushed in sterilized pestle and mortar to make 10% water extract. Tenfold dilutions of each sample were prepared and 50 μl from serial dilutions of 10^{-4} and 10^{-6} was spread on King's B agar plates [11]. The plates were incubated overnight at 37°C for 48 h. The colonies showing yellow pigmentation on King's B medium from different rhizospheric soils were picked up based on the pigment formation and fluorescence under UV light.

Purification of fluorescent & non-fluorescent bacteria

After overnight incubation, different colonies appeared on King's B agar medium were observed under ultra violet (UV) light at 254 nm. Fluorescent and non-fluorescent strains were selected and streaked on new King's B agar plates. Bacterial colonies were streaked again and again on King's B agar plates and were incubated at the same conditions as before to get pure single and isolated bacterial colonies. Pure single

colonies of bacteria were preserved in 70% glycerol stocks at -20°C for further studies.

Screening of mercury-resistant fluorescent bacteria by well plate method

Selected bacterial strains were grown in LB broth at 37°C for overnight on continuous agitation of 150 rpm. The O.D of each bacterial strain was measured by spectrophotometer at 600 nm and adjusted 0.1 O.D_{600nm} of each bacterial strain. 100 µl of each bacterial culture (O.D_{600nm} 0.1) was spread on LB plates. Four wells of 5 mm in diameter were prepared by sterilized Pasteur pipette on each LB agar plate and two different concentrations of HgCl₂ solutions (10 and 20 µg/ml) were added in each well. The plates were incubated overnight at 37°C and zone of inhibition in mm around each well was measured for each selected fluorescent bacterial isolate.

Cellular and biochemical characterization of isolated strains

Selected fluorescent and mercury-resistant bacterial isolates were subjected to different morphological, cellular and biochemical tests such as Gram staining, morphology, fluorescent King's B medium, cytochrome oxidase, DNase, triple sugar iron (TSI), Lysine decarboxylase and nitrate reduction test for characterization.

Nitrogen fixation and hydrogen sulfide production selected *Pseudomonas* spp.

For the isolation of N₂ fixing mercury-resistant fluorescent bacteria, 100 µl of each bacterial culture was spread on NFM agar plates and incubated for 2-3 days at 37 °C. For the isolation of H₂S producing mercury-resistant fluorescent rhizobacteria, Two microliter of each overnight bacterial culture (O.D_{600nm} 0.01) was spotted on LA agar plates and incubated for 3 days at 37°C. After incubation growth was observed in treatment as well as in negative control (without lead acetate) as described by Amin and Latif [12].

Genetic diversity of *Pseudomonas* spp. by DNA finger printing (SSR)

Genomic DNA extraction was performed by CTAB method as described by Shahzadi *et al.* [13]. Polymerase chain reaction (PCR) of the selected strains was performed by using SSR (short sequence repeats) primer such as ((GACA)₄). PCR reaction mixture (50 µl) was prepared by adding 5 µl of 10X PCR buffer, 3 µl of 25 mM MgCl₂, 3 µl of 2 mM dNTPs, 5 µl of primer, 0.5µl Taq polymerase enzyme, 2 µl of template DNA and 31.5 µl distill water. Automated thermocycler was used for the amplification of DNA with the amplification profile as initial denaturation at 95°C for 1 min followed by 35 cycles of denaturation at 95°C for 20 sec, annealing at 42°C for 30 sec and extension at 72°C for 20 sec. Final extension was at 72°C for 5 min. PCR products were analyzed through electrophoresis and then visualized under UV trans-illuminator. The genetic tree was constructed by using Minitab13.0 for the variability among different strains of *Pseudomonas*. Molecular weights were calculated by comparing the motifs with 100 bp DNA ladder. Dendrogram based on matrix was constructed for cluster analysis by using minitab13.0 software.

Results and Discussion

Thirty bacterial colonies were isolated from different sources on King's B agar plates and out of thirty, ten bacterial isolates were screened out on the basis of their ability to produce fluorescence under UV light at 254 nm as given in Table 1. The presence and persistence of fluorescent *Pseudomonas* in the soil ecosystem has long been established [14-16]. Three bacterial strains (SZ-5, SZ-8 and SZ-23) showed the zone of inhibition against 10 and 20 µg/ml concentration of HgCl₂ while rest of all isolates showed the resistance to HgCl₂ at given concentrations (Fig. 1 & 2). Xu, Cao [17] and

Figure 1: Screening of mercury sensitive and resistant bacterial isolates on the basis of zone of inhibition (a) Sensitive (b) Resistant.

Figure 2: Zone of inhibition (mm) of mercury sensitive strains with two concentrations of HgCl₂ (10 and 20 µg/ml).

Figure 3: Growth of bacterial strains on (a) Nitrogen free (NF) medium (b) Lead acetate (LA) medium.

Table 1. Morphological and Biochemical Characterization of selected bacterial isolates

Isolates	Gram staining	Shape	Fluorescence (King's medium) B	Oxidase	DNase production	TSI test	Lysine decarboxylase	Nitrate reduction	<i>Pseudomonas</i> spp. (<i>aeruginosa</i> , <i>cepacia</i> , <i>fluorescens</i> and <i>putida</i>)
SZ-1	-	Rods	+	+	-	R/R +	-	-	<i>P. fluorescens</i>
SZ-2	-	Rods	+	+	-	R/R +	-	-	<i>P. fluorescens</i>
SZ-3	-	Rods	+	+	-	R/R +	-	-	<i>P. fluorescens</i>
SZ-4	-	Rods	-	+	+	R/R +	-	-	<i>P. aeruginosa</i>
SZ-5	-	Rods	-	+	+	R/R +	-	-	<i>P. aeruginosa</i>
SZ-6	-	Rods	-	+	-	R/R +	+	-	<i>P. cepacia</i>
SZ-7	-	Rods	+	+	-	R/R +	-	-	<i>P. aeruginosa</i>
SZ-8	-	Rods	+	+	-	R/R +	-	-	<i>P. aeruginosa</i>
SZ-9	-	Rods	+	+	-	R/R +	-	-	<i>P. fluorescens</i>
SZ-10	-	Rods	-	+	-	R/R +	-	+	<i>P. putida</i>
SZ-11	-	Rods	-	+	-	R/R +	-	+	<i>P. putida</i>
SZ-12	-	Rods	+	+	-	R/R +	-	-	<i>P. fluorescens</i>
SZ-13	-	Rods	+	+	-	R/R +	-	-	<i>P. fluorescens</i>
SZ-14	-	Rods	+	+	-	R/R +	-	-	<i>P. fluorescens</i>
SZ-15	-	Rods	-	+	-	R/R +	-	+	<i>P. putida</i>
SZ-16	-	Rods	-	+	+	R/R +	-	-	<i>P. aeruginosa</i>
SZ-17	-	Rods	-	+	+	R/R +	-	-	<i>P. aeruginosa</i>
SZ-18	-	Rods	-	+	-	R/R +	-	+	<i>P. putida</i>
SZ-19	-	Rods	+	+	-	R/R +	-	-	<i>P. fluorescens</i>
SZ-20	-	Rods	-	+	+	R/R +	-	-	<i>P. aeruginosa</i>
SZ-21	-	Rods	+	+	-	R/R +	-	-	<i>P. fluorescens</i>
SZ-22	-	Rods	-	+	-	R/R +	-	+	<i>P. putida</i>
SZ-23	-	Rods	+	+	-	R/R +	-	-	<i>P. fluorescens</i>
SZ-24	-	Rods	+	+	-	R/R +	-	-	<i>P. fluorescens</i>
SZ-25	-	Rods	+	+	-	R/R +	-	-	<i>P. fluorescens</i>

SZ-26	-	Rods	+	+	-	R/R +	-	-	<i>P. fluorescens</i>
SZ-27	-	Rods	+	+	-	R/R +	-	-	<i>P. fluorescens</i>
SZ-28	-	Rods	+	+	-	R/R +	-	-	<i>P. fluorescens</i>
SZ-30	-	Rods	+	+	-	R/R +	-	-	<i>P. fluorescens</i>

Figure 4. Genetic diversity in *Pseudomonas* species using SSR (GACA)₄: (a) Gel electrophoresis (b) Dendrogram constructed by using SSR banding pattern.

Zeng *et al.* [18] have reported the resistance of HgCl₂ by *Pseudomonas* Spp. up to 65µg/ml. All bacterial isolates were characterized on the basis of colony morphology and biochemical tests as described by Cappuccino and Sherman [19]. All isolates were characterized as species of genera *Pseudomonas* (Table 1). Selected bacterial strains were streaked on nitrogen free medium (NFM) and incubated at 37°C for 24 hr [20, 21]. Only nitrogen fixers showed pronounced growth while nitrogen non-fixers showed no growth on NF medium as shown in Fig. 3(a). Lead Acetate (LA) Agar medium was used to study hydrogen sulfide production by selected bacterial isolates as showing dark brown

colonies on the medium after incubation at 37°C for 2-3 days. H₂S-production test is based on the relationship of mercury resistant strains with H₂S production. Results indicated that mercury resistant strains showed H₂S production and converted the mercury compounds to Hg²⁺ in the cell. Hg²⁺ reacted with H₂S to produce HgS (less toxic and highly insoluble). Black color colonies appeared due the precipitation of HgS. Mercury sensitive strains produced white colored colonies on the LA medium due to lack of their ability to produce H₂S. Bacterial strains resistant to HgCl₂ were selected from well plate method were used for H₂S production test [22]. Three strains (SZ-5, SZ-8 and SZ-23) sensitive to mercury

showed white colonies and were used as control while rest of all isolates showed H₂S production (Fig. 3(b)).

Genomic DNA of 12 bacterial strains (SZ-1, SZ-2, SZ-6, SZ-8, SZ-10, SZ-12, SZ-16, SZ-20, SZ-23, SZ-28, SZ-29 and SZ-30) identified as *Pseudomonas* spp. by cellular and biochemical test was isolated by using CTAB method. PCR amplification was done by using SSR (short sequence repeats) primer (GACA)₄ which was 16 bp. Fig. 4 Shows the banding pattern of different *Pseudomonas* spp..The phylogenetic tree was constructed in order to check the percentage homology of strains so strains SZ-2 and SZ-10; SZ-20 and SZ-29 (cluster 1) showed highest homology (100%) with other strains on the basis of banding pattern while strain SZ-16 showed 81.45% similarity with strains of cluster 1. Similarly strains SZ-30, SZ-6 and SZ-8 showed 71%, 46% and 44% homology with strains of cluster 1 and SZ-16 respectively. Over last few years, DNA fingerprinting (SSRs) is being used the most reliable technique to differentiate the most closely related strains of microbes [23-25].

Conclusion

On the basis of biochemical characterization and SSR phylogenetic analysis, it is concluded that all bacterial isolates obtained from water, soil and root nodules of various plants were found similar with *Pseudomonas* spp. with dual characteristics (1) to detoxify mercury pollutants (2) to fix atmospheric nitrogen (N₂). So, these agronomic strategies will be helpful for obtaining sustainable agriculture. Nevertheless a continued work in this area of research is needed to explore the potential of PGPRs and their ecological, genetic and biochemical relationships in habitat.

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