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Molecular Characterization of Antibiotic Producing Bacteria *Pseudomonas sp*.BP-1 from Nagavali river basin of Srikakulam, Andhra Pradesh, India

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Abstract

The *Pseudomonas* sp.BP-1 strain (NCBI gene accession number HM359121) was isolated from Nagavali river basin of Srikakulam, Andhra Pradesh India. The strain is capable of inhibiting the growth of a wide variety of Gram +ve and Gram –ve bacteria. Partial 16S rRNA gene sequence of the isolate was sequenced and compared with the sequences of representative *Pseudomonas sp.* The 16S rRNA data supported the phylogenetic position of the strain within the genus *Pseudomonas* and was closely related to *Pseudomonas sp*. 19-28; (GenBank entry: EU167964) and *Pseudomonas pseudoalcaligenes;* RW31; (EU419918) with a sequence similarity of 99%.

Key Words: *Pseudomonas sp*.BP-1 strain, Nagavali, antibiotic, 16S rRNA sequencing.

Introduction

All organisms need to compete in order to survive in their respective habitats. This biological task can be achieved by the development of competitive mechanisms such as the production of toxins, enzymes and antimicrobial agents like antibiotics. One of the areas in soil where one can find abundance in microbial populations is the rhizosphere. The high nutritional content of Nagavali river basin area promotes a high microbial colonization which includes bacteria, fungi and nematodes. Even though this area has a high nutritional content, the organisms that colonize it have to compete for space, water availability, and other physical factors (1). Therefore, these communities exhibit and maintain their competition and survival mechanisms which includes symbiotic relations, parasitism and the production of antagonistic substances such as antimicrobial agents and hydrolytic enzymes. A compilation of the microbial sources of antibiotics in the soil discovered in the United States and Japan between 1953 and 1970 revealed that approximately 85% are produced by actinomycetes, 11% by fungi and 4% by bacteria (2). Bacteria of the genus *Pseudomonas* are able to survive and prosper in a wide range of environmental conditions. This genus not only contains plant, animal and human pathogens but also accommodates species of environmental interest such as plant growth promoters, xenobiotic degraders and bio-control agents (3, 4). Among the bio-control agents, antibiotic producing strains have received considerable attention.

Antibiotics encompass a chemically heterogeneous group of organic low molecular weight compounds which are deleterious to the growth or metabolic activities of other

microorganisms at low concentrations (5, 6). The fact that there is an abundant amount of studies on antibiotics produced by *Pseudomonas* sp. has several reasons: *Pseudomonads* are common inhabitants of rhizosphere and phyllosphere are easily isolated from natural environments and utilize a wide range of substrates easy culturing and their genetic manipulations makes them more amenable to experimentation (7, 8).

Material and Methods

***Conventional identification tests*:** The strain BP-1 was isolated from Nagavali river basin (18° 10'to 19° 44' 0N lat and 82° 53' to 84° 05' 0E long) of Srikakulam District, Andhra Pradesh, India, maintained on nutrient agar and stored at 4°C. The isolate was initially evaluated by conventional tests i.e. Gram stain, growth and morphometric characteristics on nutrient agar, growth at 37°C, catalase, oxidase, motility, indole production, gelatin liquefaction, oxidative fermentative carbohydrate utilization, decarboxylation of lysine, urease activity etc. Additional tests included phenylalanine deamination, nitrate reduction, citrate utilization and H_2S production.

***Screening for antibiotic producing bacteria*:** The soil suspensions were homogenized by shaking at 200 rpm for 15 minutes at 30°C and serial dilutions were carried up to 10^{-3}. Two repetitions of each of the dilutions were inoculated on nutrient agar and the cultures (or master plates) were incubated at 37°C for 24 hours. After the incubation period, colonies that exhibited antagonism were designated as antimicrobial agent producing microbes (AAPM) and were sub cultured and purified by streaking them on nutrient agar plates. After purification the isolated AAPM's were preserved and stored at -20°C for further tests.

***Antibiograms*:** Susceptibility test with whole cell extract: Cultures were centrifuged at 3000g for 15 min, then the cell pellets were resuspended in 15% glycerol, 1% SDS, 0.1M Tris-HCl pH 6.8 and denatured by treatment at 100°C for 20 min. Non-solubilized material was removed by centrifugation at 3000g for 15 min and the resulting supernatant was used as crude whole cell extract. The antimicrobial agent producing capability of the isolate was tested by a modification of the Kirby-Bauer method

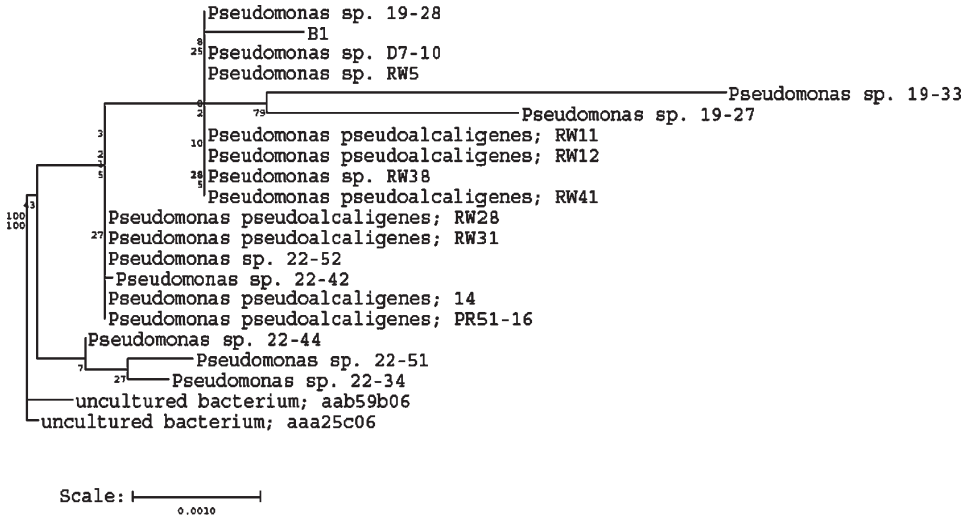

Fig. 1. Phylogenetic position based on 16S rRNA gene sequence analysis of strain BP-1

described by Boyle et al.(9). The target microorganisms used were *Escherichia coli* MTCC 40, *Pseudomonas aeruginosa* MTCC 424, *Proteus vulgaris* MTCC 426 and *Staphylococcus aureus* MTCC 87. AAPM's were incubated 24 hours at 37°C and the targets were also incubated for 24 hours at 37°C. In order to create a bacterial lawn of the targets on nutrient agar, the spread plate technique was employed by using 200 µl of each target. Wells were prepared by a sterile borer and loaded with 20 µl of the AAPM's supernatant and placed over the bacterial lawns. A positive control was included by using a sterile tetracycline antibiotic disc. Antibiograms were incubated 24-48 hours at 37°C. After this period inhibition zones were measured with a ruler using a cm scale.

***DNA preparation and PCR amplification*:** Genomic DNA was extracted from the isolates using Chromous Genomic DNA isolation kit (RKT09). Each genomic DNA used as template was amplified by PCR with the aid of 16S rDNA primers (16S Forward Primer: 5'-AGAGTRTGATCMTYGCTWAC-3' 16S Reverse Primer: 5'-CGYTAMC TTWTTACGRCT-3' and the programme consisted of denaturation at 94°C for 5 min and subsequent 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 2 min followed by final extension at 72°C for 5 min. The presence of PCR products was determined by electrophoresis of 10 µl of the reaction product in 1% agarose gel.

***16S rRNA sequencing and data analysis*:** Sequencing analysis was performed on a 1500 bp PCR product. The sequence analysis was performed using the ABI 3130 genetic analyzer and Big Dye Terminator version 3.1 cycle sequencing kit. The three 16S rRNA sequences were aligned and compared with other 16S rRNA genes in the GenBank by using the NCBI Basic Local Alignment Search tool (BLAST). A distance matrix was generated using the Jukes-cantor corrected distance model. The phylogenetic trees created using Weighbor (Weighted Neighbor Joining: A Likelihood-Based Approach to Distance-Based Phylogeny Reconstruction) with alphabet size 4 and length size 1000. The 16S rRNA gene sequences have been deposited to Genbank using BankIt submission tool and has been assigned with NCBI accession number (HM359121).

Results and Discussion

The antimicrobial activity of the genus *Pseudomonas* has been reported by many workers [10, 11] from various sources and ecological regions. The bacterial strain was isolated and identified as *Pseudomonas* sp. based on the results of 16S rRNA sequencing. The 16S rDNA amplification by forward Primer: 5'-AGAGTRTGATCMTYGCTWAC-3', Reverse Primer: 5'-CGYTAMCTTWTTACGRCT-3' followed by sequencing reveals that the strain BP-1 had highest homology (99%) with *Pseudomonas* sp. 19-28; (GenBank entry: EU167964) and *Pseudomonas pseudoalcaligenes*; RW31; (EU419918). More than 1400bp of the 16S rRNA genes of the strain BP-1 was sequenced. Analysis of the 16S rRNA sequences confirmed the strain BP-1 was found to be most similar to *Pseudomonas* sp. 19-28 (GenBank entry: EU167964) (Fig 1). The 16S rRNA gene sequence has been deposited to Genbank using BankIt submission tool and has been assigned with NCBI (National Centre for Biotechnology information) accession number HM359121.The results of inhibition zones against the target microorganisms are shown in Table 1. The strain BP-1 is a Gram negative, rod shaped bacterium with a circular, flat, translucent colony morphology with entire margin. Table 2 shows the results of the biochemical tests carried out for identification.

Table 1: Susceptibility test using filtered supernatants

	Microbial targets			
Specimen	***E.coli***	***P.aeruginosa***	***S.aureus***	***P.Vulgaris***
BP-1	1.6	1.0	1.8	1.0
Tetracycline(+ve control)	1.2	1.0	1.0	1.1

Table 2: Results of biochemical tests

Biochemical tests	**BP-1 (Remark)**
Carbohydrate fermentation	
Glucose	-ve
Adonitol	-ve
Lactose	-ve
Arabinose	-ve
Sorbitol	-ve
Citrate utilization	+ve
Nitrate reduction	-ve
Lysine utilization	+ve
Ornithine utilization	-ve
Phenylalanine deamination	-ve
Urease production	-ve
H_2S production	-ve
Indole Test	-ve
MR-VP Test	-ve

Conclusion

The authors are of the opinion that metagenomic studies are required to exploit the less known and virgin habitats for the possible new antibiotics from nature as most of the synthetic antibiotics has one or other problems including the base problem of developing multiple drug resistant microbes.

References

1. Satoshi,O.,Iked,H., Ishikawas, J.,Hanamoto, M.A., Takahashi, C., Shinose, M., Takahashi, Y., Horikawa, H., Nakazwa, H., Osonoe,T., Kikuchi,H., Shiba,T., Sakaki,Y. and Hattori, M. (2004). Genome sequence of an industrial microorganisms *Streptomyces avermitilis*: deducing the ability of producing secondary metabolites. Appl. And Env Microbiol., 70:5522-5527.
2. Tyler, V.E., Brady, L.R. and Robbers, J.E. (1988). Pharmacognosy (9th edition), Lea & Febiger. Philadelphia.
3. Palleroni, N.J. (1992). Human and animal pathogenic *Pseudomonads* In: Balos, A.,Truper, H.J., Dworkin, M., Harder. W., and Schleifer of tolaasin, a lipodepsipeptide toxin produced by mushroom pathogen *P. tolaasii*. Physiol. Molec. Plant Pathol., 39:57-70.
4. Johnsen, K., Andersen, S. and Jacobsen, C.S. (1996). Phenotypic and genotypic characterization of Phenanthrene degrading fluorescent *Pseudomonas* biovars, Appl. Environ. Microbiol., 62:3818-3825.
5. Fravel, D.R. (1988). Role of antibiosin in the biocontrol of plant diseases. Ann. Rev. Phytopathol., 26:75- 91.
6. Thomashow, L.S., Bonsall, R.F. and Weller, D.M. (1997). Antibiotic produced by soil and rhizosphere microbes Insitu:Hurst, C. J., Knudsen, G. R., McInerney, M.J. Stenjenbach, L. D., & alter, M.V. (eds). Manual of Env. Microbiology, 493-499 ASM press, Washington, D.C., USA.
7. Leisinger,T. and Margreff, R. (1979). Secondary metabolites of the fluorescent *Pseudomonas*. Microbiol. Rev., 43:422-442.

8. Whipps, J.M. (2001). Microbiological interactions and biocontrol in the rhizosphere .J. Exp. Bot, 52:487-511.

9. Boyle,J., Fancher, M. and Ross, R.Jr. (1979). Rapid modified Kirby-Bauer susceptibility test with single, high-concentration antimicrobial disks. Antimicrobial. Agents and Chemotherapy, 3:418-424.

10. Jennifer, K. and Loper, J.E. (1995). Characterization of a Genomic Region Required for Production of the Antibiotic Pyoluteorin by the Biological Control Agent *Pseudomonas fluorescens* Pf-5. Applied and Environmental Microbiology, 849–854.

11. Raaijmakers, J., Weller, D. and Thomashow, L. (1997). Frequency of Antibiotic Producing *Pseudomonas spp*. in Natural Environments. Applied and Environmental Microbiology, 881-887.